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Fluorinated Lipid Constructs Permit Facile Passage of Molecular Cargo into Living Cells

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Abstract

Fluorinated lipids get rapidly internalized into living cells and are also displayed on the cell surface. The uptake of lipids is energy dependent and is likely via the clathrin-mediated endocytic pathway. Fluorinated lipids are 3-5 fold more efficient in acting as molecular transporters of noncovalently bound proteins than their hydrocarbon counterparts. These materials could serve as efficient molecular transporters for molecules that function in the cytoplasm such as short interfering RNAs (siRNAs).

The plasma membrane enveloping mammalian cells serves a crucial gatekeeping function by careful regulation of the influx and exodus of molecules. Barring small (< 1 kDa), hydrophobic molecules that can pass through by passive diffusion, all others have to confront the impervious and selective membrane barrier to gain entry.¹ Strategies to deliver macromolecules into living cells have tremendous potential in therapeutic and imaging applications.^{2,3} Several methods have therefore been devised to achieve this end.⁴ Conjugates with amphipathic, hydrophobic or cationic polymers,⁵⁻⁸ and more recently, carbon nanotubes⁹⁻¹³ have been successfully deployed. Noncovalent assemblies of lipids and macromolecules, and liposomes that have the desired molecular consignment on the inside, have also been used.^{14,15} While these approaches have provided a powerful transporter toolkit, the quest for new classes of molecules remains of high interest. The agents must be non-toxic, efficient, and have reasonable half-lives inside cells to be general in their applicability.¹⁶ Such molecules can potentially be used to deliver selective imaging probes or be used in chemo- and genetic therapy.

Endocytosis,^{1,17} a process by which cells internalize molecules, has been recently exploited for delivering macromolecules into mammalian cells. This approach has been extremely effective and several molecules conjugated to cholesterol have been shown to traverse the membrane barrier efficiently.¹⁸⁻²⁰ In order to expand the repertoire of agents that can be used in this manner, we designed phospholipids that contain a H-phosphonate handle for further functionalization. Lipid rafts have been implicated in enhancing the efficiency of endocytosis.^{21,22} We have recently shown that fluorinated phospholipids form phase-separated microdomains of sizes 50–200 nm when mixed with hydrocarbon lipids with identical headgroup functionalities.²³ We envisioned that nanosized domains of fluorinated lipids could enhance endocytic efficiency and uptake.²¹⁻²⁴ Fluorocarbon lipids are more hydrophobic than

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Supporting Information Available. Experimental procedures, synthesis and analytical data for compounds, and movies of different focal planes in the z-axes of Fig. 2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

their hydrocarbon congeners,²⁵⁻²⁹ and are therefore expected to have higher affinities for membranes.⁴ Furthermore, fluorinated molecules have been shown to be biocompatible, and fluorinated “lipoplexes” are significantly more efficient as DNA transfection reagents compared to their hydrocarbon counterparts.³⁰

In order to investigate the ability of fluorinated lipids to act as macromolecule transport agents, compounds **1–5** were designed and synthesized (Scheme 1).^{31,32} Agents **1**, **3** and **5** are derivatives of 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine (DPPE) while **2** and **4** are related phosphodiesteres. Preparation of conjugates from the respective H-phosphonates followed previously established procedures.³³ These molecules contain either all hydrocarbon (**4** and **5**) or partially fluorinated lipid chains (**1–3**) and the head groups are adorned with different linkers attached to biotin (**1**, **2** and **4**) or to the fluorophore 7-nitrobenz-2-oxa-1,3-diazole (NBD, **3** and **5**). The agents were deemed non-toxic to cells in culture as incubation of HeLa cells with **2–5** did not show significant difference in counts after five days when compared to controls (See Supplementary Information). In addition, the complex of **2** and **4** with avidin conjugated to fluorescein isothiocyanate (**AF**, K_a to biotin $\sim 10^{15} \text{ M}^{-1}$) also had little effect on the growth profiles.

Incubation of HeLa cells with **3** at 37 °C resulted in intensely fluorescent cells as evinced by counting on a fluorescence plate reader (Figure 1). The increase in fluorescence was concentration dependent (See Supplementary Information, Fig. S9). Further inspection by fluorescence microscopy revealed that the emanating luminescence was distributed both on the cellular surface and in the interior. Similar experiments with **8** did not result in any cellular fluorescence. Jurkat and HL60 cells when treated similarly with **3** exhibited comparable levels of fluorescence suggesting that the process is general across many cell lines. Localization of **3** was further probed in HeLa cells using fluorescence microscopy.

Internalization of lipids was energy dependent suggesting that it is facilitated by endocytosis.¹⁹ When incubations of HeLa cells with **3** or the complex **2:AF** were carried out at 4 °C, fluorescence from the cells decreased significantly. Indeed, only 39 and 57% of fluorescence was detectable when compared to the experiment at 37 °C for **3** and the **2:AF** complex respectively (Figure 1). Furthermore, addition of sodium azide, an ATP depleting poison, and a known inhibitor of endocytosis, also resulted in a 21% decrease in fluorescence. These experiments implicate endocytosis as the primary mechanism of transport of the exogenous materials.^{34,35} We further interrogated whether endocytosis was orchestrated via a particular pathway. Participation of clathrin coated pits is frequently invoked in endocytic events and it can be disrupted by incubation under hypertonic conditions (400 mM sucrose).³⁶ Indeed, when HeLa cells were treated with **3** or the **2:AF** complex under 400 mM sucrose, the fluorescence from the cells was diminished by 61 and 55% respectively. These results were further corroborated with fluorescence microscopy of the resultant cells (see Supporting Information).

The ability of the transport agents to deliver macromolecules across membranes was investigated by incubation of HeLa cells with preformed complexes **1:AF**, **2:AF** or **4:AF**. The cells were pelleted by centrifugation twice and washed with PBS, re-suspended in buffer, and then examined by microscopy or fluorescence counting. All agents were effective at ferrying **AF** into the cell. In contrast, neither **AF** nor the **AF:biotin** complex were by themselves able to traverse the membrane resulting in cells that were minimally fluorescent. It has been previously established that the binding affinity of lipid-biotin conjugates to **AF** is several orders of magnitude less tighter than biotin.^{37,38} When free biotin ($\geq 5 \text{ eq}$, 1 h) was allowed to equilibrate with **2:AF** prior to incubation with HeLa cells, the cells only exhibited background levels of fluorescence.

In order to assess what fraction of **3** or the complex **2:AF** resides on the outer leaflet of the plasma membrane, we subjected cells to reduction by sodium dithionite (in the case of **3**) or to exchange with free biotin (in the case of the **2:AF** complex).¹⁹ Upon reduction with 5 mM sodium dithionite, a reagent known to extinguish NBD fluorescence, 59% of the fluorescence from the cells was lost. Biotin exchange for 1 h resulted in a similar (73%) loss of fluorescence in the case of the **2:AF** treated cells. Assuming NBD fluorescence is not affected by the environment and accounting for self-quenching in 100 μ M solutions of **3**, treatment with 100 μ M solution resulted in $\geq 10^6$ molecules on the surface of each cell and available for reduction. The localization of synthetic constructs and macromolecular cargo inside cells was investigated using microscopy done in the presence of a nucleus specific dye, 4',6-diamidino-2-phenylindole (DAPI). As the overlay images show in Figure 2, light emitted by **3** and **2:AF** originates from the cytoplasmic region and the agents are excluded entirely from the nucleus.

In general, the fluorinated lipid constructs **2** and **3** were more efficient in transport and uptake as compared to their hydrocarbon counterparts **4** and **5**. As judged from fluorescence counting, this difference was 2.6 fold. This difference in efficiency of uptake was also confirmed using flow cytometry (Figure 3). While the hydrocarbon lipids conferred a 13-fold increase in mean fluorescence intensity of cells over background, the cells treated with the fluorinated congeners were intensely fluorescent with a 63-fold increase in mean fluorescence (a difference of 4.8-fold over the hydrocarbon version). These results demonstrate the superior ability of the fluorinated lipids to trigger and act as participants in the endocytic events.

In summary, we report a new class of macromolecular transport agents capable of entering living cells through endocytosis. They are furthermore able to facilitate the entry of noncovalently bound proteins. The delivery agents were non-toxic and were distributed both on the surface and in the cytoplasm of cells but not in the nucleus. Fluorinated lipids were found to be superior in facilitating transport in this manner. A large number of molecules can be displayed on the cell surface using this methodology ($\sim 10^6$). This study paves the way for clustered display of ligands on cell surfaces and intracellular delivery of macromolecules for imaging and therapeutic applications. These constructs may be especially useful for delivering short interfering RNAs (siRNA) as the main events in RNAi are localized in the cytoplasm. Studies along these lines are in progress in our laboratories.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Relative fluorescence from HeLa cells treated with **3** or the complex **2:AF** under various conditions.

Figure 2.

Fluorescence microscopy images of HeLa cells treated with **2:AF** and **3** at 0 μM (a,d), at 10 μM (b,e) and at 100 μM (c,f) respectively. Cells were fixed with paraformaldehyde (4% w/v) and stained with DAPI (nucleus, blue channel; a-f). Overlay images clearly show that material is excluded from the nucleus. Images were taken at the same exposure for different concentrations of the lipid constructs. Bar, 10 μm (see panel a, bottom right).

Figure 3.

Flow cytometry of HeLa cells (typically 3×10^4) suspended in phosphate buffered saline treated with **8** and **3** (panel a) and with **3** and **5** (panel b). Cells treated with the fluorinated construct **5** were 4.8-fold more intensely fluorescent than those treated with the hydrocarbon analogue **3**.