Biosensors

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Monovalent Streptavidin that Senses Oligonucleotides**

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Streptavidin and its congeners occur naturally as homotetramers binding up to four molecules of biotin between monomer–monomer interfaces ($K_d \approx 10^{-14}$ M). This system is the cornerstone of a large number of bioassays, purification procedures, and imaging protocols.[1] The popularity lies in its robust nature, simplicity, and ability to couple virtually any molecule of interest to either biotin or its receptor proteins. However, the tetravalent nature of the protein can result in undesirable effects that is, crosslinking of biotinylated molecules, which may complicate synthesis of reagents (for example, streptavidin–antibody complexes become oligomeric),[2] and disrupt normal cell function in live-cell imaging.[3] The individual monomeric units of streptavidin bind biotin weakly ($K_d \approx 10^{-7}$ M)[4] and hence do not provide an optimal solution to unwanted crosslinking. A molecular biology approach to this problem has been reported: streptavidin tetramers with only one functioning biotin-binding pocket, that is, a monovalent streptavidin, can be isolated from random assemblies generated from expressed wild-type monomers and monomers containing mutations blocking biotin binding. This reagent was preferred over natural streptavidin in live-cell imaging applications.[3]

Our goal to make monovalent streptavidin easier to access and thereby available for broader applications, stemmed from the interest in rapid construction of molecular robotic elements[5,6] (for example, molecular entities called "spiders" are equivalent in their "three-legged" form to monovalent streptavidin) and previous uses of streptavidin as nanotechnology building block.[7] We now report a straightforward single-step route to a monovalent streptavidin by using a trisbiotinylated oligonucleotide to block three of streptavidin's four biotin-binding sites, thereby making use of the "chelate effect" to increase stability and yield relative to biotin or other reagents functionalized with one biotin moiety. Additionally, in the course of our experiments, an unanticipated property of the monovalent streptavidin–oligonucleotide conjugate was discovered: the complex is a sensitive sensor of single point mutations: hybridization of the cyclized oligonucleotide with a perfectly matched complementary strand triggers the dissociation of a biotin moiety (with concomitant oligomerization) and does so with greater efficiency than a complementary strand containing a single-base mismatch.

We constructed the monovalent streptavidin through a direct macrocyclization reaction with a trisbiotinylated oligonucleotide (Figure 1a). The oligonucleotide was designed with dimensions that enabled it to intramolecularly

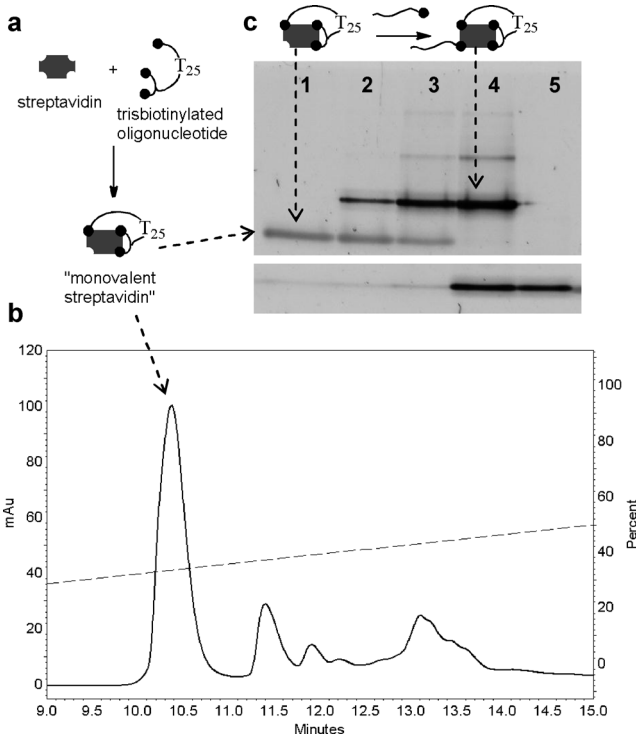

Figure 1. a) Assembly of a discrete trisbiotinylated oligonucleotide–streptavidin complex, that is, a "monovalent streptavidin", and its ability to bind only one biotin. Streptavidin (STV) is represented by a gray rectangle with a "bite" out of each corner representing biotin-binding pockets; biotin is represented by black spheres; a single-stranded oligonucleotide and organic spacers link the three biotin moieties. b) Ion-exchange HPLC trace showing the major product STV–**1** obtained when **1** and STV are combined in a 1:1 ratio at room temperature, then heated to 70°C for 15 min, and allowed to cool (minor products were composed of DNA and streptavidin, as determined by the ratio of absorbance at 260 nm/280 nm, and possess a greater charge (and hence larger mass) as their elution times indicate, but have not been characterized further). The left *y*-axis is absorbance at 260 nm and the right *y*-axis is percentage of 1 M NaCl in the mobile phase—the dashed line shows the NaCl gradient and is read off the right *y*-axis. c) Native PAGE: Lane 1 is purified STV–**1**; lanes 2–4 are a mixture of STV–**1** with 0.1, 0.5, and 2 equivalents of a monobiotinylated oligonucleotide, resulting in a single major product, thus supporting that STV–**1** is monovalent; lane 5 is the monobiotinylated oligonucleotide.

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bind to streptavidin using all three biotin moieties (Figure S1 in the Supporting Information). This is in contrast to previously reported designs, in which intramolecular cyclization was disfavored by using very short trisbiotinylated ligands.[8,9] We first tested the trisbiotinylated oligonucleotide 5-/52-Bio/TTTTTTTTTTTTTTTTTTTTTTTTT-/3BioTEG/-3 (**1**; 52-Bio = modification with two biotin moieties at the 5′-end; 3BioTEG = modification with a tetraethylene glycol spacer and one biotin moiety at the 3′-end): mixing this oligonucleotide with one equivalent of streptavidin and then heating for 15 min at 70 °C and cooling, resulted in estimated yields around 55 % for the major product STV–**1** (Figure 1 b; all yields were estimated by integration of HPLC chromatogram peak areas, see Figure S2 in the Supporting Information for details).The monovalency of the complex was confirmed by the ability of STV–**1** to accept only one additional biotinylated oligonucleotide (Figure 1 c, lanes 2–4 and Figure S3 in the Supporting Information). The yield could be increased to around 70 % by using two equivalents of streptavidin with heating the mixture to 70 °C and allowing to cool (Figure S4 in the Supporting Information).

Analogous results were obtained when the T_{25} sequence was substituted with arbitrarily chosen 24- or 20-mer nucleotide sequences, STV–**2** and STV–**3**, respectively (see the Supporting Information for sequences). The yields for these were lower, at around 30–40 %, however, they could be improved up to 70 % by using two equivalents of streptavidin, heating to 70 °C, and then cooling. Without heating, the yield was increased to 50 % by adding the trisbiotinylated oligonucleotide to streptavidin saturated with desthiobiotin ($K_d \approx 10^{-10}$; Figure S5 in the Supporting Information).[10,11] The longest nucleotide sequence tested (a 43-mer, **7**) gave the highest yield (70 %) on simply 1:1 mixing with streptavidin at room temperature (Figure S10 in the Supporting Information). The optimized yield of the monovalent streptavidin is a significant improvement when compared to statistically generated three-legged "spiders"[5] and modified streptavidin incorporating nonbinding monomers, both at approximately 35 % (the statistical maximum is 42 %).[3] Whilst the oligonucleotide linker can be substituted with any other moiety of sufficient length and flexibility, it had the important benefit of straightforward isolation of the desired product by anion-exchange HPLC.

Subsequently, we investigated the hybridization of the bound oligonucleotide linker as an approach to incorporate a fluorescent dye for imaging applications. However, when monovalent streptavidin complexes were mixed with their respective complementary oligonucleotides, we observed in PAGE experiments the appearance of an extensive "ladder" (Figure 2), indicating an oligomerization process. We hypothesized that double helix formation forced dissociation of one of the biotin moieties, thereby triggering intermolecular crosslinking (e.g. Figure S12 in the Supporting Information). Consistent with this mechanism, the extent of oligomerization observed is proportional to the amount of full-complement strand, for example **6**, added to STV–**1**; that is, substoichiometric amounts of **6** resulted in a diminished ladder, because excess STV–**1** bound dissociated biotin

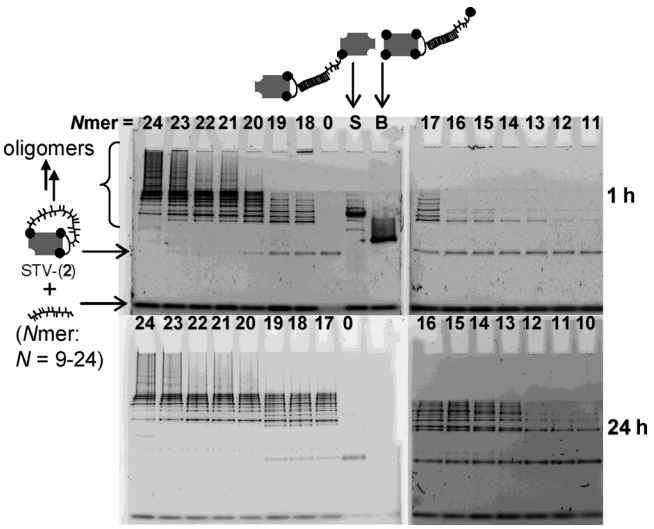

Figure 2. Native PAGE results: The effect of complementary strand length (*N*) on the extent of oligomerization after 1 h and 24 h of incubation at room temperature. For example, lane 24 shows the products obtained when a 24-mer complementary strand is added to STV–**2**; lane 23 shows the products obtained when a 23-mer complementary strand is added to STV–**2**, and so on. Lane 0 is the negative control, that is, no complementary strand is added. In lane S a 24-mer complementary strand is added in the presence of ten equivalents of streptavidin; this is a control for an approximation of a crosslinked dimer. In lane B a 24-mer complementary strand is added in the presence of 1000 equivalents of biotin; this is a control for a dissociated biotin without crosslinking.

moieties and thus stopped the oligomerization process (Figure S7 in the Supporting Information).

Oligomerization could be inhibited by introducing an additional biotin moiety at the monobiotin end (3'-end) of the oligonucleotide to give STV–**4**, that is **4** is anchored with two biotin moieties on both ends (Figure S8 in the Supporting Information). This result is consistent with the hypothesis that the biotin moiety that undergoes dissociation in STV–**1**, STV–**2**, and STV–**3** is the single biotin moiety at the 3′-end.[12] Oligomerization after hybridization can also be prevented either by "capping" the dissociated biotin with an excess of free streptavidin or by blocking incipient biotin-binding sites by an excess of free biotin (Figure S8).

The biotin dissociation is likely driven by the relief of strain caused by the increased rigidity of the resultant double helix in combination with the shortening of the length of the oligonucleotide linker upon hybridization.[13,14] The outstretched length of the single-stranded 25-mer is approximately 14 nm and its duplex is approximately 8 nm long (Figure S1). A double-helix trisbiotinylated oligonucleotide of the same length as the single-stranded system used above, that is, approximately 14 nm (measuring 43 base pairs), showed no ability to form a monovalent streptavidin when added directly to streptavidin and formed oligomers instead (Figure S9 b in the Supporting Information, lanes 2 and 3). Further, when a monovalent streptavidin was assembled by using single-stranded 43-mer **7** and then the full-complement strand was added, the dissociation rate was severely reduced (Figure S9 b, see lanes 4 and 6, and compare with Figure 2). It

was reported that force acting on the biotin–streptavidin interaction diminishes the lifetime of the interaction.[15] The proposed mechanism resembles the opening of molecular beacons,[16] or beacon-like enzyme-oligonucleotide-tethered inhibitors.[17] This oligomerization effect is also observed when shorter oligonucleotide complements are utilized (Figure 2). However, the oligomeric species produced over the time course of the experiment are smaller with respect to mass. This phenomenon can be explained by a decrease in the rate of production of dissociated biotin, thereby leading to preferential capture of starting material and also, in principle, a higher chance of cyclic oligomer formation. This hypothesis was supported by the fact that large oligomers were favored when the single vacant biotin-binding site of STV–**3** was blocked, for example, with biotinylated fluorescein (as in F-STV–**3**; Figure 3a compare lane 2 with lane 6). This observation is due to the fact that the dissociated biotin moiety of F-STV–**3** cannot form crosslinks to another F-STV–**3** (i.e. all biotin-binding sites are occupied in F-STV–**3**).

Furnished with the ability to translate the addition of a complementary oligonucleotide into a "biotin label", we were interested in the sensitivity of this process with respect to single-base mismatches—a desirable attribute for any oligonucleotide detection system. We screened fully complementary oligonucleotides ("targets") of different lengths and their single-mismatch counterparts against STV–**2** (the "probe"). The results (Figure S11 in the Supporting Information) were consistent with the following trade-off: long oligonucleotides caused opening more rapidly, while shorter oligonucleotides were more sensitive to single-point mismatches. For example, for the 24-nucleotide target strand the process was relatively rapid at about 15 min for approximately 100% dissociation, whilst for a 17-nucleotide target strand the forced biotin dissociation process took three days to reach approximately 100% dissociation (Figure S12e), but the 17-mer target was more sensitive to the mismatch than the 24-mer target. From this set of results we chose 17-nucleotide length targets with various mismatches to study their effect on the biotin dissociation and oligomerization products when added to STV–**2**, STV–**3**, and F-STV–**3** probes.

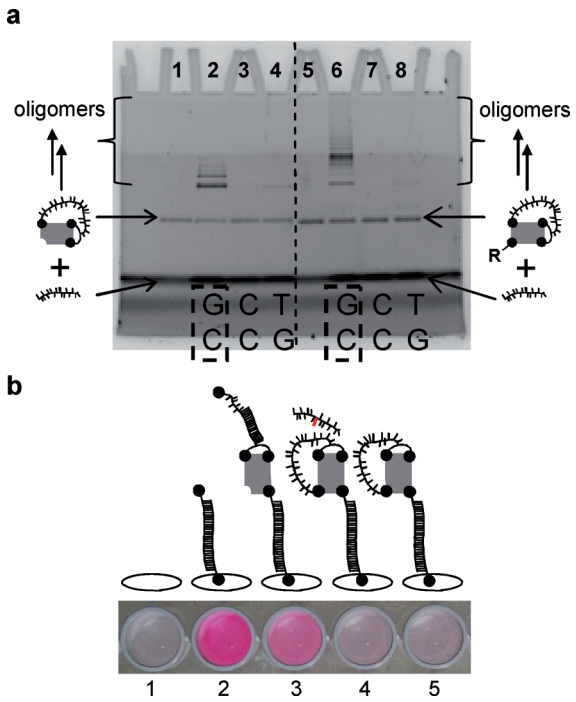

Figure 3. Sensitivity of biotin dissociation to mismatches: a) Stacking gel nondenaturing PAGE results of addition of perfectly matched (dashed box) or single-mismatch oligonucleotide to STV–**3** (lanes 1–4) compared with F-STV–**3** (where the lone vacant biotin-binding site is blocked with fluorescein biotin (●-R); lanes 5–8) after 15 min incubation at 37°C. The mismatch occurs near the mid-point of the complementary strand. b) Enzyme-linked immunosorbent assay (ELISA) results after 15 min of plate-bound STV–**3** in the presence of a perfectly matched or mismatched oligonucleotide at room temperature: 1) QuantaRed HRP substrate only; 2) positive control (biotin present); 3) perfect match; 4) single-base mismatch (CC, as in (a) above); 5) negative control (no complement added).

STV–**3** and F-STV–**3** showed high sensitivity towards 17-mer target strands with single base differences (Figure 3a and Figure S13 in the Supporting Information). STV–**2**, which contains a longer (by four bases) trisbiotinylated oligonucleotide, was less sensitive to single base differences than STV–**3** (compare Figure S12a with S12b). Interestingly, mismatch sensitivity was severely diminished (i.e. the rates of biotin dissociation for a perfectly matched complement and one with a single mismatch were similar), if the target strand is hybridized to the 3′-terminus of the probe (Figure S11). Note: over longer incubation times, the ability to discriminate single-base mismatches is gradually diminished, because all samples are moving towards equilibrium, where all starting material is oligomerized (Figure S12e).

Making use of the single vacant biotin-binding site to incorporate an additional monobiotinylated oligonucleotide provides a useful handle, for example, if the reagent needs to be attached to a solid support, or as a spatial address in various microarray applications.[18] As a preliminary demonstration, the monovalent streptavidin STV–**3** was attached through an additional monobiotinylated oligonucleotide to streptavidin-coated plates and newly dissociated biotin was detected with an horseradish peroxidase (HRP)-labeled streptavidin conjugate. We were able to distinguish between a complementary oligonucleotide and an oligonucleotide with a single point mutation, within 15 min at room temperature (Figure 3c and Figure S14 in the Supporting Information). This protocol is currently being studied and optimized for direct detection of short oligonucleotides of clinical significance.[19]

In conclusion, we present here a readily accessible monovalent streptavidin, assembled in a straightforward procedure that overcomes a statistical distribution of products, and substitutes protein expression with two commercially available reagents (streptavidin and custom made oligonucleotide). In addition, in one step one of the biotin moieties becomes available for further binding in direct response to a specific oligonucleotide sequence. This offers advantages over current applied analytical methods where biotin, or other labels are inserted requiring additional steps.[18,20] The new reagent can be fine-tuned with various functionalities, by using a wide variety of custom analogues available for synthetic oligonucleotides. The unique proper-

ties of the complex and ease of synthesis opens wide opportunities for practical applications in imaging and biosensing.

Experimental Section

Full details for experiments are provided in the Supporting Information. In general, trisbiotinylated oligonucleotide (250 μL of 1 μM) is mixed as thoroughly and as rapidly as possible with streptavidin (250 μL of 1 μM) at room temperature. The resulting mixture is purified by anion-exchange HPLC.

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[1] C. Grunwald, *Z. Phys. Chem.* **2008**, *222*, 789 – 821.
[2] M. D. Hylarides, R. W. Mallett, D. L. Meyer, *Bioconjugate Chem.* **2001**, *12*, 421 – 427.
[3] M. Howarth, D. J.-F. Chinnapen, K. Gerrow, P. C. Dorrestein, M. R. Grandy, N. L. Kelleher, A. El-Husseini, A. Y. Ting, *Nat. Methods* **2006**, *3*, 267 – 273.
[4] K. H. Lim, H. Huang, A. Pralle, S. Park, *Biochemistry* **2011**, *50*, 8682 – 8691, and references cited therein.
[5] R. Pei, S. K. Taylor, D. Stefanovic, T. E. Mitchell, M. N. Stojanovic, *J. Am. Chem. Soc.* **2006**, *128*, 12693 – 12699.
[6] K. Lund, A. J. Manzo, N. Dabby, N. Michelotti, A. Johnson – Buck, J. Nangreave, S. Taylor, R. Pei, M. N. Stojanovic, N. G. Walter, E. Winfree, H. Yan, *Nature* **2010**, *465*, 206 – 210.
[7] C. M. Niemeyer, *Angew. Chem.* **2010**, *122*, 1220 – 1238; *Angew. Chem. Int. Ed.* **2010**, *49*, 1200 – 1216.
[8] D. S. Wilbur, P. M. Pathare, D. K. Hamlin, S. A. Weerawarna, *Bioconjugate Chem.* **1997**, *8*, 819 – 832.
[9] For example: a) K. Y. Zhang, K. K.-W. Lo, *Inorg. Chem.* **2009**, *48*, 6011 – 6025; b) L. Tei, A. Barge, S. G. Crich, R. Pagliarin, V. Negri, D. Ramella, G. Cravotto, S. Aime, *Chem. Eur. J.* **2010**, *16*, 8080 – 8087.
[10] Desthiobiotin inhibits ($k_{\text{off desthiobiotin–streptavidin}} = 1.8 \times 10^{-2}\ s^{-1}$) the rapid formation ($k_{on} = 8.5 \times 10^{7}\ M^{-1} s^{-1}$) of the highly stable biotinylated oligonucleotide–streptavidin interaction ($k_{off} = 1.1 \times 10^{-6}\ s^{-1}$), thus allowing the entropically favored monovalent streptavidin product to be favored over, for example, crosslinked species (a kinetic product).
[11] M. L. B. Magalhães, C. M. Czekster, R. Guan, V. N. Malashkevich, S. C. Almo, M. Levy, *Protein Sci.* **2011**, *20*, 1145 – 1154.
[12] STV–bisbiotin-oligonulceotide complexes also produced a ladder on addition of complementary strands. Unpublished results.
[13] The persistence length for the DNA double helix is about 45.0 nm: B. Tinland, A. Pluen, J. Sturm, G. Weill, *Macromolecules* **1997**, *30*, 5763 – 5765.
[14] Bisbiotinylated double helical DNA has been shown to intramolecularly bind to streptavidin; however the double helix in such complexes contained a minimum of 87 base pairs (with a maximum yield of 28 %). Yield increased as the number of base pairs increased. C. M. Niemeyer, M. Adler, S. Gao, L. Chi, *Angew. Chem.* **2000**, *112*, 3183 – 3187; *Angew. Chem. Int. Ed.* **2000**, *39*, 3055 – 3059.
[15] a) R. Merkel, P. Nassoy, A. Leung, K. Ritchie, E. Evans, *Nature* **1999**, *397*, 50 – 53; b) P. D. Morris, K. D. Raney, *Biochemistry* **1999**, *38*, 5164 – 5171; c) A. Agarwal, P. Katira, H. Hess, *Nano Lett.* **2009**, *9*, 1170 – 1175; d) S. Guo, N. Li, N. Lad, C. Ray, B. B. Akhremitchev, *J. Am. Chem. Soc.* **2010**, *132*, 9681 – 9687.
[16] G. Bonnet, S. Tyagi, A. Libchaber, F. R. Kramer, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 6171 – 6176.
[17] A. Saghatelian, K. M. Guckian, D. A. Thayer, M. R. Ghadiri, *J. Am. Chem. Soc.* **2003**, *125*, 344 – 345.
[18] A. J. Qavi, J. T. Kindt, R. C. Bailey, *Anal. Bioanal. Chem.* **2010**, *398*, 2535 – 2549.
[19] a) J. Y. Z. Li, T. Y. Yong, M. Z. Michael, J. M. Gleadle, *Nephrology* **2010**, *15*, 599 – 608; b) A. Budhu, J. Ji, X. W. Wang, *J. Hematol. Oncol.* **2010**, *3*, 37.
[20] For example: a) A. Git, H. Dvinge, M. Salmon-Divon, M. Osborne, C. Kutter, J. Hadfield, P. Bertone, C. Caldas, *RNA* **2010**, *16*, 991 – 1006; b) W. Li, K. Ruan, *Anal. Bioanal. Chem.* **2009**, *394*, 1117 – 1124.

