Host–Guest Systems

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Molecular Printboards as a General Platform for Protein Immobilization: A Supramolecular Solution to Nonspecific Adsorption**

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The attachment of proteins to surfaces is a key step in many biotechnological processes and applications.[1–3] For many of these purposes, one needs control over the adsorption strength and reversibility, protein orientation, and retention of biological function. Such requirements can only be met when the binding of the protein to the surface is specific. Moreover, these requirements need to be addressed anew every time another protein is being immobilized. To translate these requirements from a hard-to-solve interface problem to a much more easily addressable organic synthetic task, we have recently introduced the use of β-cyclodextrin (βCD) molecular printboards as a general platform for the immobilization of proteins through small multivalent, orthogonal linker molecules.[4] In principle, this methodology allows: 1) control over the binding strength by varying the valency of the linker at the printboard, 2) control over the orientation of the protein by the bioengineering of a specific binding site for the linker at a predetermined location in the protein, 3) creation of a solution-like environment by increasing the linker length, and 4) reversibility by rinsing with solutions of mono- or multivalent competitors. One major issue that has not yet been solved in this methodology is the omnipresent problem of nonspecific protein adsorption.

Herein we introduce the use of hexa(ethylene glycol) mono(adamantyl ether) (**3**, Figure 1), which forms a dynamic, supramolecularly controlled oligo(ethylene glycol) (OEG) layer on βCD molecular printboards. This process is shown to prevent nonspecific protein adsorption. It also allows replacement by multivalent linker molecules (Figure 2), because multivalent interactions are typically orders of magnitude

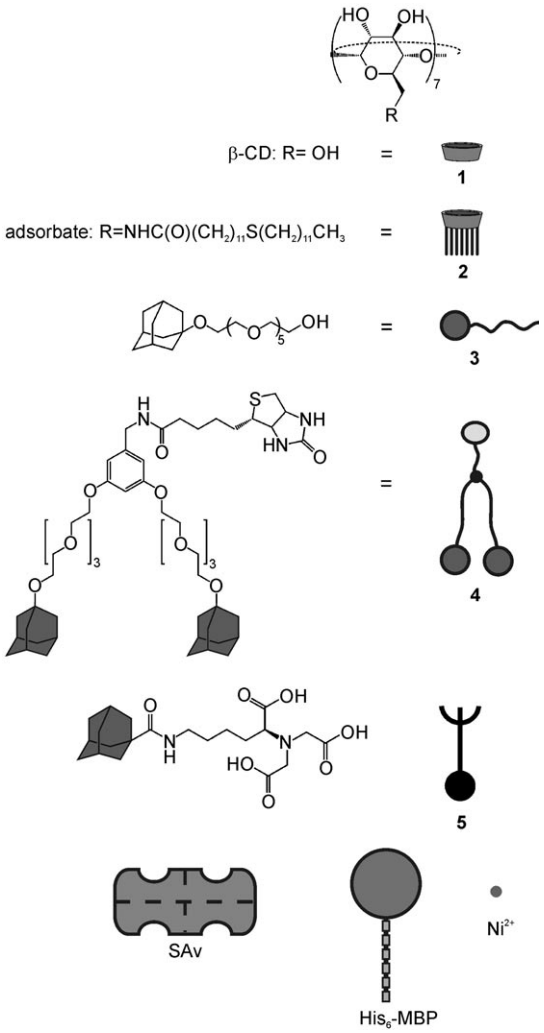

Figure 1. Host and guest compounds used in this study: βCD (**1**), βCD-heptathioether (**2**) for SAM preparation, hexa(ethylene glycol) mono(adamantyl ether) (**3**), divalent adamantyl-biotin linker (**4**), and mono(adamantyl) L-*N*-nitrilotri(acetic acid) (**5**).

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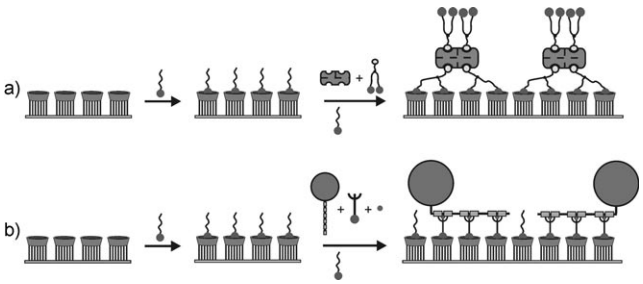

Figure 2. Adsorption schemes for the assembly of SAv(**4**)$_4$ (a) and MBP(Ni·**5**)$_3$ (b) with βCD SAMs of **2** in the presence of **3**.

stronger than monovalent ones.[5] Here we show that the methodology not only applies to the typical test protein streptavidin (SAv), but also to the histidine-tagged maltose binding protein (His$_6$-MBP), which functions here as a representative of the class of bioengineered His-tagged proteins.[6,7]

Different options exist to prevent the nonspecific adsorption of proteins onto surfaces, such as adding surfactants or bovine serum albumin (BSA) to protein solutions.[8,9] Another well known method is the use of self-assembled monolayers (SAMs) that are "protein-resistant", such as OEG SAMs.[10–12] The prevention of nonspecific interactions by using SAMs with OEG chains is attributed to loose packing and the well-hydrated nature of these SAMs.[11] SAMs consisting of hexa(ethylene glycol) appear to be the most protein-resistant, and these monolayers have been widely applied.[10,13,14]

βCD (**1**) is a well known host for various small hydrophobic organic molecules in aqueous environments.[15] We have modified βCD with seven heptathioether chains (**2**) to obtain ordered and densely packed SAMs on gold.[16,17] The binding constants of monovalent guest molecules binding to a single immobilized βCD cavity are comparable to those of the respective guest molecules binding to βCD in solution.[17] All the guest-binding sites in the βCD SAM are equivalent and independent. The use of multivalent[18,19] host–guest interactions allows the formation of kinetically stable assemblies, and thus local complex formation by patterning, for example, so that these surfaces can be viewed as "molecular printboards".[20,21]

The addition of 1 mM βCD to the phosphate-buffered saline (PBS) led to the inhibition of nonspecific interactions during the attachment of SAv to βCD SAMs.[4] For other proteins, however, this appeared to be insufficient. Non-ionic detergents such as tween 20 were not useful either, since they also interact with the βCD cavities, and do not prevent nonspecific adsorption sufficiently. Passivating the surface with BSA was possible, but this does not allow surface regeneration nor does it enable experiments to be performed in which the binding constants of proteins to the surface, attached through specifically interacting sites and/or linkers, are determined.

To solve the issue of nonspecific binding, we envisaged the use of the monovalent supramolecular blocking agent (**3**), which was designed to have a single adamantyl (Ad) group for a predictable, specific, and reversible interaction with the βCD SAMs, and a hexa(ethylene glycol) chain for preventing nonspecific protein adsorption.

The compounds used in this study are depicted in Figure 1. βCD SAMs of **2**[16,17] as well as the attachment of SAv through the divalent linker **4** to these βCD SAMs have been described in previous studies.[4] *N*-Nitrilotriacetic acid–adamantyl (NTA-Ad) linker **5** was developed for the attachment of His-tagged proteins to the βCD SAMs through their Ni^{2+} complexes.

Isothermal titration calorimetry (ITC) experiments of the complexation of **3** and **5** in solution (see the Supporting Information) showed: 1) no aggregation of **3** up to 5 mM, 2) binding constants for **3** and **5** with βCD typical for βCD-adamantyl interactions,[15] and 3) no interaction of **3** with BSA. Surface plasmon resonance (SPR) titrations were performed by adding solutions with different concentrations of **3** and **5** to βCD SAMs, with rinsing steps of 10 mM βCD in PBS applied between the additions. The SPR data (Figure 3)

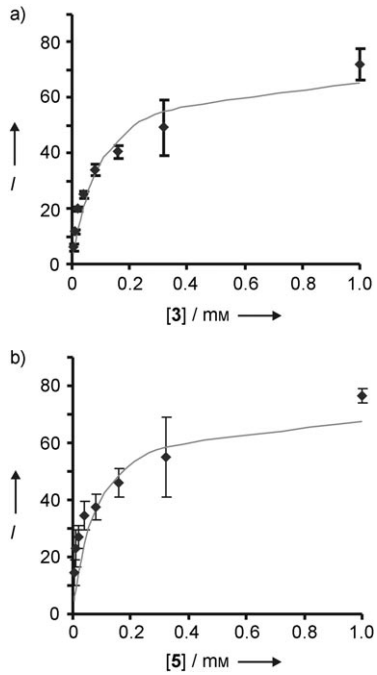

Figure 3. SPR titration (markers) and corresponding fits (solid lines) to a 1:1 Langmuir-type binding model for the binding of **3** to a βCD SAM in PBS (a), and of **5** to a βCD SAM in PBS (b). Error bars indicate the 50% confidence interval.

were fitted to a 1:1 Langmuir-type model, which gave $K_a = (2.6 \pm 0.9) \times 10^4\,M^{-1}$ for **3** and $K_a = (1.2 \pm 0.2) \times 10^4\,M^{-1}$ for **5**, which are comparable to the values found for binding to βCD in solution.

Figure 4 shows the SPR sensograms for the binding of SAv, His$_6$-MBP, and BSA in the absence and presence of **3**. Whereas SAv, MBP, and BSA showed significant nonspecific adsorption in the absence of **3** (black curves), even low concentrations (0.1 mM) of **3** seem to be sufficient for the suppression of nonspecific interactions.[22] More than 80% of all the βCD sites are already occupied by **3** at 0.1 mM, albeit in

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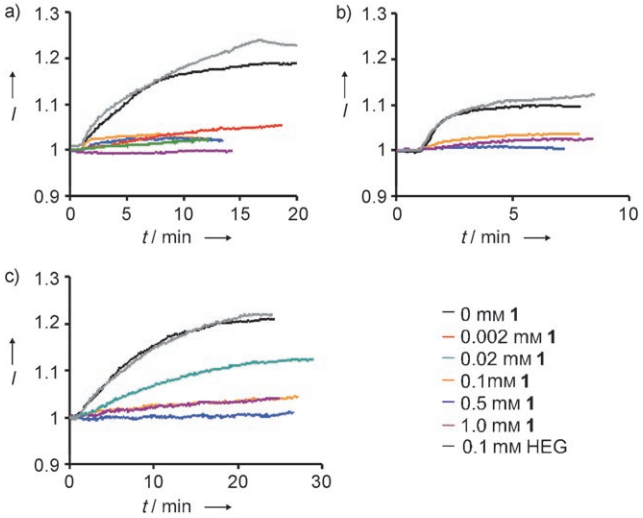

Figure 4. SPR sensograms of the adsorption of 0.1 μM SAv (a), 0.1 μM His_6-MBP (b), and 0.1 μM BSA (c) at a βCD SAM in PBS in the absence and presence of **3** or 0.1 mM hexa(ethylene glycol).

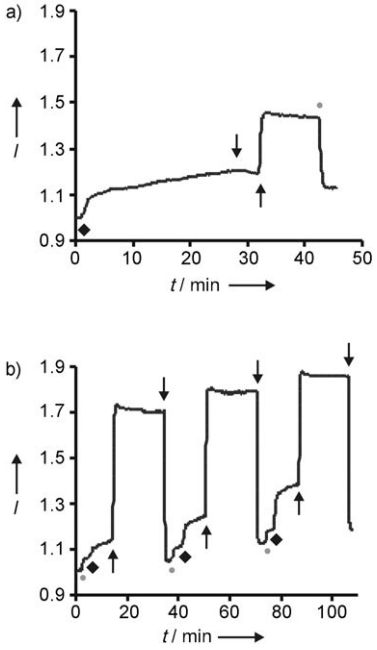

Figure 5. SPR sensograms of the adsorption of 0.1 μM SAv and **4** (ratio 1:6; a) and of different concentrations of His_6-MBP, Ni^{2+}·**5** (ratio 1:5:5; b) on βCD SAMs in the presence of 0.1 mM **3** in PBS. In the latter case, the His_6-MBP concentrations were 2.0 μM, 5.0 μM, and 10 μM, respectively. Symbols indicate switching of solutions in the SPR flow cell to SAv + **4** (1:6) + 0.1 mM **3** in PBS, or MBP + Ni^{2+} + **5** (1:5:5) + 0.1 mM **3** in PBS (black diamonds), 0.1 mM **3** in PBS (gray circles), 10 mM βCD + 0.1 mM **3** (+ 10 mM EDTA in the case of MBP) in PBS (↑) and PBS (↓).

a dynamic fashion (Figure 3). Experiments in which 0.1 mM hexa(ethylene glycol) (HEG) was used instead of **3** showed that the amount of (nonspecific) protein adsorption to the βCD SAM is comparable to that in the absence of **3**. This finding indicates that the main interaction through which nonspecific adsorption is inhibited is through the binding of **3** to the surface, thereby temporarily blocking the βCD cavities and exposing the hexa(ethylene glycol) tails to the solution. Further protein-binding experiments were performed with **3** at 0.1 mM.

To investigate whether the application of **3** still allows the specific attachment of proteins through orthogonal linkers, the binding of SAv to βCD SAMs through the orthogonal multivalent biotin-functionalized linker **4** was studied by SPR as well as the specific adsorption of His_6-MBP through the Ni^{2+}-complexed NTA-Ad linker **5** (Figure 2). SAv is a homotetrameric protein with four identical biotin-binding sites, and thus can bind four equivalents of **4**. The geometry of SAv and the length of the used divalent adamantyl-linker means that only two of the linkers, and thus four adamantyl moieties, bind four neighboring βCD cavities of the βCD SAM.[4] The multivalency effect is thus expected to make the binding of the SAv(**4**)$_4$ complex to the βCD SAM much stronger than the binding of **3**, even when **3** is used in excess. Figure 5a shows the adsorption of 0.1 μM SAv(**4**)$_4$ in the presence of 0.1 mM **3**. Most of the SAv(**4**)$_4$ complex remained after the attempted desorption with 10 mM βCD, thus proving the strong interaction of the complex with the molecular printboard. The start of the SPR curve indicates a βCD SAM already covered with **3**, which means that the absolute change in the intensity of the SPR signal is caused by the exchange of **3** for the SAv(**4**)$_4$ complex. Thus, the intensity change is lower than when SAv(**4**)$_4$ is attached to an empty βCD SAM in the presence of 1 mM βCD.[4]

In the case of His_6-MBP, the protein was premixed with a solution of Ni^{2+} ions and **5** (ratio 1:5:5) as well as 0.1 mM **3**, and this solution was flowed over the βCD SAMs already covered with **3**. Figure 5b shows the adsorption of His_6-MBP. Rinsing with 10 mM βCD and 10 mM ethylenediaminetetraacetic acid (EDTA) led to complete recovery of the baseline signal. The slight increase in the baseline is attributed to drift. This procedure was repeated at different concentrations of His_6-MBP and **5** (Figure 5b). The results show the specific binding of His_6-MBP in the presence of the monovalent blocking agent **3**. Moreover, the results show that the surface coverage of His_6-MBP can be varied.

In summary, we have developed a new supramolecular blocking agent that inhibits nonspecific protein adsorption on βCD molecular printboards. We have shown that this compound has similar binding constants to βCD in solution and at the surface, and that a 0.1 mM concentration is already sufficient to inhibit nonspecific protein adsorption, which is at a significantly lower coverage than obtained for standard protein-repelling surfaces. Moreover, it is still possible to attach proteins to the surface using multivalent orthogonal linkers, which ensure specific binding by exchange of **3**. This was shown for two proteins which are bound through differently functionalized linkers to βCD SAMs, thereby showing the versatility of this method. In conclusion, the implementation of this supramolecular nonspecific protein inhibition scheme demonstrates the strong potential for the use of molecular printboards as a general platform for the immobilization of proteins. Future directions will be to develop models for describing the multivalent thermodynam-

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ics of such orthogonal systems, and to increase the complexity of the protein architectures to antibodies and cells.

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[22] In this case, the OEG packing density is 1 – 2 orders of magnitude less than as described before (Ref. [11]); see the Supporting Information.

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