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Design of Rhenium(I) Polypyridine Biotin Complexes as a New Class of Luminescent Probes for Avidin

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This paper describes the design of a series of luminescent rhenium(I) polypyridine biotin complexes containing different spacer-arms, $[Re(N–N)(CO)_3(py\text{-}4\text{-}CH_2–NH–biotin)](PF_6)$ (py-4-CH_2–NH–biotin = 4-(biotinamidomethyl)-pyridine; N–N = 1,10-phenanthroline, phen (**1a**), 3,4,7,8-tetramethyl-1,10-phenanthroline, Me_4-phen (**2a**), 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline, Me_2–Ph_2-phen (**3a**), dipyrido[3,2-*f*:2′,3′-*h*]quinoxaline, dpq (**4a**)), $[Re(N–N)(CO)_3(py\text{-}3\text{-}CO–NH\text{-}en\text{-}NH–biotin)](PF_6)$ (py-3-CO–NH-en-NH–biotin = 3-(*N*-((2-biotinamido)ethyl)amido)pyridine; N–N = phen (**1b**), Me_4-phen (**2b**), Me_2–Ph_2-phen (**3b**), dpq (**4b**)), and $[Re(N–N)(CO)_3(py\text{-}4\text{-}CH_2–NH\text{-}cap\text{-}NH–biotin)](PF_6)$ (py-4-CH_2–NH-cap-NH–biotin = 4-(*N*-((6-biotinamido)hexanoyl)aminomethyl)pyridine; N–N = phen (**1c**), Me_4-phen (**2c**), Me_2–Ph_2-phen (**3c**), dpq (**4c**)). Upon irradiation, all of the rhenium(I)–biotin complexes exhibited intense and long-lived triplet metal-to-ligand charge-transfer (^{3}MLCT) ($d\pi(Re) \rightarrow \pi^*$(diimine)) emission in fluid solutions at 298 K. The interactions of these biotin-containing complexes with avidin have been studied by 4′-hydroxyazobenzene-2-carboxylic acid (HABA) assays, emission titrations, and competitive association and dissociation assays. On the basis of the results of these experiments, homogeneous assays for biotin and avidin have been designed.

Introduction

Avidin is a tetrameric glycoprotein with a molecular weight of ca. 66 kDa.[1] Each of the four subunits contains a substrate-binding site (at a depth of ca. 15 Å) that is specific for the small molecule biotin (vitamin H).[1c] The affinity of biotin to avidin is extraordinarily high (first dissociation constant K_d = ca. 10^{-15} M), and thus the biotin−avidin system has been widely exploited as a powerful tool in a variety of bioanalytical applications.[1] The most common strategy is to use an avidin-conjugated probe to recognize biotinylated biomolecules. Because avidin has four biotin-binding sites, biotinylated biomolecules could also be detected and quantitated by biotin−reporter conjugates when avidin is used as a bridge. However, this strategy does not apply to biotin−fluorophore conjugates because they suffer from severe fluorescence quenching upon binding to avidin, unless a long spacer is present between biotin and the fluorophore.[2] In view of the remarkable luminescence properties of rhenium(I) polypyridine complexes,[3−13] we have recently utilized a series of luminescent rhenium(I) polypyridine biotin

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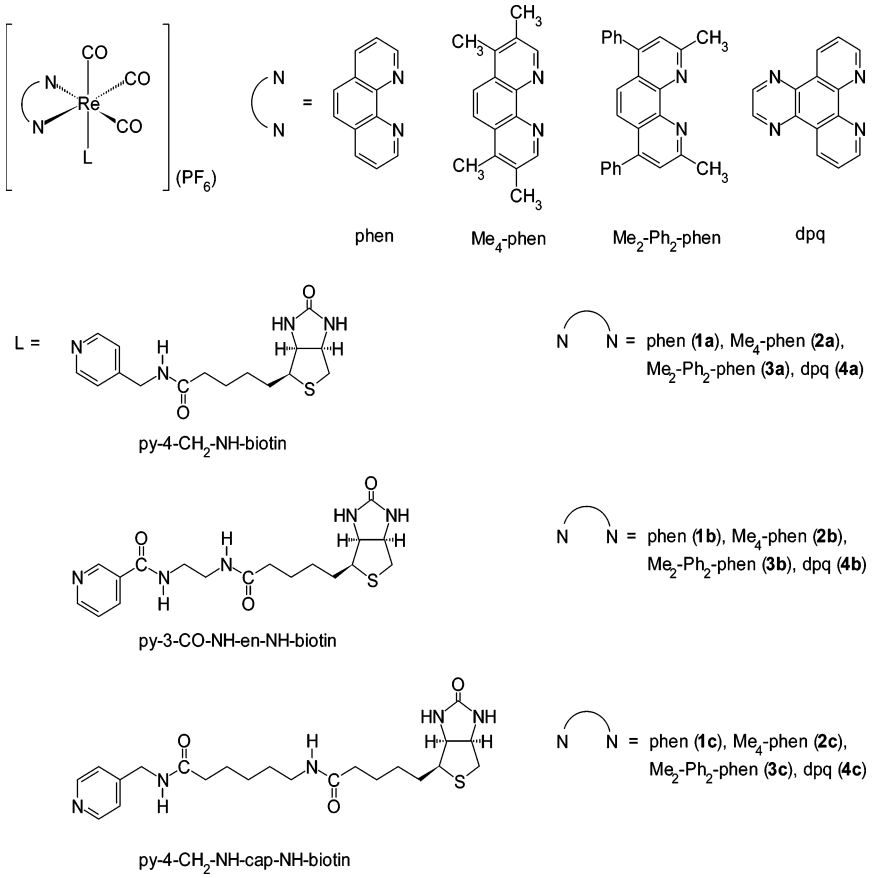
Chart 1. Structures of the Rhenium(I)−Biotin Complexes



complexes $[Re(N-N)(CO)_3(py\text{-}4\text{-}CH_2-NH-biotin)](PF_6)$ (py-4-CH_2−NH−biotin = 4-(biotinamidomethyl)pyridine; N−N = 1,10-phenanthroline, phen (**1a**), 3,4,7,8-tetramethyl-1,10-phenanthroline, Me_4-phen (**2a**), 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline, Me_2-Ph_2-phen (**3a**)) as probes for avidin.[14a] In contrast to biotin−fluorophore conjugates, these rhenium(I) complexes show increased emission intensity and lifetimes when they bind to avidin. As our continuing effort in the identification of efficient luminescent probes for avidin,[14b−d] we have investigated the photophysical and avidin-binding properties of related complexes containing a linker between the rhenium(I) luminophore and the biotin group. In this paper, we report the synthesis, characterization, and photophysical properties of a new series of luminescent rhenium(I) polypyridine biotin complexes bearing different spacer-arms, $[Re(N-N)(CO)_3(py\text{-}4\text{-}CH_2-NH-biotin)](PF_6)$ (N−N = dipyrido[3,2-*f*:2′,3′-*h*]quinoxaline, dpq (**4a**)), $[Re(N-N)(CO)_3(py\text{-}3\text{-}CO-NH\text{-}en\text{-}NH-biotin)](PF_6)$ (py-3-CO−NH-en-NH−biotin = 3-(*N*-((2-biotinamido)ethyl)-amido)pyridine; N−N = phen (**1b**), Me_4-phen (**2b**), Me_2-Ph_2-phen (**3b**), dpq (**4b**)), and $[Re(N-N)(CO)_3(py\text{-}4\text{-}CH_2-NH\text{-}cap\text{-}NH-biotin)](PF_6)$ (py-4-CH_2−NH-cap-NH−biotin = 4-(*N*-((6-biotinamido)hexanoyl)aminomethyl)pyridine; N−N = phen (**1c**), Me_4-phen (**2c**), Me_2-Ph_2-phen (**3c**), dpq (**4c**)) (Chart 1). The binding of these complexes to avidin has been studied by 4′-hydroxyazobenzene-2-carboxylic acid (HABA) assays, emission titrations, competitive association and dissociation assays, and emission quenching experiments. The effects of the polypyridine ligands and the chain lengths of the spacer-arms have been examined. On the basis of the

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Table 1. Electronic Absorption Spectral Data for the Rhenium(I)−Biotin Complexes at 298 K

complex	medium	λ_{abs}/nm (ϵ_{max}/dm^3 mol^{-1} cm^{-1})
1a	CH_2Cl_2	260 (19 290), 276 (20 630), 326 sh (4175), 364 sh (3210)
	CH_3CN	254 sh (18 630), 276 (22 635), 320 sh (5075), 360 sh (3070)
1b	CH_2Cl_2	258 (17 925), 276 (19 425), 326 sh (3905), 370 sh (2660)
	CH_3CN	254 sh (18 005), 274 (21 105), 322 sh (4995), 366 sh (2785)
1c	CH_2Cl_2	258 sh (25 255), 276 (28 310), 332 sh (5245), 380 sh (3800)
	CH_3CN	252 sh (19 375), 274 (24 135), 326 sh (4985), 368 sh (3175)
2a	CH_2Cl_2	254 (33 180), 282 (35 050), 320 sh (10 720), 370 sh (3945)
	CH_3CN	248 (25 340), 280 (29 395), 320 sh (9555), 366 sh (3135)
2b	CH_2Cl_2	250 (21 725), 280 (24 795), 328 sh (7545), 372 sh (2765)
	CH_3CN	248 (26 740), 280 (28 705), 326 sh (8750), 370 sh (2870)
2c	CH_2Cl_2	252 (29 770), 282 (37 295), 328 sh (10 325), 374 sh (4060)
	CH_3CN	248 (28 985), 280 (33 650), 326 sh (9580), 368 sh (3465)
3a	CH_2Cl_2	262 (25 660), 300 (33 895), 332 sh (13 550), 378 sh (4655)
	CH_3CN	258 (24 395), 298 (34 200), 328 sh (13 915), 378 sh (4525)
3b	CH_2Cl_2	260 (25 875), 300 (37 835), 336 sh (14 765), 394 sh (4920)
	CH_3CN	258 (26 680), 298 (36 735), 336 sh (13 220), 390 sh (4585)
3c	CH_2Cl_2	262 (23 855), 298 (36 545), 338 sh (12 460), 388 sh (4620)
	CH_3CN	258 (25 365), 298 (35 875), 336 sh (12 030), 388 sh (4315)
4a	CH_2Cl_2	260 (45 675), 292 sh (23 005), 372 sh (4685), 400 sh (3180)
	CH_3CN	258 (44 215), 290 sh (21 895), 356 sh (4625), 394 sh (2505)
4b	CH_2Cl_2	260 (44 250), 292 sh (22 135), 364 sh (4750), 396 sh (3230)
	CH_3CN	260 (41 640), 290 sh (20 990), 354 sh (4565), 390 sh (2520)
4c	CH_2Cl_2	260 (47 645), 292 sh (23 900), 364 sh (4990), 396 sh (3575)
	CH_3CN	260 (44 280), 290 sh (22 075), 354 sh (4605), 390 sh (2725)

results, homogeneous assays for biotin and avidin have been designed.

Results and Discussion

Synthesis. Two new biotin-containing pyridine ligands that possess ethylenediamine and 6-aminocaproic acid spacer-arms were synthesized in good yields. The ligand py-3-CO−NH-en-NH−biotin was obtained by reacting biotinylethylenediamine[15] with nicotinic acid *N*-hydroxysuccinimidyl ester.[16] The use of ethylenediamine as a spacer has attracted our attention because it has been employed in preparing biotin derivatives that show very strong binding to avidin.[2c,15] The ligand py-4-CH_2−NH-cap-NH−biotin was synthesized from the reaction of 4-(aminomethyl)pyridine and biotinamidohexanoic acid *N*-hydroxysuccinimidyl ester[1d] in DMF at room temperature. The luminescent rhenium(I) polypyridine biotin complexes were prepared, in moderate yields, from the reactions of $[Re(N-N)(CO)_3(CH_3CN)](CF_3SO_3)$[4a] and the corresponding biotin-containing ligands (py-4-CH_2−NH−biotin, py-3-CO−NH-en-NH−biotin, and py-4-CH_2−NH-cap-NH−biotin) in refluxing THF/methanol, followed by metathesis with KPF_6 and purification by column chromatography. The complexes were isolated as the PF_6^- salts instead of the $CF_3SO_3^-$ salts due to the hygroscopic nature of the latter. All of the new complexes were characterized by ^{1}H NMR spectroscopy, positive-ion ESI-MS, and IR spectroscopy and gave satisfactory elemental analyses.

Electronic Absorption and Luminescence Properties. The electronic absorption spectral data for the complexes are summarized in Table 1. The electronic absorption spectrum of complex **3c** in CH_3CN at 298 K is shown in Figure 1. With reference to previous photophysical studies on related rhenium(I) diimine systems,[3−9,10b−d,11a,12a] the intense high-energy absorption bands of all of the rhenium(I)−biotin complexes at ca. 248−300 nm with absorption coefficients on the order of 10^4 dm^3 mol^{-1} cm^{-1} are assigned to spin-allowed intra-ligand (^{1}IL) ($\pi \rightarrow \pi^*$) (diimine and pyridine-biotin ligands) transitions, while the less intense

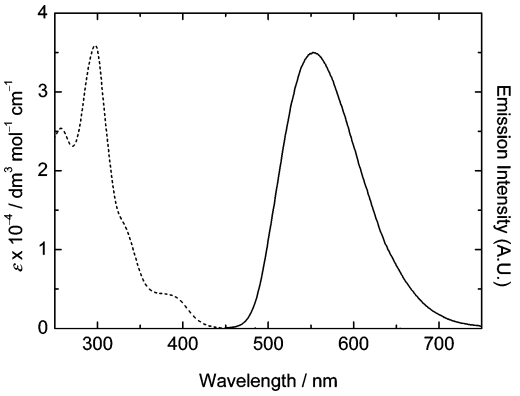

Figure 1. Electronic absorption (- - - -) and emission (−) spectra of complex **3c** in CH_3CN at 298 K.

absorption shoulders at ca. 320−400 nm are assigned to spin-allowed metal-to-ligand charge-transfer (^{1}MLCT) (dπ(Re) $\rightarrow \pi^*$(diimine)) transitions. The ^{1}IL absorption of the Me_2−Ph_2-phen complexes **3a**−**3c** occurred in a lower energy region at ca. 328−338 nm because of the electron-withdrawing phenyl substituents of the diimine ligand.

Upon photoexcitation, all of the complexes exhibited intense and long-lived greenish-yellow to yellow luminescence in fluid solutions under ambient conditions. The photophysical data of the complexes are tabulated in Table 2. The emission spectrum of complex **3c** in CH_3CN at 298 K is shown in Figure 1. This complex showed a Stokes' shift of ca. 7657 cm^{-1} from the absorption shoulder at ca. 388 nm to the emission maximum at ca. 552 nm. The long emission lifetimes of all of the complexes (in the submicrosecond to microsecond time scale), together with the large Stokes' shifts, suggest the phosphorescence nature of the emission.

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Table 2. Photophysical Data for the Rhenium(I)−Biotin Complexes at 298 K

complex	medium	λ_{em}/nm	τ_o/μs	Φ	k_r/s^{-1}	k_{nr}/s^{-1}
1a	CH_2Cl_2	536	2.69	0.25	9.3×10^4	2.8×10^5
	CH_3CN	552	1.37	0.079	5.8×10^4	6.7×10^5
	buffer[a]	552	0.79	0.027	3.4×10^4	1.2×10^6
1b	CH_2Cl_2	536	2.92	0.30	1.0×10^5	2.4×10^5
	CH_3CN	550	1.75	0.13	7.4×10^4	5.0×10^5
	buffer[a]	550	1.07	0.12	1.1×10^5	8.2×10^5
1c	CH_2Cl_2	540	2.85	0.49	1.7×10^5	1.8×10^5
	CH_3CN	554	1.46	0.16	1.1×10^5	5.8×10^5
	buffer[a]	554	0.84	0.096	1.1×10^5	1.1×10^6
2a	CH_2Cl_2	510	7.81	0.16	2.0×10^4	1.1×10^5
	CH_3CN	518	7.32	0.072	9.8×10^3	1.3×10^5
	buffer[a]	522	11.11	0.026	2.3×10^3	8.8×10^4
2b	CH_2Cl_2	516	13.54	0.19	1.4×10^4	6.0×10^4
	CH_3CN	522	9.90	0.051	5.2×10^3	9.6×10^4
	buffer[a]	518	14.06	0.041	2.9×10^3	6.8×10^4
2c	CH_2Cl_2	518	13.28	0.27	2.0×10^4	5.5×10^4
	CH_3CN	522	8.54	0.12	1.4×10^4	1.0×10^5
	buffer[a]	522	11.62	0.078	6.7×10^3	7.9×10^4
3a	CH_2Cl_2	540	7.22	0.22	3.0×10^4	1.1×10^5
	CH_3CN	550	6.78	0.079	1.2×10^4	1.4×10^5
	buffer[a]	556	6.01	0.011	1.8×10^3	1.6×10^5
3b	CH_2Cl_2	542	15.48	0.19	1.2×10^4	5.2×10^4
	CH_3CN	550	9.41	0.13	1.4×10^4	9.2×10^4
	buffer[a]	554	7.85	0.012	1.5×10^3	1.3×10^5
3c	CH_2Cl_2	542	11.65	0.18	1.5×10^4	7.0×10^4
	CH_3CN	552	6.96	0.053	7.6×10^3	1.4×10^5
	buffer[a]	558	5.08	0.028	5.5×10^3	1.9×10^5
4a	CH_2Cl_2	548	1.12	0.28	2.5×10^5	6.4×10^5
	CH_3CN	568	0.40	0.086	2.2×10^5	2.3×10^6
	buffer[a]	572	0.11	0.034	3.1×10^5	8.8×10^6
4b	CH_2Cl_2	544	1.24	0.15	1.2×10^5	6.9×10^5
	CH_3CN	562	0.52	0.065	1.3×10^5	1.8×10^6
	buffer[a]	566	0.21	0.033	1.6×10^5	4.6×10^6
4c	CH_2Cl_2	548	1.10	0.24	2.2×10^5	6.9×10^5
	CH_3CN	568	0.40	0.054	1.4×10^5	2.4×10^6
	buffer[a]	572	0.13	0.032	2.5×10^5	7.4×10^6

[a] Potassium phosphate buffer (50 mM, pH 7.4) containing 2.5% DMSO for emission wavelength and lifetime measurements, or 25% DMSO for quantum yield determinations. The use of a higher DMSO content for the quantum yield determinations was due to solubility reasons.

The emission energy of the complexes depends strongly on the diimine ligands and follows the order: Me_4-phen > phen $\approx$ Me_2-Ph_2-phen > dpq (Table 2), which is in line with the increasing π-accepting properties of the diimine ligands. All of these observations indicate that the photoluminescence of the complexes originates from an ^{3}MLCT ($d\pi(Re) \rightarrow \pi^*$-(diimine)) emissive state.[3−6,7a,c,8a,9a,c,10−11,12a,13b]

A close scrutiny of the emission data (Table 2) indicates that the complexes could be arranged into two classes: (1) The phen and dpq complexes that showed typical ^{3}MLCT emission behavior; and (2) the Me_4-phen and Me_2-Ph_2-phen complexes which displayed different emission properties. In particular, while most of the complexes showed a decrease in emission lifetimes and quantum yields upon changing the solvent from CH_2Cl_2 to CH_3CN to buffer, the Me_4-phen complexes **2a**−**2c** did not display a similar trend in emission lifetimes. Also, the emission lifetimes of these Me_4-phen complexes were exceptionally long (ca. 7.3−14 μs) especially in buffer. We believe that such findings are the consequence of a low-lying ^{3}IL (Me_4-phen) emissive state, which is supported by various reported studies. From a temperature-dependence emission investigation on the complex $[Re(Me_4\text{-phen})(CO)_3(py)]^+$,[11b] it was found that this complex possesses a low-lying ^{3}IL state which is of energy comparative to that of the ^{3}MLCT emissive state. The very long emission lifetime of this complex in CH_2Cl_2 (ca. 13 μs at 298 K) and its insensitivity toward the identity of solvents were attributed to a low-lying ^{3}IL state. In fact, rhenium(I) ^{3}IL emitters generally exhibit very long emission lifetimes and comparatively lower emission quantum yields in fluid solutions at room temperature.[6a,17] In another photophysical and theoretical study of the complexes $[M(CO)_4(Me_4\text{-phen})]$ (M = Cr or W),[18] it was found that the LUMO's of these complexes are of a_2 symmetry, while their phen counterparts are of b_1 symmetry. The exceptionally long emission lifetime of $[W(CO)_4(Me_4\text{-phen})]$ as compared to $[W(CO)_4(phen)]$ was ascribed to the different contributions of individual orbital excitations to the excited states involved.[18]

The long emission lifetimes of Me_2-Ph_2-phen complexes **3a**−**3c** are not unexpected either. For example, Lakowicz and co-workers reported that the emission lifetime of $[Re(Me_2-Ph_2\text{-phen})(CO)_3(py\text{-}COOH)]^+$ is ca. 7 μs in both CH_3OH and $CHCl_3$.[19] Another more systematic study on a range of rhenium(I) polypyridine complexes has been reported by Rillema and Wallace.[11a] Unlike other related complexes, the complexes $[Re(Me_4\text{-phen})(CO)_3(py)]^+$ and $[Re(Me_2-Ph_2\text{-phen})(CO)_3(py)]^+$ display very long emission lifetimes in

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both CH_2Cl_2 and CH_3CN. For example, the Me_4-phen complex emits with lifetimes of 12 and 13 μs in CH_2Cl_2 and CH_3CN, respectively, and the Me_2-Ph_2-phen complex emits with lifetimes of 12.5 and 10.1 μs in these two solvents. Other related complexes including 4,7-Me_2-phen, 5,6-Me_2-phen, 2,9-Me_2-phen, phen, 5-Ph-phen, and 4,7-phen all undergo a 40−60% reduction in emission lifetimes in the more polar solvent CH_3CN. Again, the different observations for the Me_4-phen and Me_2-Ph_2-phen complexes were attributed to the involvement of low-lying ^{3}IL states.[11a]

The dpq complexes **4a**−**4c** showed shorter emission lifetimes (ca. 0.1−1.2 μs) as compared to those of the other rhenium(I)−biotin complexes in this work. This observation is in line with the shorter emission lifetime of $[Ru(bpy)_2(dpq)]^{2+}$ (0.20 μs) as compared to its homoleptic analogue $[Ru(bpy)_3]^{2+}$ (0.41 μs).[20]

Most of the py-4-CH_2−NH-cap-NH−biotin complexes displayed longer emission lifetimes and higher emission quantum yields than their py-4-CH_2−NH−biotin counterparts (Table 2). The elongation of the spacer-arms can probably enhance the local hydrophobicity of the rhenium(I)−biotin complexes and thereby increase their emission lifetimes. Similar dependence of emission lifetimes on the chain length of the alkyl nitrile ligand of the complexes $[Re(bpy)(CO)_3(NC\{CH_2\}_nCH_3)]^+$ has been observed.[10a,d] Furthermore, it is interesting to note that complexes containing the *meta*-substituted pyridine ligand (py-3-CO−NH-en-NH−biotin) showed longer emission lifetimes than those containing the *para*-substituted pyridine ligands (py-4-CH_2−NH−biotin and py-4-CH_2−NH-cap-NH−biotin). With reference to related systems,[10b] the decrease in the radiationless decay rate is ascribed to the electron-withdrawing properties of the amide substituent on the py-3-CO−NH-en-NH−biotin ligand.

HABA Assays. The avidin-binding properties of the rhenium(I)−biotin complexes have been investigated by an assay for biotin and biotinylated species using the dye HABA.[1a,21] The binding of HABA to avidin is associated with an absorption feature at ca. 500 nm. Because the affinity 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 in the absorbance at 500 nm. In the current work, addition of the rhenium(I)−biotin complexes into a mixture of HABA and avidin resulted in a decrease in the absorbance at 500 nm, indicating that the bound HABA molecules were replaced by the rhenium(I)−biotin complexes. As an example, the absorption titration curves for the titrations of avidin−HABA complex with complex **2b** and unmodified biotin, respectively, are illustrated in Figure 2. The plots of $-\Delta A_{500\ nm}$ versus [Re]:[avidin] for all of the complexes show that the equivalence points occurred at [Re]:[avidin] = 4:1, which is indistinguishable from unmodified biotin. This reveals that the rhenium(I)−biotin complexes,

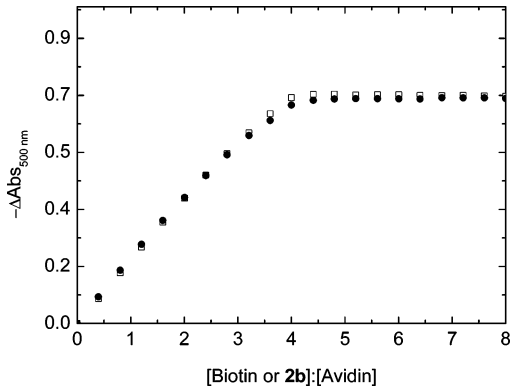

Figure 2. Absorption titration curves for the titrations of avidin−HABA complex with complex **2b** (●) and unmodified biotin (□).

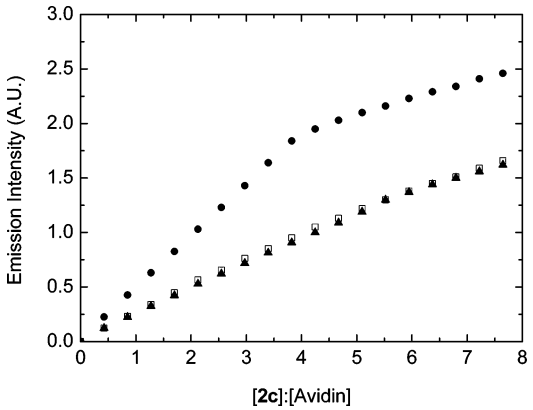

Figure 3. Luminescence titration curves for the titrations of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲), and (iii) a blank phosphate buffer solution (□) with complex **2c**.

similar to unmodified biotin, bound to the avidin molecule with a stoichiometry of 4:1.

Emission Titrations. The avidin-binding properties of the complexes have been investigated by luminescence titrations using the rhenium(I)−biotin complexes as titrants.[2,14] The titration results were compared to two control experiments in which (i) avidin was absent or (ii) avidin was presaturated with excess unmodified biotin. The luminescence titration curves for the complex **2c** are illustrated in Figure 3. Similar to the results of our previous studies on luminescent transition metal-biotin complexes,[14] all of the rhenium(I)−biotin complexes in this work displayed higher emission intensity and longer emission lifetimes in the presence of avidin (Table 3). These changes are ascribed to the binding of the biotin moieties of the complexes into the specific biotin-binding sites of avidin because no such increase was observed when excess unmodified biotin was initially present. It is likely that the observed emission enhancement and lifetime elongation is due to the increase in the hydrophobicity of the local environment of the rhenium(I)−biotin complexes after they bind to avidin. The reason is that almost all of the complexes in this work exhibited increased emission quantum yields and lifetimes upon changing the solvent from aqueous buffer to CH_2Cl_2 (Table 2).[22] The more hydrophobic Me_4-phen and

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(21) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, CA, 1996; p 591.
(22) The exceptions were complexes **2a** and **2b** that showed longer emission lifetimes in buffer than CH_2Cl_2. These different observations have been discussed in the text.

Table 3. Relative Emission Intensity and Lifetimes of the Rhenium(I)−Biotin Complexes (15.2 mM) in Aerated 50 mM Potassium Phosphate Buffer pH 7.4 at 298 K

complex	I (τ/μs)[a]	I (τ/μs)[b]	I (τ/μs)[c]
1a	1.00 (0.56)	1.42 (0.90)	0.98 (0.55)
1b	1.00 (0.69)	1.25 (1.01)	1.01 (0.68)
1c	1.00 (0.55)	1.24 (0.73)	0.96 (0.56)
2a	1.00 (1.23)	2.25 (2.96)	1.04 (1.25)
2b	1.00 (1.50)	1.26 (3.65)	1.01 (1.53)
2c	1.00 (1.31)	1.90 (2.00)	0.96 (1.36)
3a	1.00 (1.84)	2.98 (2.70)	0.96 (1.90)
3b	1.00 (2.09)	2.93 (3.25)	1.01 (2.04)
3c	1.00 (1.61)	2.27 (2.38)	0.93 (1.64)
4a	1.00 (0.11)	1.75 (0.21)	0.99 (0.11)
4b	1.00 (0.18)	1.20 (0.26)	1.03 (0.19)
4c	1.00 (0.12)	1.15 (0.16)	0.98 (0.12)

[a] [avidin] = 0 mM, [unmodified biotin] = 0 mM. [b] [avidin] = 3.8 mM, [unmodified biotin] = 0 mM. [c] [avidin] = 3.8 mM, [unmodified biotin] = 380.0 mM.

Me_2−Ph_2-phen complexes showed a higher degree of enhancement than their phen and dpq counterparts upon binding to avidin (Table 3). Also, it is interesting to note that the emission quantum yield enhancement factors from buffer to CH_2Cl_2 were the highest for complexes **3a** and **3b** (20 and 15.8, respectively) among all of the complexes (Table 2). This is apparently in line with the highest emission intensity amplification factors for these two complexes (2.98 and 2.93) upon binding to avidin (Table 3).[23] In addition to the increase in hydrophobicity, the emission enhancement could be due to the increased rigidity of the rhenium complexes upon binding to the protein. This increase in rigidity is expected to result in lower nonradiative decay rates for the complexes, and thus enhanced emission.

Concerning the effects of the chain length of the spacer-arms, we notice that the py-3-CO−NH-en-NH−biotin and py-4-CH_2−NH-cap-NH−biotin complexes (**1b**, **1c**, **2b**, **2c**, **3b**, **3c**, **4b**, and **4c**) exhibited less significant emission intensity enhancement (Table 3). It is likely that these eight complexes remain more exposed to the polar buffer after binding to the protein, as compared to their py-4-CH_2−NH−biotin analogues (complexes **1a**, **2a**, **3a**, and **4a**). Thus, the increase in hydrophobicity is less substantial. Also, the effects of increased rigidity resulting from avidin-binding are smaller for these complexes due to their longer and more flexible spacer-arms. Nevertheless, it is worth mentioning that for the titrations of avidin with these eight complexes, at [Re]:[avidin] > 4:1, the titration curves were generally parallel to (i) those of the solutions without avidin, and (ii) those with avidin and excess unmodified biotin. This is in contrast to the py-4-CH_2−NH−biotin complexes **1a**, **2a**, **3a**, and **4a**, for which unparallel curves at [Re]:[avidin] > 4:1 were observed.[14a] To illustrate this, the titration curves of complexes **3a** and **3c** are shown in Figures 4 and 5, respectively. In Figure 5, at 0 < [Re]:[avidin] < 4, the titration curve had

(23) All of the adducts showed no or very small blue shifts (≤5 nm) in emission wavelength as compared to the corresponding free complexes. Nevertheless, the hydrophobicity associated with a protein molecule in aqueous solution cannot compare to pure dichloromethane. Thus, we suppose that the enhancement of emission intensity and lifetimes of the complexes upon binding to avidin is closely related to an increase in local hydrophobicity.

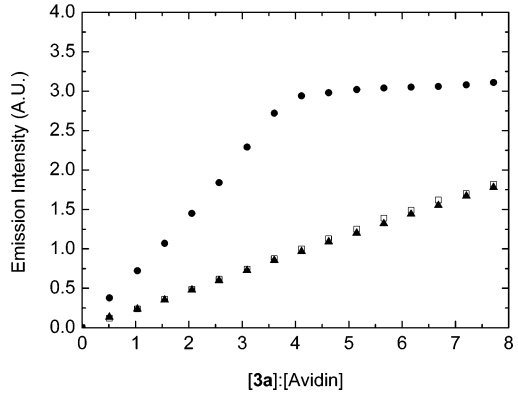

Figure 4. Luminescence titration curves for the titrations of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲), and (iii) a blank phosphate buffer solution (□) with complex **3a**.

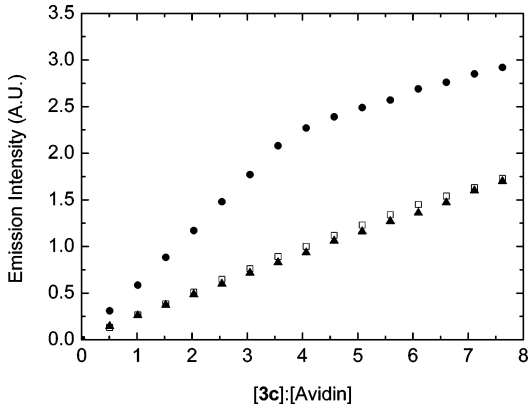

Figure 5. Luminescence titration curves for the titrations of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲), and (iii) a blank phosphate buffer solution (□) with complex **3c**.

a more positive slope than those of the control experiments, indicating that complex **3c** exhibited enhanced emission intensity upon binding to avidin. At [Re]:[avidin] > 4, the titration curve was parallel to those of the control experiments, suggesting that the excess rhenium complex molecules emitted with the same intensity as the free rhenium complex molecules in the controls. In Figure 4, however, at [Re]:[avidin] > 4, the titration curve exhibited a smaller slope than those of the control experiments, indicating that emission quenching occurred for the excess molecules of complex **3a**. It appears that this quenching can be significantly reduced when longer spacer-arms are present between the biotin and rhenium(I) luminophore. In our previous studies on related luminescent transition metal biotin complexes, similar unparallel curves were also observed for hydrophobic complexes such as $[Re(dppz)(CO)_3(py\text{-}biotin)]^{+}$[14b] and $[Ir(pq)_2(bpy\text{-}biotin)]^{+}$[14c] (dppz = dipyrido[3,2-*a*:2′,3′-*c*]-phenazine, Hpq = 2-phenylquinoline) instead of their less hydrophobic counterparts. Thus, we have reasons to believe that the emission quenching beyond the equivalence point is due to nonspecific interactions, which are probably hydrophobic in nature. Because no quenching was observed in the two control experiments (addition of rhenium complexes to buffer and to avidin preblocked with biotin, respectively), it is likely that the quenching beyond the equivalence point is due to the interactions between excess free complexes and avidin-bound complexes that are located

Table 4. First Dissociation Constants for the Rhenium−Avidin Adducts in 50 mM Potassium Phosphate Buffer pH 7.4 at 298 K and Results of Competitive Association and Dissociation Assays

complex	K_d/M	association assay/ % avidin bound	dissociation assay/ % avidin bound
1a	3.3×10^{-9}	18	22
1b	3.1×10^{-10}	30	44
1c	5.5×10^{-11}	46	85
2a	4.0×10^{-10}	22	23
2b	1.4×10^{-10}	28	36
2c	8.5×10^{-11}	46	95
3a	3.4×10^{-9}	13	12
3b	2.7×10^{-10}	23	35
3c	5.9×10^{-11}	39	79
4a	2.7×10^{-9}	27	36
4b	2.5×10^{-10}	37	73
4c	7.9×10^{-11}	41	85

closer to the protein surface (because no deviation was observed for the py-4-CH_2−NH-cap-NH−biotin complexes with long spacer-arms). Unfortunately, we are unable to explain why these interactions led to emission quenching.

The interesting observations of avidin-induced enhancement of emission intensity of the current rhenium(I)−biotin complexes are in contrast to conventional biotin−fluorophore conjugates, which suffer from severe emission quenching when they bind to avidin.[2] The quenching of biotin−fluorophore conjugates is due to efficient RET that occurs when the fluorescent molecules are in close proximity to each other after binding to the protein. Such a self-quenching effect has also been observed in protein molecules that have been highly labeled with fluorescein isothiocyanate.[24] The absence of emission quenching for the rhenium(I)−biotin complexes in the current work is a result of the negligible overlap between the absorption and emission spectra and hence the overlap integral, which renders quenching by RET not favorable.

The first dissociation constants K_d of the rhenium−avidin adducts have been estimated from the on-rates and off-rates of the rhenium−avidin adducts from kinetic experiments.[2b] The K_d value is defined as the ratio k_{off}/k_{on}, where k_{on} is the bimolecular rate constant for the binding of the fourth rhenium(I)−biotin complex to avidin−Re_3, and k_{off} is the unimolecular rate constant for the dissociation of the first rhenium(I)−biotin complex from the fully bound adduct avidin−Re_4 (as induced by addition of excess unmodified biotin molecules).[25] The K_d values ranged from ca. 5.5×10^{-11} to 3.4×10^{-9} M (Table 4), which are about 4−6 orders of magnitude larger than that of the native biotin−avidin system (K_d = ca. 10^{-15} M).[1] This difference is due to the bulkiness of the rhenium(I) polypyridine units. However, it is important to note that the binding became stronger when the biotin-containing ligands changed from py-4-CH_2−NH−biotin to py-3-CO−NH-en-NH−biotin to py-4-CH_2−NH-cap-NH−biotin (Table 4), indicating the importance of the linkers on alleviating the steric hindrance between the complexes and the protein.[26] The lowest concentration of avidin that can be detected by complex **3c** was determined to be ca. 0.15 μM from emission titrations using avidin as the titrant. This avidin concentration is lower than those determined by absorption methods using HABA (ca. 10 μM) and fluorescence methods using 2,6-anilinonaphthalene-6-sulfonic acid (2,6-ANS) (ca. 1 μM),[1e] but higher than that determined using pyrene−biotin conjugates (ca. 40 nM).[2b] However, because both HABA and 2,6-ANS are not natural substrates for avidin, their binding affinities ($K_d = 6 \times 10^{-6}$ and 2×10^{-4} M, respectively)[1a,e] are much lower as compared to those of the current rhenium(I)−biotin complexes (K_d = ca. 10^{-11} to 10^{-9} M) (Table 4). The binding of the rhenium(I)−biotin complexes to avidin was selective as no binding was observed when avidin blocked with biotin was used (Table 3). Also, other proteins of similar molecular weights such as bovine and human serum albumins did not increase the emission intensity or lifetimes of the complexes.

Competitive Association and Dissociation Assays. Competitive association and dissociation assays have been performed to study the competitive binding of the rhenium(I)−biotin complexes and native biotin to avidin.[2e,27] In the association assays, the rhenium(I)−biotin complexes competed with native biotin on binding to avidin; in the dissociation assays, the complexes bound to avidin were challenged by addition of native biotin. The percentage of the avidin molecules that bound the rhenium(I)−biotin complexes was determined by emission intensity measurements with reference to the control experiments in which native biotin was absent. The results of the association and dissociation assays are summarized in Table 4. Although the py-3-CO−NH-en-NH−biotin and py-4-CH_2−NH-cap-NH−biotin complexes **1b**, **1c**, **2b**, **2c**, **3b**, **3c**, **4b**, and **4c** exhibited less pronounced emission enhancement in the presence of avidin as compared to the py-4-CH_2−NH−biotin complexes that contain shorter spacer-arms (complexes **1a**, **2a**, **3a**, and **4a**), these eight complexes showed stronger affinities to avidin as revealed by the high percentage of avidin that remained bound by the rhenium(I)−biotin complexes in the competitive assays. For example, only ca. 18% and 22% of the avidin molecules remained bound with the py-4-CH_2−NH−biotin complex **1a** in the presence of unmodified biotin in the association and dissociation assays, respectively. However, for the py-4-CH_2−NH-cap-NH−biotin complex **1c**, ca. 46% and 85% of avidin molecules remained associated with the luminescent rhenium(I)−biotin complex in these two assays. It is conceivable that the enhanced stability for these eight rhenium−avidin adducts is a result of the longer spacer-arms of the complexes.[26] Also, it should be noted that the complex-bound avidin values for each complex should be similar in the association and dissociation assays. Apparently, the four py-4-CH_2−NH-cap-NH−biotin complexes showed larger values in the dissociation assays than

(24) Hirschfeld, T. *Appl. Opt.* **1976**, *15*, 3135−3139.
(25) The rate constants and selected linear plots are included in the Supporting Information.
(26) (a) Kam, C.-M.; Abuelyaman, A. S.; Li, Z.; Hudig, D.; Powers, J. C. *Bioconjugate Chem.* **1993**, *4*, 560−567. (b) Basak, A.; Jean, F.; Dugas, H.; Lazure, C. *Bioconjugate Chem.* **1994**, *5*, 301−305. (c) Sugawara, K.; Hoshi, S.; Akatsuka, K.; Tanaka, S.; Nakamura, H. *Anal. Sci.* **1997**, *13*, 677−679.
(27) Wilbur, D. S.; Pathare, P. M.; Hamlin, D. K.; Frownfelter, M. B.; Kegley, B. B.; Leung, W.-Y.; Gee, K. R. *Bioconjugate Chem.* **2000**, *11*, 584−598.

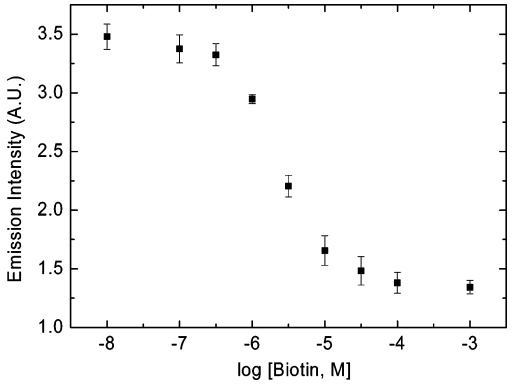

Figure 6. Results of a homogeneous competitive assay for biotin using complex **3c** and avidin. The emission intensity of the solutions is of triplicate experiments ±1 standard deviation.

in the association assays (Table 4). The reason is that the off-rates of these complexes are much lower than their counterparts of shorter spacer-arms.[25] In other words, equilibrium had not been attained during the incubation period in the dissociation assays, and the qualitative nature of the trends of these assays observed in Table 4 is thus emphasized. Nevertheless, these experiments can still serve as convenient and fast assays that allow one to have preliminary ideas on the avidin-binding properties of these new biotin derivatives.

Homogeneous Competitive Assay for Biotin. In view of the luminescence enhancement exhibited by the rhenium(I)−biotin complexes when they bind to avidin, a simple and rapid homogeneous assay for biotin has been developed. As an example, complex **3c** was used as the probe in this assay, which was based on the competition between this complex and native biotin on binding to avidin. A lower biotin analyte concentration was expected to result in a higher degree of binding of complex **3c** to avidin and thus higher emission intensity. In our experiments, the emission of the solutions was measured over a biotin analyte concentration range from 1×10^{-3} to 1×10^{-8} M, and the results are shown in Figure 6. The concentration range of biotin that can be measured by this assay was from ca. $1 \times 10^{-6.5}$ to $1 \times 10^{-4.5}$ M. The lowest concentration of biotin analyte that gave a meaningful signal (ca. $1 \times 10^{-6.5}$ M) is similar to competitive biotin assays we reported recently.[14c,d,28]

Homogeneous Avidin Assay Using QSY-Conjugated Polypeptide. The intrinsic emission intensity enhancement (I/I_o) of the complexes upon binding to avidin varied from ca. 1.2- to 3.0-fold (Table 3). To develop a sensitive assay for avidin (and biotin), it is desirable to maximize these emission enhancement factors. We were inspired by the interesting work by Whitten and co-workers on the use of fluorescent polymers and viologen-bearing biotin compounds as a probing system for avidin.[29] Our strategy is to use a quencher to selectively suppress the emission of the free rhenium(I)−biotin complex by distance-dependent RET quenching.[30] This quencher is designed in such a way that (i) when avidin is absent, it can moderately bind the luminescent complex by electrostatic attraction; (ii) it contains energy-absorbing units that can effectively reduce the emission of the free complex by RET; and (iii) its molecular size should be large, so that its quencher units cannot be in close proximity to the rhenium(I)−biotin complex after it binds to avidin. Taking all of these requirements into consideration, we exploited a water-soluble polypeptide (poly(D-Glu:D-Lys) 6:4) that had been modified with the nonfluorescent energy-acceptor dye QSY-7 hydroxysuccinimidyl ester. The reasons for using this polypeptide poly(E/K) are that (i) it consists of glutamic acid and lysine residues with an abundance ratio of ca. 6:4, and, because of the ionizable side-chain of glutamic acid, the modified polymer poly(E/K)-QSY bears an overall negative charge in aqueous solutions, which allows the macromolecule to exhibit Coulombic attraction with the free cationic rhenium(I)−biotin complex; (ii) the primary amines of the lysine residues can be readily modified by the hydroxysuccinimidyl ester derivative of the quencher; (iii) the bulky size of this polypeptide would not enable the quencher units to approach the avidin-bound rhenium(I)−biotin complex, and the RET quenching could be minimized. The organic label QSY-7 hydroxysuccinimidyl ester was chosen because the absorption maximum (ca. 560 nm) of its protein conjugate occurred at an energy similar to that of the emission maximum of the probe, complex **3c** (558 nm), leading to a Förster distance of ca. 42.1 Å.

In the absence of the quencher, upon irradiation, complex **3c** displayed intense yellow emission with a lifetime of 1.6 μs in aerated water at 298 K. The presence of poly(E/K)-QSY in a solution of the complex resulted in the reduction of emission intensity of ca. 50% and lifetime ($\tau < 0.1$ μs). This quenching is likely to occur via an RET mechanism and is facilitated by the electrostatic interaction between the negatively charged poly(E/K)-QSY and cationic complex **3c** because no such quenching was observed when the experiment was performed in 50 mM $MgSO_4$ solution or when the positively charged quencher poly(K)-QSY was used. The reason for the different decrease in emission intensity (ca. 50%) and lifetime (>1.6 fold) is not completely understood. A possible explanation is that the hydrophobicity associated with the biopolymer assisted in the "enhancement of emission intensity" back to an overall 50%. Because the emission lifetimes of the Me_2−Ph_2-phen complexes are much less sensitive to hydrophobicity as compared to their emission intensity (Table 2), a similar "restoration" of lifetime was not observed.

When avidin was added to a mixture of poly(E/K)-QSY and complex **3c** in water, both emission enhancement and lifetime elongation were observed (see below). It is likely that the rhenium(I)−biotin complex departed from the polymeric quencher and bound to the protein molecule, resulting in a longer separation between the donor (complex

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(29) (a) Chen, L.; McBranch, D. W.; Wang, H.-L.; Helgeson, R.; Wudl, F.; Whitten, D. G. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 12287−12292. (b) Jones, R. M.; Lu, L.; Helgeson, R.; Bergstedt, T. S.; McBranch, D. W.; Whitten, D. G. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 14769−14772.

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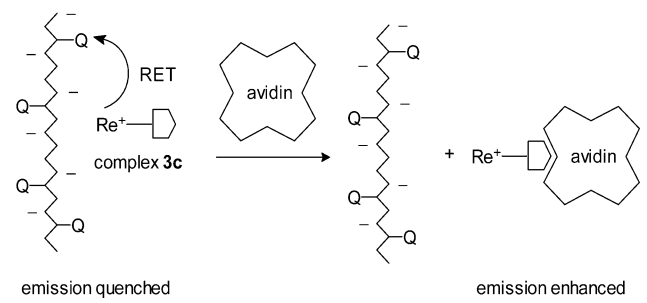
Scheme 1. Schematic Representation Showing the Emission Quenching of Complex **3c** by Poly(E/K)-QSY and Emission Enhancement upon Binding of the Complex to Avidin



3c) and acceptor (QSY-7), and hence the enhancement of emission intensity (Scheme 1). We cannot completely exclude the possibility of electrostatic interactions between the negatively charged quencher and the positively charged avidin. However, a similar increase in emission intensity and lifetime was still observed when neutral streptavidin was used instead of avidin.

On the basis of the emission enhancement, we have titrated complex **3c** in water with avidin in the absence and presence of poly(E/K)-QSY. In the presence of the polymeric quencher, the emission intensity of the complex increased significantly and showed an overall gain of 4 times at [avidin]:[**3c**] = 1/4 (Figure 7). The emission lifetimes also increased

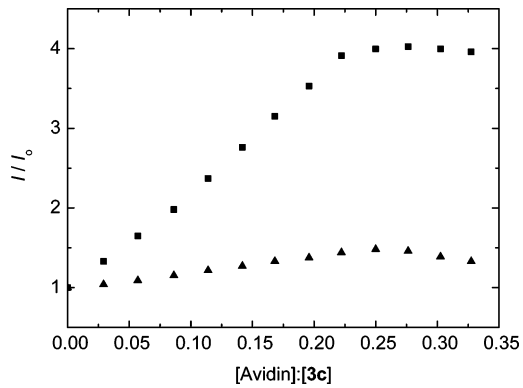


Figure 7. Luminescence titration curves for the titrations of complex **3c** (5.5 μM) with avidin in the presence (■) and absence (▲) of poly(E/K)-QSY (1.1 μM) in water at 298 K, where I and I_o are the emission intensity in the presence and absence of avidin, respectively.

from <0.1 to ca. 2.6 μs. These photophysical changes are due to the binding of the biotin moiety of the complex to the specific biotin-binding sites of avidin, because no similar changes were observed when avidin presaturated with excess biotin was used. In the absence of the quencher poly(E/K)-QSY, the complex also showed enhancement in emission intensity. However, at [avidin]:[**3c**] = 1/4, only ca. 1.5-fold of emission enhancement was observed (Figure 7).[31] Thus, using a macromolecular quencher that can selectively reduce the emission of the free rhenium(I)−biotin complex, we have successfully increased the amplification factor of the emission enhancement for avidin sensing.

Conclusions

We describe here the synthesis, characterization, photophysical, and avidin-binding properties of a series of luminescent rhenium(I)−biotin complexes. Spacer-arms of various chain lengths were introduced between the rhenium(I) polypyridine unit and the biotin moiety. We found that more hydrophobic diimine ligands can lead to a higher degree of emission enhancement and longer spacer-arms can increase the stability of the rhenium−avidin adducts. Using a negatively charged polymeric quencher, the avidin-induced emission enhancement factor of one of the complexes was increased. Our future target is to design related luminescent probes for biotin and avidin with higher detection sensitivity and to apply these systems in the development of new bioassays.

Experimental Section

Materials and General Methods. All solvents were of analytical reagent grade and purified according to literature procedures.[32] $Re(CO)_5Cl$ (Aldrich), 4-(aminomethyl)pyridine (Aldrich), nicotinoyl chloride hydrochloride (Aldrich), diimine ligands (Aldrich), biotin (Acros), KPF_6 (Acros), HABA (Sigma), avidin (Calbiochem), poly(D-Glu:D-Lys) (6:4) (Pierce), and QSY-7 hydroxysuccinimidyl ester (Molecular Probes) were used as received. Biotinylethylenediamine,[15] biotinamidohexanoic acid *N*-hydroxysuccinimidyl ester,[1d] nicotinic acid *N*-hydroxysuccinimidyl ester,[16] and dpq[33] were synthesized according to reported procedures. Complexes **1a**, **2a**, and **3a** were prepared as described previously.[14a]

^{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 an Elementar Analyzensysteme GmbH Vario EL elemental analyzer. Electronic absorption and steady-state emission/excitation spectra were recorded on a Hewlett-Packard 8453 diode array spectrophotometer and a Spex Fluorolog-2 model F111 or a Fluoromax-3 fluorescence spectrophotometer, respectively.

Unless specified otherwise, solutions for photophysical studies were degassed with no fewer than four successive freeze−pump−thaw cycles and stored in a 10-mL round-bottomed flask equipped with a 1-cm fluorescence cuvette sidearm, and sealed from the atmosphere by a Rotaflo HP6/6 quick-release Teflon stopper. Luminescence quantum yields were measured by the optically dilute method[34] using an aerated aqueous solution of $[Ru(bpy)_3]Cl_2$ (Φ = 0.028, excitation wavelength at 455 nm)[35] as the standard solution. The excitation source for emission lifetime measurements was the 355 nm output (third harmonic) of a Quanta-Ray Q-switched GCR-150-10 pulsed Nd:YAG laser. 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 digital oscilloscope, and

(31) A lower enhancement factor (1.5) in pure water than in 50 mM phosphate buffer (2.27, Table 3) is probably due to ionic-strength effects. However, a similar enhancement factor (ca. 2.28) was achieved when a more concentrated phosphate buffer (200 mM) was used in the avidin-binding studies. It is unlikely that the enhancement factor would increase further in more concentrated buffers.

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finally analyzed by using a program for exponential fits on an IBM-compatible PC-computer.

Py-3-CO−NH-en-NH−biotin. A mixture of nicotinic acid *N*-hydroxysuccinimidyl ester (232 mg, 1.05 mmol) and triethylamine (590 mL, 4.20 mmol) in 2 mL of DMF was added to biotinylethylenediamine (301 mg, 1.05 mmol) dissolved in 10 mL of DMF. The solution was stirred under nitrogen at room temperature for 12 h, after which the solution was evaporated to dryness under reduced pressure to give a pale yellow solid. Recrystallization of the solid from methanol−diethyl ether afforded py-3-CO−NH-en-NH−biotin as white crystals. Yield: 355 mg (86%). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K, relative to TMS): δ 8.96 (s, 1H, H2 of pyridine), 8.71−8.67 (m, 2H, H6 of pyridine and py-3-CO−N*H*), 8.15 (d, J = 7.9 Hz, 1H, H4 of pyridine), 7.95 (t, J = 5.4 Hz, 1H, en-N*H*−biotin), 7.49 (dd, J = 7.9 and 4.7 Hz, 1H, H5 of pyridine), 6.44 (s, 1H, NH of biotin), 6.37 (s, 1H, NH of biotin), 4.30−4.26 (m, 1H, NCH of biotin), 4.14−4.06 (m, 1H, NCH of biotin), 3.10−3.01 (m, 1H, SCH of biotin), 2.80 (dd, J_{gem} = 12.3 Hz, J_{vic} = 5.3 Hz, 1H, SCH of biotin), 2.56 (d, J_{gem} = 12.6 Hz, 1H, SCH of biotin), 2.06 (t, J = 7.3 Hz, 2H, COCH_2C$_3$H$_6$ of biotin), 1.55−1.29 (m, 6H, COCH$_2$C$_3$$H_6$ of biotin). IR (KBr, cm^{-1}): ν(NH) 3288 (s), 3073 (m), ν(CH) 2919 (m), 2848 (m), ν(CONH) 1696 (s), 1650 (s), ν(NH) 1557 (s); positive-ion ESI-MS ion clusters at m/z 392 $\{M + H^+\}^+$.

Py-4-CH$_2$−NH-cap-NH−biotin. 4-(Aminomethyl)pyridine (66 mg, 0.61 mmol) in 5 mL of DMF was added to a mixture of biotinamidohexanoic acid *N*-hydroxysuccinimidyl ester (273 mg, 0.60 mmol) and triethylamine (340 mL, 2.44 mmol) in 5 mL of DMF. The solution was stirred under nitrogen at room temperature overnight, after which the solution was evaporated to dryness under reduced pressure to give a pale yellow solid. Recrystallization of the solid from methanol−diethyl ether afforded py-4-CH$_2$−NH-cap-NH−biotin as white crystals. Yield: 237 mg (87%). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K, relative to TMS): δ 8.50 (d, J = 5.0 Hz, 2H, H2 and H6 of pyridine), 8.43 (t, J = 5.9 Hz, 1H, py-4-CH$_2$−N*H*), 7.78 (t, J = 5.1 Hz, 1H, cap-N*H*−biotin), 7.23 (d, J = 5.0 Hz, 2H, H3 and H5 of pyridine), 6.46 (s, 1H, NH of biotin), 6.39 (s, 1H, NH of biotin), 4.33−4.27 (m, 3H, NCH of biotin and py-4-CH_2−NH), 4.15−4.11 (m, 1H, NCH of biotin), 3.13−3.07 (m, 1H, SCH of biotin), 3.05−3.01 (m, 2H, CH_2−NH−biotin), 2.82 (dd, J_{gem} = 12.0 Hz, J_{vic} = 5.0 Hz, 1H, SCH of biotin), 2.58 (d, J_{gem} = 12.0 Hz, 1H, SCH of biotin), 2.17 (t, J = 7.5 Hz, 2H, CH_2C$_4$H$_8$−NH−biotin), 2.05 (t, J = 7.0 Hz, 2H, COCH_2C$_3$H$_6$ of biotin), 1.62−1.24 (m, 12H, CH$_2$C$_3$$H_6CH_2$−NH−biotin and COCH$_2C_3$$H_6$ of biotin). IR (KBr, cm^{-1}): ν(NH) 3283 (s), 3067 (m), ν(CH) 2930 (m), 2853 (m), ν(CONH) 1701 (s), 1639 (s), ν(NH) 1547 (s); positive-ion ESI-MS ion clusters at m/z 448 $\{M + H^+\}^+$.

[Re(dpq)(CO)$_3$(py-4-CH$_2$−NH−biotin)](PF$_6$) (4a). The preparation of **4a** was similar to that of **1a**,[14a] except that [Re(dpq)(CO)$_3$(CH$_3$CN)](CF$_3$SO$_3$) (222 mg, 0.32 mmol) was used. Complex **4a** was isolated as yellow crystals. Yield: 148 mg (47%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, relative to TMS): δ 10.01−9.97 (m, 2H, H4 and H4′ of pyrido rings of dpq), 9.95−9.91 (m, 2H, H6 and H6′ of pyrido rings of dpq), 9.34 (s, 2H, H2 and H3 of dpq), 8.56−8.48 (m, 4H, H5 and H5′ of pyrido rings of dpq and H2 and H6 of pyridine), 7.65 (t, J = 6.0 Hz, 1H, py-4-CH$_2$−N*H*), 7.24 (d, J = 6.5 Hz, 2H, H3 and H5 of pyridine), 5.70 (s, 1H, NH of biotin), 5.68 (s, 1H, NH of biotin), 4.52−4.48 (m, 1H, NCH of biotin), 4.30−4.26 (m, 3H, py-4-CH_2−NH and NCH of biotin), 3.19−3.12 (m, 1H, SCH of biotin), 2.93 (dd, J_{gem} = 12.6 Hz, J_{vic} = 5.0 Hz, 1H, SCH of biotin), 2.72 (d, J_{gem} = 12.6 Hz, 1H, SCH of biotin), 2.17 (t, J = 7.2 Hz, 2H, COCH_2C$_3$H$_6$ of biotin), 1.72−1.32 (m, 6H, COCH$_2$C$_3$$H_6$ of biotin). IR (KBr, cm^{-1}): ν(NH) 3441 (m), ν(CH) 2925 (m), ν(C≡O) 2034 (s), 1916 (s), ν(CONH) 1709 (m), ν(PF$_6^-$) 843 (s); positive-ion ESI-MS ion clusters at m/z: 837 $\{[Re(dpq)(CO)_3(py\text{-}4\text{-}CH_2\text{−}NH\text{−}biotin)]\}^+$, 503 $\{[Re(dpq)(CO)_3]\}^+$. Anal. Calcd for [Re(dpq)(CO)$_3$(py-4-CH$_2$−NH−biotin)](PF$_6$)·H$_2$O: C, 39.64; H, 3.23; N, 11.21. Found: C, 39.64; H, 3.52; N, 10.95.

The synthetic procedures and characterization data for the other complexes are included in the Supporting Information.

HABA Assays of Rhenium(I)−Biotin Complexes. The binding stoichiometry of the rhenium(I)−biotin complexes to avidin was assessed by HABA assays. Typically, to a mixture of HABA (300.0 μM) and avidin (7.6 μM) in 50 mM potassium phosphate buffer pH 7.4 (2 mL) were added 5 μL aliquots of the rhenium(I)−biotin complex (1.1 mM) in 1-min intervals. The formation of the rhenium−avidin adduct was indicated by a decrease in the absorbance at 500 nm due to the displacement of HABA from the avidin. By plotting $-\Delta A_{500\ nm}$ versus [Re]:[avidin], the binding stoichiometry of the rhenium(I)−biotin complex to avidin was determined.

Emission Titrations. In a typical procedure, avidin (3.8 μM) in 50 mM potassium phosphate buffer pH 7.4 (2 mL) was titrated with the rhenium(I)−biotin complexes (0.55 mM) by cumulative additions of 5 μL aliquots at 1-min intervals. The solutions were excited at 380 nm, and the emission intensity was monitored at the emission maxima of the complexes.

Competitive Association and Dissociation Assays. The binding of the rhenium(I)−biotin complexes toward avidin with respect to unmodified biotin was investigated by competitive association and dissociation assays. In the association assay, to a mixture of the rhenium(I)−biotin complex (60.0 μM) and unmodified biotin (60.0 μM) was added avidin (15.0 μM). The mixture (500 μL) was incubated at room temperature for 1 h and then loaded onto a PD-10 size exclusion column (Pharmacia) that had been equilibrated with 50 mM potassium phosphate buffer pH 7.4. The first 5 mL of the eluted solution that contained avidin was collected, and the emission spectrum was measured. The emission intensity was compared to that of the control, in which unmodified biotin was absent. In the dissociation assay, a mixture of the rhenium(I)−biotin complex (60.0 μM) and avidin (15.0 μM) was incubated at room temperature for 1 h. Unmodified biotin (60.0 μM) was then added, and the mixture (500 μL) was incubated at room temperature for 1 h. The emission intensity of the avidin-containing fraction was compared to that of the control in which no unmodified biotin was added.

Homogeneous Competitive Assay for Biotin. To samples of various concentrations of biotin analyte in 50 mM potassium phosphate buffer pH 7.4 was added complex **3c** (5.5 μM). Avidin (1.4 μM) in the same buffer was then added, and the assay mixtures (2 mL) were incubated at room temperature for 5 min. The final concentration of the biotin analyte in the assay solutions ranged from 1×10^{-3} to 1×10^{-8} M. The emission spectra of the solutions were then measured.

Conjugation of QSY-7 Hydroxysuccinimidyl Ester to Poly-(D-Glu:D-Lys) 6:4. QSY-7 hydroxysuccinimidyl ester (0.20 mg) in anhydrous DMSO (20 μL) was added to poly(D-Glu:D-Lys) 6:4 (5.0 mg) (MW = ca. 23 kDa) dissolved in 480 μL of 50 mM carbonate buffer pH 9.0. The solution was stirred gently at room temperature for 4 h. The solid residue was removed by centrifugation. The supernatant was then loaded onto a PD-10 column that had been equilibrated with Milli Q water (Millipore). The first purple band that came out from the column was collected, and the solution was extensively dialyzed against Milli Q water (2 L × 3). Finally, the solution was concentrated by centrifugation with a

YM-3 centricon (Amicon). The dye:polypeptide ratio was estimated to be ca. 0.2 from spectral data.

Homogeneous Assay for Avidin Using Poly(E/K)-QSY. Complex **3c** (5.5 μM) in Milli Q H_2O (2 mL) in the presence and absence of poly(E/K)-QSY (1.1 μM), respectively, was titrated with a solution of avidin (30.6 μM) by cumulative additions of 10 μL aliquots at 1-min intervals. The solutions were excited at 380 nm, and the emission intensity was monitored at the emission maximum of the complex (ca. 558 nm).

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Supporting Information Available: Synthetic procedures and characterization data for the rhenium(I)−biotin complexes, results of emission titrations, and determination of k_{on}, k_{off}, and K_d from kinetics experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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