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# Discrete and Active Enzyme Nanoarrays on DNA Origami Scaffolds Purified by Affinity Tag Separation

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***Abstract:*** Desired enzyme nanoarrays patterned on a DNA origami scaffold were selectively isolated by affinity tag purification from a pool of differently patterned nanoarrays, and their enzymatic activity was successfully confirmed. As few as 12 histidine residues were enough to hold a huge complex of DNA origami with multiple proteins, 260 nm in length and 5.2 MDa in molecular weight, to an immobilized metal affinity resin.

Protein nanoarrays of regularly ordered and oriented individual molecules are poised to have important roles in future proteome studies.[1] DNA nanotechnology based on the programmed assembly of branched DNA helices has been attracting great interest as a key approach to prepare scaffolds for protein nanopatterning.[2,3] To date, various DNA nanostructures have been used as scaffolds for protein nanoarrays.[4] DNA origami,[5−8] which involves the folding of long, single-stranded DNA into a designed, planar nanostructure with the aid of many short staple strands, is one of the most promising scaffolds, as the number and position of individual protein molecules in the array can be precisely controlled on a nanometer scale.[9−13] The next focus of the field should be the nanopatterning of enzymes onto a DNA origami scaffold and analysis of their activity. To date, however, no such nanoarrays have been reported,[14] mainly due to difficulties in distinguishing the activity of enzymes in nanoarrays from that of unbound enzymes. The most popular methods to separate desired DNA origami from excess staple strands and/or unbound proteins are based on size separation techniques such as size exclusion chromatography, ultrafiltration, and agarose gel electrophoresis. With these techniques, however, it has been quite difficult to purify DNA origami (ca. 4.5 MDa) from enzymes [e.g., horseradish peroxidase (HRP), 44 kDa;[15] bacterial alkaline phosphatase (AP), 94 kDa;[16] and glucose oxidase, 155 kDa][17] and conjugates with streptavidin (SA, 53 kDa)[18] or IgG (150 kDa).[19] In this study, we solved this problem by introducing hexahistidine affinity tags (His tags)[20] to DNA origami. Enzyme nanoarrays patterned in a single-molecule fashion onto DNA origami scaffolds were then successfully purified using affinity tag separation.

Figure 1 shows the structure of the His-tagged DNA origami used in this study. A stick-like DNA origami equipped with nine periodic concavities with dimensions of $7 \times 14 \times 2$ nm$^3$ was prepared as reported in our previous study.[10] When a combination of staple strands placed at the two opposite edges of a concavity (anchor strands) are biotinylated, the concavity serves as a well to accommodate exactly one SA tetramer with a diameter of 5 nm, forming robust SA nanoarrays.[10,21] The His tags were attached to the ends of two staple strands placed at the end of the punched origami (His1 and His2). The DNA portions of the His-tagged staple strands were first prepared as

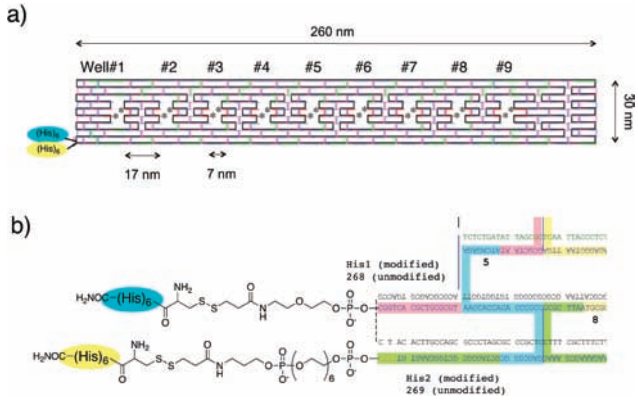


***Figure 1.*** (a) Structure of the punched DNA origami with two His tags. The positions of the biotin molecules in the anchor strands are indicated by asterisks. (b) Detailed structures of the two His-tagged staple strands (His1 and His2) and the corresponding unmodified staple strands (staples 268 and 269).

amine-bearing DNA oligomers, and the amino groups were then converted to activated disulfide bonds with a heterobifunctional cross-coupling reagent, *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP). The activated DNA oligomers were reacted with a seven-residue peptide, $H_2N$-Cys-$(His)_6$-$CONH_2$, which was prepared by solid-phase peptide synthesis using FMOC chemistry, to connect the His tags via a disulfide bond (see Supporting Information for the detailed synthetic procedure). Additional hexaethylene glycol was inserted in the linker of His2 to make it roughly twice as long as that of His1.

Metal ion affinity purification of the His-tagged DNA origami was first examined without protein. Four kinds of punched origami were prepared using the four possible combinations of the two His-tagged staple strands (His1 and His2) and the corresponding unmodified staple strands (staples 268 and 269) in order to estimate the effect of the number of His residues and the length of the linker. A cobalt-based immobilized metal affinity resin (TALON) was used for the purification.[22] Binding of the His-tagged DNA origami to the $Co^{2+}$ resin was initiated by adding the resin to a solution of punched origami annealed in 1X TA/$Mg^{2+}$ buffer (40 mM Tris, 20 mM acetic acid, and 10 mM magnesium acetate). After the supernatant was removed, the resin was washed three times with 1X TA/$Mg^{2+}$, and the bound DNA origami was eluted twice with 1X TA/$Mg^{2+}$ containing 150 mM imidazole. Figure 2 shows the analyses of each fraction in the purification steps by 1% agarose gel electrophoresis. Nonselectively bound punched origami molecules were washed from the resin by three washing steps. The desired band was observed in the first eluate, however, only when both His1 and His2 were used together and when two His tags were introduced into the punched origami molecules (Figure 2a).

  

This strongly suggests that binding of a single hexahistidine tag to $Co^{2+}$ complexes, irrespective of the length of the linker, is not enough to hold the rather large DNA origami (even larger than ribosomes) to the resin (Figure 2b,c). The low capture yield (~10%) was probably due to incomplete binding of His1 and His2 to the M13mp18 scaffold.[23] The present target site for these His-tagged staple strands is known to be a significantly self-complementary portion of the M13mp18 genome; thus, this site is often left single-stranded and excluded from DNA origami design. The recovery yield may be improved simply by attaching the His tags to other staple strands.

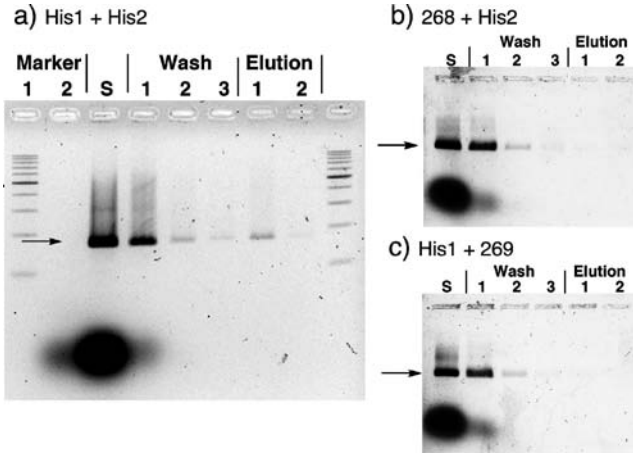


***Figure 2.*** Analyses of the binding ability of His-tagged DNA origami molecules to $Co^{2+}$ resin. A punched origami scaffold was prepared by using His1 and His2 (a), staple 268 and His2 (b), His1 and staple 269 (c), or staple 268 and 269 (data not shown). Each DNA origami molecule was mixed with a $Co^{2+}$ resin, and the supernatant was collected (S lane). The resin was washed three times with 1X TA/$Mg^{2+}$ (wash lanes 1, 2, and 3), and the DNA origami was eluted twice with 1X TA/$Mg^{2+}$ buffer containing 150 mM imidazole (elution lanes 1 and 2). Each fraction was analyzed by 1% agarose gel electrophoresis with two size markers (marker 1, a 1 kbp ladder; marker 2, a mixture of staple strands). The bands corresponding to the punched DNA origami are indicated by the arrows. Only the punched origami bearing two His tags (His1 + His2) gave a clear band in the first elution (a).

Affinity purification of two kinds of differently patterned SA nanoarrays on the punched DNA origami was examined (Figure 3a). By using appropriate anchor strands for the annealing of punched origami, one of the SA nanoarrays (motif O) was biotinylated at the five odd-numbered wells (first, third, fifth, seventh, and ninth), and the other (motif E) was biotinylated at the four even-numbered wells (second, fourth, sixth, and eighth). A 1:1 mixture of these two SA nanoarrays gave a reasonable ratio of the motifs in atomic force microscopy (AFM) images (28 for motif O and 30 for motif E, Figure 3b). When motif O alone was His-tagged using His1 and His2 in the annealing step and motif E was left unmodified with staples 268 and 269, the eluate from the $Co^{2+}$ resin contained almost pure motif O, as shown in Figure 3c. Almost 95% of the motifs (54 of 57) found in the AFM images were motif O, with five SA tetramers as bright spots. In contrast, when motif E was selectively His-tagged prior to SA patterning and motif O was left unmodified, motif E was selectively recovered after $Co^{2+}$ resin purification with nearly 94% purity (44 of 47). Thus, affinity purification of DNA origami is quite selective for successfully tagged species, and a difference in the pattern of protein nanoarrays does not alter its effectiveness.

Finally, we prepared two kinds of enzyme nanoarrays on the punched DNA origami with different enzymes and patterns and

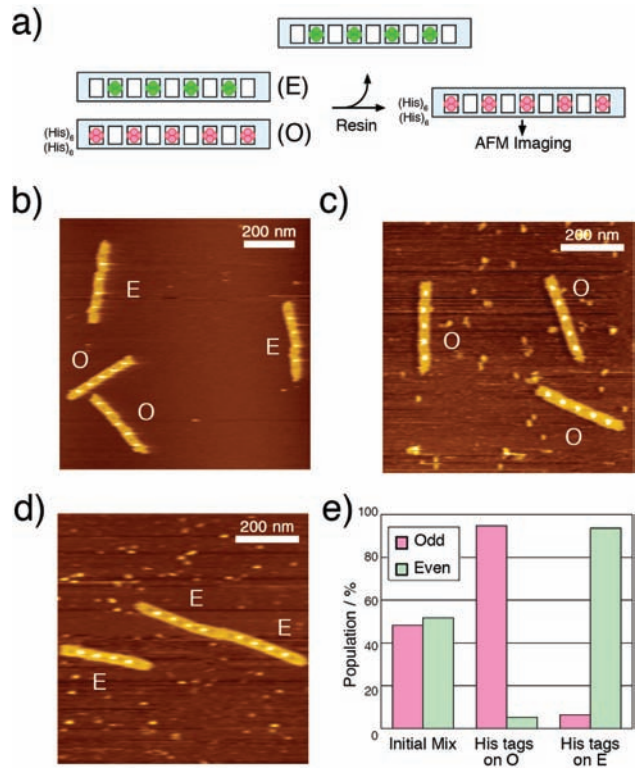


***Figure 3.*** Affinity purification of differently patterned protein nanoarrays on punched DNA origami. (a) Schematic illustration of the procedure. One of the two kinds of SA nanoarray, punched origami with SA in the odd-numbered wells (motif O) or the even-numbered wells (motif E), was modified with His tags and separated from the other nanoarray by $Co^{2+}$ resin purification. (b) A typical AFM image of a 1:1 mixture of His-tagged O motifs and unmodified E motifs. (c) A typical AFM image after purification of His-tagged O motifs from panel b. (d) Purified E motifs bearing His tags from a 1:1 mixture of His-tagged E motifs and unmodified O motifs. (e) The selectivity of the purification was estimated by counting the motifs in AFM images.

affinity purified them (Figure 4). For the patterning of motif O, an AP−SA conjugate was used in place of SA. Similarly, an HRP−SA conjugate was patterned on motif E. When only AP−SA nanoarrays patterned on motif O were His-tagged and mixed with unmodified HRP−SA nanoarrays on motif E, the eluate from the $Co^{2+}$ resin preferentially contained motif O (Figure 4a). The shape of the particles in the wells was not distinguishable from that of SA in AFM analyses.[24] This eluate, however, showed more than twice as much AP activity using an AP-specific fluorescent substrate (AttoPhos) as the eluate from a 1:1 mixture of simple AP−SA and HRP−SA conjugates without DNA origami (Figure 4c). In addition, this AP activity was 70% higher than that of the eluate from a mixture of His-tagged HRP−SA nanoarrays and unmodified AP−SA nanoarrays. On the other hand, the HRP activity of the eluate from the His-tagged HRP−SA/unmodified AP−SA nanoarray mixture was 45% higher than that of the eluate from the unmodified HRP−SA/His-tagged AP−SA nanoarray mixture in a reaction with an HRP-specific fluorescent substrate (Amplex UltraRed). The targeted enzyme nanoarray was selectively and successfully affinity purified from the mixture containing nanoarrays of different kinds of enzyme and unbound free enzymes. To our knowledge, this is the first example of verified reactions of enzymes immobilized in a single-molecule manner to DNA origami.[25]

In summary, enzyme nanoarrays on DNA origami with different enzymes and patterns were successfully prepared, and the activity

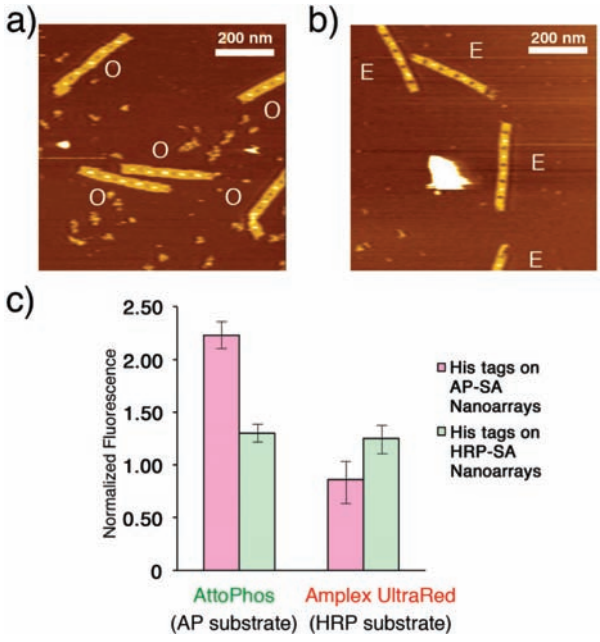


***Figure 4.*** Affinity purification of enzyme nanoarrays. AP−SA was patterned on motif O, and HRP−SA was separately patterned on motif E. (a) A typical AFM image of purified AP−SA nanoarrays from a 1:1 mixture of His-tagged AP−SA nanoarrays and unmodified HRP−SA nanoarrays. (b) A typical AFM image of HRP−SA nanoarrays. (c) The enzymatic activity of the eluate from $Co^{2+}$ resin purification of a 1:1 mixture of AP−SA nanoarrays and HRP−-SA nanoarrays (average of three independent reactions). The reaction was performed at room temperature for 20 min. The activity in the eluate from a mixture of free AP−SA and HRP−SA conjugates without punched origami was taken as unity. The amount of recovered origami was normalized on the basis of band intensity in agarose gel analyses of the eluate.

of these nanoarrays was confirmed for the first time by affinity purification. As few as 12 histidine residues could efficiently hold a huge complex of DNA origami with multiple enzymes (260 nm in length and 5.3 MDa in molecular weight for the punched origami with five AP−SA conjugates) to a $Co^{2+}$ resin. In addition to the poly-His tag used here, various peptide affinity tags have been developed to date, and many of them may be applicable to the purification of DNA origami. Greatly improved handling of DNA origami should widen the scope of the applications of protein/enzyme nanoarrays.

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**Supporting Information Available:** Experimental procedures, DNA sequences, detailed structure of the punched DNA origami, AFM images for the quantification of the motifs in the eluate, and detailed height analyses of the enzyme nanoarrays. This material is available free of charge via the Internet at http://pubs.acs.org.

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(22) This resin consists of $Co^{2+}$ carboxylmethylaspartate that binds to polyhistidine tag just like $Ni^{2+}$-NTA but exhibits less non-specific protein binding than $Ni^{2+}$-NTA.[20b] For DNA origami purification, however, popular Ni-NTA resin can be used as well (see Supporting Information, Figure S5).
(23) Even when the supernatant in S lanes, Figure 2a, was treated with additional resin, no more origami was captured (data not shown). This shows that DNA origami bearing His tags was completely captured on the resin under the present conditions.
(24) The conjugates seem to stay in the wells with their longer axes horizontal; see Supporting Information, Figure S9, for a detailed height analysis of enzyme nanoarrays.
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