ORGANIC LETTERS

2005
Vol. 7, No. 1
111−114

# Molecular Tripods Showing Fluorescence Enhancement upon Binding to Streptavidin

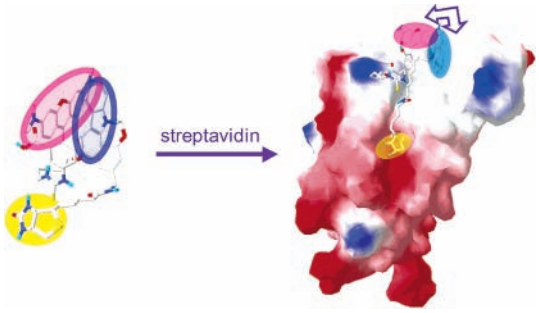
**Tae Woo Kim,[†] Hey Young Yoon,[†] Jung-hyun Park,[†] Oh-Hoon Kwon,[†] Du-Jeon Jang,*[,†] and Jong-In Hong*[,†,‡]**

*Department of Chemistry, College of Natural Sciences, Seoul National University, Seoul 151-747, Korea, and Center for Molecular Design and Synthesis, KAIST, Daejon 305−701, Korea*

*jihong@.plaza.snu.ac.kr*

**Received October 26, 2004**

## ABSTRACT




**We introduce a new approach to biotin–streptavidin assays based on a molecular tripod which consists of biotin, a fluorophore, and a quencher. The interaction between streptavidin and molecular tripods perturbs the ground-state quencher–fluorophore dimeric conformation in the absence of streptavidin and diminishes the intrinsic self-quenching of a quencher–fluorophore pair. The emission intensity of the molecular tripods plus streptavidin is 3.5–5.2 times that of molecular tripods in the absence of streptavidin.**

Since biotin shows extremely strong binding affinity toward avidin and streptavidin, the noncovalent binding interaction between biotin and (strept)avidin has found widespread bioanalytical applications such as detection of biomolecules, clinical diagnosis, and immunoassay.[1] Therefore, specific measurement of (strept)avidin is often needed. For the biotin−(strept)avidin assays, three kinds of techniques such as radioligand binding, enzyme assay, and photometrics/fluorimetrics have been developed.[2] Of these, optical and fluorescence methods offer significant advantages for analytical purposes in sensitivity and simplicity.[3] The simplest approach to the biotin−(strept)avidin monitoring would be using biotin−fluorophore conjugates. However, most biotin−fluorophore conjugates suffer from fluorescence diminishment upon binding to (strept)avidin presumably due to resonance energy transfer,[4] except for biotin−fluorophore conjugates with long spacers.[5] Therefore, it would be useful

[†] Seoul National University.
[‡] KAIST.

(1) (a) Wilchek, M.; Bayer, E. A. *Methods of Enzymology*; Academic Press: San Diego, 1990; Vol. 184. (b) Christopoulos, T. K.; Diamandis, E. P. *Immunoassay*; Academic Press: San Diego, CA, 1996; Vol. 184. (c) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, CA, 1996. (d) Guggiero, F. P.; Sheffield, J. B. J. *Histochem. Cytochem.* **1998**, *46*, 177.

(2) (a) Groman, E. V.; Rothenberg, J. M.; Bayer, E. A.; Wilchek, M. *Methods Enzymol.* **1990**, *184*, 217. (b) Barbarakis, M. S.; Qaisi, W. G.; Daunert, S.; Bachas, L. G. *Anal. Chem.* **1993**, *65*, 457. (c) Mock, D. M.; Horowitz, P. *Methods Enzymol.* **1990**, *184*, 234. (d) D'Auria, S.; Lakowicz, J. R. *Curr. Opin. Biotechnol.* **2001**, *12*, 99. (e) Harrison, C.; Traynor, J. R. *Life Sci.* **2003**, *74*, 489.

(3) (a) Lakowicz, J. R. *Principles of Fluorescence Spectroscopy*, 2nd ed.; Kluwer Academic and Plenum Publishers: New York, 1999. (b) de Silva, A. P.; Gunaratne, H. Q. N.; Gunnlaugsson, T.; Huxley, A. J. M.; McCoy, C. P.; Rademacher, J. T.; Rice, T. E. *Chem. Rev.* **1997**, *97*, 1515.

(4) (a) Kada, G.; Falk, H.; Gruber, H. J. *Biochim. Biophys. Acta* **1999**, *1427*, 33. (b) Kada, G.; Kaiser, K.; Falk, H.; Gruber, H. J. *Biochim. Biophys. Acta* **1999**, *1427*, 44.

10.1021/ol047801h CCC: $30.25 
**Published on Web 12/15/2004**

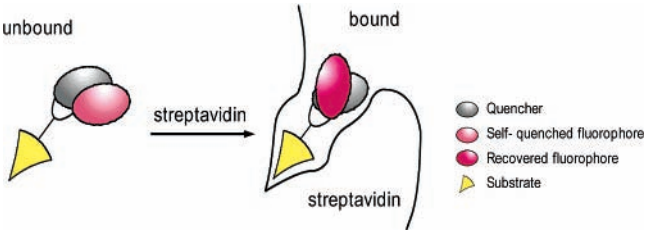



**Figure 1.** Schematic drawing for the off−on fluorescence mechanism of a molecular tripod in streptavidin binding.

to develop fluorescent probes showing fluorescence enhancement upon binding to (strept)avidin.[6] Recently, Lo and coworkers developed rhenium(I) polypyridine−biotin complexes that show luminescence enhancement and lifetime elongation upon binding to avidin.[6a,b] Sleiman recently showed that ruthenium(II) phenanthroline−biotin conjugates can be used as luminescent probes for avidin with luminescence enhancement when binding to avidin.[6e] We were interested in the development of molecular tripod (biotin−quencher−fluorophore conjugate)-based streptavidin probes with novel off−on fluorescence mechanisms.

Here we report on new fluorescent probes that are useful in the detection of streptavidin in homogeneous solution. The probes are composed of biotin, a fluorophore, a quencher, and a spacer. The quencher−fluorophore pair as a reporting group can monitor any fluorescence change before and after the biotin−streptavidin binding event.[7,8] The spacer maintains a quencher−fluorophore pair in spatial proximity, causing the fluorescence of the pair to be quenched in an aqueous, unbound state via the ground-state intramolecular complex between the fluorophore and the quencher.[9] When the probe encounters streptavidin, it forms a streptavidin−biotin complex that perturbs the ground-state intramolecular complex and eventually impedes the self-quenching of a quencher−fluorophore pair (Figure 1). We call these probes "molecular

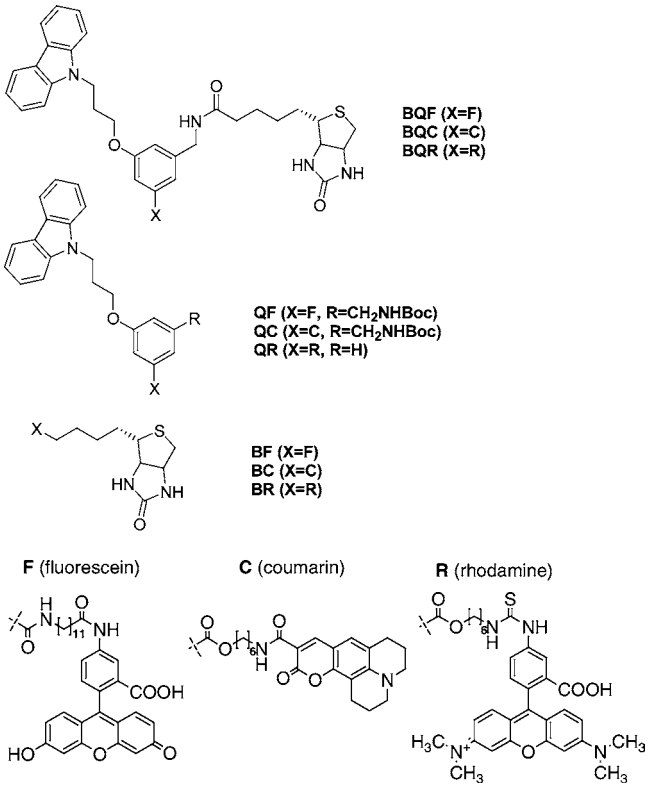



**Figure 2.** Nomenclature of the molecular tripods and reference compounds used in this work.

tripods" because they have trifurcated structures. Because streptavidin binding triggers fluorescence enhancement, in principle, molecular tripods can be used for the detection of streptavidin in homogeneous assay.

The molecular tripods consist of biotin as a substrate for streptavidin, fluorescein, coumarin 343, and rhodamine as fluorophores and carbazole as a quencher (Figure 2). A spacer based on 5-hydroxyisophthalic acid was used for ease of synthesis. Streptavidin, a nonglycosylated 52 800 Da protein with a near-neutral isoelectric point, binds four biotins per molecule with high affinity and selectivity. We also synthesized two kinds of reference compounds; quencher−fluorophore conjugates (**QF**, **QC**, **QR**) and biotin−fluorophore conjugates (**BF**, **BC**, **BR**) (Figure 2).[10,11] In the case of the biotin−fluorophore conjugates, biotin was directly connected to fluorophores without the 5-hydroxyisophthalic acid spacer.[10] To define the quenching efficiency, we also prepared alkyl derivatives of fluorophores (**F′**, **C′**, **R′**).[10]

To ensure the spontaneous formation of intramolecular ground-state dimeric complexes in the quencher−fluorophore pairs, self-quenching efficiency was measured for carbazole-containing probes (Table 1). Because the protein surface usually works as a fluorescence energy transfer sinker,[4,11,12]

(5) (a) Gruber, H. J.; Marek, M.; Schindler, H.; Kaiser, K. *Bioconjugate Chem.* **1997**, *8*, 552. (b) Marek, M.; Kaiser, K.; Gruber, H. J. *Bioconjugate Chem.* **1997**, *8*, 560. (c) Gruber, H. J.; Hahn, C. D.; Kada, G.; Riener. C. K.; Harms, G. S.; Ahrer, W.; Dax, T. G.; Knaus, H.-G. *Bioconjugate Chem.* **2000** *11*, 696.

(6) (a) Lo, K. K.-W.; Hui, W.-K.; Ng, D. C.-M. *J. Am. Chem. Soc.* **2002**, *124*, 9344. (b) Lo, K. K.-W.; Tsang, K. H.-K. *Organometallics* **2004**, *23*, 3062. (c) Lo, K. K.-W.; Chan, J. S.-W.; Lui, L.-H.; Chung, C.-K. *Organometallics* **2004**, *23*, 3108. (d) Lo, K. K.-W.; Lee, T. K.-M. *Inorg. Chem.* **2004**, *43*, 5275. (e) Slim, M.; Sleiman, H. F. *Bioconjugate Chem.* **2004**, *15*, 949.

(7) Neuweiler, H.; Schulz, A.; Vaiana, A. C.; Smith, J. C.; Kaul, S.; Wolfrum, J.; Sauer, M. *Angew. Chem., Int. Ed.* **2002**, *41*, 4769.

(8) Quencher−fluorophore pairs have been applied for sensing small molecules to high molecular weight analytes. (a) For cations: Aoki, I.; Sakai, T.; Shinkai, S. *J. Chem. Soc., Chem. Commun.* **1992**, 730. (b) For peptides: Chen, C.-T.; Wagner, H.; Still, W. C. *Science* **1998**, *279*, 851. (c) For protein−ligand binding: Wei, A.-P.; Blumenthal, D. K.; Herron, J. N. *Anal. Chem.* **1994**, *66*, 1500. (d) For cholic acid: Hossain, M. A.; Hamasaki, K.; Takahashi, K.; Mihara, H.; Ueno, A. *J. Am. Chem. Soc.* **2001**, *123*, 7435.

(9) (a) Packard, B. Z.; Toptygin, D. D.; Komoriya, A.; Brand, L. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 11640. (b) Takakusa, H.; Kikuchi, K.; Urano, Y.; Higuchi, T.; Nagano, T. *Anal. Chem.* **2001**, *73*, 939. (c) Johansson, M. K.; Fidder, H.; Dick, D.; Cook, R. M. *J. Am. Chem. Soc.* **2002**, *124*, 6950. (d) Johansson, M. K.; Cook, R. M. *Chem. Eur. J.* **2003**, *9*, 3466.

(10) See the Supporting Information.

(11) Specific binding of biotin−fluorescein conjugates towards streptavidin was accompanied by fluorescence quenching: see ref 4.

(12) Gruber, H. J.; Kada, G.; Marek, M.; Kaiser, K. *Biochim. Biopys. Acta* **1998**, *1381*, 203.

**Table 1.** Quenching Efficiency[a,b] (QE) and Enhancement Factor[c] (EF)

| A. fluorescein based[d] | | | | B. coumarin 343 based[e] | | | | C. rhodamine basd[f] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| QE | | EF | | QE | | EF | | QE | | EF | |
| **QF** | **BQF** | **BF** + S[4,11,12] | **BQF** + S | **QC** | **BQC** | **BC** + S | **BQC** + S | **QR** | **BQR** | **BR** + S | **BQR** + S |
| −99% | −97% | | 0.20 | −98% | −99% | 0.30 | 3.5 | −99% | −99% | 1.05 | 5.2 |

[a] Buffer A (100 mM NaCl, 50 mM $NaH_2PO_4$, 1 mM EDTA, pH adjusted to 7.5 with NaOH) was used. [b] QE = {[FI − $FI_0$]/$FI_0$} × 100, $FI_0$ = fluorescence intensity (FI) of free fluorophore **X** (**F** for A, **C** for B, and **R** for C) at 50 nM, FI = fluorescence intensity of the probe at 50 nM. [c] EF = $FI_{with}/FI_{without}$, $FI_{without}$ = fluorescence intensity of the probe without streptavidin, $FI_{with}$ = fluorescence intensity of the probe with streptavidin (15 nM) at [probe] = 50 nM. Values of EF greater than 1 indicate the fluorescence enhancement by streptavidin addition and values less than 1 the fluorescence quenching by streptavidin addition; [d] $\lambda^{ex}$ = 490 nm, $\lambda^{em}_{max}$ = 515 nm. [e] $\lambda^{ex}$ = 446 nm, $\lambda^{em}_{max}$ = 500 nm. [f] $\lambda^{ex}$ = 543 nm, $\lambda^{em}_{max}$ = 570 nm. S = streptavidin.

we also have to check the fluorescence change in biotin−fluorophore conjugates (**BF**, **BC**, **BR**) after streptavidin binding. The high quenching efficiency (≈ 97−99%) indicates that the designed molecules (**BQX**, **QX**, **X** = **F**, **C**, **R**) form ground-state intramolecular dimers over the 5-hydroxyisophthalic acid spacer. In the case of **BQC** and **BQR**, the quenched fluorescence of the molecular tripods was partially recovered after streptavidin binding. The emission intensity of the **BQC** + streptavidin and **BQR** + streptavidin is 3.5 and 5.2 times that of **BQC** and **BQR** in the absence of streptavidin, respectively (Table 1). However, the fluorescence of **BQF** decreased after streptavidin binding. This result implies that we have to consider the interaction between the surface of streptavidin around the binding pocket and a fluorophore in order to successfully design the molecular tripods.

The fluorescence intensity of **BC** was reduced by increasing streptavidin, while the response of **BQC** was opposite (Table 1, Figure 3). As shown in Figure 3, an enhancement

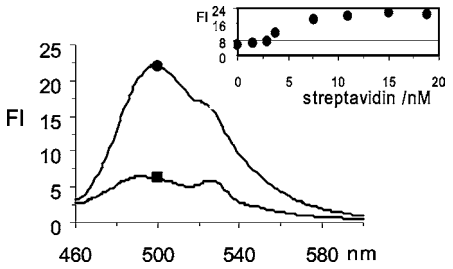



**Figure 3.** Fluorescence responses of 50 nM **BQC** to streptavidin addition (■ = 0 nM, ● = 15 nM). The inset shows the continuous fluorescence titration of **BQC** (50 nM) with streptavidin.

of 3.5 was observed in the presence of streptavidin. The results show that our strategy, namely the diminution of fluorescence self-quenching by streptavidin−biotin binding, works well in the molecular tripod.

For real-time monitoring, the time dependence of the **BQC**−streptavidin interaction was checked. It turns out that the fluorescence intensity stabilizes after a short period of vortexing and the value was hardly changed even after 1 day. It was known that the fluorimetric assay involving the biotin−fluorescein conjugate with a long 14-atom spacer possessed several drawbacks such as long assay times and moderate sensitivity, resulting from the anti-cooperative binding of streptavidin.[12] The sigmoid titration curve in the inset of Figure 3 reflects the 1:4 (streptavidin/biotin) stoichiometry and anti-cooperative binding. Therefore, we were not able to determine $K_d$ from the curve. Nevertheless, the saturation pattern above 10 nM is consistent with the known binding property of streptavidin.

To prove that the molecular tripod is a selective probe for streptavidin, we measured the fluorescence enhancement of **BQC** and **BQR** caused by the addition of streptavidin and bovine serum albumin (BSA) under the same conditions (Figure 4 and Supporting Information). While addition of

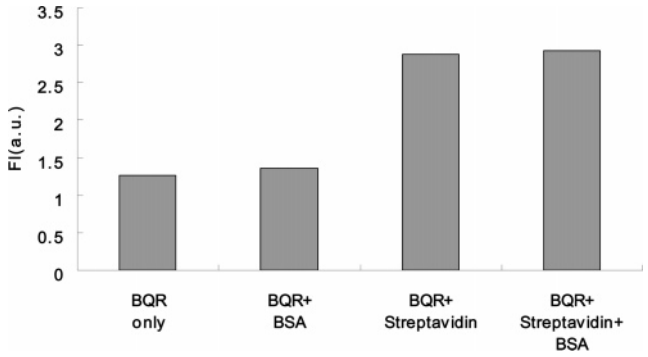



**Figure 4.** Fluorescence intensity changes of **BQR** by the addition of streptavidin, BSA, and both. [**BQR**] = 50 nM in buffer A; [streptavidin] = 400 ng $mL^{-1}$, [BSA] = 400 ng $mL^{-1}$, [streptavidin + BSA] = 400 ng $mL^{-1}$ for streptavidin and 400 ng $mL^{-1}$ for BSA.

BSA to each tripod caused little change in the fluorescence intensity, streptavidin addition resulted in larger emission enhancement. In particular, the fluorescence intensity changes little upon addition of **BQR** to the mixture of streptavidin and BSA. The results imply that the molecular tripod functions as a specific streptavidin probe.

The effect of added biotin in the emission spectrum of **BQR**−streptavidin complex is shown in Figure 5. Addition of increasing amounts of biotin to the preformed complex of **BQR**−streptavidin in more than a 4:1 ratio resulted in

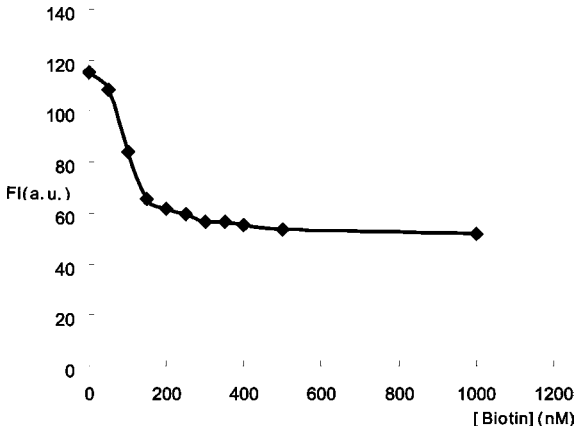



**Figure 5.** Fluorescence changes of **BQR** (500 nM) in the presence of streptavidin (100 nM) in buffer A solution with the addition of biotin solution (0−1000 nM). After each mixture of **BQR** and streptavidin was equilibrated for 1 h, a different amount of biotin was added and the mixture equilibrated for 20 h. Maximum emission intensities at 570 nm were recorded after each addition of biotin solution. The emission intensity of the free **BQR** (500 nM) is about 40 au.

gradual decrease in the fluorescence intensity, until an approximately 4:1 ratio of biotin/streptavidin was reached, upon which the fluorescence intensity of the tripod did not change even with further addition of biotin. This result indicates that **BQR** is displaced from streptavidin by biotin. Although biotin itself binds more strongly to streptavidin than **BQR**, **BQR** still strongly binds to streptavidin with fluorescence enhancement.

The fluorescence lifetime measurements show a small lifetime increase for molecular tripods (+5% for **BQC**, +8% for **BQR**) and some decrease for biotin−fluorophore conjugates (−19% for **BC**, −11% for **BR**) upon binding to streptavidin. However, the lifetime changes are too small to explain the fluorescence enhancement by energy transfer in an excitation state. Absorption spectrum of **BQR** shows that the peak at shorter wavelengths (about 520 nm) decreases after the addition of streptavidin (see the Supporting Information). The change in shape of the absorption spectrum indicates that the population of H-type ground-state intramolecular dimers decreases after streptavidin binding. The fluorescence quenching of an unbound probe results from the H-type ground-state intramolecular complex.[9] The congested environment and protein surface interaction around the protein binding pocket in the bound state disrupts the H-type ground-state intramolecular structure of the unbound state, and thus, the quenched fluorescence is recovered.

A qualitative prospect for the interaction between the streptavidin surface and a tripod can be obtained from X-ray crystal structure (PDB ID 1STP).[13] Lys-121 of streptavidin−biotin complex is placed within 15 Å from the carboxy terminal of biotin. The positive charge around the streptavidin binding pocket gives a clue to understanding why coumarin 343 and rhodamine work for molecular tripods and why fluorescein does not. A net negatively charged, fluorescein-based probe may undergo electrostatic attraction with Lys-121, so the time/space proximity between the protein surface and the fluorescein-based probe makes the protein surface function as a fluorescence sinker. Compared with a fluorescein-based one, neutral coumarin 343-based, and net positively charged rhodamine-based probes have no attractive interaction with Lys-121. Although this explanation may be too simplified, this rough approach suggests a guideline for molecular tripod design. From the protein surface analysis, we can rule out fluorophores which might interact with hot residues around the protein−substrate binding pocket.

In summary, we have developed a new biotin−streptavidin assay technique using a molecular tripod based on a biotin−streptavidin interaction. The interaction between streptavidin and the molecular tripod perturbs ground-state quencher−fluorophore dimeric conformation and diminishes the intrinsic self-quenching of the quencher−fluorophore pair. The fluorescence enhancement brought about by streptavidin binding is comparable to the fluorescence quenching of biotin−4-fluorescein.[4] Positive fluorescence response, rapid equilibrium, saturated titration behavior, and streptavidin specificity show that the molecular tripod is suitable for detecting streptavidin in homogeneous solutions. This new approach is potentially useful for the study of various protein−substrate interactions.

**Acknowledgment.** Financial support from the Basic Research Program of the KOSEF (Grant No. R02-2003-000-10055-0) is gratefully acknowledged. T.W.K., J.-h.P., and H.Y.Y. thank the Ministry of Education for the award of the BK 21 fellowship. D.-J.J. is thankful for the Strategic National R&D Program.

**Supporting Information Available:** Synthetic scheme and spectral data of the molecular tripods and reference compounds in Figure 2, fluorescence enhancement factor by the addition of streptavidin, BSA, and both, and absorption spectra of **BQR** and **BQR** + streptavidin. This material is available free of charge via the Internet at http://pubs.acs.org.

OL047801H

(13) Weber, P. C.; Ohlendorf, D. H.; Wendoloski, J. J.; Salemme, F. R. *Science* **1989**, *243*, 85.