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Supramolecular Polymers as Dynamic Multicomponent Cellular Uptake Carriers

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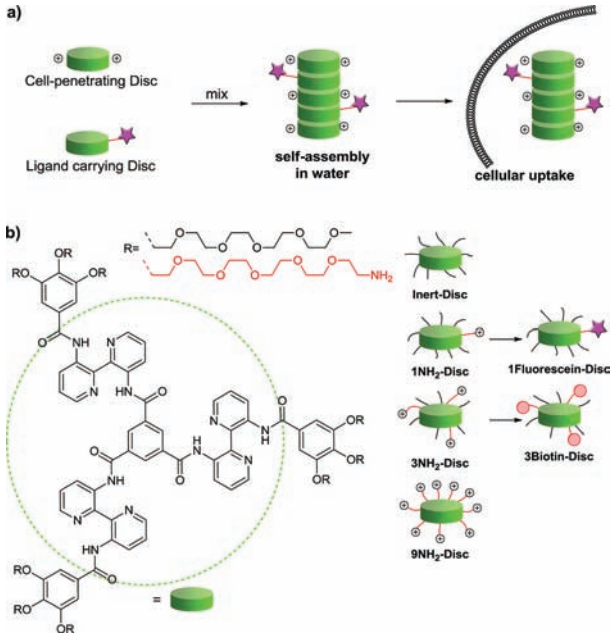
Supporting Information

ABSTRACT: Supramolecular synthesis represents a flexible approach to the generation of dynamic multicomponent materials with tunable properties. Here, cellular uptake systems based on dynamic supramolecular copolymers have been developed using a combination of differently functionalized discotic molecules. Discotics featuring peripheral amine functionalities that endow the supramolecular polymer with cellular uptake capabilities were readily synthesized. This enabled the uptake of otherwise cell-impermeable discotics via cotransport as a function of supramolecular coassembly. Dynamic multicomponent and multifunctional supramolecular polymers represent a novel and unique platform for modular cellular uptake systems.

The highly selective permeability of the plasma membrane, while essential for cell function and survival, represents as well a major challenge for the intracellular delivery of cargo, such as imaging agents, therapeutics, and reporter molecules.[1] For cargo delivery, two strategies have been widely used: one is based on ligand−receptor interactions,[2] and the other uses cell-penetrating peptides (CPPs).[3,4] A key feature of CPPs[5,6] is their positive charge due to their high arginine and lysine content, which favors binding to cell-membrane-bound proteoglycans and leads to cellular uptake via endocytosis.[7,8] This phenomenon is not restricted only to polycationic peptides; polyamine dendrimers,[9,10] foldamers,[11,12] and polymers[13,14] are similarly capable of crossing the cell membrane. Synthetic supramolecular polymers[15] have yet to be explored as intracellular carriers, although their unique self-assembly properties combined with the modular nature of their synthesis, as in liposome-based systems,[16] makes supramolecular polymers attractive systems for cellular uptake. Dynamic and adaptable multifunctional and multivalent self-assembled scaffolds can be conveniently prepared without the need for repeated syntheses,[17,18] as is the case for conventional polymer-based systems. We envisaged that the supramolecular synthesis of heterogeneous noncovalent supramolecular polymers comprising cell-penetrating monomers and non-cell-permeable monomers would enable cellular uptake of the intermixed system (Chart 1a). Here we demonstrate the use of supramolecular synthesis to access in a rapid manner dynamic multicomponent polymers of diverse composition with powerful cell-penetrating properties.

Chart 1. (a) Concept of Supramolecular Cellular Uptake Carriers; (b) Library of Amine- And Ligand-Functionalized Discotics



The supramolecular polymers used herein are C_3-symmetric amphiphilic discotics that self-assemble into columnar stacks at dilute micromolar concentrations in water.[19] The monomers consist of an aromatic core shielded by nine biocompatible and water-soluble poly(ethylene oxide) (PEO) chains, which can be readily functionalized at the peripheral regions to enable, for example, the attachment of bioactive ligands.[20,21] The self-assembly of these discotic molecules into columnar assemblies induces strong autofluorescence with a large Stokes shift[22,23] that is convenient for cellular imaging. For this work, we designed a library of structurally related discotic molecules differing only in the number of peripheral amine groups (**1NH$_2$-Disc**, **3NH$_2$-Disc**, and **9NH$_2$-Disc**; Chart 1b) with the purpose of studying their cellular uptake properties. Together with intrinsically non-cell-permeable discotic monomers featuring either a single fluorescein fluorophore, or three biotins, or only inert glycol side chains (**1Fluorescein-Disc**,

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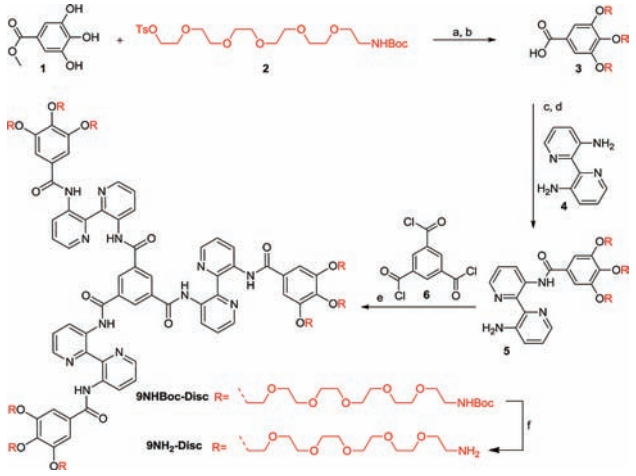
3Biotin-Disc, or **Inert-Disc**, respectively), a library of six supramolecular discotic monomers was synthesized.

For example, the facile convergent synthesis of **9NH$_2$-Disc** (Scheme 1) commenced with the reaction of methyl 3,4,5-trihydroxybenzoate (**1**) with tosylate **2** and subsequent saponification to yield wedge molecule **3** in an efficient 85% yield over two steps. Acid chloride formation[24] followed by monoacylation of **4** afforded bipyridine wedge **5**, whose subsequent reaction with trimesic chloride **6** followed by amine deprotection using trifluoroacetic acid (TFA) afforded the desired **9NH$_2$-Disc** [for the syntheses of **1NH$_2$-Disc** and **3NH$_2$-Disc**, see the Supporting Information (SI)].

Scheme 1. Synthesis of 9NH$_2$-Disc[a]



[a]Reagents and conditions: (a) K_2CO_3, DMF, 70 °C, 10 h, 86%; (b) KOH, 1:1 EtOH/H_2O, 80 °C, 3 h, 97%; (c) 1-chloro-*N*,*N*-2-trimethylpropenylamine, CH_2Cl_2, rt, 2 h, quant.; (d) 2,2′-bipyridine-3,3′-diamine, NEt_3, CH_2Cl_2, rt, 18 h, 61%; (e) **5**, NEt_3, CH_2Cl_2, 5 °C, 18 h, 32%; (f) TFA, CH_2Cl_2, rt, 2 h, 97%.

The cellular uptake of the supramolecular homopolymers by adherent HeLa cells was evaluated using live-cell multiphoton fluorescence microscopy. HeLa cells were incubated for 1 h with different concentrations of **Inert-Disc**, **1NH$_2$-Disc**, **3NH$_2$-Disc**, and **9NH$_2$-Disc** and imaged after washing. At high concentrations (5 μM), no uptake could be observed for **Inert-Disc** or **1NH$_2$-Disc**, which might be explained by the low number of amines of these discotics. In contrast, cells incubated with **3NH$_2$-Disc** or **9NH$_2$-Disc** featured cellular uptake, as determined by the detection of discotic fluorescence, at both high (5 μM) and low (0.5 μM) concentrations (Figure 1 and Figure S2 in the SI). The effectiveness of the cellular uptake of **3NH$_2$-Disc** and **9NH$_2$-Disc** did not significantly differ in the range tested (0.5−10 μM). The surprising excellent uptake efficiency of **3NH$_2$-Disc** might be explained by the increased density of peripheral positive charge upon self-assembly into columnar stacks. This explanation is supported by recent studies of polyarginine peptides, which revealed that in the case of linear peptides, a minimum of seven or eight arginines is necessary for successful cellular uptake,[25,26] whereas three arginines presented on self-assembling vesicles were as effective as the natural TAT sequence.[27] Supramolecular assembly thus allows for the generation of high-density positive charge sufficient for effective cellular uptake using discotic monomers featuring only a limited number of peripheral amine groups.

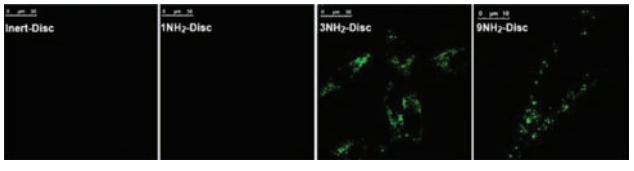


Figure 1. Confocal microscopy images of the cellular uptake of **Inert-Disc**, **1NH$_2$-Disc**, **3NH$_2$-Disc**, and **9NH$_2$-Disc** (all 5 μM) by HeLa cells.

The supramolecular homopolymers were nontoxic toward HeLa cells up to 10 μM (MTT assay; Figure S3).[28] The cellular uptake of the supramolecular polymers and their potential cytotoxicity were further investigated for two additional cell lines: HEK 293 and the difficult to transfect suspension cell line THP-1. In both cases, similar effective uptake and low toxicity was observed (Figures S4−S7). These results underline the general cellular applicability of the supramolecular polymers.

Time-lapse confocal microscopy was performed to determine the subcellular localization of the supramolecular polymers (Figure 2a). Within 10 min of incubation with **3NH$_2$-Disc** or **9NH$_2$-Disc**, binding to the outer leaflet of the cell membrane was observed, most probably resulting from electrostatic interactions with cell-surface proteoglycans.[8] Additionally, cellular internalization, as indicated by increased fluorescence within the cell, was observed after 30 min and increased over time, leading to more punctuated fluorescence in the cytoplasm that localized around the perinuclear region after 90 min. Co-staining with the membrane and endosomal marker 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) and the lysosomal marker LysoTracker Red strongly indicated that the uptake of the positively charged supramolecular polymers occurred via endocytosis (Figure 2b and Figure S8). In addition to the efficient uptake of amine-functionalized discotics, the introduction of guanidinium groups might also be expected to enhance the uptake and modulate localization, as has been seen for CPPs.[29,30]

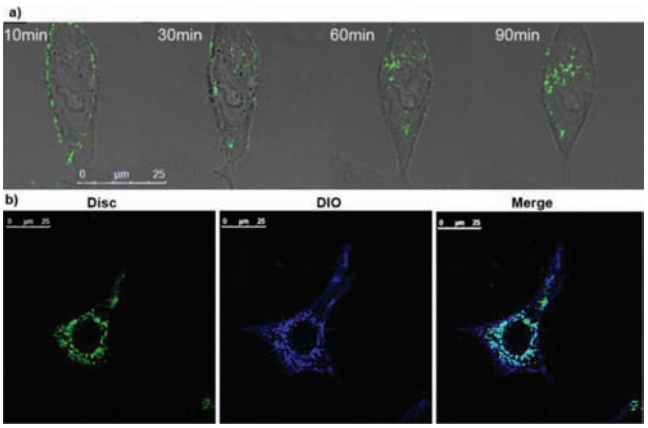


Figure 2. (a) Cellular uptake of **3NH$_2$-Disc** (5 μM) by live HeLa cells over time. (b) Confocal microscopy image of live HeLa cells incubated with **3NH$_2$-Disc** (5 μM) overnight and co-stained with DiO, a marker for cellular membranes that stains early endosomes as well as the cell membrane.

A potentially significant advantage of supramolecular polymers over conventional polymers and small molecules is their dynamic, multicomponent nature. Coassembly of different monomers or the dynamic intermixing of supramolecular

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polymers leads to dynamic supramolecular copolymers.[31,32] To investigate the potential of this concept, supramolecular copolymers were prepared via mixing of a cell-penetrating discotic monomer (**3NH$_2$-Disc** or **9NH$_2$-Disc**) with a non-cell-permeable monomer. If effective, the amine-functionalized discotics might act as carrier molecules, facilitating the cellular uptake of different non-cell-permeable discotics by virtue of their combined presence in the supramolecular copolymer. **1Fluorescein-Disc** alone was not taken up by HeLa cells, nor was there any unspecific binding to the outer leaflet of the cell membrane (Figure S9). HeLa cells were therefore incubated with different copolymers of **9NH$_2$-Disc** and non-cell-permeable **1Fluorescein-Disc**.[33] In contrast to **1Fluorescein-Disc** alone, cellular imaging after 30 min coincubation showed a clear fluorescence signal along the cell membrane for both the discotic fluorophore and the fluorescein (Figure S10). The cells were subsequently washed and imaged 24 h after incubation, at which point the copolymers had been internalized and colocalization of the discotic and fluorescein signals was observed inside the cell (Figure 3a). Similar results were

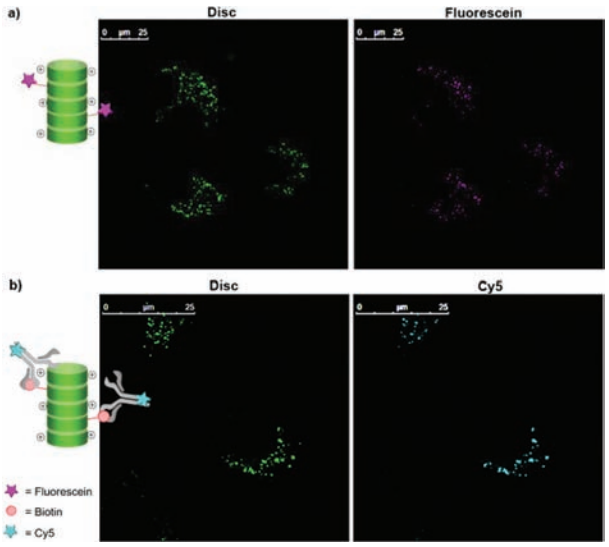

Figure 3. Discotic copolymers as cellular uptake carriers. (a) Confocal images of living HeLa cells incubated for 1 h with a 5 μM mixture of **9NH$_2$-Disc** and **1Fluorescein-Disc** (80:20) and imaged after 24 h. (b) Confocal images of HeLa cells incubated for 1 h with a 5 μM mixture of **9NH$_2$-Disc** and **3Biotin-Disc** (80:20) and then fixed and stained with a Cy5-labeled antibiotin antibody after 24 h.

observed for HEK 293 cells (Figure S11). These results demonstrate that it is necessary for the **1Fluorescein-Disc** to assemble with **9NH$_2$-Disc** as a supramolecular copolymer in order for cellular uptake to occur.[34]

The **3Biotin-Disc** was non-cell-permeable when alone (Figure S13) but successfully taken up when present as a supramolecular copolymer with **9NH$_2$-Disc**. To visualize the cellular uptake in this case, the cells were fixed and stained with a Cy5-labeled antibiotin antibody (Figure 3b). Several copolymers were prepared using different ratios of **9NH$_2$-Disc** in combination with either **1Fluorescein-Disc** or **3Biotin-Disc** (50:50, 80:20, 95:5). For each of these copolymers, internalization of the normally cell-impermeable discotic was observed. The 80:20 mixtures gave the best results in terms of uptake and visualization. Similar results were obtained for **3NH$_2$-Disc** (Figure S14).

Finally, the versatility of the approach was highlighted by evaluating a supramolecular copolymer consisting of three different discotics: **9NH$_2$-Disc**, **1Fluorescein-Disc**, and **3Biotin-Disc** (80:10:10). The copolymers were added to the cells and incubated for 1 h, after which the cells were washed and then fixed and immunostained after 24 h. Confocal microscopy revealed the presence and colocalization of all three fluorescent signals corresponding to the discotic scaffold, fluorescein, and biotin inside the cell (Figure 4). These results highlight the

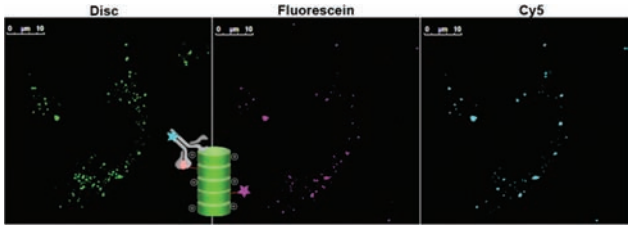

Figure 4. Confocal images of HeLa cells incubated for 1 h with a 5 μM mixture of **9NH$_2$-Disc**, **1Fluorescein-Disc**, and **3Biotin-Disc** (80:10:10). After 24 h, the cells were fixed and stained with a Cy5-labeled antibiotin antibody.

potential of the supramolecular synthesis approach as a highly flexible and effective means to internalize differently functionalized discotic monomers and to express their functionality in the combined system of a copolymer.

In conclusion, a library of cell-permeable and non-cell-permeable ligand-functionalized discotic molecules was synthesized to enable the supramolecular synthesis of a variety of dynamic multicomponent copolymers with tunable properties. Supramolecular homopolymers of amine-functionalized discotics demonstrated efficient cellular uptake that was detected by means of their autofluorescence using live-cell multiphoton fluorescence microscopy. Translocation through the cell membrane by otherwise non-cell-permeable ligand-functionalized discotics was induced by mixing them with amine-functionalized discotics, leading to the in situ supramolecular synthesis of cell-permeable supramolecular copolymers. The rapid generation of copolymers with different compositions by simple intermixing of a variety of readily synthesized discotic monomers led to multifunctional supramolecular polymers consisting of up to three different discotic monomers. After efficient cellular uptake, each of the components could be individually visualized, highlighting the potential of dynamic multicomponent supramolecular polymers. In this example, these polymers represent a unique and flexible platform for modular cellular uptake systems, but other applications can be similarly envisioned.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Materials, methods, detailed experimental procedures (synthesis and cellular assays), compound characterization, and supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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(33) To ensure intermixing of the discotics, the disc mixtures were pre-equilibrated overnight at room temperature.
(34) As evidence that cellular uptake was caused by the supramolecular copolymerization of the discotic monomers and not, for example, by permeabilization of the membrane through the addition of **9NH$_2$-Disc**, HeLa cells were incubated with a mixture of **9NH$_2$-Disc** and the carboxyfluorescein fluorophore alone. When the same incubation and imaging procedure as before was used, no fluorescein signal was detected inside the cell (Figure S12).