Site-Specific Attachment of Proteins onto a 3D DNA Tetrahedron through Backbone-Modified Phosphorothioate DNA

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One of the most significant advances in nanoscale science and technology of the last decade is the successful demonstration of DNA as a programmable material to form increasingly complex and well-defined nanostructures.[1–4] In particular, several groups have explored a variety of strategies for assembling a range of geometrically well-defined 3D DNA structures such as polyhedrons.[5–17] For example, three-point star motifs have been designed to assemble into tetrahedra, dodecahedra, and buckyballs, while five-point star motifs have been prepared to form icosahedra and large nanocages.[11,18–20] With these beautiful and complex nanostructures in hand, the next challenge is to functionalize these structures with functional materials such as nanoparticles or proteins in order to realize their full potential in applications such as nanoelectronics/photonics, sensing, imaging, and nanomedicine.[21–29] However, due to the carefully optimized sequence and rigidities of these structures, functionalization and decoration of 3D DNA structures with proteins and other nanocargos has remained difficult to date.[20,30,31]

We have previously demonstrated the use of backbone phosphorothioate (PS)-modified DNA as anchor sites for precise control of the position of and distance between multiple gold nanoparticles and proteins on 1D double-helical DNA's backbone through the engineering of different bifunctional linkers.[32,33] Phosphorothioate is a derivative of DNA in which one of the nonbridging oxygen atoms is replaced by a more reactive sulfur atom (**Figure 1**A). Since the PS group can be programmably placed along a strand of DNA with single base resolution, does not affect the Watson–Crick pairing, and enables precise distance control between nanoconjugates, it is an ideal technology for specific functionalization of complex 3D DNA nanostructures without altering the design or stability of the structures. Recently, the Mao group has demonstrated incorporation of hairpin structures onto the vertices of a DNA tetrahedron by carefully optimizing both the location and sequence of the hairpin as well as the rigidity of the final structure.[30] However, since hairpins cannot provide "sticky end" functionality to the structure, it is also important to explore other means of functionalization that do not require sticky ends or DNA functionalization of the cargo. Herein, we present a novel technique for attaching protein to a 3D DNA tetrahedron through a simple and versatile technique via PS backbone-modified DNA and the use of short bifunctional linkers. This technique is chemically and synthetically simple, can be adapted to other DNA nanostructures, exhibits high yields, and the modification can be programmably placed within the structure.

The tetrahedron's design consists of three unique strands of DNA that make up the three-point star motif: a central strand (78 base pairs), a middle strand (42 base pairs), and a short strand (21 base pairs).[11] At concentrations below 60 nM, the strands anneal into the familiar three-point motif and subsequently self-assemble into a tetrahedron structure in a one-pot synthesis. Of particular interest to this study is the long, central strand containing five consecutive thymine (T) residues that form the flexible vertices of the tetrahedron. By replacing three adjacent phosphate backbones in the thymines of the central strand with PS, we can selectively and effectively decorate the vertices of the DNA tetrahedron through the careful engineering of a bifunctional linker (Figure 1A). The PS-modified central strand was first chemically modified with a biotin polyethyleneoxide iodoacetamide bifunctional linker in 1× Tris/acetic acid/ethylenediaminetetraacetic acid (EDTA)-Mg^{2+} (TAE-Mg^{2+}) buffer at 50 °C for 5 h and the excess linker was removed by filtration. The conjugation was confirmed by mass spectrometry and 100% conjugation was achieved after purification (see Supporting Information).

Assembly of the DNA tetrahedron containing the PS modification and bifunctional linker was adapted from a previously reported protocol.[11] Briefly, the three DNA strands were mixed at a ratio of 1:3:3 in a TAE-Mg^{2+} buffer and the mixture was slowly cooled from 95 to 25 °C over 24 h. The assembled DNA structures were characterized by nondenaturing polyacrylamide gel electrophoresis (PAGE). In

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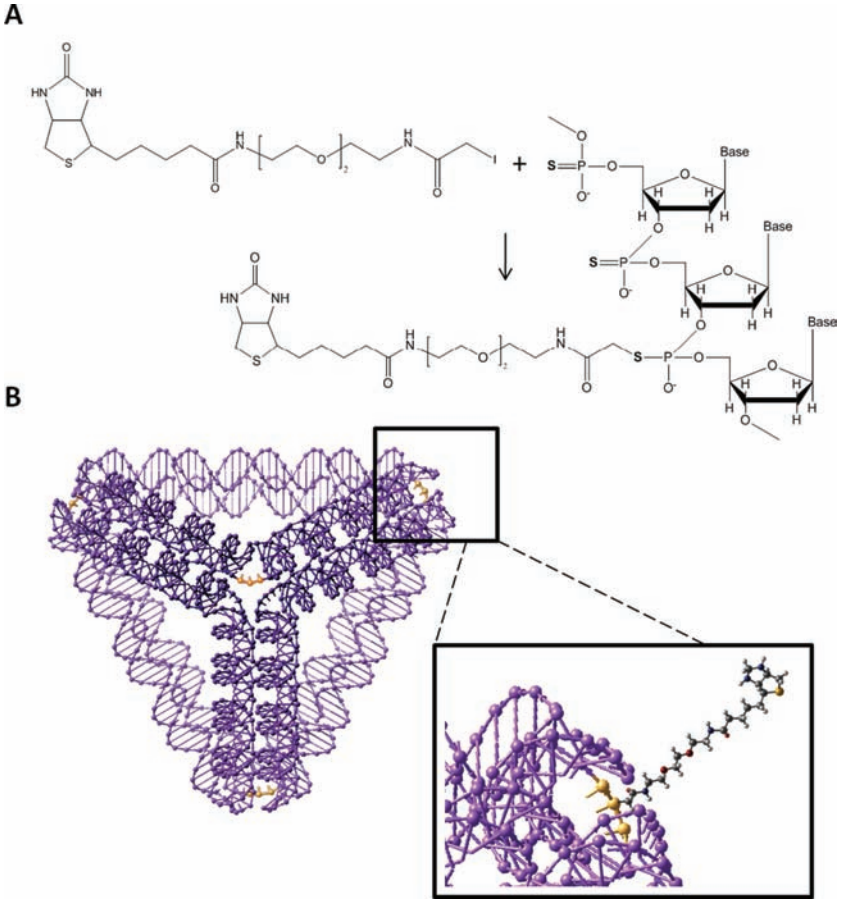

Figure 1. A) Chemical structure of a phosphorothioated DNA backbone and its subsequent covalent conjugation with the biotin polyethyleneoxide iodoacetamide bifunctional linker. B) 3D schematic of the formed tetrahedron structure with PS bonds represented in orange before and after conjugation.

higher molecular weights appear instead. These results suggest successful conjugation of one, two, three, and four proteins onto the DNA nanostructure. Since the streptavidin protein has four binding sites for biotin, the concentration of protein added was 16 times the concentration of tetrahedra to encourage the binding of multiple proteins to a single nanostructure while discouraging the formation of single protein bound to multiple nanostructures. It is interesting to note that while we achieved 100% conjugation of protein to single-stranded PS-DNA, the yield of tetrahedron structures modified with four streptavidin molecules was significantly lower than expected (see Supporting Information). This is due to a combination of steric effects and thermal hydrolysis of the biotin linker during the annealing process.

To further confirm the successful conjugation of protein to the discrete nanostructures, the unmodified structure and the band corresponding to a tetrahedron with one protein attachment was purified by nondenaturing PAGE, recovered, and analyzed using AC-AFM. In the absence of streptavidin, the AFM image of the assembled DNA strands containing PS modification and bifunctional linker shows well-dispersed and uniform structures with a height of approximately 2 nm and a lateral dimension of 25 nm (**Figure 3**). These results are similar to what

Figure 2, only a single band was observed in lanes 1, 2, and 3, suggesting that both the PS-modified and biotin-linker-functionalized central strands self-assembled with the middle and short strands into a single complex, in good agreement with previous reports and confirms that non-linker-functionalized PS-DNA structures do not interact with streptavidin.[11]

After successfully attaching biotin bifunctional linkers on the DNA tetrahedron, we further explored their applications in protein assembly. The biotin-linker-modified DNA tetrahedron was incubated with an excess of streptavidin overnight and characterized by nondenaturing PAGE and alternating current atomic force microscopy (AC-AFM). As shown in lane 4 of Figure 2, the intensity of the band corresponding to the DNA tetrahedron without conjugated proteins decreases and four discrete bands with

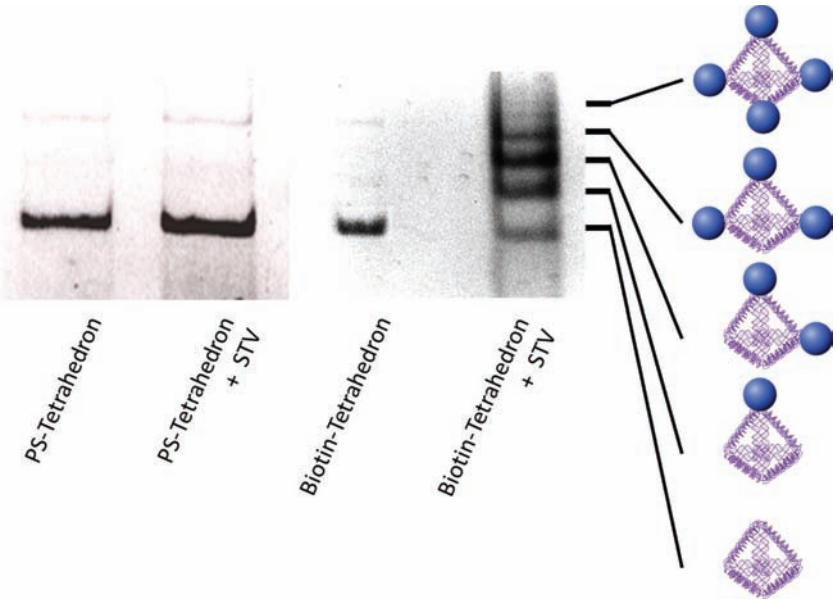

Figure 2. Gel shift assay showing the completed PS-tetrahedron before and after streptavidin (STV) incubation (lanes 1 and 2) and the biotin-functionalized tetrahedron before and after STV incubation (lanes 3 and 4).

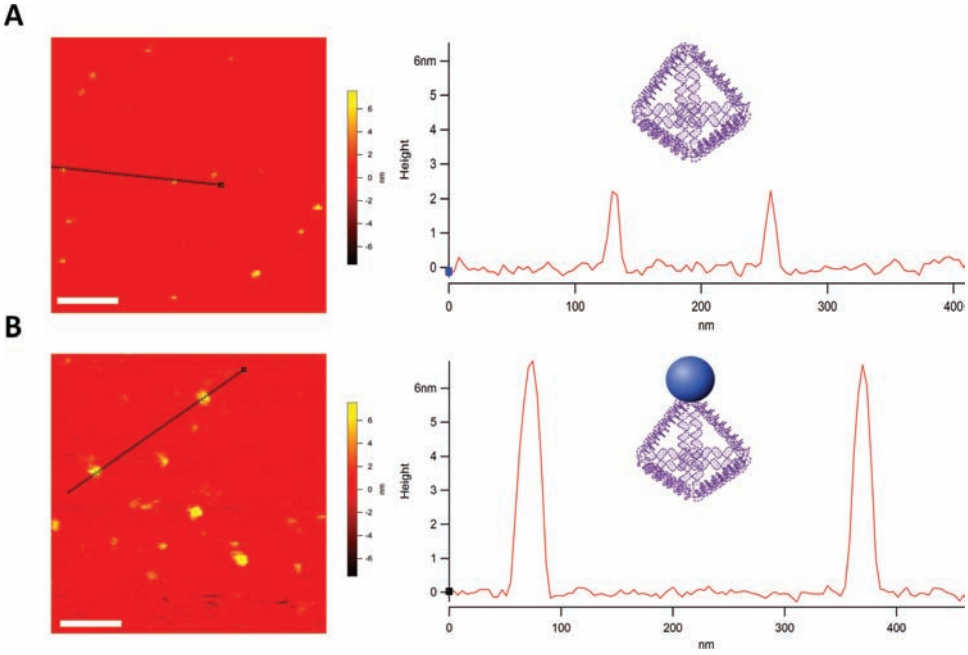

Figure 3. AFM and cross-sectional analysis of DNA tetrahedron A) before and B) after incubation with streptavidin. The nanostructures without protein are well dispersed with average height of ≈2 nm. After incubation, the nanostructure size increases by ≈4 nm to ≈6 nm, which indicates successful protein(s) conjugation, in good agreement with a measured protein size of ≈4.5 nm. The surface roughness is <0.2 nm. Scale bar: 100 nm.

was observed for a previously reported DNA tetrahedron without the PS modification or the bifunctional linker, and are consistent with a collapsed tetrahedron due to its dried state and the tapping force.[11] After incubation with protein, nanostructures recovered from the band corresponding to a tetrahedron with one bound streptavidin show structures with an increase in height to ≈6 nm, which correlates well with the addition of a ≈4 nm protein onto the tetrahedron. Additional evidence for protein conjugation to the DNA tetrahedron scaffold can be obtained from observation of 2D structures when the same samples were imaged in fluid, which shows larger, unfolded hexagonal arrays formed from three-point star components of the tetrahedron and selective protein attachment to the vertices (see Supporting Information).

In conclusion, we have demonstrated a novel functionalization and protein decoration of a 3D DNA polyhedron via a phosphothioated DNA backbone and short bifunctional linkers. Linker-functionalized PS-DNA was introduced as a component to the DNA tetrahedron without further optimization in the design of the structure; the modified structure exhibited similar stability and conformation to the unmodified structure. Phosphorothioate modification of DNA is a simple and general technique for the immobilization of nanomaterials onto any DNA structure and could become a general platform in the development of structural DNA nanotechnology.

Experimental Section

Materials: All oligonucleotides were purchased from IDT, Inc. (Coralville, IA) with HPLC purification. Biotin polyethyleneoxide iodoacetate and buffer components were purchased from Sigma–Aldrich and used as received. Illustra NAP-5 columns were purchased from GE Healthcare (17–0853-01). Streptavidin was purchased from Roche Applied Science (11721666001). Avidin agarose for purification of biotin-modified DNA was purchased from Thermo Scientific (20219).

Formation of DNA Complexes: The TAE-Mg^{2+} buffer contained 40 mM Tris base (pH 8.0), 20 mM acetic acid, 2 mM EDTA, and 12.5 mM magnesium acetate. The three component strands of the tetrahedron (central, middle, and short strands) were mixed in a 1:3:3 ratio, heated to 95 °C, and allowed to cool to room temperature over 24 h. DNA samples were annealed at 60 nM concentration and directly used after assembly for AFM imaging and concentrated to 2 μM for gel electrophoresis experiments. For gel purification experiments, DNA tetrahedra were concentrated to 2 μM, treated with a 16× excess of streptavidin, and allowed to incubate at 4 °C for 12 h.

Nondenaturing PAGE: Gels containing 4% polyacrylamide (19:1 acrylamide/bisacrylamide) were run on an Owl Adj2 electrophoresis unit at 4 °C (4 W, constant wattage). The running buffer was 1× TAE-Mg^{2+} buffer. After electrophoresis, the gels were stained with ethidium bromide (Sigma) and imaged under UV illumination. DNA recovery was performed by cutting and soaking gel pieces with 1× TAE-Mg^{2+} buffer for 12 h at 4 °C.

AFM Imaging: A drop of DNA solution (3 μL) was spotted onto a freshly cleaved mica surface, and kept for 10 s to achieve strong adsorption. The mica surface was then washed with 30 mM magnesium acetate solution and dried by nitrogen. DNA samples were imaged in AC mode on an Asylum Cypher AFM instrument in air, using oxide-sharpened silicon probes having a resonance frequency in the range of 280–320 kHz (MikroMasch–NSC15). Fluid imaging was performed using nitride probes having a resonance frequency of 21–52 kHz (Olympus TR400PSA). The tip–surface interaction was minimized by optimizing the scan set-point to the

highest possible value. AFM imaging was performed at 22 °C. AFM data were processed with IGOR Pro software.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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