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Orthogonal supramolecular protein assembly on patterned bifunctional surfaces†

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We report successful and selective dual protein assembly on patterned bifunctional β CD-Ni(II)NTA surfaces, using red fluorescent protein variants with hexahistidine-tags and teal fluorescent protein variants conjugated with a peptide containing three adamantyl groups. We show that dual protein patterns can only be assembled, when opposing supramolecular interactions have been optimized and nonspecific interactions have been sufficiently suppressed.

Supramolecular assembly affords precise, highly tuneable interactions that can be controlled independently and simultaneously, making orthogonal self-assembly feasible.^{1,2} Synthetic supramolecularly controlled protein dimerization, protein functioning and protein assembly, all occurring orthogonally to natural recognition events, have recently demonstrated great potential for the modulation of biomolecular assemblies in solution.^{3–10} Control over the selective, orthogonal assembly of proteins on surfaces has also attracted a great deal of interest, due to its applicability in biosensors, bioengineering and fundamental biological studies. Notable examples include the electroresponsive assembly of ferrocene-modified yellow fluorescent proteins (YFPs) on β -cyclodextrin (β CD) monolayers¹¹ and the oligonucleotide-directed anchoring of proteins for the study of protein–protein interactions.^{12,13} The push to achieve more complex assembly of multiple proteins on surfaces, has prompted a fast-paced advancement in surface chemistry, as it is paramount for fundamental research on, and development of sophisticated

biodevices. A variety of methods *via* covalent bond formation and supramolecular interactions has been employed to control the assembly of multiple types of proteins on surfaces, mostly integrated with advanced lithographic techniques.^{14–20} While it is desirable to anchor proteins with control over orientation, affinity, reversibility and coverage, the above-mentioned protein-functionalized surfaces, documented in the literature, evidence that orthogonal supramolecular assembly of proteins on surfaces is not yet readily accessible and requires further urgent attention.

Here, we report the successful orthogonal, supramolecular assembly of two different proteins on patterned bifunctional surfaces. Contrary to the expectation of a straightforward simultaneous assembly, our dual protein patterns could only be assembled under optimized incubation and washing conditions and in a specific order of sequential assembly.

As supramolecular interaction pairs hexahistidine (H_6)/nickel(II) nitrilotriacetic acid (Ni(II)NTA) and β CD/adamantane (ad) were employed (Chart 1). Our recent studies on the oriented assembly of H_6 -tagged monomeric variants of an *Entacmaea quadricolor* red fluorescent protein, TagRFP, on Ni(II)NTA-functionalized surfaces, yielded association binding constants varying across two orders of

Chart 1 Structures of triadamantyl-functionalized ad_3 TFP and hexahistidine-functionalized $1H_6$ TagRFP and $3H_6$ TagRFP.

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magnitude for variants containing one ($K = 3 \times 10^6 \text{ M}^{-1}$), two ($4 \times 10^7 \text{ M}^{-1}$) or three ($3 \times 10^8 \text{ M}^{-1}$) H_6 -tags.²¹ Depending on the location of the H_6 -tags on the protein, surface coverage and protein orientation could be controlled.²¹ However, key to the present work is the insight that the reversibilities of the different variants' binding show considerable differences, depending on their respective number of H_6 -tags. The larger the number of H_6 -tags the more stringent the conditions necessary to reverse the binding. Binding of TagRFP with a single H_6 -tag to $\text{Ni}(\text{II})\text{NTA}$ surfaces was reversed by washing with mere buffer, binding of TagRFP *via* two H_6 -tags required washing with buffers containing competitors such as imidazole and EDTA, to be reversibly or irreversibly broken, resp., while triply H_6 -tagged TagRFP resisted complete removal from $\text{Ni}(\text{II})\text{NTA}$ surfaces, even after extensive washing in the presence of competitors.²¹ Therefore, we selected for the current study two of the above-mentioned TagRFP variants, specifically, with one ($1\text{H}_6\text{TagRFP}$) and three ($3\text{H}_6\text{TagRFP}$) H_6 -tags (Chart 1, for more details see Chart S1, ESI†). These TagRFP variants were engineered to contain one H_6 -tag at the N-terminus, in the case of $1\text{H}_6\text{TagRFP}$.²¹ In the case of $3\text{H}_6\text{TagRFP}$, both termini (located at the same end of the β -barrel), bore each one H_6 -tag, while a third was located on a mutation site in a flexible loop at opposite end of the β -barrel.²¹

Furthermore, we constructed a trivalent adamantyl-modified TFP (a monomeric variant of *Clavularia cyan fluorescent protein*) (ad_3TFP , Chart 1) for the current study. ad_3TFP was produced by conjugating a maleimide-capped triadamantyl-heptapeptide to a site-selectively mutated TFP, which contained a single cysteine residue, G174C, situated in one of the loops at the end of the β -barrel (see more details in ESI†). The triadamantyl-heptapeptide ($-\text{GE}(\text{ad})\text{RE}(\text{ad})\text{DE}(\text{ad})\text{K}$) was synthesized, using a glutamic acid with adamantyl-modified side chain ($\text{E}(\text{ad})$), by automated solid phase peptide synthesis with standard Fmoc-chemistry and purified by column chromatography (see ESI†). In order to optimize the geometry for multiple adamantyl moieties binding to a βCD self-assembled monolayers (βCD -SAMs), the adamantyl moieties were coupled to the glutamic acid side chains *via* C_6 -linkers (Chart 1).

Surface plasmon resonance (SPR) experiments were performed to determine the binding affinity of ad_3TFP to βCD -SAMs. βCD -SAMs were prepared on gold substrates and blocked in a flow of 0.1 mM mono-adamantyl hexaethylene glycol (adHEG) as described previously.^{22,23} Then, solutions containing different concentrations of ad_3TFP were passed over these SAMs, while monitoring the change of the plasmon resonance angle ($\Delta\alpha$) upon protein binding. Fig. 1 shows the increasing equilibrium angle shifts, reached for increasing concentrations of ad_3TFP . An overall binding constant of $5.4 \times 10^7 \text{ M}^{-1}$ for the new trivalent ad_3TFP - βCD interaction pair was determined, using a multivalent binding model corrected for competing adHEG ²⁴ (see ESI†). The value of the binding constant of ad_3TFP lies between that of the pairs $1\text{H}_6\text{TagRFP}$ - $\text{Ni}(\text{II})\text{NTA}$ and $3\text{H}_6\text{TagRFP}$ - $\text{Ni}(\text{II})\text{NTA}$.

In order to study the specificity of the assembly of ad_3TFP only on βCD moieties on substrates, dual line-patterns of βCD , backfilled with PEG-silane, were fabricated employing

Fig. 1 Equilibrium resonance angle shifts (■) for ad_3TFP binding to a βCD -SAM, in the presence of 0.1 mM monovalent adHEG , and simulated points (+) using a multivalent binding model (see ESI†).

nano- imprint lithography (NIL) as depicted in Scheme 1, but replacing $\text{Ni}(\text{II})\text{NTA}$ -functionalized lines with PEG-silane. These βCD -PEG surfaces were incubated with ad_3TFP or with a reference TFP construct lacking adamantyl moieties (E_3TFP , a conjugate of -GEREDEK to $\text{G}^{174\text{C}}$ TFP, Chart S1 and ESI†). Specific binding was achieved for ad_3TFP , while nonspecific binding of E_3TFP was negligible (Fig. S1, ESI†). We note that addition of βCD leads to negligible disassembly of ad_3TFP , but improves specific host-guest interactions.^{11,23} The specific binding of $1\text{H}_6\text{TagRFP}$ and $3\text{H}_6\text{TagRFP}$ to $\text{Ni}(\text{II})\text{NTA}$ -PEG surfaces had previously been demonstrated (Fig. S1, ESI†), while control experiments with $0\text{H}_6\text{TagRFP}$, lacking H_6 -tags, led to no discernible binding to $\text{Ni}(\text{II})\text{NTA}$ surfaces.²¹

These results clearly show that specific assembly of each of the constructs could be achieved after optimizing incubation and washing parameters. Generally speaking, there is a number of parameters that can be tuned to facilitate specific protein assembly *via* supramolecular interactions. On the most basic level, the necessary binding strength for successful protein assembly can be achieved by a judicious choice of interaction pair and valency, whereas the specificity of protein assembly, can generally be optimized by an addition of competitors and/or blocking agents. In addition, there is a number of other parameters that typically require careful optimization when dealing with protein assembly, in order to reduce nonspecific

Scheme 1 Procedure to fabricate NIL-patterned bifunctional βCD - $\text{Ni}(\text{II})\text{NTA}$ glass surfaces (see ESI† for details), poly(methyl methacrylate) (PMMA), N -[3-(trimethoxysilyl)propyl] ethylenediamine (TPEDA), phenyl diisothiocyanate (DITC) and trifluoroacetic acid (TFA).

Fig. 2 Fluorescence images of NIL-patterned bifunctional β CD-Ni(II)NTA substrates (β CD: narrower, 5 μ m wide lines; Ni(II)NTA: wider lines) after incubation with 100 nM 3H₆TagRFP (left), 500 nM ad₃TFP (middle) or 3 μ M 1H₆TagRFP (right) and subsequent washing overnight with PBS containing 5% v/v Tween20 (left and right) or 0.1 mM adHEG and 10 mM β CD (middle). Left, right: red channel; middle: green channel.

interactions sufficiently, while specific interactions remain adequate. Such parameters include the concentration of the protein construct during incubation, the use of detergents and the stringency of washing steps. It will obviously become more challenging, with each additional protein construct, to meet all limitations and requirements, for successful and selective multiple protein assembly. Here, in order to obtain reliable, specific protein assembly on patterned bifunctional surfaces, with minimal nonspecific binding, we optimized the following parameters for each of the constructs, ad₃TFP, 1H₆TagRFP and 3H₆TagRFP, separately: the concentration of the protein constructs during incubation, the concentration of detergent Tween20 in the incubation and wash buffers, incubation time, washing time and the addition of adHEG and β CD as competitors to incubation and wash buffers (Fig. 2).

We found that, generally, a concentration of between 100 nM and 5 μ M, an incubation time of at least 2 h and washing times of up to 16 h worked well for the surface assembly of each separate construct, on bifunctional β CD-Ni(II)NTA surfaces. The addition of high concentrations of Tween20 (5% v/v) to incubation and wash buffer turned out to be crucial for the specific assembly of both TagRFP variants, while it impaired the specific assembly of ad₃TFP. Reliable, specific assembly of ad₃TFP on bifunctional β CD-Ni(II)NTA surfaces was only possible with much lower concentrations of Tween20 (0.005% v/v) and in the presence of adHEG (0.1 mM) and it further benefitted from the addition of β CD (10 mM) to the wash buffer (Fig. S2, ESI†).

Where multiple protein assembly requires unique, optimized conditions, small molecules are less prone to nonspecific interactions and can, therefore, generally be readily assembled under a wide range of conditions. Fig. 3 shows fluorescence images of bifunctional β CD-Ni(II)NTA surfaces on which a diadamantyl-modified fluorescein (ad₂Fl)²⁵ and 1H₆TagRFP were assembled sequentially, but under the same conditions (in PBS + 5% v/v Tween20). Dual patterns were readily achieved, not requiring any additional washing steps. Notably, dual patterns were formed, even during simultaneous (one-pot) assembly of ad₂Fl and 1H₆TagRFP under the above-mentioned conditions (Fig. S3, ESI†). However, as discussed above, for the surface-assembly of multiple proteins, requirements are much more restrictive and it, then, comes as no great surprise that the orthogonal assembly of multiple proteins on multi-functional patterns proved challenging. As expected, the conflicting needs for incubation and washing conditions, when assembling the TagRFP variants and ad₃TFP

Fig. 3 Fluorescence images of NIL-patterned bifunctional β CD-Ni(II)NTA substrates (β CD: narrower, 5 μ m wide lines; Ni(II)NTA: wider lines) after incubation, first, with 10 μ M ad₂Fl and then, with 5 μ M 1H₆TagRFP and subsequent rinsing with PBS with Tween20. All solutions contained Tween20 (5% v/v). Top left: red channel; top right: green channel; bottom left: overlay of top row images; bottom right: intensity profiles in channel colour.

on bifunctional β CD-Ni(II)NTA (Scheme 1) surfaces, caused a dramatic decrease in specificity and binding strength in our attempts to co-assemble both proteins from a mixture. We attribute this to the incompatibility of the assembly conditions. Obviously, each protein assembled on complementary regions of the bifunctional surface needs to withstand all incubation and washing steps required for the assembly of the following protein, which vastly reduced the range of possible conditions to achieve dual protein assembly.

Consistently, the binding of 1H₆TagRFP when assembled last, was hardly sufficiently stable for the reproducible fabrication of dual protein patterns. Furthermore, no attempt at assembling first 1H₆TagRFP, then ad₃TFP, yielded any noticeable dual pattern formation (Fig. S4, ESI†). Moreover, the presence of high concentrations of Tween20, necessary to ensure the specific surface assembly of TagRFP variants, impaired the specific surface assembly of ad₃TFP (Fig. S2 and S5, ESI†), while the requirement of adHEG and β CD wash for specific assembly of ad₃TFP, had a negative effect on assembling the TagRFP variants (Fig. S2 and S5, ESI†). Likewise, the order of assembly was crucial to the successful formation of orthogonal protein patterns when assembling 3H₆TagRFP and ad₃TFP. Only when first assembling 3H₆TagRFP, then ad₃TFP, could dual protein patterns be reproducibly formed (Fig. 4), while first assembling ad₃TFP, followed by 3H₆TagRFP, never led to the observation of intact dual protein patterns (Fig. S4, ESI†). This cannot easily be rationalized with buffer incompatibility, when taking into account that, when assembling ad₃TFP first, and then 1H₆TagRFP, specific dual protein patterns were observed, albeit of poor quality and reproducibility. These findings could, however, be caused by differences in (nonspecific) binding of the TagRFP variants' to β CD surfaces. Indeed, residual fluorescence of 3H₆TagRFP bound to β CD surfaces, was at least twice that of 1H₆TagRFP, indicating that the nonspecific binding of 3H₆TagRFP was higher than that of 1H₆TagRFP. This result explains why 3H₆TagRFP might replace ad₃TFP from the β CD-functionalized parts of the pattern, while 1H₆TagRFP does not. It follows, then, that ad₃TFP competes with already nonspecifically bound

Fig. 4 Fluorescence images of NIL-patterned β CD-Ni(II)NTA substrates (β CD: narrower, 5 μ m wide lines; Ni(II)NTA: wider lines) after incubation with 100 nM $3H_6$ TagRFP and washing for 10 h with PBS containing 5% v/v Tween20 and 0.1 mM adHEG, then incubation with 500 nM ad_3 TFP and washing overnight with PBS containing 0.1 mM adHEG as well as 10 mM β CD. Top left: red channel; top right: green channel; bottom left: overlay of top row images; bottom right: intensity profiles in channel colour.

$3H_6$ TagRFP for binding to β CD surfaces. Regardless, first assembling $3H_6$ TagRFP, then ad_3 TFP, was the only reliable procedure to achieve dual protein assembly on bifunctional β CD-Ni(II)NTA surfaces. The use of optimized incubation and washing procedures for the assembly of ad_3 TFP, apparently proves sufficient for ad_3 TFP binding to prevail over nonspecific $3H_6$ TagRFP interactions with β CD. This, again, demonstrates the delicate balance of conditions that must be found for successful, orthogonal assembly of proteins on surfaces.

In conclusion, we have developed a procedure to reproducibly assemble proteins on patterned bifunctional surfaces using supramolecular interactions. Two different fluorescent proteins modified site-selectively with supramolecular binding moieties were employed. Assembly of each protein construct on patterned surfaces proved to be governed, to a large extent, by the balance between specific and nonspecific interactions and required optimization of incubation and washing conditions. Achieving dual protein assembly required a careful choice and fine-tuning of the assembly conditions, such as choice of buffer, blocking agents, detergents, order of assembly, concentration and timing. In our case, sequential orthogonal protein assembly on our patterned, bifunctional surfaces was successful, though with a strong influence of the stability of the first interaction pair, on the specific binding of the second.

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Conflicts of interest

There are no conflicts to declare.

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