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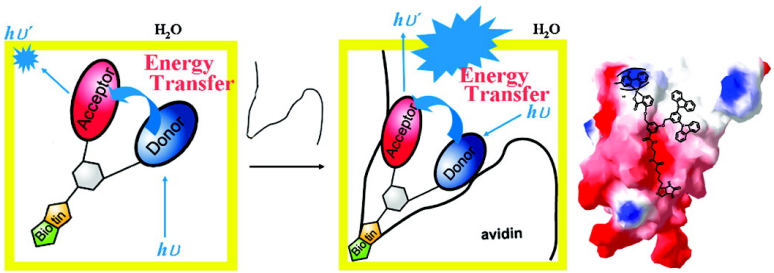

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Signal Amplification via Intramolecular Energy Transfer Using Tripodal Neutral Iridium(III) Complexes upon Binding to Avidin

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The extremely strong binding interaction between biotin and (strept)avidin has widespread bioanalytical applications such as the detection of biomolecules, clinical diagnosis, and immunoassays.[1] Of the many techniques for biotin−(strept)avidin assays,[2] there has been considerable interest in transition metal complexes using phosphorescence to overcome the problems of organic fluorophores such as the strong pH dependence, low photostability, small Stokes shifts, and short lifetimes.[3−6] Among them, Ir(III) complexes are promising candidates in various bioanalytical applications on account of their intense emission, wide range of emission energies, and high luminescence quantum yield.[5a] However, most ionic transition metal complexes developed as biological labeling reagents did not show high sensitivity upon binding to (strept)avidin.[3−5] For example, the luminescence enhancement factor of previous transition metal complexes upon binding to (strept)avidin is usually in the range of *ca.* 1−3 fold. This is because the luminescence enhancement only results from the increased hydrophobicity of the surrounding environment of the bound probes, compared with the environment of the free probes.[3−5]

This study reports a tripod system (**1**) for biotin−avidin assays, which consists of an energy acceptor, an energy donor, and biotin (Chart 1). This system is expected to provide a dramatic increase in luminescence intensity due to the intramolecular energy transfer between the donor and acceptor, as well as the increased hydrophobicity of the neutral probe, upon binding to avidin. To the best of our knowledge, this is the first luminescent biotin−transition metal complex conjugate using intramolecular energy transfer for luminescence enhancement. Neutral systems can offer a more hydrophobic environment than ionic probes. Therefore, they are expected to show a high binding affinity[3] and reduce the nonspecific interaction[7] that can exist between the protein surface and ionic transition metal probe. Iridium(III) bis[(4,6-difluorophenyl)-pyridinato-*N*,*C*$^{2'}$]picolinate (FIrpic), which is a well-known sky blue dopant in OLEDs, was selected as an acceptor on account of its high quantum efficiency, and *N*,*N*′-dicarbazolyl-3,5-benzene (mCP) was chosen as an energy donor that demonstrates higher singlet and triplet energies than those of the acceptor.[8a] Control probe **2** without a donor part, which has a similar structure to that of previous transition metal probes but is electronically neutral, was also prepared (Figure 1). The luminescence of probes **1** and **2** results from both ^{1}MLCT (dπ(Ir)→π*(N−O)) and ^{3}LC in the FIrpic moiety and has a similar emission spectrum (λ_{max} = 472 nm) to that of FIrpic.[8b] There is a good overlap between the emission spectrum of the donor (mCP unit) and the absorption spectrum of FIrpic over 350 nm (^{1}MLCT and ^{3}LC region), which ensures singlet−singlet energy transfer from mCP to FIrpic.[9−11] Furthermore, the distance between the donor and acceptor (∼15 Å, under the assumption that all the bonds are linked through a *trans* geometry) in the optimized geometric structure of probe **1** and density functional theory (DFT) calculations suggest that the effective triplet−triplet energy transfer between the donor and acceptor can also occur.[9,10,12] Indeed, upon

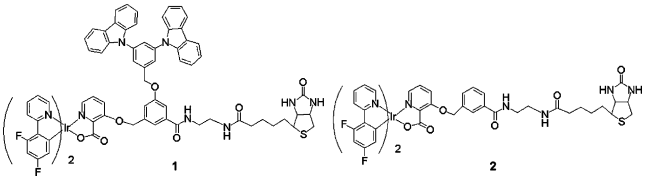


Figure 1. Molecular structures of the neutral probes with an energy donor (**1**) and without an energy donor (**2**).

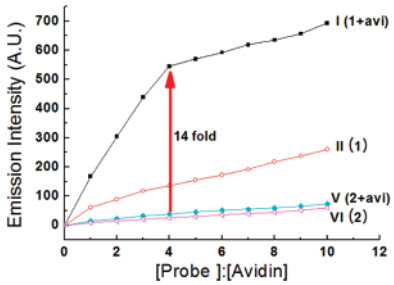
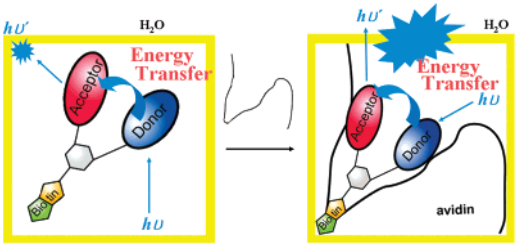


Figure 2. Luminescence intensity changes for (I) the titration of avidin (1.66 μM) with **1**, (II) **1** in the buffer A solution[9] at 310 nm excitation, (V) the titration of avidin (1.66 μM) with **2**, and (VI) **2** in the buffer A solution, upon excitation at 380 nm.

Chart 1. Schematic Diagram of a Neutral Tripod Probe System



binding to avidin, the neutral tripod **1** showed a dramatic increase in emission intensity when excited at the donor absorption peak (310 nm), rather than at the MLCT region (380 nm) of the acceptor (Chart 1 and Figure 2, *vide infra* for details).

The avidin binding properties of probes **1** and **2** were investigated using the standard HABA (4,4′-hydroxyazobenzene-2-carboxylic acid) assays,[1,3−5] which are based on the competition between biotin and HABA for binding to avidin, and luminescence titration experiments. The addition of probe **1** or **2** into a mixture of HABA (1 mM) and avidin (1.66 μM) results in a decrease in absorbance at 500 nm.[9] This shows that the bound HABA molecules are replaced by probes **1** and **2** with the same stoichiometry as that of free biotin (4 (**1** or **2**):1 (avidin)).

Luminescence titration using probe **1**[13] showed the turning point at 4 equiv of **1** due to the strong interaction of the biotin moiety[9,14] with the four binding sites of avidin (I, Figure 2). Luminescence titration using probe **1** also shows a remarkable increase in emission intensity (I) (*ca.* 14-fold) at **1**/avidin = 4:1, when excited at the

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Table 1. Photophysical Properties and Lifetime of **1** and **2** at 298 K

	medium	λ_{max}/nm (Φ_{PL}[b])	τ (μs)[c]	τ (μs)[d]	τ (μs)[e]
1	CH_2Cl_2	472, 519 (0.28)	1.01		
	CH_3CN	473, 498 (0.14)	0.49		
	H_2O[a]	474, 505 (0.01)	0.28	0.56	0.28
2	CH_2Cl_2	469, 496 (0.10)	0.92		
	CH_3CN	472, 493 (0.03)	0.45		
	H_2O[a]	463, 501 (0.01)	0.26	0.49	0.25

[a] pH 7.5, buffer A solution (H_2O/DMSO = 9:1). [b]Quantum yield was measured using FIrpic (Φ_{PL}=0.42) as a reference. [c][**1**] = [**2**] = 6.64 μM, [avidin] = 0 μM. [d][**1**] = [**2**] = 6.64 μM, [avidin] = 1.66 μM. [e][**1**] = [**2**] = 6.64 μM, [avidin] = 1.66 μM, [biotin] = 166 μM.

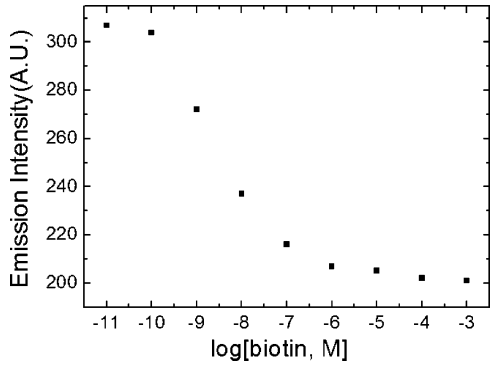

Figure 3. Homogeneous competitive assay for biotin using probe **1** (6.64 μM) and avidin (1.66 μM). The emission intensity was measured at 472 nm.

donor (mCP unit) absorption peak (310 nm), compared with that of compound **2** (V) without an energy donor upon 4:1 binding with avidin when excited at the MLCT region (380 nm) (Figure 2). Furthermore, the emission intensity of the probe **1**−avidin complex dramatically increases with increasing concentration of probe **1** until it fully binds to avidin and is *ca*. 4-fold higher than that of probe **1** itself (II) when excited at 310 nm. At over 4 equiv, the slope of the probe **1**−avidin complex (I) becomes similar to that of probe **1** (II) without avidin. This means that the increase in emission with more than 4 equiv of probe **1** just reflects the increase in the probe concentration. Therefore, a nonspecific interaction between the free probes and the protein surface can be excluded.[4b,7] Compound **8** without a biotin moiety[9] showed no increase in emission intensity in the presence of avidin (Figure S9).

In contrast, the luminescence titration with probe **2** shows that the emission intensity of the probe **2**−avidin complex (V) is *ca*. 1.6 times that of probe **2** in the absence of avidin (VI).[9] The emission intensity of probe **1** (II) excited at 310 nm was significantly larger than that of probe **2** (VI) excited at 380 nm and increased with increasing concentration of the probe due to intramolecular energy transfer. This indicates that intramolecular energy transfer can be an effective method for increasing the sensitivity (Φ_{ET} > 74%, in avidin + **1**).[9] In addition, as shown in the Supporting Information, the emission intensity of probe **1** (IV) in the presence of avidin was much lower when excited at 380 nm (MLCT region of FIrpic) compared to 310 nm (I).[9] The lifetime of probes **1** and **2** in the presence of avidin was elongated *ca*. 2.0- and 1.9-fold, respectively, due to the hydrophobic environment of the biotin binding sites of avidin (Table 1). This was supported by the fact that the lifetimes of probes **1** and **2** decrease with increasing solvent polarity (Table 1). The addition of excess biotin to a 1:4 mixture of avidin and probe **1** (or **2**) restored the original lifetime of probe **1** (or **2**) before complexation. Therefore, the increase in emission intensity results from intramolecular energy transfer and the hydrophobicity associated with the binding sites of avidin. Therefore, this new tripod system can be used effectively for labeling of biomolecules.

A homogeneous competitive biotin assay was carried out by adding free biotin in the range of 10^{-11} to 10^{-3} M to a mixture of probe **1** (6.64 μM) and avidin (1.66 μM) in buffer A solution. After 1 h of incubation, the emission intensity of probe **1** decreased gradually according to the concentration of free biotin because the binding of free biotin to avidin (K_d = ca. 10^{-15} M) is much stronger than that of probe **1** (Figure 3). Due to the high emission intensity of the **1**−avidin complex, the linear range for the detection of the free biotin analyte was increased greatly to $10^{-6.5}$−$10^{-10.5}$ M. Compared with ionic transition metal probes, the detection limit of the current system is higher by 2 or 3 orders of magnitude.[3−5]

In summary, we developed the first phosphorescent sensing system that can overcome intrinsic limitations in the sensitivity of the previous biotin−avidin assays. The neutral sensing system for biotin−avidin assays offers remarkable sensitivity over traditional transition metal based probes, due to the intramolecular energy transfer and increased hydrophobicity associated with the avidin binding site and neutral probe **1**, and can be used as a homogeneous competitive assay for biotin. New biomolecule probe systems can be produced if biotin is replaced with other recognition elements for various biomolecules.

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Supporting Information Available: Experimental procedures, spectral data, UV and PL data, measurement of ET efficiency, and titration data. This material is available free of charge via the Internet at http://pubs.acs.org.

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(11) The singlet−singlet intramolecular energy transfer in **1** from mCP to FIrpic exhibits a high efficiency of 92% via the transient PL method.[9,10]

(12) The triplet−triplet energy transfer efficiency of **1** was estimated to be 99% via the transient PL method.[9,10]

(13) Due to the solubility problem of probes **1** and **2** in aqueous solvent, H_2O/DMSO (9:1, v/v) was used in all the titrations.

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