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Multivalent Binding of Small Guest Molecules and Proteins to Molecular Printboards inside Microchannels

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Abstract: β-Cyclodextrin (β-CD) monolayers have been immobilized in microchannels. The host–guest interactions on the β-CD monolayers inside the channels were comparable to the interactions on β-CD monolayers on planar surfaces, and a divalent fluorescent guest attached with a comparable binding strength. Proteins were attached to these monolayers inside microchannels in a selective manner by employing a strategy that uses streptavidin and orthogonal linker molecules. The design of the chip, which involved a large channel that splits into four smaller channels, allowed the channels to be addressed separately and led to the selective immobilization of antibodies. Experiments with labeled antibodies showed the selective immobilization of these antibodies in the separate channels.

Keywords: cyclodextrins · host–guest systems · lab-on-a-chip devices · monolayers · proteins

Introduction

There has been considerable interest in building bionanostructures at surfaces for sensing purposes. The main factors are to control orientation, functionality, and specificity of protein adsorption.[1–8] Examples of complex bionanostructures at surfaces are, for example, the development of a human chorionic gonadotropin sensor by Knoll et al.,[9] and the sensor types described in a review by Wilchek and Bayer.[10]

Antibodies (ABs) are often present in sensors because they can be used as medical diagnostic tools.[11,12] Control over orientation when immobilizing ABs to surfaces for sensor purposes is of the utmost importance because this constitutes, for a large part, the effectiveness of the ABs to detect antigens.[13–18] One way to achieve this is by using Fc (fragment crystallizable) receptors, such as protein A (PA), protein G (PG), or protein A/G (PA/G).[19–22] An AB binds with its Fc fragment to PA or PG, and therefore, the Fab (fragment antigen binding) fragments of the AB are directed towards the solution, and as such, are capable of binding antigens from solution.

The strive for miniaturization is important in biological assays because it allows faster diagnostics with small amounts of sample, and therefore, lower costs. There are currently numerous applications for these microchips.[23–25] Microchips can be used in medical diagnostic systems in which all of these criteria are important.[26,27] Protein functionality and the inhibition of nonspecific adsorption are key issues in this field. Immunoassays, which involve the immobilization of ABs in microchannels, are an important class of biological assays because small quantities of antigens can be detected.[28–33]

We have previously shown that it is possible, by means of host–guest chemistry, to control the binding properties of proteins to β-cyclodextrin (β-CD) monolayers[34] by using orthogonal linkers.[35] Such linkers can be designed with different numbers of guest groups, which results in protein complexes with a varying number of interactions, and thereby, a tunable binding strength to the β-CD monolayer. Protein–linker complexes with a low number of interactions (e.g., 2) to the surface can be removed from the surface in a competition experiment with β-CD in solution (β-CD$_l$), whereas a

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higher number of interactions (=4) yields very stable systems.[35] This method also allows control over the orientation of the proteins to the surface because the linker can be designed in such a way that it will bind to a specific region of the protein. Protein adsorption onto the host–guest binding platform can be conducted with complete suppression of nonspecific interactions, by employing a monovalent oligo(ethylene glycol) masking molecule during the adsorption.[36]

Herein, we show that microchannels can be functionalized in a stepwise manner with β-CD monolayers. We have shown that it is possible to exploit the host–guest chemistry, developed for planar substrates, in microchannels. Subsequently, previously introduced protein immobilization procedures were applied to the attachment of ABs in these microchannels. Furthermore, the addressability of individual channels for localized AB assembly was investigated, and the selectivity of the AB recognition was studied.

Results and Discussion

For the immobilization of β-CD monolayers and the subsequent specific attachment of proteins separately inside these channels, a microchip was fabricated with one large channel (width 390 μm, height 50 μm) that splits into four smaller channels (width 60 μm, height 50 μm), which are separated by 50 μm (Figure 1). The chip was made of glass on silicon. The compounds used in this study are depicted in Figure 2.

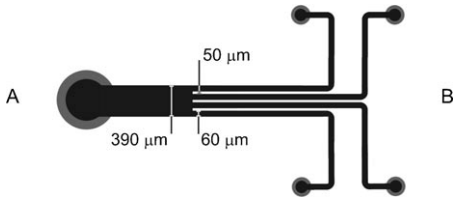


Figure 1. Design of the chip used in this study.

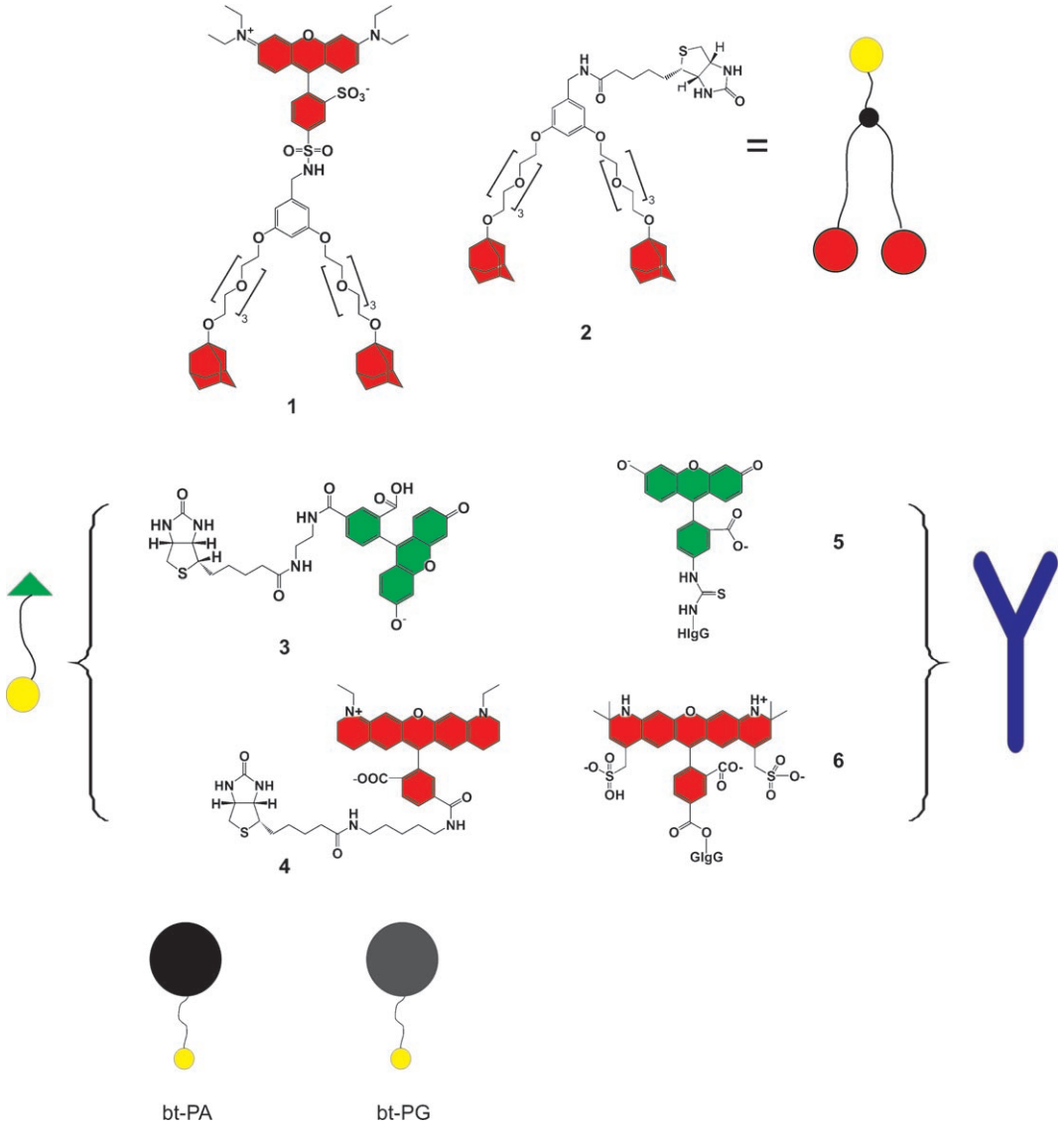


Figure 2. Compounds used in the chip study: lissamine–rhodamine dendritic wedge (**1**), divalent adamantyl biotin linker (**2**), biotin-4-fluorescein (**3**), Atto 565-biotin (**4**), human IgG-fluorescein (**5**), goat Alexafluor 568-IgG (**6**), bt-PA, and bt-PG.

The immobilization of β-CD monolayers in the microchannels was carried out in three subsequent reaction steps (Scheme 1) similar to the procedure previously described.[37] Cleaning was performed by flushing the channels with piranha solution then water and drying with N_2. Functionalization was achieved by subsequent treatment with *N*-[3-(trimethoxysilyl)propyl]ethylenediamine (TPEDA), 1,4-phenylene diisothiocyanate (DITC), and β-CD-heptamine. No blocked channels that result from polymerization were observed by microscopy at any stage.

To test whether or not β-CD immobilization in the channels was successful, static contact angles were recorded after every functionalization step (Figure 3). The results of these

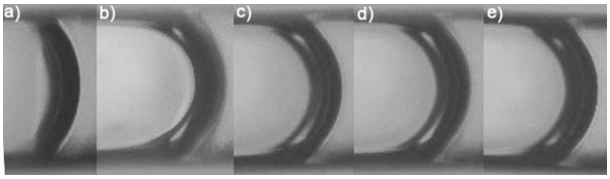


Figure 3. Contact angles measured in a) an unfunctionalized microchannel, b) after cleaning with piranha solution, c) after subsequent functionalization with a TPEDA, d) after subsequent functionalization with DITC, and e) after subsequent functionalization with β-CD-heptamine (see Scheme 1).

measurements are listed in Table 1. The contact angles measured followed the same trend as the advancing contact angles measured on planar substrates described by Onclin et al.[37] The absolute values are, however, smaller. This is in part owing to the static nature of the measurement, but possibly also owing to a larger surface roughness[38] as a result of the lithographic fabrication method.

Table 1. Static water contact angles of the monolayers in the microfluidic channels.

Monolayer	θ [°]
NH_2	36±2
NCS	42±2
β-CD	23±2

Sulforhodamine B acid chloride was used to prove the formation of the amino monolayer. From Figure 4a and b it

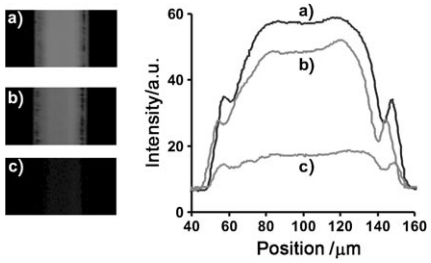
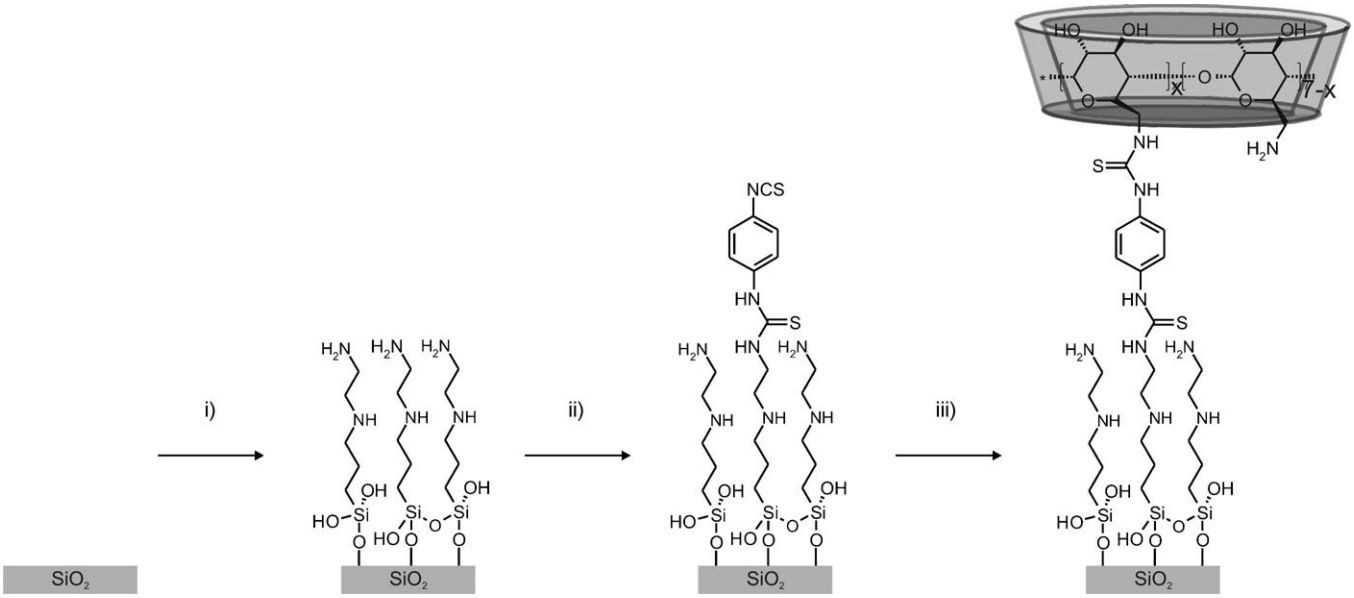


Figure 4. Fluorescence microscopy images (left) and intensity profiles (right) of the attachment of sulforhodamine B acid chloride onto NH_2-functionalized (a), piranha-cleaned (b), and CH_3-functionalized (c) microfluidic channels.

can be concluded that the sulforhodamine B acid chloride reacted with the NH_2 groups of the silane monolayer and the silanol groups present at the piranha-cleaned surface inside the microchannels. Therefore, monolayers of dodecyltriethoxysilane, which has similar adsorption properties to TPEDA with regards to the reactivity of the silane head group and the length of the backbone, were formed inside the microchannels. The CH_3 head group did not react with sulforhodamine B acid chloride (Figure 4c). This observation



Scheme 1. Synthetic scheme for the preparation of β-CD monolayers inside microchannels composed of SiO_2 and glass: i) piranha solution, followed by TPEDA in freshly distilled toluene, RT, 4 h, ii) DITC in ethanol, iii) RT, 2 h, β-CD-heptamine in Millipore water pH 8.5, RT, 2 h.

indirectly proves that a silane monolayer, and also a TPEDA monolayer, is formed inside the microchannels.

To test whether or not host–guest interactions of β-CD monolayers inside microchannels are comparable to those on planar substrates, adamantyl-terminated dendritic wedge **1** was immobilized in the channels by rinsing a solution of **1** (0.1 mM) in β-CD (1 mM) through the channels for 30 min. The fluorescence image that was recorded after subsequent rinsing for 10 min with water (Figure 5a) shows clearly that **1** is present in the channels. Subsequently, rinsing with β-CD (1 mM) was carried out for 10 min, followed by rinsing with water for 10 min. The fluorescence image after this treatment (Figure 5b) showed a slightly lower intensity. Subsequently, β-CD (10 mM) flowed through the chip for 20 min followed by a water rinse for 10 min. The fluorescence image that was recorded thereafter (Figure 5c) showed that the intensity had dropped significantly, but **1** was not completely removed. Rinsing for 10 min with ethanol did not result in significant changes (Figure 5d), and the fluorescence of the channels dropped to almost zero only after rinsing with methanol for 10 min followed by rinsing with water for 10 min (Figure 5e). These results are comparable to the results obtained from experiments in which divalent adamantyl guests were attached to β-CD monolayers on gold and glass.[39,40] The possibility of disrupting the host–guest assemblies by organic solvents, such as methanol, has also previously been shown.[41] Therefore, it can be concluded that the interaction between **1** and the channel wall is governed by supramolecular interactions, which also strongly indicates the presence of the β-CD monolayer inside the channel.

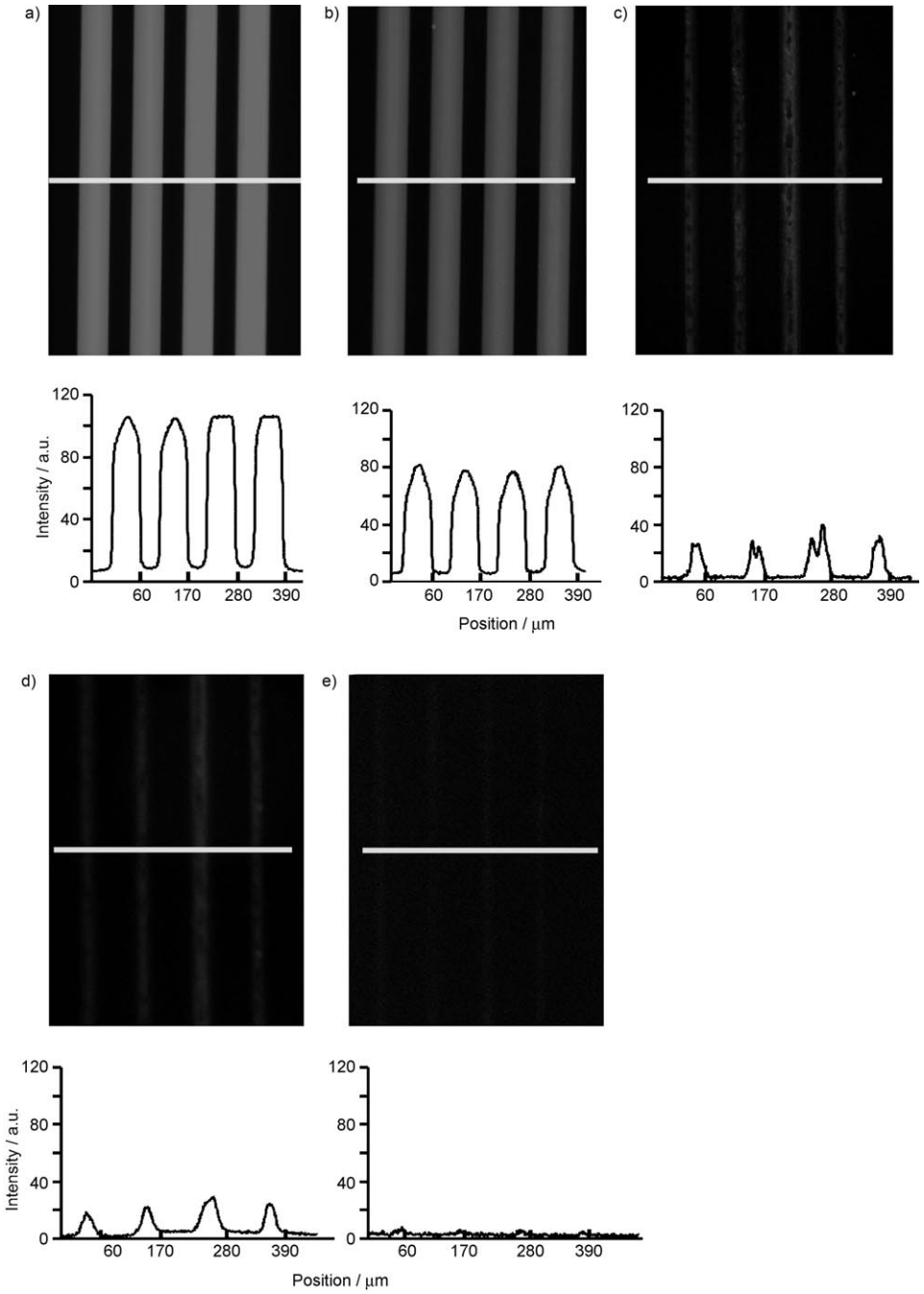


Figure 5. Fluorescence microscopy images after the attachment of **1** onto β-CD monolayers inside the channels (30 min), and the sequential flow of a) water (10 min), b) β-CD (1 mM; 10 min) and water (10 min), c) β-CD (10 mM; 20 min) and water (10 min), d) ethanol (10 min) and water (10 min), and e) methanol (10 min) and water (10 min) through the microchannels.

The binding constant of the host–guest interaction of **1** to the β-CD monolayer was studied in a fluorescence titration experiment (Figure 6a). Fitting of the data (Figure 6b) to a sequential binding model[42] (see the Experimental Section) yielded an intrinsic binding constant for the surface ($K_{i,s}$; which represents the binding of one of the adamantyl groups of **1** to the β-CD monolayer in an overall independent divalent binding mode) of $2.5 \times 10^4\,M^{-1}$, which is in good agreement with the literature.[39] From these results, it can be concluded that immobilization and the properties of the

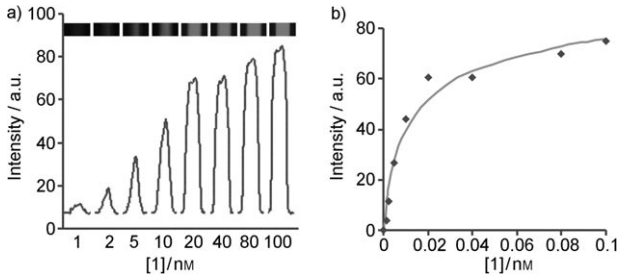


Figure 6. Fluorescence microscopy images and fluorescence intensity profiles of the adsorption of **1** onto a molecular printboard in a microchannel at different concentrations (a; from left to right: 0.001, 0.002, 0.005, 0.01, 0.02, 0.04, 0.08, and 0.1 μM). Fluorescence titration data points (◆) and the fit to the sequential binding model (——) of **1** on molecular printboards in a microfluidic device at different concentrations (b).

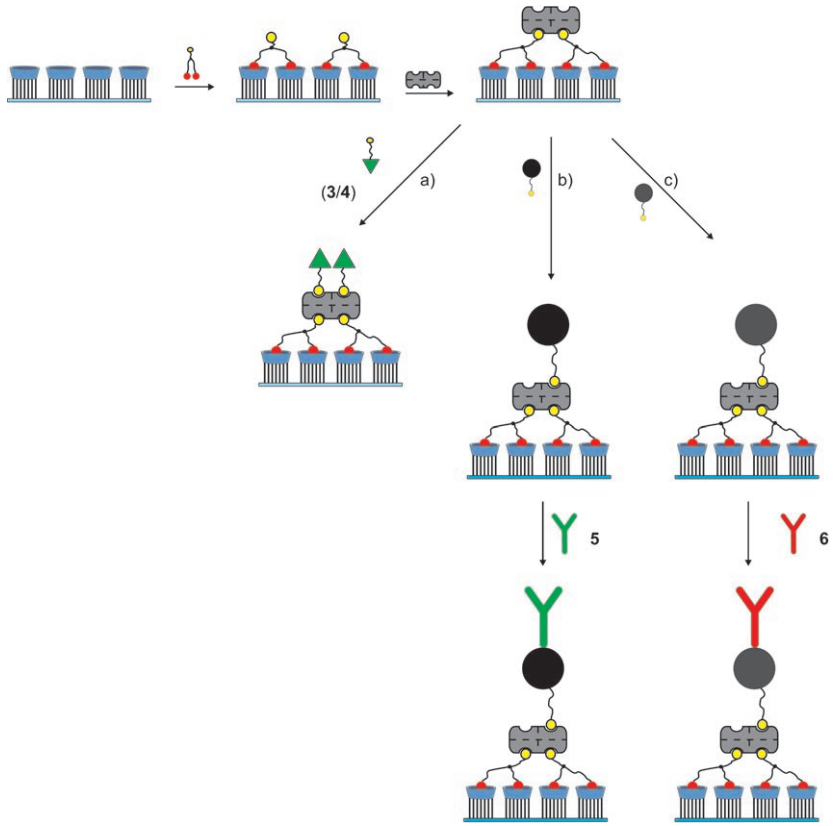
β-CD monolayers in the channels are comparable to β-CD monolayers on planar substrates.

Immobilization of streptavidin (SAv) at the β-CD monolayers was performed as described before (see Scheme 2).[35] Linker **2**, which consists of two adamantyl functionalities to ensure binding to β-CD monolayers and a biotin functionality to ensure binding to SAv, was washed over the surface followed by a wash with SAv. This assembly process leaves two biotin binding pockets available for further functionalization with, for example, fluorescently labeled biotin moieties (**3** and **4**; Scheme 2a), or biotinylated protein A (bt-PA) or protein G (bt-PG), which in turn can bind to fluorescently labeled human-IgG (**5**) and goat-IgG (**6**), respectively (Scheme 2b and c).

To show that the channels can be addressed individually, SAv was assembled in all channels through divalent linker **2**. The latter was adsorbed from inlet A (see Figure 1) and from the same side SAv subsequently flowed through the channel. Two different fluorescently labeled biotin derivatives (**3** and **4**) were introduced from the small inlets at side B through alternating channels to create assemblies according to the procedure shown in Scheme 2a. The flow rate in this experiment was set such that there was sufficient back-pressure to prevent mixing or back-diffusion of the different biotin derivatives in the small channels. After 30 min Millipore water was washed through the channels, also from side B.

Imaging with green excitation light showed that **3** was immobilized in two channels (Figure 7). Imaging the channels with blue excitation light showed that **4** was immobilized in the other two channels. The combined image shows the four channels, with alternating **3** and **4**, which proved the possibility of individual channel functionalization by using intrinsically reversible supramolecular interactions.

Similarly, according to Scheme 2b and c, **5**, which was labeled with a fluorescein isothiocyanate (FITC) label, and **6**, which was labeled with Alexa fluor-568, were assembled through bt-PA and bt-PG, respectively.



Scheme 2. The attachment of proteins to β-CD monolayers: the attachment of SAv through **2**, and the subsequent attachment to SAv of a a) fluorescently labeled biotin derivative (**3**, **4**), or of b) IgG **5**, or c) IgG **6** through biotinylated PA or PG.

To achieve this, bt-PA and bt-PG were simultaneously introduced into the channels from side B on the SAv–**2**-coated β-CD monolayer, in the alternating fashion shown above for **3** and **4**. Subsequently, compounds **5** and **6** were simultaneously passed through the channels in an alternating fashion from side B. The results of these experiments are depicted in Figure 8a. Similar to the results obtained from the previous experiment, there is a clear difference between the different channels, which indicates that these more complex bionanostructures can also be selectively immobilized in different channels.

For protein assays it is important to show that only specific ABs are detected. This prevents the appearance of, for example, false positives. To prove that this is possible in

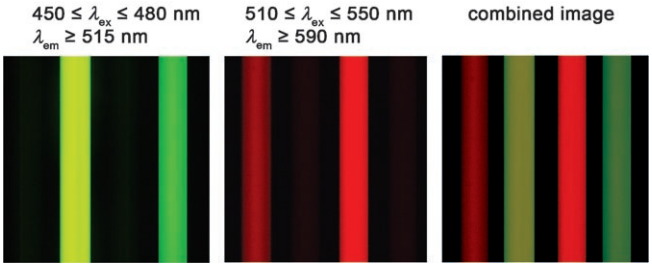


Figure 7. Fluorescence microscopy images recorded with blue (left) and green (center) excitation light of the chip functionalized with biotin derivatives (**3** and **4**) in alternating channels (Scheme 2a). The combined image is also shown (right).

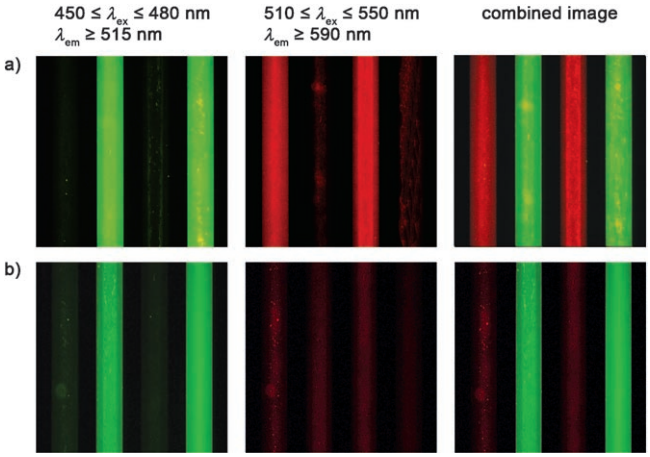


Figure 8. Fluorescence microscopy images recorded with blue (left) and green (center) excitation light of the chip functionalized with a) bt-PA–**5** and bt-PG–**6** in alternating channels (Scheme 2b and c), and b) with bt-PA+**5** and bt-PA+**6** in alternating channels. The overlaid images are also shown (right).

our system, divalent linker **2** and SAv were immobilized in the β-CD-covered channels from side A followed by the immobilization of bt-PA also from this side, which results in all channels being covered with bt-PA (Scheme 2b). Subsequently, compounds **5** and **6** were simultaneously introduced through alternating channels of the chip in the reverse direction (from side B) for 30 min. After rinsing with water for 20 min it became apparent that only two channels had been modified with a fluorescent IgG (Figure 8b): only immobilization of **5** was observed. Compound **6** was not immobilized, as expected, because **6** does not bind to PA. These experiments showed that the channels can be addressed separately and that the channels can be modified in such a manner that the immobilization of ABs is specific.

Conclusion

We have shown that β-CD monolayers can be assembled in microchannels, and that the host–guest recognition properties of these β-CD monolayers are comparable to those on planar substrates. The assembly of bionanostructures inside microchannels was proven by fluorescence microscopy, and the separate addressability of the four small channels was also shown. ABs were shown to attach in a specific manner. The tools presented herein can potentially be used for the development of more complex diagnostic systems to be used, for example, in medical or environmental applications.

Experimental Section

General: All chemicals were used as received. Compounds **1**, **2**, and β-CD-heptamine were synthesized as described previously.[35,39,43] Freshly distilled toluene was used for microchannel functionalization. SAv- and FITC-labeled IgG from human serum were obtained from Sigma Aldrich. Alexa-fluor 568 labeled goat anti-rabbit IgG (H+L) was obtained from Invitrogen, the Netherlands. bt-PA and bt-PG were obtained from Sigma Aldrich. PBS (10 mM, pH 7.5) that contained NaCl (150 mM) was used during experiments.

Microchips: The microchip used for this study is composed of one large channel (width 390 μm, height 50 μm) that splits into four smaller channels (width 60 μm, height 50 μm), which are separated by 50 μm.

Microchips were prepared as follows: After standard cleaning and treatment with HNO_3, a silicon wafer (<100>, p-type) was coated with photoresist (1.7 μm; Olin 907.17) and baked for 90 s at 90 °C. Subsequently, contact photolithography with an exposure time of 4 s was performed, followed by a 1 min post-exposure bake at 120 °C. The photoresist layer was developed by immersion of 30 s in "dirty" developer and 25 s in "clean" developer. Subsequently, the Bosch process[44] was applied at a rate of 20 μm min^{-1}. Photoresist stripping was carried out by rinsing with acetone, followed by treatment with HNO_3 for 20 min, and a few minutes of O_2 plasma exposure. To create the inlets, powder blasting foil (BF410) was applied on the other side of the wafer by laminating it at 130 °C. Photolithography through a mask was applied for 20 s. After this the photoresist was developed for 3 min. The inlets were made by powder blasting with Al_2O_3 grains (29 μm; viahole formation). The inlets had a size of 1 mm at the outside, and 360 μm at the bottom. The powder blasting foil was stripped with acetone and soda. After this procedure the wafer was cleaned in an ultrasonic bath in acetone (20 min) and standard cleaning. Pyrex glass was cleaned by standard cleaning and attached by anodic bonding at 400 °C for 3 min at 400 V, 3 min at 600 V, 3 min at 800 V, and finally 10 min at 1000 V. Dicing to separate the microchips on the wafer by a disco dicing saw was carried out after laminating the wafer with a transparent foil on the silicon side. After dicing, the foil was detached by 3 min UV irradiation for final cleaning.

Microchip holders were fabricated from black Delrin blocks and Teflon. The chip was placed in a black Delrin custom-made holder onto which syringes could be connected by nanoports to create pressure drives flow with a CMA/102 Microdialysis Pump on which 250 μL flat Hamilton syringes were mounted. Syringes were connected to fused silica capillaries (100 μm in diameter) by means of nanoports. The applied flow rate was 2 μL min^{-1} in the experiments for the assembly of the β-CD monolayer.

Functionalization of the microchip channels: In a preceding cleaning procedure, fresh piranha solution (approximately 250 μL) was flushed through the chip every 5 min for 45 min. After the last piranha flush, water was flushed through the channels followed by drying in a stream of N_2. Thereafter, freshly distilled toluene was flushed through the chip for 10 min. Subsequently, a solution of TPEDA (5 mM) in toluene was passed through the chip for 4 h at room temperature. After this step, distilled toluene was flushed through for 15 min, followed by drying of the channels in a stream of N_2. No blocked channels that result from polymerization were observed by microscopy. The channels were flushed with ethanol, followed by DITC (10 mM) in ethanol for 2 h at room temperature and rinsed with ethanol. After drying the chip, Millipore water was passed through the chip, followed by β-CD-heptamine (10 mM) in Millipore water at pH 8.5 for 2 h at room temperature. This was again followed by rinsing with water, and drying the channels in a stream of N_2.

Thermodynamic data modeling: The intrinsic binding constant $K_{i,s}$ of the divalent guest to the β-CD monolayer inside the microchannels was determined by using a stepwise adsorption model implemented in a spreadsheet.[42] In this model, a stepwise binding of the divalent guest to the monolayer is expected in which both binding events are considered to be independent. The increased stability of the divalent guest bound to the β-CD surface (β-CD_s) when compared with the binding between the divalent guest and β-CD in an aqueous solution (β-CD_l) is caused by the higher effective concentration of β-CD_s compared with β-CD_l.[42]

Protein binding to β-CD monolayers inside microchannels: All protein concentrations used in the experiments in which protein binding to the monolayer inside the channels was the target were 1×10^{-7} M. For the assembly of the different proteins and biotin derivatives a flow rate of 4 μL min^{-1} for 30 min was used. In between and after the different assembly steps, PBS buffer (see above) was passed through the chip at a rate of 2 μL min^{-1}.

Optical microscopy: For optical microscopy an Olympus BH-2 microscope equipped with a CCD camera was used.

Fluorescence microscopy: Fluorescence microscopy images were made by using an Olympus inverted research microscope IX71 equipped with a mercury burner U-RFL-T as a light source and an Olympus DP70 digital camera (12.5 million pixel cooled digital color camera) for image acquisition. Blue excitation ($450\le\lambda_{ex}\le480$ nm) and green emission ($\lambda_{em}\ge515$ nm) was filtered by using a U-MWB Olympus filter cube. Green excitation ($510\le\lambda_{ex}\le550$ nm) and red emission ($\lambda_{em}\ge590$ nm) was filtered by using a U-MWG Olympus filter cube.

Acknowledgements

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