DOI: 10.1002/ejic.200900518

Luminescent Polypyridinerhenium(I) Bis-Biotin Complexes as Crosslinkers for Avidin

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Keywords: Luminescence / Imaging agents / Sensors / Crosslinking / Rhenium / Cytotoxicity

Two novel luminescent polypyridinerhenium(I) bis-biotin complexes $[Re(N^\wedge N)(CO)_3(pyridine)](CF_3SO_3)$ {N^N = 4,4′-bis{[2-(biotinamido)ethyl]aminocarbonyl}-2,2′-bipyridine, bpyC2B2 (**1**), 4,4′-bis[(2-{[6-(biotinamido)hexanoyl]amino}ethyl)aminocarbonyl]-2,2′-bipyridine, bpyC2C6B2 (**2**)} and their biotin-free counterpart [N^N = 4,4′-bis(*n*-butylaminocarbonyl)-2,2′-bipyridine, bpyC4 (**3**)] have been synthesized and characterized. Upon irradiation, all the complexes exhibited triplet metal-to-ligand charge-transfer (^{3}MLCT) emission in fluid solutions at room temperature and alcohol glass at 77 K. The avidin-binding properties of the bis-biotin complexes **1** and **2** have been studied by 4′-hydroxyazobenzene-2-carboxylic acid (HABA) assays, emission titrations, and dissociation assays. The potential use of the complexes as signal amplifiers for heterogeneous recognition assays has been demonstrated using avidin-coated microspheres and one of the complexes. Additionally, the cytotoxicity of these rhenium(I) complexes towards the human cervix epithelioid carcinoma (HeLa) cell line has been evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assays. Furthermore, the cellular uptake of the complexes has been examined by laser-scanning confocal microscopy.

Introduction

The avidin-biotin system is a powerful tool in various bioanalytical applications due to the extraordinarily high affinity of biotin to avidin (first dissociation constant $K_d \approx 10^{-15}$ M).[1–3] Owing to the 1:4 (avidin:biotin) binding stoichiometry, small molecules with two or more biotin moieties are anticipated to have the potential to crosslink avidin. Some of these multiple-biotin compounds have been used to pretarget antibody-bound tumors,[4] increase the number of biotin-binding sites,[4a,4b,5a] construct polymeric architectures,[4a,4b,5a] and amplify detection signals in bioassays.[4a,4b,5] In view of the interesting emission behavior[6–26] and efficient cellular uptake properties[13a,14,26l] of luminescent polypyridinerhenium(I) complexes, and our interest in the use of transition metal complexes in biological studies,[27,28] we have developed a number of biological labels and probes using these complexes, including a series of biotin-containing complexes.[26c,26e,26g,26i,26l] Thus, we envisage that polypyridinerhenium(I) complexes funtionalized with two biotin units can serve as new luminescent crosslinkers for avidin.[28]

Herein, we report the synthesis, characterization, and photophysical properties of two luminescent polypyridinerhenium(I) bis-biotin complexes $[Re(N^\wedge N)(CO)_3(pyridine)](CF_3SO_3)$ {N^N = 4,4′-bis{[2-(biotinamido)ethyl]-aminocarbonyl}-2,2′-bipyridine, bpyC2B2 (**1**), 4,4′-bis[(2-{[6-(biotinamido)hexanoyl]amino}ethyl)aminocarbonyl]-2,2′-bipyridine, bpyC2C6B2 (**2**)} and their biotin-free counterpart $[Re(N^\wedge N)(CO)_3(pyridine)](CF_3SO_3)$ [N^N = 4,4′-bis(*n*-butylaminocarbonyl)-2,2′-bipyridine, bpyC4 (**3**)] (Scheme 1). Although we have previously reported a number of luminescent polypyridinerhenium(I) biotin complexes,[26c,26e,26g,26i,26l] none of the biotin pendants is attached to the diimine ligand, which is directly involved in the metal-to-ligand charge-transfer (MLCT) emissive states. Since the MLCT emission of polypyridinerhenium(I) complexes is usually very sensitive to their local environment,[8a,10a,12a] it is possible that the avidin-binding may have stronger influence on the emission properties of the complexes. In this work, the two biotin units are connected to the 2,2′-bipyridine ligand. The amide linkage attached to the bpy is expected to lower the emission energy, which is desirable for biological applications. The avidin-binding properties of the bis-biotin complexes have been studied by 4′-hydroxyazobenzene-2-carboxylic acid (HABA) assays, emission titrations, and dissociation assays. The potential use of the bis-biotin complexes as detection signal amplifiers for heterogeneous recognition assays has been demonstrated using avidin-coated microspheres and one of the complexes. Additionally, the cytotoxicity of these polypyridinerhenium(I) complexes towards the human cervix epithelioid carcinoma (HeLa) cell line has been evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assays. Furthermore, the cellular uptake properties of the complexes have been examined by laser-scanning confocal microscopy.

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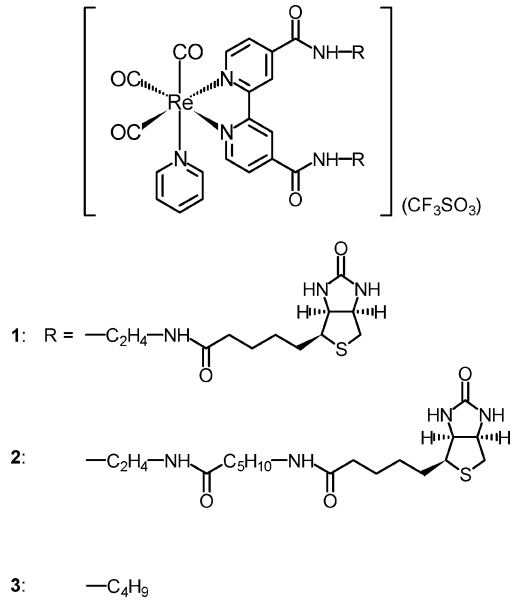

Scheme 1. Structures of complexes **1**–**3**.

Results and Discussion

Synthesis

The biotin units of complexes **1** and **2** were introduced to the C4 and C4′ positions of 2,2′-bipyridine via spacer-arms of different chain lengths.[28b] The synthesis involved the reaction of 4,4′-bis(methoxycarbonyl)-2,2′-bipyridine with excess ethylenediamine in CH_2Cl_2, yielding 4,4′-bis[(2-aminoethyl)aminocarbonyl]-2,2′-bipyridine. This compound was then reacted with biotinyl-*N*-hydroxysuccinimidyl ester and *N*-hydroxysuccinimidyl 6-biotinamidohexanoate,[29] to form the diimine ligands bpyC2B2 and bpyC2C6B2, respectively. The diimine ligand of complex **3**, bpyC4, was synthesized from the substitution reaction of 4,4′-bis(methoxycarbonyl)-2,2′-bipyridine with *n*-butylamine. Complexes **1**–**3** were synthesized by refluxing a pyridine solution of $[Re(N^N)(CO)_3(CH_3CN)](CF_3SO_3)$[30] and the samples were purified by column chromatography and recrystallization from MeOH/diethyl ether. The complexes were characterized by ^1H NMR spectroscopy, positive-ion ESI-MS, IR spectroscopy, and gave satisfactory microanalysis. All three complexes were yellow in color and very soluble in polar solvents such as alcohols and water.

Electronic Absorption and Luminescence Spectroscopy

The electronic absorption spectroscopic data of complexes **1**–**3** in MeOH are summarized in Table 1 and the electronic absorption spectrum of complex **2** in MeOH at 298 K is shown in Figure 1. All three complexes showed intense absorption bands at about 252–333 nm. With reference to the absorption studies of related polypyridinerhenium(I) complexes,[6a,7,8a,8b,9a,9b,10,11b,12,15–19,21–26] these bands have been assigned to spin-allowed intraligand (^1IL) ($\pi \rightarrow \pi^*$) (N^N and pyridine) transitions. The complexes also displayed less intense absorption peaks at ca. 373–376 nm, which have been assigned to spin-allowed MLCT [$d\pi(Re) \rightarrow \pi^*(N^N)$] transitions.[6a,7,8a,8b,9a,9b,10,11b,12,15,16b,17–19,20a,21–26] As expected, the absorption characteristics of the bis-biotin complexes **1** and **2** highly resembled those of their biotin-free counterpart complex **3** (Table 1) since they have the same core chromophoric unit.

Table 1. Electronic absorption spectroscopic data of complexes **1**–**3** in MeOH at 298 K.

Complex	λ_{abs} [nm] (ε [$dm^3\,mol^{-1}\,cm^{-1}$])
1	252 (16615), 285 sh (13575), 318 sh (9060), 332 sh (7630), 376 (3750)
2	252 (21530), 288 sh (17485), 318 sh (12350), 333 (10170), 373 (4780)
3	254 (18960), 283 sh (16155), 320 sh (9940), 332 sh (8820), 373 (4040)

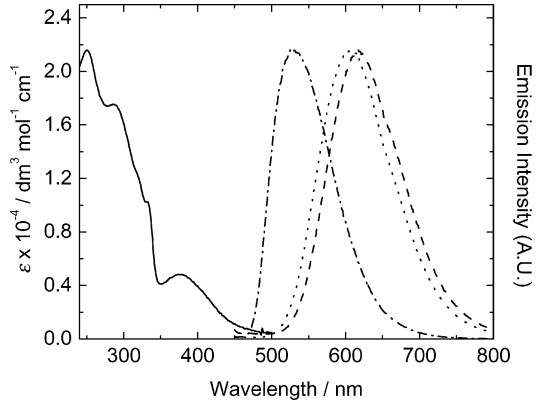

Figure 1. Electronic absorption (solid line) and emission spectra of complex **2** in MeOH (dotted line) and 50 mM phosphate buffer (dashed line) at 298 K and in EtOH/MeOH (4:1, v/v) (dash-dotted line) at 77 K.

Upon photoexcitation, all the complexes exhibited yellow to red luminescence in fluid solutions under ambient conditions and in low-temperature alcohol glass. The photophysical data of the complexes are summarized in Table 2. The emission spectra of complex **2** at 298 K in degassed solutions and in alcohol glass at 77 K are shown in Figure 1. In fluid solutions at room temperature, complexes **1**–**3** displayed a structureless emission band at about 560–616 nm. The emission of the complexes has been assigned to a spin-forbidden MLCT [$d\pi(Re) \rightarrow \pi^*(N^N)$] excited state. This assignment has been supported by (1) the strong dependence of the emission energies, quantum yields, and excited-state lifetimes on the polarity of the solvents, and (2) the large blue-shifts of emission maxima upon cooling the samples to 77 K due to rigidochromism.[6a,7,9,11b,12,13b,14,16,17,18b,19,20a,21,22,25,26] The emission properties of all three complexes are very similar, revealing that the two biotin pendants and the spacer-arms do not significantly perturb the electronic structures of the complexes.

Table 2. Photophysical data of complexes **1**–**3**.

Complex	Medium (T [K])	λ_{em} [nm]	τ_o [μs]	Φ_{em}
1	CH_3CN (298)	596	0.13	0.016
	MeOH (298)	604	0.088	0.011
	Buffer[a] (298)	615	0.071	0.0039
	Glass[b] (77)	527	5.52	
2	CH_3CN (298)	597	0.15	[c]
	MeOH (298)	606	0.072	0.0089
	Buffer[a] (298)	616	0.040	0.0054
	Glass[b] (77)	528	5.64	
3	CH_2Cl_2 (298)	560	0.55	0.20
	CH_3CN (298)	598	0.14	0.027
	MeOH (298)	604	0.090	0.011
	Buffer[a] (298)	613	0.052	0.0053
	Glass[b] (77)	528	5.58	

[a] 50 mM potassium phosphate buffer pH 7.4. [b] EtOH/MeOH (4:1, v/v). [c] The quantum yield cannot be determined due to insufficient solubility of the complex.

HABA Assays

The avidin-binding properties of complexes **1** and **2** have been studied by HABA assays, which are based on the competition between unmodified biotin or biotinylated species and HABA molecules upon binding to avidin.[3a] The binding of HABA to avidin is associated with an absorption feature at ca. 500 nm. Addition of biotin displaces the bound HABA molecules from avidin due to the much weaker avidin-binding of HABA ($K_d \approx 6 \times 10^{-6}$ M) compared to that of biotin ($K_d \approx 10^{-15}$ M),[1,3a] leading to a decrease of absorbance at 500 nm. Addition of complexes **1** and **2**, respectively, to a mixture of avidin and HABA resulted in a decrease of absorbance at 500 nm, indicating the binding of the biotin moieties of the rhenium(I) complexes to avidin. The results of spectrophotometric titrations of a mixture of avidin and excess HABA with complex **2** and unmodified biotin are shown in Figure 2. If both biotin moieties of the same complex can displace the avidin-bound HABA molecules, the equivalence point (eq. pt.) should occur at [Re]:[avidin] = 2 for these bis-biotin complexes. If only one of the two biotins functions, the eq. pt. will occur at 4. We found that both complexes **1** and **2** showed an eq. pt. at [Re]:[avidin] ≈ 2.5, which is < 4, suggesting that both biotins are functional. However, the values being larger than the ideal value 2 indicates that the binding is not sufficiently strong and/or not both biotin moieties of the complex are functional simultaneously, possibly due to steric hindrance.

Emission Titrations

The avidin-binding properties of the bis-biotin complexes **1** and **2** have been investigated by emission titrations, in which avidin was titrated with the complexes. The titration results have been compared to two control experiments in which (1) avidin was absent, and (2) avidin was presaturated with excess unmodified biotin. The titration curves of complexes **1** and **2** are shown in Figures 3 and 4, respectively, and the titration results are summarized in Table 3. Similar to our previous studies,[26c,26e,26g,26i,26l,28] both complexes **1** and **2** exhibited emission enhancement and lifetime extension in the presence of avidin (Table 3 and Figures 3 and 4). Additionally, the emission wavelengths of complexes **1** and **2** were blue-shifted from ca. 610 nm to 589 and 601 nm, respectively. These findings have been attributed to binding of the biotin moieties of the complexes to avidin because similar changes were not observed in the control experiments and in the case of the biotin-free complex **3**. These observed changes for complexes **1** and **2** have been attributed to the increased hydrophobicity and rigidity of the local environment of the complexes after they bind to avidin, which is in agreement with the emission data of the complexes in solvents of different polarity (Table 2). The eq. pt. of the titrations occurred at [Re]:[avidin] ≈ 2.5 (Figures 3 and 4), which is < 4, indicating that both biotin moieties of the same complex can bind to avidin. However, it is important to point out that the results of the HABA assays and emission titrations do not provide information on whether the binding is intermolecular or intramolecular with respect to avidin.

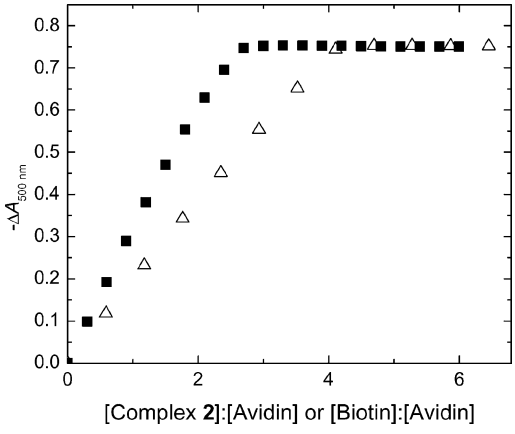

Figure 2. Spectrophotometric titrations of a mixture of avidin and excess HABA with complex **2** (solid squares) and unmodified biotin (hollow triangles).

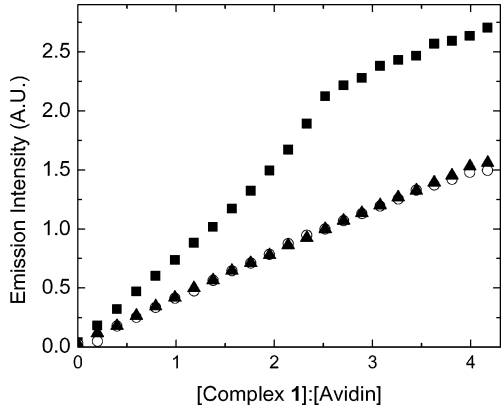

Figure 3. Emission titration curves for the titrations of (i) 3.8 μM avidin (solid squares), (ii) 3.8 μM avidin and 380 μM unmodified biotin (solid triangles), and (iii) a blank phosphate buffer solution (hollow circles) with complex **1**.

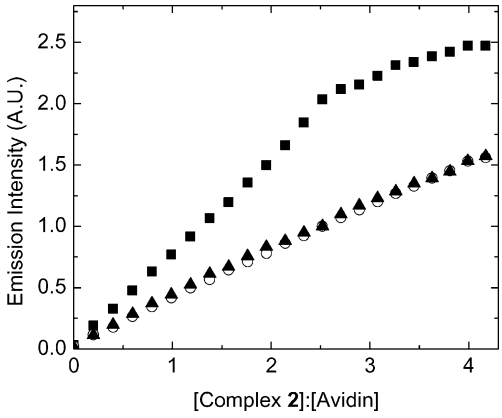

Figure 4. Emission titration curves for the titrations of (i) 3.8 μM avidin (solid squares), (ii) 3.8 μM avidin and 380 μM unmodified biotin (solid triangles), and (iii) a blank phosphate buffer solution (hollow circles) with complex **2**.

Table 3. Relative emission intensities and emission lifetimes of complexes **1** and **2** in the absence and presence of avidin (and excess biotin) in aerated 50 mM potassium phosphate buffer pH 7.4/MeOH (9:1, v/v) at 298 K.

Complex[a]	I (τ [μs])[b]	I (τ [μs])[c]	I (τ [μs])[d]
1	1.00 (0.070)	2.12 (0.24)	1.00 (0.072)
2	1.00 (0.043)	2.03 (0.12)	1.07 (0.046)

[a] [Re] = 9.9 μM. [b] [avidin] = 0 M, [biotin] = 0 M. [c] [avidin] = 3.8 μM, [biotin] = 0 M. [d] [avidin] = 3.8 μM, [biotin] = 380 μM.

Interestingly, the emission titration curves showed abnormal "extra" emission enhancement in the region when [Re]:[avidin] was between 2 and 2.5 (Figures 3 and 4). One possible reason is that the emission of these bis-biotin complexes is lower when both biotins bind to avidin, (biotin$_{bound}$–Re–biotin$_{bound}$, Form I) (regardless of inter- or intramolecular binding) whereas it is higher when only one of the two biotins binds to avidin (biotin$_{bound}$–Re–biotin$_{free}$, Form II). In the early part of the titrations, most of the complex molecules are in Form I. When the eq. pt. approaches, an increasing number of molecules exist in Form II. It is possible that when both biotins bind to the substrate-binding sites of avidin (Form I), the rhenium(I) diimine core is more exposed to the polar aqueous environment, giving rise to a lower emission intensity of the complex in this Form. To gain further insight, we have performed "backward" emission titrations in which avidin was used to titrate the bis-biotin complexes. The titration results for complexes **1** and **2** are shown in Figures 5 and 6, respectively. As shown in the figures, the emission intensity of the complexes was enhanced upon addition of avidin. Interestingly, the eq. pt. occurred at ca. 0.25 in both titrations ($I/I_o \approx 2.5$), indicating that the 1:4 adduct avidin-(Re)$_4$ (which is in Form II) is the predominant product in both cases. This is reasonable because the complexes were present in excess in early part of the titrations. At the end points, the emission wavelengths of complexes **1** and **2** (Form II) were ca. 585 and 583 nm, respectively, which are shorter than those of the products (Form I) in the "forward" titrations (589 and 601 nm, respectively), suggesting a more hydrophobic environment of the complexes in Form II than in Form I. Most importantly, beyond the eq. pt., the emission intensity of both complexes shows a substantial decrease (Figures 5 and 6), which can be ascribed to the conversion of Form II (higher intensity) to Form I (lower intensity) of the complexes. Nevertheless, it is important to emphasize that these pictures are based on the assumption that binding of the biotin units of the complexes to avidin is highly reversible.

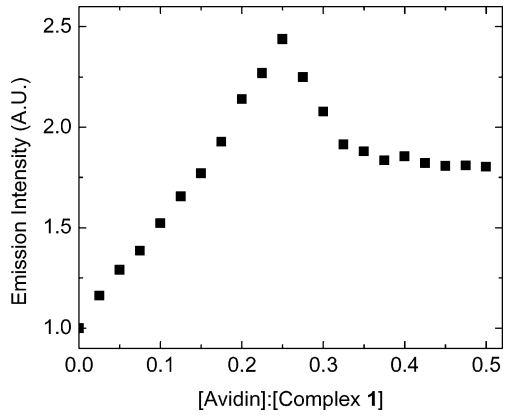

Figure 5. Emission titration curves for the titrations of 15.2 μM complex **1** with avidin.

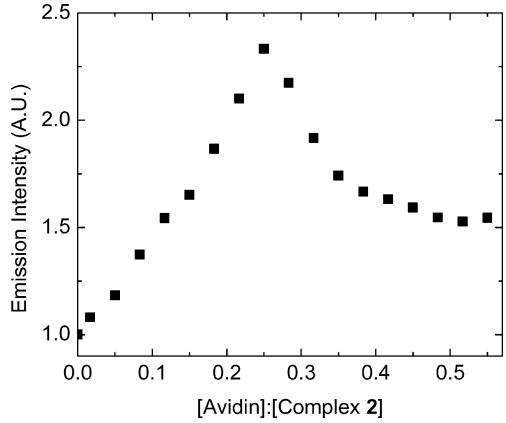

Figure 6. Emission titration curves for the titrations of 15.2 μM complex **2** with avidin.

Dissociation Assays

The stability of the avidin adducts of the bis-biotin complexes has been examined by dissociation assays. Dissociation of the avidin-bound rhenium(I) bis-biotin complex was induced by addition of excess unmodified biotin and the emission intensity of the solution was measured. The off-rate constants (k_{off}) for complexes **1** and **2** are 3.1×10^{-4} and 4.1×10^{-4} s^{-1}, respectively. The similar values reflect that the two spacer-arms of different chain lengths did not substantially affect the stability of the avidin complex adducts. These k_{off} values are similar to those of related rhenium(I) biotin complexes [Re(phen)(CO)$_3$(4-(*N*-((6-biotinamido)hexanoyl)aminomethyl)pyridine)]$^+$ (phen = 1,10-

phenanthroline) ($k_{off} = 2.8 \times 10^{-4}$ s^{-1})[26g] but about one order of magnitude smaller than those of [Re(phen)(CO)$_3$(4-(biotinamidomethyl)pyridine)]$^+$ ($k_{off} = 7.5 \times 10^{-3}$ s^{-1})[26g] which contained a shorter spacer-arm.

Signal Amplification

One of the targets of this work is the exploration of the bifunctional (luminescence and avidin-crosslinking) properties of the rhenium(I) bis-biotin complexes in emission signal amplification for the detection of biotinylated molecules. In this work, microspheres coated with avidin were successively incubated with complex **2**, with stringent washing after each incubation step. Laser-scanning confocal microscopy images of typical microspheres upon immobilization of zero to four layers of complex **2** are shown in Figure 7. It can be seen that the microsphere samples acquired increasing emission intensities upon successive immobilization of avidin and complex **2** (Figures 7 and 8). After coating of four layers of the complex, the average emission intensity of the microspheres was about 6.5 times higher than that of those immobilized with only one layer. It is important to point out that similar results were not obtained when a model protein bovine serum albumin (BSA) was used instead of avidin or when the avidin was presaturated with biotin from the outset (Figure 8), indicating that the increasing emission intensity resulted from the avidin-crosslinking properties of complex **2**. We anticipate that this interesting emission signal amplification can be applied in different heterogeneous assays for a wide range of biological interactions such as antigen/hapten-antibody recognition and DNA hybridization.

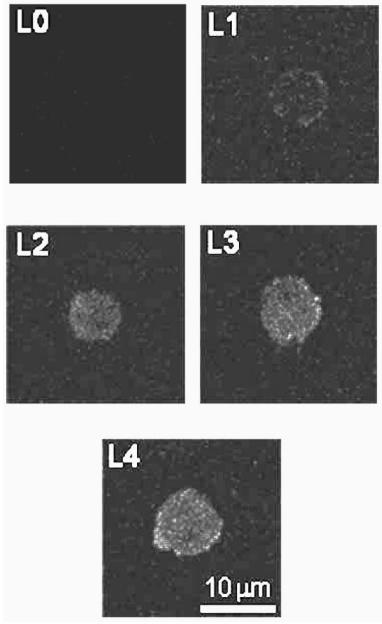

Figure 7. Confocal microscopy images of avidin-coated microspheres upon immobilization of zero (L0), one (L1), two (L2), three (L3), and four (L4) layers of complex **2**.

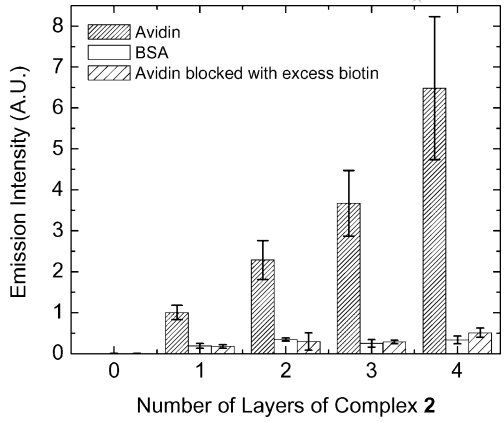

Figure 8. Averaged emission intensities of avidin-modified microspheres ($N = 6$) upon incubation of zero to four layers of complex **2**. The results of the control experiments (BSA was used instead of avidin or when the avidin was presaturated with biotin from the outset) are included.

Cytotoxicity

We have studied the cytotoxicity of complexes **1**–**3** by the MTT assay using HeLa cells as the model cell line.[31] The dose-dependence of surviving cells after exposure to the complexes for 48 h has been evaluated. Unfortunately, the solubility of the bis-biotin complexes **1** and **2** in aqueous solution was not sufficiently high for evaluation of their IC_{50} values. However, from the limited data points, it appears that these complexes are basically noncytotoxic (IC_{50} values estimated to be > 250 μM). In contrast, the biotin-free complex **3** exhibited a relatively low IC_{50} value (7.7 ± 1 μM), which is almost 3.3-fold smaller than that of cisplatin (25.6 ± 2.3 μM) under the same experimental conditions. The cytotoxicity of this complex is comparable to that of the diphosphanerhenium(I) complexes [Re(CO)$_3$(diphosphane)Br][32a] but much higher than that of related polypyridinerhenium(I) complexes [Re(2-appt)(CO)$_3$Cl] [2-appt = 2-amino-4-phenylamino-6-(2-pyridyl)-1,3,5-triazine] ($IC_{50} \approx 50$ μM) and [Ru(tBu$_2$-bpy)$_2$(2-appt)]$^{2+}$ (tBu$_2$-bpy = 4,4′-di-*tert*-butyl-2,2′-bipyridine) ($IC_{50} = 59.7$ μM), both of which have been found to bind to the minor groove of double-stranded DNA.[32b] Overall, complexes **1**–**3** are relatively noncytotoxic compared to the organometallic areneruthenium complexes [(η^6-arene)Ru(ethylenediamine)(X)](PF$_6$)$_n$ (X = substituted pyridines and halides), some of which exhibit fast hydrolysis kinetics and high cytotoxicity toward the human ovarian cancer cell line A2780.[32c] Interestingly, the polypyridinerhenium(I) biotin complexes [Re(N^N)(CO)$_3$(py-biotin-TU-Et)](PF$_6$) (py-biotin-TU-Et = 3-ethylthioureidyl-5-{*N*-[(2-biotinamido)ethyl]aminocarbonyl}pyridine, N^N = phen, 3,4,7,8-tetramethyl-1,10-phenanthroline, 4,7-diphenyl-1,10-phenanthroline) (IC_{50} = 17.5–28.5 μM)[26l] are more cytotoxic than complexes **1** and **2**. It seems that the number of biotin moieties on the polypyridinerhenium(I) complexes is related to their cytotoxicity. The more biotin pendants the complexes have, the less

cytotoxic the complexes are. It is conceivable that this is a consequence of the lower lipophilicity of the complexes brought about by the polar biotin units.

Live-Cell Confocal Imaging

Exploitation of luminescent polypyridinerhenium(I) complexes in in vivo biological applications has attracted much attention recently.[13a,14,26l] In view of the interesting luminescence properties of complexes **1**–**3**, the possibility of using these complexes as imaging reagents for live cells has been investigated using laser-scanning confocal microscopy. The microscopy images of HeLa cells treated with complexes **1**–**3** (10 μM, 6 h) are illustrated in Figure 9. The complexes retained their luminescence properties within the cells. However, the emission intensities of cells loaded with the bis-biotin complexes **1** and **2** are considerably weaker than that treated with the biotin-free complex **3** (Figure 9). We have tentatively attributed these observations to the lower cellular uptake efficiency of complexes **1** and **2** because all the complexes displayed comparable emission quantum yields in buffer solution (Table 2). The lower cellular uptake could be a result of the lower lipophilicity of the complexes. We have studied the cellular uptake of the avidin adducts of complexes **1** and **2** but the microscopy images were similar to those of the free complexes. HeLa cells treated with all the free complexes showed localization in the perinuclear region with much weaker or no emission from the nuclei, indicative of negligible nuclear uptake or net exclusion of the complexes from the nucleus (Figure 9). Similar observations have been observed in the polypyridinerhenium(I) mono-biotin complexes $[Re(N^N)(CO)_3(py\text{-}biotin\text{-}TU\text{-}Et)](PF_6)$.[26l] Based on these images, it appears that the bis-biotin complexes **1** and **2** bound to the Golgi apparatus. However, we cannot exclude that the complexes also binds to other lipophilic organelles such as endoplasmic reticulum and mitochrondria.

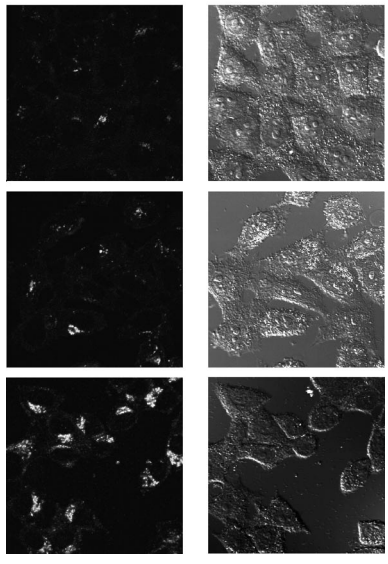

Figure 9. Laser-scanning confocal microscopy images of HeLa cells incubated with complexes **1** (top row), **2** (middle row) and **3** (bottom row) (10 μM) at 37 °C for 6 h.

Conclusions

In this work, two luminescent rhenium(I) bis-biotin complexes and their biotin-free counterpart have been synthesized and characterized. The photophysical properties of these complexes have been investigated. The avidin-binding properties of the bis-biotin complexes have been studied by HABA assays, emission titrations, and dissociation assays. The two bis-biotin complexes displayed emission enhancement and lifetimes elongation upon binding to avidin. The avidin-crosslinking properties have been demonstrated by microscopy studies using avidin-coated microspheres. Additionally, the cytotoxicity of all the complexes towards HeLa cells has been examined using the MTT assay. The results showed that the bis-biotin complexes were basically noncytotoxic while complex **3** was fairly cytotoxic, which could be related to the lipophilicity of the complexes. The cellular uptake of the complexes by HeLa cells has been investigated using laser-scanning confocal microscopy. The microscopic images revealed that the complexes retained their luminescence properties within the cells. We believe that these new biotin reagents with interesting bifunctional properties (luminescence and avidin-crosslinking) will be useful in the studies of biological interactions such as antigen/hapten-antibody recognization and DNA hybridization, and in the development of new luminescent transition metal-based live-cell imaging agents.

Experimental Section

Materials and Reagents: All buffer components were of biological grade and used as received. All solvents were of analytical reagent grade and were purified according to standard procedures. Ethylenediamine (Acros), triethylamine (Acros), and *n*-butylamine (Acros) were purified according to standard procedures. Silver trifluoromethanesulfonate (Acros), *N*-hydroxysuccinimide (Acros), *N*,*N*′-dicyclohexylcarbodiimide (Acros), chromium(VI) oxide (Acros), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) (Acros), cisplatin (Acros), biotin (Acros), 4,4′-dimethyl-2,2′-bipyridine (Aldrich), $Re(CO)_5Cl$ (Aldrich), avidin (Calbiochem), BSA (Calbiochem), HABA (Sigma), MTT (Sigma) were used as recevied. Biotinyl-*N*-hydroxysuccinimidyl ester and *N*-hydroxysuccinimidyl 6-biotinamidohexanoate were prepared according to reported procedures.[29] Microspheres modified with carboxyl groups were supplied by Bangs Laboratories. HeLa cells were obtained from American Type Culture Collection. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), phosphate buffered saline (PBS), trypsin-EDTA, and penicillin/streptomycin were purchased from Invitrogen. Unless specified, the growth medium for cell culture contained DMEM with 10% FBS and 1% penicillin/streptomycin.

General Procedures for the Synthesis of Polypyridinerhenium(I) Complexes 1–3: The synthetic procedures of the polypyridine ligands (N^N = bpyC2B2, bpyC2C6B2, and bpyC4)[28b] and $[Re(N^N)(CO)_3(CH_3CN)](CF_3SO_3)$[30] have been reported. Com-

plexes **1**–**3** were obtained from the reactions of $[Re(N^\wedge N)(CO)_3(CH_3CN)](CF_3SO_3)$ (88 μmol) and pyridine (10 mL) under refluxing conditions for 4 h. The resultant reaction mixture was evaporated to dryness to give a yellow solid. The complex was purified by column chromatography on alumina. The desired product was eluted with CH_3CN/MeOH (3:1, v/v). Recrystallization of the crude product from MeOH/diethyl ether afforded the complex as air-stable yellow crystals.

[Re(bpyC2B2)(CO)$_3$(pyridine)](CF$_3$SO$_3$) (1): Yield 72 mg (62%). ^{1}H NMR (300 MHz, [D$_6$]DMSO, 298 K, relative to TMS): δ = 9.48 (d, J = 5.7 Hz, 2 H, 6-H and 6-H* of bpy), 9.32–9.26 (m, 2 H, bpyCON*H*), 8.99 (s, 2 H, 3-H and 3-H* of bpy), 8.42 (d, J = 6.0 Hz, 2 H, 2-H and 6-H of pyridine), 8.19 (d, J = 6.3 Hz, 2 H, 5-H and 5-H* of bpy), 7.99–7.93 (m, 3 H, 4-H of pyridine, C_2H_4-N*H*-biotin), 7.42 (t, J = 6.0 Hz, 2 H, 3-H and 5-H of pyridine), 6.42 (s, 2 H, NH of biotin), 6.37 (s, 2 H, NH of biotin), 4.32–4.24 (m, 2 H, NCH of biotin), 4.14–4.09 (m, 2 H, NCH of biotin), 3.07–3.03 (m, 2 H, SCH of biotin), 2.89–2.74 (m, 2 H, SCH of biotin), 2.08–2.03 (m, 4 H, COCH_2 of biotin), 1.65–1.15 (m, 12 H, $COCH_2C_3H_6$ of biotin) ppm. IR (KBr): $\tilde{\nu}$ = 3406 (m, N–H), 2919 (s, C–H), 2034 (s, C≡O), 1926 (s, C≡O), 1695 (m, C=O), 1157 (m, $CF_3SO_3^-$), 1029 (m, $CF_3SO_3^-$) cm^{-1}. Positive-ion ESI-MS: ion cluster at m/z 1130 $[M - CF_3SO_3]^+$. $C_{45}H_{53}F_3N_{11}O_{12}ReS_3{\cdot}2.5H_2O$ (1324.4): calcd. C 40.81, H 4.41, N 11.63; found C 40.66, H 4.64, N 11.51.

[Re(bpyC2C6B2)(CO)$_3$(pyridine)](CF$_3$SO$_3$) (2): Yield 69 mg (52%). ^{1}H NMR (300 MHz, [D$_6$]DMSO, 298 K, relative to TMS): δ = 9.48 (d, J = 6.0 Hz, 2 H, 6-H and 6-H* of bpy), 9.44–9.35 (m, 2 H, bpyCON*H*), 9.04 (s, 2 H, 3-H and 3-H* of bpy), 8.41 (d, J = 5.1 Hz, 2 H, 2-H and 6-H of pyridine), 8.19 (d, J = 6.0 Hz, 2 H, 5-H and 5-H* of bpy), 8.01–7.93 (m, 3 H, 4-H of pyridine, C_2H_4N*H*CO), 7.74 (m, 2 H, C_6H_{12}-N*H*-biotin), 7.42 (t, J = 6.3 Hz, 2 H, 3-H and 5-H of pyridine), 6.43 (s, 2 H, NH of biotin), 6.37 (s, 2 H, NH of biotin), 4.30–4.26 (m, 2 H, NCH of biotin), 4.14–4.09 (m, 2 H, NCH of biotin), 3.10–3.03 (m, 2 H, SCH of biotin), 2.97–2.92 (m, 4 H, CH_2NH-biotin), 2.82–2.74 (m, 2 H, SCH of biotin), 2.08–1.97 (m, 8 H, COCH_2), 1.63–1.11 (m, 24 H, $CONHCH_2C_3H_6CH_2NHCOCH_2C_3H_6$) ppm. IR (KBr): 3411 (m, N–H), 2934 (s, C–H), 2033 (s, C≡O), 1931 (s, C≡O), 1659 (m, C=O), 1163 (m, $CF_3SO_3^-$), 1029 (m, $CF_3SO_3^-$). Positive-ion ESI-MS: ion cluster at m/z 1356 $[M - CF_3SO_3]^+$. $C_{57}H_{75}F_3N_{13}O_{14}ReS_3{\cdot}2.5H_2O$ (1550.7): calcd. C 44.15, H 5.20, N 11.74; found C 44.04, H 5.20, N 11.72.

[Re(bpyC4)(CO)$_3$(pyridine)](CF$_3$SO$_3$) (3): Yield 70 mg (56%). ^{1}H NMR (300 MHz, [D$_6$]DMSO, 298 K, relative to TMS): δ = 9.45 (d, J = 6.0 Hz, 2 H, 6-H and 6-H* of bpy), 9.27–9.16 (m, 2 H, bpyCON*H*), 9.00 (s, 2 H, 3-H and 3-H* of bpy), 8.40 (d, J = 4.8 Hz, 2 H, 2-H and 6-H of pyridine), 8.19 (d, J = 6.0 Hz, 2 H, 5-H and 5-H* of bpy), 7.95 (t, J = 7.8 Hz, 1 H, 4-H of pyridine), 7.41 (t, J = 7.5 Hz, 2 H, 3-H and 5-H of pyridine), 4.11 (q, J = 5.4 Hz, 4 H, $NHCH_2C_3H_7$), 1.51 (p, J = 7.8 Hz, 4 H, $NHCH_2CH_2C_2H_5$), 1.33 (se, J = 7.2 Hz, 4 H, $NHC_2H_5CH_2CH_3$), 0.89 (t, J = 7.5 Hz, 6 H, CH_3) ppm. IR (KBr): $\tilde{\nu}$ = 3457 (m, N–H), 2960 (s, C–H), 2028 (s, C≡O), 1915 (s, C≡O), 1654 (m, C=O), 1158 (m, $CF_3SO_3^-$), 1030 (m, $CF_3SO_3^-$) cm^{-1}. Positive-ion ESI-MS: ion cluster at m/z 703 $[M - CF_3SO_3]^+$. $C_{29}H_{31}F_3N_5O_8ReS$ (852.9): calcd. C 40.84, H 3.66, N 8.21; found C 41.09, H 3.52, N 8.12.

Instrumentation and Methods: NMR spectra were recorded on a Varian Mercury 300 MHz NMR spectrometer at 298 K. Positive-ion ESI mass spectra were recorded on a Perkin–Elmer Sciex API 365 mass spectrometer. IR spectra were recorded on a Perkin–Elmer 1600 series FT-IR spectrophotometer. Elemental analyses were carried out on a Vario EL III CHN elemental analyzer. Electronic absorption, steady-state emission spectra were recorded on a Hewlett–Packard 8453 diode array spectrophotometer and a SPEX FluoroLog 3-TCSPC spectrophotometer, respectively. The emission lifetimes were measured in the Fast MCS or a TCSPC lifetime mode with a NanoLED N-340 or NanoLED N-375 as the excitation source, respectively. Unless specified, all the solutions for photophysical studies were degassed with no fewer than four successive freeze-pump-thaw cycles and stored in a 10-cm^3 round-bottomed flask equipped with a sidearm 1-cm fluorescence cuvette and sealed from the atmosphere by a Rotaflo HP6/6 quick-release Teflon® stopper. Luminescence quantum yields were measured by the optically dilute method[33] with an aerated aqueous solution of $[Ru(bpy)_3]Cl_2$ (Φ_{em} = 0.028, λ_{ex} = 455 nm)[34] as the standard solution.

HABA Assays: To a mixture of avidin (7.6 μM) and HABA (300 μM) in 50 mM potassium phosphate buffer pH 7.4 (2 mL) were added 5 μL aliquots of the bis-biotin complex (1.1 mM) in MeOH at 1-min intervals. The formation of the avidin complex adduct was indicated by a decrease in the absorbance at 500 nm due to the displacement of HABA molecules from the avidin by the complex. A plot of $-\Delta A_{500nm}$ vs. [Re]:[avidin] was constructed. Titrations using unmodified biotin were also performed.

Emission Titrations: Avidin (3.8 μM) in 50 mM potassium phosphate buffer pH 7.4 (2 mL) was titrated with the bis-biotin complex (0.30 mM) in MeOH by additions of 5 μL aliquots at 1-min intervals. The solution was excited at 355 nm, and the emission intensity was measured. The equivalence points were determined by linear fitting of the initial and final parts of the titration curves. In the "backward" titrations, the complex (15.2 μM) in 50 mM potassium phosphate buffer pH 7.4/MeOH (9:1, v/v) (2 mL) was titrated with avidin (0.15 mM) in 50 mM potassium phosphate buffer pH 7.4.

Dissociation Assays: The dissociation of the avidin-bound bis-biotin complex was induced by addition of excess biotin (2 μmol) in 50 mM potassium phosphate buffer pH 7.4 (200 μL) to a mixture of the bis-biotin complex (20 nmol) and avidin (7.6 nmol) in 50 mM potassium phosphate buffer pH 7.4/MeOH (9:1, v/v) (1.8 mL). The kinetics of the dissociation was monitored by the decrease in emission intensity. The reaction proceeded under pseudo-first-order conditions. The pseudo-first-order rate constants, k_{off}, were obtained by nonlinear least-square fits of I_{obs} vs. time t according to the following equation:[35]

$$I_{obs} = I_{min} + (I_{max} - I_{min}) \exp(-k_{off}\, t)$$

where I_{obs}, I_{max}, and I_{min} are the emission intensities of the apparent, initial, and final forms of the bis-biotin complex, respectively.

Signal Amplification Studies: A 10-μL portion of the microsphere sample was centrifuged and the solid was washed with 50 mM potassium phosphate buffer pH 7.4 (4 × 1 mL). The microspheres were then resuspended in 1 mL phosphate buffer. Avidin (60 nmol) and EDC (0.3 nmol) in 1 mL phosphate buffer were added to the microsphere suspension. The mixture was incubated at room temperature for 12 h. The conjugated microspheres were then collected by centrifugation, washed with phosphate buffer (6 × 1 mL), and resuspended in 200 μL of the same buffer to give a microsphere stock solution. The avidin-coated microspheres were resuspended in 1 mL of phosphate buffer as a stock mixture. A 15-μL portion of the mixture was pipetted onto a glass slide and examined by a laser-scanning confocal microscope (Leica TCS SPE) (λ_{ex} = 405 nm; λ_{em} > 532 nm). The stock mixture was incubated with complex **2** (0.6 mmol) dissolved in a mixture of 100 μL anhydrous DMSO and 900 μL phosphate buffer for 15 min. The microspheres

were then washed with DMSO/phosphate buffer (1:9, v/v) (1 mL) and phosphate buffer containing 0.1% BSA (1 mL) to remove excess complex and nonspecifically-bound complex. The supernatant was removed after centrifugation. Then, the microspheres were incubated with an avidin solution in phosphate buffer (10 mg/1 mL) for 15 min and then washed with phosphate buffer (3 × 1 mL) to remove excess avidin. Four cycles of alternative incubation with complex **2** and then with avidin were performed. After each cycle, the microspheres were resuspended in 1 mL of phosphate buffer, and a 15-μL portion of the mixture was examined by confocal microscopy.

MTT Assays: Cytotoxicity assays were conducted in 96-well, flat-bottomed microtiter plates. The supplemented culture medium (100 μL) with about 10,000 cells per well was incubated at 37 °C under a 5% CO_2 atmosphere for 24 h. The polypyridinerhenium(I) complex was dissolved in the culture medium with 1% DMSO and the solutions were added to the wells. After the microtiter plate was incubated for 48 h, MTT in PBS (5 mg/mL, 10 μL) was added to each well. The microplate was incubated for another 3 h. The medium was removed carefully and 2-propanol (200 μL) was added to each well. All the assays were run in parallel with a positive control, in which cisplatin was used as a cytotoxic agent. The absorbance of all the solutions at 570 nm was measured with a SPECTRAmax 340 microplate reader (Molecular Devices Corporation, California). The IC_{50} values of the complexes were evaluated based on the percentage cell survival in a dose-dependent manner relative to the controls.

Live-Cell Confocal Imaging: HeLa cells in growth medium (ca. 100,000 cells/mL) were seeded on a sterilized coverslip in a 35-mm tissue culture dish and grown at 37 °C under a 5% CO_2 atmosphere for 48 h. The culture medium was then removed and replaced with medium/DMSO (99:1, v/v) containing the polypyridinerhenium(I) complex (5 μM). After incubation for 1 h, the medium was removed, and the cell layer was washed gently with PBS (5 × 1 mL). The coverslip was mounted onto a sterilized glass slide and then imaged using a Leica TCS SPE confocal microscope with the excitation wavelength at 405 nm and the emission was measured using a 532-nm long-pass filter.

Acknowledgments

We thank the City University of Hong Kong for financial support (Project Numbers 7002098 and 7002483). M.-W. L. acknowledges the receipt of a Postgraduate Studentship administered by the City University of Hong Kong.

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Received: June 10, 2009
Published Online: August 28, 2009

