Bioconjugate Chemistry

Communication

pubs.acs.org/bc

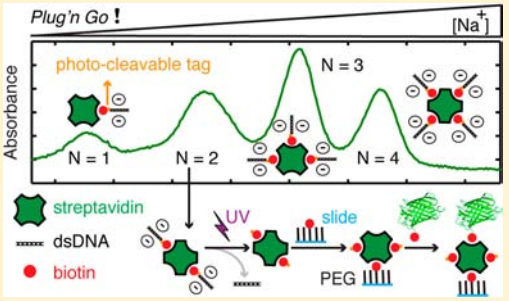
"Plug-and-Go" Strategy To Manipulate Streptavidin Valencies

Xun Sun, Daniel Montiel,[†] Hao Li, and Haw Yang*

Department of Chemistry, Princeton University, Princeton, New Jersey 08544, United States

Supporting Information

ABSTRACT: The streptavidin–biotin set is one of the most widely utilized conjugation pairs in biotechnological applications. The tetravalent nature of streptavidin and its homologues, however, tends to result in such undesirable complications as cross-linking or ill-defined stoichiometry. Here, we describe a mutagenesis-free strategy to manipulate the valencies of wild-type streptavidin that only requires commercially available reagents. The basic idea is simple: one obtains the desired streptavidin valency by blocking off unwanted binding sites using ancillary biotin ("plug"); this way, the extraordinary fM-biotin-binding affinity is fully retained for the remaining sites in streptavidin. In the present implementation, the ancillary biotin is attached to an auxiliary separation handle, negatively charged DNA or His-tagged protein, via a photochemically or enzymatically cleavable linker. Mixing streptavidin with the ancillary biotin construct produces a distribution of streptavidin valencies. The subsequent chromatographic separation readily isolates the construct of desired streptavidin valency, and the auxiliary handles are easily removed afterward ("go"). We demonstrate how this "plug-and-go" strategy allows a precise control for the compositions of streptavidin–biotin conjugates at the single-molecule level. This low-entry-barrier protocol could further expand the application scope of the streptavidin technology.



INTRODUCTION

The tetrameric *S. avidinii* streptavidin and chicken avidin are widely used "hubs" for bioconjugation in biochemistry, bio- and nano-technology, and medicine.[1,2] At the heart of these applications are the extraordinarily strong and specific non-covalent interaction (dissociation constant $K_d \approx 0.1-10$ fM),[3,4] and the near-diffusion-limited reaction rate ($k_{on} \approx 10^6-10^7$ M^{-1} s^{-1}) between streptavidin/avidin and its ligand, biotin.[5,6] The streptavidin/avidin–biotin pair enables a broad spectrum of applications, including affinity-tagged protein purification,[7,8] surface immobilization,[9,10] nanofabrication,[11,12] protein microarray,[13] among others. Extensive efforts have been devoted to expanding the functionality of this pair, which has one of the strongest protein–ligand interactions in nature. A new line of homologous proteins from various organisms with the similar biotin-binding capacity has been reported, such as rhizavidin from *R. etli*,[14] tamavidin from *P. cornucopiae*,[15] and xenavidin from *X. tropicalis*.[16] In addition, more than 100 mutants around the biotin-binding pocket in streptavidin and avidin[17] and assorted single-chain streptavidin/avidin constructs from circular permutation[18−20] have provided valuable information for fine-tuning this protein–ligand interaction.

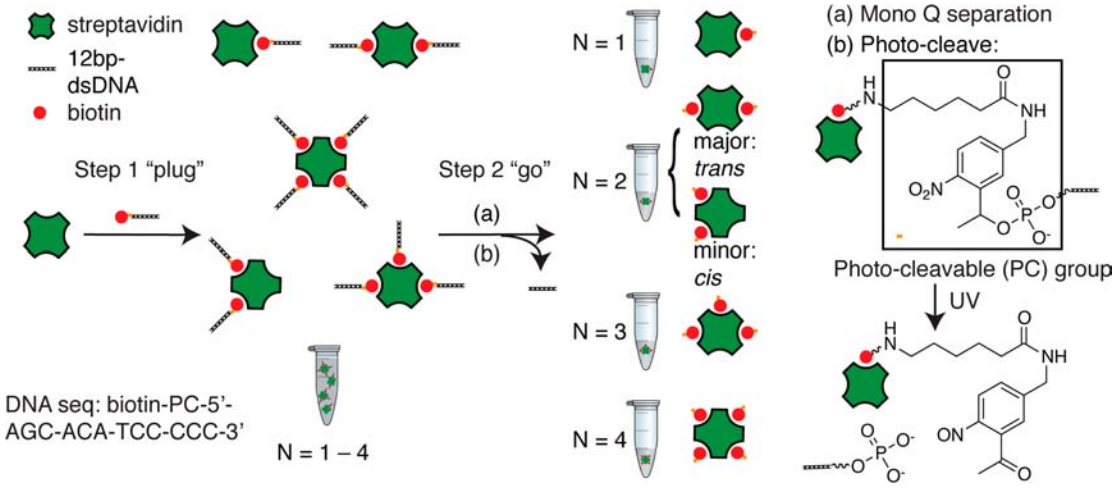
Though it is easy to implement the streptavidin/avidin–biotin conjugation, the fact that a streptavidin/avidin molecule has four binding sites could result in undesirable multimerization of biotinylated molecules on a single tetramer,[21] preventing the precise control of conjugate compositions. Two general approaches have been demonstrated to circumvent this issue; both involve genetic engineering of the streptavidin/avidin. The basic idea of the first method is to alter the oligomerization state of streptavidin/avidin by genetic modifications. Kulomaa and co-workers generated a monomeric avidin mutein by introducing two mutations at the subunit–subunit interface.[22] Park and co-workers engineered a novel monomeric construct by combining sequences from rhizavidin and streptavidin.[23,24] Wong and co-workers converted the streptavidin tetramer to soluble monomers via site-directed mutagenesis of structure-maintaining residues.[25−27] Along a similar vein, a handful of dimeric streptavidin muteins has been reported by Cantor and co-workers by altering the subunit interface.[28−30] Of note, the K_d's for the aforementioned constructs (~nM at best) are many orders of magnitude lower than that of the wild-type streptavidin, corroborating the notion that intersubunit interactions are likely a contributing factor to the extraordinary biotin-binding affinity.[31,32] While the greatly elevated binding reversibility and the relatively greater dissociation rate constant are useful in certain applications, e.g., affinity-tag-based purification, these constructs are not suitable for applications where the prolonged immobilization is a must, e.g., protein or DNA array or single-molecule experiments.

The basic idea of the second approach, developed by Ting and co-workers, is to genetically engineer a biotin-binding-incapable "dead" streptavidin subunit and to mix it with the wild-type streptavidin subunit with a polyhistidine tag (His-tag).[33−35] The subsequent refolding of such mixed subunits afforded a distribution of multivalent streptavidin. The unaltered subunit retained the fM K_d affinity toward biotin binding. In this approach, a Ni column was used to separate the

Received: March 19, 2014
Revised: July 26, 2014
Published: July 31, 2014

   dx.doi.org/10.1021/bc500296p | *Bioconjugate Chem.* 2014, 25, 1375−1380

Scheme 1. "Plug-and-Go" Strategy To Prepare Streptavidin with Well-Defined Valencies



desired streptavidin valency based on the number of His-tag's. More recently, Howarth and co-workers extended this "dead"/wild-type mixture approach and used ion exchange to separate out different stoichiometric ratios using the genetically engineered negatively charged poly glutamic/aspartic acid handles; they have observed a better separation resolution than that from using the Ni column.[36]

Not only have these pioneering works offered solutions to controlling the valency of streptavidin/avidin, but they also have provided additional insights for the streptavidin−biotin interaction. Yet, for a practitioner whose main interests are in utilizing the stoichiometric or structural aspect of streptavidin with well-defined binding sites, the aforementioned methods might still represent a relatively high technical barrier. Because streptavidin with clearly specified valencies is still not commercially available, one will have to make the genetically modified constructs in house; the investment in resources could be significant even for those who are familiar with molecular biology techniques. As such, a robust and easy-to-implement method is expected to further expand the application scope of the streptavidin/avidin technology.

Here, we introduce another strategy to manipulating streptavidin valency generally, which does not require any genetic modification to the core streptavidin. Inspired by the successful separation of streptavidin using different number of negatively charged handles,[36] we reasoned that a simple statistical mixing of wild-type streptavidin and biotin tagged with negatively charged scaffolds could afford a similar anion-exchange separation. In this way, the precise control over streptavidin's valency could be achieved by "counting" the number of tagged biotin on one streptavidin. Since the native binding surface of streptavidin would not be altered, the resultant construct should retain the same high affinity toward biotin as the unmodified streptavidin. For this proof-of-concept experiment, we chose short double-strand DNA (dsDNA) as the auxiliary handle; dsDNA was selected to prevent the oligomerization of streptavidin−DNA conjugates by DNA hybridization as shown by Talyor et al. in their work using trisbiotinylated single-strand oligonucleotides to block three biotin-binding sites in streptavidin.[37] The constitutive negative charge from DNA under neutral pH has been used to separate quantum dots or gold nanoparticles by gel electrophoresis.[38] To permit the removal of the auxiliary DNA handles after purification, we also included a photocleavable (PC) 2-nitrobenzyl linker[39] in the design (Scheme 1). This strategy proved to be convenient because the biotinylated forms of DNA with and without PC are commercially available. We showed that our scheme could accurately manipulate the valency of streptavidin. We further generalized the scope of separation handles to a His-tagged tobacco etch virus (TEV) protease-cleavable auxiliary protein to achieve the similar valency control. To showcase the use of such conjugates in biotechnological work, we applied the DNA-cleaved *trans*-divalent streptavidin to stoichiometrically immobilize one protomer from a dimeric *R. reniformis* green fluorescence protein (*rr*GFP)[40] at the single-molecule level.

■ RESULTS AND DISCUSSION

A short 12-base-pair 5′-biotinylated DNA with the PC group between the biotin and the 5′ position was chosen in this work as the auxiliary DNA handle to assist valency selection (Scheme 1, DNA handle abbreviated as bioPC12bpdsDNA). The DNA sequence was from the bacteriophage M13mp18 vector. Only one strand of the dsDNA carried the photocleavable biotin. The DNA was annealed prior to the mixing with streptavidin. The DNA sequence was such that its melting temperature (~45 °C) was higher than room temperature (25 °C) to ensure thermal stability of the auxiliary DNA handle. A prepacked Mono Q column (1 mL bed volume) was used as the anion-exchange medium for this study. The use of Mono Q to separate and to purify DNA fragments is a well-established protocol that has a resolution of ~10 bp.[41,42] We applied a low Na^+ gradient slope (0−1 M Na^+ in 400 mL during a time course of 200 min) to improve the separation resolution based on the number of auxiliary DNA handles, where constructs of high DNA handle number are expected to be eluted under high Na^+ concentrations due to better Debye screening (see Supporting Information for details).

We first characterized individual components to establish the baseline chromatogram readout. The completely annealed bioPC12bpdsDNA was injected to the Mono Q column and eluted as a single peak centered around 187 mL (~38 mS/cm peak conductance, Figure S1). As a control, we also annealed another design of the auxiliary DNA handle without the PC moiety (designated as bio12bpdsDNA); we observed that it was eluted in a similar fashion (Figure S1). In our design, we anticipated that the elution position of unconjugated auxiliary

dx.doi.org/10.1021/bc500296p | *Bioconjugate Chem.* 2014, 25, 1375−1380

DNA would be far away from that of streptavidin alone. In another two control runs, we showed that indeed this was the case: The wild-type core streptavidin (E14-S136, ProSpec, Figure S2) was eluted much earlier regardless of whether it contained biotin or not (20−30 mL elution volume, Figure S3).

We next turned to the main experiments. The auxiliary bioPC12bpdsDNA was incubated with streptavidin at a 5:2 molar ratio at room temperature for 10 h. The mixture was then injected into a Mono Q column for separation, monitored at both 260 nm (DNA) and 280 nm (streptavidin). Since the binding of biotin to streptavidin is noncooperative,[43,44] we expected streptavidin−DNA conjugates with a distribution of streptavidin/DNA ratios. Indeed, the Mono Q chromatogram in Figure 1a clearly shows a well-separated mixture of four

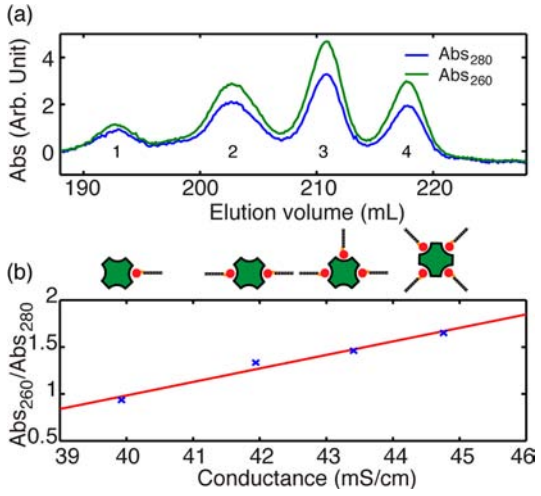


Figure 1. Anion-exchange separation of streptavidin with different valencies. (a) Mono Q chromatogram of the streptavidin−DNA conjugates. The relative absorbance is offset to the beginning of elution (~188 mL) for a better visual presentation. (b) The 260 nm/280 nm absorbance ratio, which is proportional to DNA/protein ratio, scales linearly with the peak conductance which is a direct measure of salt concentration.

components. The 260 nm/280 nm (DNA/streptavidin) absorbance ratio is seen to scale linearly with the peak conductance (Figure 1b) that is a direct measure of the salt (Na^+) concentration, suggesting that anion-exchange separation was dependent on the number of DNA handles. A similar elution profile was seen for bio12bpdsDNA conjugated with streptavidin under the same condition (Figure S4), indicating that the presence of the PC group did not affect the separation of streptavidin−DNA conjugates. On the basis of the evidence presented above and the finite possible combinations of streptavidin binding-site occupancy, we assigned a streptavidin/DNA ratio to each peak on the chromatogram as 1:1, 1:2, 1:3, and 1:4, from early elution to late elution.

The structures for the 1:1, 1:3, and 1:4 streptavidin−DNA conjugates are self-evident (cf. the cartoon shown as interpanel inset of Figure 1). The 1:2 conjugate, however, could have two possible geometrical arrangements; the D_2 symmetry of streptavidin architecture[45] affords *cis*- or *trans*-divalent streptavidin (we refer to 1,3 and 1,4 constructs as the *trans*-divalent streptavidin in our experimental scheme, as the distances between the numbered biotin-binding pockets are very similar in these two constructs;[36] also, see Scheme 1). As demonstrated by Fairhead et al.,[36] the purified *trans*-1,3-divalent streptavidin readily reacted with biotinylated DNA whereas the *cis*-divalent streptavidin clearly showed a negative cooperativity in binding biotinylated DNA. We reasoned that the analogous negatively cooperative binding between streptavidin and biotinylated DNA would occur in our work (presumably because of the steric repulsion) such that the *trans*-divalent streptavidin would be favored in reactions with a short incubation time (denoted as the major species in Scheme 1). We went on to test this idea by increasing the incubation time to increase the population of the less-favored *cis*-divalent streptavidin. As expected, a minor peak (compared to the *trans*-divalent streptavidin) was observed in our control experiment and its peak conductance was around 41 mS/cm (after the trivalent and before the *trans*-divalent streptavidin). This peak was assigned to the *cis*-divalent streptavidin (Figure S5). The valency assignments for all peaks were further supported by biotin-4-fluoresceine titration (Figure S6).

Having assigned stoichiometries and structures to the chromatogram peaks, we next proceeded to photocleave off the auxiliary DNA from the conjugates. The photocleaving approach is advantageous over a chemical-cleaving method because the steric effect could be a constraint for the latter,[46] whereas for the former the distance between biotin and the cleavable tag is not critical.[47] We analyzed the DNA off-loading reaction using native gel electrophoresis. The biotin-bound streptavidin exhibited a faster mobility (lanes 1 and 2 in Figure 2a), consistent with the additional negative changes on the

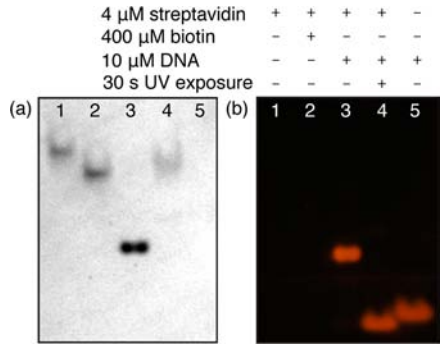


Figure 2. Native gel electrophoresis to resolve the UV-cleaved conjugates: (a) 8% native gel stained by Coomassie blue; (b) the same gel stained by 0.1% ethidium bromide. DNA refers to bio-PC12bpdsDNA.

biotin carboxylate[43,48] (also see Figure S3) and possibly the reported binding-induced structural compactness.[49] The binding of biotinylated DNA further increased the mobility of streptavidin (lane 3 in Figure 2a), owing to the high density of negative charges on DNA.[50] For DNA of such a short length, the 8% native gel could not resolve the number of DNA handles.[51] The colocalization of the DNA with streptavidin, stained by ethidium bromide (lane 3 in Figure 2b), supported this interpretation. We then optimized the UV exposure time to be 30 s under our current experimental condition, which was sufficient to cleave off DNA. We point out that it is necessary to tune the UV exposure time because overexposure to UV could lead to aggregation or misfolding of streptavidin (Figure S7). After a 30 s UV exposure, the photocleaved released DNA (without the PC group) was seen to migrate further to a position similar to that of the uncleaved DNA control (lanes 4 and 5 in Figure 2b). Because of the loss of the negatively charged auxiliary DNA, the streptavidin post-UV-exposure

dx.doi.org/10.1021/bc500296p | *Bioconjugate Chem.* 2014, 25, 1375−1380

(lane 4 in Figure 2a) was migrated faster than the free streptavidin and slower than the biotin-bound streptavidin. The smearing of this band supported the noncooperative binding between biotin and streptavidin,[44] which was also supported by a biotin titration experiment (Figure S8). A separate SDS–PAGE analysis also revealed a similar migration pattern (Figure S9). The purity of Mono Q purified multivalent streptavidin constructs were confirmed by a native PAGE analysis (Figure S10).

As an example illustrating the application of streptavidin with well-defined valencies, we utilized *trans*-divalent streptavidin to stoichiometrically immobilize individual protomers of the dimeric *rr*GFP on the single-molecule level and visualized via confocal fluorescence microscopy (Figure 3a,b and also see

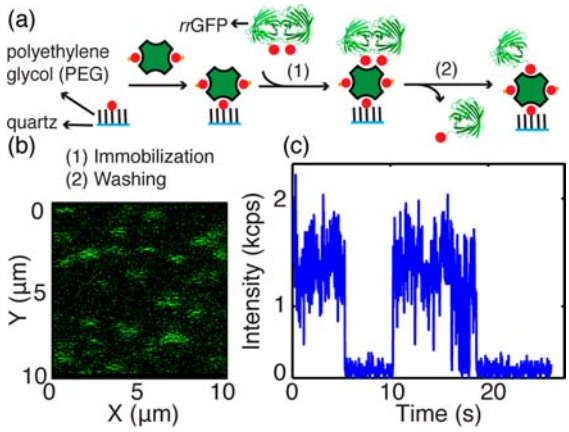


Figure 3. Application of the *trans*-divalent streptavidin to imaging of single protomer *rr*GFP: (a) scheme to stoichiometrically immobilize one *rr*GFP (PDB code 2RH7); (b) a representative scanning image of immobilized *rr*GFP; (c) a representative blinking trajectory of a single *rr*GFP.

Supporting Information for experimental details). We chose *rr*GFP because fluorescent proteins have been widely used for labeling and, in increasing cases, for assaying assembly stoichiometry. We were able to observe reversible blinking events for single *rr*GFP (Figures 3c), similar to the findings of the more studied GFP from *A. victoria*.[52] We also immobilized *rr*GFP by trivalent streptavidin and observed a distinct histogram of emission intensity that was shifted to higher photon counts (Figure S11), indicative of the relative enrichment of the immobilized dimeric *rr*GFP, as also seen in individual trajectories with the two-step bleaching behavior (Figure S12).

As suggested by previous studies, the biotin–streptavidin interaction at the solution–surface interface may be weaker than that in solution.[53] Therefore, we also tested the stability of our divalent streptavidin for prolonged surface immobilization and found that the biotin–streptavidin interaction was stable up to 3 or 4 days even under the single-molecule conditions (data not shown). The high stability of our divalent streptavidin is most likely due to the two bound ancillary biotin molecules, stoichiometrically delivered by the auxiliary DNA to the construct; it is known that streptavidin is significantly stabilized if all its four binding sites are occupied by biotin.[44,54]

In summary, we have demonstrated a robust and easy-to-implement method to produce streptavidin of well-defined valency. The central concept is intuitive: The valency of the four-site streptavidin can be specified by plugging up unwanted sites using ancillary biotin. Such a "plug-and-go" strategy does not require any genetic manipulation, uses commercially available reagents, and involves only a simple two-step mixing and separation. Our strategy consists of three basic elements: the ancillary biotin, the cleavable linker bridge, and the chromatographically resolvable auxiliary handle. The ancillary biotin modifies the available sites in streptavidin and confers stability to the streptavidin such that the resulting valence-specified streptavidin retains the strong biotin-binding capability. The cleavable bridge linking the ancillary biotin and the auxiliary handle allows the easy removal of the handle after purification. Finally, the auxiliary handle enables resolution of different streptavidin valencies via a standard chromatography separation.

We point out that this plug-and-go strategy is general. For example, the cleavable bridge does not have to be photo-activated; similarly, the auxiliary handle does not have to be DNA. As an example of generalizing the idea, we showed that streptavidin valency could also be manipulated using ancillary biotin linked to a TEV protease-cleavable bridge linked to a His-tagged ~30 kDa protein, where the His tag on the auxiliary protein allows the isolation using a Ni affinity column (Figure S13). By integration with various reported multibiotinylated "linkers",[37,55,56] this strategy could be potentially extended to manipulate the valency of cross-linked streptavidin/avidin or other homologous proteins that go beyond tetravalency.

While an immediate biophysical application of the plug-and-go strategy has been demonstrated in this work, we speculate that it would find further direct applications in bio- and nanotechnology as well as materials sciences, fields that will benefit from a precise composite and structural control of conjugated modules. The relatively low-entry-barrier strategy reported here to manipulate streptavidin valencies and to control bioconjugation compositions would greatly accelerate expansion of the application scope of the streptavidin/avidin technology and stimulate further innovations.

■ ASSOCIATED CONTENT

Supporting Information

Experimental details and supplementary figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +1-609-258-3578. Fax: +1-609-258-3708. E-mail: hawyang@princeton.edu.

Present Address

[†]D.M.: Laboratory of Genetically Encoded Small Molecules, The Rockefeller University, New York, NY 10065.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Simon McManus for helpful discussions. This work is supported by Human Frontiers Science Program No. RGP0031/2010-C202 and the financial support from Princeton University.

REFERENCES

(1) Dundas, C. M., Demonte, D., and Park, S. (2013) Streptavidin-biotin technology: Improvements and innovations in chemical and biological applications. *Appl. Microbiol. Biotechnol. 97*, 9343−9353.

(2) Laitinen, O. H., Nordlund, H. R., Hytönen, V. P., and Kulomaa, M. S. (2007) Brave new (strept)avidins in biotechnology. *Trends Biotechnol. 25*, 269−277.

(3) Sano, T., Vajda, S., Reznik, G. O., Smith, C. L., and Cantor, C. R. (1996) Molecular engineering of streptavidin. *Ann. N.Y. Acad. Sci. 799*, 383−390.

(4) Sano, T., and Cantor, C. R. (2000) Streptavidin-containing chimeric proteins: Design and production. *Methods Enzymol. 326*, 305−311.

(5) Buranda, T., Jones, G. M., Nolan, J. P., Keij, J., Lopez, G. P., and Sklar, L. A. (1999) Ligand receptor dynamics at streptavidin-coated particle surfaces: A flow cytometric and spectrofluorimetric study. *J. Phys. Chem. B 103*, 3399−3410.

(6) Srisa-Art, M., Dyson, E. C., deMello, A. J., and Edel, J. B. (2008) Monitoring of real-time streptavidin−biotin binding kinetics using droplet microfluidics. *Anal. Chem. 80*, 7063−7067.

(7) Rybak, J. N., Scheurer, S. B., Neri, D., and Elia, G. (2004) Purification of biotinylated proteins on streptavidin resin: A protocol for quantitative elution. *Proteomics 4*, 2296−2299.

(8) Chodosh, L. A., and Buratowski, S. (2001) Purification of DNA-binding proteins using biotin/streptavidin affinity systems. *Current Protocols in Protein Science* Chapter 9, Unit 9.7, Wiley, Brooklyn, NY.

(9) Rasnik, I., Myong, S., Cheng, W., Lohman, T. M., and Ha, T. (2004) DNA-binding orientation and domain conformation of the *E. coli* rep helicase monomer bound to a partial duplex junction: Single-molecule studies of fluorescently labeled enzymes. *J. Mol. Biol. 336*, 395−408.

(10) Niemeyer, C. M., Boldt, L., Ceyhan, B., and Blohm, D. (1999) DNA-directed immobilization: Efficient, reversible, and site-selective surface binding of proteins by means of covalent DNA-streptavidin conjugates. *Anal. Biochem. 268*, 54−63.

(11) Rief, M., Oesterhelt, F., Heymann, B., and Gaub, H. E. (1997) Single molecule force spectroscopy on polysaccharides by atomic force microscopy. *Science 275*, 1295−1297.

(12) Christman, K. L., Requa, M. V., Enriquez-Rios, V. D., Ward, S. C., Bradley, K. A., Turner, K. L., and Maynard, H. D. (2006) Submicron streptavidin patterns for protein assembly. *Langmuir 22*, 7444−7450.

(13) Geho, D., Lahar, N., Gurnani, P., Huebschman, M., Herrmann, P., Espina, V., Shi, A., Wulfkuhle, J., Garner, H., Petricoin, E., III, Liotta, L. A., and Rosenblatt, K. P. (2005) Pegylated, steptavidin-conjugated quantum dots are effective detection elements for reverse-phase protein microarrays. *Bioconjugate Chem. 16*, 559−566.

(14) Meir, A., Helppolainen, S. H., Podoly, E., Nordlund, H. R., Hytönen, V. P., Määttä, J. A., Wilchek, M., Bayer, E. A., Kulomaa, M. S., and Livnah, O. (2009) Crystal structure of rhizavidin: Insights into the enigmatic high-affinity interaction of an innate biotin-binding protein dimer. *J. Mol. Biol. 386*, 379−390.

(15) Takakura, Y., Tsunashima, M., Suzuki, J., Usami, S., Kakuta, Y., Okino, N., Ito, M., and Yamamoto, T. (2009) Tamavidins—novel avidin-like biotin-binding proteins from the Tamogitake mushroom. *FEBS J. 276*, 1383−1397.

(16) Määttä, J. A., Helppolainen, S. H., Hytönen, V. P., Johnson, M. S., Kulomaa, M. S., Airenne, T. T., and Nordlund, H. R. (2009) Structural and functional characteristics of xenavidin, the first frog avidin from *Xenopus tropicalis*. *BMC Struct. Biol. 9*, 63.

(17) Laitinen, O. H., Hytönen, V. P., Nordlund, H. R., and Kulomaa, M. S. (2006) Genetically engineered avidins and streptavidins. *Cell. Mol. Life Sci. 63*, 2992−3017.

(18) Nordlund, H. R., Hytönen, V. P., Hörhä, J., Määttä, J. A., White, D. J., Halling, K., Porkka, E. J., Slotte, J. P., Laitinen, O. H., and Kulomaa, M. S. (2005) Tetravalent single-chain avidin: From subunits to protein domains via circularly permuted avidins. *Biochem. J. 392*, 485−491.

(19) Hytönen, V. P., Nordlund, H. R., Hörhä, J., Nyholm, T. K., Hyre, D. E., Kulomaa, T., Porkka, E. J., Marttila, A. T., Stayton, P. S., Laitinen, O. H., and Kulomaa, M. S. (2005) Dual-affinity avidin molecules. *Proteins 61*, 597−607.

(20) Hytönen, V. P., Hörhä, J., Airenne, T. T., Niskanen, E. A., Helttunen, K. J., Johnson, M. S., Salminen, T. A., Kulomaa, M. S., and Nordlund, H. R. (2006) Controlling quaternary structure assembly: Subunit interface engineering and crystal structure of dual chain avidin. *J. Mol. Biol. 359*, 1352−1363.

(21) Howarth, M., Takao, K., Hayashi, Y., and Ting, A. Y. (2005) Targeting quantum dots to surface proteins in living cells with biotin ligase. *Proc. Natl. Acad. Sci. U.S.A. 102*, 7583−7588.

(22) Laitinen, O. H., Nordlund, H. R., Hytönen, V. P., Uotila, S. T. H., Marttila, A. T., Savolainen, J., Airenne, K. J., Livnah, O., Bayer, E. A., Wilchek, M., and Kulomaa, M. S. (2003) Rational design of an active avidin monomer. *J. Biol. Chem. 278*, 4010−4014.

(23) Lim, K. H., Huang, H., Pralle, A., and Park, S. (2013) Stable, high-affinity streptavidin monomer for protein labeling and monovalent biotin detection. *Biotechnol. Bioeng. 110*, 57−67.

(24) Demonte, D., Drake, E. J., Lim, K. H., Gulick, A. M., and Park, S. (2013) Structure-based engineering of streptavidin monomer with a reduced biotin dissociation rate. *Proteins 81*, 1621−1633.

(25) Qureshi, M. H., and Wong, S. L. (2002) Design, production, and characterization of a monomeric streptavidin and its application for affinity purification of biotinylated proteins. *Protein Expression Purif. 25*, 409−415.

(26) Qureshi, M. H., Yeung, J. C., Wu, S. C., and Wong, S. L. (2001) Development and characterization of a series of soluble tetrameric and monomeric streptavidin muteins with differential biotin binding affinities. *J. Biol. Chem. 276*, 46422−46428.

(27) Wu, S. C., Ng, K. K., and Wong, S. L. (2009) Engineering monomeric streptavidin and its ligands with infinite affinity in binding but reversibility in interaction. *Proteins 77*, 404−412.

(28) Aslan, F. M., Yu, Y., Mohr, S. C., and Cantor, C. R. (2005) Engineered single-chain dimeric streptavidins with an unexpected strong preference for biotin-4-fluorescein. *Proc. Natl. Acad. Sci. U.S.A. 102*, 8507−8512.

(29) Aslan, F. M., Yu, Y., Vajda, S., Mohr, S. C., and Cantor, C. R. (2007) Engineering a novel, stable dimeric streptavidin with lower isoelectric point. *J. Biotechnol. 128*, 213−225.

(30) Sano, T., Vajda, S., Smith, C. L., and Cantor, C. R. (1997) Engineering subunit association of multisubunit proteins: A dimeric streptavidin. *Proc. Natl. Acad. Sci. U.S.A. 94*, 6153−6158.

(31) Sano, T., and Cantor, C. R. (1995) Intersubunit contacts made by tryptophan-120 with biotin are essential for both strong biotin binding and biotin-induced tighter subunit association of streptavidin. *Proc. Natl. Acad. Sci. U.S.A. 92*, 3180−3184.

(32) Lim, K. H., Huang, H., Pralle, A., and Park, S. (2011) Engineered streptavidin monomer and dimer with improved stability and function. *Biochemistry 50*, 8682−8691.

(33) Howarth, M., Chinnapen, D. J., Gerrow, K., Dorrestein, P. C., Grandy, M. R., Kelleher, N. L., El-Husseini, A., and Ting, A. Y. (2006) A monovalent streptavidin with a single femtomolar biotin binding site. *Nat. Methods 3*, 267−273.

(34) Howarth, M., Liu, W., Puthenveetil, S., Zheng, Y., Marshall, L. F., Schmidt, M. M., Wittrup, K. D., Bawendi, M. G., and Ting, A. Y. (2008) Monovalent, reduced-size quantum dots for imaging receptors on living cells. *Nat. Methods 5*, 397−399.

(35) Howarth, M., and Ting, A. Y. (2008) Imaging proteins in live mammalian cells with biotin ligase and monovalent streptavidin. *Nat. Protoc. 3*, 534−545.

(36) Fairhead, M., Krndija, D., Lowe, E. D., and Howarth, M. (2014) Plug-and-play pairing via defined divalent streptavidins. *J. Mol. Biol. 426*, 199−214.

(37) Taylor, S. K., Wang, J. X., Kostic, N., and Stojanovic, M. N. (2013) Monovalent streptavidin that senses oligonucleotides. *Angew. Chem., Int. Ed. 52*, 5509−5512.

(38) Fu, A., Micheel, C. M., Cha, J., Chang, H., Yang, H., and Alivisatos, A. P. (2004) Discrete nanostructures of quantum dots/Au with DNA. *J. Am. Chem. Soc. 126*, 10832−10833.
(39) Bai, X., Kim, S., Li, Z., Turro, N. J., and Ju, J. (2004) Design and synthesis of a photocleavable biotinylated nucleotide for DNA analysis by mass spectrometry. *Nucleic Acids Res. 32*, 535−541.
(40) Ward, W. W., and Cormier, M. J. (1979) An energy transfer protein in coelenterate bioluminescence. Characterization of the *Renilla* green-fluorescent protein. *J. Biol. Chem. 254*, 781−788.
(41) Müller, W. (1986) Fractionation of DNA restriction fragments with ion-exchangers for high-performance liquid-chromatography. *Eur. J. Biochem. 155*, 203−212.
(42) Westman, E., Eriksson, S., Låås, T., Pernemalm, P. A., and Sköld, S. E. (1987) Separation of DNA restriction fragments by ion-exchange chromatography on FPLC columns Mono-P and Mono-Q. *Anal. Biochem. 166*, 158−171.
(43) Jones, M. L., and Kurzban, G. P. (1995) Noncooperativity of biotin binding to tetrameric streptavidin. *Biochemistry 34*, 11750−11756.
(44) González, M., Bagatolli, L. A., Echabe, I., Arrondo, J. L., Argarana, C. E., Cantor, C. R., and Fidelio, G. D. (1997) Interaction of biotin with streptavidin. Thermostability and conformational changes upon binding. *J. Biol. Chem. 272*, 11288−11294.
(45) Le Trong, I., Wang, Z. Z., Hyre, D. E., Lybrand, T. P., Stayton, P. S., and Stenkamp, R. E. (2011) Streptavidin and its biotin complex at atomic resolution. *Acta Crystallogr. D 67*, 813−821.
(46) Herman, T. M., Lefever, E., and Shimkus, M. (1986) Affinity chromatography of DNA labeled with chemically cleavable biotinylated nucleotide analogs. *Anal. Biochem. 156*, 48−55.
(47) Elia, G. (2008) Biotinylation reagents for the study of cell surface proteins. *Proteomics 8*, 4012−4024.
(48) Sano, T., and Cantor, C. R. (1990) Cooperative biotin binding by streptavidin. Electrophoretic behavior and subunit association of streptavidin in the presence of 6 M urea. *J. Biol. Chem. 265*, 3369−3373.
(49) Freitag, S., Le Trong, I., Klumb, L., Stayton, P. S., and Stenkamp, R. E. (1997) Structural studies of the streptavidin binding loop. *Protein Sci. 6*, 1157−1166.
(50) Funabashi, H., Ubukata, M., Ebihara, T., Aizawa, M., Mie, M., and Kobatake, E. (2007) Assessment of small ligand-protein interactions by electrophoretic mobility shift assay using DNA-modified ligand as a sensing probe. *Biotechnol. Lett. 29*, 785−789.
(51) Chory, J., and Pollard, J. D., Jr. (2001) Separation of small DNA fragments by conventional gel electrophoresis. *Current Protocols in Molecular Biology*, Chapter 2, Unit 2.7, Wiley, Brooklyn, NY.
(52) Dickson, R. M., Cubitt, A. B., Tsien, R. Y., and Moerner, W. E. (1997) On/off blinking and switching behaviour of single molecules of green fluorescent protein. *Nature 388*, 355−358.
(53) Swift, J. L., Heuff, R., and Cramb, D. T. (2006) A two-photon excitation fluorescence cross-correlation assay for a model ligand-receptor binding system using quantum dots. *Biophys. J. 90*, 1396−1410.
(54) Bayer, E. A., Ehrlich-Rogozinski, S., and Wilchek, M. (1996) Sodium dodecyl sulfate-polyacrylamide gel electrophoretic method for assessing the quaternary state and comparative thermostability of avidin and streptavidin. *Electrophoresis 17*, 1319−1324.
(55) Wilbur, D. S., Pathare, P. M., Hamlin, D. K., and Weerawarna, S. A. (1997) Biotin reagents for antibody pretargeting. 2. Synthesis and in vitro evaluation of biotin dimers and trimers for cross-linking of streptavidin. *Bioconjugate Chem. 8*, 819−832.
(56) Farrera, J. A., Hidalgo-Fernández, P., Hannink, J. M., Huskens, J., Rowan, A. E., Sommerdijk, N. A., and Nolte, R. J. (2005) Divalent ligand for intramolecular complex formation to streptavidin. *Org. Biomol. Chem. 3*, 2393−2395.