Nanostructures

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Increasing the Complexity of Periodic Protein Nanostructures by the Rolling-Circle-Amplified Synthesis of Aptamers**

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DNA scaffolds[1–4] for the precise orientation of proteins and nanoparticles are a subject of research interest because of their potential utility in catalysis[2] and biosensing[3] as well as their ability to act as templates for hierarchical nanostructures and nanomachines.[4] The justification for using DNA and proteins to form such scaffolds is twofold. Firstly, the specific interactions of Watson–Crick base pairs and in protein–ligand binding have been and will continue to be used to make a menagerie of diverse, self-assembling architectures. Secondly, the preexisting toolbox of biomachinery can be exploited to impart topological complexity onto these systems. Encoded information in DNA has been used to organize chemical and biochemical materials such as small molecules,[2,5] macromolecules,[6] nanotubes,[7] nanoparticles,[8] and proteins.[9] These three-dimensional, hierarchical structures, in which complexity is added through bottom-up assembly, have generally been the result of selective biochemical interactions such as biotin–streptavidin,[1a,9a] DNA base-pairing,[2,8c–g,j,9b] and protein–aptamer[2f,3c,9c,10] binding, and have resulted in periodic arrays of the nanostructures immobilized onto a DNA template.

Herein we report the preparation of one-dimensional DNA–protein/DNA–protein–nanoparticle hierarchical assemblies created with protein–aptamer[10] interactions on DNA scaffolds that are formed using the biomachinery of rolling-circle amplification (RCA).[2d,f,8f,g,j,9e,11] RCA is a technique in which a circular oligonucleotide sequence serves as a template to create a complementary single-stranded DNA (ssDNA) chain that contains periodic repeats of the sequence coded for by the circular oligonucleotide. RCA has been used previously to make star-shaped[8g] as well as linear[8f,j] periodic assemblies of nanoparticles in which nanoparticles modified with complementary ssDNA bind to the periodic scaffolds. Using RCA, we have created linear DNA chains containing aptamers for the protein thrombin[2f,9c,10] and also both thrombin and lysozyme.[3c] Aptamer–protein binding can be used to increase the complexity of nanostructures, while simultaneously simplifying their preparation. Because native proteins can be used without any prior modification, the use of aptamers represents a versatile methodology for creating nanostructures. Herein we demonstrate the self-assembly of these one-dimensional, periodic protein–DNA composites by atomic force microscopy (AFM) and confocal fluorescence microscopy (CFM). These DNA–protein conjugates can themselves serve as further templates for hierarchical structures, and this is demonstrated by immobilization of gold nanoparticles onto the amine-rich proteins.

The circular oligonucleotides **1** and **2** were subjected to RCA to create linear ssDNA tapes (Figure 1a) that contain periodic repeats of the aptamers for thrombin and lysozyme (in **3**) or only the aptamer for thrombin (in **4**). Oligonucleotide **1** contains four distinct segments which each impart a specific property to the resulting periodic DNA tape **3**. Circular DNA **1** contains a 20-base-pair (bp) sequence to which the primer **5** binds to initiate RCA; in the resulting tape **5** acts as a spacer between the two aptamer units. The next segment of **1** codes for the aptamer for the protein thrombin (blue). The following sequence is another spacer of 20 bp, followed ultimately by the aptamer for lysozyme. This design programs for the DNA tape to which two proteins can bind; the separation between the two proteins can be manipulated by modulating the number of bps in the spacers. Similarly, **4** codes for two separated aptamers, albeit both are the aptamers for the protein thrombin with a separation of 30 bp.

The RCA assembly of a periodic, programmable DNA tape that contains aptamers for the proteins thrombin and lysozyme is depicted in Figure 1a. Control of the length of these periodic tapes is accomplished by regulating the dNTP (dNTPs = four-base deoxyribonucleotide triphosphate mixture) to oligonucleotide ratio as well as the number of dNTPs added during the RCA process. The successful preparation of **3** by RCA was confirmed by gel electrophoresis and AFM. In the gel (Figure 1b) a strong band corresponding to chains in excess of the maximum marker length of 10000 bp per chain was observed, which would lead to chains of DNA many micrometers in length. This result is consistent with our target length of 100000 bp per chain set by the conditions of the RCA, which would result in chains of **3** approximately 34 μm long. After drop-casting a dilute solution of this DNA onto a freshly cleaved mica surface, we observed long, coiled DNA chains micrometers in length (Figure 1c). The height of the coiled strings is 6 Å, which is the expected magnitude for ssDNA, and the lengths appear to be within the range we predict, although exact determination is difficult owing to the serpentine nature of the ssDNA.

The ability of the resulting ssDNA **3** to form functional nanostructures that contain both thrombin and lysozyme (Figure 1a) was visualized by confocal fluorescence microscopy (CFM). ssDNA **3** was exposed to a solution of

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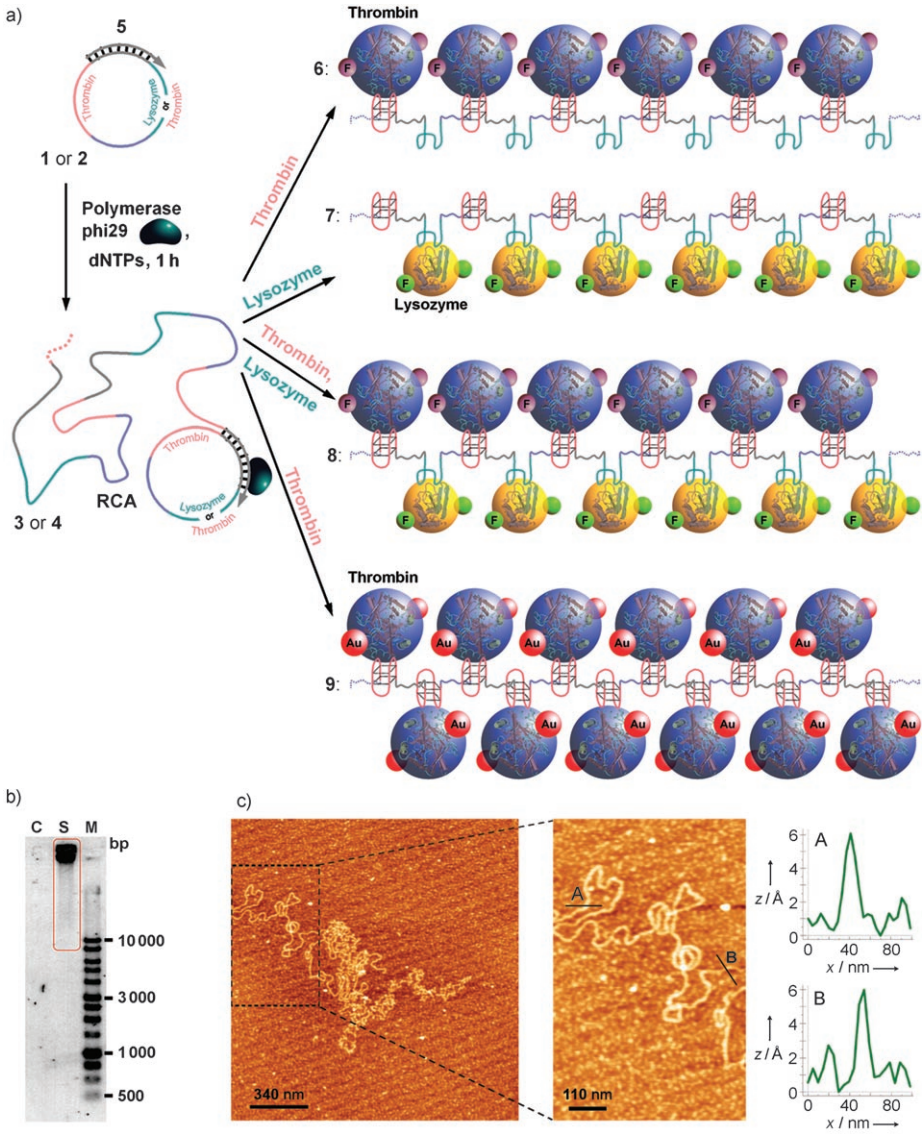

Figure 1. Preparation and characterization of periodic DNA tapes created using rolling-circle amplification. a) A circular template (**1** or **2**) is subjected to polymerization to create the periodic tapes (**3** or **4**), which in turn can be reacted with the appropriate enzyme to form the protein-immobilized nanostructures **6**–**9**. b) Gel electrophoresis of periodic DNA tape **3**. C = control (all components without polymerase), S = sample with all components, M = 1 kb ladder. c) AFM images of DNA tape **3** and associated height profiles.

tetramethylrhodamine carboxylic acid (TAMRA)-labeled thrombin (→**6**), fluorescein-labeled lysozyme (→**7**), or both simultaneously (→**8**). In the case of **6**, red nanowires were observed by CFM (Figure 2a) following excitation of the TAMRA dye, and in the case of **7**, similar green nanowires were observed (Figure 2b) following excitation of the fluorescein dye. The RCA tape simultaneously exposed to both fluorescein-labeled lysozyme and TAMRA-labeled thrombin formed the bifunctionalized DNA tape **8** in a single step. Confocal microscopy on the resulting sample showed that all the observed wires had both red (Figure 2c) and green (Figure 2d) fluorescence. These two images could be overlaid, which results in yellow nanowires (Figure 2e), thereby demonstrating the presence of both thrombin and lysozyme on each tape and confirming the one-step self-assembly of multiple proteins onto a programmed DNA scaffold.

The successful assembly of proteins onto the ssDNA scaffold was also demonstrated by AFM. After immersion of the DNA into a thrombin solution and drop-casting of this solution of **6** onto a freshly cleaved mica slide, long chains with periodic round bumps, 1.2 nm in height, were observed (Figure 3a). Following an identical deposition protocol with **7**, we observed similar bumps, 1–2 nm in height (Figure 3b). The spacing between individual proteins could not be resolved because the resolution determined by the radius of the AFM tip is larger than the programmed spacing between the individual proteins. After exposure of **3** to both proteins, we again resolved long nanowires of **8** with periodic bumps along their lengths (Figure 3c). These AFM studies provide further support that periodic DNA tapes formed by RCA provide a robust scaffold onto which multiple proteins can be immobilized with precise spatial control in a single step.

We then exploited these proteins for further hierarchical self-assembly. The amino residues on the surface of the regularly spaced enzymes can be used to increase the complexity of the nanostructures by acting as anchoring sites for Au nanoparticles, thereby forming inorganic nanowires. To create such structures, periodic DNA tape **4** was immersed in a solution of thrombin that had been modified with 1.4-nm mono(*N*-hydroxysuccinimide)(NHS)–gold nanoparticles to form the Au nanowire **9**. The DNA–protein–nanoparticle assembly was characterized by AFM (Figure 4a). Prior to modification with Au, periodic bumps, 1.2 nm in height appear along the wires. Following modification with gold, the height of the bumps increases to 2.5 nm, a gain of 1.3 nm, which corresponds well to the nanoparticle height of 1.4 nm. Nanowire **9** could also be resolved using transmission electron microscopy (TEM; Figure 4b). The TEM images show black dots, approximately 1.4 nm in diameter, that form long chains

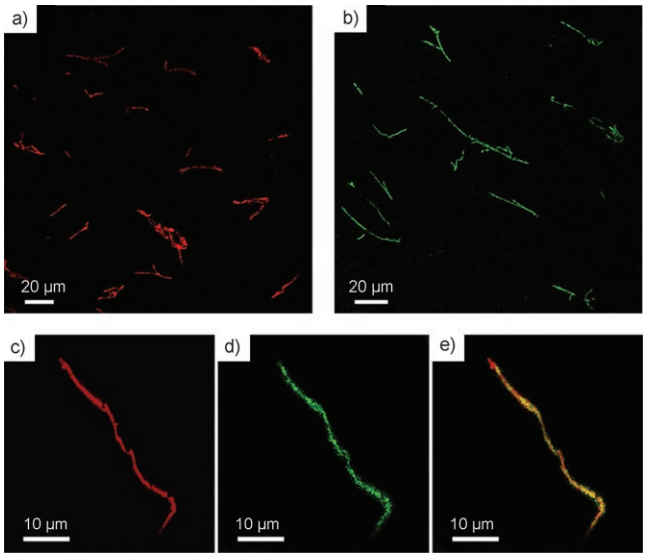

Figure 2. CFM images of periodic DNA tapes **6**–**8** immobilized with TAMRA-labeled thrombin and fluorescein-labeled lysozyme. a) Periodic tape **6** with TAMRA-labeled thrombin ($\lambda_{ex}=543$ nm, $\lambda_{em}=570$ nm), b) **7** with fluorescein-labeled lysozyme ($\lambda_{ex}=488$ nm, $\lambda_{em}=520$ nm), c) **8** with TAMRA-labeled thrombin and fluorescein-labeled lysozyme ($\lambda_{ex}=543$ nm, $\lambda_{em}=570$ nm), d) **8** modified with TAMRA-labeled thrombin and fluorescein-labeled lysozyme ($\lambda_{ex}=488$ nm, $\lambda_{em}=520$ nm), and e) overlay of CFM images in (c) and (d) taken for **8**.

with a regular spacing of 4.0 ± 0.9 nm. The hybrid nanowires seen in the TEM images are several hundred nanometers in length, which agrees well with both fluorescence and AFM measurements.

By exploiting the biomachinery of RCA, we have prepared two modular DNA scaffolds onto which proteins and nanoparticles can be fixed with precise control. The immobilization of both proteins onto a single DNA strand was confirmed by AFM and confocal fluorescence microscopy. While the two scaffolds are unique in their architectures, they share the ability to bind multiple nanostructures simultaneously. In addition, by modifying the DNA sequence of these scaffolds, we can change the nature of the assembly. These systems are unique in their use of robust protein–aptamer binding along with efficient RCA to create complex nanostructurures. The potential of these modular hierarchical nanoassemblies in biosensing, catalyzing enzymatic cascades, and the formation of complex nanowires is just part of the promise of DNA scaffolds.

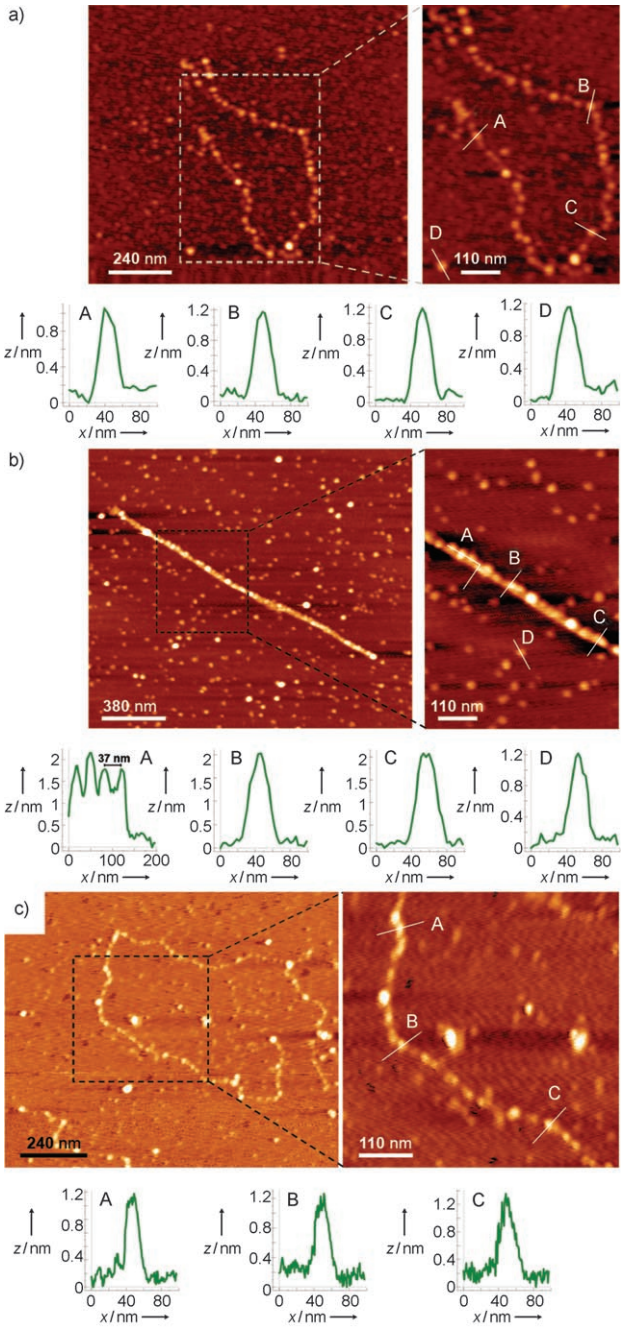

Figure 3. AFM topographical images of protein-functionalized DNA tapes and associated height profiles: a) nanostructure **6**, b) nanostructure **7**, and c) nanostructure **8**.

Experimental Section

General methods: Tunneling electron microscopy (TEM) images were recorded on an FEI Tecnai F20 G^2 instrument with 0.24-nm resolution operating in bright-field mode on 300-mesh copper grids (Electron Microscopy Sciences, USA). All AFM imaging measurements were performed at room temperature using a multimode scanning probe microscope with a Nanoscope 3A controller (Digital Instruments, Veeco Probes, USA). Gel electrophoresis was carried out with 0.6% agarose gel and SYBR Green I stain (Molecular Probes, Inc., USA). Confocal fluorescence microscopy was carried out on either an LSM 410 Zeiss confocal laser microscope with DlanApochromat × 63/1.4 oil lens or an Olympus FluoView FV300 confocal laser scanning microscope with a UIS PLAPO 63 × 1.4 oil lens with a 488-nm Ar laser and a 543-nm He–Ne laser. AFM topographical images were recorded on samples deposited on freshly cleaved mica surfaces (Structure Probe, Inc., USA) that were first pacified with a 5 mM $MgCl_2$ solution for 1 min before the solution of interest was drop-cast. Images were recorded with Ultrasharp SiN AFM tips (Mikromasch, Germany) in tapping mode at their resonant frequency, and these images were analyzed with WsXM SPIP software (Nanotec, Inc., Spain).[12] The portion of the sequences complementary to the aptamers are highlighted in bold.

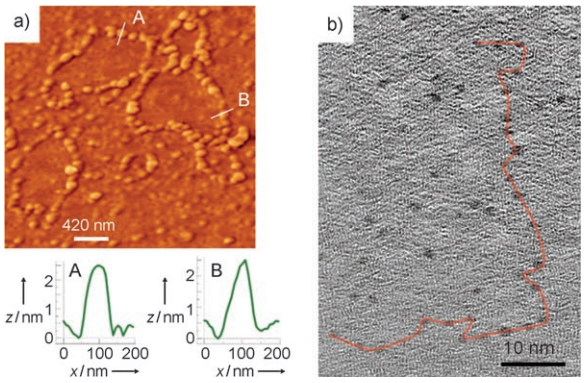

Figure 4. DNA–protein–nanoparticle hybrid nanostructures. a) AFM topographical image of **9**. b) TEM image of nanoparticle-labeled nanostructure **9**. The red contour connecting the DNA scaffold was determined by identifying a linear array of nanoparticles for which the separation between adjacent particles was less than 4 nm.

Dye-labeled proteins: A 5 μM solution of thrombin in *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer (0.1M, pH 7.4) was treated with 10 μM TAMRA-NHS for 50 min at room temperature to form TAMRA-labeled thrombin. The reaction mixture was then purified and separated from the excess dye by aptamer-binding protein buffer (20 mM Tris-HCl, (pH 7.4), 140 mM NaCl, 5 mM KCl, 5 mM $CaCl_2$, and 1 mM $MgCl_2$) using a centricon filtration device (10000 Da cutoff). A 5 μM solution of lysozyme in HEPES buffer (0.1M, pH 7.4) was treated with 10 μM fluorescein-NHS for 50 min at room temperature to form fluorescein-labeled lysozyme. The reaction mixture was then purified and separated from the excess dye by treatment with aptamer-binding protein buffer using a centricon filtration device (10000 Da cutoff).

Preparation of circular DNA template **1**: A 0.8×10^{-6}M solution of the phosphorylated linear DNA (5′-GATCCTAACTAA-GTAACTCTG**CACTCTTTAGCCCTGAT**AAAAAAAAAAAA-AAAAAAAA**CCAACCACACCAACC**AAAAAACCACAC-3′) was treated with a 9×10^{-6}M solution of the ligation template **5** (5′-TTAGGATCGTGTGGTT-3′) in the Quick Ligation Kit buffer at 25°C for 30 min. The synthesis was completed with the Quick Ligation Kit, using the manufacturer-supplied protocol. The enzymes were denatured by heating at 65°C for 10 min. The ligated circular DNA **1** was then treated for 30 min at 37°C with exonuclease I (2 U μL^{-1}) to degrade excess single-stranded primers that had not hybridized with the phosphorylated linear DNA. The enzymes were denatured by heating at 80°C for 20 min. DNA template **1** was isolated using a centricon filtration device (30000 Da cutoff).

Preparation of circular DNA template **2:** A 0.8×10^{-6}M of the phosphorylated linear DNA (5′-GATCCTAAAAAAAAA**CCAAC-CACACCAACC**AAAAAAAAAAAAAAAAAAAAAAAAAA-AAAA**CCAACCACACCAACC**AAAAAAAAAACCACAC-3′) was treated with a 9×10^{-6}M solution of the ligation template **5** (5′-TTAGGATCGTGTGGTT-3′) in the Quick Ligation Kit buffer at 25°C for 30 min. The synthesis was completed with the Quick Ligation Kit, using the manufacturer-supplied protocol. The enzymes were denatured by heating at 65°C for 10 min. The ligated circular DNA **2** was then treated with exonuclease I (2 U μL^{-1}) for 30 min at 37°C to degrade excess single-stranded primers that had not hybridized with the phosphorylated linear DNA. The enzyme was denatured by heating at 80°C for 20 min. DNA **2** was isolated using a centricon filtration device (30000 Da cutoff).

Preparation of periodic DNA tape **3**: Ligated circular DNA **1** (50×10^{-9}M) and template **5** (50×10^{-9}M) were mixed with phi29 DNA polymerase (0.4 U μL^{-1}) and dNTPs (0.5 mM). The RCA process was carried out in RCA buffer (50 mM Tris-HCl (pH 7.5), 10 mM $(NH_4)_2SO_4$, 10 mM $MgCl_2$ and 4 mM 1,4-dithiothreitol (DTT)) in a final volume of 50 μL for 1 h at 30°C. The phi29 DNA polymerase was denatured by heating at 65°C for 10 min.

Preparation of periodic DNA tape **4**: Ligated circular DNA **2** (50×10^{-9}M) and template **5** (50×10^{-9}M) were mixed with phi29 DNA polymerase (0.4 U μL^{-1}) and dNTPs (0.5 mM). The RCA process was carried out in RCA buffer in a final volume of 50 μL for 1 h at 30°C. The phi29 DNA polymerase was denatured by heating at 65°C for 10 min.

Protein-labeled RCA tapes **6**–**8**: RCA product **3** (1×10^{-9}M) was treated with 100 nM of either TAMRA-labeled thrombin, fluorescein-labeled lysozyme, or both in aptamer-binding protein buffer in a final volume of 50 μL for 2 h at 37°C.

Au-labeled thrombin: Thrombin (1.5 nmol) was reacted with 1.4-nm Au-NHS particles (3 nmol) in HEPES buffer for 85 min at room temperature to form Au-labeled thrombin. The reaction mixture was then purified and separated from the excess Au nanoparticles by the aptamer-binding protein buffer using a centricon filtration device (50000 Da cutoff).

Au-thrombin-labeled RCA tape **9**: RCA product **4** (1×10^{-9}M) was treated with 100 nM Au-labeled thrombin in the aptamer-binding protein buffer in a final volume of 50 μL for 2 h at 37°C.

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