Molecular Boxes on a Molecular Printboard: Encapsulation of Anionic Dyes in Immobilized Dendrimers**

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***F**ifth-generation poly(propylene imine) dendrimers, modified with 64 apolar adamantyl groups, have been immobilized on cyclodextrin host monolayers ("molecular printboards") on glass by supramolecular microcontact printing. The immobilized dendrimers retain their guest-binding properties and function as "molecular boxes" that can be filled with fluorescent dye molecules from solution. Alternatively, part of the immobilized dendrimers were filled with dye molecules by cross-microcontact printing while the remaining, empty dendrimers were filled with a different dye from solution, resulting in alternating patterns of dye molecules. In addition, we demonstrate that encapsulation of dyes in immobilized dendrimers is reversible: immobilized molecular boxes can be filled with a dye, emptied, and subsequently refilled with a different dye.*

Keywords:
- cyclodextrins
- dendrimers
- host–guest chemistry
- microcontact printing
- self-assembled monolayers

1. Introduction

Dendrimers are highly branched, well-defined macromolecules consisting of a core, an interior region, and numerous end-groups. In recent years, this class of molecules has attracted increasing attention because of their molecular-recognition properties.[1–4] Moreover, dendrimers can be tailored into biocompatible compounds with low cytotoxicity, which makes them promising candidates as drug-delivery systems.[5] The molecular-recognition properties are based on the presence of voids in the interior of the dendrimers and/or on interactions with functionalities at the periphery. Higher-generation dendrimers, in particular, can efficiently encapsulate guests as the densely packed end-groups seclude the internal voids. Meijer has introduced the term "molecular box" for a dendrimer that encapsulates one or more guest molecules,[4] and there are numerous examples of guest encapsulation inside dendrimers. One well-known example is the complexation of anionic dyes by dendrimer hosts.[6–13] It is believed that this recognition process is primarily governed by favorable electrostatic interactions between protonated tertiary amines in the dendrimer core and the anionic dyes. However, a recent study has suggested that a good match in shape is also required.[11] Dendrimers modified with an apolar periphery can be employed as extractants of anionic dye molecules from water into organic solvents,[6] although there has been some discussion regarding the maximum number of dye molecules that can be encapsulated inside a dendrimer molecular box.[6,8,10,14,15] Neutral molecules can be bound inside dendrimer molecules by hydrophobic interactions,[16–18] or by hydrogen bonds.[19–22]

The controlled immobilization of dendrimers on a surface provides a handle to study and manipulate these molecules at a fixed position in space and could be of considerable importance for future applications, such as the development of biochips. Immobilization of dendrimers on a surface

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[**] The authors are grateful to Dr. Mária Péter for performing the AFM measurements and to Christian A. Nijhuis for the synthesis of dendrimer **1**. The Microchemical Systems (MiCS) program of the MESA+ Institute for Nanotechnology and the European FP6 Integrated Project NaPa (contract no. NMP4-CT-2003-500120) are acknowledged for financial support. The content of this work is the sole responsibility of the authors.

DOI: 10.1002/smll.200500078

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has been reported based on covalent chemistry,[11,23] physisorption,[24] and by hydrogen-bond formation.[25] Chen et al. have studied the complexation behavior of immobilized dendritic molecules by surface plasmon resonance.[11] Recently, our group has reported the immobilization of single dendrimer molecules on gold by insertion of dendrimer molecules or dendrimer precursors in a monolayer,[26,27] and by host–guest interactions on a "molecular printboard" on gold.[28] A molecular printboard is a monolayer of cyclodextrin (CD) host molecules that can bind suitable guest molecules (such as adamantyl derivatives) by hydrophobic inclusion.[28,29] The interaction of the guest molecules with the immobilized hosts can be tuned and controlled according to the principles of multivalency.[30,31] We introduced the concept of "supramolecular microcontact printing" for a form of microcontact printing (μCP) where guest molecules are printed in patterns on a molecular printboard.[32–34] Dendrimers with multiple guest end-groups can be used for nanofabrication with gold and silica nanoparticles.[35,36] In this paper, a molecular printboard on glass[29] is used to bind adamantyl-terminated dendrimer molecules by specific supramolecular interactions. The dendrimer boxes are immobilized in microscale patterns by supramolecular μCP. The dendrimers can subsequently be employed as hosts for the encapsulation of fluorescent molecules.

2. Results and Discussion

2.1. Patterning with Dendrimers

Fifth-generation poly(propylene imine) (G5 PPI) dendrimer **1**, functionalized with 64 adamantyl groups,[13] was selected for immobilization on β-cyclodextrin (β-CD) monolayers on glass.[29] The molecular structure of this dendrimer is shown in Figure 1A. Dendrimer **1** contains 62 tertiary amines that can be protonated to attract and encapsulate anionic guest molecules by electrostatic interactions. To solubilize **1** in water, an acidic solution (pH 2) was employed to protonate the tertiary amines. This results in improved water solubility and also ensures that the dendrimer adopts a fully extended conformation due to electrostatic repulsion. In addition, the hydrophobic adamantyl groups were complexed with β-CD.[13,37] By this method, stable aqueous dendrimer solutions could be prepared with concentrations of up to 0.15 mM. Immobilization of **1** on a β-CD monolayer was accomplished by supramolecular microcontact printing (μCP)[32–34] using an oxidized PDMS stamp and an aqueous solution of **1** as ink. The μCP procedure is depicted schematically in Figure 1B. The ink solution used for μCP contained 0.15 mM of **1** and 10 mM of β-CD in aqueous solution at pH 2. μCP was performed with PDMS stamps with a relief of 10-μm lines spaced 5 μm apart. Prior to inking, the stamps were oxidized to improve the wettability of the stamp with the aqueous ink solution.

Bringing the inked stamps into conformal contact with the molecular printboard for one minute resulted in the transfer of dendrimers from the stamp to the surface, as observed by atomic force microscopy (AFM; Figure 2). A regular pattern of 10-μm lines spaced 5 μm apart was observed both in the height and the friction images. We could therefore conclude that μCP of the dendrimers results in a faithful replication of the stamp features on the printboard. AFM indicated the transfer of more than one monolayer of dendrimer, but extensive rinsing with an acidic β-CD solution resulted in a decreased height consistent with approximately one monolayer of dendrimers. The height observed by AFM was about 1 nm, indicating that the dendrimers are in a flattened conformation on the surface, or are flattened by the load of the AFM tip. In contrast to μCP with divalent molecules, as reported previously,[32–34] rinsing with β-CD solution did not result in removal of the patterns, even after prolonged rinsing (Figure 2). This is a strong indication that **1** interacts by multiple supramolecular interactions with,

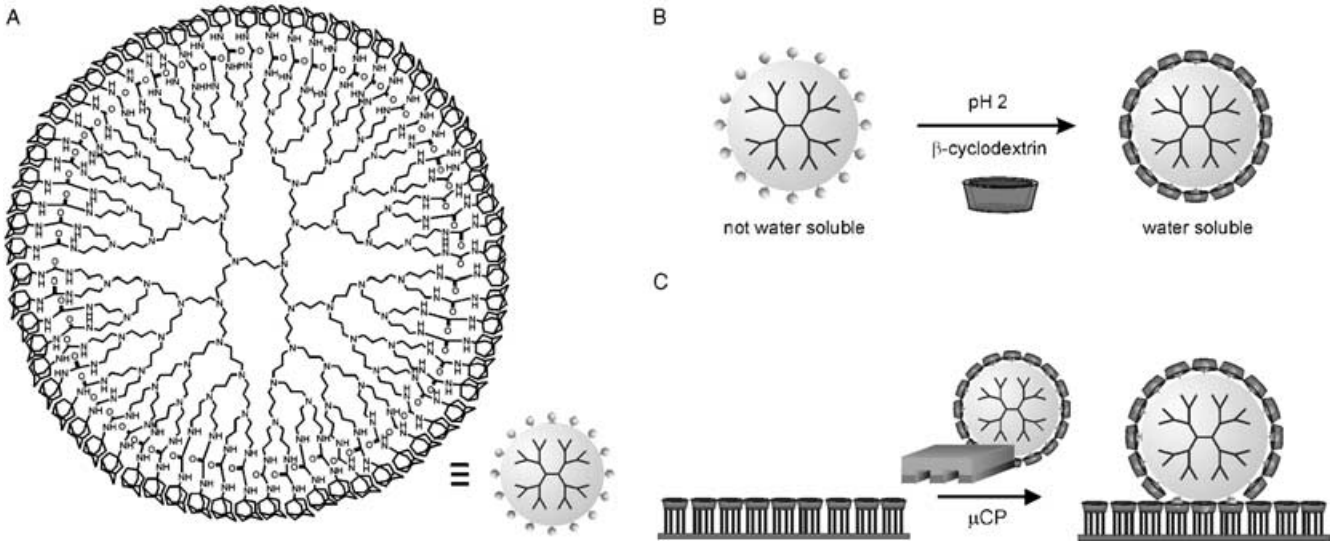

Figure 1. A) Molecular structure of a fifth-generation adamantyl-terminated PPI dendrimer **1**. B) Solubilization of **1** in water with β-CD at pH 2. C) Schematic representation of microcontact printing of **1** on a β-CD molecular printboard.

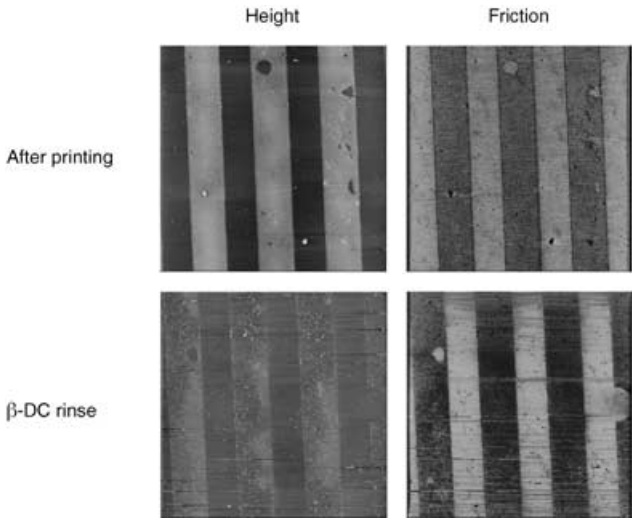

Figure 2. Contact-mode AFM images ($50 \times 50\ \mu m^2$) after microcontact printing of the β-CD complex of dendrimer **1** on the molecular printboard. The *z*-scale is 20 nm in the top-left image and 10 nm in the bottom-left image. Friction forces (a.u.) increase from dark to bright. Images were obtained directly after printing (top row) and after rinsing with 300 mL of 10 mM aqueous β-CD solution (bottom row).

and binds in a quasi-irreversible fashion to, the molecular printboard of β-CD. In fact, recent electrochemical measurements, using a G5 PPI dendrimer with 64 ferrocene endgroups, suggest that approximately seven out of 64 endgroups are involved in binding to the molecular printboard on gold.[37]

2.2. Encapsulation of Fluorescent Anionic Guests

To encapsulate anionic dye molecules in immobilized dendrimers, the dendrimers were printed on the molecular printboard. After μCP, the patterns were rinsed extensively with aqueous β-CD solutions at pH 2 and subsequently dipped in a 10^{-4} M aqueous dye solution for one minute. To remove physisorbed dye molecules the substrates were rinsed with an aqueous phosphate buffer solution (pH 7). This procedure is outlined in Figure 3.

Bengal Rose and fluorescein were chosen for encapsulation in the dendrimer boxes as both are known to interact with PPI dendrimers modified with apolar groups.[6] Bengal Rose and fluorescein are doubly negatively charged dyes, thus assuring an electrostatic interaction with the protonated interior of the dendrimers. Both dyes are water-soluble

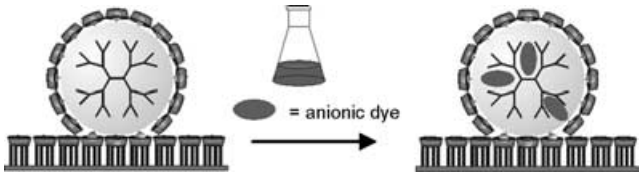

Figure 3. Schematic representation of the filling of immobilized dendrimer patterns with anionic dyes.

and fluorescent at neutral and high pH. After exposure to the dye solution and extensive rinsing, the substrates were imaged with a laser scanning confocal microscope (LSCM). Bengal Rose was excited at 543 nm and fluorescein at 488 nm. The LSCM images in Figure 4 show an accurate replication of the stamp features, indicating that dye complexation takes place selectively in the areas where the dendrimers were deposited by the stamp.

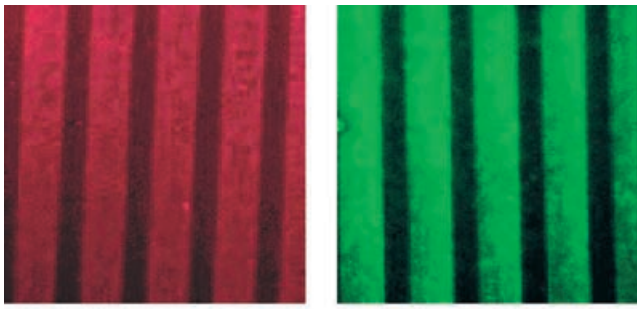

Figure 4. Confocal microscopy images ($65 \times 65\ \mu m^2$) after microcontact printing of **1** on a molecular printboard, followed by filling of the immobilized dendrimers with Bengal Rose and fluorescein.

To demonstrate the wider scope of this methodology a more sophisticated procedure was carried out. After printing the dendrimers in 10-μm lines on the molecular printboard, a second identical stamp was inked with an aqueous 10^{-4} M Bengal Rose solution. This stamp was brought into contact with the substrate *perpendicular* to the printed dendrimer pattern ("cross-printing"), causing the transfer of Bengal Rose molecules from the stamp to the surface both on the bare β-CD monolayer and on the dendrimer-modified monolayer. Next, the substrate was briefly rinsed with water. Following the double μCP procedure, the substrate was dipped into an aqueous 10^{-4} M fluorescein solution and finally rinsed with an aqueous phosphate buffer solution (pH 7). Imaging of the cross-printed substrate was performed by simultaneously exciting both dyes and monitoring the emission at different wavelengths (Figure 5). The left-hand image in this figure was obtained by exciting at 543 nm and monitoring the emission above 600 nm, both of which are suitable wavelengths for Bengal Rose. The image shows that Bengal Rose has been selectively deposited only in the regions where dendrimers are immobilized and where Bengal Rose was cross-printed. The center image was acquired by exciting at 488 nm and monitoring the emission between 500 and 530 nm, where fluorescein emits. It is evident that adsorption of fluorescein takes place mainly in the dendrimers at the areas where Bengal Rose is not printed, that is, in "empty" immobilized dendrimers. Simultaneous imaging (right) shows alternating parts of dendrimer patterns selectively filled with the two dyes.

This experiment demonstrates that, in addition to complexation from solution, immobilized dendrimers can also be filled by μCP of anionic dyes. The observation that dipping the substrate in a fluorescein solution results in prefer-

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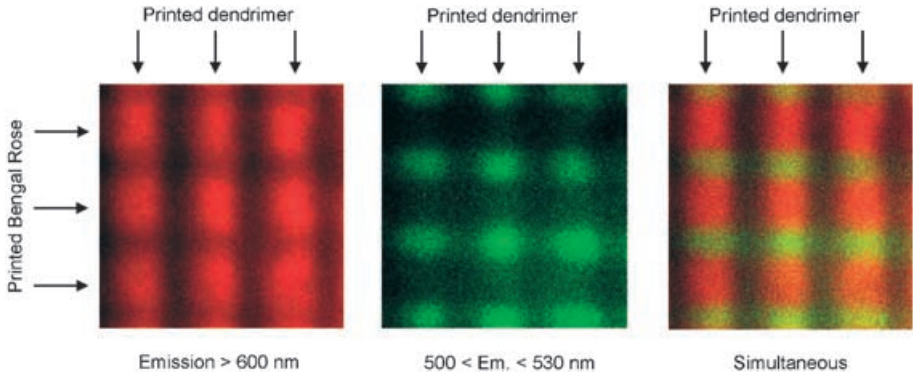

Figure 5. Confocal microscopy images (50 × 50 μm²) after microcontact printing of **1** on a molecular printboard, followed by cross-printing of Bengal Rose and subsequent filling with fluorescein. The substrate was simultaneously excited at 488 nm and 543 nm and images were recorded by measuring the emission above 600 nm (left), between 500 and 530 nm (center), and both simultaneously (right).

ential deposition in the "empty" dendrimers is an indication that μCP of Bengal Rose results in efficient filling of the dendritic molecules. Also, it suggests that the dyes are bound fairly strongly inside the dendrimers, as only limited exchange of the two dyes is observed under these experimental conditions.

One potential application of dendrimers is in controlled drug release.[5] For this purpose, the encapsulation process needs to be reversible and the dendrimers should be able to release their guests upon an external stimulus. One clever example has been reported by Paleos et al.,[17] who were able to release encapsulated pyrene molecules from the interior of dendrimers by lowering the pH of the solution. Here, the reversibility of the encapsulation process was tested with dendrimers immobilized on the molecular printboard. Printed dendrimers were filled with fluorescein by dipping in an aqueous fluorescein solution (Figure 6, left image). Next, the substrate was rinsed with an aqueous 100 mM phosphate buffer solution at pH 9 to deprotonate the dendritic molecules and/or exchange the fluorescein molecules for nonfluorescent phosphates. LSCM indicated a large decrease in fluorescence intensity (Figure 6, center), reflecting the reversibility of the complexation process. Subsequently, the substrate was rinsed with an aqueous HCl solution to protonate the tertiary amines in the interior of the dendrimer, and dipped in an aqueous Bengal Rose solution. The image on the right (Figure 6) shows the substrate after refilling with Bengal Rose. Clearly, Bengal Rose is adsorbed in the dendrimer patterns, thus demonstrating the reversibility of guest encapsulation on a surface. In addition, these experiments indicate that the immobilized dendrimer boxes allow many consecutive handling and rinsing steps without degradation of the μCP pattern.

3. Conclusions

Fifth-generation PPI dendrimers have been immobilized on β-CD molecular printboards on glass. The dendrimer molecules are anchored to the substrate by multiple supramolecular interactions and form an essentially irreversible bond in aqueous solutions. The host potential of the immobilized dendrimers was investigated by encapsulating anionic dyes like Bengal Rose and fluorescein, and confocal microscopy images showed that dendrimers can also function as molecular boxes when they are immobilized. Moreover, more sophisticated strategies are possible: dyes were delivered to dendrimers by μCP, and the remaining areas with empty dendrimers could be filled selectively with a different dye from solution. Additionally, we have shown that the encapsulation process on the β-CD surface is reversible by emptying fluorescein-filled dendrimers and subsequently refilling them with Bengal Rose.

This work shows that dendrimers remain versatile molecules when immobilized onto a surface. Since these macromolecules can carry many functionalities, their application as building blocks and templates in nanofabrication is promising.[35,36] In addition, the reversibility of the encapsulation process makes these types of immobilized molecular boxes attractive as potential drug-delivery systems.

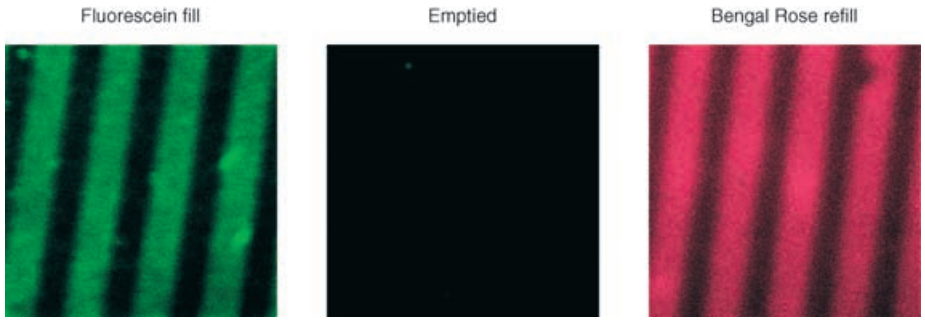

Figure 6. Confocal microscopy images (60 × 60 μm²) after microcontact printing of **1** on a molecular printboard, followed by filling of the immobilized dendrimers with fluorescein (left). The dendrimers were subsequently emptied by rinsing with an aqueous buffer solution at pH 9 (center), reprotonated by rinsing with an HCl solution, and refilled by dipping in a Bengal Rose solution (right). The left and center images were obtained at identical confocal microscope settings.

4. Experimental Section

Materials and methods: The G5 PPI dendrimer with 64 adamantyl groups was prepared following the procedure reported by Meijer et al.[39] According to MALDI-TOF, the dendrimer is monodisperse and contains only a very small fraction with less than 64 adamantyl groups. Aqueous dendrimer solutions were prepared by adding a β-CD solution (10 mM, pH 2) to the dendrimers, followed by extensive sonication.[13] Dyes were obtained from Aldrich and used as received.

Substrate preparation: β-CD monolayers on glass were prepared as described previously.[29]

Microcontact printing: μCP stamps (10-μm lines, spaced 5 μm apart) were prepared by casting a 10:1 (v/v) mixture of poly(dimethylsiloxane) and curing agent (Sylgard 184, Dow Corning) against a silicon master. After overnight curing at 60 °C the stamps were mildly oxidized in a UV/ozone reactor for 30 to 60 min to improve wetting by aqueous ink solutions, and subsequently inked by soaking them in an aqueous adsorbate solution for at least 5 min. The stamps were inked in a dendrimer solution (0.15 mM) containing β-CD (10 mM) followed by drying in a stream of nitrogen. Printing was performed for 60 s by bringing the stamp into conformal contact with the substrate by hand, without the use of external pressure.

Atomic force microscopy: AFM experiments were carried out with a Nanoscope III multimode AFM (Digital Instruments, Santa Barbara, CA, USA) in contact mode using V-shaped Si_3N_4 cantilevers (Nanoprobes, Digital Instruments) with a spring constant of 0.1 $N\,m^{-1}$. Images were acquired in ambient atmosphere (≈30–40 % relative humidity, 25 °C).

Laser scanning confocal microscopy: Confocal microscopy images of the microcontact-printed substrates were taken on a Carl Zeiss LSM 510 microscope. The beam was focused on the substrate using an oil immersion lens (N.A. 1.4, 63×). Bengal Rose was excited at 543 nm, while fluorescein was excited at 488 nm. The emitted fluorescence was collected on a PMT R6357 spectrophotometer. All confocal microscopy images were acquired in air.

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Received: March 9, 2005
Published online on July 1, 2005

