

Controlled Alignment of Multiple Proteins and Nanoparticles with Nanometer Resolution via Backbone-Modified Phosphorothioate DNA and Bifunctional Linkers

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Proteins are an important class of biomolecules with a wide range of applications in fields such as therapeutics, diagnostics, and catalysis.¹ While their use in the ensemble solution state has been well established, the precise control and alignment of proteins on the nanometer scale have only recently been explored.² Such protein assemblies hold great promise in proteomics, medicine, bioenergy, nanoelectronics, and photonics.

To achieve the precise, nanoscale alignment of proteins, one needs both a suitable template and an efficient conjugation method. DNA nanostructures are ideal choices as templates because of their programmable and readily modifiable properties.³ Proteins are commonly attached to the ends of DNA templates.⁴ While technically easy to synthesize, end-modified DNA is limited in its versatility, as it is not readily applicable to place proteins at any position, such as in the middle, of the DNA strand. To overcome this problem, researchers have used modified DNA bases such as biotin-dT to introduce functional groups inside the strand.^{2b,c} However, this modification significantly disrupts the Watson–Crick base pairing of DNA and dramatically inhibits the formation of more complex nanostructures. To reduce base-pairing disruptions, proteins have also been conjugated at positions modified with enzymes⁵ or with polyamide conjugates in the minor groove of DNA.⁶ While these modifications increase the number of positions where DNA can conjugate, there are still limitations. For example, the enzymes used to internally modify DNA strands recognize specific DNA sequences, which restricts the position where the protein can be placed.

Phosphorothioate (PS) DNA is a derivative of DNA which has one of the nonbridging oxygens replaced by a sulfur atom. Thanks to the advances in biotechnology, the replacement can be readily achieved at any position on the DNA with little effect on the overall stability of the DNA. This modification results in an enhanced resistance to nuclease degradation.⁷ Since the PS group is more reactive than phosphate,⁸ we reported a short thiol-containing bifunctional linker that was attached to a gold nanoparticle (AuNP) on one end and was PS-functionalized on the other end.⁹ This method enables specific binding to PS on one end while attaching to a gold nanoparticle (AuNP) on the other via Au–thiol interaction, resulting in the directed assembly of a AuNP dimer whose distance can be controlled within a few nanometers. To realize the true potential of this method, however, one needs to develop different bifunctional linker chemistries for the precise positioning of different species onto the same DNA template beyond dimer control and AuNP conjugation.

Herein we present the assembly of multiple proteins onto the backbone of a double-stranded DNA (dsDNA) using bifunctional

Scheme 1. Schematic and Chemical Representations of Protein Assembly onto a Phosphorothioate DNA Template Using Short Bifunctional Linkers (BL)^a

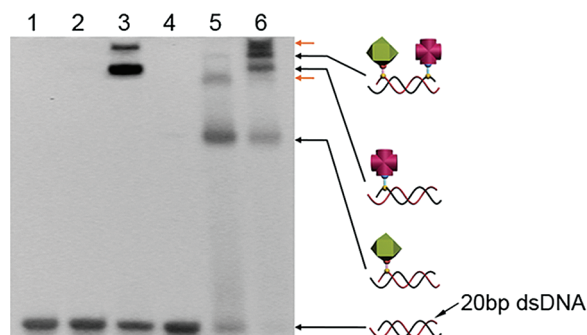
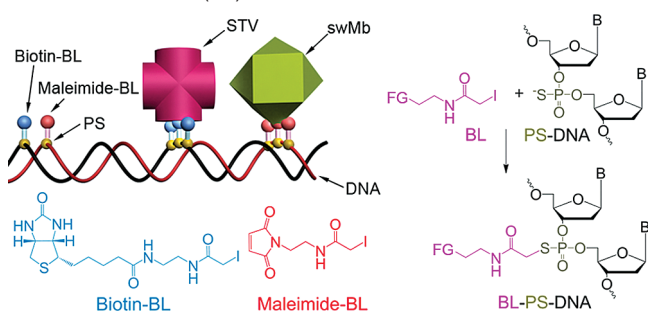


Figure 1. Gel shift assay (4–20% native PAGE, EtBr stained) showing the separation between the DNA–protein conjugate from free DNA. Lane 1: PS-dsDNA control. Lane 2: PS-dsDNA with STV (no BL). Lane 3: biotin-BL-dsDNA with STV. Lane 4: PS-dsDNA with swMb (no BL). Lane 5: maleimide-BL-dsDNA with swMb. Lane 6: dsDNA containing both biotin and maleimide BLs with STV and swMb. The gold arrows on the right indicate bands of minor components of multiple STV-DNA and swMb-DNA conjugates (such as in lanes 3, 5, 6).

linkers (BL) with unique binding ends. By introducing multiple linkers on dsDNA, the numbers and positions of proteins conjugated could be controlled. Furthermore, we showed the activity of conjugated proteins by binding biotinylated AuNPs onto preassembled STV–dsDNA complexes, resulting in AuNP dimers and trimers with controlled distances. More importantly, through a melting temperature study, we showed that the protein–DNA assembly conjugated via our backbone modified PS-DNA system is much more stable than the base-modified system placed in the middle of a dsDNA strand, demonstrating superior properties of this system for practical applications.

To demonstrate the controlled and specific alignment of different proteins using different bifunctional linker chemistries, we chose streptavidin (STV, 52.8 kDa) and sperm whale myoglobin (swMb,

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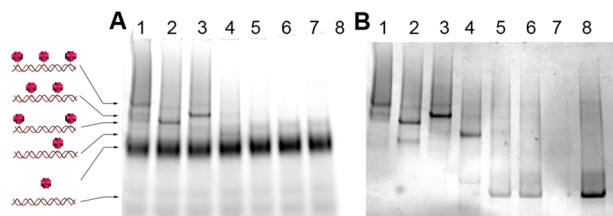


Figure 2. Multiple STV attachment onto 100 bp dsDNA, demonstrated by native PAGE. Images of (A) fluorescein-labeled STV ($\lambda_{\text{excit}} = 473$ nm and $\lambda_{\text{em}} = 520$ nm) and (B) EtBr-treated DNA ($\lambda_{\text{excit}} = 530$ nm and $\lambda_{\text{em}} = 580$ nm) after conjugation. Both gel images were taken from the same gel using different excitation and emission wavelengths. Lanes 1–5: 3BS-dsDNA (3 binding sites (BS) on DNA with 42 bp distances), 2BS-dsDNA (80) (2BSs on DNA with 80 bp distance), 2BS-dsDNA (50) (2BSs on DNA with 50 bps distance), 1BS-dsDNA (1BS on DNA), and Biotin-BL treated 0BS-dsDNA (no BS on DNA) treated with STV. Lane 6: Biotin-BL untreated 0PS-dsDNA with STV. Lane 7 and 8: Unreacted STV and 0BS-dsDNA, respectively. Once dsDNA binds to STV, the conjugate should be observed in both gel images A and B.

16.7 kDa) as model proteins. Our linker contained an iodoacetamide group on one end and a biotin on the other (biotin-BL, Scheme 1) to conjugate STV. By replacing the biotin with a maleimide group on the binding end, the second bifunctional linker (maleimide-BL) can be used to conjugate swMb with a cysteine mutation on the surface. To investigate the interaction between biotin-BL-treated PS-dsDNA (biotin-BL-dsDNA) and STV, the 20 base-pair (20 bp) DNA was reacted with STV and the mixture was imaged by native 4–20% polyacrylamide gel electrophoresis (PAGE). Distinct bands of the STV–dsDNA conjugate (~65 kDa) were observed in the presence of biotin-BL, but not in its absence (Figure 1, lanes 2, 3). Similarly, distinct bands of swMb–dsDNA (~29 kDa) appeared in the presence of maleimide-BL but not in its absence (lanes 4, 5). This result confirms the conjugation of both proteins onto dsDNA with BL treatment. More importantly, a dsDNA containing biotin-BL and maleimide-BL in each complementary strand could interact with both proteins, forming an even larger heteroprotein structure (~82 kDa, lane 6).¹⁰

Since multiple PS modifications can be introduced at any desired place on a DNA backbone, we sought to use the PS-DNA to control the numbers and positions of proteins conjugated to a DNA strand. To demonstrate the versatility of this technique, 100 bp dsDNAs (~34 nm) with 1–3 binding sites (BSs)¹¹ were reacted with STV and purified by native 4–20% PAGE (see Figure 2). The fluorescein label on STV (STV-FITC) made it possible to track the STV's position independently from the DNA. As shown in lanes 1–4, if a DNA with at least one BS reacts with STV, an identical band appears in both Figure 2A and B, indicating the assembly of an STV–dsDNA conjugate. In contrast, control samples without a PS modification showed negligible interaction between the biotin-BL and DNA (lanes 5, 6). More importantly, as the number of BSs placed on the DNA increased from zero to one, two, or three, a series of increasing higher molecular weight bands were formed (lanes 1–4). This result suggests that as the number of BSs placed on the DNA increases, the number of STVs conjugated to DNA increases as well. Interestingly, when DNA strands that were identical in length, sequence, and number of BSs were used to conjugate STV, as the distance between the two BSs placed on DNA varied from 80 bp (2BS-dsDNA (80)) to 50 bp (2BS-dsDNA (50)), their electrophoretic mobility changed. The distinct positions of the bands may be due to different conformations of the complexes or possibly the bending of DNA after its conjugation with a protein.

Once attached to DNA, it is important for the proteins to maintain their activities so the protein–dsDNA complex is available as a

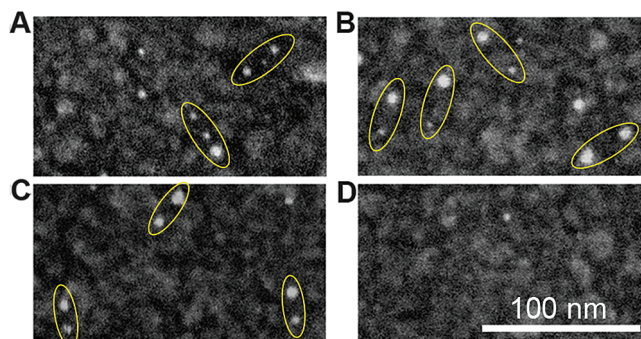


Figure 3. Electron microscopic images of AuNP trimers and dimers formed on STV and dsDNA conjugates. Biotinylated AuNPs were assembled on (A) 3BS-dsDNA, (B) 2BS-dsDNA (80), (C) 2BS-dsDNA (50), and (d) 0BS-dsDNA, all after treatment with STV.

template for other functions. To explore the conjugated proteins' activity, biotinylated AuNPs were treated with STV and 100 bp dsDNA conjugates preimmobilized on silicon surfaces. Figure 3A shows an electron microscope image of AuNP trimers formed on STV–3BS-dsDNA templates. A large portion of AuNPs formed trimers separated by ~13 nm on average, which is close to the predicted distance of ~14.3 nm. To show distance control, 2BS-dsDNA (80) and 2BS-dsDNA (50) with the binding sites separated by 80 and 50 bps were used (Figure 3B and C). The average distances between AuNPs of dimers were ~26 and ~16 nm, respectively, which is close to the predicted distances between the two binding sites of 2BS-dsDNA (80) (~27.2 nm) and 2BS-dsDNA (50) (~17 nm) (see Figure S7 for statistical analyses). Without the PS modification, only a few AuNPs were observed (Figure 3D). These results indicate that STVs can indeed bind to DNA templates and form trimers and dimers with controlled distances.

The stability of the protein–dsDNA assembly is important for practical applications. Under identical conditions (5 mM phosphate, 10 mM NaCl buffer, pH 7), the STV–dsDNA conjugate with biotin-BL and PS modification had a melting temperature ~17 °C higher than that with biotin-dT (Figure S8). This surprisingly large difference strongly suggests that protein–DNA assemblies formed with PS modifications and BLs are more stable than those formed with modified bases.

In summary, we have demonstrated a general method for the precise and specific alignment of different proteins using phosphorothioate DNA and different short bifunctional linkers. This method allows for high yield conjugation of multiple proteins to DNA with nanometer resolution. The linker can be designed to conjugate any proteins and nanomaterials specifically for a wide range of applications.

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Supporting Information Available: Experimental details and additional data from PAGE, electron microscopy, distance control statistics, and melting curves are included. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (10) The top band in lane 6 may be composed of either one or both minor components of the proteins that have a larger mass.
- (11) Each BS contains triple biotin-BL labels on three adjacent PSs since triple labeling on one BS significantly increased the reaction yield to ~100% on 20 bp dsDNA (see Figure S3). Only one STV binds to one BS (see Figure S4).

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