Protein Nanoarrays

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Accommodation of a Single Protein Guest in Nanometer-Scale Wells Embedded in a "DNA Nanotape"**

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Remarkable progress in scanning probe microscope technology has enabled us to visualize various chemical or biological molecules at single-molecule resolutions. One attractive application of this technology is nanopatterning of proteins.[1] Such protein nanoarrays would be essential tools in future diagnosis, proteome analysis, and many other biological research fields. Recently, more precise nanoarrays, in which each protein molecule is individually arrayed on a DNA scaffold, have been reported.[2] Such DNA scaffolds are fruits of DNA nanotechnology, which is based on programmed assembly of branched DNA helices.[3] Various DNA nanostructures, for example, 2D double crossover (DX) crystals,[4] 1D triple crossover (TX) arrays,[5] DNA nanogrids,[6] kagome lattices,[7] and DNA origami,[8] have been used as the scaffold.

The next important target of protein nanoarray study is to control the orientation of each protein molecule. Such nanoarrays of regularly ordered and oriented protein molecules in the nanometer range should realize more advanced systems for use in the above fields. However, previous fixation of proteins to the scaffold was carried out mostly by connecting the protein and DNA with a single flexible linker or by introducing an aptamer in a hairpin, and those proteins were placed on the surface of the scaffold. It is not easy to regulate precisely the orientation of such proteins.

Herein, we propose a new strategy that leads to more robust and regulated protein nanoarrays: making nanometer-scale wells embedded in a DNA sheet for the selective capture of a target protein in a single-molecule manner, by "anchoring" it with two linkers (Figure 1). We made a tapelike DNA scaffold (a "DNA nanotape") by bundling nine DNA helices, and placed regularly arranged nanometer-scale wells in it. By attaching two biotin residues at two edges of each well, we successfully and size-selectively captured just one streptavidin (SA) tetramer in a well, and formed 28-nm-period SA nanoarrays.

The structure of the DNA motifs used in this study is shown in Figure 2. Two types of U-shaped motif were designed (Figure 2a). Motif **1** consists of four helices each eight turns long (84 bp (base pairs)) and five helices each six turns long (63 bp). These helices are connected to the adjacent helices through immobile four-way junctions at two positions, which are two or four helical turns apart. Figure 2b shows the connection pattern of each strand in **1**. Consequently, a flat, raftlike, nine-helix DNA bundle is formed. The longer helices are placed two by two in the edges of the motif, and thus **1** has a rectangular concavity two turns wide and five helices long at one side. The 5′ ends of the longer helices are sticky ends of five bases (dashed lines in Figure 2b).

When the sticky ends on the same helix are complementary to each other, the resulting self-complementary **1**

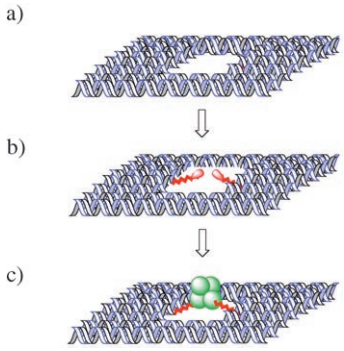

Figure 1. Strategy for the assembly of a protein nanoarray. a) Formation of a nanometer-scale well in a DNA scaffold. b) Introduction of two (or more) linkers to the edges of the well. c) Size-selective capturing of a single protein molecule in a well by "anchoring" the molecule with the linkers. In the present study, the DNA scaffold is a flat, nine-helix DNA bundle, the linker (red) is a biotin-TEG residue, and the protein (green) is a SA tetramer. TEG = triethylene glycol.

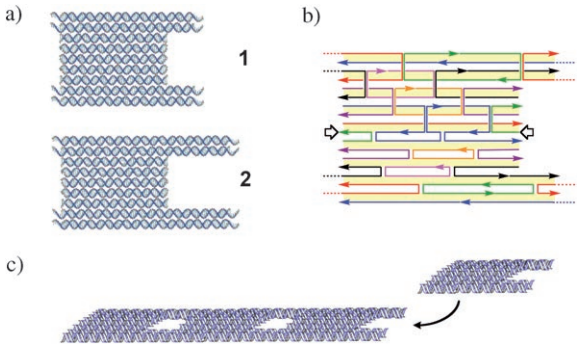

Figure 2. a) Structures of the DNA motifs. b) Connection pattern of each DNA strand in **1**. The dashed lines represent sticky ends. For the SA capturing experiment, biotin-TEG residues were attached to the ends of the strands indicated by the open arrowheads. See the Supporting Information for the sequence. c) Self-assembly of **1**.

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assembles into a DNA nanotape (Figure 2c) and the concavity becomes a nanometer-scale well with a theoretical dimension of $6.8 \times 10.0 \times 2.0$ nm (a "two-turn well" from its width). Non-self-complementary **1** remains a single U-shaped motif. Motif **2** consists of four long helices and five short helices, as for **1**, but the longer helices are extended to 105 bp (ten helical turns). Consequently, the nanotape formed with **2** provides larger "four-turn wells" of dimension $13.6 \times 10.0 \times 2.0$ nm.

The formation of the motifs and DNA nanotapes was first confirmed by nondenaturing PAGE analyses of annealed mixtures containing all the DNA components (Figure 3). As

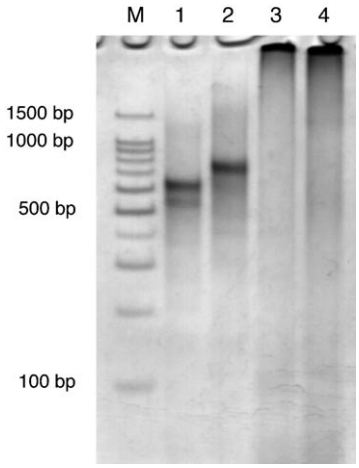

Figure 3. Nondenaturing 4% PAGE analysis of non-self-complementary and self-complementary motifs. Lane 1, non-self-complementary **1**; lane 2, non-self-complementary **2**; lane 3, self-complementary **1**; lane 4, self-complementary **2**. Lane M is the 100-bp marker.

seen in lanes 1 and 2, mixtures of non-self-complementary motifs gave fairly sharp major bands of similar mobility with corresponding size markers (motif **1** is 651 bp in total and **2** is 735 bp),[9] which shows that neither undesired aggregation of the motif nor critical incomplete complexation occurred within and after the annealing procedure. When mixtures of self-complementary motifs were loaded (Figure 3, lanes 3 and 4), only the bottom of the wells in the gel were darkly stained, and the bands corresponding to the single motifs completely disappeared. This result suggests that the motifs self-assembled into a 1D array as designed.

Successful formation of DNA nanotapes was further confirmed by AFM analyses of the mixtures. Flat, tapelike structures with an average height of 2.5 ± 0.5 nm, width of 26 ± 2 nm, and length around 300 nm were imaged on mica (Figure 4). The width of a helix in a DX motif is often observed to be up to 3 nm by AFM, and thus these numbers are in good agreement with the original design. In addition, well-defined, periodic, rectangular holes are observed in the tapes. The size of the holes clearly represents the length of the arms in the motifs.

The observed dimension of the holes in the array of **2** is approximately 10×10 nm at the half-height position, which is slightly smaller than the expected size, probably because of the tip shape (Figure 4b). One of the edges appears every

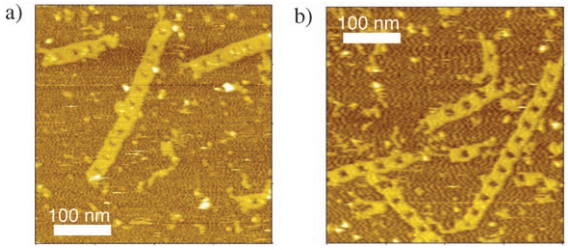

Figure 4. AFM images of DNA nanotapes made of a) **1** with two-turn wells and b) **2** with four-turn wells. The range of the images is 400×400 nm. The apparent period of the wells is 26 ± 2 and 33 ± 2 nm, respectively.

33 ± 2 nm, which is exactly ten times as long as one helical pitch of a canonical B-type helix (3.4 nm). On the other hand, the holes in the array of **1** are relatively narrow, and the average period of the holes is 26 ± 2 nm (Figure 4a). This value is also in good accordance with the length of eight helical turns (27 nm).

We then made SA nanoarrays. Two biotin residues were attached to the middle of two edges of each well by using modified strands bearing biotin-TEG (see Figure S3 in the Supporting Information) at the ends indicated by the open arrowheads in Figure 2b. After the biotinylated DNA nanotape was annealed as for normal nanotape, three equivalents (per individual tile) of SA tetramer were added to the solution. After overnight complexation, the mixture was deposited on mica and imaged by AFM.

When SA was added to the 1D array of doubly biotinylated **1**, many periodic bright spots were observed at the positions of the wells (Figure 5a). According to the height profile (Figure 5a, right), these bright spots appear every 28 ± 2 nm, which is exactly the expected pitch of two-turn wells. Moreover, these spots are about 3 nm higher than the DNA nanotape around them. As the diameter of the SA tetramer is 5 nm,[6a] the spots are attributed to individual tetramers that are accommodated in the 2-nm-deep two-turn wells.

Interestingly, almost 96% of the examined two-turn wells were filled with only one SA tetramer, even though two biotin residues were placed in a well and three equivalents of tetramer were added to the system. A total of 242 well-resolved wells were examined and only three wells were found with two SA tetramers crowded onto the wells (see Figure S3 in the Supporting Information). Another six wells were empty, and the rest contained a single SA tetramer. Presumably, this high selectivity is because the size of the well precisely fits just one tetramer, and is not big enough to accommodate two SA tetramers.

In addition, the length of the TEG linker that binds biotin to DNA is about 2.3 nm,[10] and the linkers are connected to the edges 6.8 nm apart. Thus, the two biotin residues can come as close as 2.2 nm in a two-turn well. This is sufficiently close for these biotins to "anchor" one SA tetramer, which has four biotin-binding sites, cooperatively to the well (Figure 5b). On the other hand, a four-turn well is twice as large as two-turn wells, and the two connections of the linkers are 13.6 nm apart. The minimum distance between the two biotin residues in the four-turn wells is thus far larger than the diameter of a tetramer, and anchoring is impossible here (Figure 5d).

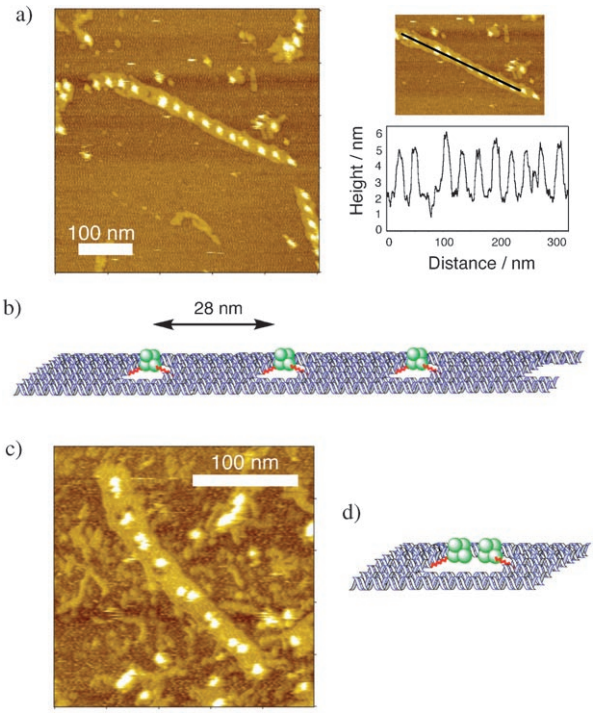

Figure 5. a) Left: AFM image (500×500 nm) of a SA nanoarray prepared from doubly biotinylated **1**. Right: cross-sectional analysis of the image. b) The SA nanoarray observed in (a), which is prepared by size-selective capturing of a single SA tetramer in a two-turn well. c) Image (250×250 nm) of a SA nanoarray prepared from doubly biotinylated **2**. d) Two SA tetramers trapped in a four-turn well shown in (c).

Consistently, many four-turn wells were found with two SA tetramers inside (Figure 5c).

To evaluate the effectiveness of this unique anchoring on the trapping of SA, alternate 1D arrays of doubly and singly biotinylated wells were prepared by assembling two differently biotinylated motifs (Figure S3 in the Supporting Information), and the arrays were analyzed by AFM (Figure S4). After five successive AFM scans, several defects in the SA arrays appeared in alternate wells, which might be assigned to the singly biotinylated wells. This result shows that the dual modification is preferable to obtain robust SA arrays. However, many consecutive SA arrays were still observed, which suggests that tetramers in singly biotinylated wells are also reasonably stable, presumably being nested by the wells of DNA helices. This is an advantage of the present capture-in-a-well strategy. In contrast, tetramers in four-turn wells were relatively unstable, regardless of the number of biotin modifications, and were readily scratched off within several scanning experiments (data not shown). The importance of the size fitting is apparent.

In conclusion, we have successfully constructed a new U-shaped DNA motif that can be assembled into 1D arrays with regularly arranged nanometer-scale wells. The size-selective capture of just one SA tetramer in each well was achieved by using two linkers together. The present strategy may be applicable not only to SA but also to other proteins by using other noncovalent and/or covalent interactions. Such an attempt is now under way in our laboratory.

Experimental Section

DNA strands were purchased from Sigma-Genosys (Japan) and used without further purification. The motifs were formed with 1 μM DNA (for each strand) in a solution containing 40 mM Tris, 20 mM acetic acid, 2 mM ethylenediaminetetraacetic acid, and 12.5 mM magnesium acetate (1× TAE/Mg buffer, total volume 100 μL). This mixture was cooled from 90 to 25 °C at a rate of −1 °C min^{-1} using a PCR thermal cycler to anneal the strands. The mixtures were then analyzed by 4% nondenaturing PAGE; the gel was stained with Stains-all (Sigma–Aldrich).

AFM imaging of DNA nanotapes was performed on a SPA-300HV system (SII, Japan). Annealed mixtures of 1 μM DNA strands were first diluted with 1× TAE/Mg buffer solution (50 parts), and then the solution (3 μL) was deposited on freshly cleaved mica. Further 1× TAE/Mg buffer solution (200 μL) was added, and imaging was carried out in the fluid dynamic force mode with an OMCL-TR400-PSA tip (Olympus, Japan).

For the preparation of SA nanoarrays, three equivalents (per single tile, final concentration 3 μM) of SA were added to the solution after the biotinylated DNA nanotape was annealed as for normal nanotapes. After overnight complexation, the mixture was deposited on mica and imaged by AFM as above.

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