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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.

1. 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 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 Fig. 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

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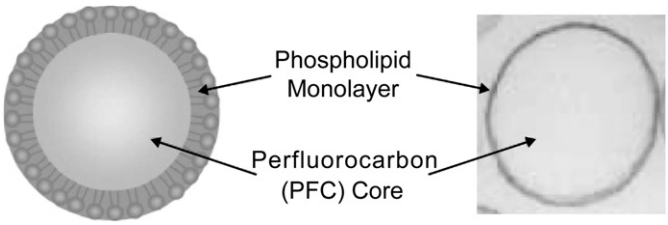

Fig. 1. *Perfluorocarbon nanoparticles*. Side by side cartoon and transmission electron microscopy images demonstrate the structural features of perfluorocarbon (PFC) nanoparticles. The particle (~200 nm) contains a liquid PFC core that is surrounded by a phospholipid monolayer, where drugs, targeting ligands, and imaging agents can be incorporated by the hydrophobic effect or covalent linkage to lipid anchors.

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.

2. Materials and methods

2.1. 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 mol% 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 PEG_{2000} linker to facilitate targeting and limit steric hindrance. The nanoparticles were fluorescently labeled by including 0.2 mol% fluorescein, 4.56 mol% nitrobenzoxadiazole (NBD), 0.1 mol% rhodamine (Avanti Polar Lipids, Inc.), or 0.3 mol% 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 4 min 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 242 nm (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).

2.2. 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% CO_2 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.

2.3. 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 h. 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 (20 mM sodium azide, 50 mM 2-deoxyglucose) [17], cholesterol-disrupted (50 μg/mL filipin) [18], macropinocytosis-inhibited (5 mM amiloride) [19], and actin-disrupted (25 μM 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 min or for quantitative assessment by removing adherent cells with trypsin.

The specificity of inhibitors was determined by 1 h incubations with common pathway markers for clathrin-mediated endocytosis with transferrin-AlexaFluor488 (10 μg/mL), caveolae/lipid raft-mediated endocytosis with cholera toxin subunit B-AlexaFluor488 (1 μg/mL) (Molecular Probes), and macropinocytosis with 70 kDa dextran–FITC (2.5 mg/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 s).

2.4. 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 1 mL of 20% potassium hydroxide in methanol. The suspension was then combined with 2 mL of internal standard (0.01% octane in Freon), vigorously vortexed, and agitated continuously with a shaker for 1 h. GC (Agilent, 6890 Series) analysis was performed using a flame ionization detector and bonded phase column (30 m × 0.53 mm, J&W Scientific). Samples were normalized by protein content determined by measuring the absorbance at 280 nm with a UV spectrometer (UV-1601, Shimadzu Corp., Kyoto, Japan).

For confocal imaging cells were grown on #1.5 glass bottom culture dishes (Bioptechs 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.

2.5. 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.

3. Results

3.1. 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