DOI: 10.1002/cbic.200900229

Precisely Programmed and Robust 2D Streptavidin Nanoarrays by Using Periodical Nanometer-Scale Wells Embedded in DNA Origami Assembly

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A new punched DNA origami assembly with periodic nanometer-scale wells has been successfully designed and constructed. Through the attachment of two biotins at the two edges of each well, just one streptavidin (SA) tetramer ($d = 5$ nm) was size-selectively captured in each $6.8 \times 12 \times 2.0$ nm well; this allowed formation of a 28 nm-period SA nanoarray of individual molecules. The position of SA capture can be fully controlled by placement of biotins in the nanoarray well. Moreover, construction of a 2D nanoarray of individual SA tetramers through selective positioning of SA tetramers in any desired wells in a complex of such punched origami motifs is also possible. The stability of the SA captured by this fixation strategy (DNA wells and two biotin linkers) was directly compared on the same molecule with the stability of SA captured with other possible strategies that do not employ wells or two linkers. In this way, the robustness of this means of fixation was clearly established.

Introduction

Nanoarrays of regularly ordered and oriented individual protein molecules should play indispensable roles in future proteome studies. DNA nanotechnology,[1,2] which is based on programmed assembly of branched DNA helices, has recently attracted great interest as a key technology in the preparation of scaffolds for the nanopatterning of proteins. Various DNA nanostructures have been used as scaffolds for protein nanoarrays and analyzed by atomic force microscopy (AFM).[3–16] Mostly, fixation of proteins to the scaffold has been achieved by connecting protein and DNA through a single flexible linker, and those proteins are then situated on the surface of the scaffold. However, it is not easy to obtain well-resolved, clear AFM images of such protein molecules because they are often scratched off of the array under repetitive AFM-tip scanning. This may become a critical problem in the development of future practical applications of protein nanoarrays, such as single-molecule immunoassay with use of AFM imaging for signal detection.

We have recently proposed a new fixation strategy that leads to more robust and regulated protein nanoarrays: production of nanometer-scale wells embedded in a DNA sheet and selective capture of the target protein there in a single-molecule manner, by "mooring" it with two linkers.[17] We prepared a tape-like DNA scaffold by assembling U-shaped DNA tiles made of nine DNA helices, and positioned regularly arranged nanometer-scale wells in it. By attaching two biotins at the two edges of each well, we successfully and size-selectively captured just one SA tetramer in that well to form 28 nm-period SA nanoarrays. Although the means of fixation are precise and robust, limited control over the 1D-scaffold assembly has been the major obstacle to achieving further application of the system. Uniformity of the object of interest is one of the essential target requirements for a practical analytical system. To overcome this and to develop useful protein nanoarray systems, as well as to validate the generality of our fixation strategy, we focused on DNA origami assemblies in which long single-stranded DNA is folded into a designed planer nanostructure with the aid of many short staple strands.[18] We have designed a new punched DNA origami with periodic nanometer-scale wells, have successfully positioned exactly one SA tetramer in each predetermined well in a complex of such punched origami motifs, and have formed robust 2D nanoarrays of individual SA tetramers, just like putting marbles in any desired indentations on a peg solitaire board (Figure 1).

Results and Discussion

Design and construction of the punched DNA origami

The structure of the monomeric punched origami is shown in Figure 2. The circular 7249-nucleotide single-stranded M13mp18 genome is used as the scaffold. Figure 2A shows the folding pattern of this viral DNA. A total of 267 staple strands (18–46 nucleotides, Figure 2B) are used to fold it into a

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900229.

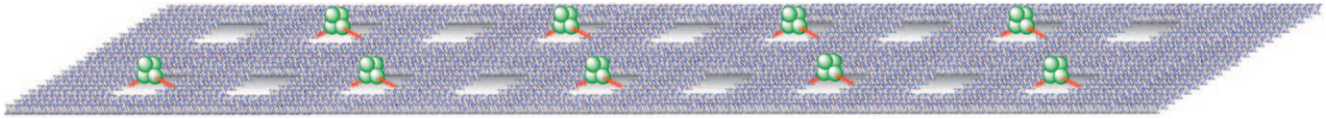

Figure 1. Illustration of the 2D SA nanoarray constructed in this study.

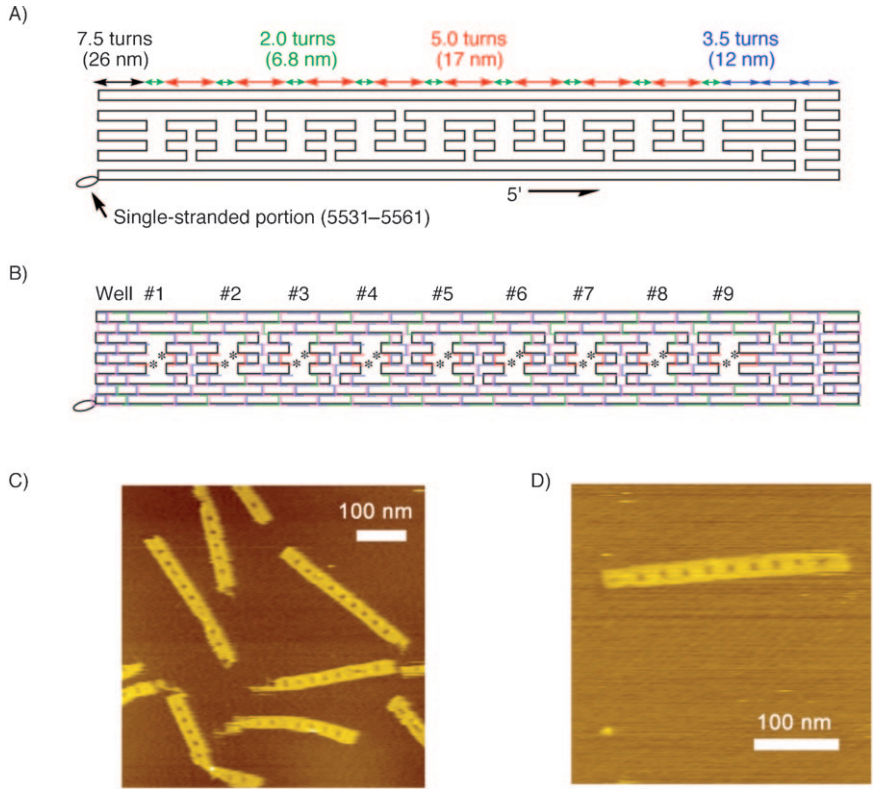

Figure 2. The structure of the punched DNA origami assembly designed in this study. A) Folding pattern of single-stranded M13mp18 viral DNA. Dimensions of the motif are 76 turns (theoretically 260 nm) wide and ten helices long (30 nm). B) Hybridization pattern of the staple strands. In total, 267 strands are used (see Figures S1–S3 and Table S1 in the Supporting Information for details). Positions at which 5′-biotin-TEG residues were attached for streptavidin capturing are indicated by the asterisks. C) An AFM image of the punched origami motifs. The range of the image is 600×600 nm. D) A zoomed image of a motif. The range of the image is 350×350 nm. Periodic rectangular holes are clearly observable. The apparent period of the holes is 26±1 nm.

narrow, rectangle, 76 turns wide (theoretically 260 nm) and ten helices long (30 nm). Inside of this motif, nine hollow sections, each two turns wide and four helices long (theoretical dimensions are 6.8×12×2.0 nm), are periodically located every seven turns (24 nm). These hollow sections are used as nanometer-sized wells to capture single SA tetramers inside. Successful formation of the origami was confirmed by AFM analyses of annealed mixtures of viral DNA and staple strands in 1×TAE/Mg buffer. Flat, tape-like structures with an average height of 2.4±0.5 nm, width of 29±1 nm, and regular length of about 300 nm, were clearly imaged on mica (Figure 2C, D). In addition, well-defined, periodic rectangular holes were observable in the motifs. The observed periodicity of the holes was 26±1 nm; this is in good accordance with the expected pitch of the wells.

Size-selective capturing of single SA molecules in wells

Formation of SA nanoarrays on the punched origami was performed as follows: Pairs of staple strands in the set intended for placement adjacent to the wells were replaced with 5′-biotinylated pairs (a total of nine pairs/18 strands, red strands in Figure 2B). These biotinylated strands each bear a biotin-triethyleneglycol (TEG) residue at the 5′-end, and two biotins are accordingly fixed in each well. After the staples had been annealed with viral DNA, excess SA was added to the solution, and the solution was immediately deposited on freshly cleaved mica for AFM imaging. The length of the TEG linker connecting biotin and DNA is about 2.3 nm,[19] and the linkers are connected to the edges of a well such that they are 6.8 nm (two helical turns) apart. The two biotins in a well can thus come as close as 2.2 nm, so that the TEG linkers act like anchors and cooperatively moor a SA tetramer to the well (Figure 3A). Figure 3B shows a typical AFM image of a SA nanoarray formed on the punched origami. Exactly nine periodically appearing bright spots are observed in each motif. According to the height profile (Figure 3B, right), these bright spots appear every 28±1 nm, which is quite close to the observed pitch of the wells. Moreover, these spots are 2–3 nm higher than the DNA helices around them. Since SA tetramer's diameter is 5 nm,[11] they are attributed to individual tetramers accommodated in the 2 nm-deep wells (Figure 3B, bottom). The occupancy of the wells in 60 reasonably resolved motifs (a total of 540 wells) was checked, and only seven wells were found to be empty (Figure S5 in the Supporting Information). The estimated yield of SA capture is thus 98.7%.

The biggest advantage of using DNA origami as the scaffold for a protein nanoarray is that each of the wells in the motif is easily distinguishable. By simply selecting the staple to be biotinylated, the well in which a tetramer is to be captured can be freely chosen. Figure 3C–E shows some examples of the se-

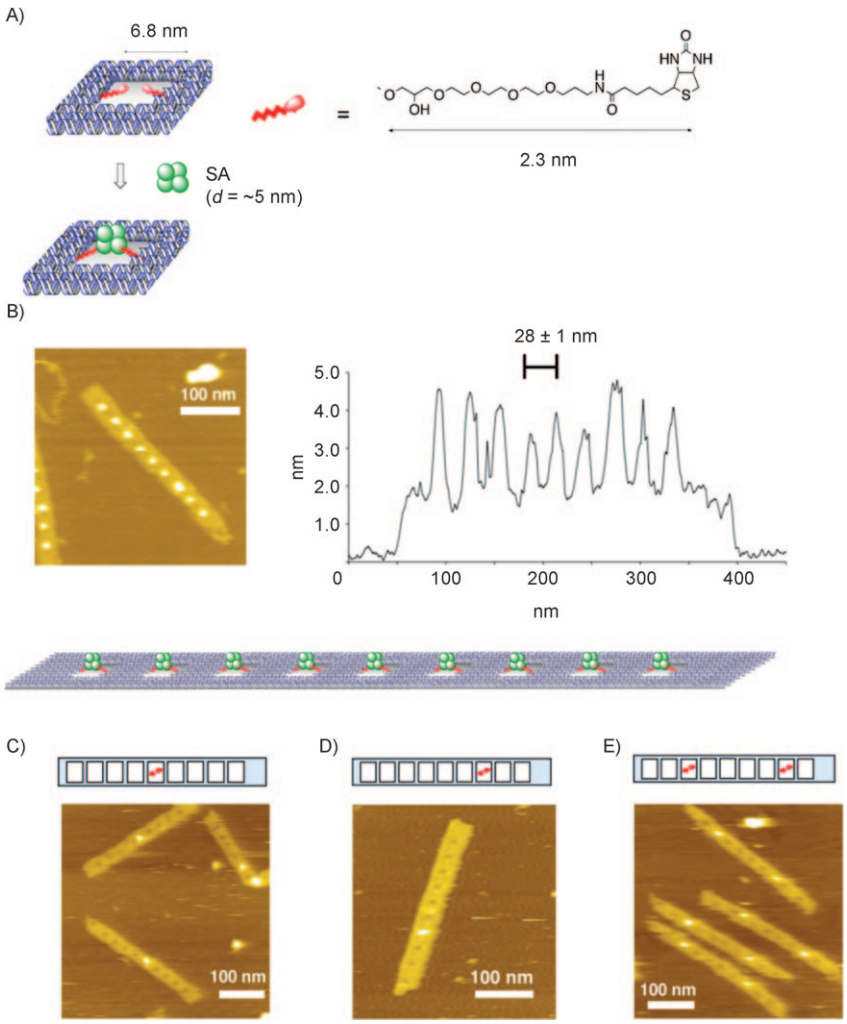

Figure 3. Construction of a streptavidin nanoarray by size-selective capture of one streptavidin tetramer in each well. A) Strategy of capture. Two biotin-TEG residues are attached to the two edges of each well, and cooperatively moor one streptavidin tetramer to the well. B) A 350×350 nm AFM image of nanoarrays (left), a height profile of a nanoarray (right), and a schematic illustration of the nanoarray (lower). C) A 450×450 nm AFM image of a punched origami with biotin modification only in the central well. Exactly one SA molecule is selectively captured there. D) Selective capture of SA in the seventh well. The range of the image is 350×350 nm. E) Selective capture of two SA molecules in the third and the eighth wells. The range of this image is 450×450 nm.

lective capture of one (or more) tetramers in a predetermined well. When only the fifth well was modified with biotins, SA was only found in the fifth well, as shown in Figure 3C. In Figure 3E, both the third and the eighth wells were modified with biotins. The yields of the desired products were quite high. A total of 52 motifs with biotin modifications in the third and the eighth wells were examined, for example, and 50 of them successfully captured exactly two SA tetramers in the third and the eighth wells (see Figure S6 in the Supporting Information; the other two motifs were each found with one additional SA tetramer). The number of SA units and the positions of capture are precisely controllable.

Formation of a 2D SA nanoarray on a punched origami assembly

Nanoarrays made with punched origami are not limited to 1D arrays: 2D arrangements are also possible by merging two (or more) punched origami motifs together. Figure 4A shows a complex of two motifs that are separately annealed and then joined together at their longer edges. In one of the motifs in the complex, staple strands that face one of the longer edges were exchanged with ten pairs of joint strands possessing eight or ten additional nucleotides complementary to the corresponding region in the longer edge in the other motif (see Figure S7 and Table S2 in the Supporting Information for details). The other motif in the complex was annealed without the corresponding staple strands to accept the sticky-ends on the first motif.

Construction of 2D SA nanoarrays by positioning SA tetramers selectively in desired wells is also possible. A hetero-complex of two punched origami motifs—one biotinylated only in the even-numbered wells and the other only in the odd-numbered wells—was prepared, and SA was added to the solution. As clearly shown in Figure 4B, the desired 2D SA nanoarray in a zigzag arrangement was achieved in fairly high yield.

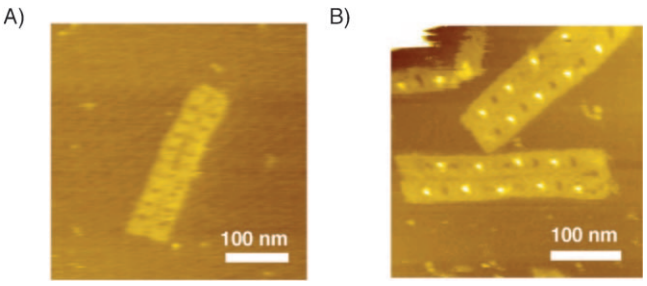

Figure 4. Streptavidin 2D nanoarray formed as a complex of two punched origami motifs. A) A 400×400 nm AFM image of the complexes of two punched origami motifs. B) A 350×350 nm AFM image of 2D streptavidin nanoarrays in zigzag arrangement.

Direct comparison with other fixation strategies

One of the most important features of this fixation strategy is the robustness of the resulting nanoarray. For purposes of comparison, SA tetramers in the even-numbered positions were placed "on" the origami, but not through accommodation in a well. To position all the tetramers on one side of the motif, biotins for the even-numbered positions were instead attached to dumbbell hairpins sticking out of the same origami surface (Figure 5A). The structures of mono- or bis-biotinylated dumbbell hairpins employed in this study are shown in Figure 5B. Additional four strands and 24 extended staple strands were used for the annealing of the motif to fill out the even-numbered wells with four DNA helices, and the dumbbell was attached to the second one from the top (Figure 5C, see Figure S9 and Tables S3 and S4 in the Supporting Information for details). As can be seen in Figure 5D, a 1D array of SA tetramers was also successfully obtained with this motif. However, most of the tetramers trapped on the mono-biotinylated dumbbells disappeared over the course of a few AFM measurements. One interesting finding for the bis-biotinylated dumbbell system is that only one SA tetramer is trapped on a dumbbell, although two biotins are attached to it (Figure 5E). Cooperative binding of biotins is also suggested for the dumbbell system. Such tetramers were fairly stable, but were still more likely to be scratched off the array with repeated AFM scanning than those accommodated in the odd-numbered wells.

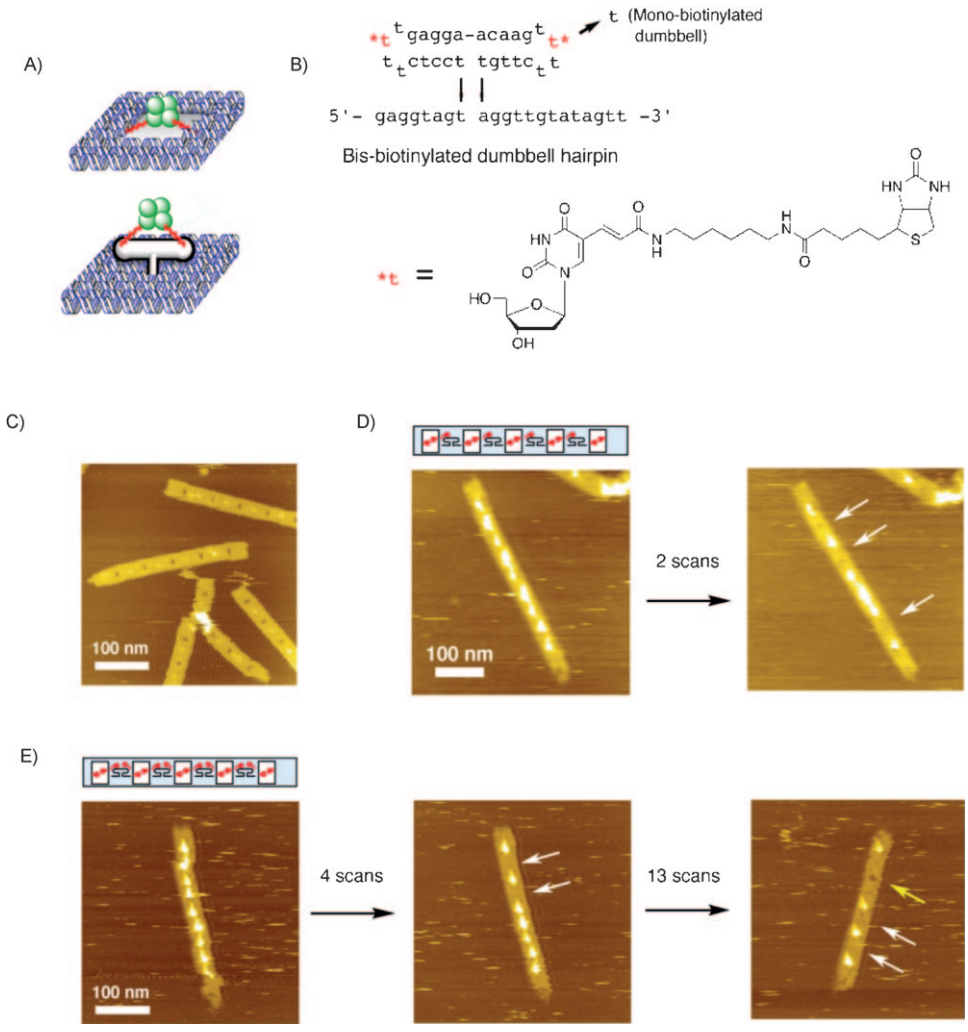

Figure 5. Stabilities of the SA tetramers accommodated in the wells. A) Schematic illustration of the two systems. In this dual-anchor strategy, the SA tetramer is accommodated in a 2 nm-deep DNA well (upper). For comparison, the SA tetramer was also attached to a dumbbell hairpin sticking out of an origami surface (lower). B) The structure of the dumbbell hairpin used in this experiment. The even-numbered wells were each filled with four DNA helices, and this dumbbell hairpin was attached to the second one. Biotins were attached to the nucleobases in the T4 loops (indicated by the asterisks). C) A 400×400 nm AFM image of punched origami motifs with dumbbell hairpins in the even-numbered positions. No SA was added to the solution. Small projections can be observed between the wells. D) Dual-anchor vs. dumbbell hairpin with one biotin. SA tetramers trapped by the dumbbell hairpins, which look slightly larger, disappeared after a few scans (indicated by the arrows). The range of the image is 450×450 nm. E) Dual-anchor vs. dumbbell hairpin with two biotins. SA tetramers on the dumbbell hairpins preferentially disappeared (indicated by white arrows), whereas only one SA tetramer disappeared from a well (yellow arrow). The range of the image is 400×400 nm.

We then checked the necessity of the two-linker system and compared the dual-anchor strategy to the simpler single-anchor strategy. Both involve molecular accommodation in a well but the latter uses only one biotin to capture the SA. Here, odd-numbered wells were modified with two biotins as described above, whereas for each even-numbered well only one biotinylated staple strand was used. As shown in Figure 6, even if all of the wells were initially filled with SA tetramers, all of those in the even-numbered wells had disappeared after as few as four successive AFM scans, whereas those in the odd-numbered wells were still clearly imaged. This result shows that the dual-anchor strategy is preferable. A direct comparison between the single-anchor strategy and that based on dumbbell hairpins with one biotin was also performed (Figure S10 in the Supporting Information). Each strategy involves only one biotin, but the former places it in a well and the latter on the origami surface. As would be expected, SA on dumbbell hairpins disappeared more rapidly in AFM imaging. Overall, the order of robustness of fixation is as follows: dual-anchor in a well > a dumbbell hairpin with two biotins ≫ single-anchor in a well > a dumbbell hairpin with one biotin.

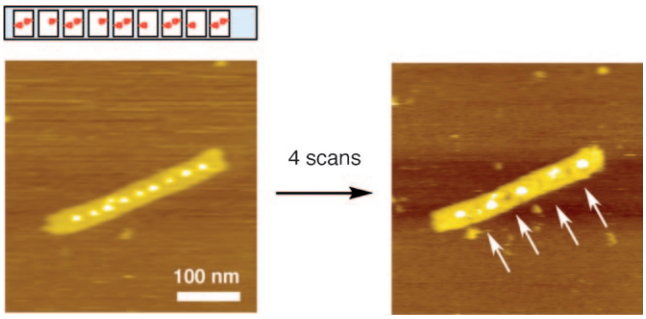

Figure 6. Direct comparison of the dual-anchor fixation strategy and single-anchor strategy. The odd-numbered wells in the motif were each modified with two biotin-TEG residues and the even-numbered wells with only one biotin. After four successive AFM scans, all of the SA tetramers in the odd-numbered wells had disappeared (indicated by the arrows). The range of the image is 400×400 nm.

Conclusions

We have successfully designed a new punched DNA origami assembly that can selectively capture exactly one SA tetramer each of any of its predetermined wells and stably accommodate them. One of the latest topics in DNA nanotechnology is whether proteins attached to DNA scaffolds still preserve their intrinsic structures and functions.[20,21] Although the possibility of trivalent capturing of one SA tetramer in a well has been confirmed (data not shown), it is still unclear whether the empty binding sites in an SA embedded in a well are accessible from the external solvent and maintain their strong biotin binding activity in that environment. If this is the case, however, attachment of secondary biotinylated proteins to SA to establish future applications of nanoarrays would be quite feasible. The native uniformity of DNA origami should make it much easier to purify desired protein nanoarrays by various conventional methods such as size-exclusion chromatography.

Experimental Section

Staple DNA strands were purchased from Integrated DNA Technologies (Coralville, IA, USA) and were used without further purification. Formation of the motifs was carried out with M13mp18 ssDNA (5 nM, Takara, Japan) and staples (50 nM for each strand) in a solution containing Tris (40 mM), acetic acid (20 mM), EDTA (2 mM), and magnesium acetate (12.5 mM, 1×TAE/Mg buffer, 50 μL). This mixture was cooled from 90 °C to 25 °C at a rate of $-1.0\,^{\circ}C\,min^{-1}$ by use of a PCR thermal cycler to anneal the strands.

AFM imaging of DNA origami was performed with a SPA-300HV system (SII, Japan). Annealed mixture (3 μL) was deposited on freshly cleaved mica, additional 1×TAE/Mg buffer (200 μL) was added, and the imaging was performed by use of the fluid DFM scanning mode with a BL-AC40TS tip (Olympus, Japan).

Biotin-TEG-modified staple strands and the strands for biotinylated dumbbell hairpins were chemically synthesized from appropriate phosphoramidite monomers (Glen Research, VA, USA) and were purified by denaturing polyacrylamide gel electrophoresis or reversed-phase HPLC. The annealed mixture of a biotinylated origami was first treated with a gel-filtration micro spin column equilibrated to 1×TAE/Mg buffer (Microspin S-400HR, GE Healthcare, UK) to remove excess staple strands, and SA (3 equiv to the number of wells; for example, 135 nM for Figure 3B) was added to the solution. The mixture was immediately deposited on mica and imaged by AFM.

Merging of two origami motifs was carried out as follows: firstly, punched origami motifs (10 nM) were separately annealed with or without joint staple strands, and treated on a gel-filtration micro spin column. Eluted fractions were then mixed together and concentrated to half volume under vacuum. After overnight incubation at room temperature, SA was added to the solution, and imaging was immediately carried out.

Acknowledgement

This work was partially supported by the Ministry of Education, Science, Sports, Culture and Technology, Japan in the form of a Grant-in-Aid for Specially Promoted Scientific Research (18001001) and a Grant-in-Aid for Young Scientists (B) (20750126).

Keywords: DNA · nanoarrays · nanotechnology · proteins · streptavidin

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Received: April 15, 2009
Published online on June 27, 2009

