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Review

Luminescent transition metal complex biotin conjugates

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Contents

Abstract

The avidin–biotin system is a classic protein–ligand model and is widely employed in bioanalytical applications. Although biotin has been linked to various reporter units, such as fluorescent organic compounds, luminescent transition metal-based biotin conjugates have not been explored. We have recently incorporated biotin into a series of luminescent rhenium(I), iridium(III) and ruthenium(II) polypyridine complexes to form new sensors for avidin. The most important observations were the enhanced emission intensities and extended lifetimes of these luminescent transition metal biotin complex conjugates when they bound to the protein. These changes resulted from the increased hydrophobicity and rigidity of the local surroundings of the probes after the binding event. The effects of the polypyridine and cyclometallating ligands and spacer-arms between the luminophores and biotin on protein-binding were examined. On the basis of the characteristic photophysical properties of these luminescent transition metal biotin complexes, new assays for avidin and biotin were developed.

Keywords: Avidin–biotin system; Protein–ligand model; Luminescent

1. Introduction

Avidin is a tetrameric glycoprotein (MW = 68 kDa) with four identical subunits, each containing 128 amino acids. It can bind up to four biotin molecules (Vitamin H) with an exceptionally high affinity (first dissociation constant, K_d = ca. 10^{-15} M) [1,2]. The X-ray structure of avidin showed that the size of the tetrameric avidin molecule is ca. 56 Å × 50 Å × 40 Å and the four identical subunits are arranged in a quaternary structure which positions the two pairs of the binding sites on opposite

Abbreviations: bpy, 2,2′-bipyridine; bpy-CH_2-NH-C2-NH-biotin, 4-(*N*-((2-biotinamido)ethyl)aminomethyl)-4′-methyl-2,2′-bipyridine; bpy-CO-NH-C2-NH-biotin, 4-(*N*-((2-biotinamido)ethyl)amido)-4′-methyl-2,2′-bipyridine; bpy-CO-NH-C6-NH-biotin, 4-(*N*-((6-biotinamido)hexyl)amido)-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; Hbsb, 2-((1,1′-biphenyl)-4-yl)benzothiazole; Hbsn, 2-(1-naphthyl)benzothiazole; Hbt, 2-phenylbenzothiazole; Hbtth, 2-(2-thienyl)benzothiazole; Hbzq, 7,8-benzoquinoline; Hmppy, 2-(4-methylphenyl)pyridine; Hmppz, 3-methyl-1-phenylpyrazole; Hppy, 2-phenylpyridine; Hppz, 1-phenylpyrazole; Hpq, 2-phenylquinoline; IL, intraligand; Me_2-Ph_2-phen, 2,9-dimethyl-4,7-diphenyl-1,10-phenanthroline; Me_4-phen, 3,4,7,8-tetramethyl-1,10-phenanthroline; MLCT, metal-to-ligand charge-transfer; phen, 1,10-phenanthroline; py-3-CO-NH-C2-NH-biotin, 3-(*N*-((2-biotinamido)ethyl)amido)pyridine; py-4-CH_2-NH-biotin, 4-((biotinamido)methyl)pyridine; py-4-CH_2-NH-C6-NH-biotin, 4-(*N*-((6-biotinamido)hexanoyl)aminomethyl)pyridine; RET, resonance-energy transfer

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sides of the protein [3]. The biotin-binding sites of avidin are located at ca. 9 Å below the surface of the avidin molecule and contain both hydrophobic and polar residues for the recognition of biotin [3].

The avidin–biotin system has been widely employed in many bioanalytical applications [4,5]. In general, biomolecules can be biotinylated and then detected by avidin that has been labelled with reporters such as fluorescent compounds or enzymes [6]. Since avidin has four biotin-binding sites, biotinylated biomolecules can also be recognised by biotin–reporter conjugates when avidin is used as a bridge. Thus, various biotin compounds containing a reporter or functional unit such as a transition metal complex have been developed: for example, a tricarbonyl(η^5-cyclopentadienyl)manganese biotin compound has been used as an IR-active tracer in competitive bioassays [7]. Redox-active ferrocene–biotin conjugates containing a poly(ethylene oxide) chain have been designed as electrochemical sensors for avidin [8]. Recently, a biotinylated iron(II) diimine complex $[Fe(N–N)_3]^{2+}$ has been employed as a redox-active biotin-bridge for the immobilisation of avidin layers on an electrode surface [9]. Additionally, a nickel(salen)–biotin conjugate that can couple with highly accessible guanine residues in DNA has been designed [10]. Incubation of a model oligonucleotide with this conjugate yielded a high molecular weight DNA adduct that can be isolated using avidin.

Fluorescent organic–biotin conjugates have been designed. Most of these molecules experience self-quenching upon binding to the protein [11–15]. The quenching appears to be RET (or homotransfer) in nature [16]. The small Stokes' shifts of these compounds result in large overlap integrals. Thus, when the organic fluorophores come to close proximity, effective RET occurs, leading to substantial emission quenching. These properties allow the organic–biotin derivatives to serve as probes for avidin. However, for the purpose of a recognition assay, we believe that luminescent transition metal complexes could be used as markers or affinity labels in view of their characteristic photophysical properties, in particular, the large Stokes' shifts due to the phosphorescent nature of their emission. The insignificant overlap integrals prevent them from self-quenching when the complexes bind to avidin. In fact, by virtue of their variable oxidation states, flexible coordinating geometry, and rich photophysical and electrochemical properties, many transition metal complexes have been covalently linked to biomolecules for various purposes [17–23]. We have exploited the possibility of using luminescent transition metal complexes, such as those of rhenium(I), iridium(III) and rhodium(III) [24–31], as biological probes because many of these complexes show intense and long-lived luminescence with tunable emission energy in the visible region. Importantly, the emission of these systems is very sensitive to their local surroundings, 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. This review article describes our recent work in the design of luminescent transition metal biotin complexes.

2. Rhenium(I) polypyridine biotin complexes

We synthesised a family of luminescent rhenium(I) polypyridine biotin complexes $[Re(N–N)(CO)_3(py\text{-}spacer\text{-}biotin)](PF_6)$ (N–N = phen, Me_4-phen, Me_2-Ph_2-phen, dpq; py-spacer-biotin = py-4-CH_2-NH-biotin, py-3-CO-NH-C2-NH-biotin, py-4-CH_2-NH-C6-NH-biotin) (Fig. 1) [32,33]. The identity of the diimine ligands was changed to perturb the spectroscopic and emission properties of the complexes, and to study the effects of the hydrophobicity of the complexes on avidin-binding. Spacer-arms of different chain lengths were introduced to evaluate their influence on the protein-binding strength of the complexes. Upon irradiation, the complexes exhibited orange to green triplet MLCT ($d\pi(Re) \rightarrow \pi^*(N–N)$) emission in fluid solutions at 298 K [34–36]. The photophysical data of the complexes are collected in Table 1. Most of the complexes displayed a decrease in emission lifetimes and quantum yields upon changing the solvent from CH_2Cl_2 to CH_3CN to buffer (Table 1). Interestingly, the Me_4-phen complexes did not show a similar trend, and their emission lifetimes in fluid solutions were exceptionally long (ca. 7.3–14 μs) especially in aqueous buffer. This could be the consequence of a low-lying ^{3}IL (Me_4-phen) emissive state [37]. It could also be attributed to the different contributions of individual orbital excitations to the excited states involved [38].

The binding of the complexes to avidin was studied using the standard HABA assay [1,4,5]. The binding of HABA to avidin is associated with an absorption feature at ca. 500 nm [1,4,5]. Since 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) [1], addition of biotin will replace the bound HABA molecules from the protein, leading to a decrease in the absorbance at 500 nm. In our 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. We found that all these complexes bound to avidin with the same stoichiometry as unmodified biotin ([Re]:[avidin] = 4:1).

The most remarkable observations were that the complexes displayed enhanced emission intensities and extended lifetimes upon binding to avidin. At [Re]:[avidin] = 4:1, the emission intensities were enhanced by ca. 1.2–3.0-fold and the emission lifetimes were extended by ca. 1.3–2.4-fold (Table 1). Emission titration curves for $[Re(Me_2\text{-}Ph_2\text{-}phen)(CO)_3(py\text{-}4\text{-}CH_2\text{-}NH\text{-}C6\text{-}NH\text{-}biotin)](PF_6)$ 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 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 unit [11–15]. The self-quenching is in line with the fact that biomolecules multiply labelled with organic fluorophores do not become correspondingly more fluorescent [39]. The absence of emission quenching for the rhenium(I) biotin complexes is

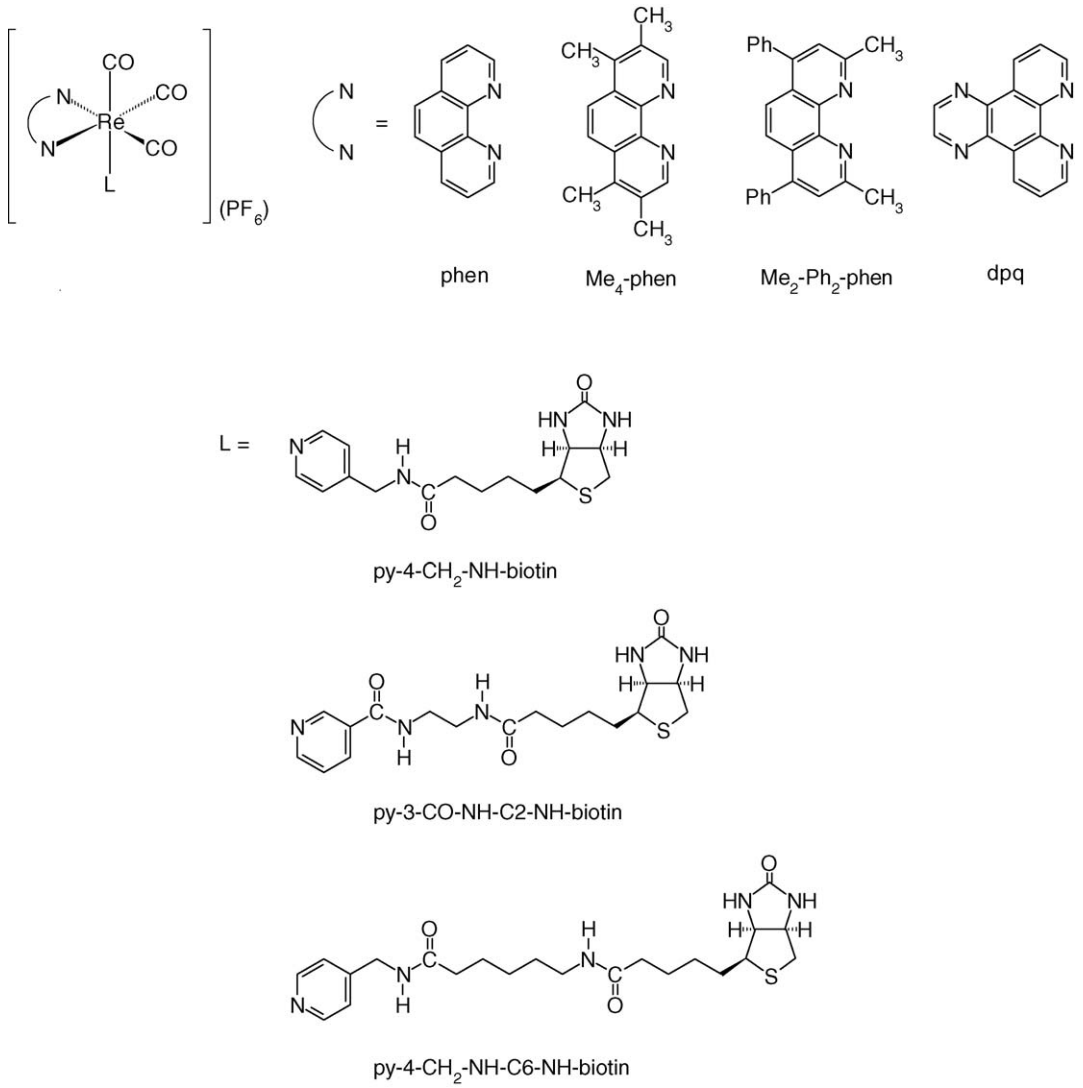

Fig. 1. Structures of $[Re(N–N)(CO)_3(py\text{-}spacer\text{-}biotin)](PF_6)$. Reproduced from [33], with permission of The American Chemical Society.

because of the insignificant overlap between their absorption and emission spectra, which disfavours RET quenching. Since both the emission intensities and lifetimes of the excited complexes are 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. Another 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.

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 1). The emission quantum yield enhancement factors from aqueous buffer to CH_2Cl_2 were the highest for $[Re(Me_2\text{-}Ph_2\text{-}phen)(CO)_3(py\text{-}4\text{-}CH_2\text{-}NH\text{-}biotin)](PF_6)$ and $[Re(Me_2\text{-}Ph_2\text{-}phen)(CO)_3(py\text{-}3\text{-}CO\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)$ (20 and 15.8, respectively) among all of the complexes. These observations are in line with

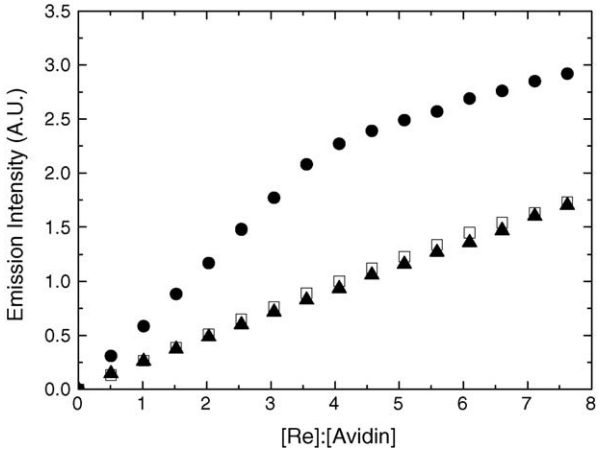

Fig. 2. Luminescence titration curves for the titrations of: (i) 3.8 μM avidin (●); (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲); and (iii) a blank phosphate buffer solution (□) with $[Re(Me_2\text{-}Ph_2\text{-}phen)(CO)_3(py\text{-}4\text{-}CH_2\text{-}NH\text{-}C6\text{-}NH\text{-}biotin)](PF_6)$. Reproduced from [33], with permission of The American Chemical Society.

Table 1
Photophysical data of $[Re(N–N)(CO)_3(py\text{-}spacer\text{-}biotin)](PF_6)$ at 298 K

N–N	Py-spacer-biotin	Medium	λ_{em} (nm[a])	τ_0 (μs[a])	Φ_{em}[a]	I (τ (μs))[b,c]	I (τ (μs))[b,d]	I (τ (μs))[b,e]
phen	py-4-CH_2-NH-biotin	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			
		Buffer[f]	552	0.79	0.027			
phen	py-3-CO-NH-C2-NH-biotin	CH_2Cl_2	536	2.92	0.30	1.00 (0.69)	1.25 (1.01)	1.01 (0.68)
		CH_3CN	550	1.75	0.13			
		Buffer[f]	550	1.07	0.12			
phen	py-4-CH_2-NH-C6-NH-biotin	CH_2Cl_2	540	2.85	0.49	1.00 (0.55)	1.24 (0.73)	0.96 (0.56)
		CH_3CN	554	1.46	0.16			
		Buffer[f]	554	0.84	0.096			
Me_4-phen	py-4-CH_2-NH-biotin	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			
		Buffer[f]	522	11.11	0.026			
Me_4-phen	py-3-CO-NH-C2-NH-biotin	CH_2Cl_2	516	13.54	0.19	1.00 (1.50)	1.26 (3.65)	1.01 (1.53)
		CH_3CN	522	9.90	0.051			
		Buffer[f]	518	14.06	0.041			
Me_4-phen	py-4-CH_2-NH-C6-NH-biotin	CH_2Cl_2	518	13.28	0.27	1.00 (1.31)	1.90 (2.00)	0.96 (1.36)
		CH_3CN	522	8.54	0.12			
		Buffer[f]	522	11.62	0.078			
Me_2-Ph_2-phen	py-4-CH_2-NH-biotin	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			
		Buffer[f]	556	6.01	0.011			
Me_2-Ph_2-phen	py-3-CO-NH-C2-NH-biotin	CH_2Cl_2	542	15.48	0.19	1.00 (2.09)	2.93 (3.25)	1.01 (2.04)
		CH_3CN	550	9.41	0.13			
		Buffer[f]	554	7.85	0.012			
Me_2-Ph_2-phen	py-4-CH_2-NH-C6-NH-biotin	CH_2Cl_2	542	11.65	0.18	1.00 (1.61)	2.27 (2.38)	0.93 (1.64)
		CH_3CN	552	6.96	0.053			
		Buffer[f]	558	5.08	0.028			
dpq	py-4-CH_2-NH-biotin	CH_2Cl_2	548	1.12	0.28	1.00 (0.11)	1.75 (0.21)	0.99 (0.11)
		CH_3CN	568	0.40	0.086			
		Buffer[f]	572	0.11	0.034			
dpq	py-3-CO-NH-C2-NH-biotin	CH_2Cl_2	544	1.24	0.15	1.00 (0.18)	1.20 (0.26)	1.03 (0.19)
		CH_3CN	562	0.52	0.065			
		Buffer[f]	566	0.21	0.033			
dpq	py-4-CH_2-NH-C6-NH-biotin	CH_2Cl_2	548	1.10	0.24	1.00 (0.12)	1.15 (0.16)	0.98 (0.12)
		CH_3CN	568	0.40	0.054			
		Buffer[f]	572	0.13	0.032			

Reproduced from [32,33], with permission of The American Chemical Society.
[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] 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 highest emission intensity amplification factors for these two complexes (2.98 and 2.93) upon binding to avidin (Table 1). Concerning the effects of the chain length of the spacer-arms, we noted that the py-3-CO-NH-C2-NH-biotin and py-4-CH_2-NH-C6-NH-biotin complexes exhibited less significant emission intensity enhancement (Table 1). It is likely that these eight complexes remain 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 is less substantial. Additionally, the effects of increased rigidity resulting from avidin-binding are 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 experiments [12]. The K_d-values ranged from ca. 5.5×10^{-11} to 3.4×10^{-9} M, 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 results from the bulkiness of the rhenium(I) polypyridine units. The binding became stronger when the biotin-containing lig-

ands changed from py-4-CH_2-NH-biotin to py-3-CO-NH-C2-NH-biotin to py-4-CH_2-NH-C6-NH-biotin (data not shown), indicating the importance of the spacer-arms on alleviating the steric hindrance between the complexes and the protein [40–42].

The intrinsic emission intensity enhancement (I/I_0) of the complexes upon binding to avidin varied from ca. 1.2 to 3.0-fold (Table 1). To develop sensitive assays for avidin and biotin, it is desirable to maximise these emission enhancement factors. Our strategy is to use a quencher to selectively suppress the emission of the free rhenium(I) biotin complex by distance-dependent RET quenching [43]. The quencher we used was a water-soluble polypeptide (poly(D-Glu:D-Lys) 6:4) modified with the non-fluorescent 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 thus it 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; and (iii) the bulky size of this polypeptide would not enable the quencher units to approach the avidin-bound rhenium(I) biotin complex and quench its emission by RET. In the absence of the quencher, upon irradiation, the complex [Re(Me_2-Ph_2-phen)$(CO)_3$(py-4-CH_2-NH-C6-NH-biotin)](PF_6) 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 the cationic complex 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. When avidin was added to a mixture of poly(E/K)–QSY and the rhenium(I) biotin complex 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 (rhenium(I) complex) and acceptor (QSY-7®), and hence the enhancement of emission intensity (Fig. 3). On the basis of the emission enhancement, we titrated the complex in water with avidin in the absence and presence of poly(E/K)–QSY. In the absence of the quencher poly(E/K)–QSY, the complex showed enhancement in emission intensity upon addition of avidin. At [avidin]:[Re] = 1/4, only ca. 1.5-fold of emission enhancement was observed (Fig. 4). In the presence of the polymeric quencher, the emission intensity of the complex increased significantly, and showed an overall gain of four times at [avidin]:[Re] = 1/4 (Fig. 4). The emission lifetimes also increased from <0.1 to ca. 2.6 μs. Thus, using a macromolecular quencher that can preferentially reduce the emission of the free rhenium(I) biotin complex, the amplification factor of the emission enhancement for avidin sensing was increased.

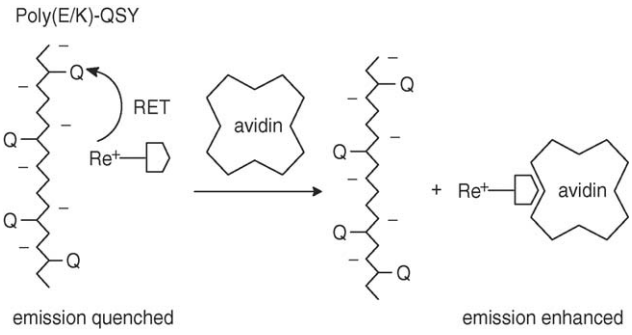

Fig. 3. Schematic representation showing the emission quenching of [Re(Me_2-Ph_2-phen)$(CO)_3$(py-4-CH_2-NH-C6-NH-biotin)](PF_6) by poly(E/K)–QSY and emission enhancement upon binding of the complex to avidin. Reproduced from [33], with permission of The American Chemical Society.

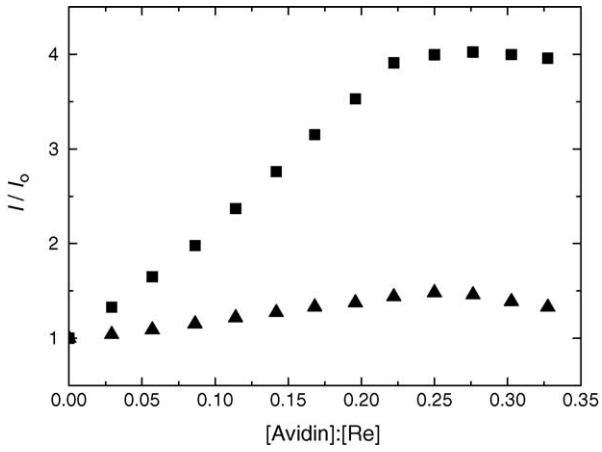

Fig. 4. Luminescence titration curves for the titrations of [Re(Me_2-Ph_2-phen)$(CO)_3$(py-4-CH_2-NH-C6-NH-biotin)](PF_6) (5.5 μM) in the absence (▲) and presence (■)of poly(E/K)–QSY (1.1 μM) in water at 298 K, where I_0 and I are the emission intensities in the absence and presence of avidin, respectively. Reproduced from [33], with permission of The American Chemical Society.

To explore bifunctional biological probes, we prepared new rhenium(I) biotin complexes containing the extended planar diimine ligands dppz and dppn (Fig. 5) [44]. These extended planar diimine ligands were expected to allow the complexes to intercalate into the base-pairs of double-stranded DNA molecules [18,45,46], and the biotin moieties would enable the complexes to bind to avidin. Absorption and emission titration results revealed that all the complexes bound to double-stranded DNA by intercalation. The HABA assays showed that all the complexes bound to avidin with a stoichiometry of 4:1 ([Re]:[avidin]). Similar to other rhenium(I) biotin com-

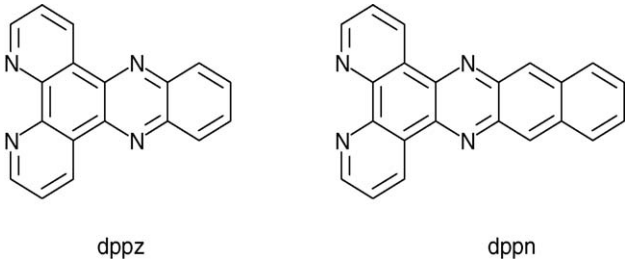

Fig. 5. Structures of the ligands dppz and dppn.

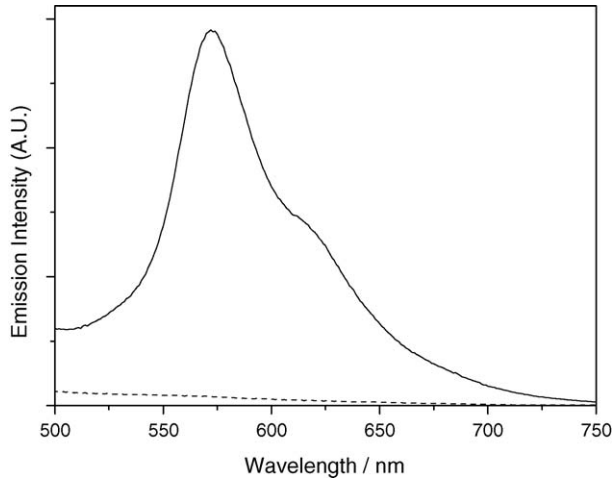

Fig. 6. Emission spectra of [Re(dppz)(CO)$_3$(py-4-CH$_2$-NH-biotin)](PF$_6$) in the absence (- - -) and presence (—) of avidin in degassed potassium phosphate buffer. Reproduced from [44], with permission of The American Chemical Society.

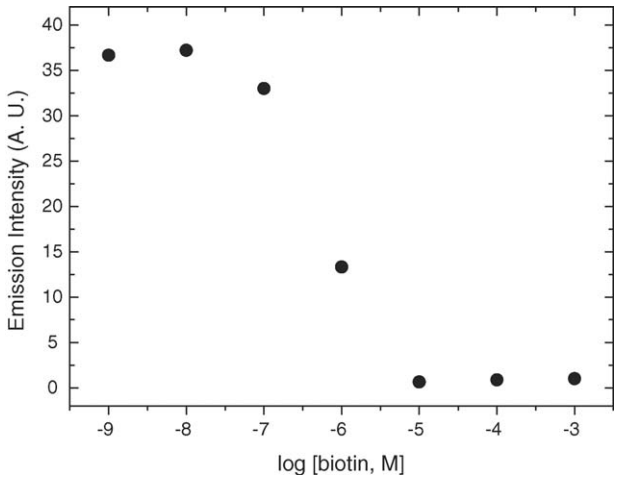

Fig. 7. Homogeneous competitive assay for biotin using [Re(dppz)(CO)$_3$(py-4-CH$_2$-NH-biotin)](PF$_6$) and avidin.

plexes described above [32,33], the emission intensities and lifetimes of these complexes also increased in the presence of avidin. In particular, the complex [Re(dppz)(CO)$_3$(py-4-CH$_2$-NH-biotin)](PF$_6$) exhibited a large emission enhancement factor of ca. 40 (Fig. 6). The avidin-induced emission enhancement of these rhenium(I) biotin complexes was 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 assay, avidin was added to a solution of the rhenium(I) polypyridine biotin complex and the biotin analyte. The solution was incubated at room temperature for 1 h and its emission intensity was then measured. A lower biotin analyte concentration was expected to result in a higher degree of binding of the rhenium(I) polypyridine biotin complex to avidin, and thus a higher emission intensity. The results of the assay using [Re(dppz)(CO)$_3$(py-4-CH$_2$-NH-biotin)](PF$_6$) are shown in Fig. 7. The concentration of biotin that could be determined by this assay was between ca. $1 \times 10^{-7.5}$ and 1×10^{-5} M. The lowest concentration of biotin analyte that gave a meaningful signal (ca. $1 \times 10^{-7.5}$ M) was about 1.5 orders of magnitude

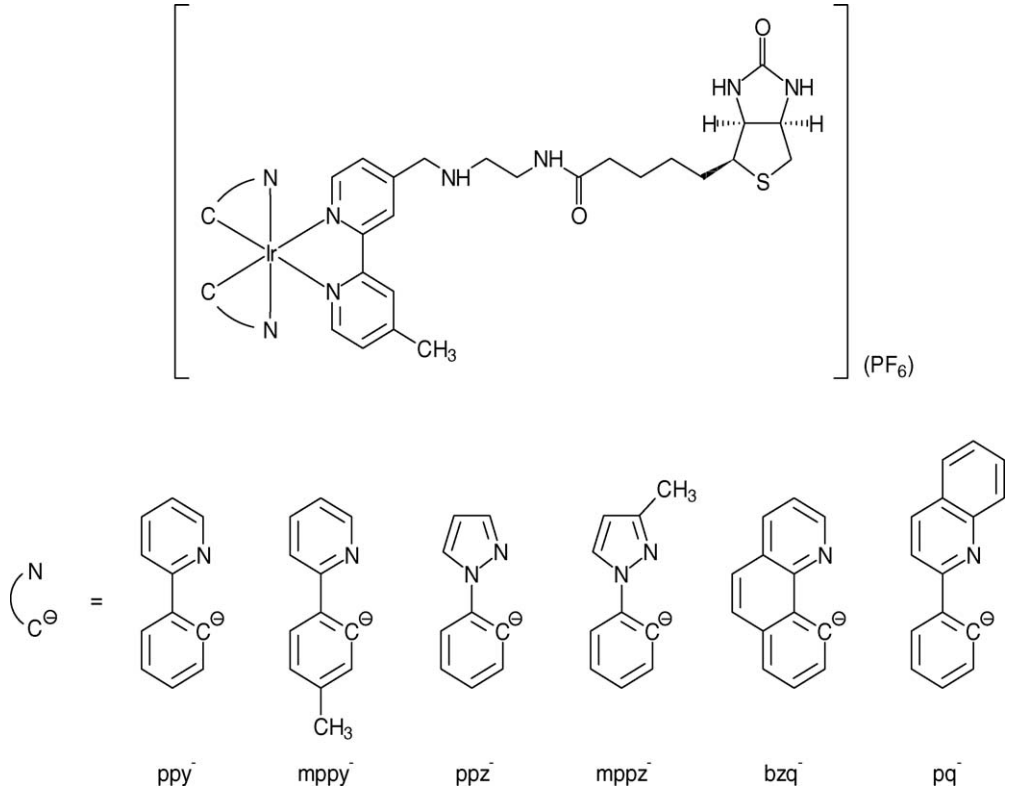

Fig. 8. Structures of [Ir(N–C)$_2$(bpy-CH$_2$-NH-C2-NH-biotin)](PF$_6$). Reproduced from [52], with permission of The American Chemical Society.

lower than that of a heterogeneous competitive biotin assay we reported previously (ca. 1×10^{-6} M) [28] but comparable to that of a competitive assay based on the change of electrode response of copper enhanced by a thiourea–biotin compound (ca. $1 \times 10^{-7.54}$ M) [47].

3. Iridium(III) polypyridine biotin complexes

The interesting emission properties of iridium(III) polypyridine complexes [34,48–51], and our recent interest in using these complexes as biological labelling reagents [25,27–29,31] prompted us to design new luminescent cyclometallated iridium(III) polypyridine biotin complexes $[Ir(N–C)_2(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)$ (HN–C = Hppy, Hmppy, Hppz, Hmppz, Hbzq, Hpq) (Fig. 8) [52]. 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 2). The emission spectra of complexes $[Ir(ppy)_2(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)$ and $[Ir(bzq)_2(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)$ in CH_3CN at 298 K and in alcohol glass at 77 K are shown in Fig. 9. An excited-state assignment of ^{3}MLCT ($d\pi(Ir) \rightarrow \pi^*(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)$) was supported by the observations that the complex $[Ir(ppy)_2(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)$ emitted at higher energy than the model complex $[Ir(ppy)_2(bpy)](PF_6)$ (λ_{em} = 588 nm in both CH_3CN and MeOH at 298 K). The electron-donating methyl and aminomethyl substituents of $bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin$ 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 also supported by the fact that the emission of $[Ir(ppy)_2(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)$ and $[Ir(ppz)_2(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)$ occurred at slightly higher energy than their $mppy^-$ and $mppz^-$ counterparts, respectively (Table 2). The electron-donating methyl groups of the cyclometallating ligands $mppy^-$ and $mppz^-$ enriched the electron density of the iridium(III) centres, and thus stabilised the ^{3}MLCT ($d\pi(Ir) \rightarrow \pi^*(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)$) emissive states of these two complexes. The complex $[Ir(pq)_2(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)$ showed structured emission spectra and very long emission lifetimes (τ_0 = ca. 2–3 μs) in fluid solutions at 298 K, suggestive of substantial ^{3}IL ($\pi \rightarrow \pi^*$) (pq^-) character in its emissive state [29,31,51].

Binding of these iridium(III) biotin complexes to avidin was confirmed by the HABA assays [1,4,5]. The equivalence points

Table 2
Photophysical data of $[Ir(N–C)_2(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)$

HN–C	Medium (T (K))	λ_{em} (nm[a])	τ_0 (μs[a])	Φ_{em}[a]	I (τ (ns))[b,c]	I (τ (ns))[b,d]	I (τ (ns))[b,e]
Hppy	CH_3CN (298)	576	0.48	0.16	1.00 (90)	1.85 (140)	1.05 (82)
	MeOH (298)	577	0.36	0.11			
	H_2O/DMSO[f] (298)	584	0.21	0.035			
	Glass[g] (77)	473 sh, 506, 537 sh	4.74				
Hmppy	CH_3CN (298)	587	0.35	0.081	1.00 (83)	1.88 (148)	0.91 (87)
	MeOH (298)	580	0.24	0.065			
	H_2O/DMSO[f] (298)	589	0.17	0.053			
	Glass[g] (77)	475 sh, 513, 542 sh	4.57				
Hppz	CH_3CN (298)	560	0.92	0.19	1.00 (71)	1.48 (232)	0.99 (79)
	MeOH (298)	561	0.62	0.11			
	H_2O/DMSO[f] (298)	567	0.28	0.12			
	Glass[g] (77)	496, 527 sh	5.47				
Hmppz	CH_3CN (298)	574	0.39	0.093	1.00 (59)	1.95 (166)	1.04 (65)
	MeOH (298)	577	0.28	0.079			
	H_2O/DMSO[f] (298)	576	0.17	0.058			
	Glass[g] (77)	509, 538 sh	4.38				
Hbzq	CH_3CN (298)	577	0.47	0.12	1.00 (73)	1.81 (147)	0.97 (80)
	MeOH (298)	580	0.29	0.054			
	H_2O/DMSO[f] (298)	583	0.17	0.054			
	Glass[g] (77)	502 (max), 541, 583 sh	41.36 (33%), 5.12 (67%)				
Hpq	CH_3CN (298)	554, 605 sh	2.95	0.37	1.00 (1533)	3.29 (2343)	1.04 (1529)
	MeOH (298)	551, 605 sh	2.90	0.37			
	H_2O/DMSO[f] (298)	559, 604 sh	1.88	0.24			
	Glass[g] (77)	540 (max), 582, 632 sh	4.78				

Reproduced from [52], with permission of The American Chemical Society.

[a] In degassed solvents.

[b] Relative emission intensities in aerated 50 mM potassium phosphate buffer pH 7.4.

[c] [Ir] = 15.2 μM, [avidin] = 0 μM, [unmodified biotin] = 0 μM.

[d] [Ir] = 15.2 μM, [avidin] = 3.8 μM, [unmodified biotin] = 0 μM.

[e] [Ir] = 15.2 μM, [avidin] = 3.8 μM, [unmodified biotin] = 380.0 μM.

[f] H_2O/DMSO (1:1, v/v).

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

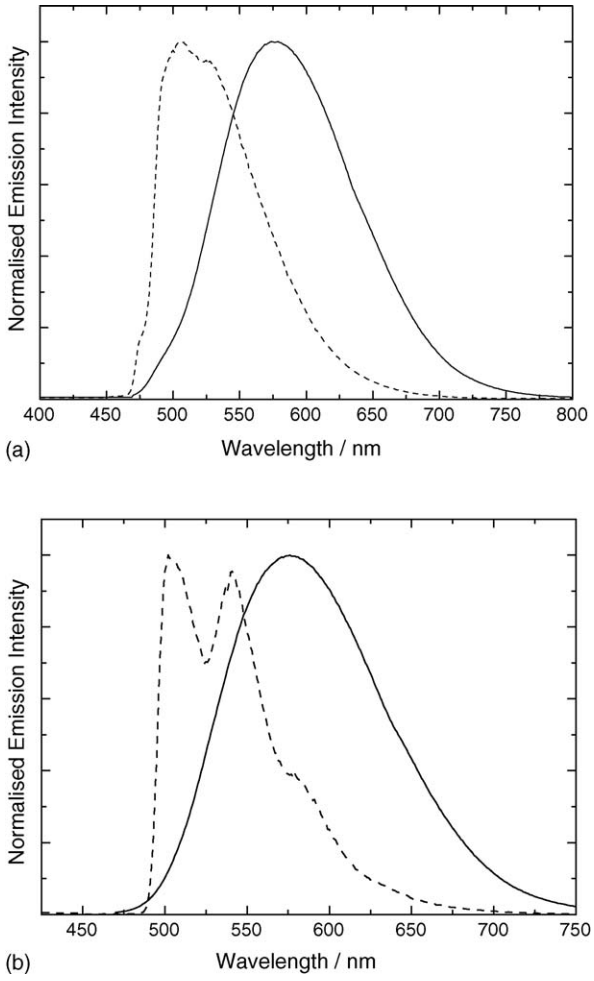

Fig. 9. Emission spectra of: (a) $[Ir(ppy)_2(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)$ and (b) $[Ir(bzq)_2(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)$ in CH_3CN at 298 K (—) and in EtOH/MeOH (4:1, v/v) at 77 K (- - -). Reproduced from [52], with permission of The American Chemical Society.

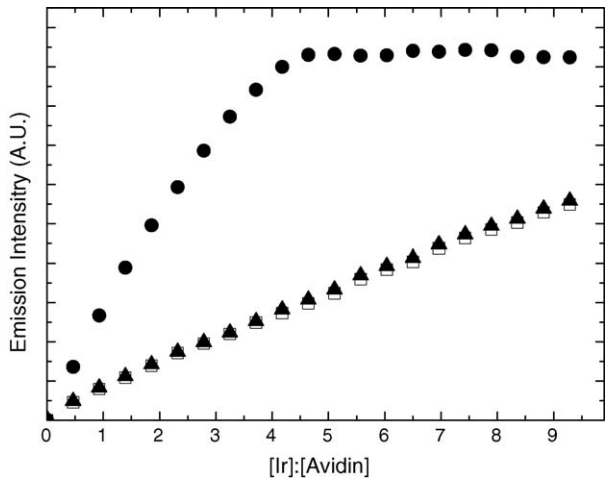

Fig. 10. Luminescence titration curves for the titrations of: (i) 3.8 μM avidin (●); (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲); and (iii) a blank phosphate buffer solution (□) with $[Ir(pq)_2(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)$. Reproduced from [52], with permission of The American Chemical Society.

occurred at [Ir]:[avidin] = from ca. 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 displayed enhanced emission intensities upon binding avidin. At the equivalence points, the emission intensities and lifetimes of the complexes increased by factors of ca. 1.5–3.3 (Table 2). 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 2). Another reason is the increased rigidity of the local surroundings of the complexes provided by the protein. The complex $[Ir(pq)_2(bpy\text{-}CH_2\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)$, 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. 10). It is likely that these observations are 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.

Although these iridium(III) biotin complexes could function as new luminescent probes for avidin, their emission maxima were somewhat confined to ca. 550–590 nm, limiting their use as multi-colour probes. The reason is that the excited states are essentially triplet metal-to-ligand charge-transfer ^{3}MLCT ($d\pi(Ir) \rightarrow \pi^*(N\text{–}N)$) in nature. Thus, changing the cyclometallating ligand cannot significantly alter the emission energy of the systems. Recent studies have shown that introduction of arylbenzothiazole ligands to iridium(III) centre can give efficient triplet intraligand ^{3}IL ($\pi \rightarrow \pi^*$) (arylbenzothiazole) emitters that show very intense and long-lived emission [51]. In fact, the use of such hydrophobic ligands is attractive because more hydrophobic transition metal biotin conjugates provide higher degrees of emission enhancement (see above) [32,33,44,52]. In consideration of these factors, we designed a new series of luminescent cyclometallated iridium(III) arylbenzothiazole biotin complexes $[Ir(N\text{–}C)^2(bpy\text{-}CO\text{-}NH\text{-}C6\text{-}NH\text{-}biotin)](PF^6)$ (HN–C = Hbt, Hbsb, Hbtth, Hbsn) (Fig. 11) [53].

Upon irradiation, these complexes displayed intense red to green emission under ambient conditions and in alcohol glass at 77 K (Table 3). The emission spectra of these four complexes in degassed CH_2Cl_2 at 298 K are shown in Fig. 12. In solutions at 298 K, these complexes exhibited long-lived (in the microsecond timescale) and structured emission bands at ca. 528–712 nm. In view of very long emission lifetimes and rich structural features of the emission bands, the emission is assigned to an ^{3}IL ($\pi \rightarrow \pi^*$) ($N\text{–}C^-$) excited state, perhaps with mixing of some ^{3}MLCT ($d\pi(Ir) \rightarrow \pi^*(N\text{–}C^-)$) character. This assignment is supported by the observations that $[Ir(bsn)_2(bpy\text{-}CO\text{-}NH\text{-}C6\text{-}NH\text{-}biotin)](PF_6)$, which contained a more conjugated bsn^- ligand, emitted at lower energy than

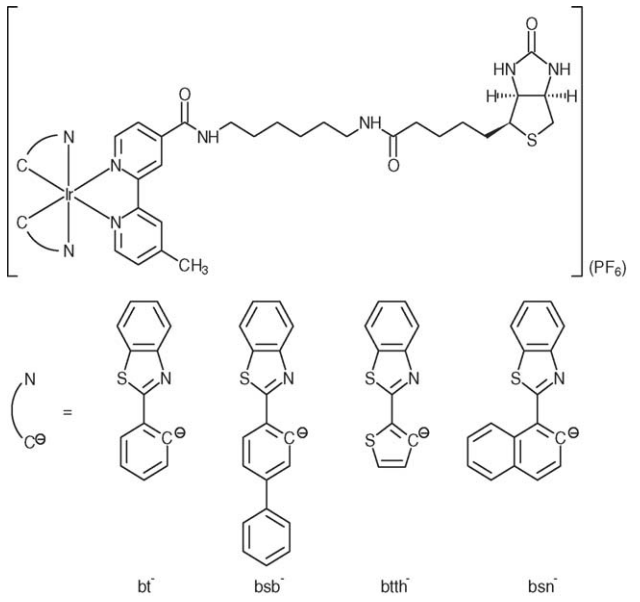

Fig. 11. Structures of $[Ir(N–C)_2(bpy\text{-}CO\text{-}NH\text{-}C6\text{-}NH\text{-}biotin)](PF_6)$. Reproduced from [53], with permission of The American Chemical Society.

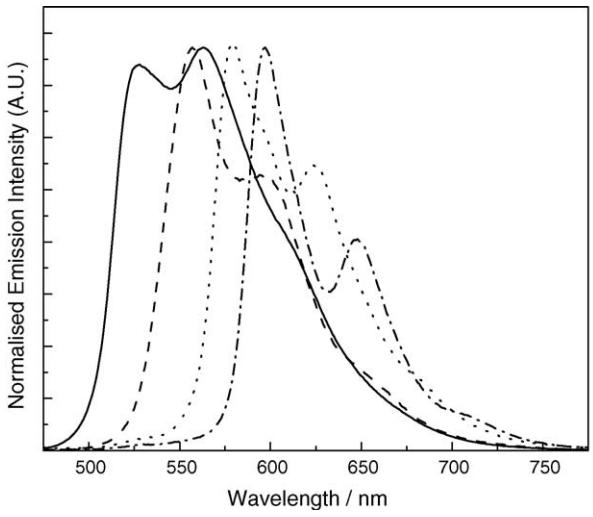

Fig. 12. Emission spectra of: $[Ir(N–C)_2(bpy\text{-}CO\text{-}NH\text{-}C6\text{-}NH\text{-}biotin)](PF_6)$ ($N–C^- = bt^-$ (—); bsb^- (- - -); $btth^-$ (· · ·); and bsn^- (-·-·-) in degassed CH_2Cl_2 at 298 K. Reproduced from [53], with permission of The American Chemical Society.

$[Ir(bt)_2(bpy\text{-}CO\text{-}NH\text{-}C6\text{-}NH\text{-}biotin)](PF_6)$, which had a less conjugated bt^- ligand. Actually, ^{3}IL emission has been commonly observed in related iridium(III) arylbenzothiazole complexes with acetylacetonate and other *O*,*O* and *O*,*N* ligands [51]. Interestingly, using arylbenzothiazole as the ligands, the emissive-state of these cyclometallated iridium(III) polypyridine complexes shifted from ^{3}MLCT ($d\pi(Ir) \rightarrow \pi^*(N–N)$) to ^{3}IL ($\pi \rightarrow \pi^*$) ($N–C^-$)/^{3}MLCT ($d\pi(Ir) \rightarrow \pi^*(N–C^-)$) in character, resulting in much more intense and long-lived luminescence. Similar observations have been made in the cyclometallated iridium(III) polypyridine complexes containing a conjugated 2-phenylquinoline ligand [29,31,51,52].

The avidin-binding properties of the iridium(III) biotin complexes were investigated by luminescence titrations using the complexes as the titrants. The emission titration curves

Table 3
Photophysical data of $[Ir(N–C)_2(bpy\text{-}CO\text{-}NH\text{-}C6\text{-}NH\text{-}biotin)](PF_6)$

HN–C	Medium (T (K))	λ_{em} (nm[a])	τ_0 (μs[a])	Φ_{em}[a]	I (τ (ns))[b,c]	I (τ (ns))[b,d]	I (τ (ns))[b,e]
Hbt	CH_2Cl_2 (298)	528, 563 (max), 612 sh	3.21	0.51	1.00 (0.051)	2.38 (0.12)	1.03 (0.049)
	CH_3CN (298)	528 sh, 570, 616 sh	1.31	0.33			
	Buffer[f] (298)	566, 612 sh	0.12	0.019			
	Glass[g] (77)	516 (max), 530 sh, 557, 574 sh, 604, 628 sh, 664 sh	5.68				
Hbsb	CH_2Cl_2 (298)	557 (max), 596, 638 sh	5.80	0.39	1.00 (0.39)	8.09 (1.94)	0.97 (0.48)
	CH_3CN (298)	556 (max), 595, 636 sh	5.84	0.40			
	Buffer[f] (298)	564 (max), 597, 637 sh	1.90	0.051			
	Glass[g] (77)	543 (max), 589, 640	8.25				
Hbtth	CH_2Cl_2 (298)	580 (max), 624, 685 sh	9.29	0.25	1.00 (0.12)	2.47 (0.45)	1.02 (0.13)
	CH_3CN (298)	580 (max), 624, 685 sh	8.53	0.23			
	Buffer[f] (298)	582 (max), 625, 685 sh	0.27	0.016			
	Glass[g] (77)	572 (max), 592, 622, 644 sh, 678	11.39				
Hbsn	CH_2Cl_2 (298)	598 (max), 648, 710 sh	4.53	0.11	1.00 (1.24)	5.84 (1.83)	1.05 (1.00)
	CH_3CN (298)	598 (max), 648, 710 sh	3.92	0.10			
	Buffer[f] (298)	598 (max), 648, 712 sh	1.91	0.014			
	Glass[g] (77)	590 (max), 608, 642, 664 sh, 702	6.42				

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[a] In degassed solvents.

[b] Relative emission intensities in aerated 50 mM potassium phosphate buffer pH 7.4. [Ir] = 3.7, 4.1, 3.4 and 5.7 μM for the bt^-, bsb^-, $btth^-$ and bsn^- complexes, respectively.

[c] Iridium(III) complex only.

[d] [avidin] = 0.67 μM.

[e] [avidin] = 0.67 μM, [unmodified biotin] = 67 μM.

[f] Potassium phosphate buffer (50 mM, pH 7.4) containing 25% DMSO.

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

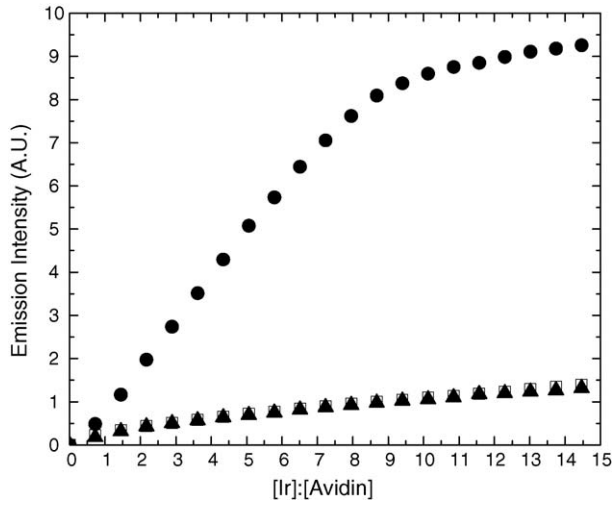

Fig. 13. Luminescence titration curves for the titrations of: (i) 0.67 μM avidin (●); (ii) 0.67 μM avidin and 67.0 μM unmodified biotin (▲); and (iii) a blank phosphate buffer solution (□) with [Ir(bsb)$_2$(bpy-CO-NH-C6-NH-biotin)](PF$_6$). Reproduced from [53], with permission of The American Chemical Society.

for the complexes [Ir(N–C)$_2$(bpy-CO-NH-C6-NH-biotin)](PF$_6$) (N–C$^-$ = bsb$^-$ and bsn$^-$) are illustrated in Figs. 13 and 14, respectively. As expected, all the current iridium(III) biotin complexes displayed enhanced emission intensities and extended emission lifetimes in the presence of avidin (Table 3). Note that under our experimental conditions, the equivalence points of the titrations varied from ca. 5.1 to 8.5 for these complexes (see, for example, Figs. 13 and 14). The possibility of non-specific binding is excluded on the basis of the control experiment results. The occurrence of equivalence points beyond the expected stoichiometry ([biotin]:[avidin] = 4) suggests that the concentrations of the complexes used in these titration experiments were not substantially larger than the K_d-values of these systems. Unfortunately, higher concentrations of complexes could not be employed in these titrations due to precipitation problems. Importantly, the more hydrophobic biphenyl and naphthyl complexes showed very significant emission enhancement factors (8.1 and 5.8, respectively, Table 3). Thus, it is apparent that employing more hydrophobic luminescent biotin conjugates provides higher detection sensitivity, although lower solubility may be a concern.

The K_d-values of the adducts formed from avidin and all the iridium(III) biotin complexes described here varied from ca. 10^{-10} to 10^{-8} M, which are about five to seven orders of magnitude larger than that of the native biotin-avidin system [1,2]. It appears that the lack of a long spacer-arm and/or the bulky [Ir(N–C)$_2$] moieties led to the diminished binding strength.

The possibility of utilising the current iridium(III) complexes as probes for biotin and biotinylated species on the basis of the RET principle [43] was investigated. Malachite Green-modified avidin (MG-Av) was used as the energy acceptor given the considerable overlap between the absorption band of the dye (λ_{abs} = 629 nm) and the emission bands of the donor, complex [Ir(btth)$_2$(bpy-CO-NH-C6-NH-biotin)](PF$_6$) (λ_{em} = 582 (max), 625, and 685 nm). On the basis of the spectral data of the donor and acceptor, a Förster distance of ca. 38.5 Å was estimated. The emission intensity of the complex (3.7 μM) in degassed buffer was enhanced by 20-fold (Fig. 15) and the lifetime was extended from 0.27 to 1.82 μs in the presence of avidin (1.4 μM). However, the emission intensity of the complex only increased by 1.5-fold and the lifetime was reduced from 0.27 to 0.15 μs when MG-Av was used instead of avidin. The lower emission intensity and shorter emission lifetime was a consequence of the binding of the complex to MG-Av, leading to shorter distances between the donor and the acceptor molecules. As a result, emission quenching due to RET occurred. This explanation is supported by the fact that no such decrease in emission lifetime (τ = 0.29 μs) was observed when MG-Av was pre-blocked with unmodified biotin (370 μM). The results of these experiments could form the basis of detection of biotin and biotinylated molecules using emission lifetime measurements.

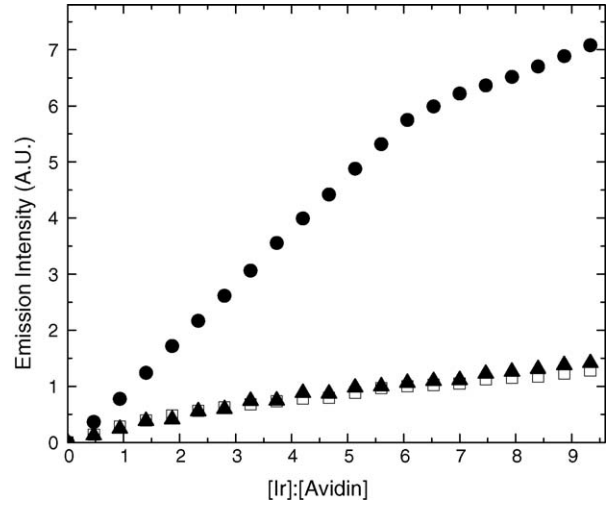

Fig. 14. Luminescence titration curves for the titrations of: (i) 0.67 μM avidin (●); (ii) 0.67 μM avidin and 67.0 μM unmodified biotin (▲); and (iii) a blank phosphate buffer solution (□) with [Ir(bsn)$_2$(bpy-CO-NH-C6-NH-biotin)](PF$_6$). Reproduced from [53], with permission of The American Chemical Society.

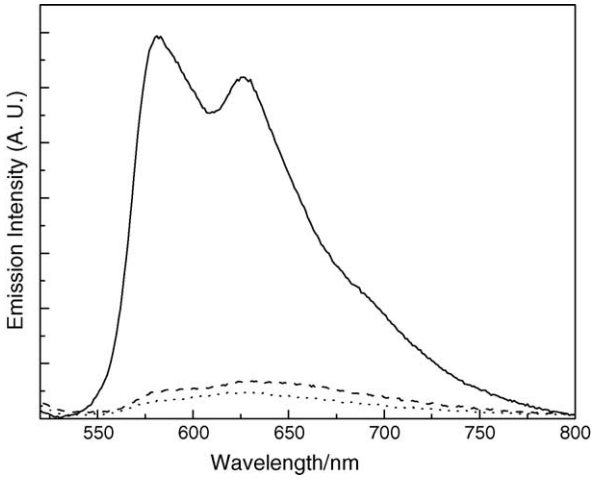

Fig. 15. Emission spectra of [Ir(btth)$_2$(bpy-CO-NH-C6-NH-biotin)](PF$_6$) (3.7 μM) in a degassed: (i) 1.4-μM avidin solution (—); (ii) 1.4-μM MG-Av solution (- - -); and (iii) blank phosphate buffer solution (· · ·) at 298 K. Reproduced from [53], with permission of The American Chemical Society.

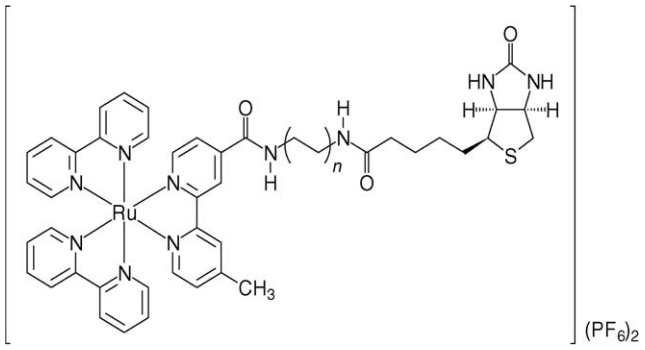

Fig. 16. Structures of $[Ru(bpy)_2(N–N)](PF_6)_2$ (N–N = bpy-CO-NH-C2-NH-biotin, $n = 1$; bpy-CO-NH-C6-NH-biotin, $n = 3$). Reproduced from [54], with permission of The American Chemical Society.

Table 4
Photophysical data of $[Ru(bpy)_2(N–N)](PF_6)_2$ (N–N = bpy-CO-NH-C2-NH-biotin and bpy-CO-NH-C6-NH-biotin)

N–N	Medium (T (K))	λ_{em} (nm[a])	τ_0 (μs[a])	Φ_{em}[a]
bpy-CO-NH-C2-NH-biotin	CH_3CN (298)	632	1.39	0.065
	MeOH (298)	636	1.09	0.055
	Glass[b] (77)	595, 651 sh	5.82	
bpy-CO-NH-C6-NH-biotin	CH_3CN (298)	629	1.45	0.072
	MeOH (298)	633	1.16	0.069
	Glass[b] (77)	595, 648 sh	5.64	

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[a] In degassed solvents.
[b] EtOH/MeOH (4:1, v/v).

4. Ruthenium(II) polypyridine biotin complexes

Since the ^{3}MLCT emission of ruthenium(II) polypyridine complexes is very well characterised [34], we are interested in the design of luminescent ruthenium(II)-based biological probes. Two ruthenium(II) polypyridine biotin complexes $[Ru(bpy)_2(N–N)](PF_6)_2$ (N–N = bpy-CO-NH-C2-NH-biotin, bpy-CO-NH-C6-NH-biotin) (Fig. 16) were synthesised and characterised [54]. The complexes exhibited intense and long-lived orange-red ^{3}MLCT ($d\pi(Ru) \rightarrow \pi^*(N–N)$) luminescence upon irradiation in fluid solutions at 298 K and in alcohol glass at 77 K (Table 4). Since the emission energy of these complexes is slightly lower than that of $[Ru(bpy)_3]^{2+}$ (λ_{em} = ca. 621 nm in CH_3CN and 616 nm in MeOH at 298 K) [55], the acceptor orbitals should possess predominant π^*(bpy-CO-NH-C2-NH-biotin or bpy-CO-NH-C6-NH-biotin) character, given the lower-lying π^* orbitals of the biotin-containing diimine ligands than those of bpy owing to the electron-withdrawing amide substituents.

The binding of both complexes to avidin was confirmed by the HABA assays [1,4,5]. The K_d-values for these two complexes were determined to be 4.8×10^{-10} and 3.1×10^{-11} M, respectively. Luminescence titration results showed that at [Ru]:[avidin] = 4, the emission intensities and lifetimes of the complexes increased by ca. 1.2–1.4-fold (Fig. 17 and Table 5). Unfortunately, the intrinsic increase in emission intensity of both complexes upon binding to avidin was rather small. Similar to the emission quenching work described above, we aimed at

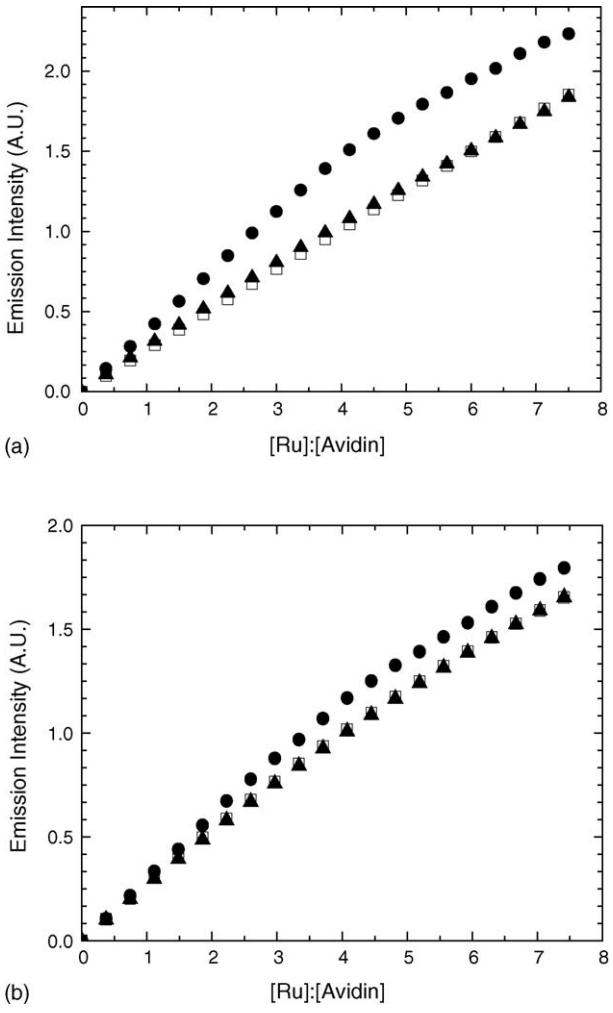

Fig. 17. Luminescence titration curves for the titrations of: (i) 3.8 μM avidin (●); (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲); and (iii) a blank phosphate buffer solution (□) with (a) $[Ru(bpy)_2$(bpy-CO-NH-C2-NH-biotin)$](PF_6)_2$ and (b) $[Ru(bpy)_2$(bpy-CO-NH-C6-NH-biotin)$](PF_6)_2$. Reproduced from [54], with permission of The American Chemical Society.

increasing these enhancement factors by addition of a quencher that can preferentially reduce the luminescence of 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 [34]. In view of the highly positively charged avidin molecule (pI = ca. 10) [1], we anticipated that the emission quenching of the avidin-bound ruthenium(II) biotin complexes by the cationic MV^{2+} ions would be less effective. 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 [56]. 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 $[Ru(bpy)_2(N–N)](PF_6)_2$ (N–N = bpy-CO-NH-C2-NH-biotin, bpy-CO-NH-C6-NH-biotin) was quenched by MV^{2+} in the absence of avidin, with Stern–Volmer constants, K_{SV}, of ca. 137 and 172 M^{-1}, respectively. In the presence of avidin, the emission quenching became less efficient

Table 5
Relative emission intensities and emission lifetimes of $[Ru(bpy)_2(N–N)](PF_6)_2$ in the absence and presence of avidin (and excess biotin) with various concentrations of methyl viologen (MV^{2+}) and KCl[a]

N–N	$[MV^{2+}] = 0$ M, [KCl] = 0 M			$[MV^{2+}] = 15.0$ mM, [KCl] = 0 M			$[MV^{2+}] = 15.0$ mM, [KCl] = 2.0 M		
	I (τ (ns))[b]	I (τ (ns))[c]	I (τ(ns))[d]	I (τ (ns))[b]	I (τ (ns))[c]	I (τ (ns))[d]	I (τ (ns))[b]	I (τ (ns))[c]	I (τ (ns))[d]
bpy-CO-NH-C2-NH-biotin	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)
bpy-CO-NH-C6-NH-biotin	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)

Reproduced from [54], with permission of The American Chemical Society.
[a] Relative emission intensities in aerated 50 mM potassium phosphate buffer pH 7.4.
[b] [Ru] = 15.0 μM, [avidin] = 0 μM, [unmodified biotin] = 0 μM.
[c] [Ru] = 15.0 μM, [avidin] = 3.8 μM, [unmodified biotin] = 0 μM.
[d] [Ru] = 15.0 μM, [avidin] = 3.8 μM, [unmodified biotin] = 380.0 μM.

(K_{SV} = ca. 49 and 67 M^{-1} for the bpy-CO-NH-C2-NH-biotin and bpy-CO-NH-C6-NH-biotin 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 di-cationic MV^{2+} ion, it did not seem to play a very important role in the diminished quenching efficiency because the K_{SV} constants of the Ru_4-avidin adducts were essentially independent on the ionic strength of the solutions (data not shown). On the basis of these interesting results, the emission titration experiments were repeated with MV^{2+} being a quencher present in the bulk solution. Under low-salt conditions, in the presence of MV^{2+}, both complexes displayed more significant enhancement in emission intensities (ca. 2.4- and 2.0-fold for the bpy-CO-NH-C2-NH-biotin and bpy-CO-NH-C6-NH-biotin complexes, respectively, at [Ru]:[Avidin] = 4 (Table 5)) upon binding to avidin. The emission lifetimes were also extended upon the binding (Table 5), and the elongation factors showed improvement (ca. 2.5 and 2.1 for the bpy-CO-NH-C2-NH-biotin and bpy-CO-NH-C6-NH-biotin complexes, respectively) compared to the case in which the quencher was absent (ca. 1.4 and 1.2 for the bpy-CO-NH-C2-NH-biotin and bpy-CO-NH-C6-NH-biotin complexes, respectively) (Table 5). Under high-salt conditions, due to the more efficient quenching of the emission of the free complexes by MV^{2+}, higher amplification factors of both emission intensities (I/I_0) and lifetimes (τ/τ_0) at [Ru]:[Avidin] = 4 were anticipated. The titration curves for the bpy-CO-NH-C2-NH-biotin and bpy-CO-NH-C6-NH-biotin complexes in the presence of MV^{2+} under high-salt conditions are shown in Fig. 18. Our results clearly showed that the emission intensities of the bpy-CO-NH-C2-NH-biotin and bpy-CO-NH-C6-NH-biotin complexes were significantly enhanced by ca. 3.2- and 3.0-fold (Table 5), respectively, which were larger than those in the previous two cases. Meanwhile, the emission lifetime elongation factors (ca. 2.8 and 2.5 for the bpy-CO-NH-C2-NH-biotin and bpy-CO-NH-C6-NH-biotin complexes, respectively) were also the most significant among all three conditions (Table 5).

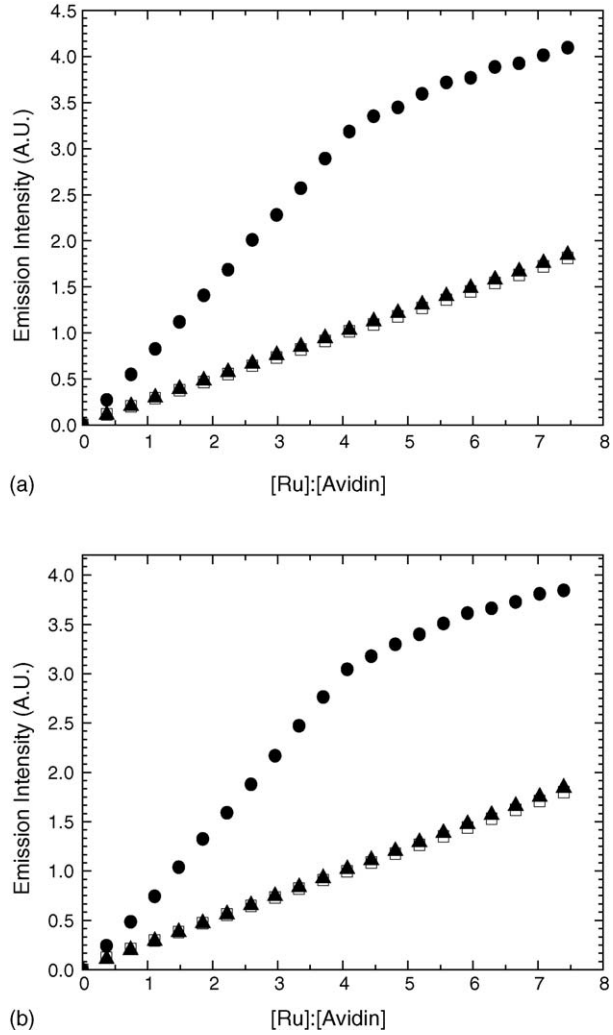

Fig. 18. Luminescence titration curves for the titrations of: (i) 3.8 μM avidin (●); (ii) 3.8 μM avidin and 380.0 μM unmodified biotin (▲); and (iii) phosphate buffer (□), in the presence of 15.0 mM MV^{2+} and 2.0 M KCl, with (a) $[Ru(bpy)_2(bpy\text{-}CO\text{-}NH\text{-}C2\text{-}NH\text{-}biotin)](PF_6)_2$ and (b) $[Ru(bpy)_2(bpy\text{-}CO\text{-}NH\text{-}C6\text{-}NH\text{-}biotin)](PF_6)_2$. Reproduced from [54], with permission of The American Chemical Society.

5. Conclusion

This review article summarises our recent work on the design of luminescent transition metal biotin complexes. It is obvious that avidin-induced emission enhancement is not limited

to rhenium(I), iridium(III) and ruthenium(II) but is common to luminescent transition metal complexes that show environment-sensitive emission with large Stokes' shifts. Our results showed that more hydrophobic complexes can offer a higher emission enhancement factor, but too high hydrophobicity will substantially lower the solubility of complexes in aqueous solution. Another observation was that 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_0) and lifetime (τ/τ_0), limiting their detection sensitivity. We are currently designing systems that show stronger binding to avidin with a higher emission enhancement factor. Our ultimate target is to identify light-switches for avidin, which could provide insight in the development of luminescent probes for other biological molecules.

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