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Luminescent Ruthenium(II) Polypyridine Biotin Complexes: Synthesis, Characterization, Photophysical and Electrochemical Properties, and Avidin-Binding Studies

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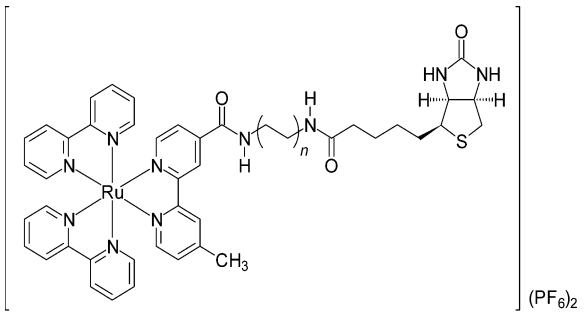
Two luminescent ruthenium(II) polypyridine complexes containing a biotin moiety $[Ru(bpy)_2(L1)](PF_6)_2$ (**1**) and $[Ru(bpy)_2(L2)](PF_6)_2$ (**2**) (bpy = 2,2′-bipyridine; L1 = 4-(*N*-((2-biotinamido)ethyl)amido)-4′-methyl-2,2′-bipyridine; L2 = 4-(*N*-((6-biotinamido)hexyl)amido)-4′-methyl-2,2′-bipyridine) have been synthesized and characterized, and their photophysical and electrochemical properties have been studied. Upon photoexcitation, complexes **1** and **2** display intense and long-lived triplet metal-to-ligand charge-transfer (^{3}MLCT) ($d\pi(Ru) \rightarrow \pi^*$(L1 or L2)) emission in fluid solutions at 298 K and in low-temperature glass. We have studied the binding of these ruthenium(II) biotin complexes to avidin by 4′-hydroxyazobenzene-2-carboxylic acid (HABA) assays, luminescence titrations, competitive assays using native biotin, and quenching experiments using methyl viologen. On the basis of the results of these experiments, a homogeneous competitive assay for biotin has been investigated.

Introduction

The binding of biotin to avidin is one of the strongest protein−ligand interactions in nature (first dissociation constant K_d = ca. 10^{-15} M).[1] Owing to the strong and specific interaction, avidin molecules labeled with fluorophores and enzymes are widely used in detecting biotinylated biomolecules.[2−4] In theory, biotinylated biomolecules could be recognized by fluorophore−biotin conjugates using avidin as a bridge because this protein has four biotin-binding sites. However, this approach is not feasible since common organic biotin-containing fluorophores exhibit severe emission quenching due to resonance-energy transfer (RET) upon binding to avidin, unless long spacers are present between the biotin and fluorophore units.[5,6]

We have recently reported a series of luminescent rhenium(I) and iridium(III) polypyridine biotin complexes that showed emission intensity enhancement and lifetime elongation upon binding to avidin, rendering them a new class of luminescent probes for this protein.[7] The biotin conjugates of other luminescent transition metal complexes, especially those with an environment-sensitive charge-transfer excited state, are anticipated to exhibit similar properties. In this paper, we report the synthesis, characterization, and photophysical and electrochemical properties of two luminescent ruthenium(II) polypyridine complexes containing a biotin moiety $[Ru(bpy)_2(L1)](PF_6)_2$ (**1**) and $[Ru(bpy)_2(L2)](PF_6)_2$ (**2**) (bpy = 2,2′-bipyridine; L1 = 4-(*N*-((2-biotinamido)ethyl)-amido)-4′-methyl-2,2′-bipyridine; L2 = 4-(*N*-((6-biotinamido)hexyl)amido)-4′-methyl-2,2′-bipyridine) (Chart 1). The avidin-binding properties of the complexes have been studied by 4′-hydroxyazobenzene-2-carboxylic acid (HABA) assays,

Chart 1. Structures of Complexes **1** and **2**



(**1**): n = 1, (**2**): n = 3

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Table 1. Electronic Absorption Spectral Data of Complexes **1** and **2** at 298 K

complex	medium	λ_{abs}/nm (ϵ/dm^3 mol^{-1} cm^{-1})
1	CH_3CN	245 (22,815), 255 sh (20,335), 288 (57,965), 360 sh (5,445), 393 sh (4,745), 423 sh (8,555), 458 (11,280)
	MeOH	245 (27,585), 256 sh (24,085), 289 (68,780), 361 sh (6,660), 395 sh (6,095), 424 sh (10,805), 456 (13,830)
2	CH_3CN	245 (26,855), 256 sh (24,185), 288 (70,475), 359 sh (6,205), 394 sh (5,730), 423 sh (10,795), 458 (14,420)
	MeOH	246 (28,945), 254 sh (27,375), 288 (74,440), 361 sh (6,560), 395 sh (6,465), 424 sh (12,205), 455 (15,710)

luminescence titrations, competitive assays using native biotin, and quenching experiments using methyl viologen. On the basis of the results of these experiments, a homogeneous competitive assay for biotin has been investigated.

Results and Discussion

Synthesis. The biotin-containing diimine ligands, L1 and L2, are synthesized from the reactions of succinimidyl-4-carboxy-4′-methyl-2,2′-bipyridine[8a] with biotinylethylenediamine[9] and *N*-biotinyl-1,6-diaminohexane,[10] respectively. Complexes **1** and **2** are obtained from the reactions of *cis*-$[Ru(bpy)_2Cl_2]\cdot 2H_2O$[8b] with L1 and L2, respectively, in refluxing ethanol, followed by metathesis with KPF_6 and recrystallization from acetone/diethyl ether. Both complexes are characterized by ^{1}H NMR spectroscopy, positive-ion ESI-MS, and IR spectroscopy, and give satisfactory microanalysis.

Electronic Absorption Spectroscopy. The electronic absorption spectral data of complexes **1** and **2** in CH_3CN and MeOH are listed in Table 1. The electronic absorption spectrum of complex **1** is shown in Figure 1. The absorption

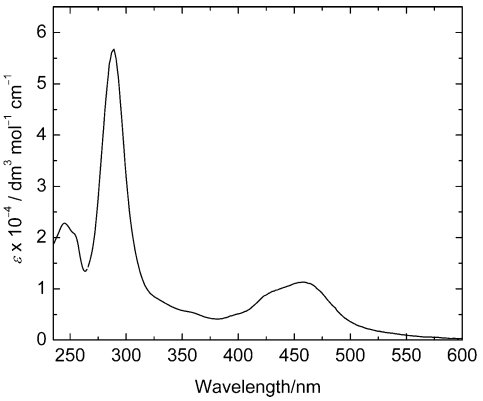

Figure 1. Electronic absorption spectrum of complex **1** in CH_3CN at 298 K.

Table 2. Photophysical Data of Complexes **1** and **2**, and $[Ru(bpy)_3]^{2+}$

complex	medium (T/K)	λ_{em}/nm	τ_o/μs	Φ_{em}
1	CH_3CN (298)	632	1.39	0.065
	MeOH (298)	636	1.09	0.055
	Glass[a] (77)	595, 651 sh	5.82	
2	CH_3CN (298)	629	1.45	0.072
	MeOH (298)	633	1.16	0.069
	Glass[a] (77)	595, 648 sh	5.64	
$[Ru(bpy)_3]^{2+}$	CH_3CN[b] (298)	621	0.84	0.061
	MeOH[b] (298)	616	0.81	0.045
	Glass[c] (77)	580 (max), 633, 692	5.20	

[a] EtOH/MeOH (4:1, v/v). [b] From ref 13. [c] From ref 11b.

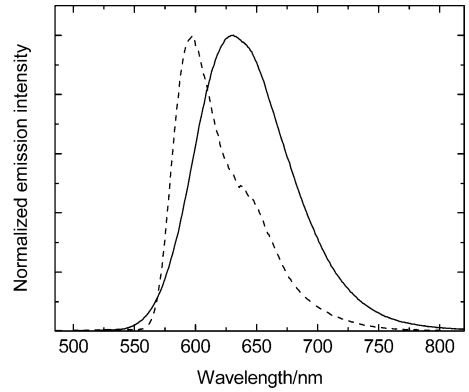

Figure 2. Emission spectra of complex **1** in CH_3CN at 298 K (—) and EtOH/MeOH (4:1, v/v) at 77 K (- - -).

spectra of both complexes resemble those of $[Ru(bpy)_3]^{2+}$ and its derivatives.[8c,11,12] The intense absorption bands at ca. 288 nm (ϵ on the order of 10^4 dm^3 mol^{-1} cm^{-1}) are assigned to intraligand (^{1}IL) transitions ($\pi \rightarrow \pi^*$)(bpy and L1 or L2). The absorption bands in the visible region (ca. 423−458 nm) are assigned to metal-to-ligand charge-transfer (^{1}MLCT) transitions (dπ(Ru) $\rightarrow$ π^*(bpy and L1 or L2)).

Luminescence Properties. The complexes exhibit intense and long-lived orange-red luminescence upon irradiation in fluid solutions at 298 K and in alcohol glass at 77 K. The photophysical data of both complexes and $[Ru(bpy)_3]^{2+}$ are summarized in Table 2. The emission spectra of complex **1** in CH_3CN at 298 K and in low-temperature glass are shown

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(6) The compound biotin−$NHCH_2CH_2$−Cy3 reported in ref 5e is different from common fluorophore−biotin conjugates. The emission of this compound increases at a [Cy3]:[avidin] ratio (n) between 0 and 2. At $n = 1$, the enhancement factor is about 1.5. The increase has been ascribed to the binding of the Cy3 moiety of the conjugate to a "Cy3-philic" site of the protein. Nevertheless, emission quenching occurs from $n > 2$ onwards. At $n = 4$, the emission intensity of the solution is about half that of the control solutions.

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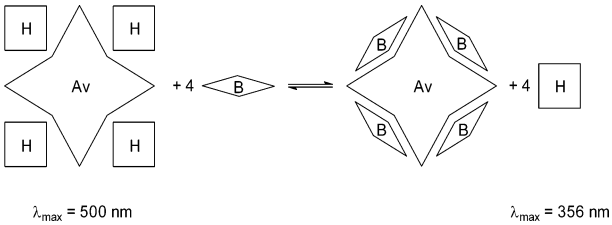
Table 3. Electrochemical Data of Complexes **1** and **2**, and $[Ru(bpy)_3]^{2+}$ [a]

complex	oxidation, $E_{1/2}$ or E_a/V	reduction, $E_{1/2}$ or E_c/V
1	+1.28	−1.25, −1.50, −1.76, −2.21[b]
2	+1.26	−1.27, −1.51, −1.79, −2.24[b]
$[Ru(bpy)_3]^{2+}$ [c]	+1.35	−1.33, −1.52, −1.76, −2.40[b]

[a] In CH_3CN (0.1 mol dm^{-3} $^nBu_4NPF_6$) at 298 K, glassy carbon electrode, sweep rate = 0.1 V s^{-1}, all potentials versus SCE. [b] Irreversible wave. [c] From ref 14.

Scheme 1. Replacement of Avidin-Bound HABA Molecules by Free Biotin Molecules Leads to a Decrease of Absorbance at 500 nm (Av = avidin, H = HABA, B = biotin)



in Figure 2. The emission maxima of the complexes occur at ca. 629−632 nm in CH_3CN and at ca. 633−636 nm in MeOH at 298 K. The emission is likely to originate from an excited state of ^{3}MLCT (dπ(Ru) → π*(diimine)) character. Since the emission energy of these complexes is slightly lower than that of $[Ru(bpy)_3]^{2+}$ (λ_{em} = ca. 621 nm in CH_3CN and 616 nm in MeOH at 298 K),[13] the acceptor orbitals should possess predominant π*(L1 or L2) character, given the lower-lying π* orbitals of the biotin-containing diimine ligands than those of bpy owing to the electron-withdrawing amide substituents. While the luminescence quantum yields of complexes **1** and **2** are comparable to those of $[Ru(bpy)_3]^{2+}$ under similar conditions,[13] the emission lifetimes of complexes **1** and **2** are longer (from ca. 1.1 to 1.5 μs), which can be ascribed to the extended π-conjugation of the biotin-containing diimine ligands.[8d] An extended π system allows a greater delocalization of the excited electron, which reduces the adjustments in local bond displacements and modulates the vibrational overlap between states. As a result, the nonradiative decay rate constant decreases and the lifetime of the ^{3}MLCT state increases.[8d] Complexes **1** and **2** also display intense and long-lived ^{3}MLCT (dπ(Ru) → π*(L1 or L2)) emission in alcohol glass at 77 K. The emission maxima (ca. 595 nm) also occur at lower energy than that of $[Ru(bpy)_3]^{2+}$ (ca. 580 nm).[11b]

Electrochemical Properties. The electrochemical properties of complexes **1** and **2** have been studied by cyclic voltammetry. The electrochemical data of both complexes and $[Ru(bpy)_3]^{2+}$ are listed in Table 3. Complexes **1** and **2** show a reversible ruthenium(III/II) oxidation couple at ca. +1.27 V vs SCE. Another reversible couple is observed at ca. −1.26 V for both complexes, and it is ascribed to the reduction of the biotin-containing diimine ligands. This assignment is supported by the fact that this couple occurs at a slightly higher potential than that of the first bpy-based reduction of $[Ru(bpy)_3]^{2+}$ (ca. −1.33 V)[14] due to the electron-withdrawing amide groups on the ligands L1 and L2. The reversible couples of complexes **1** and **2** at ca. −1.50 V and −1.80 V are assigned to the reduction of the ancillary bpy ligands.

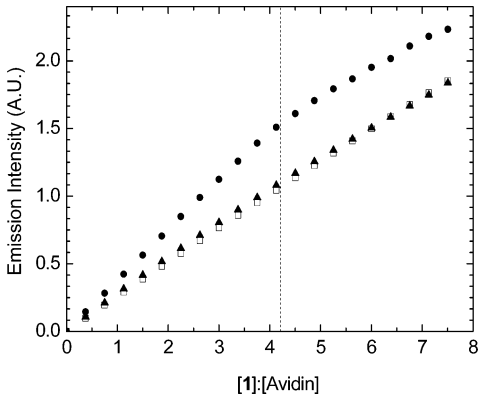


Figure 3. Luminescence titration curves for the titrations (complex **1** as titrant) of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲), and (iii) a blank phosphate buffer solution (□).

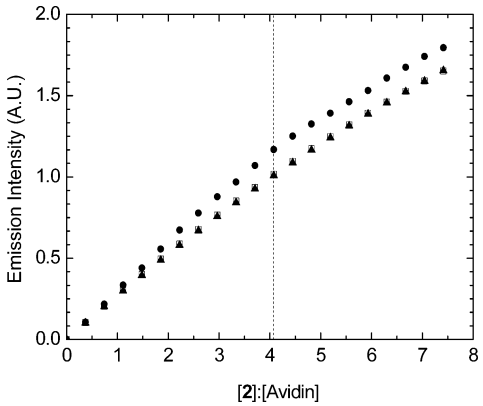


Figure 4. Luminescence titration curves for the titrations (complex **2** as titrant) of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲), and (iii) a blank phosphate buffer solution (□).

HABA Assays. The binding of complexes **1** and **2** to avidin has been studied by HABA assays, which are based on the competition between biotin and HABA on binding to avidin.[1,4] The binding of HABA to avidin is associated with an absorption feature at ca. 500 nm. Since the binding of HABA to avidin ($K_d = 6 \times 10^{-6}$ M) is much weaker than that of biotin (K_d = ca. 10^{-15} M), addition of biotin will replace the bound HABA molecules from the protein, leading to a decrease of the absorbance at 500 nm (Scheme 1). In this work, addition of complexes **1** or **2** to a mixture of avidin and HABA results in a decrease in absorbance at 500 nm, suggesting the binding of the biotin moieties of the ruthenium(II) complexes to avidin. The plots of $-\Delta A_{500\ nm}$ vs [Ru]:[avidin] for complexes **1** and **2** show that the equivalence points occur at [Ru]:[avidin] = ca. 4. These observations indicate that these ruthenium(II) biotin complexes bind to avidin with stoichiometry the same as that of native biotin. To gain further insights into the avidin-binding properties of these complexes, luminescence titrations and competitive assays have been performed.

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Table 4. Relative Emission Intensities and Emission Lifetimes of Complexes **1** and **2** in the Absence and Presence of Avidin (and Excess Biotin) with Various Concentrations of Methyl Viologen (MV^{2+}) and KCl[a]

	$[MV^{2+}] = 0$ M, [KCl] = 0 M			$[MV^{2+}] = 15.0$ mM, [KCl] = 0 M			$[MV^{2+}] = 15.0$ mM, [KCl] = 2.0 M		
complex	$I(\tau$/ns)[b]	$I(\tau$/ns)[c]	$I(\tau$/ns)[d]	$I(\tau$/ns)[b]	$I(\tau$/ns)[c]	$I(\tau$/ns)[d]	$I(\tau$/ns)[b]	$I(\tau$/ns)[c]	$I(\tau$/ns)[d]
1	1.00 (382)	1.41 (541)	1.04 (379)	1.00 (164)	2.39 (407)	1.03 (169)	1.00 (145)	3.18 (401)	1.03 (142)
2	1.00 (408)	1.16 (484)	0.99 (390)	1.00 (159)	1.97 (338)	0.96 (162)	1.00 (130)	2.98 (324)	1.02 (132)

[a] Relative emission intensities in aerated 50 mM potassium phosphate buffer pH 7.4, [Ru] = 15.0 μM. [b] [avidin] = 0 μM, [unmodified biotin] = 0 μM. [c] [avidin] = 3.8 μM, [unmodified biotin] = 0 μM. [d] [avidin] = 3.8 μM, [unmodified biotin] = 380.0 μM.

Table 5. First Dissociation Constants and Results of Association and Dissociation Assays for the Adducts Formed from the Binding of Complexes **1** and **2** to Avidin[a]

complex	K_d/M	association assay/ % avidin bound	dissociation assay/ % avidin bound
1	4.8×10^{-10}	24 ± 3.1	38 ± 2.7
2	3.1×10^{-11}	31 ± 2.9	45 ± 2.8

[a] In 50 mM potassium phosphate buffer pH 7.4 at 298 K.

Luminescence Titrations and Competitive Assays. The binding of complexes **1** and **2** to avidin has been investigated by luminescence titrations using the complexes as the titrants.[5,7] The results are compared to two series of control titrations in which (i) avidin is absent, and (ii) the avidin solution is saturated with excess unmodified biotin. The luminescence titrations of complexes **1** and **2** are shown in Figures 3 and 4, respectively. The luminescence titration results show that both complexes display enhanced emission intensities in the presence of avidin. At [Ru]:[avidin] = 4, both the emission intensities and lifetimes of the complexes increase by factors of ca. 1.2- to 1.4-fold (Table 4). These observations are in line with our previous results on luminescent rhenium(I) and iridium(III) polypyridine biotin conjugates.[7] We ascribe the emission enhancement and lifetime elongation to the specific binding of the biotin moieties of complexes **1** and **2** to the biotin-binding sites of avidin because similar observations are not noticed when excess unmodified biotin is initially present in the avidin solution (Table 4). It is likely that the observed enhancement is associated with the hydrophobicity of the biotin-binding sites of the avidin molecule. While fluorophore−biotin conjugates exhibit significant emission quenching due to RET upon binding to avidin,[5,6] the ruthenium(II) biotin complexes in the current work do not suffer from self-quenching even when they are in close proximity. We reason that the insignificant overlap between absorption and emission spectra of the ruthenium(II) polypyridine complexes disfavors the self-quenching effects.

The first dissociation constants (K_d) of the adducts formed from the binding of complexes to avidin have been determined from the emission titration experiments.[15] The K_d values for complexes **1** and **2** are ca. 4.8×10^{-10} and 3.1×10^{-11} M, respectively (Table 5), which are about 4−5 orders of magnitude larger than that of the native biotin−avidin system.[1] The lower binding affinities of these complexes to avidin can be accounted for by the relatively bulky ruthenium polypyridine unit. Nevertheless, the K_d value for complex **2** is an order of magnitude smaller than that for complex **1**. It

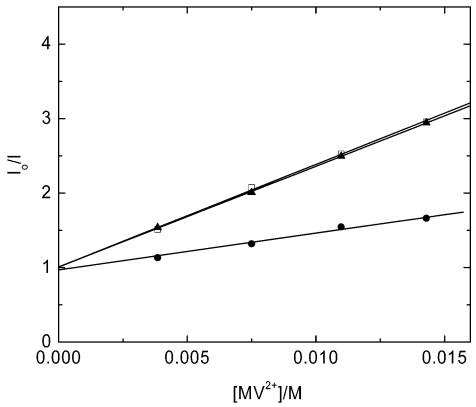

Figure 5. Stern−Volmer plots for the titrations (MV^{2+} as titrant) of (i) 15.0 μM complex **1** and 3.8 μM avidin (●), (ii) 15.0 μM complex **1**, 3.8 μM avidin, and 380.0 μM unmodified biotin (▲), and (iii) 15.0 μM complex **1** (□).

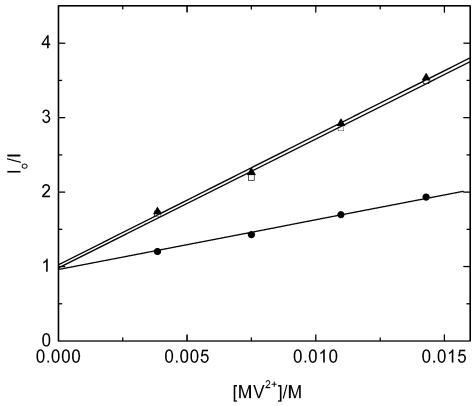

Figure 6. Stern−Volmer plots for the titrations (MV^{2+} as titrant) of (i) 15.0 μM complex **2** and 3.8 μM avidin (●), (ii) 15.0 μM complex **2**, 3.8 μM avidin, and 380.0 μM unmodified biotin (▲), and (iii) 15.0 μM complex **2** (□).

appears that the introduction of a longer spacer between the biotin and ruthenium(II) luminophore can improve the binding stability of the ruthenium−avidin adduct. The competitive binding of complexes **1** and **2** with native biotin to avidin has been investigated by competitive association and dissociation assays.[5e,16] In the association assays, complexes **1** and **2** compete with native biotin on binding to avidin; in the dissociation assays, the ruthenium(II) complexes bound to avidin are challenged by addition of native biotin. The results of the assays (Table 5) show that ca. 24−45% avidin molecules remain bound by the complexes in the presence of biotin. The avidin-bound percentage of

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Table 6. Stern-Volmer Constants for the Quenching of Complexes **1** and **2** by Methyl Viologen (MV^{2+}) in the Absence and Presence of Avidin (and Excess Biotin)[a]

	[KCl] = 0 M			[KCl] = 2.0 M		
complex	K_{sv}/M^{-1} [b]	K_{sv}/M^{-1} [c]	K_{sv}/M^{-1} [d]	K_{sv}/M^{-1} [b]	K_{sv}/M^{-1} [c]	K_{sv}/M^{-1} [d]
1	137	49	135	288	59	292
2	172	67	174	313	99	309

[a] In aerated 50 mM potassium phosphate buffer pH 7.4, [Ru] = 15.0 μM. [b] [avidin] = 0 μM, [unmodified biotin] = 0 μM. [c] [avidin] = 3.8 μM, [unmodified biotin] = 0 μM. [d] [avidin] = 3.8 μM, [unmodified biotin] = 380.0 μM.

complex **2** is higher, and this observation is in line with the smaller K_d value for its adduct with avidin (Table 5).

Emission Quenching Studies. To develop an assay for avidin (and biotin), it is desirable to maximize the difference in emission between the free and avidin-bound forms of complexes **1** and **2**. Unfortunately, the intrinsic increase in emission intensity of both complexes upon binding to avidin is small. Addition of a quencher will improve the luminescence increase if it preferentially quenches the free form of the complexes compared to the avidin-bound form. Methyl viologen, MV^{2+}, is a good candidate because it can effectively quench the emission of common ruthenium(II) polypyridine complexes via reductive quenching mechanism. In view of the highly positively charged avidin molecule (pI = ca. 10),[1] less effective emission quenching of the avidin-bound ruthenium(II) biotin complexes by the dicationic MV^{2+} ion is also anticipated. Since the quencher and the complexes are all cationic, it may also help to add a high salt concentration to improve the accessibility of the free complexes to the quencher. This will be useful if the salt affects quenching of the free form more than the avidin-bound form of the complexes.

As the first step, the emission quenching of complexes **1** and **2** by MV^{2+} has been studied in the absence and presence of avidin in 50 mM potassium phosphate buffer pH 7.4. The Stern−Volmer plots for complexes **1** and **2** are shown in Figures 5 and 6, respectively. Our results show that the luminescence of both complexes **1** and **2** is quenched by MV^{2+} in the absence of avidin, with Stern−Volmer constants, K_{SV}, of ca. 137 and 172 M^{-1}, respectively (Table 6). In the presence of avidin, however, the emission quenching becomes less efficient (K_{SV} = ca. 49 and 67 M^{-1} for complexes **1** and **2**, respectively) (Table 6 and Figures 5 and 6). Apparently, the difference in emission quenching efficiency is a consequence of the specific binding of complexes **1** and **2** to avidin because when excess unmodified biotin is initially present in the avidin solution, the emission quenching is indistinguishable from that in the absence of the protein (Table 6 and Figures 5 and 6). It is conceivable that the decrease of K_{SV} originates primarily from the shielding of the complexes by the protein matrix and, to a certain extent, by the immobilization of the complexes by the protein, rendering the quenching by MV^{2+} more difficult to occur. Although Coulombic repulsion exists between the positively charged avidin molecule and dicationic MV^{2+} ion, it does not seem to play a very important role in the diminished quenching efficiency. The reason is that the K_{SV} constants of both complexes in the presence of avidin show a relatively small increase upon changing from low-salt (LS) ([KCl] = 0 M) to high-salt (HS) ([KCl] = 2.0 M) conditions

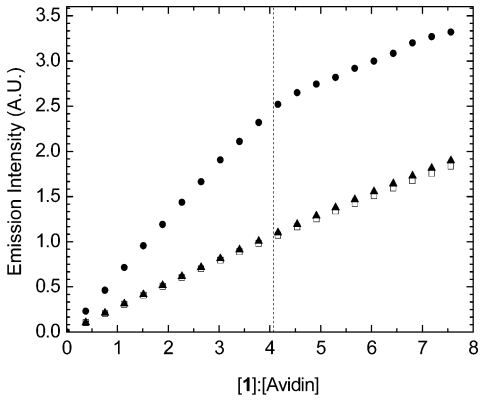

Figure 7. Luminescence titration curves for the titrations (complex **1** as titrant) of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲), and (iii) phosphate buffer (□), in the presence of 15.0 mM MV^{2+}.

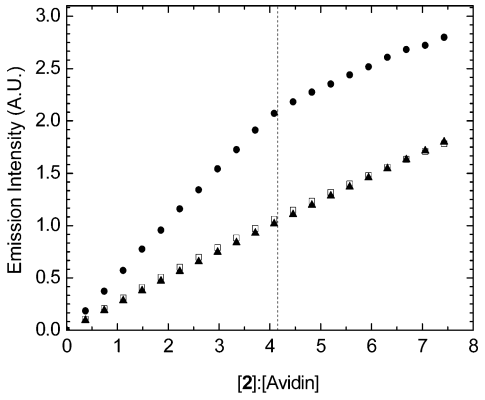

Figure 8. Luminescence titration curves for the titrations (complex **2** as titrant) of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲), and (iii) phosphate buffer (□), in the presence of 15.0 mM MV^{2+}.

(K_{SV}(HS)/K_{SV}(LS) = 1.20 and 1.48 for avidin-bound complexes **1** and **2**, respectively) (Table 6). These increases of K_{SV} are less substantial compared to those of the free complexes **1** and **2** (K_{SV}(HS)/K_{SV}(LS) = 2.10 and 1.82, respectively) and complexes **1** and **2** in the presence of biotin-blocked avidin (K_{SV}(HS)/K_{SV}(LS) = 2.16 and 1.78, respectively). These results, however, suggest that electrostatic repulsion between the positively charged free complexes and MV^{2+} ion is significantly reduced under HS conditions.[17] On the basis of these findings, we have repeated the emission titration experiments with MV^{2+} being a quencher present in the bulk solution. Under LS conditions, the titration curves for complexes **1** and **2** are shown in Figures 7 and 8, respectively. Our results show that in the presence of MV^{2+} both complexes display a more significant enhancement in emission intensities (ca. 2.4- and 2.0-fold for complexes **1** and **2**, respectively, at [Ru]:[Avidin] = 4) upon binding to

(17) Gaines, G. L., Jr. *J. Phys. Chem.* **1979**, *83*, 3088.

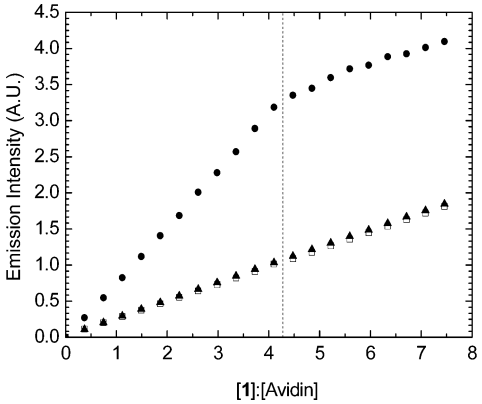

Figure 9. Luminescence titration curves for the titrations (complex **1** as titrant) of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲), and (iii) phosphate buffer (□), in the presence of 15.0 mM MV^{2+} and 2.0 M KCl.

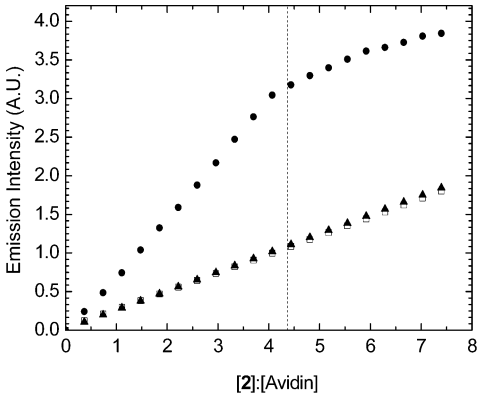

Figure 10. Luminescence titration curves for the titrations (complex **2** as titrant) of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲), and (iii) phosphate buffer (□), in the presence of 15.0 mM MV^{2+} and 2.0 M KCl.

avidin (Table 4). The emission lifetimes are also extended upon the binding (Table 4), and the elongation factors show improvement (ca. 2.5 and 2.1 for complexes **1** and **2**, respectively) compared to the case in which the quencher is absent (ca. 1.4 and 1.2 for complexes **1** and **2**, respectively) (Table 4). Under HS conditions, due to the more efficient quenching of the emission of both free complexes by MV^{2+}, higher amplification factors of both emission intensities (I/I_o) and lifetimes (τ/τ_o) at [Ru]:[Avidin] = 4 are anticipated. The titration curves for complexes **1** and **2** in the presence of MV^{2+} under HS conditions are shown in Figures 9 and 10, respectively. Our results clearly show that the emission intensities of complexes **1** and **2** are significantly enhanced by ca. 3.2- and 3.0-fold (Table 4), respectively, which are larger than those in the previous two cases. Meanwhile, the emission lifetime elongation factors (ca. 2.8 and 2.5 for complexes **1** and **2**, respectively) are also the most significant among all three conditions (Table 4).

Homogeneous Competitive Assay for Biotin. Since the emission intensities of the complexes are enhanced upon binding to avidin, this property has been exploited in the design of a new homogeneous assay for biotin. The assay is based on the competition between complex **1** and unmodified biotin on binding to avidin. In the assays, avidin is added to a solution of complex **1** and the biotin analyte in the presence

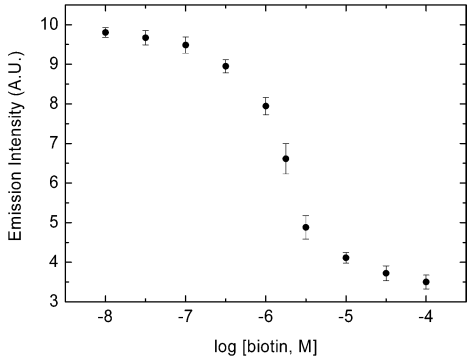

Figure 11. Homogeneous competitive assay for biotin using complex **1** and avidin in the presence of 4.0 mM MV^{2+} and 2.0 M KCl. The emission intensity of the supernatant is average of triplicate experiments ± 1 standard deviation.

of MV^{2+} under HS conditions. A lower biotin analyte concentration is expected to result in a higher degree of binding of complex **1** to avidin, and hence a higher emission intensity. In our experiments, the emission of the solutions is measured over a biotin analyte concentration range from 1×10^{-4} to 1×10^{-8} M, and the results are shown in Figure 11. The concentration range of biotin that can be determined by this assay is between ca. $1 \times 10^{-6.5}$ and $1 \times 10^{-5.5}$ M. Our future target is to design a system which can display a significant change in emission properties in the presence of avidin, and to develop related homogeneous and heterogeneous assays for biotinylated species.

Conclusion

Two luminescent ruthenium(II) polypyridine biotin complexes have been synthesized and characterized, and their photophysical and electrochemical properties have been investigated. The photophysical data indicate that the emission of the complexes originates from an ^{3}MLCT ($d\pi$(Ru) $\rightarrow \pi^*$(L1 or L2)) excited state. The binding of complexes **1** and **2** to avidin has been studied by HABA assays, luminescence titration experiments, competitive association and dissociation assays, and quenching experiments. First dissociation constants have been determined for the ruthenium−avidin adducts from luminescence titrations. A homogeneous competitive assay for biotin has been developed using complex **1** and avidin. In this work, we have developed new luminescent probes for avidin by attaching a biotin moiety to a ruthenium(II) polypyridine luminophore. We anticipate that the avidin-binding properties of related luminescent biotin−transition metal complex conjugates can be utilized in the development of different bioanalytical applications.

Experimental Section

Materials and Synthesis. All solvents were of analytical reagent grade and purified according to the literature procedures.[18] Succinimidyl-4-carboxy-4′-methyl-2,2′-bipyridine,[8a] biotinylethylenediamine,[9] *N*-biotinyl-1,6-diaminohexane,[10] and *cis*-$[Ru(bpy)_2Cl_2]\cdot 2H_2O$[8b] were prepared by reported methods.

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Physical Measurements and Instrumentation. ^{1}H NMR spectra were recorded on a Varian Mercury 300 MHz NMR spectrometer at 298 K. Positive-ion ESI mass spectra were recorded on a Perkin-Elmer Sciex API 365 mass spectrometer. IR spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrophotometer. Elemental analyses were carried out on a Carlo Erba 1106 elemental analyzer at the Institute of Chemistry, Chinese Academy of Sciences. Electronic absorption, steady-state excitation/emission spectra were recorded on a Hewlett-Packard 8453 diode array spectrophotometer and a Spex Fluorolog-2 model F111 fluorescence spectrophotometer, respectively. Unless specified, all the solutions for photophysical studies were degassed with no fewer than four successive freeze−pump−thaw cycles and stored in a 10-cm^3 round-bottomed flask equipped with a sidearm 1-cm fluorescence cuvette and sealed from the atmosphere by a Rotaflo HP6/6 quick-release Teflon stopper. Luminescence quantum yields were measured using the optically dilute method[19] with an aerated aqueous solution of [Ru(bpy)$_3$]Cl$_2$ ($\Phi_{em} = 0.028$, $\lambda_{ex} = 455$ nm)[20] as the standard solution. The 355-nm output (third harmonic) of a Quanta-Ray Q-switched GCR-150-10 pulsed Nd:YAG laser was the excitation source for emission lifetime measurements. Luminescence decay signals from a Hamamatsu R928 photomultiplier tube were converted to potential changes by a 50-Ω load resistor and then recorded on a Tektronix model TDS 620A (500 MHz, 2 GS/s) digital oscilloscope, and analyzed using a program for exponential fits on an IBM-compatible PC. The electrochemical measurements were performed on a CH Instruments Electrochemical Workstation CHI750A. The cyclic voltammetry experiments were carried out at room temperature using a two-compartment glass cell with a working volume of 500 μL. A platinum gauze counter electrode was accommodated in the working electrode compartment. The working and reference electrodes were a glassy carbon electrode and a Ag/AgNO$_3$ (0.1 mol dm^{-3} ^{n}Bu$_4$NPF$_6$ in CH$_3$CN) electrode, respectively. The reference electrode compartment was connected to the working electrode compartment via a Luggin capillary. Solutions for electrochemical measurements were degassed with prepurified argon gas. All potentials were referred to SCE.

Ligand L1. A mixture of succinimidyl-4-carboxy-4′-methyl-2,2′-bipyridine (103 mg, 0.33 mmol), biotinylethylenediamine (95 mg, 0.33 mmol), and triethylamine (1 mL, 7.12 mmol) in 10 mL of DMF was stirred at room temperature for 12 h. The yellow solution was evaporated under vacuum to give a brownish yellow solid. The solid was washed with CHCl$_3$ and then recrystallized from methanol/diethyl ether to give yellow crystals. Yield: 93 mg (60%). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K, TMS): δ 8.94 (t, $J = 5.5$ Hz, 1H, bpy-4-CON*H*), 8.78 (d, 1H, $J = 4.7$ Hz, H6 of pyridyl ring), 8.73 (s, 1H, H3 of pyridyl ring), 8.55 (d, 1H, $J = 4.7$ Hz, H6′ of pyridyl ring), 8.24 (s, 1H, H3′ of pyridyl ring), 7.96 (t, 1H, $J = 5.1$ Hz, N*H*-biotin), 7.76 (d, 1H, $J = 4.7$ Hz, H5 of pyridyl ring), 7.32 (d, 1H, $J = 4.7$ Hz, H5′ of pyridyl ring), 6.42 (s, 1H, NH of biotin), 6.36 (s, 1H, NH of biotin), 4.32−4.19 (m, 1H, NCH of biotin), 4.14−3.98 (m, 1H, NCH of biotin), 3.29−3.22 (m, 4H, NHC$_2$*H*$_4$NH), 3.13−2.95 (m, 1H, SCH of biotin), 2.78 (dd, 1H, $J_{gem} = 12.4$ Hz, $J_{vic} = 4.9$ Hz, SCH of biotin), 2.57 (d, 1H, $J_{gem} = 12.4$ Hz, SCH of biotin), 2.39 (s, 3H, CH$_3$ on C4′ of pyridyl ring), 2.03 (t, 2H, $J = 6.9$ Hz, COC*H*$_2$C$_3$H$_6$ of biotin), 1.65−1.18 (m, 6H, COCH$_2$C$_3$*H*$_6$ of biotin). IR (KBr) (ν/cm^{-1}): 3307 (br, NH), 1696 (s, C═O). Positive-ion ESI-MS ion clusters at *m*/*z* 483 {M + H$^+$}$^+$, 505 {M + Na$^+$}$^+$.

Ligand L2. The procedure for L2 was similar to that for the preparation of L1, except that *N*-biotinyl-1,6-diaminohexane (113 mg, 0.33 mmol) was used instead of biotinylethylenediamine. The brownish yellow product was recrystallized from methanol/diethyl ether to give yellow crystals. Yield: 100 mg (58%). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K, TMS): δ 8.90 (t, $J = 5.4$ Hz, 1H, bpy-4-CON*H*), 8.78 (d, 1H, $J = 4.7$ Hz, H6 of pyridyl ring), 8.73 (s, 1H, H3 of pyridyl ring), 8.56 (d, 1H, $J = 4.7$ Hz, H6′ of pyridyl ring), 8.25 (s, 1H, H3′ of pyridyl ring), 7.78−7.74 (m, 2H, H5 of pyridyl ring and N*H*-biotin), 7.31 (d, 1H, $J = 4.7$ Hz, H5′ of pyridyl ring), 6.43 (s, 1H, NH of biotin), 6.36 (s, 1H, NH of biotin), 4.32−4.22 (m, 1H, NCH of biotin), 4.15−4.05 (m, 1H, NCH of biotin), 3.29−3.25 (m, 2H, bpy-4-CONHC*H*$_2$), 3.10−2.92 (m, 3H, SCH of biotin and C*H*$_2$NH-biotin), 2.78 (dd, 1H, $J_{gem} = 12.3$ Hz, $J_{vic} = 4.8$ Hz, SCH of biotin), 2.54 (d, 1H, $J_{gem} = 12.3$ Hz, SCH of biotin), 2.41 (s, 3H, CH$_3$ on C4′ of pyridyl ring), 2.02 (t, 2H, $J = 6.9$ Hz, COC*H*$_2$C$_3$H$_6$ of biotin), 1.67−1.12 (m, 14H, NHCH$_2$C$_4$*H*$_8$CH$_2$NH and COCH$_2$C$_3$*H*$_6$ of biotin). IR (KBr) (ν/cm^{-1}): 3298 (br, NH), 1696 (s, C═O). Positive-ion ESI-MS ion clusters at *m*/*z* 539 {M + H$^+$}$^+$, 561 {M + Na$^+$}$^+$.

[Ru(bpy)$_2$(L1)](PF$_6$)$_2$ (1). A mixture of *cis*-[Ru(bpy)$_2$Cl$_2$]·2H$_2$O (76 mg, 0.15 mmol) and L1 (85 mg, 0.18 mmol) in 15 mL of ethanol was heated at reflux for 12 h. The solution turned from purple to deep red. The mixture was evaporated to dryness to give a red solid. The solid was then dissolved in 10 mL of water and heated to ca. 50 °C. Excess KPF$_6$ was added to the solution and the mixture was stirred for 30 min. After cooling to room temperature, the red precipitate was collected, washed with cold water, and recrystallized from acetone/diethyl ether to give complex **1** as red crystals. Yield: 188 mg (74 mg, 43%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ 9.07 (s, 1H, H3 of pyridyl ring of L1), 8.84−8.81 (m, 5H, H3′ of pyridyl ring of L1, and H3 and H3′ of bpy), 8.61 (br, 1H, bpy-4-CON*H* of L1), 8.24−8.19 (m, 5H, H6 of pyridyl ring of L1, and H4 and H4′ of bpy), 8.10−8.04 (m, 4H, H6 and H6′ of bpy), 7.89 (d, 1H, $J = 5.6$ Hz, H6′ of pyridyl ring of L1), 7.82 (d, 1H, $J = 5.9$ Hz, H5 of pyridyl ring of L1), 7.60−7.56 (m, 5H, N*H*-biotin of L1, and H5 and H5′ of bpy), 7.45 (d, 1H, $J = 5.6$ Hz, H5′ of pyridyl ring of L1), 5.95 (s, 1H, NH of biotin), 5.72 (s, 1H, NH of biotin), 4.50−4.46 (m, 1H, NCH of biotin), 4.32−4.27 (m, 1H, NCH of biotin), 3.50−3.36 (m, 4H, NHC$_2$*H*$_4$NH of L1), 3.17−3.15 (m, 1H, SCH of biotin), 2.67−2.59 (m, 4H, SCH of biotin and CH$_3$ on C4′ of pyridyl ring of L1), 2.22 (t, 2H, $J = 7.2$ Hz, COC*H*$_2$C$_3$H$_6$ of biotin), 1.61−1.18 (m, 6H, COCH$_2$C$_3$*H*$_6$ of biotin). IR (KBr) (ν/cm^{-1}): 3437 (br, NH), 1696 (s, C═O), 835 (s, PF$_6^-$). Positive-ion ESI-MS ion clusters at *m*/*z* 448 {M − 2PF$_6^-$}$^{2+}$, 1041 {M − PF$_6^-$}$^+$; Anal. Calcd for RuC$_{44}$H$_{46}$N$_{10}$O$_3$SP$_2$F$_{12}$·2H$_2$O: C, 43.25; H, 4.12; N, 11.46. Found: C, 43.18; H, 3.93; N, 11.51.

[Ru(bpy)$_2$(L2)](PF$_6$)$_2$ (2). The procedure was similar to that described for the preparation of complex **1**, except that L2 (94 mg, 0.18 mmol) was used instead of L1. The crude product was recrystallized from acetone/diethyl ether to give complex **2** as red crystals. Yield: 114 mg, 63%. ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ 9.24 (s, 1H, H3 of pyridyl ring of L2), 8.85−8.82 (m, 5H, H3′ of pyridyl ring of L2, and H3 and H3′ of bpy), 8.53 (br, 1H, bpy-4-CON*H* of L2), 8.25−8.17 (m, 5H, H6 of pyridyl ring of L2, and H4 and H4′ of bpy), 8.12−8.05 (m, 4H, H6 and H6′ of bpy), 7.95−7.93 (m, 1H, H6′ of pyridyl ring of L2), 7.90−7.88 (m, 1H, H5 of pyridyl ring of L2), 7.61−7.56 (m, 4H, H5 and H5′ of bpy), 7.46 (d, 1H, $J = 5.9$ Hz, H5′ of pyridyl ring of L2), 7.23 (br, 1H, N*H*-biotin of L2), 6.01 (s, 1H, NH of biotin), 5.79 (s, 1H, NH of biotin), 4.51−4.49 (m, 1H, NCH of biotin), 4.40−4.30 (m, 1H, NCH of biotin), 3.44−3.36 (m, 2H, bpy-4-CONHC*H*$_2$ of L2), 3.29−3.15 (m, 3H, SCH of biotin and C*H*$_2$-NH-biotin of L2), 2.94 (dd, 1H, $J_{gem} = 12.6$ Hz, $J_{vic} = 5.4$ Hz,

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SCH of biotin), 2.69 (d, 1H, J_{gem} = 12.6 Hz, SCH of biotin), 2.61 (s, 3H, CH_3 on C4′ of pyridyl ring of L2), 2.21−2.15 (m, 2H, $COCH_2C_3H_6$ of biotin), 1.70−1.23 (m, 14 H, $COCH_2C_3H_6$ of biotin and $NHCH_2C_4H_8CH_2NH$ of L2). IR (KBr) (ν/cm^{-1}): 3432 (br, NH), 1696 (s, C═O), 835 (s, PF_6^-). Positive-ion ESI-MS ion clusters at m/z 476 $\{M - 2PF_6^-\}^{2+}$, 1097 $\{M - PF_6^-\}^+$. Anal. Calcd for $RuC_{48}H_{54}N_{10}O_3SP_2F_{12}\cdot(CH_3)_2CO\cdot3H_2O$: C, 45.23; H, 4.91; N, 10.34. Found: C, 45.20; H, 4.71; N, 10.57.

HABA Assays. In a typical procedure, aliquots (5 μL) of the ruthenium(II) biotin complex (1.1 mM) were added cumulatively to a mixture of avidin (7.6 μM) and HABA (0.3 mM) in 50 mM potassium phosphate buffer pH 7.4 (2 mL) at 1-min intervals. The binding of the ruthenium(II) biotin complex to avidin was indicated by the decrease of the absorbance at 500 nm due to the displacement of HABA from the avidin molecule by the ruthenium(II) biotin complex. The value $-\Delta A_{500\ nm}$ was plotted against [Ru]:[avidin].

Luminescence Titrations. In a typical procedure, aliquots (5 μL) of the ruthenium(II) biotin complex (0.55 mM) were added cumulatively to avidin (3.8 μM) in 50 mM potassium phosphate buffer pH 7.4 (2 mL) at 1-min intervals. The emission spectrum of the solution was then measured. The titration results were compared to two sets of control titrations in which (i) avidin was absent, and (ii) the avidin solution was saturated with excess biotin (380.0 μM). The luminescence titrations were repeated in the presence of MV^{2+} (15.0 mM) under low-salt ([KCl] = 0 M) and high-salt ([KCl] = 2.0 M) conditions.

Competitive Assays. The competition between the ruthenium(II) biotin complexes and unmodified biotin on binding to avidin was investigated by competitive association and dissociation assays. In the association assays, avidin was added to a mixture of ruthenium(II) biotin complex (16 μM) and unmodified biotin (16 μM) to a concentration of 4 μM in 50 mM potassium phosphate buffer pH 7.4. The solution was incubated at room temperature for 1 h, and then diluted to 500 μL. The solution was then loaded onto a PD-10 size exclusion column (Pharmacia) that had been equilibrated with the same buffer. The first 5 mL of the eluted solution that contained avidin was collected and the emission intensity of this solution was measured. The emission intensity was compared to that of the control, in which unmodified biotin was absent. In the dissociation assays, a mixture of avidin (4 μM) and ruthenium(II) biotin complex (16 μM) in 50 mM potassium phosphate buffer pH 7.4 was incubated at room temperature for 1 h. Then unmodified biotin was added to the solution to a concentration of 16 μM and the mixture was incubated for 1 h. The mixture was then diluted to 500 μL and loaded onto a PD-10 size exclusion column (Pharmacia) that had been equilibrated with the same buffer. The first 5 mL of the eluted solution that contained avidin was collected and the emission intensity of this solution was measured. The emission intensity was compared to that of the control, in which unmodified biotin was absent.

Emission Quenching Studies. In a typical procedure, aliquots (50 μL) of MV^{2+} (150.0 mM) were added cumulatively to a mixture of ruthenium(II) biotin complex (15.0 μM) and avidin (3.8 μM) in 50 mM potassium phosphate buffer pH 7.4 (2 mL). The emission spectrum of the solution was then measured. The titration results were compared to two sets of control titrations in which (i) avidin was absent, and (ii) the avidin solution was saturated with excess biotin (380.0 μM). The titrations were repeated under high-salt conditions ([KCl] = 2.0 M).

Homogeneous Competitive Assay for Biotin. A biotin analyte solution (500 μL) in 50 mM potassium phosphate buffer pH 7.4 with KCl (2.0 M) was mixed with complex **1** (10 μL, 0.4 mM) dissolved in DMSO. Avidin (50 μL, 20 μM) and MV^{2+} (30 μL, 133.3 mM) in 50 mM potassium phosphate buffer pH 7.4 was then added to the solution. The solution was diluted to 1.0 mL with the same buffer. The final concentration of the biotin analyte in the solutions ranged from 1×10^{-4} to 1×10^{-8} M. The solutions were incubated at room temperature for 1 h and their emission intensity was then measured.

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