Published in final edited form as:

Angew Chem Int Ed Engl. 2013 May 17; 52(21): 5509–5512. doi:10.1002/anie.201209948.

Monovalent Streptavidin that Senses Oligonucleotides**

Dr. Steven K. Taylor,

Department of Medicine, Division of Experimental, Therapeutics, Columbia University, 630 W. 168th St., Box 84, New York, NY 10032 (USA), Fax: (+1) 212-305-3475

Jingxian Wang,

Department of Medicine, Division of Experimental, Therapeutics, Columbia University, 630 W. 168th St., Box 84, New York, NY 10032 (USA), Fax: (+1) 212-305-3475

Natasa Kostic, and

Department of Medicine, Division of Experimental, Therapeutics, Columbia University, 630 W. 168th St., Box 84, New York, NY 10032 (USA), Fax: (+1) 212-305-3475

Prof. Dr. Milan N. Stojanovic

Department of Medicine, Division of Experimental, Therapeutics, Columbia University, 630 W. 168th St., Box 84, New York, NY 10032 (USA), Fax: (+1) 212-305-3475. Department of Biomedical Engineering, Columbia University (USA)

Steven K. Taylor: st2171@columbia.edu

Abstract

We report a straightforward chemical route to monovalent streptavidin, a valuable reagent for imaging. The one-step process is based on a (tris)biotinylated-oligonucleotide blocking three of streptavidin's four biotin binding sites. Further, the complex is highly sensitive to single-base differences - whereby perfectly matched oligonucleotides trigger dissociation of the biotin-streptavidin interaction at higher rates than single-base mismatches. Unique properties and ease of synthesis open wide opportunities for practical applications in imaging and biosensing.

Keywords

DNA; sensors; proteins

Streptavidin and its congeners occur naturally as homotetramers binding up to four molecules of biotin between monomer-monomer interfaces ($K_d \sim 10^{-14}$ 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 i.e. crosslinking of

**This work was supported by NSF (CBET-1033288 and 1026592) and NIH (RGM-104960). We thank Dr. Francine Katz for expert advice, and Prof. Henry Hess for advice in writing this paper.

Correspondence to: Steven K. Taylor, st2171@columbia.edu.

Supporting information for this article is available on the WWW under <http://www.angewandte.org> or from the author.

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 ($K_d \sim 10^{-7}$ M)^[4] and hence do not provide an optimal solution to unwanted cross-linking. A molecular biology approach to this problem has been reported: streptavidin tetramers with only one functioning biotin-binding pocket, i.e. 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 more flexible in 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 blocks.^[7] We now report a straightforward single-step route to a monovalent streptavidin using a trisbiotinylated-oligonucleotide to block three of streptavidin’s four biotin-binding sites, thereby capitalizing on the ‘chelate effect’ to increase stability and yield relative to biotin or other monobiotin-functionalized reagents. 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 via a direct macrocyclization reaction with a tris-biotinylated oligonucleotide, see Figure 1a. The oligonucleotide was designed with dimensions that enabled it to intramolecularly bind to streptavidin using all three biotin moieties (see Supporting Information Figure S1). This is in contrast to previously reported designs, in which intramolecular cyclization was disfavored by using very short tris-biotinylated ligands.^[8, 9] We first tested the tris-biotinylated oligonucleotide 5-/52-Bio/TTT TTT TTT TTT TTT TTT T-3BioTEG/-3 (**1**): mixing this oligonucleotide with one equivalent of streptavidin and then heating for 15mins at 70°C and cooling, resulted in estimated yields around 55% for the major product STV-(**1**) as shown in Figure 1b (all yields were estimated by integration of HPLC chromatogram peak areas, see Supporting Information Figure S2 for details). The monovalency of the complex was confirmed by the ability of STV-(**1**) to accept only one additional biotinylated oligonucleotide (Figure 1c [lanes 2–4] and Supporting Information Figure S3). 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, see Supporting Information Figure S4.

Analogous results were obtained when the T₂₅ sequence was substituted with arbitrarily chosen 24- or 20-mer nucleotide sequences, STV-(**2**) and STV-(**3**), respectively. But yields for these were lower, at around 30–40%, however, they could be improved up to 70% using two equivalents of streptavidin and heating to 70°C then cooling. Without heating, yield was increased to 50% by adding the tris-biotinylated-oligonucleotide to desthiobiotin (K_d

~10⁻¹⁰)-saturated streptavidin.^[10, 11] (Supporting Information Figure S5). In terms of yield, the longest nucleotide sequence tested (a 43-mer, **7**) gave the highest (70%) on simply 1:1 mixing with streptavidin at room temperature, (Supporting Information Figure S10). The optimized yield of the monovalent streptavidin is a significant improvement when compared to statistically generated three-legged “spiders”,^[5] and modified streptavidin incorporating non-binding monomers, both at c.a. 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 via 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 streptavidins were mixed with their respective complementary oligonucleotides, we observed in PAGE experiments the appearance of an extensive ‘ladder’ as shown in Figure 2, indicating an oligomerization process. We hypothesized that double helix formation forced dissociation of one of the biotin moieties, triggering intermolecular crosslinking (e.g. Supporting Information Figure S12). Consistent with this mechanism, the extent of oligomerization observed is proportional, for example, to the amount of full-complement strand (**6**) added to STV-(**1**), i.e. sub-equivalent amounts of (**6**) resulted in a diminished ‘ladder’ due to excess STV-(**1**) binding dissociated biotin moieties and thus ‘capping’ propagation of the oligomerization process (Supporting Information Figure S7).

Oligomerization could be inhibited by introducing an additional biotin moiety at the monobiotin end (3' end) of the oligonucleotide to give STV-(**4**), i.e. (**4**) is anchored with two biotins on both ends (Supporting Information Figure S8). This result is consistent with the hypothesis that the biotin moiety undergoing dissociation in STV-(**1**, **2**, and **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 blocking incipient biotin binding sites by an excess of free biotin (Supporting Information 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 oligonucleotide linker's length upon hybridization.^[13, 14] The outstretched length of the single stranded 25-mer is ~14nm and its duplex is ~8nm, see Supporting Information Figure S1. A double-helix tris-biotinylated-oligonucleotide of the same length as the single stranded system used above i.e. ~14nm (measuring 43 base pairs), showed no ability to form a monovalent streptavidin when added directly to streptavidin, and formed oligomers instead (Supporting Information Figure S9b, c.f. lanes 2 and 3). Further, when a monovalent streptavidin was assembled using single-stranded 43mer (**7**) and then the full-complement added, the dissociation rate is severely reduced (Supporting Information Figure S9b, see lanes 4, 6, and compare with Figure 2). It has been 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, 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)), see Figure 3a cf. lane 2 with lane 6. This observation is due to the dissociated biotin moiety of F-STV-(3) not having the option of crosslinking 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 (Supporting Information Figure S11) 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 15mins for ~100% dissociation, whilst for a 17-nucleotide 'target' strand the forced biotin dissociation process took three days to reach ~100% dissociation (Supporting Information Figure S12e) but with the 17-mer 'target' 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'.

STV-(3) and F-STV-(3) showed high sensitivity towards 17mer 'target' strands with single base differences, see Figure 3a and Supplementary Information Figure S13. STV-(2), which contains a longer (by four bases) trisbiotinylated oligonucleotide, was less sensitive to single base differences than STV-(3) (compare Supporting Information Figure S12a with S12b). Interestingly, mismatch sensitivity was severely diminished (i.e. the rate of biotin dissociation for a perfectly matched complement and one with a single mismatch was similar) if 'target' base-paired from the 3'-terminus of the 'probe' c.f. Supporting Information Figure S11. Note: over longer incubation times, the ability to discriminate single base mismatches is gradually diminished due to all samples moving towards equilibrium where all starting material is oligomerized (Supporting Information 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 via an additional monobiotinylated-oligonucleotide to streptavidin-coated plates and newly dissociated biotin detected with an HRP-labeled streptavidin conjugate. We were able to distinguish between a complementary oligonucleotide and an oligonucleotide with a single-point mutation, within 15 minutes at room temperature (see Figure 3c and Supporting Information Figure S14). 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 off-the-shelf reagents (e.g., streptavidin and custom made oligonucleotide). In addition, in one-step a biotin 'flag' can be 'hoisted' 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, using a wide variety of custom analogs available for synthetic oligonucleotides. The unique properties 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, tris-biotinylated oligonucleotide ($250\ \mu\text{L} \times 1\ \mu\text{M}$) is mixed as thoroughly and as rapidly as possible with streptavidin ($250\ \mu\text{L} \times 1\ \mu\text{M}$) at room temperature. The resulting mixture is purified by anion exchange HPLC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

References

1. Grunwald C. *Z Phys Chem.* 2008; 222:789–821.
2. Hylarides MD, Mallett RW, Meyer DL. *Bioconjugate Chem.* 2001; 12:421–427.
3. Howarth M, Chinnapen DJF, Gerrow K, Dorrestein PC, Grandy MR, Kelleher NL, El-Husseini A, Ting AY. *Nat Methods.* 2006; 3:267–273. [PubMed: 16554831]
4. Lim KH, Huang H, Pralle A, Park S. *Biochemistry.* 2011; 50:8682–8691. and references within. [PubMed: 21892837]
5. Pei R, Taylor SK, Stefanovic D, Mitchell TE, Stojanovic MN. *J Am Chem Soc.* 2006; 128:12693–12699. [PubMed: 17002363]
6. Lund K, Manzo AJ, Dabby N, Michelotti N, Johnson-Buck A, Nangreave J, Taylor S, Pei R, Stojanovic MN, Walter NG, Winfree E, Yan H. *Nature.* 2010; 465:206–210. [PubMed: 20463735]
7. Niemeyer CM. *Angew Chemie Int Ed.* 2010; 49:1200–1216.
8. Wilbur DS, Pathare PM, Hamlin DK, Weerawarna SA. *Bioconjugate Chem.* 1997; 8:819–832.
9. For example: Zhang KY, Lo KKW. *Inorg Chem.* 2009; 48:6011–6025. [PubMed: 19480459] Tei L, Barge A, Crich SG, Pagliarin R, Negri V, Ramella D, Cravotto G, Aime S. *Chem Eur J.* 2010; 16:8080–8087. [PubMed: 20533461]
10. Desthiobiotin inhibits ($k_{\text{off}} \text{ desthiobiotin-streptavidin} = 1.8 \times 10^{-2} \text{ s}^{-1}$) the rapid formation ($k_{\text{on}} = 8.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$) of the highly stable biotinylated-oligonucleotide-streptavidin interaction ($k_{\text{off}} = 1.1 \times 10^{-6} \text{ s}^{-1}$), thus allowing the entropically favored monovalent streptavidin product to be favored over, for example, cross-linked species (a kinetic product).
11. Magalhães MLB, Czekster CM, Guan R, Malashkevich VN, Almo SC, Levy M. *Protein Sci.* 2011; 20:1145–1154. [PubMed: 21520321]
12. STV-bisbiotin-oligonucleotide 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: Tinland B, Pluen A, Sturm J, Weill G. *Macromolecules.* 1997; 30:5763–5765.

14. Bis-biotinylated 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. Niemeyer CM, Adler M, Gao S, Chi L. *Angew Chemie Int Ed*. 2000; 39:3055–3059.
15. (a) Merkel R, Nassoy P, Leung A, Ritchie K, Evans E. *Nature*. 1999; 397:50–53. [PubMed: 9892352] (b) Morris PD, Raney KD. *Biochemistry*. 1999; 38:5164–5171. [PubMed: 10213622] (c) Agarwal A, Katira P, Hess H. *Nanolett*. 2009; 9(3):1170–1175. (d) Guo S, Li N, Lad N, Ray C, Akhremitchev BB. *J Am Chem Soc*. 2010; 132:9681–9687. [PubMed: 20583795]
16. Bonnet G, Tyagi S, Libchaber A, Kramer FR. *Proc Natl Acad Sci USA*. 1999; 96:6171–6176. [PubMed: 10339560]
17. Saghatelian A, Guckian KM, Thayer DA, Ghadiri MR. *J Am Chem Soc*. 2003; 125:344–345. [PubMed: 12517141]
18. Qavi AJ, Kindt JT, Bailey RC. *Anal Bioanal Chem*. 2010; 398:2535–2549. [PubMed: 20680616]
19. (a) Li JYZ, Yong TY, Michael MZ, Gleadle JM. *Nephrology*. 2010; 15:599–608. [PubMed: 20883280] (b) Budhu A, Ji J, Wang XW. *J Hematol Oncol*. 2010; 3:37. [PubMed: 20925959]
20. For example: Git A, Dvinge H, Salmon-Divon M, Osborne M, Kutter C, Hadfield J, Bertone P, Caldas C. *RNA*. 2010; 16:991–1006. [PubMed: 20360395] Li W, Ruan K. *Anal Bioanal Chem*. 2009; 394:1117–1124. [PubMed: 19132354]

Figure 1.

(a) Assembly of a discrete tris-biotinylated oligonucleotide-streptavidin complex, i.e. a “monovalent streptavidin”, and its ability to bind only one biotin (in this : streptavidin (STV) is represented by a grey 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 15mins and stood to cool (minor products were composed of DNA and streptavidin as determined by ratio of absorbance at 260nm/280nm and possess a greater charge (and hence mass) due to their elution times, but have not been characterized further). (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.

Figure 2.

Native PAGE results: The effect of complementary strand length (N) on the extent of oligomerization after 1 hour and 24 hours of incubation at room temperature. E.g. 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 no complementary strand added i.e. negative control. Lane S is 24mer complementary strand added in the presence of ten equivalents of streptavidin – this being a control for an approximation of a crosslinked dimer; and Lane B is 24mer complementary strand added in the presence of 1000 equivalents of biotin – this being a control for a dissociated biotin without crosslinking.

Figure 3. Sensitivity of biotin dissociation to mismatches: (a) Stacking gel non-denaturing PAGE results of addition of perfectly matched (dashed box) or single mismatch oligonucleotide to STV-(3) (LHS) compared with F-STV-(3) (where the lone vacant biotin-binding site is blocked with fluorescein-biotin (●-R)) (RHS) after 15mins incubation at 37°C. The mismatch occurs near the mid-point of the complementary strand. (b) ELISA results after 15mins of plate-bound STV-(3) in the presence of a perfectly matched or mismatched oligonucleotide at room temperature: i –QuantaRed HRP substrate only; ii – positive control, i.e. biotin present; iii –perfect match; iv – single base mismatch (CC, as in a above); v – negative control i.e. no complement added.