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Exploiting lipid raft transport with membrane targeted nanoparticles: A strategy for cytosolic drug delivery

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Abstract

The ability to specifically deliver therapeutic agents to selected cell types while minimizing systemic toxicity is a principal goal of nanoparticle-based drug delivery approaches. Numerous cellular portals exist for cargo uptake and transport, but after targeting, intact nanoparticles typically are internalized via endocytosis prior to drug release. However, in this work, we show that certain classes of nanoparticles, namely lipid-coated liquid perfluorocarbon emulsions, undergo unique interactions with cells to deliver lipophilic substances to target cells without the need for entire nanoparticle internalization. To define the delivery mechanisms, fluorescently-labeled nanoparticles complexed with $\alpha_v\beta_3$ -integrin targeting ligands were incubated with $\alpha_v\beta_3$ -integrin expressing cells (C32 melanoma) under selected inhibitory conditions that revealed specific nanoparticle-to-cell interactions. We observed that the predominant mechanism of lipophilic delivery entailed direct delivery of lipophilic substances to the target cell plasma membrane via lipid mixing and subsequent intracellular trafficking through lipid raft-dependent processes. We suggest that local drug delivery to selected cell types could be facilitated by employing targeted nanoparticles designed specifically to utilize alternative membrane transport mechanisms.

INTRODUCTION

The ability to safely and specifically deliver potent drugs to selected cell types, while concomitantly monitoring local drug concentrations and efficacy against pathological molecular epitopes invokes a challenge originally conceptualized in Paul Ehrlich's "magic bullet" analogy [1]. One approach entails the use of nanoparticulate agents that might concentrate potent drugs at sites of pathology through molecular targeting of high-capacity therapeutic carriers[2]. This approach requires the design of nanoparticles that recognize and bind to selected molecular epitopes, and subsequently maximize therapeutic delivery to a targeted site while minimizing adverse effects elsewhere [3].

Several delivery systems have been proposed and used for this purpose including liposomes, micelles, dendrimers, and copolymers [4]. Two clinically available examples include Doxil® and Abraxane®, which represent liposomal or albumin-bound nanoparticulates, respectively. Although the mechanisms of action for these two carriers are not well delineated, non-targeted systems typically accumulate within the interstitial space of target sites (i.e., cancer) through

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the passive endothelial retention and permeability (EPR) mechanism [5]. In the case of Abraxane, albumin-linked receptor-mediated transcytosis across endothelial cells has been proposed as an additional mechanism of interstitial accumulation [6]. Regardless, once at the target site these particles either slowly degrade and deliver their payloads by diffusion [7], or can undergo cellular endocytosis, which can be enhanced by targeting receptor mediated endocytosis or incorporating membrane penetrating peptides [8]. However, endocytosis of drug delivery vehicles requires an endosomal escape step to gain entry into the cell cytosol and avoid inactivation of delivered compounds by lysosomal degradation [2,9,10]. A drug delivery mechanism for targeted nanoparticles that utilizes directed cytoplasmic delivery into a targeted cell while avoiding endosomal incorporation could potentially provide an avenue for delivering greater amounts of agent with more efficient and immediate access to intracellular targets. Accordingly, we have developed and demonstrated a synthetic lipid-based nanoparticle delivery vehicle that utilizes existing biological delivery pathways, and can achieve rapid deposition of large amounts of active agent directly into cellular membrane compartments without incorporation into the endosomal milieu.

In this study, we report the delivery mechanism of a synthetic, molecularly-targeted nanovesicle (see Figure 1) comprising a lipid/surfactant monolayer surrounding a dense lipophobic, hydrophobic perfluorocarbon (PFC) core. This construction permits direct mixing of lipids with the target cell plasma membrane through lipid exchange and lipid particle fusion. We have observed previously that targeted liquid PFC nanoparticles perform well as sensitive and specific molecular imaging contrast agents that operate by incorporation of ligands directed towards specific molecular markers (e.g., fibrin, tissue factor, $\alpha_v\beta_3$ -integrin) [11–13]. PFC nanoparticles can additionally serve as drug delivery vehicles, where lipophilic drugs can be dissolved into the outer lipid monolayer, or complexed to lipid anchors in the monolayer for subsequent delivery to target cells for site-targeted therapeutic action [12,13].

To define the specific delivery mechanisms of lipophilic substances from nanoparticles into target cells, we sought to elucidate the mechanism of cellular delivery for each component of the synthetic carrier: 1) the PFC core materials, and 2) the drug/lipid monolayer components. To model the mechanism of drug delivery, we defined the transport of particle lipid moieties, because available data regarding handling of hydrophobic drugs suggests similar transport mechanisms [14]. We observed that upon nanoparticle binding to the targeted surface, both lipid and PFC core materials can be delivered directly to the plasma membrane through lipid exchange and/or lipid particle fusion without requiring energy or the involvement of complex cell transport mechanisms. However, further intracellular transport of the lipophilic (or drug) materials into the cytoplasm is an active, energy-dependent process that is mediated fundamentally by lipid raft-based transport.

MATERIALS AND METHODS

Nanoparticle Formulation

Liquid PFC nanoparticles were formulated using methods previously developed in our laboratories [11]. Briefly, the emulsions comprised 20% (v/v) perfluorooctylbromide (PFOB), 2% (w/v) of a surfactant/lipid co-mixture, and 1.7% (w/v) glycerin in distilled, deionized water. The surfactant co-mixture primarily contained lecithin (Avanti Polar Lipids, Inc., Alabaster, AL), in addition to the following phosphatidylethanolamine conjugates. For targeted nanoparticles, 0.01 mole% of a peptidomimetic vitronectin antagonist of the $\alpha_v\beta_3$ -integrin linked to phosphatidylethanolamine (Kereos Inc., St. Louis, MO) was included in the surfactant layer. The targeting ligand was separated from the nanoparticle with a PEG₂₀₀₀ linker to facilitate targeting and limit steric hindrance. The nanoparticles were fluorescently labeled by including 0.2 mole% fluorescein, 4.56 mole% nitrobenzoxidaizol (NBD), 0.1 mole % rhodamine (Avanti Polar Lipids, Inc.), or 0.3 mole% of Alexa Fluor® 488-conjugated

phosphatidylethanolamine in the surfactant mixture. As described previously, Alexa Fluor® 488 carboxylic acid, succinimidyl ester (Molecular Probes, Eugene, OR) was coupled to caproylamine-dipalmitoyl-phosphatidylethanolamine (Avanti Polar Lipids, Inc.) in an overnight incubation and was purified using thin layer chromatography [15]. Control (non-targeted) nanoparticles were produced that lacked only the ligand targeted to $\alpha_v\beta_3$. The mixture of surfactant components, PFC and water was blended, and then emulsified at 20,000 PSI for four minutes in an ice bath (S110 Microfluidics emulsifier, Microfluidics, Newton, MA). Particle size analysis by laser light scattering (Brookhaven Instruments Corp., Holtsville, NY) measured a nominal nanoparticle size of 242nm (polydispersity = 0.17). Zeta potential measurements at 37°C were similar for non-targeted and $\alpha_v\beta_3$ -targeted nanoparticles (-23.14 ± 4.74 vs. -29.21 ± 1.52 mV, respectively).

Cell Culture

Human C32 melanoma cells (Washington University Tissue Culture Support Center, St. Louis, MO) were maintained in a humidified atmosphere of 95% air and 5% CO₂ in MEM containing Earle's salts and 10% (v/v) FCS (Sigma, St. Louis, MO). C32 cells were utilized due to their high expression of the $\alpha_v\beta_3$ -integrin in culture [16], which was confirmed by our laboratory with flow cytometry.

Mechanism of Delivery

C32 melanoma cells were incubated with a 100 pM concentration of fluorescently-labeled, non-targeted or $\alpha_v\beta_3$ -targeted nanoparticles for 1 hr. The nanoparticle concentration was estimated from nominal particle size determined by laser light scattering and the total volume of perfluorocarbon incorporated into the formulation. To define the role of active, energy-dependent, and endocytic cellular processes that might characterize nanoparticle-to-cell interactions, experiments were performed under cold (4°C and 18°C), ATP-depleted (20mM sodium azide, 50mM 2-deoxyglucose) [17], cholesterol-disrupted (50µg/mL filipin) [18], macropinocytosis-inhibited (5mM amiloride) [19], and actin-disrupted (25µM cytochalasin D) conditions [20]. Cells were incubated with the inhibitors for 15 min before addition of nanoparticles. After incubation and removal of free or unbound nanoparticles with PBS washing, cells were prepared for direct observation with 2% paraformaldehyde fixation for 30 minutes or for quantitative assessment by removing adherent cells with trypsin.

The specificity of inhibitors was determined by 1 hr incubations with common pathway markers for clathrin-mediated endocytosis with transferrin-AlexaFluor488 (10µg/mL), caveolae/lipid raft-mediated endocytosis with cholera toxin subunit B-AlexaFluor488 (1µg/mL) (Molecular Probes), and macropinocytosis with 70kD dextran-FITC (2.5mg/mL) (Sigma) [21]. Simultaneous incubation with pathway markers and nanoparticles was used to determine the location of intracellular nanoparticle signal. The location of nanoparticle signal within the plasma membrane was assessed by staining for lipid rafts (Molecular Probes Kit) after nanoparticle incubation, which involved staining lipid rafts with cholera toxin B and crosslinking the label in place with anti-cholera toxin B antibody. Lipid location was also assessed by fluorescence recovery after photobleaching (FRAP) in live cells by bleaching a portion of the plasma membrane (50 iterations at 50% laser power output) and measuring change in fluorescent signal over time (170 sec).

Cellular Assessment

PFC content measured by gas chromatography (GC) was used as a tracer to quantify delivery of particles to cells. To determine PFOB content with GC, cells were suspended in 1mL of 20% potassium hydroxide in methanol. The suspension was then combined with 2mL of internal standard (0.01% octane in freon), vigorously vortexed, and agitated continuously with a shaker for 1 hr. GC (Agilent, 6890 Series) analysis was performed using a flame ionization

detector and bonded phase column (30m X 0.53mm, J&W Scientific). Samples were normalized by protein content determined by measuring the absorbance at 280nm with a UV spectrometer (UV-1601, Shimadzu Corp., Kyoto, Japan).

For confocal imaging cells were grown on #1.5 glass bottom culture dishes (Bioprotech Inc., Butler, PA). Fluorescence imaging of cell sections was conducted with a confocal microscope (Zeiss Meta 510, Thornwood, NY) using standard filter sets. The location of nanoparticles with respect to the cell was determined with simultaneous differential interference contrast (DIC) imaging.

Analysis of confocal images was conducted with Image J software. To determine the extent of cytosolic lipid delivery, two regions of interest (~450 pixels) were drawn: 1) inside the cell cytosol and 2) within the background outside the cell. The average pixel value was calculated for each region and the average cytosolic pixel value is reported as the overall signal above the background level (i.e., background signal subtracted from cytosol signal). Images of colocalized pixels were created using the RG2B colocalization plugin with auto thresholding.

Statistical Analysis

Differences between the treatment groups were evaluated for significance using analysis of variance (ANOVA) with statistical software (JMP IN by SAS, Cary, NC). An experimental repetition of 3 or greater with a p-value less than 0.05 was considered statistically significant.

RESULTS

Cellular delivery of nanoparticle lipids

To elucidate the mechanism of cellular delivery of lipophilic materials contained within the phospholipid surface monolayer of a PFC-core nanoparticle vesicle, nanoparticles were formulated with various fluorescent labels conjugated to the headgroup of phosphatidylethanolamine, including carboxyfluorescein (FITC), Alexa Fluor 488®, nitrobenzoxadiazole (NBD), and rhodamine. To mimic the *in vivo* application [13] and ensure cellular delivery of fluorescent lipid materials from the nanoparticles, a small molecule ligand to the $\alpha_v\beta_3$ -integrin was included, which permitted specific binding of the nanoparticles to target cells expressing surface $\alpha_v\beta_3$ -integrin (C32 melanoma), as demonstrated directly with confocal microscopy (Fig. 2). In the absence of targeting ligand (i.e. non-targeted nanoparticles), minimal fluorescent signal was associated with the cells.

By 1 hr, targeting of nanoparticles and delivery of lipids onto the plasma membrane was apparent regardless of the fluorescent label. We observed similar levels and distributions of targeted nanoparticle binding with all fluorescent lipid labels. Additionally, further intracellular transport of all labels was observed, characterized by diffuse staining throughout the cytosol for FITC and AlexaFluor488 labels and diffuse staining with additional punctate characteristics for NBD and rhodamine labels. The punctate nature of the staining observed for NBD and rhodamine represented only a portion (roughly <30%) of the total cytoplasmic signal for these agents, and hypothetically might be attributable to either internalization of intact nanoparticles, or to cellular trafficking of the labels (or the lipid membranes that confine them) to specific intracellular compartments. The patterns for the FITC and AlexaFluor488 labels did not manifest any such features of intracellular compartmentalization.

Intracellular transport via lipid rafts

The role of active, energy-dependent, and endocytic cellular processes that might characterize nanoparticle-to-cell interactions were investigated with both broad and specific inhibition of transport pathways using temperature or drug intervention (for inhibitor specificity see, Suppl.

Fig. 1). Active cellular processes were broadly inhibited with cold (4°C) temperature and depletion of cellular ATP by the combined inhibition of ATP production (i.e., mitochondrial ATPases) and glycolysis with sodium azide and 2-deoxyglucose, respectively [17]. General endocytic processes were inhibited with cold temperature (18°C) and disruption of transport mechanisms mediated by actin filaments with cytochalasin D (CytD) [17, 20]. Inhibitor specificity studies indicated macropinocytosis was more sensitive to general endocytic inhibition than clathrin- and caveolae/lipid raft-mediated endocytosis (Suppl. Fig. 1). Finally, the specific endocytic pathways of caveolae/lipid-raft-mediated endocytosis and macropinocytosis were inhibited by caveolar pit/lipid raft disassembly with the cholesterol binding action of filipin and by prevention of membrane ruffling with inhibition of the Na/H transporter using amiloride, respectively [18, 19]. Intracellular delivery of fluorescent, rhodamine-labeled lipid materials from nanoparticles to target cells under the various inhibitory conditions was visualized with confocal microscopy (Fig. 3).

The deposition of lipid moieties derived from nanoparticles onto the plasma membrane depended explicitly on specific cellular targeting, because no substantive delivery was observed for non-targeted nanoparticles. For nanoparticles targeted to $\alpha_v\beta_3$ -expressing cells, specific binding of targeted nanoparticles to the cell plasma membrane and transport of lipophilic materials into the cell cytoplasm, was not affected by: 18°C, macropinocytosis-inhibition (amiloride), and actin-disruption (CytD).

Under conditions of biochemical energy depletion (4°C and ATP depleted), and lipid raft-inhibition (filipin), targeting and delivery of lipids to the plasma membrane was maintained, indicating that delivery occurs through lipid particle fusion and/or lipid mixing. However, lipid transport into the cell cytoplasm beyond the cell membrane was markedly reduced, suggesting that lipophilic agent delivery into the cell interior is an active, lipid raft-dependent process that utilizes existing cellular machinery for transport.

Distinct mechanism of PFC delivery

To further elucidate PFC nanoparticle-to-cell interactions, the cytosolic pixel intensities of fluorescent material transported into the cell was determined for each group and compared directly with the PFC component measured by gas chromatography. Quantification of the intracellular lipid signal confirmed the visual interpretation that intracellular transport was maintained under 18°C, amiloride, and CytD conditions, although the overall level was slightly reduced compared to 37°C (Fig. 4a). The intracellular transport of lipids was inhibited most effectively by 4°C, ATP depletion, and lipid raft disruption (filipin). This was confirmed by statistical analysis, which separated the endocytic blocking treatments into two groups: 1) where cytosolic delivery was achieved (18°C, amiloride, cytochalasin D, $p < 0.001$) and 2) where cytosolic delivery was dramatically inhibited (4°C, ATP depletion, filipin, $p < 10^{-10}$).

Quantification of the PFC component associated with the cells after 1 hour of incubation revealed a nearly 6-fold increase in PFC levels achieved by targeting to the cell surface (Fig. 4b: 4.4 ± 0.6 vs. 25.7 ± 1.1 $\mu\text{g PFC/mg protein}$, non-targeted vs. $\alpha_v\beta_3$ -targeted, respectively). In contrast to the confocal image readouts, quantification of the PFC component revealed no change in the concentration of PFC (or nanoparticles) that was present under conditions of filipin treatment or ATP depletion in stark contrast to the concomitant inhibition of lipid delivery (Fig. 4a). However, a nearly 50 percent reduction in overall PFC levels was observed when the temperature was further reduced to 4°C (13.1 ± 0.9 $\mu\text{g PFC/mg protein}$). The observed temperature dependence might be attributable to energy/ATP-independent processes, such as an alteration of “passive” targeting kinetics (i.e., overall number of bound nanoparticles), or a change in membrane characteristics (via disturbed fusion, or limited lipid mixing, or limited diffusion) that might have facilitated PFC delivery at the higher temperatures. To further examine the temperature dependence of “passive” targeting, the bound PFC component was

assessed at an early (10 min) time point, and binding at 37°C and 4°C (6.3 ± 0.4 vs. 6.8 ± 1.0 μg PFC/mg protein, respectively) was observed to be equivalent. These findings likely implicate altered mechanical properties of the membrane as the primary mechanism preventing PFC delivery to cells, which again is consistent with the hypothesis that stored energy in the nanoparticle membrane itself drives the PFC core delivery as suggested above, through fusion, lipid mixing, and lateral diffusion, and not through endocytosis.

Direct delivery to target membrane

The mechanism responsible for the areas of diffuse staining observed within the plasma membrane of target cells (Fig. 2 & 3) was examined to verify that lipids can “stream” from the nanoparticle lipid monolayer directly onto the cell plasma membrane. To confirm direct lipid delivery to the plasma membrane, we used fluorescence recovery after photobleaching (FRAP) to differentiate between lipids confined within bound nanoparticles and lipids free to diffuse within the cell plasma membrane. After incubating with NBD-labeled, $\alpha_v\beta_3$ -targeted nanoparticles, live cells were imaged over time with confocal microscopy at room temperature, during which a small portion of the plasma membrane was bleached. Images clearly reveal a loss in signal with the bleach sequence as compared to the prebleached image, which over time was able to recover as lipids diffused into the bleached area of the plasma membrane (Fig. 5). Direct measurements of signal intensity over time revealed a nearly 60 percent loss in signal in the bleached area, which recovered over 80 percent of the original signal intensity in approximately 170 seconds. These results prove that lipophilic materials located in the nanoparticle monolayer can be directly delivered to the plasma membrane through mechanisms of lipid particle fusion and lipid mixing, followed by subsequent trafficking into the cell via active lipid raft transport mechanisms.

Cellular location of nanoparticle lipids

To evaluate compartments involved in transmembrane and intracellular transport and trafficking, targeted nanoparticles and cells were incubated simultaneously with transferrin, cholera toxin subunit B (CT-B), and 70kD FITC-dextran to assess colocalization of nanoparticle lipids with clathrin-mediated, caveolae-mediated, and macropinocytic pathways, respectively [21]. We observed only minor intracellular colocalization with clathrin-mediated or caveolae-mediated pathways (Fig. 6). Some nanoparticle lipid signal seemed to colocalize with dextran in the cell cytosol, indicating that some intact nanoparticles can be internalized via macropinocytosis. Alternatively, this colocalization could also be due to internalization of portions of the membrane where nanoparticle lipids already have been deposited, since macropinosomes are known to contain lipid rafts [22]. Although macropinocytosis appears to play a role in transport, an alternate pathway is clearly the predominant mechanism since inhibition by amiloride does not disrupt PFC or lipid delivery (Fig. 3).

Additionally, we stained the plasma membrane for lipid rafts after incubation with nanoparticles by labeling the glycosphingolipid GM1, the receptor of CT-B predominantly located in lipid rafts [23]. The lipid raft staining was punctate in nature (i.e., not uniform throughout the plasma membrane), indicating the label was specific for lipid rafts. We observed significant colocalization in discrete regions of the cell plasma membrane between the nanoparticle lipid label and the stained lipid rafts. Although both the nanoparticle lipid label and CT-B initially colocalize on the plasma membrane itself, intracellular transport must occur through different pathways since little overlap was observed inside the cell (see caveosome labeling). These results confirm that nanoparticle lipids within the plasma membrane are primarily localized and transported into the cell from the membrane compartment by way of lipid rafts and that internalization of intact nanoparticles is not the predominant mechanism of intracellular lipid delivery.

DISCUSSION

Site targeted drug delivery is poised to assume a prominent role in pharmacotherapeutics as a partial solution to systemic toxicity of potent drugs delivered by conventional means. By packaging these agents into cell targeted therapeutic carriers, systemic toxicity may be reduced because much lower serum levels can be administered, but therapeutic benefit is still achieved because of high accumulation at the target site. Most therapeutic carriers deliver drugs through diffusion across the plasma membrane as the drug is slowly released due to carrier degradation, or through endocytosis of the carrier, which requires endosomal escape for drugs to reach the cell cytosol and circumvent lysosomal degradation [8]. In contrast, PFC nanoparticle emulsions utilize lipid-drug transport mechanisms that require close apposition to the target cell surface to permit transfer of lipophilic nanoparticle substances directly to the cell plasma membrane with subsequent intracellular trafficking via lipid rafts without requiring internalization of intact nanoparticles.

Previous results in our laboratory have shown targeted PFC nanoparticles with drugs incorporated into the outer lipid monolayer of the nanoparticle are highly effective both *in vitro* and *in vivo* [12,13]. To delineate the mechanism of intracellular drug transport from PFC nanoparticles to target cells, we examined the mechanisms that mediated the cellular delivery of nanoparticle components (i.e. lipid and PFC). In our study, we concluded that endocytosis of intact PFC nanoparticles played only a minor role as a mechanism of intracellular drug transport, which was evidenced by the finding of distinct delivery mechanisms for the outer monolayer lipids as compared with the PFC core (Fig. 4), and the observation of minimal intracellular colocalization with endocytic markers (Fig. 6). We observed that delivery of both nanoparticle components was dependent on the feature of specific ligand-based targeting to the cell surface, whereas very little cellular interaction was observed with non-targeted nanoparticles (Fig. 2), consistent with previous observations of minimal therapeutic benefit with non-targeted, drug-loaded nanoparticles [12,13].

Upon specific targeting to the cell surface, the lipophilic contents in the monolayer were delivered directly to the cell plasma membrane (Fig. 5) and subsequently trafficked to the cytosol via lipid raft-mediated mechanisms (Fig. 3). In contrast, PFC delivery to the cell was only mediated by conditions inhibiting direct lipid-particle interactions with the plasma membrane such as fusion (4°C) and not cell transport mechanisms (Fig. 4). The mechanisms that mediate direct delivery to the cell membrane are the contact-mediated pathways of intermembrane lipid exchange or mixing and lipid particle fusion [24,25].

Similar to our observations, others have described that lipids delivered to cells via these mechanisms are distributed throughout the cell cytoplasm, with little lipophilic material present in the nucleus [24]. Although similar transport mechanisms for lipophilic drugs and lipid molecules have been observed, lipophilic drug transport is less restrained (i.e., access to the nucleus) and is not subject to specific cell sorting mechanisms, as observed with NBD- and rhodamine-labeled lipids (Fig. 2). After initial delivery to the plasma membrane, trafficking of the lipophilic substances to intracellular compartments could be mediated by several lipid-raft dependent mechanisms, including vesicular transport of caveosomes or macropinosomes [26], shuttling of lipids through transporters, or other non-vesicular pathways [27]. Given that intracellular transport was not mediated by actin (Fig. 3) and did not overlap with caveosomes (Fig. 5), the data suggest that another cellular mechanism besides vesicular transport could be mediating the distribution of lipids throughout the cytosol.

The biophysical process that drives the fusion and lipid mixing after nanoparticle binding does not require cellular sources of energy, and likely represents a combination mainly of the free energy stored in the nanoparticle membrane itself (i.e., monolayer bending modulus) and the

diffusional potential. For the latter, the FRAP membrane studies reveal the particle lipids delivered to the plasma membrane are able to diffuse laterally (Fig. 4), so barriers to diffusion appear minimal. The basis for the first case involves the higher energy state associated with increased membrane curvature as compared to the low energy state associated with “relaxed” planar (i.e., flat) membranes. This difference in energy states is evidenced by the phenomenon of “Ostwald ripening” (i.e., molecular diffusion), the thermodynamic aging process of emulsions, where mass transfer promotes increased particle size (i.e. reduced membrane curvature), indicating higher inherent energy levels associated with increased particle curvatures [28,29]. Upon binding and close association with the plasma membrane, the energy stored within the lipid membrane of the nanoparticle could be sufficient to overcome the energy barriers to fusion [30]. Additionally, cellular delivery of the particle core (PFC), as evidenced by cellular PFC measurements (Fig. 3b), could further promote lipid mixing and fusion, since the surface tension exerted by the PFC to balance the membrane bending energy would no longer hold the lipids in a spherical shape. We can only speculate about any other potential roles the PFC component may play in the observed delivery mechanism. Although PFCs are known to be chemically and biologically inert, studies of lipid mixing with perfluorocarbon gases have revealed that at least when presented initially in gaseous form, perfluorocarbon can intercalate within the tails of phospholipids [31]. Since liposomes can also deliver lipids to cells via contact-mediated mechanisms, it is likely that the presence of the PFC does not play a vital role in transport.

Given these considerations, other lipid-based carriers could potentially deliver drugs through similar mechanisms. Both micelles and liposomes primarily deliver drugs via endocytosis of intact carriers, but might be able to deliver drugs directly to the outer leaflet of the plasma membrane. These mechanisms were first studied with non-targeted liposomal carriers as a method to introduce exogenous lipids to cells [24,25]. Koning *et al.* has reported the ability to deliver lipid-linked “prodrugs” from targeted liposomes without requiring intact nanoparticle internalization, although the drugs were still delivered to lysosomes [32,33]. Otherwise, for targeted liposomes delivery is still primarily through nanoparticle endocytosis [8], which could be attributed to the target, usually receptors internalized upon ligand binding, or steric hindrance due to the pegylation of the liposome surface to prevent clearance through the reticuloendothelial system.

The importance of “intracellular pharmacokinetics” is highlighted by the vast array of approaches developed to either optimize delivery upon endocytosis or divert delivery through alternate pathways [10,34,35]. Multiple methods have been successful in achieving increased delivery of active compounds at the site of action and have been shown to help overcome multidrug resistance [36]. Typically, the characteristics of the particle are modified in order to influence the route of internalization. For example by controlling particle size, cellular internalization can be achieved in a manner that avoids trafficking to lysosomes [37,38]. Significant work has been conducted to optimize drug delivery from liposomes, including targeting to cell epitopes that result in particle internalization (e.g., folate, transferrin, and LDL receptors) and triggering of drug release by controlled carrier instability (e.g., pH-, temperature-, and light-sensitive liposomes) [39–41]. Others have diverted the mechanism of delivery by formulating liposomes to fuse directly with the membrane by incorporating membrane-destabilizers (e.g., cell penetrating peptides, viral proteins) [10,42]. Similarly, future PFC nanoparticle formulations, as well as other lipid-based carriers, could be designed to promote lipid exchange and lipid particle fusion (e.g., incorporation of higher concentrations of phosphatidylethanolamine) [43]. In addition, we have reported that applying exogenous energy to the system with the use of modest nondisruptive levels of ultrasound can dramatically augment lipophilic delivery to cells through contact-mediated mechanisms [44]. Ultimately, such strategies may further improve the therapeutic benefit of carrier systems by improving the number of active drug compounds that reach their site of action.

CONCLUSIONS

Targeted PFC nanoparticles deliver lipophilic substances directly to the target cell membrane with subsequent trafficking via lipid raft-dependent pathways. Since the delivered lipophilic substances ultimately localize to the cellular phospholipid bilayer and not inside endosomes, no additional escape mechanism is required for drug activity. The delivery event only occurs after binding and close apposition to the target cell surface, previously dubbed “contact-facilitated” drug delivery [12]. This mechanism of drug delivery to cells has proven to be effective *in vivo* by permitting over a 50,000 reduction in the overall required drug dose [13]. Overall, the materials of the nanoparticulate drug carrier dictate the type of interactions capable with the cell surface, where close cellular association of a lipid-based nanoparticle enables delivery of drugs via unique contact-mediated methods. We hypothesize that by employing both molecular targeting and existing cellular mechanisms of membrane transport lower systemic doses of selected drugs might be required to achieve therapeutic benefit at the target site.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Perfluorocarbon nanoparticles

Side by side cartoon and transmission electron microscopy images demonstrate the structural features of perfluorocarbon (PFC) nanoparticles. The particle (~200nm) contains a liquid PFC core that is surrounded by a phospholipid monolayer, where drugs, targeting ligands, and imaging agents can be incorporated by the hydrophobic effect or covalent linkage to lipid anchors.

Figure 2. Cellular transport of nanoparticle lipid labels

Cells (C32 melanoma) overexpressing the $\alpha_v\beta_3$ -integrin were incubated for 1 hr with non-targeted or $\alpha_v\beta_3$ -targeted nanoparticles containing various fluorescent lipid labels. Confocal micrographs demonstrate that regardless of the lipid label non-targeted nanoparticles have minimal cellular association. Upon targeting to the $\alpha_v\beta_3$ -integrin, nanoparticles bind to the cell surface and deliver lipids to the plasma membrane and throughout the cell cytoplasm, but not the nucleus. Scale bar, 10 μ m.

Figure 3. Mechanism of lipophilic delivery

C32 cells were incubated with rhodamine-labeled, non-targeted (NT NPs) or $\alpha_v\beta_3$ -targeted ($\alpha_v\beta_3$ NPs) nanoparticles for 1hr under physiologic or inhibitory conditions. Confocal micrographs reveal lipid delivery to the cell plasma membrane is dependent on direct targeting to the cell surface, and further intracellular transport of lipids is an active (4°C), ATP-dependent, lipid raft-mediated (filipin) process, that does not rely on macropinocytosis (amiloride) or actin microfilaments (CytD). Scale bar, 10 μ m.

Figure 4. Distinct mechanisms of lipid and PFC delivery

The mechanisms for lipid and PFC cellular delivery from non-targeted (NT NPs) or targeted ($\alpha_v\beta_3$ NPs) nanoparticles were directly compared under physiologic and inhibitory conditions. a.) Analysis of pixel intensities within the cell cytosol confirms delivery from the nanoparticle lipid monolayer to the cell interior is dependent on cellular energy and mediated by lipid rafts (n=10). b.) Direct measurement of PFC levels reveals PFC delivery to cells is dependent on specific targeting to the cell surface, reduced at cold (4°C) temperatures, and independent of cell transport mechanisms (n=8). Error bars represent SEM. *p<0.001. **p<10⁻¹⁰

Figure 5. Direct lipophilic delivery to target cell plasma membrane

Fluorescence recovery after photobleaching (FRAP) was conducted on the plasma membrane (see inset) of a cell incubated with NBD-labeled, $\alpha_v\beta_3$ -targeted nanoparticles (NPs). The NBD lipid signal within the plasma membrane was bleached (see white box). The signal recovered as new lipids diffused within the area, proving nanoparticle lipids are located within the plasma membrane and not restrained within nanoparticles. Scale bar, 5 μ m (entire cell image) and 1 μ m (insets of plasma membrane).

Figure 6. Nanoparticle lipids colocalize with lipid rafts

Cells were incubated with rhodamine-labeled nanoparticles and markers for clathrin pits (transferrin), caveosomes (CT-B), macropinosomes (70kD dextran), and lipid rafts (CT-B, post-stained). Nanoparticle rhodamine lipids were found within lipid-raft stained plasma membranes (caveosomes and lipid rafts), but few were found within the intracellular compartments of clathrin pits, caveosomes, or macropinosomes. Scale bar, 10 μ m.