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

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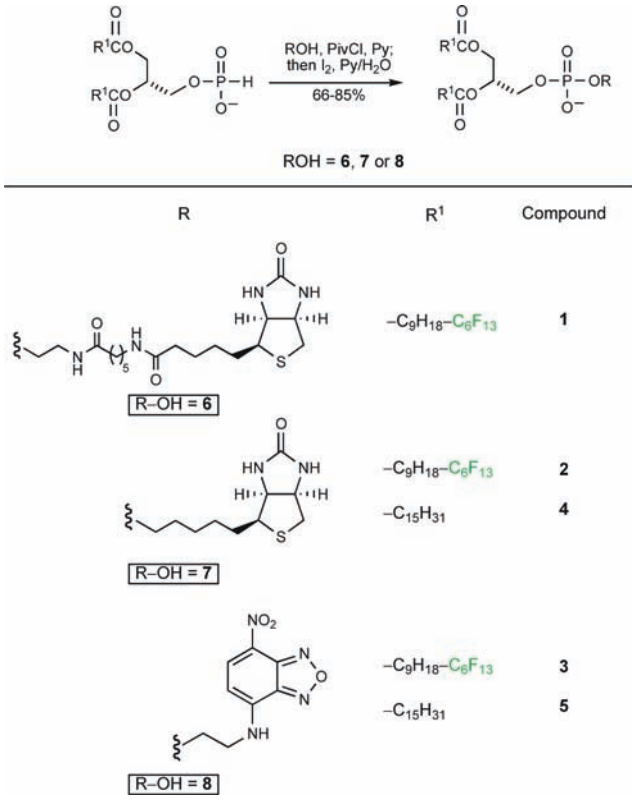
The plasma membrane enveloping mammalian cells serves a crucial gatekeeping function by careful regulation of the influx and exodus of molecules. Barring small (<1 kDa), hydrophobic molecules that can pass through by passive diffusion, all others have to confront the impervious and selective membrane 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 nanotubes[9−13] 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 nontoxic and 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 chemotherapy 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] 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 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]

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 phosphodiesters. Preparation of conjugates from the respective H-phosphonates followed previously established procedures.[33] These molecules contain

Scheme 1. Structures of Phospholipids **1**−**5** and Synthesis from the Corresponding H-Phosphonates



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 nontoxic to cells in culture, as incubation of HeLa cells with **2**−**5** did not show significant difference in counts after 5 days when compared to controls (see Supporting Information). In addition, the complex of **2** and **4** with avidin conjugated to fluorescein isothiocyanate (**AF**, K_a to biotin $\sim 10^{15}$ 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 Supporting Information, Figure 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

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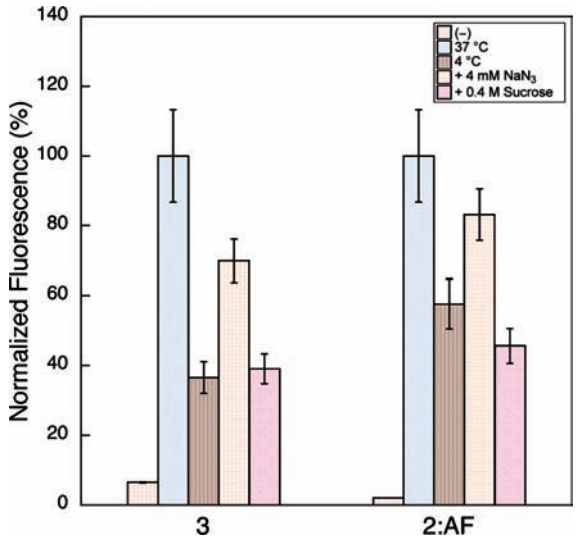

Figure 1. Relative fluorescence from HeLa cells treated with **3** (100 μM) or the complex **2**:**AF** (100 μM) under various conditions.

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 each of the preformed complexes **1**:**AF**, **2**:**AF**, and **4**:**AF** in separate experiments. The cells were pelleted by centrifugation twice and washed with PBS, resuspended 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 equiv, 1 h) was allowed to equilibrate with **2**:**AF** prior to incubation with HeLa cells, the cells only exhibited background levels of fluorescence.

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

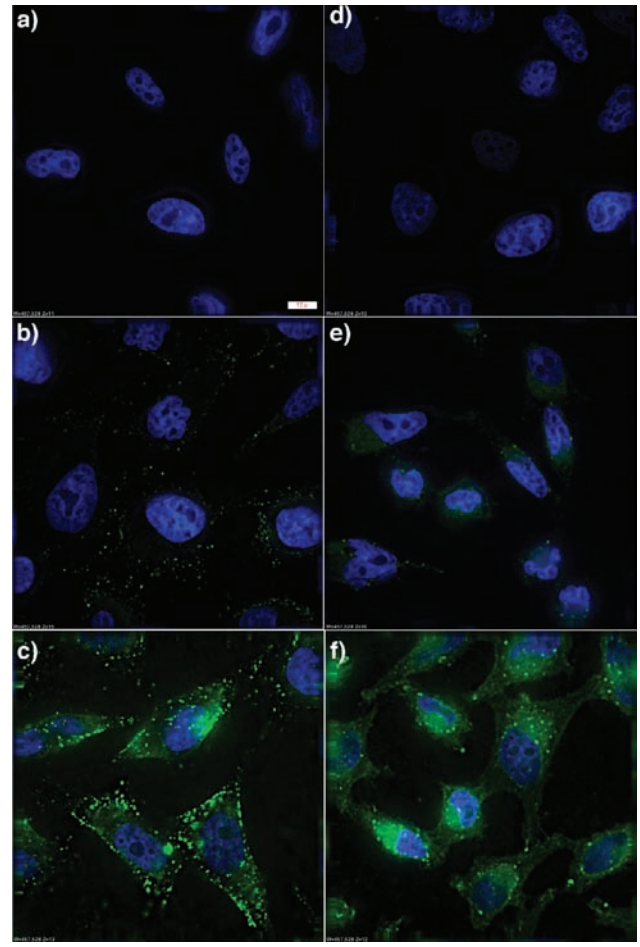

Figure 2. Fluorescence microscopy images of HeLa cells treated with **2**:**AF** and **3** at 0 μM (a,d), 10 μM (b,e), and 100 μM (c,f), respectively. Cells were fixed with paraformaldehyde (4% w/v) and stained with DAPI (nucleus, blue channel; a−f). Overlay images clearly show that material is excluded 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).

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 μM solutions of **3**, treatment with a 100 μM solution resulted in $\geq 10^6$ molecules on the surface of each cell, 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

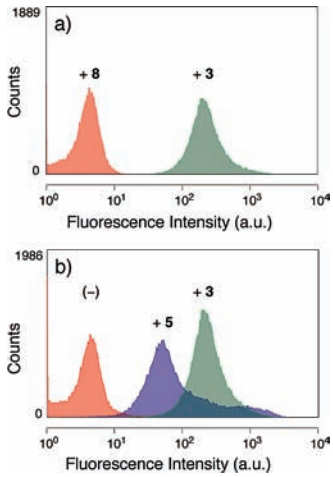

Figure 3. Flow cytometry of HeLa cells (typically 3×10^4) suspended in phosphate buffered saline treated with **8** and **3** (panel a) and with **3** and **5** (panel b). Cells treated with the fluorinated construct **3** were 4.8-fold more intensely fluorescent than those reated with the hydrocarbon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

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 nontoxic 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 the 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 (siRNAs), as the main events in RNAi are localized in the cytoplasm. Studies along these lines are in progress in our laboratories.

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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 Figure 2. This material is available free of charge via the Internet at http://pubs.acs.org.

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