

Progress toward Plug-and-Play Polymer Strings for Optical Tweezers Experiments: Concatenation of DNA Using Streptavidin Linkers

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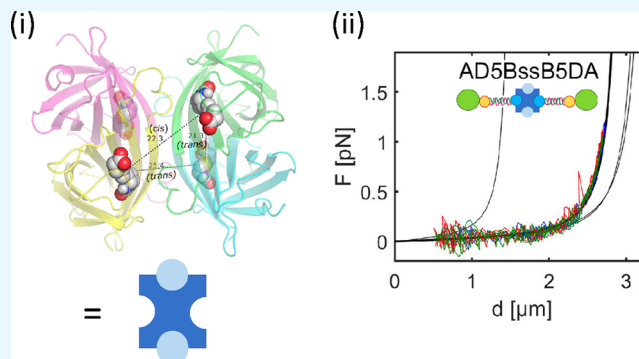
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ABSTRACT: Streptavidin is a tetrameric protein that is renowned for its strong binding to biotin. The robustness and strength of this noncovalent coupling has led to multitudinous applications of the pairing. Within the streptavidin tetramer, each protein monomer has the potential to specifically bind one biotin-bearing moiety. Herein, by separating various streptavidin species that have had differing numbers of their four potential binding sites blocked, several different types of “linking hub” were obtained, each with a different valency. The identification of these species and the study of the plugging process used to block sites during their preparation were carried out using capillary electrophoresis. Subsequently, a specific species, namely, a *trans*-divalent linker, in which the two open biotin-binding pockets are approximately opposite one another, was used to concatenate two ~ 5 kb pieces of biotin-terminated double-stranded DNA. Following the incubation of this DNA with the prepared linker, a fraction of ~ 10 kb strings was identified using gel electrophoresis. Finally, these concatenated DNA strings were stretched in an optical tweezer experiment, demonstrating the potential of the methodology for coupling and extending molecules for use in single-molecule biophysical experiments.



INTRODUCTION

Single-molecule studies have continued to grow in popularity as technological capability improves and experimental platforms for their application become more available. While typically challenging compared to traditional bulk techniques, single-molecule studies offer unprecedented access to the world of molecular biology and deep insights into the functioning of molecular machines in the noisy thermal bath in which they operate. Optical tweezers (OT) have provided a key experimental tool for the pursuit of such studies, acknowledged recently by the award of a Nobel prize.

For several decades, DNA has been the workhorse of single-molecule experiments, especially those carried out with OT. This is not only due to DNA's intrinsic biological relevance but also due to the facile control of its size and sequence and the simplicity of attaching “handles” to the chain termini. DNA–protein conjugates have also previously been employed to help understand the behavior of proteins at the single-molecule level.^{1–5} However, similar studies on other important biological macromolecules, such as polysaccharides, have somewhat fallen behind. This is despite the clear need for such experiments in samples where, more often than not, subpopulations of heterogeneous samples exhibit different properties that are masked in bulk studies. A generic strategy that enables the generation of a “plug-and-play” string format and facilitates the study of shorter molecules with more

challenging attachment chemistry would represent significant progress toward ameliorating this difficulty.

Streptavidin–biotin is one of the most widely known noncovalent binding pairs and can be used, among other things, to perform biomolecule conjugation. With a dissociation constant, K_d , of ~ 0.1 – 10 fM, streptavidin–biotin conjugation has found wide versatility in biotechnology, including in applications from biosensors to cell biology.⁶ Streptavidin is a homotetrameric protein, with each monomer able to bind one biotin-displaying molecule.⁷ The binding of biotin to streptavidin has been found to be noncooperative, but with every additional binding of biotin, the structural and thermodynamic stability of the protein itself increases.^{8–10} Being able to control the number of active valencies that the tetramer displays opens up potential pathways to further applications by allowing hubs of known valency (1, 2, 3, or 4) to be formed. Previous attempts of this type have largely involved using genetic modification techniques to produce

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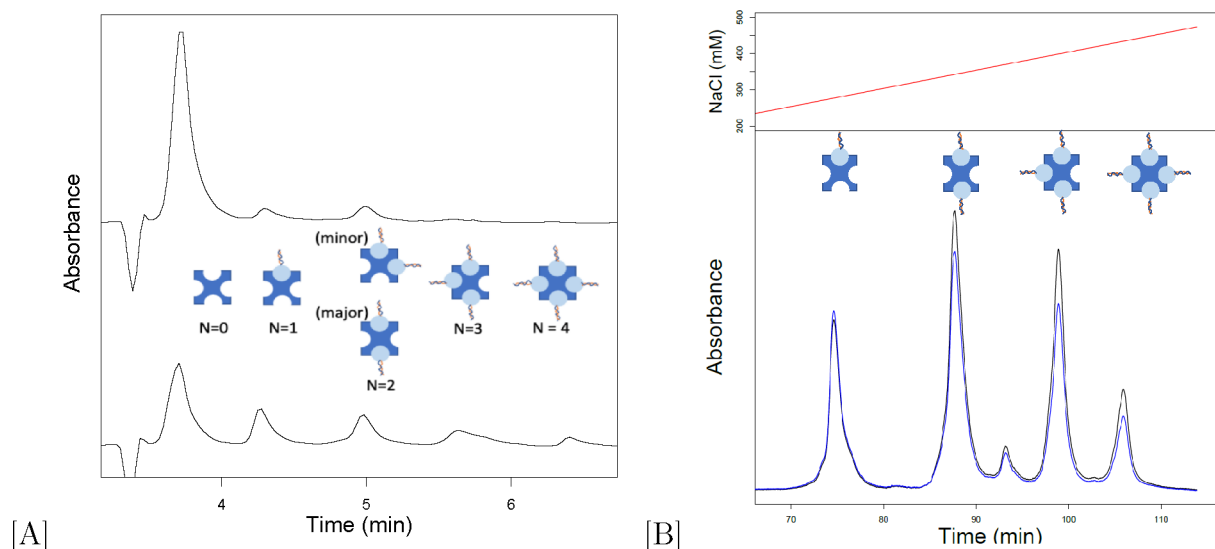


Figure 1. (A) Species formed when biotin-terminated 12 bp dsDNA was incubated with tetravalent streptavidin at different ratios (1:1 (top) and 4:1 (bottom)). Absorbance at 192 nm was recorded. (B) Separation of the different tetrameric species formed after overnight incubation of streptavidin with biotin-terminated 12 bp dsDNA, by anion-exchange chromatography, permitting fraction collection. The weakest band is tentatively assigned to a *cis*-divalent species. Absorbances at 260 nm (black) and 280 nm (blue) were recorded.

monomeric mutants that are not capable of biotin binding but are still successfully incorporated into the tetramer.^{11,12} Furthermore, designed modifications of the binding properties of monomeric and dimeric streptavidin have also been carried out.^{13–16} Other methodologies that have sought to reduce the possible number of biotin-bearing molecules that can bind to tetramers have used preliminary incubations of streptavidin with biotin-terminated “plugging molecules”. In this way, by controlling the stoichiometric ratios of components and the incubation time, partially filled monovalent, divalent, and trivalent streptavidin species can all be formed.^{17,18}

A separation technique using anion-exchange chromatography for isolating different tetrameric “hubs” from streptavidin samples with different amounts of the potential binding sites blocked has previously been reported.¹⁷ Specifically, after incubating streptavidin with 12 bp double-stranded DNA (dsDNA) molecules carrying a biotin moiety at one end, species with different numbers of the binding sites filled were successfully separated using an anion-exchange chromatography column. By incorporating a photocleavable (PC) 2-nitrobenzyl linker between the 12 bp oligonucleotide and the biotin, it was then possible to remove the separation-facilitating DNA oligomers post fraction-collection, leaving just the “biotin plugs”. This enabled the generation of a series of “preplugged” streptavidin species with certain sites passivated and others available for binding biotin-displaying species.

Herein, we perform experiments of this type but monitor the valency of the different species and the site-plugging process, using capillary electrophoresis (CE), in contrast to traditional gel chromatography. Based on our results, different valency species were resolved much more clearly in CE than in gels. Moreover, the *trans*-divalent streptavidin species collected here were incubated with ~5 kb biotin-terminated DNA strands. Following the incubation, concatenated ~10 kb strings were detected using gel electrophoresis and, furthermore, were successfully stretched between beads in a traditional dual-trap dumbbell-style DNA-stretching experiment.

RESULTS AND DISCUSSION

Observing Streptavidin–dsDNA (12 bp) Conjugates with CE. Streptavidin was mixed at varying stoichiometric ratios with 12 bp dsDNA oligomers (functionalized with biotin at one end via a photocleavable linker). Different ratios of dsDNA/tetravalent streptavidin (approximately 1:1 and 4:1) were incubated overnight and then analyzed by CE. The resulting electropherograms are shown in Figure 1A. By considering the different possible products of the incubation, such as free streptavidin ($N = 0$) and streptavidin with one ($N = 1$), two ($N = 2$), three ($N = 3$), or four ($N = 4$) biotin-terminated DNA oligomers bound and how the relative concentrations of these species would be expected to change as more DNA is added, the five peaks in the electropherogram were preliminarily identified (see schematics in the figure). As shown in due course (Figure 6), there are three possible configurations (two *trans*-divalent and one *cis*-divalent) for the divalent product. Considering the evidence that the *cis*-divalent species is less stable due to steric hindrance,¹⁹ the *trans*-divalent products were expected to be dominant, with a minor quantity of *cis*-divalent streptavidin. Both possible *trans*-divalent species would be expected (i) to be populated similarly, as neither offers steric advantage, (ii) to be indistinguishable by electrophoretic techniques, and (iii) to stretch similarly in OT experiments. Statistically in plugging *trans* sites, both interdimer and intradimer *trans* arrangements are equally likely. The presence of distinct *trans* species, resulting from substantial tetrahedral distortion of the streptavidin homotetramer from the canonically depicted square-planar arrangement, does not appear to have been considered in prior work of tethering oligomers to streptavidin.

At the neutral pH of the CE separation, the streptavidin ($pI = 5$) is slightly negatively charged.²⁰ Under the CE conditions used, anionic species are dragged toward the cathode by the electro-osmotic flow (EOF). The downward peak at around 3.5 min provides a neutral marker (formed here by the refractive index change from injection plug solvent passing the detection window) and monitors the value of the EOF.²¹ Peaks

from positively charged species will migrate earlier than the EOF, and peaks from negatively charged species will be observed later. As the negative charge density of the complexes increases as an increased number of biotin-terminated nucleotides become bound, the migration time increases. Any unreacted DNA oligomers present elute considerably later owing to their high negative charge and small size and are not seen here. Based on the electrophoregrams (Figure 1A), a roughly 4:1 ratio of oligonucleotide species to streptavidin was adopted to produce an ideal distribution of multivalent species and a high yield of divalent species for use in further trials.

The migration of species in the CE experiments was monitored using UV irradiation at several wavelengths including 192, 260, and 280 nm. DNA has a characteristic absorbance at 260 nm owing to its highly conjugated purine and pyrimidine bases, whereas proteins have a characteristic absorbance at 280 nm specific to the tryptophan and tyrosine moieties in proteins.^{22,23} Although using 260 and 280 nm is advantageous in monitoring DNA and proteins, respectively, the maximum UV absorbance over the wavelengths monitored was found at 192 nm (Figure 1A), reflecting the presence of the peptide bonds in the amino acids at ~205–220 nm.^{24,25} Hence, the absorbances at 192 nm were selected for rendering the electrophoregram figures (unless specified otherwise). The electrophoretic mobilities of the species observed in Figure 1A were calculated according to

$$\mu_{\text{ep}} = \frac{L_{\text{tot}} \cdot L_{\text{eff}}}{V} \cdot \left(\frac{1}{t_{\text{mig}}} - \frac{1}{t_{\text{EOF}}} \right) \quad (1)$$

where μ_{ep} is the electrophoretic mobility of species, L_{tot} is the total length of the capillary (0.485 m), L_{eff} is the distance between the point of injection and point of detection (0.40 m), V is the applied voltage (25 kV), t_{mig} is the migration time of species, and t_{EOF} is the migration time of neutral species (electro-osmotic flow) (3.35 min), as shown in Table 1:

Table 1. Electrophoretic Mobilities, μ_{ep} , of the Various Streptavidin/DNA Conjugates Shown in Figure 1A (Uncertainties Are Estimated at ~5%)

species (N)	t_{mig} (min)	$\mu_{\text{ep}}/10^{-7}$ ($\text{m}^2 \text{V}^{-1} \text{s}^{-1}$)
0	3.68	-2.07
1	4.23	-4.82
2	4.93	-7.42
3	5.56	-9.21
4	6.32	-10.89

Fraction-Collecting Streptavidin–dsDNA (12 bp) Conjugates of Specific Valency. While CE requires only tiny amounts of sample and resolves the differing species of interest well, in a relatively short time, its application as a preparative technique is limited. To collect specific partially plugged species types, anion exchange chromatography was performed as described in Experimental Procedures. The resulting chromatogram following injection of the incubated sample is shown in Figure 1B.

This result compares well with that previously reported¹⁷ and allowed known partially plugged species types to be collected. As expected, there are two peaks observed for the divalent species, and following the original paper, the major peak is taken to be the *trans*-divalent species. The collected specific valency streptavidin–DNA oligomer complexes were

then run in CE experiments to confirm the previous peak assignment shown in Figure 1A. A selection of these experiments is shown in Figure 2, which serves to highlight the utility of CE for the separation and monitoring of these species.

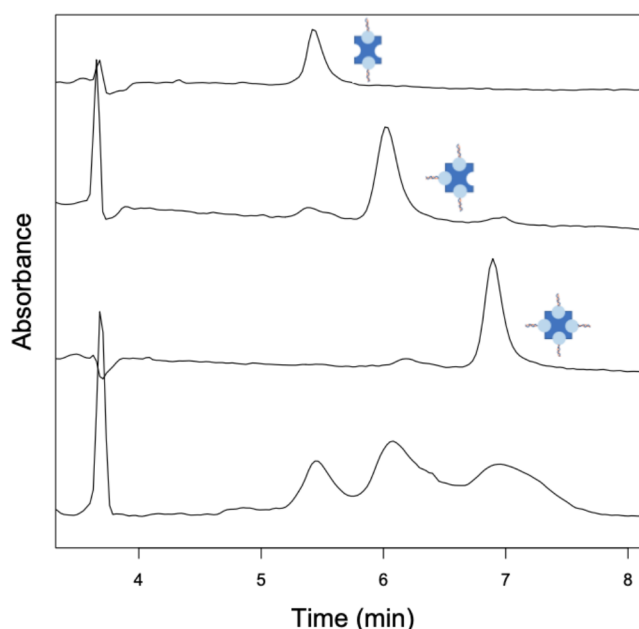


Figure 2. Electrophoregrams of the fraction-collected divalent ($N = 2$), monovalent ($N = 3$), and fully filled streptavidin ($N = 4$) fractions (obtained from the HPAEC column and labeled schematically), injected separately and co-injected (bottom) into the CE setup.

When comparing Figures 1A and 2, it is worthwhile to mention a few points relevant to the interpretation of CE electrophoregrams. First, it is not uncommon for the magnitude of the EOF to vary slightly, even between runs with nominally identical conditions, owing to its exquisite sensitivity to the zeta-potential of the capillary walls that is difficult to regenerate exactly between successive runs.^{26,27} This means that the migration times of peaks representing species of interest (and thereby the observed resolution of different species) may be slightly different between runs. It should be noted, however, that the physical parameter distinguishing the electrophoretic transport of the analytes, the electrophoretic mobility, is of course unchanged, as can be confirmed by its calculation, which includes the migration time of the EOF. Second, depending on the relative composition (and thereby refractive index) of the sample and the background electrolyte (BGE), the EOF-reporting peak can appear different in size and even negative or positive.²⁸ Here, it is seen negative-going in Figure 1A but positive-going in Figure 2, owing to the different solution conditions for the diluted fraction-collected samples.

Post Fraction-Collection Cleavage of dsDNA (12 bp) from Streptavidin Conjugates. Once the fractions were successfully collected, the oligonucleotide tails were cleaved off the separated species by UV irradiation to avoid any possible undesired interactions of subsequent molecules targeted for binding with those used in passivating the other sites. Figure 3A shows the products expected to be formed during the time course of UV radiation exposure when starting with the divalent species (a, top), the monovalent (b, middle), and the

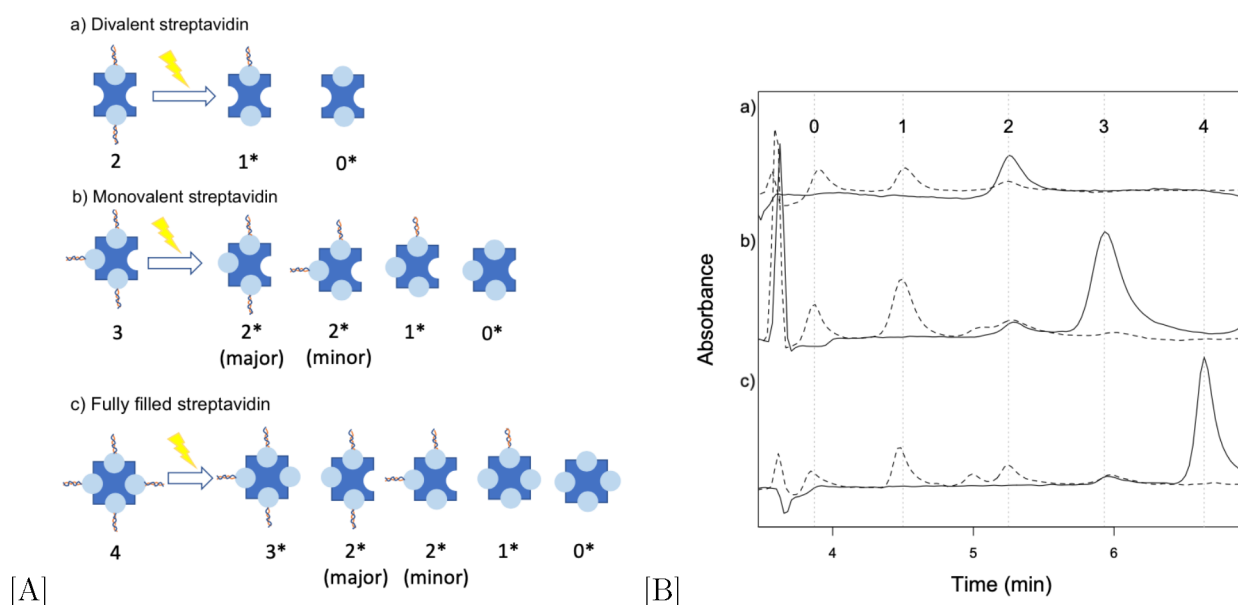


Figure 3. (A) Multiple species expected to be formed during UV irradiation of the different starting streptavidin–12 bp dsDNA conjugates. (B) Electrophoregrams monitoring the species present after 60 s UV irradiation of different starting streptavidin–12 bp dsDNA conjugates (solid line represents the electrophoregram of reactants before UV, and dotted line represents the electrophoregrams of products after UV).

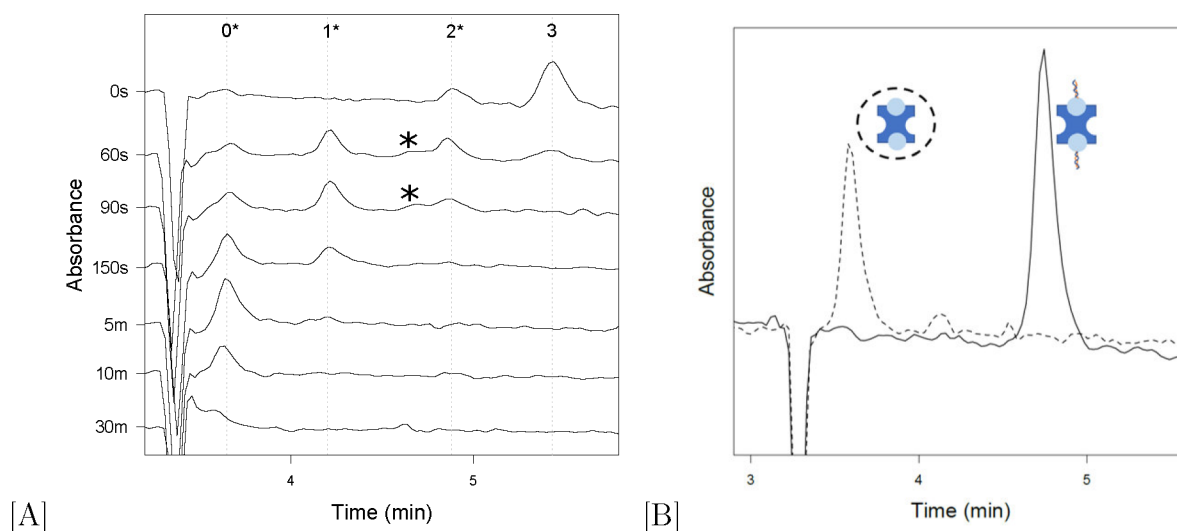


Figure 4. Electrophoregrams monitoring the species present (A) with different times of UV irradiation of monovalent streptavidin–dsDNA (12 bp) ($N = 3$) conjugates (* indicates presence of *cis*-divalent species), (B) after 5 min UV irradiation of divalent streptavidin–dsDNA (12 bp) ($N = 2$) conjugates.

fully filled streptavidin conjugate (c, bottom). On cleaving the linkage between the biotin and the DNA, this process leaves each streptavidin binding site that was originally occupied by the 12 bp dsDNA oligomer with only the biotin stub in the pocket. This essentially plugs the binding site so that it is not available for further interaction. The figure shows the possible mixture of species that would be expected to be found during the cleavage process before its completion. For example, as the oligonucleotide is shaved off the divalent streptavidin species (Figure 3A(a,top)), a maximum of three species can be present (specifically, the original streptavidin carrying two biotin–12 bp dsDNA tails; streptavidin carrying one biotin–12 bp dsDNA tail and one biotin plug; and streptavidin carrying two biotin plugs). Similarly for monovalent streptavidin, a total of five possible products are expected (Figure 3A(b), middle), and for the initially fully filled streptavidin, there are six (Figure

3A(c), bottom). All of the species that could possibly be obtained upon UV irradiation are labeled as N^* , where N is the number of 12 bp dsDNA oligomers still present in each species, and * denotes that UV was instrumental in the generation of the species.

Figure 3B shows the results from the corresponding experiment, using CE to monitor the species present and their relative concentrations after irradiating the sample for 60 s, as described in Experimental Procedures (electrophoregrams of starting and irradiated samples are shown as solid and dotted lines, respectively). As alluded to earlier, the electrophoretic mobility of the species along with the electro-osmotic flow determines the migration time in CE, depending primarily on the ratio of the charge to the hydrodynamic friction coefficient of the analytes. Once the 12 bp dsDNA is removed, the streptavidin binding site it once occupied simply contains a

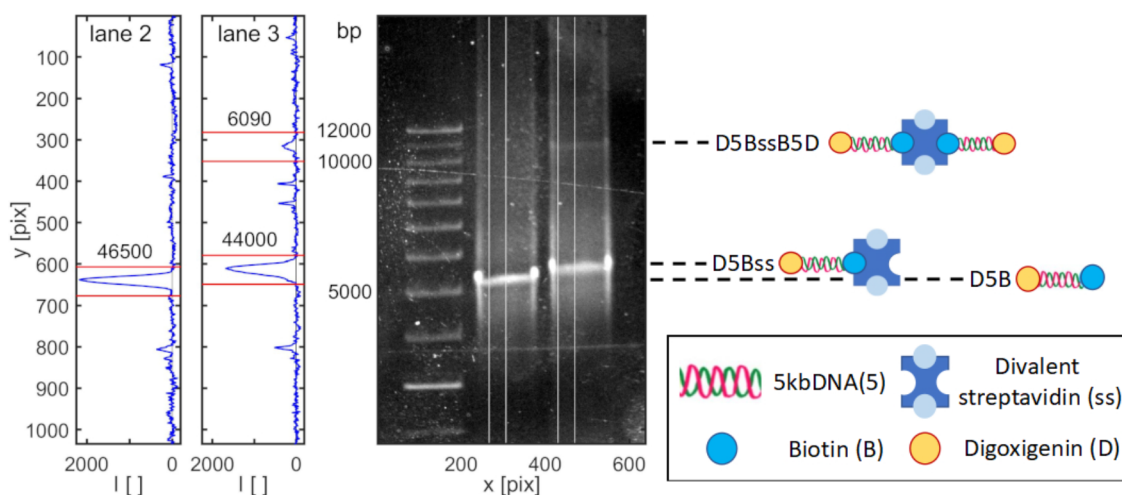


Figure 5. Gel electrophoresis experiment showing a standard size ladder (Invitrogen 1 kb + DNA ladder) in lane 1, the 5 kb biotin-terminated DNA in lane 2, and the results of an incubation of the divalent streptavidin with the 5 kb biotin-terminated DNA in lane 3. Background-corrected integrated intensity profiles for lanes 2 and 3, between the pairs of vertical white lines indicated on the image of the gel, are shown to the left. Integrated intensities along these profiles, between the red lines indicated, are reported in the figure. Schematics of the species observed are also shown on the right.

neutral biotin stub, which is not expected to significantly affect the electrophoretic transport behavior of the complex in which it resides. Hence, when cleavage of all the oligonucleotides is complete, all the streptavidin conjugates, no matter how many plugged sites they contain, would be expected to migrate past the detection window at the same time, with a mobility comparable to pure streptavidin (position 0). Similarly, species that at some point during the cleavage process only possess one nucleotide tail (and any number of biotin plugs in other sites) would be expected to have the same electrophoretic mobility as the starting trivalent streptavidin (position 1) species, and so on and so forth. The experimental results shown in Figure 3B indeed follow the expected progression mapped out in Figure 3A, confirming the generation and correct identification of the starting species, as well as the UV-induced removal of the oligonucleotides. It is worth noting that the irradiation of fully filled and monovalent streptavidin yields an additional peak intermediate between those designated 1 and 2. Based on the position of the peak, it seems likely that this is a *cis*-divalent streptavidin species, which could not be distinguished on a traditional gel.

Time Course of Removal of dsDNA (12 bp) from Streptavidin Conjugates. Figure 3B still shows a variety of different streptavidin–biotin–dsDNA conjugates present in samples after 60 s of UV irradiation, indicating that this reaction time was insufficient to cleave all oligonucleotides from the conjugates. Differing UV exposure times were subsequently applied to find the time required to remove all oligonucleotide tails and thereby generate the sought-after streptavidin species with known numbers of biotin-plugged or available biotin-binding sites. The oligonucleotide removal is illustrated further in Figure 4A for the HPAEC-collected monovalent streptavidin species (the tetramer with three biotin–dsDNA oligomers initially bound). The complex was subjected to UV irradiation for a total of 30 min, and the progress of the photocleavage of the DNA tails was monitored by taking 30 μ L aliquots of the sample at different time intervals and running the sample in the CE. Figure 4A clearly shows how the peaks move to lower charge density as time progresses, and the 12 bp nucleotide tails are removed, with

the expected progression through the intermediate species. After 5 min, the majority of the oligonucleotides has been cleaved, and the streptavidin itself (now containing three “passivating” biotin plugs) still remains intact (as inferred from the similarity of the peak shape to that obtained with unadulterated streptavidin). Furthermore, the protein seems relatively stable even after 10 min of UV irradiation. Some modification of the protein itself is, however, evident at 30 min, as changes in the peak shape indicate. An interesting feature is seen \sim 4.6 min in the electrophoregrams recorded after 60 and 90 s UV irradiation. A peak manifests as a slight shoulder toward the 2* peak and is presumed to be the *cis*-equivalent of the double tail. This peak is only visible during a short time frame in the process of cleaving the tails and is not clearly observed in the formation of the different valencies, confirming that *cis*-configuration is much more unstable than any of the other configurations as previously suggested.²⁹

As the primary interest here is producing divalent streptavidin species that can be used as linking molecules, the fraction-collected *trans*-divalent streptavidin ($N = 2$) sample was subsequently exposed to UV irradiation for 5 min. Figure 4B shows the pre- and post-irradiation electrophoregrams, again confirming that, under these conditions, the oligonucleotides are removed, leaving simple biotin stubs plugging two sites and with the protein intact.

Using Divalent Streptavidin as a Linker. The results of the CE experiments performed on incubations of streptavidin with 12 bp dsDNA and on the fraction-collected samples and their subsequent UV-generated species provide confidence that specific divalent streptavidin species can be generated in large amounts and investigated for their potential as a linker molecule as described below.

Investigating the Yield of Concatenated 10 kb DNA. Initial tests of this linking functionality were carried out by incubating the *trans*-divalent streptavidin species with biotin-terminated DNA that could be visualized straightforwardly using a standard agarose gel, as described in the Experimental Procedures. For this purpose, \sim 5 kb DNA that would, upon successful concatenation, effectively result in the formation of a \sim 10 kb DNA strand was selected. Figure 5 does indeed show

the presence of a ~ 10 kb band post-incubation. The visualized band is relatively faint, however, suggesting that the yield of the concatenated species was lower than might be expected ($\sim 12\%$) for the $\sim 2:1$ ratio of streptavidin/DNA used in this experiment. To investigate the hypothesis that the first of the ~ 5 kb DNA strands binding to the streptavidin obscures the remaining binding sites, the sequential filling of the streptavidin sites with biotin-terminated DNA oligomers of different lengths was investigated. While *cis*-divalent streptavidin attachment of one DNA molecule has previously been shown to sterically and electrostatically hinder the binding of another moiety,²⁹ it seems less likely with the *trans*-divalent streptavidin used here.

The distance between the two biotin binding faces of a streptavidin tetramer is ~ 2.0 nm¹⁹ (Figure 6). Two different lengths of biotin-terminated DNA oligomer (12 bases (~ 4.0 nm) and 28 bases (~ 9.5 nm)) were used briefly to investigate the role that steric and electrostatic effects might play in the consecutive binding of biotin-terminated DNA into potential valencies (data not shown). It was found that when tetravalent streptavidin was incubated with 12 bp biotin-terminated DNA, the DNA oligomers seamlessly filled the open valencies until nearly all of the sites in the streptavidin conjugate species were completely filled. However, substantially more valencies remained unfilled when the same experiment was repeated with 28 bp DNA oligomers. This observation does suggest that, as the length of the molecules targeted to be coupled increases, steric and/or electrostatic effects will limit the yield of the concatenated species derived.²⁹ It is worth noting that, despite the low yield, two large (micron-sized) DNA pieces can be successfully concatenated in this way in sufficient quantities to undertake single-molecule stretching experiments, for example, by optical tweezers.

Stretching Streptavidin-Linked Strings in a Dual-Trap Optical Tweezers Setup. DNA concatenation via the divalent streptavidin linker (ss) was additionally verified by stretching the formed DNA string using optical tweezers. The setup for the experiment is illustrated in Figure 7A,B. Initially, standard samples, nominally 5 or 10 kb DNA, terminated by either biotin (B) or digoxigenin (D) at the termini, are bound to streptavidin (S)- or anti-digoxigenin (A)-coated beads, which are optically trapped in the tweezers. This bound DNA can be stretched by separating the beads (Figure 7A). By tracking the position of the beads, as shown in Figure 7B, a force $F = k(x_b - x_s)$ can be determined (where k is the optical trap stiffness, with extension $d = x_s - x_b - r_s - r_b$, and r_s and r_b are the radii of the beads, and the subscripts s and b relate to the small(er) and big(ger) of the beads, respectively).

Four samples were examined: (1) A-D-10kb-B-S (reference DNA) (Figure 7C); (2) A-D-5kb-B-S (Figure 7D); (3) A-D-10kb-D-A (Figure 7E); and (4) A-D-5kb-B-ss-B-5kb-D-A (concatenated DNA) (Figure 7F). Sample 1 is a standard DNA stretch with the two DNA strands terminated by biotin at one end and digoxigenin at the other; where one streptavidin-coated and one anti-digoxigenin-coated bead are utilized in the experiment. Sample 2 is a standard 5 kb stretch of the same nature as 1. Sample 3 is similar to 1 but has both bead-facing ends terminated with digoxigenin, as a prelude to sample 4, in which the biotin-displaying ends of both chains are free to couple to the divalent streptavidin linker. Typical single-duplex force–extension curves for the four samples are shown in Figure 7C–F, respectively. These force–extension curves were fitted to the wormlike chain model (WLC):

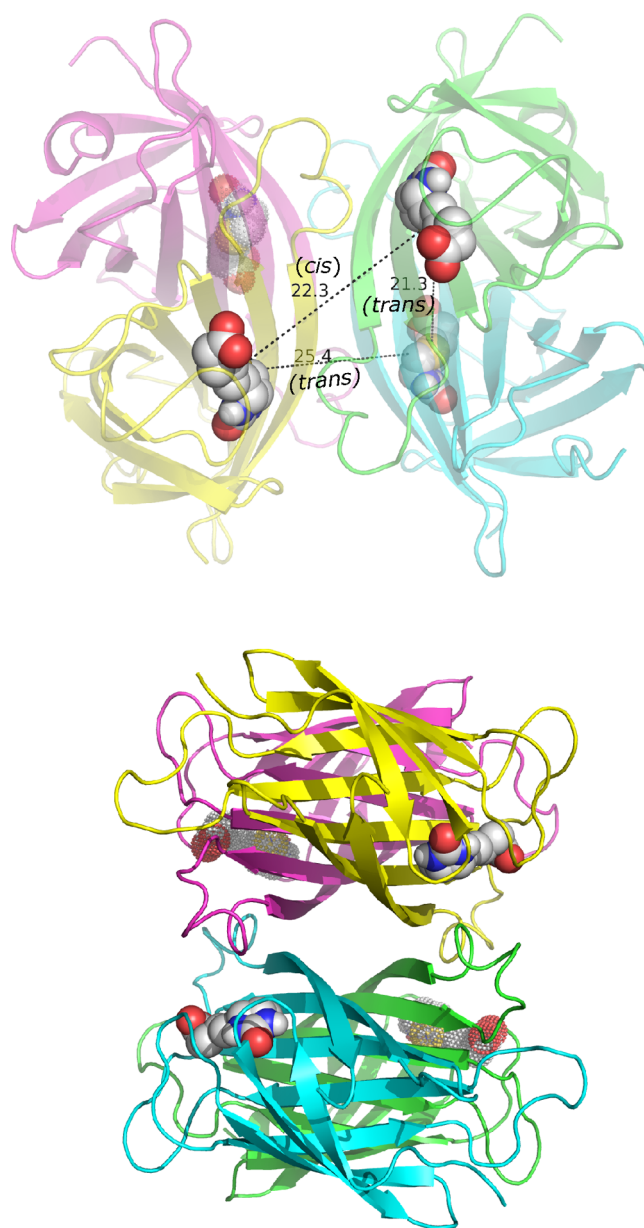


Figure 6. Completely filled biotin–streptavidin conjugate with the S–S distances between the *cis* and possible *trans* sites marked as 22.3 and 21.3 Å, and 25.4 Å, respectively), and possible *trans* configuration of divalent streptavidin (solid indicates biotin “plugs”, whereas the dotted renderings of the biotin indicate the potential, currently vacant biotin-binding sites).

$$F = \frac{k_B T}{l_p} \left(\frac{1}{4} \left(1 - \frac{d}{l_c} \right)^{-2} + \frac{d}{l_c} - \frac{1}{4} \right) \quad (2)$$

to determine the chain’s persistence length, l_p (the length over which the chain might be considered straight), and the contour length, l_c (the end-to-end length along the chain). The statistics of the fitted parameters are shown in Table 2. Note that a single trap stiffness k was assumed for all measurements; this value was chosen so that the mean l_p value for the D–10 kb–B measurements matched that previously found for this reference sample.^{30,31}

Based on these statistics, the following can be concluded: (1) All DNA tested has the same l_p . (2) The ratio of the

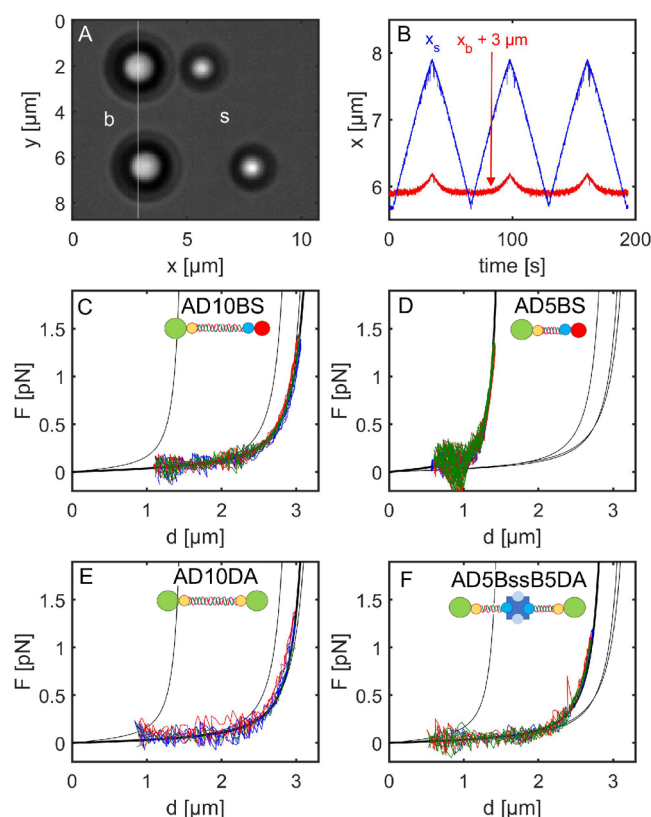


Figure 7. Optical tweezers experiments: Stretching control and concatenated DNA. (A) Micrograph of the setup showing a big (b) and small (s) bead tethering DNA, their separation using OT, and the consequential movement of the left-hand bead in response to moving the right-hand one of the pair. (B) Displacements of the beads as they are made to approach and retract from each other. (C–F) Derived force–extension curves for the four samples described in the text are illustrated in the respective panes.

extracted contour lengths of the 5 and 10 kb dsDNA samples, $l_c(\text{D-5kb-B})/l_c(\text{D-10kb-B}) = 0.46$ (0.438–0.490) is consistent with the known ratio of their base pairs $4682/10051 = 0.466$. (3) The ratio of the extracted contour lengths of two 10 kb dsDNA samples, with different termini, $l_c(\text{D-10kb-D})/l_c(\text{D-10kb-B}) = 0.98$ (0.943–1.01), is consistent with the known ratio of their base pairs $10051/10051 = 1.00$. (4) The ratio of the extracted contour lengths of the string resulting from the incubation of 5 kb DNA with *trans*-divalent streptavidin, and the 5 kb DNA, $l_c(\text{D-5kb-B-ss-B-5kb-D})/l_c(\text{D-5kb-B}) = 1.93$ (1.81–2.07), is consistent with the expected ratio of base pairs, $9364/4682 = 2.00$, if concatenation occurs. This observation confirms that the concatenated structure with the intervening biotin–streptavidin bridge (D-5kb-B-ss-B-5kb-D) was formed and is robust enough to run intact in an agarose gel and to be held in an OT experiment.

CONCLUSION

CE provides a rapid methodology for studying the results of incubating streptavidin with biotin-terminated DNA oligomers, requiring minimal amounts of sample and consumables. Using HPAEC to collect fractions and UV irradiation to remove separation-facilitating DNA oligomers, specific divalent streptavidin species can be generated and have the potential to be used as linker molecules to create “plug-and-play” strings for single-molecule experiments. This divalent streptavidin, when incubated with ~ 5 kb biotin-terminated DNA, produces a ~ 10 kb concatenated species which can be observed on an agarose gel. Furthermore, in optical tweezers experiments, the ratio of the extracted contour lengths of the string resulting from the incubation of ~ 5 kb DNA with divalent streptavidin and the ~ 5 kb DNA, $l_c(\text{D-5kb-B-ss-B-5kb-D})/l_c(\text{D-5kb-B}) = 1.93$ (1.81–2.07), is consistent with the known ratio of base pairs, $9364/4682 = 2.00$, which is to be expected if concatenation occurs. This observation confirms that the concatenated structure D-5kb-B-ss-B-5kb-D was formed. Moreover, the force–extension curve of the concatenated string was experimentally indistinguishable from that which would be expected from a single DNA chain of the same length, showing that, at least at these forces, the tightly bound divalent streptavidin linker does not modify the stretching behavior significantly. It is hoped that as an extension to this work, using two streptavidin linkers, any biotin-terminated polymer might be inserted between two sections of DNA of substantial length to facilitate single-molecule experiments on molecules that are currently difficult to address by other means.

EXPERIMENTAL PROCEDURES

Streptavidin–DNA Oligonucleotide Binding. DNA oligonucleotides (Integrated DNA Technologies, Inc., Coralville, Iowa/Singapore) and streptavidin (PRO-283, ProSpec-Tany TechnoGene, Ltd., Rehovot, Israel) were purchased and used following the protocols described in detail in Sun et al.¹⁷ Briefly, a primer with a photocleavable biotin moiety attached via the 5′ end (biotin-PC-AGC-ACA-TCC-CCC) was annealed to its complement (GGG-GGA-TGT-GCT) in 10 mM sodium phosphate buffer, 50 mM NaCl pH 7.5 at 94 °C for 2 min and then cooled slowly for a final concentration of 450 μM double-stranded DNA. We combined this 12 bp DNA (40–160 μM final concentration) with streptavidin first dissolved in water (40 μM final concentration) overnight at room temperature in 100 mM sodium phosphate buffer pH 6.5. We tested various concentrations of 12 bp DNA with streptavidin to determine the optimal ratio for a maximum yield of streptavidin species with exactly two bound DNA oligomers. A ratio of 4:1 DNA to streptavidin (equivalently the total number of DNA oligos equal to the total number of binding sites on streptavidin) was used for sample preparation

Table 2. OT Experimental Results for the Samples Described in the Text and Illustrated in Figure 7C–F^a

sample	stretches	mean l_p (nm)	mean l_c (μm)	bp
A-D-10kb-B-S	28	59 (± 10)	3.425 (± 0.047)	10 051
A-D-5kb-B-S	9	54 (± 9.8)	1.588 (± 0.066)	4682
A-D-10kb-D-A	23	72 (± 12)	3.342 (± 0.066)	10 051
A-D-5kb-B-ss-B-5kb-D-A	13	75 (± 12)	3.069 (± 0.075)	9364

^aIndividual stretches were independently fitted to the WLC model, and means and standard deviations of the fitted l_p and l_c for each DNA sample were calculated.

for running through the anion exchange column. Throughout, primer with photocleavable biotin was kept in the dark.

Anion-Exchange Chromatography. To separate the streptavidin with different numbers of DNA oligos attached, we used a Uno Q column 5/50 (GE Life Sciences/Cytiva, Marlborough, Massachusetts) anion exchange column run in a NGC Quest 10 Plus chromatography system (Bio-Rad Laboratories, Inc., Hercules, California). Following the protocol in Sun et al.,¹⁷ we used a linear salt gradient beginning with 100% buffer A (20 mM Tris/HCl pH 8) and ending with 100% buffer B (buffer A with 1 M NaCl) over 400 mL at a rate of 2 mL/min.

Capillary Electrophoresis. Experiments were carried out using an automated Agilent CE system (HP 3D), equipped with a diode array detector. Electrophoresis was carried out in a fused silica capillary with an internal diameter of 50 μm and a total length of 48.5 cm (40 cm from inlet to detector), unless otherwise stated. The capillary incorporated an extended light-path detection window (150 μm) and was thermostated at 25 $^{\circ}\text{C}$. All new capillaries were conditioned by rinsing for 30 min with 1 M NaOH, 30 min with a 0.1 M NaOH solution, 15 min with water, and 30 min with BGE. A total of 50 mM sodium phosphate buffer at pH 7.0 was used as a CE BGE and filtered through 0.2 μm filters (Whatman) before use. Between runs, the capillary was washed for 2 min with 1 M NaOH, 2 min with 0.1 M NaOH, 1 min with water, and 2 min with BGE. Detection was carried out using UV absorbance typically at 192 or 260 nm with a bandwidth of 2 nm. Samples were loaded hydrodynamically (various injection times at 5000 Pa, typically giving injection volumes of the order of 10 nL) and typically electrophoresed across a potential difference of 25 kV. All experiments were carried out at normal polarity (inlet anodic) unless otherwise stated.

UV Irradiation. UV-induced cleavage of the 12 bp oligonucleotides from their biotin termini was performed using a multiband (254/366 nm) Mineralight UV lamp (Ultra-Violet Products, Inc., San Gabriel, California). Samples in a quartz cuvette were subjected to UV irradiation for different times, and the photocleavage was monitored by capillary electrophoresis.

DNA Gel Electrophoresis. To produce ~ 5 kb DNA with biotin attached at one end, we performed PCR according to the manufacturer's recommendations (PCR Extender System, QuantaBio, Beverly, Massachusetts) using Lambda phage DNA as a template (New England Biolabs Inc., Ipswich, Massachusetts), a biotinylated primer and an unmodified primer (forward 5'-biotinTEG-CTGATGAGTTCGTGCC-GTACAACCTGGCGTAATC-3', reverse 5'-GTTTGTACT-CCAGCGTCTCATCTTTATGCGCC-3', Integrated DNA Technologies). This produced a single band in a conventional agarose gel electrophoresis experiment at the expected 5031 bp. The cleaned PCR product was combined with biotin-plugged divalent streptavidin, where the oligonucleotides of the two plugging molecules had been cleaved off with 5 min UV irradiation, and incubated overnight in 100 mM sodium phosphate buffer pH 6.5. Products of the incubation were visualized on a 0.5% agarose gel using staining with ethidium bromide.

Optical Tweezers. DNA stretching experiments were carried out on an inverted microscope (Nikon Eclipse TE2000-U) equipped with holographic optical tweezers (Arryx, Chicago, USA). The setup includes a fixed 5 W (1032 nm) infrared laser, a spatial light modulator steered

(SLM, Boulder NLS phase only) 2 W (1064 nm) infrared laser, and a high-speed camera (Andor NEO). A high numerical aperture water immersion objective (Nikon plan apo, magnification = 60 \times , NA = 1.2) was used for focusing and trapping. The DNA studied here comprised ~ 10 kb (10051 bp) pieces,³² ~ 5 kb (4682 bp) pieces, and ~ 10 kb assemblies of two of the 5 kb pieces concatenated with *trans*-divalent streptavidin. Each strand of the double-stranded duplex was terminated by either biotin or digoxigenin (preattached to the primers used in the PCR production process, 5'BiotinTEG or 5'DIGN, Integrated DNA Technologies) that bind to streptavidin- or anti-digoxigenin-coated beads, respectively, by physisorption. DNA was incubated in TSB (50 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.6) with 1:26 μm diameter streptavidin-coated beads (SVP-10-5, Spherotech, Lake Forest, Illinois) for at least an hour and combined with 2:12 μm diameter anti-digoxigenin-coated beads (DIGP-20-2, Spherotech) in TSB in a well slide.

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Notes

The authors declare no competing financial interest.

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