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Selective Cavitand-Mediated Endocytosis of Targeted Imaging Agents into Live Cells

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Abstract

A water-soluble synthetic receptor molecule is capable of selective, controlled endocytosis of a specifically tagged target molecule in different types of living human cells. The presence of suitable choline-derived binding handles is essential for the molecular recognition and transport process, allowing selective guest transport and imaging of cancer cells.

Selective recognition and transport of small molecules across living cell membranes is vital for the development of more effective drug therapies. Natural systems employ membrane-embedded ionophores that allow small charged species to pass through the membrane, but larger organic molecules are transported via different mechanisms.¹ There are three major biological mechanisms for transport across cell membranes: phagocytosis, pinocytosis, and receptor-mediated endocytosis (RME).² RME is the most prevalent and efficient for the transport of relatively small molecules such as drug candidates and proteins/antibodies.³ Synthetic molecules that can mimic this function and *selectively* transport small organic species across membranes have many uses.⁴ Effective methods of transport include the use of molecular umbrellas⁵ or covalent attachment of a receptor motif to a cholesterol or lipid anchor, allowing receptor display above the bilayer interface and inducing RME of the target.⁶ The recognition event in this case refers to a strongly specific interaction between known biological partners, e.g., peptide:protein^{6a} or drug:bioreceptor,^{6c} and each target must be transported by its own designed synthetic receptor molecule.

While efforts toward synthetic RME have mimicked the natural process by covalently appending a known biological receptor to a steroid or lipid derivative, we offer a different strategy: the use of fully synthetic, *shape-selective* receptor molecules that can self-incorporate into membrane lipid bilayers, recognize specific targets, and promote their transport by endocytosis. Recognition events mediated solely by *shape-based* interactions are usually insufficiently strong to compete in the complex environment of a living cell.⁷

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ASSOCIATED CONTENT

Supporting Information

Experimental details and characterization data. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Purely shape- and charge-based guest recognition is rarely observed outside highly controlled synthetic environments, and its application in living cells is unprecedented.

Recently, we described a new method of molecular recognition at a biomimetic supported bilayer interface.⁸ Deep cavitands,⁹ small-molecule receptors capable of non-covalently binding molecules of suitable size and shape, can be incorporated into supported lipid bilayers, displaying their targets at the membrane:water interface. These species are capable of molecular recognition and reaction promotion at the bilayer interface.¹⁰ Their application has been limited to biomimetic constructs; little is known about their behavior in living cells.⁷ Here we show that synthetic host molecules can be incorporated in living eukaryotic cell membranes and transport suitable targets across the cell membrane into the cell interior via “cavitand-mediated endocytosis”.

Incorporating a host molecule in a cell membrane requires a certain lipid-like quality in the host, and this is supplied by the water-soluble tetracarboxylate deep cavitand **1**.¹¹ **1** is synthesized in four steps from simple starting materials and is soluble in water at millimolar concentrations, existing in the “vase”-shaped conformation shown in Figure 1c. It displays all the properties of lipids, including a hydrophobic body and a charged terminus, and can be incorporated into larger aggregates like lipid micelles,¹² bilayer vesicles,¹³ and supported lipid bilayers.^{8,10} The amphiphilic nature of the host allows it to self-incorporate in synthetic membranes⁸ while maintaining a single orientation in the upper leaflet of a bilayer. This presents the open cavity to the exterior of the membrane bilayer, allowing recognition of suitable targets while *inside* the membrane itself. Binding selectivity is retained, and the host molecule acts as an anchor for substrates containing a suitable binding handle. Hydrophobic anchors are obviously unsuitable, but excellent selectivity for substituted trimethylammonium ($R-NMe_3^+$) salts is possible in pure water.^{8,10} The challenge for extending this concept to *living* cells is the presence of numerous competitive guests in the machinery of a cell. Open-ended shape-based receptors show wide target scope but notoriously poor target *selectivity*, and the often-exploited crutch of the hydrophobic effect becomes ineffective when tailoring receptors for work in lipophilic membrane environments.

Cavitand **1** is well-suited to function in natural systems, however: the negatively charged carboxylates at the rim prevent phosphocholine binding while favoring choline and acetylcholine binding, allowing function in phospholipid bilayers. The binding of choline derivatives is strong, and even challenging targets such as large hydrophilic proteins can be immobilized by presenting biotin functions at a bilayer interface.⁸ These abilities suggest that the cavitand can incorporate into *natural* cell membranes and bind targets at the cell surface.

Directly visualizing **1** in living cells is challenging, so an indirect method was exploited. Fluorescently tagged choline derivative **2** can be synthesized in one step from commercially available fluorescein isothiocyanate and is well-precedented to bind in lipid bilayer–cavitan **1** complexes in aqueous solution, allowing visualization by confocal fluorescence microscopy (CFM).⁸ Human cervical cancer (HeLa) cells were used to determine the function of **1** in living cells, for their large size and ease of visualization. HeLa cells were incubated in Dulbecco’s Modified Eagle Medium containing 10% fetal bovine serum (FBS) at 37 °C with 5% CO₂. The cell cultures were exposed to the transport system with variable incubation times before visualization. After incubation, the cultures were washed three times with phosphate-buffered saline (PBS) buffer to remove unincorporated additives from the system, the cells fixed, and the cell nuclei stained with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI). CFM and differential interference contrast (DIC) microscopy were

used to image the cells, and the three images (DIC, 405 nm DAPI, 488 nm guest **2**) were combined to form the images in Figure 2.

Initially, four experiments were performed: the cells were exposed to 50 μM guest **2** in the presence and absence of 50 μM cavitand **1**. Two incubation periods were tested for each system, 1 and 24 h. Figure 2a,b shows that the presence of **1** vastly increases the incorporation rate of the fluorescent guest molecule. After only 1 h, significant incorporation of the target guest is observed in the cell interior. No guest is observed in the nucleus, and the guest is not localized in the cell membrane but has been transported across the membrane bilayer into the cytosol (see Supporting Information (SI) for full images). The guest molecule has no affinity for the cell bilayer itself: no fluorescence is seen in the cell membrane, and minimal transport is observed in the absence of **1**. This is consistent with previous observations that **2** is strongly water-soluble and shows little lipophilicity.⁸ Guest **2** is capable of internalization after extensive (24 h) incubation, but the incorporation is vastly slower than observed in the presence of **1**. The transport efficiency shows a slight dependence on cavitand concentration; the experiment was repeated at $[\mathbf{1}] = 10$ and 20 μM , and while accelerated transport was observed in both cases, it was less effective than at $[\mathbf{1}] = 50 \mu\text{M}$. Importantly, neither **1** nor **2** displays overt cytotoxicity at the concentrations used. Qualitatively, a small number of cells become non-viable at $[\mathbf{1}] = 100 \mu\text{M}$, but no loss of cell viability is observed at 50 μM or lower. When the cells were exposed to **1** under far more stringent conditions (minimum essential medium and only 0.5% FBS), a cell proliferation/viability assay showed 75% of the cells were viable after 24 h incubation with 10 μM **1**. There was no effect on proliferation, membrane permeability, or caspase activity (induction of apoptosis) in cells exposed to **1** at concentrations as high as 1 μM (see SI for full data).

The transport process is not only limited to cancerous cell lines. Figure 2c,d shows the effect of treating GM00637 human skin fibroblast cells with the cavitand–guest mixture, using identical incubation and visualization procedures. In this case, the background rate of incorporation of guest in the absence of **1** was observed, but the process was still significantly accelerated by adding the host molecule, and maximal internalization of added guest **2** was observed after only 1 h incubation.

The microscopy results were corroborated and quantitated with flow cytometry experiments using a HeLa population of 1×10^6 cells with incubation times of 1 and 24 h. Following 1 h incubation, cells incubated with cavitand **1** endocytosed guest **2** at a significantly greater rate than untreated cells in the presence of guest **2** alone (Figure 3a). After 24 h, cells treated with **1** displayed a 10-fold greater fluorescence than cells simply incubated with **2** (Figure 3b). The singly peaked histograms clearly indicate that all cells in the sample incorporate fluorescence and that **1** is not acting on a subpopulation of cells.

One of the advantages of using a molecular receptor for transport purposes is the *selectivity* of the process. Only species with a suitable binding handle can be recognized by the host; those with no R-NMe₃⁺ group show no binding affinity. To illustrate that the transport process is both mediated by the cavitand host and *selective* for the targeted imaging agent displaying the requisite binding handle, the HeLa cells were exposed to fluorescein **3** in the presence and absence of cavitand **1** (Figure 4a,b). Fluorescein (a fluorophore with no cavitand-binding handle) is suitably water-soluble at the concentrations used (50 μM) and shows imaging properties almost identical to those of guest **2**. After both 1 and 24 h incubation, no incorporation of fluorescein to the cellular interior was observed. The propensity for transfection of fluorescein is significantly lower than that of **2** (no incorporation was observed at all, even after 24 h), but it is notable that the cavitand is not a nonspecific transfection agent: in the absence of a suitable binding handle, adding cavitand has no effect on the introduction of small molecule guests. The NMe₃⁺ anchor is essential

for transport, and species that cannot be recognized by the cavitand are not incorporated in the cell.

This selectivity could be disadvantageous in cell types that contain significant concentrations of choline derivatives: choline and acetylcholine are essential neurotransmitters in CNS cells, after all. This was illustrated by adding choline to the system as a competitive inhibitor for the cavitand-mediated transport system. An excess of choline chloride **4** (100 or 200 μM) was added to the HeLa cell culture medium in the presence of 50 μM cavitand **1** and 50 μM guest **2**. The cells were incubated for both 1 and 24 h to study the effect of occupying the host cavities on the transport process (Figure 4c,d). Adding the competitive guest slowed the transport of targeted guest **2** but, importantly, did not stop it completely. After 1 h incubation, very little internalized **2** was observed in the cell interior (Figure 4c). The transport was not prevented, however: after 24 h incubation, significant transport was observed (albeit less than in the absence of choline). The reversible binding event allows competitive guests to bind in the cavitand host, but they are not irreversibly bound and do not prevent transport of the imaging agent. Fortunately, the binding constants of choline **4** and guest **2** are similar. While we have been unable to determine the binding affinity of choline for **1** in membrane environments, the binding affinity is 600 M^{-1} in PBS buffer,^{11b} similar to that of substituted choline derivatives such as **2** in supported lipid bilayers (542 M^{-1}).⁸ Previous results showed that the affinity of guests for host **1** in a bilayer membrane can be approximated by their affinity in salt solutions such as PBS.

These results show that the cavitand is capable of selective transfection of cellular imaging agents that are suitably derivatized with the requisite binding anchor motif. The simple microscopic imaging studies are not sufficient to accurately determine the *mechanism* of this transport process, however. Among precedented cellular transport mechanisms, pinocytosis and phagocytosis are unlikely, as the transported species are too small. As cavitand **1** is well-precedented to self-incorporate into lipid bilayers, two mechanistic pathways are realistic possibilities (see Figure 5): either membrane translocation via a “flip-flop” mechanism (with or without bound substrate), similar to that of cholesterol and phospholipids,¹⁴ or the desirable outcome, cavitand-mediated endocytosis. Simple visual examination of the cell images suggests that incorporation occurs via endocytosis: the fluorescence is punctate, indicating that the fluorophore is contained in endosomes rather than evenly distributed in the cytosol.¹⁵ This is not an uncommon problem with RME of drug candidates, as observed for the transport of small molecules by cholesterol-linked receptors in the absence of endosome-disrupting peptides.¹⁵ Cellular endocytosis can be slowed by forcing depletion of ATP production. If the transport process is analyzed in the presence of ATP-depleting conditions¹⁶ (50 mM 2-deoxy-D-glucose and 10 mM NaN₃; see SI for data), no transport of **2** is observed in the presence of 50 μM **1**.

As observation of the cavitand itself in the system is indirect, it is not entirely clear whether the endocytosis is *triggered* by the molecular recognition event or the host is constantly endo**-/exocytosed by the cell under the incubation conditions and allows endocytosis of the target guest upon complex formation. If the cell is pre-incubated with 50 μM **1** before addition of guest **2**, guest transport is still observed, suggesting (although not proving) the latter explanation (see SI for figures).

Further evidence for a cavitand-mediated endocytosis mechanism can be obtained by analyzing membrane-containing vesicles without the clathrin-based endocytic machinery present in living cells. Giant unilamellar vesicles (GUVs) are excellent mimics of cell membranes, displaying a bilayer membrane with suitably sized (~100 nm diameter) internal cavity. They are incapable of endocytosis, but translocation via “flip-flop” is well-known and occurs rapidly at room temperature.¹⁴ Suitable GUVs were prepared from POPC lipids

via literature methods.¹⁷ The vesicles were doped with 5% Rhodamine-labeled DOPE lipids to aid visualization and to confirm the unilamellar structure (Figure 5c,d).¹⁸ The vesicles were incubated with either 20 μM guest **2** alone (Figure 5e) or 20 μM cavitand **1** and 20 μM guest **2** (Figure 5f) for both 1 and 24 h (see SI for full images). CFM imaging shows that, in both cases, no incorporation of the fluorescent guest to the vesicle interior occurs. It is entirely possible that the cavitand undergoes flip-flop while in the vesicle bilayer, but this mechanism is ineffective for *transport* of bound guest and supports the evidence that the cell transport mechanism is a cavitand-mediated endocytosis event.

In conclusion, we have shown that a water-soluble synthetic receptor molecule is capable of selective, controlled endocytosis of a specifically tagged target molecule in different types of living human cells with little observed cytotoxicity. The presence of suitable choline-derived binding handles is essential for the molecular recognition and transport process, and no transport occurs without it. The cavitand-mediated delivery system described here provides a novel method of small-molecule transmembrane transport, controlled by highly selective (and yet highly flexible) size and shape-based molecular recognition. The ability to induce selective endocytosis of designed targets has the potential to provide a crucial advance in targeted drug delivery methodologies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

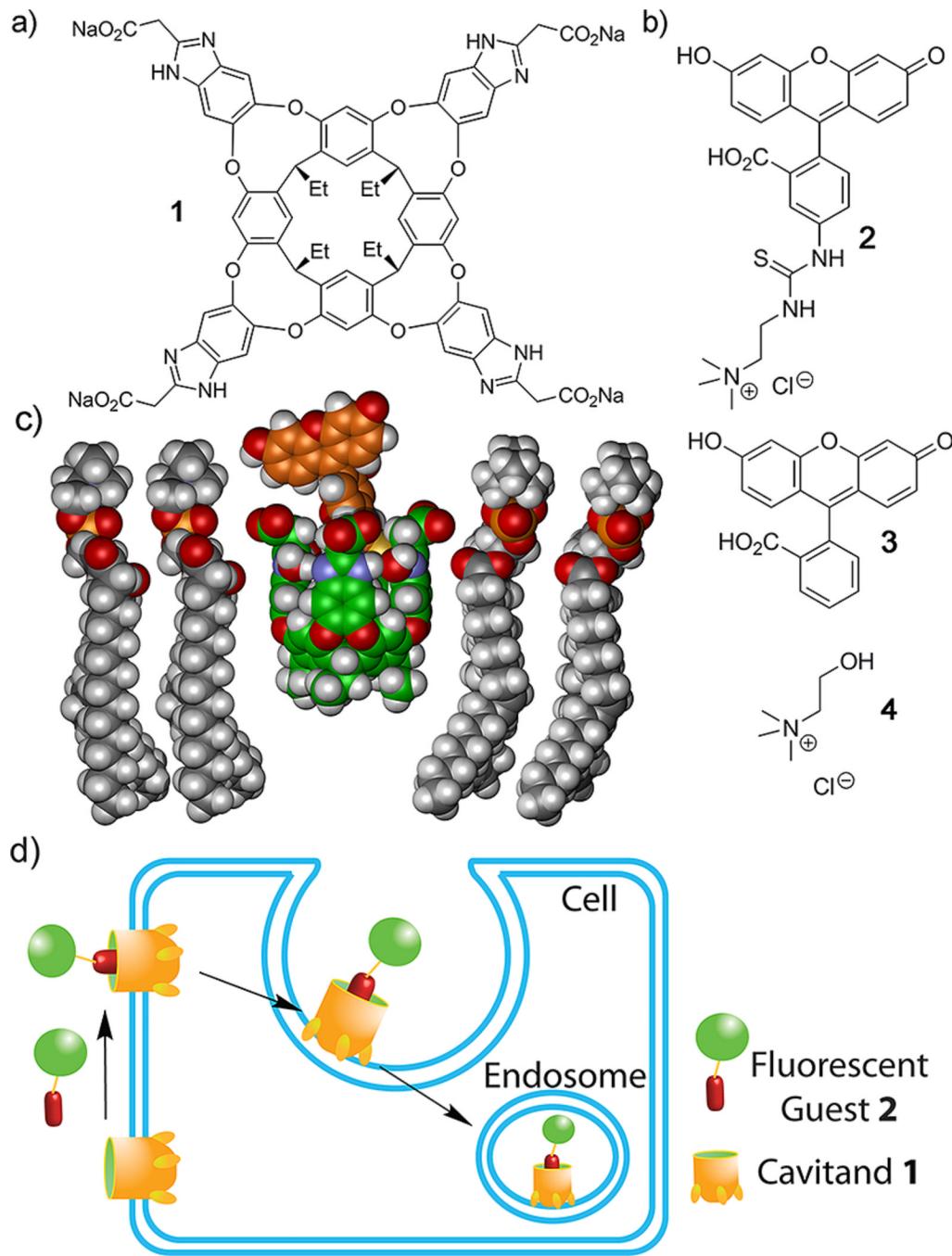
Acknowledgments

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**Figure 1.**

(a) Tetracarboxylate cavitand **1**. (b) Guests used in this study. (c) Representation of **1** binding tagged guest **2** in a membrane bilayer. (d) Cartoon representation of the transport process.

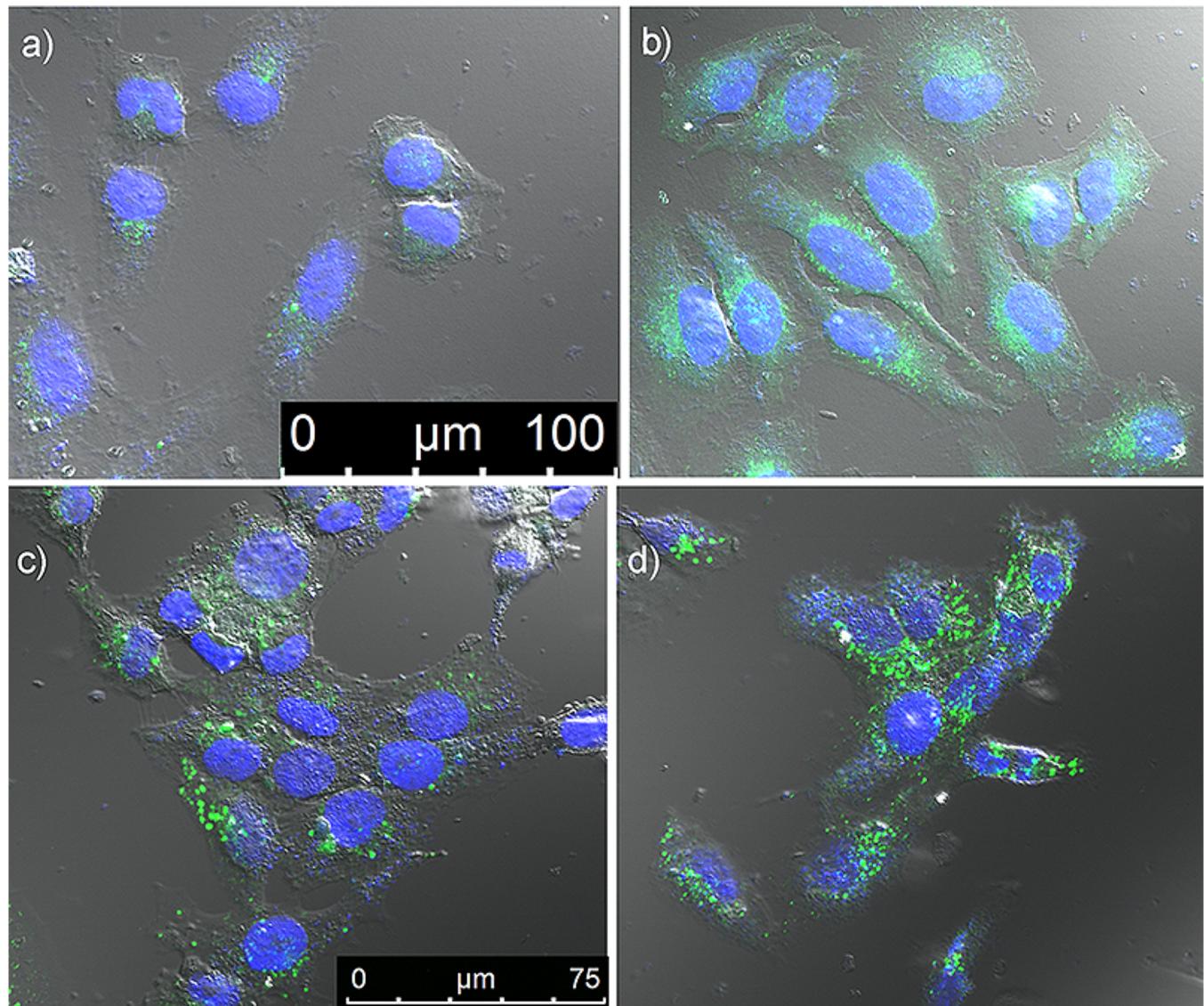


Figure 2.

Cavitand-mediated endocytosis. DIC/CFM images of the addition of guest **2** to live cells (nuclei stained with DAPI): (a) HeLa cells, 50 μ M guest **2** only, 1 h incubation; (b) HeLa cells, 50 μ M guest **2**, 50 μ M cavitand **1**, 1 h incubation; (c) GM00637 cells, 50 μ M guest **2** only, 1 h incubation; (d) GM00637 cells, 50 μ M guest **2**, 50 μ M cavitand **1**, 1 h incubation.

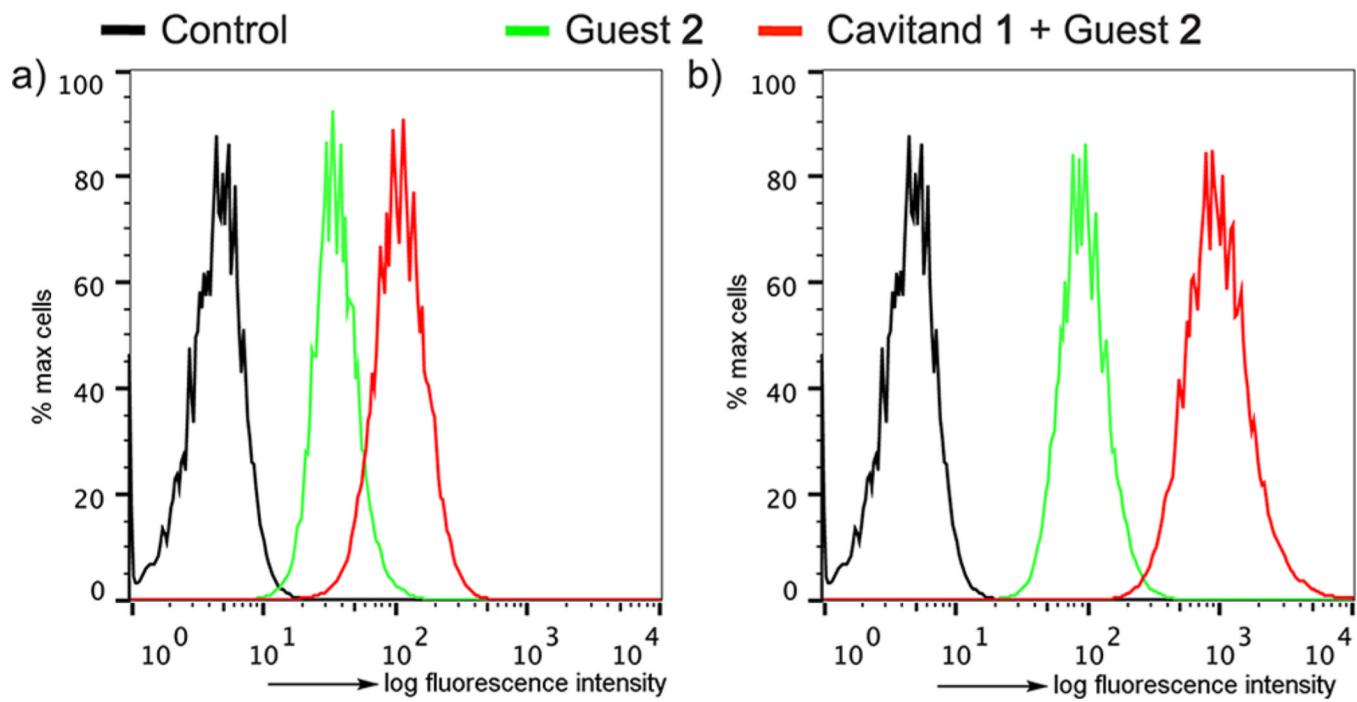
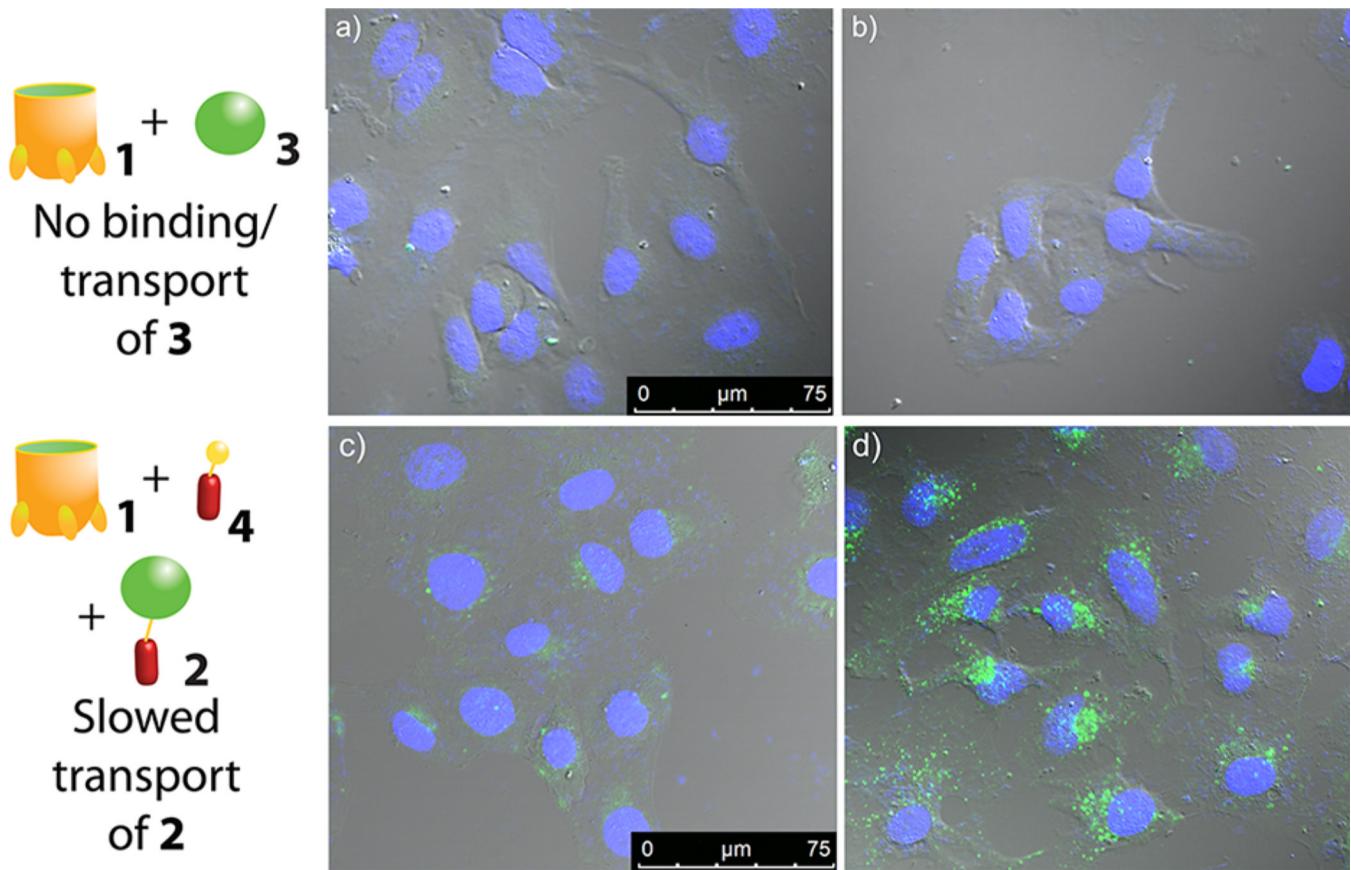


Figure 3.

Flow cytometry plots of guest **2** fluorescent intensity following incubation of guest **2** alone (green) and cavitand **1** incubation with guest **2** (red) for (a) 1 or (b) 24 h. Unstained HeLa cells plotted in black. These are representative plots of two independent experiments conducted using cells from triplicate wells. Cell population = 1×10^6 .

**Figure 4.**

Competition and selectivity. DIC/CFM images of the addition of guest **2** to HeLa cells (nuclei stained with DAPI): (a) 50 μ M fluorescein **3** only, 1 h incubation; (b) 50 μ M fluorescein **3**, 50 μ M cavitand **1**, 1 h incubation; (c) 50 μ M guest **2**, 50 μ M cavitand **1**, 100 μ M choline **4**, 1 h incubation; (d) 50 μ M guest **2**, 50 μ M cavitand **1**, 100 μ M choline **4**, 24 h incubation.

