Review Article

Luminescent Transition Metal Polypyridine Biotin Complexes

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In view of the fact that many transition metal complexes display intense and long-lived metal-to-ligand charge-transfer emission with a large Stokes' shift, we have incorporated biotin into a series of luminescent rhenium(I), iridium(III) and ruthenium(II) polypyridine complexes to form new probes for the protein avidin. All these luminescent transition metal polypyridine biotin complexes displayed enhanced emission intensities and extended lifetimes upon binding to avidin. These changes are closely related to the hydrophobic biotin-binding pockets of avidin and the increased rigidity of the local environment of the probes after they bind to the protein. We have exploited the characteristics of these luminescent transition metal biotin complexes in the development of various assays.

Keywords: Biotin; Iridium(III); Luminescence; Rhenium(I); Ruthenium(II).

INTRODUCTION

Avidin is a glycoprotein (MW = 68 kDa) that can bind up to four biotin molecules with exceptionally high affinity (first dissociation constant, K_d = approximately 10^{-15} M). The avidin-biotin system has been widely employed in many bioanalytical applications.[1,2] Biotinylated biomolecules can be detected by avidin molecules that have been labelled with reporters such as fluorescent compounds or enzymes.[3] Because avidin has four biotin-binding sites, it appears that biotinylated biomolecules can also be recognised by fluorophore–biotin conjugates when avidin is used as a bridge. Unfortunately, this approach is not practicable because most fluorophore–biotin molecules experience self-quenching upon binding to avidin, unless long spacers are present between the fluorophore and biotin.[4–8]

We anticipate that this problem can be solved using luminescent transition metal complexes as the reporter in view of their characteristic photophysical properties, in particular, the large Stokes' shifts. In fact, by virtue of their variable oxidation states, flexible coordinating geometry, and rich photophysical and electrochemical properties, many transition metal complexes have been conjugated to biomolecules for various purposes.[9–23] The possibility of using luminescent transition metal complexes, such as rhenium(I), iridium(III) and ruthenium(II) polypyridine complexes, as biological probes is very attractive because many of these complexes show intense and long-lived luminescence with tunable emission energy in the visible region. Importantly, the emission of many of these systems is very sensitive to the local surroundings of the probes, such as the hydrophobicity of the environment. Incorporation of biologically important molecules into these luminescent complexes could thus generate a new class of biological probes.

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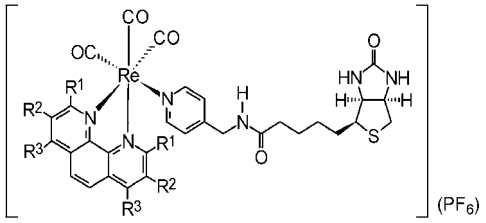
Abbreviations: BSA, bovine serum albumin; bpy, 2,2′-bipyridine; bpy-en-biotin, 4-(*N*-((2-biotinamido)ethyl)aminomethyl)-4′-methyl-2,2′-bipyridine; dppn, benzo[*i*]dipyrido[3,2–*a*:2′,3′–*c*]phenazine; dppz, dipyrido[3,2–*a*:2′,3′–*c*]phenazine; dpq, dipyrido-[3,2–*f*:2′,3′–*h*]quinoxaline; HABA, 4′-hydroxyazobenzene-2-carboxylic acid; Hbzq, 7,8-benzoquinoline; Hmppy, 2-(4-methyl-phenyl)pyridine; Hmppz, 3-methyl-1-phenylpyrazole; Hppy, 2-phenylpyridine; Hppz, 1-phenylpyrazole; Hpq, 2-phenylquinoline; HSA, human serum albumin; IL, intraligand; L1, 4-(*N*-((2-biotinamido)ethyl)amido)-4′-methyl-2,2′-bipyridine; L2, 4-(*N*-((6-biotin-amido)hexyl)amido)-4′-methyl-2,2′-bipyridine; Me_4-phen, 3,4,7,8-tetramethyl-1,10-phenanthroline; Me_2-Ph_2-phen, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline; MLCT, metal-to-ligand charge-transfer; phen, 1,10-phenanthroline; py-4-CH_2-NH-biotin, 4-((biotinamido)methyl)pyridine; py-3-CO-NH-en-NH-biotin, 3-(*N*-((2-biotinamido)ethyl)amido)pyridine; py-4-CH_2-NH-cap-NH-biotin, 4-(*N*-((6-biotinamido)hexanoyl)aminomethyl)pyridine; RET, resonance-energy transfer.

Although there have been reports on conjugation of the biotin moiety to different transition metal complexes,[24–30] the use of transition metal biotin complexes as luminescent probes has not been investigated. Our recent effort in the design of luminescent transition metal polypyridine biotin complexes is described in this review article.

Rhenium(I) Polypyridine Biotin Complexes

In 2002, we reported the design of three luminescent rhenium(I) polypyridine biotin complexes $[Re(N\text{-}N)(CO)_3(py\text{-}4\text{-}CH_2\text{-}NH\text{-}biotin)](PF_6)$ (N-N = phen, Me_4-phen, Me_2-Ph_2-phen) (Fig. 1).[31] Similar to their biotin-free counterparts, these rhenium(I)-biotin complexes exhibited intense and long-lived ^{3}MLCT ($d\pi(Re) \rightarrow \pi^*(N\text{-}N)$) luminescence upon irradiation (Table 1).[32–34] The binding of the complexes to avidin has been studied using the standard HABA assay.[35] The results revealed that all three complexes bound to avidin with the same stoichiometry as unmodified biotin



[Re(phen)(CO)$_3$(py-4-CH$_2$-NH-biotin)](PF$_6$): R^1 = R^2 = R^3 = H
[Re(Me$_4$-phen)(CO)$_3$(py-4-CH$_2$-NH-biotin)](PF$_6$): R^1 = H; R^2 = R^3 = CH$_3$
[Re(Me$_2$-Ph$_2$-phen)(CO)$_3$(py-4-CH$_2$-NH-biotin)](PF$_6$): R^1 = CH$_3$; R^2 = H; R^3 = C$_6$H$_5$

Fig. 1. Structures of $[Re(N\text{-}N)(CO)_3(py\text{-}4\text{-}CH_2\text{-}NH\text{-}biotin)](PF_6)$. Reproduced from Reference 31, with permission of the American Chemical Society.

(Re:avidin = 4:1). The most remarkable observation was that all the complexes displayed enhanced emission intensities and extended lifetimes upon binding to avidin. At [Re]:[avidin] = 4:1, the emission intensities were enhanced by approximately 1.4 to 3.0 fold and the emission lifetimes were extended by approximately 1.5 to 2.4 fold (Table 1). Emission titration curves for the Me_2-Ph_2-phen complex are shown in Fig. 2. Since no similar changes were observed when excess biotin was initially present, the increase in emission intensities and lifetimes was a consequence of the specific binding of the complexes to the biotin-binding sites of avidin (Table 1). These observations

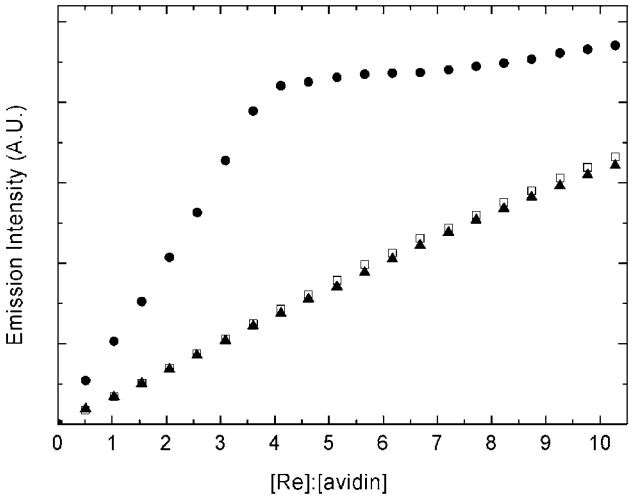


Fig. 2. Luminescence titration curves for the titrations of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲), and (iii) a blank phosphate buffer solution (□) with $[Re(Me_2\text{-}Ph_2\text{-}phen)(CO)_3(py\text{-}4\text{-}CH_2\text{-}NH\text{-}biotin)](PF_6)$. Reproduced from Reference 31, with permission of the American Chemical Society.

Table 1. Photophysical data of $[Re(N\text{-}N)(CO)_3(py\text{-}4\text{-}CH_2\text{-}NH\text{-}biotin)](PF_6)$ at 298 K. Reproduced from Reference 31, with permission of The American Chemical Society

Complex	medium	λ_{em}/nm[a]	τ_o/μs[a]	Φ_{em}[a]	I (τ/μs)[b,c]	I (τ/μs)[b,d]	I (τ/μs)[b,e]
$[Re(phen)(CO)_3(py\text{-}4\text{-}CH_2\text{-}NH\text{-}biotin)](PF_6)$	CH_2Cl_2	536	2.69	0.25	1.00 (0.56)	1.42 (0.90)	0.98 (0.55)
	CH_3CN	552	1.37	0.079			
$[Re(Me_4\text{-}phen)(CO)_3(py\text{-}4\text{-}CH_2\text{-}NH\text{-}biotin)](PF_6)$	CH_2Cl_2	510	7.81	0.16	1.00 (1.23)	2.25 (2.96)	1.04 (1.25)
	CH_3CN	518	7.32	0.072			
$[Re(Me_2\text{-}Ph_2\text{-}phen)(CO)_3(py\text{-}4\text{-}CH_2\text{-}NH\text{-}biotin)](PF_6)$	CH_2Cl_2	540	7.22	0.22	1.00 (1.84)	2.98 (2.70)	0.96 (1.90)
	CH_3CN	550	6.78	0.079			

[a] In degassed solvents. [b] Relative emission intensities in aerated 50 mM potassium phosphate buffer pH 7.4. [c] [Re] = 15.2 μM, [avidin] = 0 μM, [unmodified biotin] = 0 μM. [d] [Re] = 15.2 μM, [avidin] = 3.8 μM, [unmodified biotin] = 0 μM. [e] [Re] = 15.2 μM, [avidin] = 3.8 μM, [unmodified biotin] = 380.0 μM.

are in contrast to most fluorophore–biotin conjugates, which suffer from severe emission quenching upon binding to avidin due to RET, unless exceptionally long spacers such as poly(ethylene glycol) are present between the fluorophore and the biotin units.[4–8] The self-quenching is in line with the findings that biomolecules multiply labelled with organic fluorophores do not become correspondingly more fluorescent.[36] The absence of emission quenching in the present case is due to the insignificant overlap between the absorption and emission spectra of the rhenium(I) polypyridine biotin complexes, which disfavours RET quenching. Because both the emission intensities and lifetimes of the excited complexes were sensitive to the hydrophobicity of the environment (Table 1), it is likely that the enhancement results from the hydrophobicity associated with the biotin-binding pockets of avidin. This is supported by the observations that the emission intensity enhancement factors (1.42, 2.25, 2.98 for the phen, Me_4-phen and Me_2-Ph_2-phen complexes, respectively) increased with the lipophilicity of the complexes (the log $P_{o/w}$ values of these three complexes were 0.94, 2.08 and 4.05, respectively). Another possible reason for the emission enhancement and lifetime extension is the increased rigidity of the surroundings of the complexes upon the binding event because such an increase can lead to lower non-radiative decay efficiency and hence more intense and long-lived emission.

We have prepared new rhenium(I) polypyridine biotin complexes by making use of the extended planar diimine ligands dppz and dppn (Fig. 3).[37] These extended planar diimine ligands were expected to allow the complexes to intercalate into the base-pairs of double-stranded DNA molecules,[10,38] and the biotin moieties would enable the complexes to bind to avidin. Upon irradiation, the complexes exhibited intense and long-lived greenish-yellow to orange luminescence in solutions at room temperature and in low-temperature alcohol glass (Table 2). In the presence of double-stranded calf thymus DNA, the low-energy absorption bands of all the complexes displayed pronounced hypochromism and a small bathochromic shift, and the emission of the complexes was substantially enhanced.

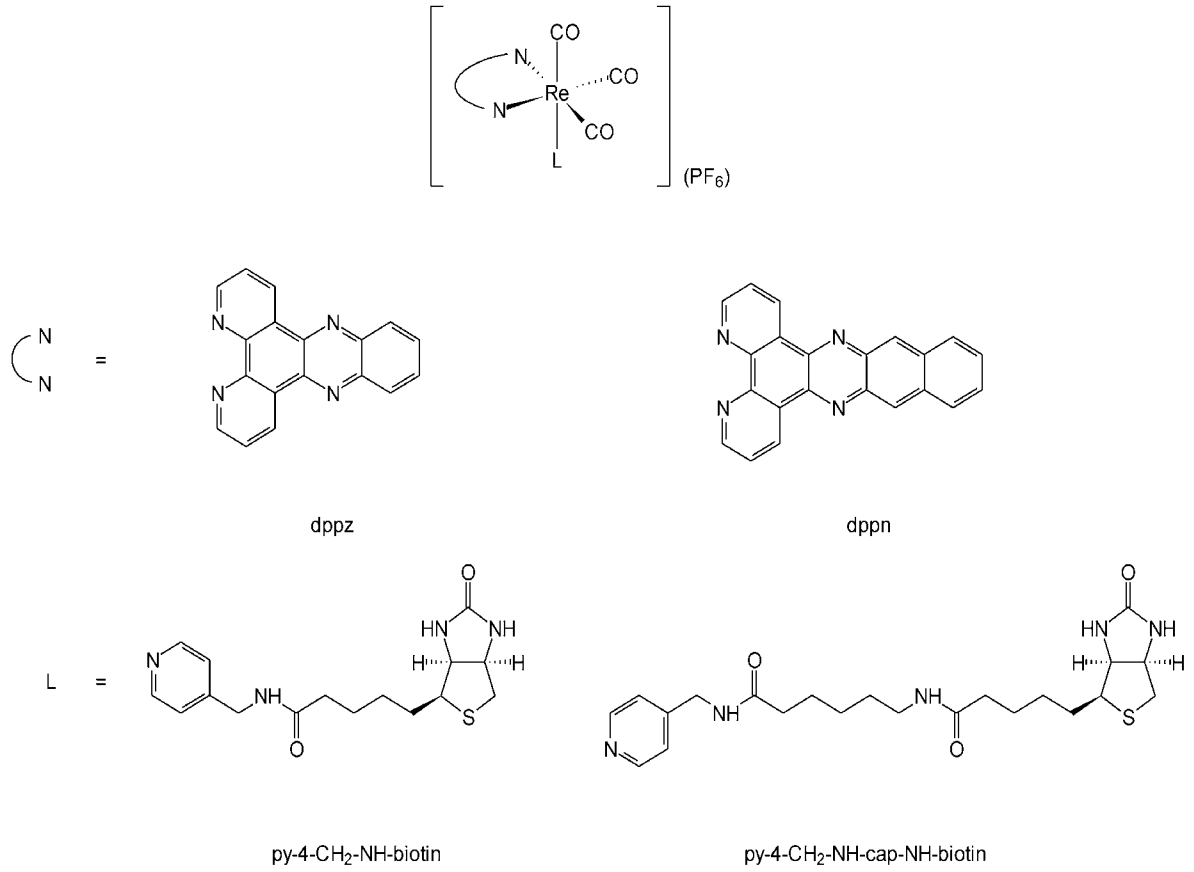


Fig. 3. Structures of rhenium(I)-biotin complexes containing extended planar diimine ligands. Reproduced from Reference 37, with permission of the American Chemical Society.

Table 2. Photophysical data of rhenium(I)-biotin complexes containing extended planar diimine ligands. Reproduced from Reference 37, with permission of The American Chemical Society

Complex	medium (T/K)	λ_{em}/nm	τ_o/μs	Φ_{em}	I (τ/μs)^{a,b}	I (τ/μs)^{a,c}	I (τ/μs)^{a,d}
[Re(dppz)(CO)$_3$(py-4-CH$_2$-NH-biotin)](PF$_6$)	CH_2Cl_2 (298)	558, 601 sh	4.90	0.0049	1.00	39.87	0.85
	CH_3CN (298)	556, 599 sh	7.12	0.0016	(0.39)	(0.91)	(0.39)
	Glass[e] (77)	547 (max), 562 sh, 592, 614 sh, 645 sh	12887				
[Re(dppz)(CO)$_3$(py-4-CH$_2$-NH-cap-NH-biotin)](PF$_6$)	CH_2Cl_2 (298)	556, 607 sh	4.50	0.0049	1.00	1.92	0.89
	CH_3CN (298)	554, 597 sh	8.01	0.0031	(0.28)	(0.50)	(0.26)
	Glass[e] (77)	544 (max), 559 sh, 590, 612 sh, 638 sh	12405				
[Re(dppn)(CO)$_3$(py-4-CH$_2$-NH-biotin)](PF$_6$)	CH_2Cl_2 (298)	587	17.88	0.20	1.00	3.65	1.28
	CH_3CN (298)	595	31.47	0.13	(0.26)	(0.40)	(0.27)
	Glass[e] (77)	517, 556 (max), 603 sh	5.77				
[Re(dppn)(CO)$_3$(py-4-CH$_2$-NH-cap-NH-biotin)](PF$_6$)	CH_2Cl_2 (298)	588	22.15	0.23	1.00	2.39	1.09
	CH_3CN (298)	595	33.09	0.095	(0.22)	(0.29)	(0.20)
	Glass[e] (77)	524, 558 (max), 607 sh	6.72				

[a] Relative emission intensities in degassed 50 mM potassium phosphate buffer at pH 7.2. [b] [Re] = 15.2 μM, [avidin] = 0 μM, [unmodified biotin] = 0 μM. [c] [Re] = 15.2 μM, [avidin] = 3.8 μM, [unmodified biotin] = 0 μM. [d] [Re] = 15.2 μM, [avidin] = 3.8 μM, [unmodified biotin] = 380.0 μM. [e] EtOH/MeOH (4:1, v/v).

These changes are attributable to the binding of the complexes to the DNA molecules by intercalation.[10,38–47] The HABA assays showed that all the complexes bound to avidin with a stoichiometry of 4:1 (Re:avidin). The emission intensities and lifetimes of the complexes also increased in the presence of avidin (Table 2). The emission intensity enhancement factors varied from approximately 1.9 to 40. The emission spectral changes of [Re(dppz)(CO)$_3$(py-4-CH$_2$-NH-biotin)](PF$_6$) upon addition of avidin are shown in Fig. 4. The results of the titrations revealed that the complexes bound to both double-stranded DNA with an intrinsic binding constant of approximately 10^4 M^{-1}, and avidin with a dissociation constant in the order of approximately 10^{-9} M. To the best of our knowledge, these complexes are the first luminescent probes that respond to both DNA molecules and avidin.

Recently, we have expanded this family of rhenium(I) polypyridine biotin complexes by varying the identity of the diimine ligands and the introduction of spacer-arms of various chain lengths between the luminophore and the biotin unit (Fig. 5).[48] Upon photoexcitation, all the complexes exhibited intense and long-lived greenish-yellow to yellow ^{3}MLCT luminescence in fluid solutions under ambient conditions. The photophysical data of selected complexes are tabulated in Table 3. Solvents of different polarity have been chosen to study their effects on the photophysical properties of the complexes. In general, the emission lifetimes of the complexes decreased upon increasing solvent polarity. However, the Me$_4$-phen complexes showed exceptionally long emission lifetimes in aqueous buffer, which is due to the mixed ^{3}IL/^{3}MLCT emissive-state character of these complexes. Results of the HABA assays revealed that these rhenium(I) polypyridine biotin complexes bound to avidin with a stoichiometry of 4:1 (Re:avidin). Again, all the complexes displayed higher emission intensity and longer emission lifetimes upon binding to avidin

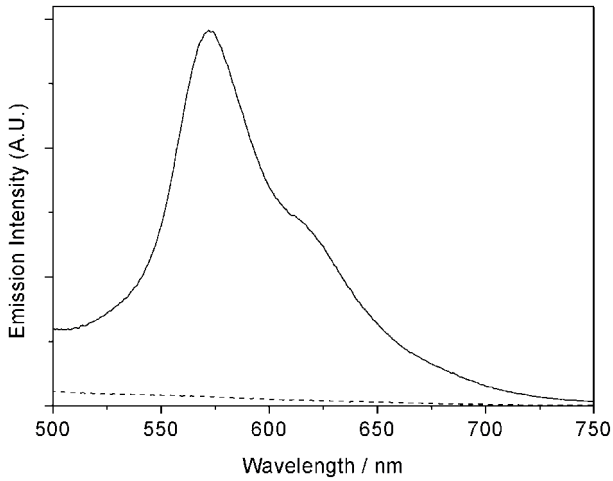


Fig. 4. Emission spectra of [Re(dppz)(CO)$_3$(py-4-CH$_2$-NH-biotin)](PF$_6$) in the absence (---) and presence (—) of avidin in degassed potassium phosphate buffer. Reproduced from Reference 37, with permission of the American Chemical Society.

(Table 3). These observations were a consequence of the increased hydrophobicity and rigidity of the environment of the rhenium(I) polypyridine complexes. It is important to note that the more hydrophobic Me_4-phen and 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, the emission quantum yield enhancement factors from aqueous buffer to CH_2Cl_2 were the highest for [Re(Me_2-Ph_2-phen)$(CO)_3$(py-4-CH_2-NH-biotin)](PF_6) and [Re(Me_2-Ph_2-phen)$(CO)_3$(py-3-CO-NH-en-NH-biotin)](PF_6) (20 and 15.8, respectively) among all of the complexes. These findings were in line with the highest emission intensity amplification factors for these two complexes (2.98 and 2.93) upon binding to avidin (Table 3). Concerning the effects of the chain length of the spacer-arms, the py-3-CO-NH-en-NH-biotin and py-4-CH_2-NH-cap-NH-biotin complexes exhibited less significant emission intensity enhancement (Table 3). It is likely that these eight complexes remained more exposed to the polar buffer after binding to the protein compared to their py-4-CH_2-NH-biotin analogues. Thus, the increase in hydrophobicity associated with protein-binding was less substantial. Also, the effects of increased rigidity resulting from avidin-binding were smaller for these complexes due to their longer and more flexible spacer-arms.

The first dissociation constants K_d of the rhenium–avidin adducts were estimated from the on-rates and off-rates of the rhenium–avidin adducts from kinetic experi-

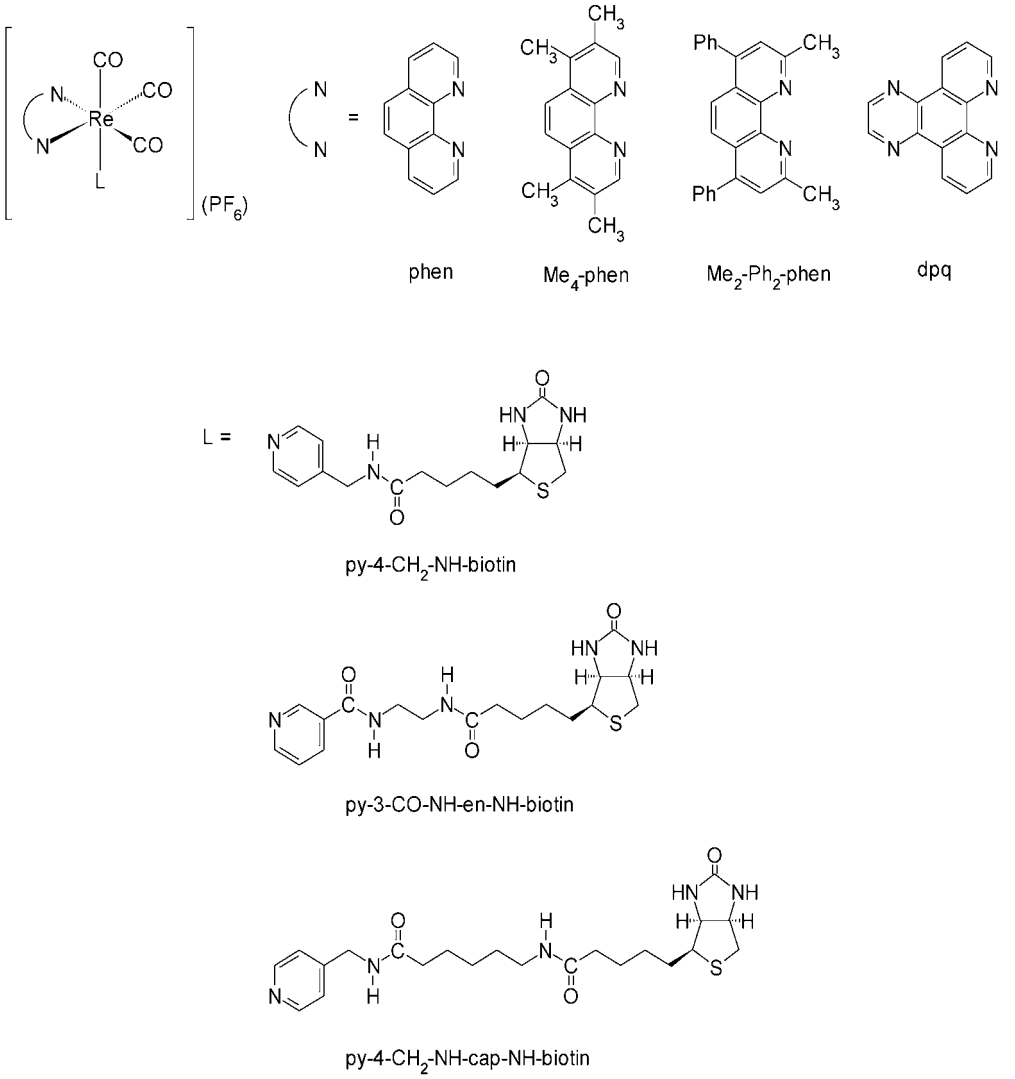


Fig. 5. Structures of rhenium(I) polypyridine biotin complexes. Reproduced from Reference 48, with permission of the American Chemical Society.

Table 3. Photophysical data of selected rhenium(I) polypyridine biotin complexes. Reproduced from Reference 48, with permission of The American Chemical Society

Complex	medium	λ_{em}/nm[a]	τ_o/μs[a]	Φ_{em}[a]	I (τ/μs)[b,c]	I (τ/μs)[b,d]	I (τ/μs)[b,e]
[Re(phen)(CO)$_3$(py-3-CO-NH-en-NH-biotin)](PF$_6$)	CH_2Cl_2	536	2.92	0.30	1.00	1.25	1.01
	CH_3CN	550	1.75	0.13	(0.69)	(1.01)	(0.68)
	Buffer[f]	550	1.07	0.12			
[Re(phen)(CO)$_3$(py-4-CH$_2$-NH-cap-NH-biotin)](PF$_6$)	CH_2Cl_2	540	2.85	0.49	1.00	1.24	0.96
	CH_3CN	554	1.46	0.16	(0.55)	(0.73)	(0.56)
	Buffer[f]	554	0.84	0.096			
[Re(Me$_4$-phen)(CO)$_3$(py-3-CO-NH-en-NH-biotin)](PF$_6$)	CH_2Cl_2	516	13.54	0.19	1.00	1.26	1.01
	CH_3CN	522	9.90	0.051	(1.50)	(3.65)	(1.53)
	Buffer[f]	518	14.06	0.041			
[Re(Me$_4$-phen)(CO)$_3$(py-4-CH$_2$-NH-cap-NH-biotin)](PF$_6$)	CH_2Cl_2	518	13.28	0.27	1.00	1.90	0.96
	CH_3CN	522	8.54	0.12	(1.31)	(2.00)	(1.36)
	Buffer[f]	522	11.62	0.078			
[Re(Me$_2$-Ph$_2$-phen)(CO)$_3$(py-3-CO-NH-en-NH-biotin)](PF$_6$)	CH_2Cl_2	542	15.48	0.19	1.00	2.93	1.01
	CH_3CN	550	9.41	0.13	(2.09)	(3.25)	(2.04)
	Buffer[f]	554	7.85	0.012			
[Re(Me$_2$-Ph$_2$-phen)(CO)$_3$(py-4-CH$_2$-NH-cap-NH-biotin)](PF$_6$)	CH_2Cl_2	542	11.65	0.18	1.00	2.27	0.93
	CH_3CN	552	6.96	0.053	(1.61)	(2.38)	(1.64)
	Buffer[f]	558	5.08	0.028			
[Re(dpq)(CO)$_3$(py-4-CH$_2$-NH-biotin)](PF$_6$)	CH_2Cl_2	548	1.12	0.28	1.00	1.75	0.99
	CH_3CN	568	0.40	0.086	(0.11)	(0.21)	(0.11)
	Buffer[f]	572	0.11	0.034			
[Re(dpq)(CO)$_3$(py-3-CO-NH-en-NH-biotin)](PF$_6$)	CH_2Cl_2	544	1.24	0.15	1.00	1.20	1.03
	CH_3CN	562	0.52	0.065	(0.18)	(0.26)	(0.19)
	Buffer[f]	566	0.21	0.033			
[Re(dpq)(CO)$_3$(py-4-CH$_2$-NH-cap-NH-biotin)](PF$_6$)	CH_2Cl_2	548	1.10	0.24	1.00	1.15	0.98
	CH_3CN	568	0.40	0.054	(0.12)	(0.16)	(0.12)
	Buffer[f]	572	0.13	0.032			

[a] In degassed solvents. [b] Relative emission intensities in aerated 50 mM potassium phosphate buffer pH 7.4. [c] [Re] = 15.2 μM, [avidin] = 0 μM, [unmodified biotin] = 0 μM. [d] [Re] = 15.2 μM, [avidin] = 3.8 μM, [unmodified biotin] = 0 μM. [e] [Re] = 15.2 μM, [avidin] = 3.8 μM, [unmodified biotin] = 380.0 μM. [f] 50 mM potassium phosphate buffer 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.

ments.[5] The K_d values ranged from approximately 5.5 × 10^{-11} to 3.4 × 10^{-9} M, which were about 4 to 6 orders of magnitude larger than that of the native biotin–avidin system (K_d = approximately 10^{-15} M).[1–2] This difference was due to the bulkiness of the rhenium(I) polypyridine units. 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, indicating the importance of the linkers on alleviating the steric hindrance between the complexes and the protein.[49–51]

Iridium(III) Polypyridine Biotin Complexes

The interesting emission properties of iridium(III) polypyridine complexes,[32,52] and our recent interest in using these complexes as biological labelling reagents[17,19–21,23] have prompted us to design new luminescent cyclometallated iridium(III) polypyridine biotin complexes [Ir(N-C)$_2$(bpy-en-biotin)](PF$_6$) (HN-C = Hppy, Hmppy, Hppz, Hmppz, Hbzq, Hpq) (Fig. 6).[53]

Upon excitation, all the complexes showed intense and long-lived orange to greenish-yellow luminescence in fluid solutions under ambient conditions and in low-temperature glass (Table 4). The emission spectra of complex [Ir(ppy)$_2$(bpy-en-biotin)](PF$_6$) in CH_3CN at 298 K and in alcohol glass at 77 K are shown in Fig. 7. In fluid solutions at room temperature, the complexes emitted at approximately 551 – 589 nm and the emission intensities and lifetimes decreased with increasing solvent polarity. As expected, in rigid glass at low temperature, the emission lifetimes were much longer. The emission of the complexes

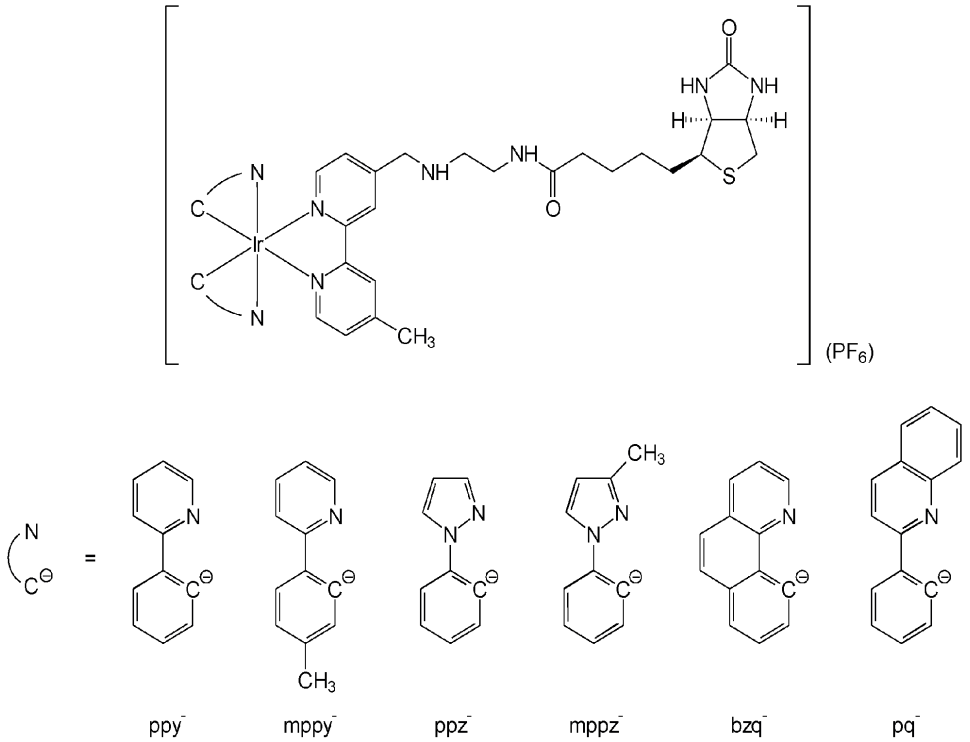


Fig. 6. Structures of iridium(III) polypyridine biotin complexes. Reproduced from Reference 53, with permission of the American Chemical Society.

was assigned to an ^{3}MLCT ($d\pi(Ir) \rightarrow \pi^*$(bpy-en-biotin)) excited state.[32,52] This assignment was supported by the observations that the complex $[Ir(ppy)_2(bpy\text{-}en\text{-}biotin)](PF_6)$ emitted at higher energy than the model complex $[Ir(ppy)_2(bpy)](PF_6)$ (for example, λ_{em} = 588 nm in both CH_3CN and MeOH at 298 K) because the electron-donating methyl and aminomethyl substituents of bpy-en-biotin destabilised the π^* orbitals of this diimine ligand, and thus led to a higher ^{3}MLCT ($d\pi(Ir) \rightarrow \pi^*$(N-N)) emission energy for the biotin complex. The ^{3}MLCT assignment was further supported by the findings that the emission of $[Ir(ppy)_2(bpy\text{-}en\text{-}biotin)](PF_6)$ and $[Ir(ppz)_2(bpy\text{-}en\text{-}biotin)](PF_6)$ occurred at slightly higher energy than $[Ir(mppy)_2(bpy\text{-}en\text{-}biotin)](PF_6)$ and $[Ir(mppz)_2(bpy\text{-}en\text{-}biotin)](PF_6)$, respectively (Table 4). It is likely that the electron-donating methyl groups of the cyclometallating ligands $mppy^-$ and $mppz^-$ rendered the iridium(III) centres more electron rich, and thus stabilised the ^{3}MLCT ($d\pi(Ir) \rightarrow \pi^*$(bpy-en-biotin)) emissive states of these two complexes compared to those of $[Ir(ppy)_2(bpy\text{-}en\text{-}biotin)](PF_6)$ and $[Ir(ppz)_2(bpy\text{-}en\text{-}biotin)](PF_6)$, respectively. The complex $[Ir(pq)_2(bpy\text{-}en\text{-}biotin)](PF_6)$ showed structured emission spectra and very long emission lifetimes (τ_o = approximately 2 – 3 μs) in fluid solutions at 298 K, suggesting of substantial ^{3}IL ($\pi \rightarrow \pi^*$) (pq^-) character in its emissive state.

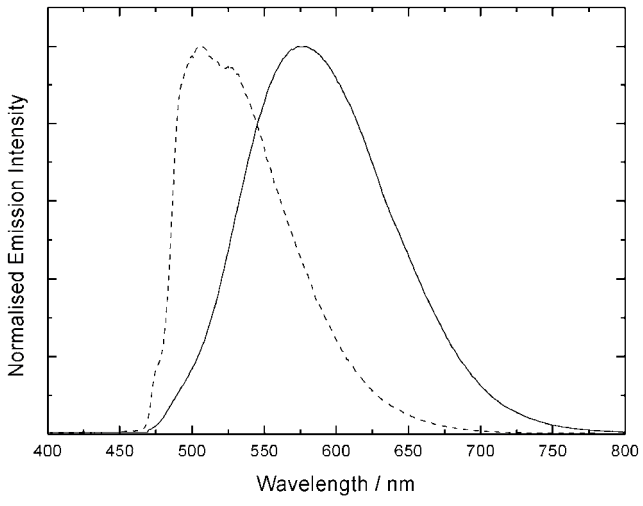


Fig. 7. Emission spectra of $[Ir(ppy)_2(bpy\text{-}en\text{-}biotin)](PF_6)$ in CH_3CN at 298 K (—) and in EtOH/MeOH (4:1, v/v) at 77 K (---). Reproduced from Reference 53, with permission of the American Chemical Society.

Table 4. Photophysical data of $[Ir(N\text{-}C)_2(bpy\text{-}en\text{-}biotin)](PF_6)$. Reproduced from Reference 53, with permission of The American Chemical Society

Complex	medium (*T*/K)	λ_{em}/nm	τ_o/μs	Φ_{em}	I (τ/ns)[a,b]	I (τ/ns)[a,c]	I (τ/ns)[a,d]
$[Ir(ppy)_2(bpy\text{-}en\text{-}biotin)](PF_6)$	CH_3CN (298)	576	0.48	0.16	1.00	1.85	1.05
	MeOH (298)	577	0.36	0.11	(90)	(140)	(82)
	H_2O/DMSO[e] (298)	584	0.21	0.035			
	Glass[f] (77)	473 sh, 506, 537 sh	4.74				
$[Ir(mppy)_2(bpy\text{-}en\text{-}biotin)](PF_6)$	CH_3CN (298)	587	0.35	0.081	1.00	1.88	0.91
	MeOH (298)	580	0.24	0.065	(83)	(148)	(87)
	H_2O/DMSO[e] (298)	589	0.17	0.053			
	Glass[f] (77)	475 sh, 513, 542 sh	4.57				
$[Ir(ppz)_2(bpy\text{-}en\text{-}biotin)](PF_6)$	CH_3CN (298)	560	0.92	0.19	1.00	1.48	0.99
	MeOH (298)	561	0.62	0.11	(71)	(232)	(79)
	H_2O/DMSO[e] (298)	567	0.28	0.12			
	Glass[f] (77)	496, 527 sh	5.47				
$[Ir(mppz)_2(bpy\text{-}en\text{-}biotin)](PF_6)$	CH_3CN (298)	574	0.39	0.093	1.00	1.95	1.04
	MeOH (298)	577	0.28	0.079	(59)	(166)	(65)
	H_2O/DMSO[e] (298)	576	0.17	0.058			
	Glass[f] (77)	509, 538 sh	4.38				
$[Ir(bzq)_2(bpy\text{-}en\text{-}biotin)](PF_6)$	CH_3CN (298)	577	0.47	0.12	1.00	1.81	0.97
	MeOH (298)	580	0.29	0.054	(73)	(147)	(80)
	H_2O/DMSO[e] (298)	583	0.17	0.054			
	Glass[f] (77)	502 (max), 541, 583 sh	41.36 (33%), 5.12 (67%)				
$[Ir(pq)_2(bpy\text{-}en\text{-}biotin)](PF_6)$	CH_3CN (298)	554, 605 sh	2.95	0.37	1.00	3.29	1.04
	MeOH (298)	551, 605 sh	2.90	0.37	(1533)	(2343)	(1529)
	H_2O/DMSO[e] (298)	559, 604 sh	1.88	0.24			
	Glass[f] (77)	540 (max), 582, 632 sh	4.78				

[a] Relative emission intensities in aerated 50 mM potassium phosphate buffer pH 7.4. [b] [Ir] = 15.2 μM, [avidin] = 0 μM, [unmodified biotin] = 0 μM. [c] [Ir] = 15.2 μM, [avidin] = 3.8 μM, [unmodified biotin] = 0 μM. [d] [Ir] = 15.2 μM, [avidin] = 3.8 μM, [unmodified biotin] = 380.0 μM. [e] H_2O/DMSO (1:1, v/v). [f] EtOH/MeOH (4:1, v/v).

Addition of the complexes to a mixture of avidin and HABA resulted in a decrease in absorbance at 500 nm, suggesting the binding of the biotin moieties of the iridium(III) complexes to avidin. The plots of $-\Delta Abs_{500\,nm}$ vs. [Ir]:[avidin] for the complexes showed that the equivalence points occurred at [Ir]:[avidin] = from approximately 4.1 to 5.2. Assuming that avidin can only specifically bind the complexes at the four biotin-binding sites, the occurrence of the equivalence points at [Ir]:[avidin] > 4 suggested that the binding of these iridium(III) biotin complexes to avidin was not substantially stronger than that of HABA.

Luminescence titrations using the complexes as titrants showed that all the complexes display enhanced emission intensities upon binding avidin. At the equivalence points, the emission intensities and lifetimes of the complexes were increased by factors of approximately 1.5 to 3.3 (Table 4). The observed enhancement was associated with the hydrophobicity of the biotin-binding sites of the avidin molecule, given that the emission quantum yields and lifetimes of the complexes were higher and longer in more non-polar solvents (Table 4). It is noteworthy that $[Ir(pq)_2(bpy\text{-}en\text{-}biotin)](PF_6)$, being more hydrophobic than the other complexes, exhibited a higher degree of emission enhancement after binding to avidin. Also, unlike the other complexes, beyond the equivalence point, the emission titration curve of this complex was not parallel to those of the two control solutions, and the emission intensity remained essentially constant (Fig. 8). We believe that these observations were related to the high hydrophobicity of this complex. The possibility of non-specific interactions between the avidin-bound complex and excess free complex could not be excluded.

The K_d values of the complexes formed between avidin and the complexes varied from approximately 2.0×10^{-10} to 2.0×10^{-8} M, which are about five to seven orders of magnitudes larger than that of the native biotin-avidin system.[1–2] It appears that the lack of a spacer-arm between the iridium(III) luminophore and the biotin moiety led to the diminished binding strength.

The avidin-induced emission enhancement has been

exploited in the design of a simple homogeneous assay for biotin. The assay was based on the competition between complexes and unmodified biotin on binding to avidin. In the assays, avidin was added to a solution of the iridium(III) polypyridine biotin complex and the biotin analyte. The solution was incubated at room temperature for one hour and its emission intensity was then measured. A lower biotin analyte concentration was expected to result in a higher degree of binding of the iridium(III) polypyridine biotin complex to avidin, and thus a higher emission intensity. The results for the assay using $[Ir(ppy)_2(bpy\text{-}en\text{-}biotin)](PF_6)$ are shown in Fig. 9. The concentration of biotin that could be determined by this assay was between approximately $1 \times 10^{-7.5}$ and $1 \times 10^{-5.5}$ M. The lowest concentration of biotin analyte that gave a meaningful signal (approximately $1 \times 10^{-7.5}$ M) was about 1.5 order of magnitude lower than that of a heterogeneous competitive biotin assay we reported previously (approximately 1×10^{-6} M)[20] but comparable to that of a competitive assay based on the change of electrode response of copper enhanced by a thiourea–biotin compound (approximately $1 \times 10^{-7.54}$ M).[54]

Ruthenium(II) Polypyridine Biotin Complexes

Since the ^{3}MLCT emission of ruthenium(II) polypyridine complexes is very well characterised,[32] we are interested in the design of luminescent ruthenium(II)-based biological probes. Two ruthenium(II) polypyridine biotin complexes $[Ru(bpy)_2(N\text{-}N)](PF_6)_2$ (N-N = L1, L2) (Fig. 10) have been synthesised and characterised.[55] The complexes exhibited intense and long-lived orange-red luminescence upon irradiation in fluid solutions at 298 K and in alcohol glass at 77 K. The emission maxima of the complexes occurred at approximately 629 – 632 nm in CH_3CN and at approximately 633 – 636 nm in MeOH at 298 K (Ta-

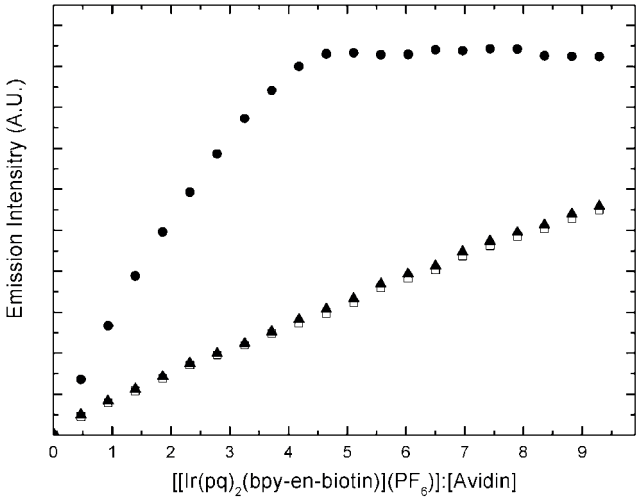


Fig. 8. Luminescence titration curves for the titrations of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲), and (iii) a blank phosphate buffer solution (□) with $[Ir(pq)_2(bpy\text{-}en\text{-}biotin)](PF_6)$. Reproduced from Reference 53, with permission of the American Chemical Society.

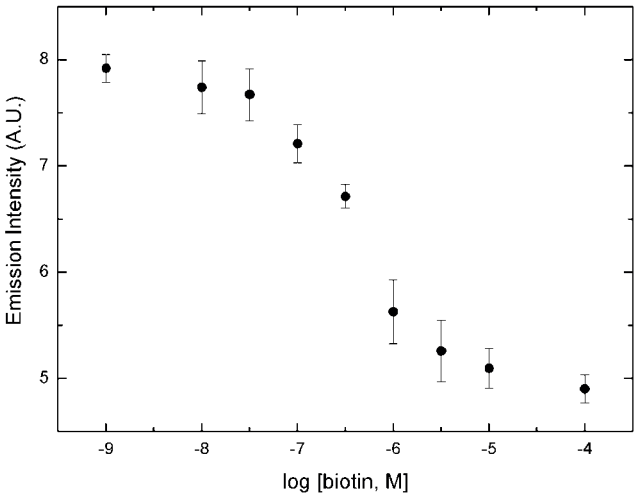
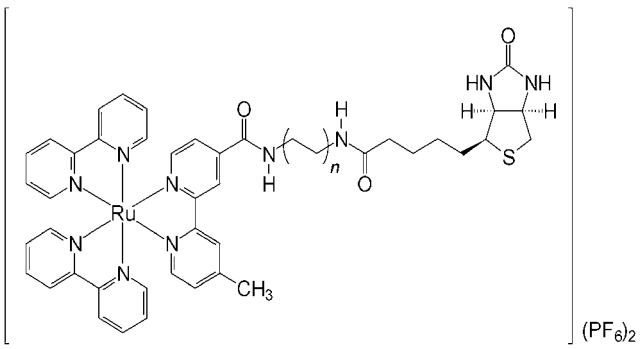


Fig. 9. Homogeneous competitive assay for biotin using $[Ir(ppy)_2(bpy\text{-}en\text{-}biotin)](PF_6)$ and avidin. The emission intensity of the supernatant is an average of triplicate experiments ± 1 standard deviation. Reproduced from Reference 53, with permission of the American Chemical Society.



$[Ru(bpy)_2(L1)](PF_6)_2$: n = 1
$[Ru(bpy)_2(L2)](PF_6)_2$: n = 3

Fig. 10. Structures of ruthenium(II) polypyridine biotin complexes. Reproduced from Reference 55, with permission of the American Chemical Society.

Table 5. Photophysical data of $[Ru(bpy)_2(L1)](PF_6)$ and $[Ru(bpy)_2(L2)](PF_6)$. Reproduced from Reference 55, with permission of The American Chemical Society

Complex	medium (*T*/K)	λ_{em}/nm	τ_o/μs	Φ_{em}
$[Ru(bpy)_2(L1)](PF_6)$	CH_3CN (298)	632	1.39	0.065
	MeOH (298)	636	1.09	0.055
	Glass[a] (77)	595, 651 sh	5.82	
$[Ru(bpy)_2(L2)](PF_6)$	CH_3CN (298)	629	1.45	0.072
	MeOH (298)	633	1.16	0.069
	Glass[a] (77)	595, 648 sh	5.64	

[a] EtOH/MeOH (4:1, v/v).

ble 5). The emission is likely to originate from an excited state of ^{3}MLCT ($d\pi(Ru) \rightarrow \pi^*$(diimine)) character. Since the emission energy of these complexes is slightly lower than that of $[Ru(bpy)_3]^{2+}$ (λ_{em} = approximately 621 nm in CH_3CN and 616 nm in MeOH at 298 K),[56] 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. These two complexes also displayed intense and long-lived ^{3}MLCT ($d\pi(Ru) \rightarrow \pi^*$(L1 or L2)) emission in alcohol glass at 77 K.

The plots of $-\Delta Abs_{500\ nm}$ vs. [Ru]:[avidin] for both complexes in the HABA assays showed that the equivalence points occurred at [Ru]:[avidin] = approximately 4. These observations suggest that these ruthenium(II) biotin complexes bind to avidin with the same stoichiometry as native biotin.

The luminescence titration results showed that both complexes displayed enhanced emission intensities in the presence of avidin. At [Ru]:[avidin] = 4, both the emission intensities and lifetimes of the complexes increased by factors of approximately 1.2 to 1.4 fold (Fig. 11 and Table 6). In order to develop an assay for avidin, it is desirable to maximise the difference in emission between the free and avidin-bound forms of both complexes. Unfortunately, the intrinsic increase in emission intensity of both complexes upon binding to avidin was rather 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 oxidative quenching mechanism. In view of the highly positively-charged avidin molecule (pI = about 10),[1–2] less effective emission quenching of the avidin-bound ruthenium(II) biotin complexes by the cationic MV^{2+} ions 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.

The luminescence of both complexes was quenched by MV^{2+} in the absence of avidin, with Stern-Volmer constants, K_{SV}, of about 137 and 172 M^{-1} for the L1 and L2 complexes, respectively. In the presence of avidin, the emission quenching became less efficient (K_{SV} = about 49

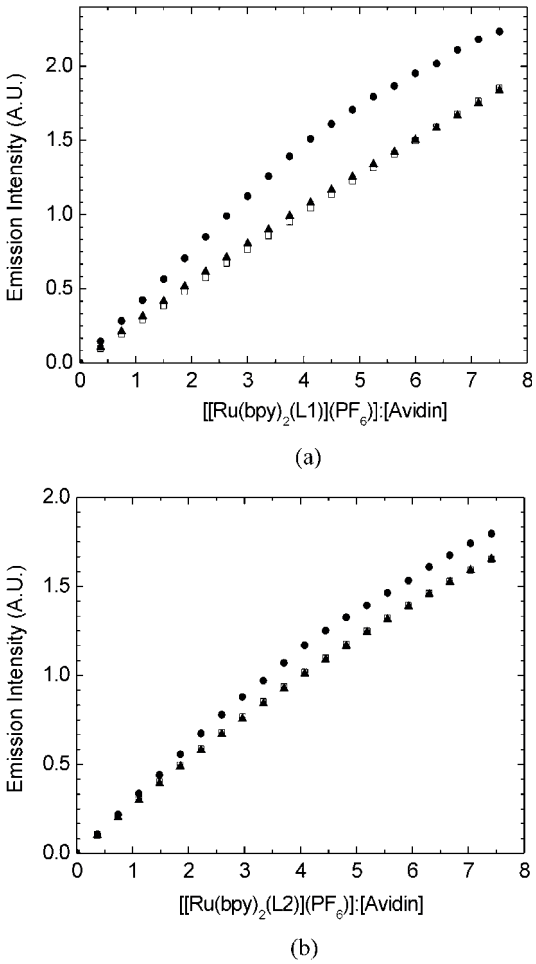


Fig. 11. Luminescence titration curves for the titrations of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲), and (iii) a blank phosphate buffer solution (□) with (a) $[Ru(bpy)_2(L1)](PF_6)_2$ and (b) $[Ru(bpy)_2(L2)](PF_6)_2$. Reproduced from Reference 55, with permission of the American Chemical Society.

Table 6. Relative emission intensities and emission lifetimes of $[Ru(bpy)_2(L1)](PF_6)$ and $[Ru(bpy)_2(L2)](PF_6)$ in the absence and presence of avidin (and excess biotin) with various concentrations of methyl viologen (MV^{2+}) and KCl.[a] Reproduced from Reference 55, with permission of The American Chemical Society

	$[MV^{2+}]$ = 0 M, [KCl] = 0 M			$[MV^{2+}]$ = 15.0 mM, [KCl] = 0 M			$[MV^{2+}]$ = 15.0 mM, [KCl] = 2.0 M		
Complex	$I(\tau/ns)^b$	$I(\tau/ns)^c$	$I(\tau/ns)^d$	$I(\tau/ns)^b$	$I(\tau/ns)^c$	$I(\tau/ns)^d$	$I(\tau/ns)^b$	$I(\tau/ns)^c$	$I(\tau/ns)^d$
$[Ru(bpy)_2(L1)](PF_6)$	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)
$[Ru(bpy)_2(L2)](PF_6)$	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.

and 67 M^{-1} for the L1 and L2 complexes, respectively). It is likely that the decrease of K_{SV} originated primarily from the shielding of the complexes by the protein matrix and, to a certain extent, by the immobilisation of the complexes by the protein, rendering the quenching by MV^{2+} more difficult to occur. Although coulombic repulsion existed between the positively-charged avidin molecule and dicationic MV^{2+} ion, it did not seem to play a very important role in the diminished quenching efficiency. The reason was that the K_{SV} constants of both complexes in the presence of avidin showed a relatively small increase upon changing from low-salt (LS) ([KCl] = 0 M) to high-salt (HS) ([KCl] = 2.0 M) conditions (K_{SV}(HS)/K_{SV}(LS) = 1.20 and 1.48 for the avidin-bound L1 and L2 complexes, respectively). These increases of K_{SV} were less substantial compared to those of the free L1 and L2 complexes (K_{SV}(HS)/K_{SV}(LS) = 2.10 and 1.82, respectively) and the L1 and L2 complexes in the presence of biotin-blocked avidin (K_{SV}(HS)/K_{SV}(LS) = 2.16 and 1.78, respectively). These results indicated that electrostatic repulsion between the positively-charged free complexes and MV^{2+} ion was significantly reduced under HS conditions.[57] On the basis of these interesting findings, the emission titration experiments were repeated with MV^{2+} being a quencher present in the bulk solution. Under LS conditions, in the presence of MV^{2+}, both complexes displayed a more significant enhancement in emission intensities (about 2.4 and 2.0 fold for the L1 and L2 complexes, respectively, at [Ru]:[Avidin] = 4 (Table 6)) upon binding to avidin. The emission lifetimes were also extended upon the binding (Table 6), and the elongation factors showed improvement (about 2.5 and 2.1 for the L1 and L2 complexes, respectively) compared to the case in which the quencher was absent (about 1.4 and 1.2 for the L1 and L2 complexes, respectively) (Table 6). 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] =

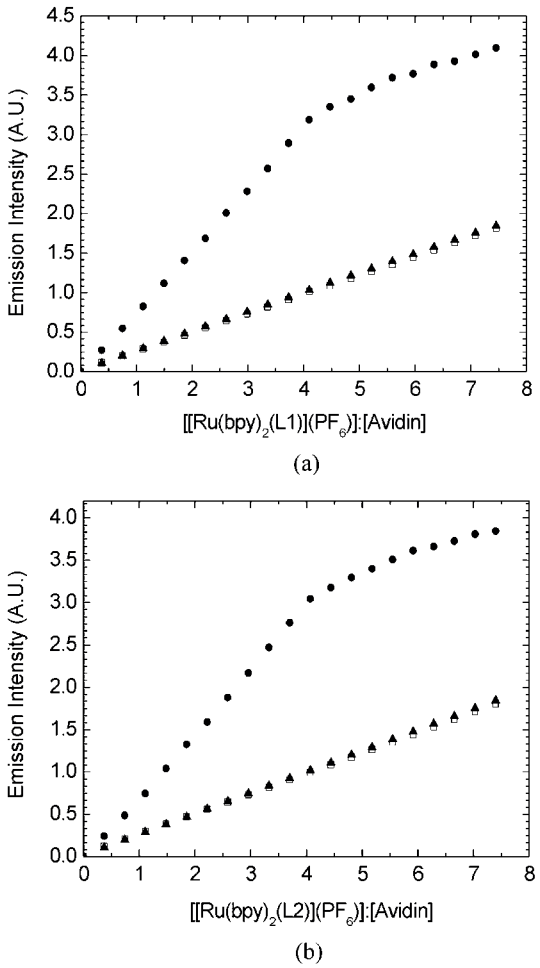


Fig. 12. Luminescence titration curves for the titrations of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲), and (iii) phosphate buffer (□), in the presence of 15.0 mM MV^{2+} and 2.0 M KCl, with (a) $[Ru(bpy)_2(L1)](PF_6)_2$ and (b) $[Ru(bpy)_2(L2)](PF_6)_2$. Reproduced from Reference 55, with permission of the American Chemical Society.

4 were anticipated. The titration curves for the L1 and L2 complexes in the presence of MV^{2+} under HS conditions are shown in Fig. 12, respectively. Our results clearly showed that the emission intensities of the L1 and L2 complexes were significantly enhanced by about 3.2 and 3.0 fold (Table 6), respectively, which were larger than those in the previous two cases. Meanwhile, the emission lifetime elongation factors (about 2.8 and 2.5 for the L1 and L2 complexes, respectively) were also the most significant among all three conditions (Table 6).

CONCLUSION

This review article summarises our recent work on the design of luminescent transition metal biotin complexes. We believe that the avidin-induced emission enhancement is not limited to rhenium(I), iridium(III) and ruthenium(II) polypyridine complexes, but is common to other transition metal complexes that show environment-sensitive emission with large Stokes' shifts. From the results, we found that more hydrophobic complexes can give rise to stronger binding to the protein, but too high hydrophobicity will substantially lower the solubility of complexes in aqueous solution. Whilst a longer spacer-arm between the luminophore can enhance the binding affinity, it renders the probe more exposed to the bulk solution after the binding and thus lowers the enhancement factors of emission intensity (I/I_o) and lifetimes (τ/τ_o). These remarks will be taken into consideration in the design of related luminescent probes for avidin.

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REFERENCES

1. Wilchek, M.; Bayer, E. A. *Anal. Biochem*. **1988**, *171*, 1.
2. Wilchek, M.; Bayer, E. A. *Methods Enzymol*. **1990**, *184*, 5.
3. Kricka, L. J. *Nonisotopic DNA Probe Techniques*; Academic Press: San Diego, 1992.
4. Gruber, H. J.; Marek, M.; Schindler, H.; Kaiser, K. *Bioconjugate Chem*. **1997**, *8*, 552.
5. Marek, M.; Kaiser, K.; Gruber, H. J. *Bioconjugate Chem*. **1997**, *8*, 560.
6. Kada, G.; Falk, H.; Gruber, H. J. *Biochim. Biophys. Acta* **1999**, *1427*, 33.
7. Kada, G.; Kaiser, K.; Falk, H.; Gruber, H. J. *Biochim. Biophys. Acta* **1999**, *1427*, 44.
8. Gruber, H. J.; Hahn, C. D.; Kada, G.; Riener, C. K.; Harms, G. S.; Ahrer, W.; Dax, T. G.; Knaus, H.-G. *Bioconjugate Chem*. **2000**, *11*, 696.
9. Gray, H. B.; Winkler, J. R. *Annu. Rev. Biochem*. **1996**, *65*, 537.
10. Erkkila, K. E.; Odom, D. T.; Barton, J. K. *Chem. Rev.* **1999**, *99*, 2777.
11. Cohen, S. M.; Lippard, S. J. *Prog. Nucleic Acid Res*. **2001**, *67*, 93.
12. Beilstein, A. E.; Tierney, M. T.; Grinstaff, M. W. *Comments Inorg. Chem*. **2000**, *22*, 105.
13. Terpetschnig, E.; Szmacinski, H.; Lakowicz, J. R. *Methods Enzymol*. **1997**, *278*, 295.
14. Perrin, D. M.; Mazumder, A.; Sigman, D. S. *Prog. Nucleic Acid Res*. **1996**, *52*, 123.
15. Winkler, J. R.; Gray, H. B. *Chem. Rev.* **1992**, *92*, 369.
16. Lo, K. K.-W.; Ng, D. C.-M.; Hui, W.-K.; Cheung, K.-K. *J. Chem. Soc., Dalton Trans*. **2001**, 2634.
17. Lo, K. K.-W.; Ng, D. C.-M.; Chung, C.-K. *Organometallics* **2001**, *20*, 4999.
18. Lo, K. K.-W.; Hui, W.-K.; Ng, D. C.-M.; Cheung, K.-K. *Inorg. Chem*. **2002**, *41*, 40.
19. Lo, K. K.-W.; Chung, C.-K.; Ng, D. C.-M.; Zhu, N. *New. J. Chem*. **2002**, *26*, 81.
20. Lo, K. K.-W.; Chung, C.-K.; Zhu, N. *Chem. Eur. J*. **2003**, *9*, 475.
21. Lo, K. K.-W.; Chung, C.-K.; Lee, T. K.-M.; Lui, L.-H.; Tsang, K. H.-K.; Zhu, N. *Inorg. Chem*. **2003**, 6886.
22. Lo, K. K.-W.; Li, C.-K.; Lau, K.-W.; Zhu, N. *Dalton Trans*. **2003**, 4682.
23. Lo, K. K.-W.; Chan, J. S.-W.; Chung, C.-K.; Tsang, V. W.-H.; Zhu, N. *Inorg. Chim. Acta* **2004**, *357*, 3109.
24. Arterburn, J. B.; Rao, K. V.; Goreham, D. M.; Valenzuela, M. V.; Holguin, M. S. *Organometallics* **2000**, *19*, 1789.
25. Zhou, X.; Shearer, J.; Rokita, S. E. *J. Am. Chem. Soc*. **2000**,

12, 9046.
26. Salmain, M.; Fischer-Durand, N.; Cavalier, L.; Rudolf, B.; Zakrzewski, J.; Jaouen, G. *Bioconjugate Chem*. **2002**, *13*, 693.
27. Rudolf, B.; Makowska, M.; Domagala, A.; Rybarczyk-Pirek, A.; Zakrzewski, J. *J. Organomet. Chem*. **2003**, *668*, 95.
28. Haddour, N.; Gondran, C.; Cosnier, S. *Chem. Commun*. **2004**, 324.
29. Hofmeier, H.; Pahnke, J.; Weidl, C. H.; Schubert, U. S. *Biomacromolecules* **2004**, *5*, 2055.
30. Jhaveri, S. D.; Trammell, S. A.; Lowy, D. A.; Tender, L. M. *J. Am. Chem. Soc*. **2004**, *126*, 6540.
31. Lo, K. K.-W.; Hui, W.-K.; Ng, D. C.-M. *J. Am. Chem. Soc*. **2002**, *124*, 9344.
32. Kalyanasundaram, K. *Photochemistry of Polypyridine and Porphyrin Complexes*; Academic Press: San Diego, CA, **1992**.
33. Lee, A. J. *Chem. Rev*. **1987**, *4*, 711.
34. Vlček, Jr., A. *Coordin. Chem. Rev*. **2002**, *230*, 225.
35. Green, N. M. *Adv. Protein Chem*. **1975**, *29*, 85.
36. Hirschfeld, T. *Appl. Opt*. **1976**, *15*, 3135.
37. Lo, K. K.-W.; Tsang, K. H.-K. *Organometallics* **2004**, *23*, 3062.
38. Metcalfe, C.; Thomas, J. A. *Chem. Soc. Rev*. **2003**, *32*, 215.
39. Hartshorn, R. M.; Barton, J. K. *J. Am Chem. Soc*. **1992**, *114*, 5919.
40. Holmlin, R. E.; Yao, J. A.; Barton, J. K. *Inorg. Chem*. **1999**, *38*, 174.
41. Lincoln, P.; Broo, A.; Nordén, B. *J. Am. Chem. Soc*. **1996**, *118*, 2644.
42. Barker, K. D.; Benoit, B. R.; Bordelon, J. A.; Davis, R. J.; Delmas, A. S.; Mytykh, O. V.; Petty, J. T.; Wheeler, J. F.; Kane-Maguire, N. A. P. *Inorg. Chim. Acta* **2001**, *322*, 74.
43. Arounaguiri, S.; Maiya, B. G. *Inorg. Chem*. **1996**, *35*, 4267.
44. Herebian, D.; Sheldrick, W. S. *J. Chem. Soc., Dalton Trans*. **2002**, 966.
45. Stoeffler, H. D.; Thornton, N. B.; Temkin, S. L.; Schanze, K. S. *J. Am. Chem. Soc*. **1995**, *117*, 7119.
46. Yam, V. W.-W.; Lo, K. K.-W.; Cheung, K.-K., Kong, R. Y.-C., *J. Chem. Soc., Chem. Commun*. **1995**, 1191.
47. Yam, V. W.-W.; Lo, K. K.-W.; Cheung, K.-K.; Kong, R. Y.-C. *J. Chem. Soc., Dalton Trans*. **1997**, 2067.
48. Lo, K. K.-W.; Hui, W.-K. *Inorg. Chem*. **2005**, *44*, 1992.
49. Kam, C.-M.; Abuelyaman, A. S.; Li, Z.; Hudig, D.; Powers, J. C. *Bioconjugate Chem*. **1993**, *4*, 560.
50. Basak, A.; Jean, F.; Dugas, H.; Lazure, C. *Bioconjugate Chem*. **1994,** *5*, 301.
51. Sugawara, K.; Hoshi, S.; Akatsuka, K.; Tanaka, S.; Nakamura, H. *Anal. Sci*. **1997**, *13*, 677.
52. Dixon, I. M.; Collin, J.-P.; Sauvage, J.-P.; Flamigni, L.; Encinas, S.; Barigelletti, F. *Chem. Soc. Rev*. **2000,** *29*, 385.
53. Lo, K. K.-W.; Chan, J. S.-W.; Lui, L.-H.; Chung, C.-K. *Organometallics* **2004**, *23*, 3108.
54. Kuramitz, H.; Natsui, J.; Tanaka, S.; Hasebe, K. *Electroanalysis* **2000**, *12*, 588.
55. Lo, K. K.-W.; Lee, T. K.-M. *Inorg. Chem*. **2004**, 5275.
56. Carvalho, I. M. M. D.; Moreira, I. D. S.; Gehlen, M. H. *Inorg. Chem*. **2003**, *42*, 1525.
57. Gaines, G. L., Jr. *J. Phys. Chem*. **1979**, *83*, 3088.