Inorg. Chem. **2007**, *46*, 700–709

Cyclometalated Iridium(III) Diimine Bis(biotin) Complexes as the First Luminescent Biotin-Based Cross-Linkers for Avidin

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Received July 3, 2006

Four luminescent cyclometalated iridium(III) diimine complexes $[Ir(N–C)_2(N–N)](PF_6)$ (HN–C = 2-(4-(*N*-((2-biotinamido)ethyl)aminomethyl)phenyl)pyridine, Hppy-4-CH_2NHC_2NH-biotin, N–N = 3,4,7,8-tetramethyl-1,10-phenanthroline, Me_4-phen (**1a**); N–N = 4,7-diphenyl-1,10-phenanthroline, Ph_2-phen (**2a**); HN–C = 2-(4-(*N*-((6-biotinamido)hexyl)aminomethyl)phenyl)pyridine, Hppy-4-CH_2NHC_6NH-biotin, N–N = Me_4-phen (**1b**); N–N = Ph_2-phen (**2b**)), each containing two biotin units, have been synthesized and characterized. The photophysical and electrochemical properties of these complexes have been investigated. Photoexcitation of these iridium(III) diimine bis(biotin) complexes in fluid solutions at 298 K and in alcohol glass at 77 K resulted in intense and long-lived luminescence. The emission is assigned to a triplet metal-to-ligand charge-transfer (^{3}MLCT) ($d\pi(Ir) \rightarrow \pi^*(N–N)$) excited state. The emissive states of complexes **1a**,**b** are probably mixed with some ^{3}IL ($\pi \rightarrow \pi^*$) (Me_4-phen) character. The interactions of these iridium(III) diimine bis(biotin) complexes with avidin have been studied by 4′-hydroxyazobenzene-2-carboxylic acid (HABA) assays and emission titrations. The potential for these complexes to act as cross-linkers for avidin has been examined by resonance-energy transfer- (RET-) based emission quenching experiments, microscopy studies using avidin-conjugated microspheres, and HPLC analysis.

Introduction

The biotin−avidin system has been widely exploited as a powerful tool in a variety of bioanalytical applications because the affinity of biotin to avidin is extremely high (first dissociation constant K_d = ca. 10^{-15} M) and the properties of various biomolecules are usually retained after biotinylation.[1,2] Small molecules equipped with two or more biotin moieties have been used to pretarget antibody-bound tumors, to increase the number of biotin-binding sites, and to afford polymeric architectures.[3,4] However, avidin cross-linkers which possess useful reporting properties such as fluorescence, to the best of our knowledge, have not been explored. In view of the remarkable photophysical properties of luminescent iridium(III) polypyridine complexes[5−20] and our recent interest in transition metal complex-based biological

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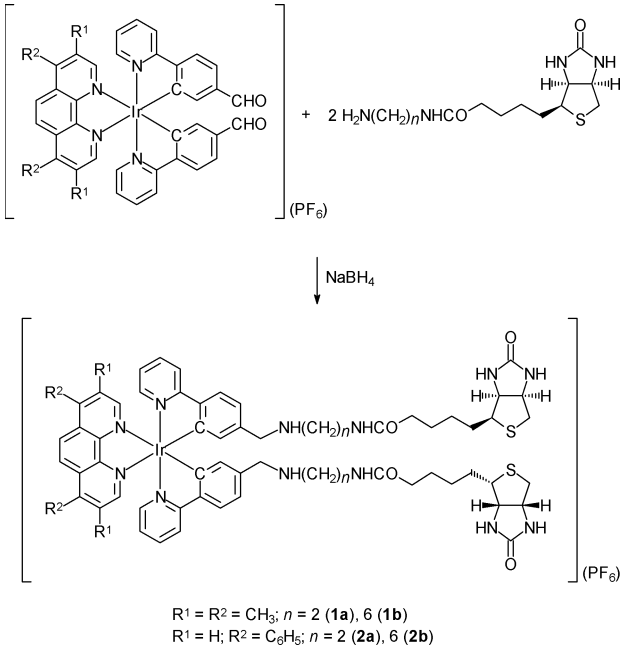
10.1021/ic0612202 CCC: $37.00
Published on Web 01/12/2007

labels and probes,[20,21] we anticipate that iridium(III) polypyridine complexes could be exploited as new luminescent cross-linkers for avidin. We have previously shown that luminescent transition metal biotin complexes, unlike their organic counterparts, display emission enhancement after binding to avidin.[20d−f,21b,d−f,h] Related luminescent avidin cross-linkers are expected to enable the formation of macromolecular reagents offering higher sensitivity in bioassays.

This paper describes the synthesis, characterization, and photophysical and electrochemical properties of four luminescent cyclometalated iridium(III) diimine complexes [Ir(N−C)$_2$(N−N)](PF$_6$) (HN−C = 2-(4-(*N*-((2-biotinamido)-ethyl)aminomethyl)phenyl)pyridine, Hppy-4-CH$_2$NHC$_2$NH-biotin, N−N = 3,4,7,8-tetramethyl-1,10-phenanthroline, Me$_4$-phen (**1a**); N−N = 4,7-diphenyl-1,10-phenanthroline, Ph$_2$-phen (**2a**); HN−C = 2-(4-(*N*-((6-biotinamido)hexyl)-aminomethyl)phenyl)pyridine, Hppy-4-CH$_2$NHC$_6$NH-biotin, N−N = Me$_4$-phen (**1b**); N−N = Ph$_2$-phen (**2b**)), each containing two biotin units; the synthesis of these complexes is outlined in Scheme 1. The interactions of these complexes with avidin have been studied by 4′-hydroxyazobenzene-2-carboxylic acid (HABA) assays and emission titrations. The potential for these luminescent complexes to act as cross-linkers for avidin has been examined by resonance-energy transfer- (RET-) based emission quenching experiments, microscopy studies using avidin-conjugated microspheres, and HPLC analysis.

Scheme 1. Synthesis of the Iridium(III) Diimine Bis(biotin) Complexes



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Experimental Section

Materials and Synthesis. All solvents were of analytical grade and purified according to standard procedures.[22] All buffer components were of biological grade and used as received. $NaBH_4$ (Acros), biotin (Acros), HABA (Sigma), avidin (Calbiochem), 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC) (Acros), and QSY-7 hydroxysuccinimidyl ester (Molecular Probes) were used without further purification. Green fluorescent and nonfluorescent microspheres that were modified with carboxyl groups were supplied by Bangs Laboratories. *N*-Biotinyl-1,6-diaminohexane,[23] *N*-biotinyl-1,2-diaminoethane,[24] and $[Ir(pba)_2(N-N)](PF_6)$ (Hpba = 4-(2-pyridyl)benzaldehyde)[20b] were synthesized according to literature procedures. Tetra-*n*-butylammonium hexafluorophosphate (TBAP) was obtained from Aldrich and was recrystallized from hot ethanol and dried in vacuo at 110 °C before use. NAP-5 size-exclusion columns and YM-30 microcons were purchased from Pharmacia and Amicon, respectively.

$[Ir(ppy\text{-}4\text{-}CH_2NHC_2NH\text{-}biotin)_2(Me_4\text{-}phen)](PF_6)$ (1a). A mixture of $[Ir(pba)_2(Me_4\text{-}phen)](PF_6)$ (100 mg, 0.11 mmol), *N*-biotinyl-1,2-diaminoethane (63 mg, 0.22 mmol), and 200 μL of triethylamine in 50 mL of methanol was refluxed under an inert atmosphere of nitrogen in the dark for 2 h. After the yellow solution was cooled to room temperature, solid $NaBH_4$ (33 mg, 0.88 mmol) was added to the solution. The solution was stirred under an inert atmosphere of nitrogen for 12 h and evaporated to dryness. The solid was redissolved in CH_2Cl_2 (50 mL) and then washed with distilled water (30 mL × 3). The CH_2Cl_2 layer was dried over anhydrous $MgSO_4$, filtered, and evaporated to dryness to give a yellow solid. Subsequent recrystallization of the product from a methanol/diethyl ether mixture afforded complex **1a** as yellow crystals. Yield = 80 mg (49%). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K, TMS): δ = 8.47 (s, 2H; H2 and H9 of Me_4-phen), 8.17 (d, J = 8.2 Hz, 2H; H3 of pyridyl ring of ppy$^-$), 7.87−7.79 (m, 6H; H5 and H6 of Me_4-phen and H4 of pyridyl ring and H6 of phenyl ring of ppy$^-$), 7.67 (t, J = 5.5 Hz, 2H; N*H*-biotin), 7.41 (d, J = 6.2 Hz, 2H; H6 of pyridyl ring of ppy$^-$), 7.03 (d, J = 7.9 Hz, 2H; H5 of phenyl ring of ppy$^-$), 6.93 (t, J = 6.3 Hz, 2H; H5 of pyridyl ring of ppy$^-$), 6.42 (s, 2H; NH of biotin), 6.36 (s, 2H; NH of biotin), 6.23 (s, 2H; H3 of phenyl ring of ppy$^-$), 4.27−4.25 (m, 2H; NCH of biotin), 4.10−4.08 (m, 2H; NCH of biotin), 3.46 (d, J = 4.4 Hz, 4H; $^-$ppy-4-C*H*$_2$NH), 3.08−3.01 (m, 6H; SCH of biotin and C*H*$_2$NH-biotin), 2.79 (s, 6H; CH_3 at C4 and C7 of Me_4-phen), 2.75−2.71 (m, 2H; SCH of biotin), 2.55 (s, 2H; SCH of biotin), 2.40 (t, J = 6.2 Hz, 4H; NHC*H*$_2$CH$_2$NHCO), 2.29 (s, 6H; CH_3 at C3 and C8 of Me_4-phen), 2.01 (t, J = 7.3 Hz, 4H; COC*H*$_2$C$_3$H$_6$ of biotin), 1.56−1.21 ppm (m, 12H; COCH$_2$C$_3$*H*$_6$ of biotin). IR (KBr) (ν/cm^{-1}): 3424 (br, N−H), 3278 (br, N−H), 1700 (s, C═O), 844 (s, PF_6^-). A positive-ion ESI-MS ion cluster was found at *m*/*z* 678, $\{M^+ + Na^+\}^{2+}$. Anal. Calcd for $C_{64}H_{76}N_{12}O_4S_2F_6PIr\cdot H_2O$: C, 51.36; H, 5.25; N, 11.23. Found: C, 51.42; H, 5.23; N, 11.10.

$[Ir(ppy\text{-}4\text{-}CH_2NHC_6NH\text{-}biotin)_2(Me_4\text{-}phen)](PF_6)$ (1b). The procedure was similar to that employed for the preparation of complex **1a**, except that *N*-biotinyl-1,6-diaminohexane (75 mg, 0.22 mmol) was used instead of *N*-biotinyl-1,2-diaminoethane. Complex **1b** was isolated as orange yellow crystals. Yield = 85 mg (49%). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K, TMS): δ = 8.47 (s, 2H; H2 and H9 of Me_4-phen), 8.16 (d, J = 7.9 Hz, 2H; H3 of pyridyl ring of ppy$^-$), 7.86−7.79 (m, 6H; H5 and H6 of Me_4-phen and H4 of pyridyl ring and H6 of phenyl ring of ppy$^-$), 7.72 (t, J = 5.5 Hz, 2H; N*H*-biotin), 7.39 (d, J = 5.6 Hz, 2H; H6 of pyridyl ring of ppy$^-$), 6.99 (d, J = 7.3 Hz, 2H; H5 of phenyl ring of ppy$^-$), 6.92 (t, J = 6.6 Hz, 2H; H5 of pyridyl ring of ppy$^-$), 6.42 (s, 2H; NH of biotin), 6.36 (s, 2H; NH of biotin), 6.25 (s, 2H; H3 of phenyl ring of ppy$^-$), 4.28−4.24 (m, 2H; NCH of biotin), 4.09−4.07 (m, 2H; NCH of biotin), 3.45 (d, J = 3.5 Hz, 4H; $^-$ppy-4-C*H*$_2$NH), 3.10−2.95 (m, 6H; SCH of biotin and C*H*$_2$NH-biotin), 2.79 (s, 6H; CH_3 at C4 and C7 of Me_4-phen), 2.75−2.73 (m, 2H; SCH of biotin), 2.54 (s, 2H; SCH of biotin), 2.29−2.26 (m, 10H; NHC*H*$_2$C$_4$H$_8$-CH$_2$NHCO and CH_3 at C3 and C8 of Me_4-phen), 2.00 (t, J = 7.0 Hz, 4H; COC*H*$_2$C$_3$H$_6$ of biotin), 1.52−1.16 ppm (m, 28H; NHCH$_2$C$_4$*H*$_8$CH$_2$NHCO and COCH$_2$C$_3$*H*$_6$ of biotin). IR (KBr) (ν/cm^{-1}): 3424 (br, N−H), 3280 (br, N−H), 1699 (s, C═O), 844 (s, PF_6^-). A positive-ion ESI-MS ion cluster was found at *m*/*z* 734, $\{M^+ + Na^+\}^{2+}$. Anal. Calcd for $C_{72}H_{92}N_{12}O_4S_2F_6PIr$: C, 54.36; H, 5.83; N, 10.57. Found: C, 54.61; H, 5.93; N, 10.27.

$[Ir(ppy\text{-}4\text{-}CH_2NHC_2NH\text{-}biotin)_2(Ph_2\text{-}phen)](PF_6)$ (2a). The procedure was similar to that employed for the preparation of complex **1a**, except that $[Ir(pba)_2(Ph_2\text{-}phen)](PF_6)$ (111 mg, 0.11 mmol) was used instead of $[Ir(pba)_2(Me_4\text{-}phen)](PF_6)$. Complex **2a** was isolated as orange crystals. Yield = 82 mg (47%). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K, TMS): δ = 8.26−8.20 (m, 6H; H2 and H5 of Ph_2-phen and H3 of pyridyl ring of ppy$^-$), 8.05 (d, J = 5.3 Hz, 2H; H3 of Ph_2-phen), 7.91−7.86 (m, 4H; H3 of pyridyl ring and H6 of phenyl ring of ppy$^-$), 7.65−7.58 (m, 14H; Ph of Ph_2-phen, H6 of pyridyl ring of ppy$^-$ and N*H*-biotin), 7.05−7.00 (m, 4H; H5 of pyridyl ring and H5 of phenyl ring of ppy$^-$), 6.42 (s, 2H; NH of biotin), 6.36 (s, 2H; NH of biotin), 6.25 (s, 2H; H3 of phenyl ring of ppy$^-$), 4.27−4.24 (m, 2H; NCH of biotin), 4.09−4.07 (m, 2H; NCH of biotin), 3.47 (s, 4H; $^-$ppy-4-C*H*$_2$NH), 3.10−3.02 (m, 6H; SCH of biotin and C*H*$_2$NH-biotin), 2.76 (dd, J_{gem} = 12.6 Hz, J_{vic} = 5.6 Hz, 2H; SCH of biotin), 2.55 (s, 2H; SCH of biotin), 2.42 (t, J = 6.2 Hz, 4H; NHC*H*$_2$CH$_2$NHCO), 2.00 (t, J = 7.2 Hz, 4H; COC*H*$_2$C$_3$H$_6$ of biotin), 1.56−1.25 ppm (m, 12H; COCH$_2$C$_3$*H*$_6$ of biotin). IR (KBr) (ν/cm^{-1}): 3424 (br, N−H), 3277 (br, N−H), 1698 (s, C═O), 841 (s, PF_6^-). A positive-ion ESI-MS ion cluster was found at *m*/*z* 726, $\{M^+ + Na^+\}^{2+}$. Anal. Calcd for $C_{72}H_{76}N_{12}O_4S_2F_6PIr\cdot H_2O$: C, 54.29; H, 4.94; N, 10.55. Found: C, 54.18; H, 4.90; N, 10.68.

$[Ir(ppy\text{-}4\text{-}CH_2NHC_6NH\text{-}biotin)_2(Ph_2\text{-}phen)](PF_6)$ (2b). The procedure was similar to that employed for the preparation of complex **1b**, except that $[Ir(pba)_2(Ph_2\text{-}phen)](PF_6)$ (111 mg, 0.11 mmol) was used instead of $[Ir(pba)_2(Me_4\text{-}phen)](PF_6)$. Complex **2b** was isolated as orange crystals. Yield = 96 mg (52%). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K, TMS): δ = 8.27−8.19 (m, 6H; H2 and H5 of Ph_2-phen and H3 of pyridyl ring of ppy$^-$), 8.04 (d, J = 5.6 Hz, 2H; H3 of Ph_2-phen), 7.89−7.85 (m, 4H; H3 of pyridyl ring and H6 of phenyl ring of ppy$^-$), 7.73 (t, J = 5.3 Hz, 2H; N*H*-biotin), 7.64−7.57 (m, 12H; Ph of Ph_2-phen and H6 of pyridyl ring of ppy$^-$), 7.02−6.99 (m, 4H; H5 of pyridyl ring and H5 of phenyl ring of ppy$^-$), 6.41 (s, 2H; NH of biotin), 6.35 (s, 2H; NH of biotin), 6.27 (s, 2H; H3 of phenyl ring of ppy$^-$), 4.28−4.24 (m, 2H; NCH of biotin), 4.09−4.07 (m, 2H; NCH of biotin), 3.47 (s, 4H; $^-$ppy-4-C*H*$_2$NH), 2.99−2.97 (m, 6H; SCH of biotin and C*H*$_2$-NH-biotin), 2.78−2.74 (m, 2H; SCH of biotin), 2.55 (s, 2H; SCH of biotin), 2.31 (t, J = 6.7 Hz, 4H; NHC*H*$_2$C$_4$H$_8$CH$_2$NHCO), 2.00 (t, J = 7.2 Hz, 4H; COC*H*$_2$C$_3$H$_6$ of biotin), 1.53−1.18 ppm (m, 28H; NHCH$_2$C$_4$*H*$_8$CH$_2$NHCO and COCH$_2$C$_3$*H*$_6$ of biotin). IR (KBr) (ν/cm^{-1}): 3431 (br, N−H), 3277 (br, N−H), 1701 (s, C═O), 842 (s, PF_6^-). A positive-ion ESI-MS ion cluster was found at *m*/*z* 783,

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Table 1. Electronic Absorption Spectral Data for the Iridium(III) Diimine Bis(biotin) Complexes at 298 K

complex	medium	λ_{abs}/nm (ϵ/dm^3 mol^{-1} cm^{-1})
1a	CH_2Cl_2	254 (52 485), 274 (62 965), 407 sh (4605), 471 sh (545)
	CH_3CN	252 (55 560), 272 (64 775), 407 sh (4690), 470 sh (465)
1b	CH_2Cl_2	253 sh (52 450), 274 (62 440), 405 sh (5060), 470 sh (790)
	CH_3CN	252 (56 815), 272 (66 295), 407 sh (4910), 468 sh (585)
2a	CH_2Cl_2	258 sh (59 845), 274 (71 810), 285 sh (66 370), 330 sh (24 500), 385 sh (12 685), 470 sh (1590)
	CH_3CN	259 sh (52 945), 274 (60 520), 284 sh (56 730), 382 sh (10 560), 470 sh (1425)
2b	CH_2Cl_2	260 sh (53 610), 275 (63 600), 285 sh (58 695), 330 sh (21 785), 386 sh (11 130), 470 sh (1415)
	CH_3CN	259 sh (61 590), 274 (71 120), 284 sh (66 275), 383 sh (12 425), 470 sh (1820)

$\{M^+ + Na^+\}^{2+}$. Anal. Calcd for $C_{80}H_{92}N_{12}O_4S_2F_6PIr\cdot 0.5H_2O$: C, 56.66; H, 5.53; N, 9.91. Found: C, 56.60; H, 5.78; N, 10.14.

Instrumentation and Methods. Instruments for the characterization and photophysical and electrochemical measurements have been described previously.[20c] Details on the HABA assays, emission titrations, and competitive association and dissociation assays have also been reported.[21f] An intramolecular binding mode ([Ir]:[avidin] = 2) was assumed in order to determine the K_d for all the complexes.[21f]

Binding of Excess Complexes 2a,b to Avidin for RET-Based Emission Quenching Assays. An excess amount of complex **2a** (1.2 mg, 0.76 μmol) or **2b** (1.3 mg, 0.76 μmol) dissolved in 40 μL of anhydrous DMSO was added rapidly to avidin (1 mg, 15 nmol) in 360 μL of 50 mM potassium phosphate buffer pH 7.4. The mixture was incubated at room temperature for 12 h. The solid residue was removed by centrifugation. The products **2a**−avidin and **2b**−avidin were purified using a NAP-5 size-exclusion column, concentrated with a YM-30 microcon to a volume of 250 μL, and stored at 4 °C before use.

Conjugation of QSY-7 Hydroxysuccinimidyl Ester to Avidin for RET-Based Emission Quenching Assays. QSY-7 hydroxysuccinimidyl ester (2.4 mg, 3.03 μmol) in 80 μL of anhydrous DMSO was added to avidin (4 mg, 60 nmol) in 720 μL of 50 mM potassium phosphate buffer pH 7.4. The mixture was incubated at room temperature for 12 h. The solid residue was removed by centrifugation. The supernatant was then loaded onto a NAP-5 size-exclusion column. The purple band containing the conjugate QSY−avidin was collected, successively washed with phosphate buffer using a YM-30 microcon, diluted to 1 mL to give a stock solution, and stored at 4 °C before use.

Preparation of Microsphere Samples for Confocal Microscopy and Scanning Electron Microscopy Studies. Two microsphere samples were used in these studies. The first consisted of carboxyl-functionalized microspheres (diameter, 10 μm; concentration, 1.05 g cm^{-3}) embedded with a green fluorescent dye (λ_{ex}, 480 nm; λ_{em}, 520 nm), and the second, nonfluorescent carboxyl-functionalized microspheres (diameter, 0.78 μm; concentration, 1.07 g cm^{-3}). A 100-μL portion of either sample was centrifuged, and the solid was washed with 50 mM potassium phosphate buffer pH 7.4 (1 mL × 4). The microspheres were resuspended in 1 mL of phosphate buffer. Avidin (60 nmol) and EDC (0.3 nmol) in 1 mL of phosphate buffer were then added to the microsphere suspension, and the mixture was incubated at room temperature for 12 h. The conjugated microspheres were collected by centrifugation, washed with phosphate buffer (1 mL × 6), and resuspended in 200 μL of the same buffer to give a microsphere stock solution. The iridium(III) diimine bis(biotin) complex (0.9 nmol) in a mixture of 5 μL of anhydrous DMSO and 155 μL of phosphate buffer was added to 40 μL of the microsphere stock solution. The mixture was incubated at room temperature for 12 h. The microspheres were collected by centrifugation, washed gently with phosphate buffer (1 mL × 3), and resuspended in 40 μL of the same buffer to give a microsphere sample solution. In the confocal microscopy studies, a 20-μL portion of the green fluorescent microsphere sample solution was pipetted onto a glass slide and examined by a laser-scanning confocal microscope (Carl Zeiss, LSM510) with an excitation wavelength of 488 nm and emission wavelength longer than 505 nm. In the scanning electron microscopy studies, a 20-μL portion of the nonfluorescent microsphere sample solution was pipetted onto a polycarbonate polymer membrane filter with a pore size of 0.4 μm and dried. The membrane was examined by a scanning electron microscope (Philips, XL30) operating at 10 kV.

HPLC Analysis. A 125-μL aliquot of the iridium(III) diimine bis(biotin) complex (10.3 nmol) in a mixture of 7 μL of DMSO and 118 μL of phosphate buffer was added slowly, with a Hamilton syringe, to a stirred solution of avidin (10.3 nmol) in 50 μL of phosphate buffer. After the solution was incubated at room temperature for 30 min, 20 μL of the solution was analyzed by size-exclusion HPLC. The chromatographic system consisted of a Water 600 pump (Waters Corp., Milford, MA) equipped with a Rheodyne 7725i injector (Rohnert Park, CA) with a 20-μL sample loop. The column was a Waters Protein Pak Glass 300SW size-exclusion column (8.0 × 300 mm, Waters Corp., Milford, MA). The UV detector (Waters 996 photodiode array) was set at 280 nm. The mobile phase used was composed of 50 mM potassium phosphate pH 6.8, 300 mM NaCl, 1 mM EDTA, and 1 mM NaN_3 in deionized water at a flow rate of 0.75 mL min^{-1}. Under these conditions, unmodified avidin was eluted at ca. 12.3 min.

Results and Discussion

Synthesis. The two aldehyde groups of the iridium(III) diimine complexes $[Ir(pba)_2(N{-}N)](PF_6)$ (N−N = Me_4-phen, Ph_2-phen) can react with primary amine groups to form stable secondary amines after reductive amination.[20b] These complexes have been used to cross-link L-alanine and avidin, respectively, resulting in luminescent bioconjugates. In this work, reactions of $[Ir(pba)_2(N{-}N)](PF_6)$ and 2 equiv of biotin-containing amines namely *N*-biotinyl-1,2-diaminoethane and *N*-biotinyl-1,6-diaminohexane in the presence of $NaBH_4$ afforded iridium(III) diimine bis(biotin) complexes **1a**,**b** and **2a**,**b** (Scheme 1). These were characterized by ^{1}H NMR spectroscopy, positive-ion ESI-MS, IR spectroscopy, and microanalysis.

Electronic Absorption and Emission Properties. The electronic absorption spectral data of the iridium(III) diimine bis(biotin) complexes are listed in Table 1. Likewise, the electronic absorption spectra of complexes **1a** and **2a** in CH_2Cl_2 are shown in Figures 1 and 2, respectively. All the complexes showed intense spin-allowed intraligand (^{1}IL) ($\pi \rightarrow \pi^*$) (N−N and N−C$^-$) absorption bands at ca. 252−330 nm (ϵ of the order of 10^4 dm^3 mol^{-1} cm^{-1}) and spin-allowed metal-to-ligand charge-transfer (^{1}MLCT) (dπ(Ir) → π^*(N−N and N−C$^-$)) absorption shoulders at ca. 382−471 nm.[5−20] Due to spin−orbital coupling associated with the iridium-

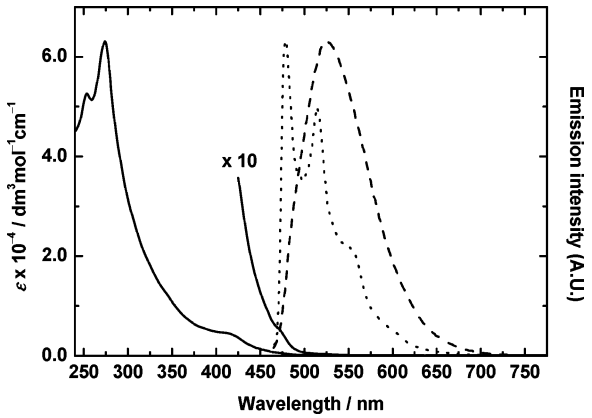

Figure 1. Electronic absorption (—, CH_2Cl_2, 298 K) and emission spectra (----, CH_2Cl_2, 298 K; ····, EtOH/MeOH 4:1 v/v, 77 K) of complex **1a**.

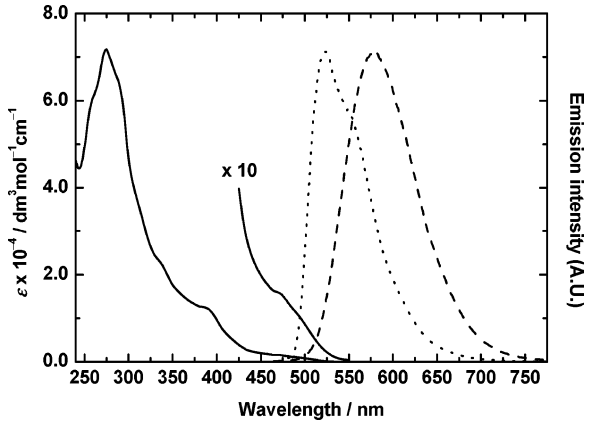

Figure 2. Electronic absorption (—, CH_2Cl_2, 298 K) and emission spectra (----, CH_2Cl_2, 298 K; ····, EtOH/MeOH 4:1 v/v, 77 K) of complex **2a**.

(III) center, spin-forbidden ^{3}MLCT (dπ(Ir) → π^*(N−N and N−C$^-$)) absorption tailing was also observed at a lower energy region (ca. 480 − 550 nm) (Figures 1 and 2). These absorption features are similar to other related iridium(III) polypyridine systems.[5−20]

Upon irradiation, all the complexes displayed intense and long-lived green to orange luminescence in fluid solutions under ambient conditions and in low-temperature alcohol glass. The emission data are summarized in Table 2, and the emission spectra of complexes **1a** and **2a** in degassed CH_2Cl_2 at 298 K and in EtOH/MeOH glass at 77 K are shown in Figures 1 and 2, respectively. The emission of the complexes under ambient conditions has been assigned to a ^{3}MLCT (dπ(Ir) → π^*(N−N)) excited state.[5,6b−e,9b,10,11,12c,f,14a,15−19,20a,c,d,f] This assignment is supported by lower emission energy, shorter excited-state lifetimes, and lower emission quantum yields of the complexes in more polar CH_3CN solutions compared with those in CH_2Cl_2 solutions (Table 2). The ^{3}MLCT assignment is further substantiated by the finding that the emission of the Me_4-phen complexes **1a**,**b** occurred at higher energy than that of the Ph_2-phen complexes **2a**,**b**, which may be explained by the higher energy of the π^* levels of the Me_4-phen ligand. However, the Me_4-phen complexes **1a**,**b** showed very long excited-state lifetimes, even in aqueous buffer at 298 K (τ_o = 2.09 and 3.13 μs for complexes **1a**,**b**, respectively), suggesting that the emissive states of these complexes possess some ^{3}IL (π → π^*) (Me_4-phen) character. Similar assignments have been made in other Me_4-phen complexes of iridium(III), rhenium(I), and tungsten(0).[20b,25−27] In aqueous buffer, complexes **1a** and **2a** which possess shorter spacer arms displayed shorter excited-state lifetimes and longer emission wavelengths compared to their analogous complexes **1b** and **2b** with longer spacer arms (Table 2). We argue that the longer spacer arms provide a more nonpolar local environment to complexes **1b** and **2b** thereby increasing their excited-state lifetimes and inducing a blue shift in emission wavelength. In less polar solvents such as CH_2Cl_2 the effect of varying the spacer arms is negligible. Similar dependence of emission lifetime and emission wavelength on the chain length of the nitrile ligand of the luminescent rhenium(I) complexes $[Re(bpy)(CO)_3(NC\{CH_2\}_n CH_3)]^+$ has been observed.[28] Upon cooling of the samples to 77 K, all the current iridium(III) complexes showed hypsochromic shifts in their emission maxima, which is a typical feature of common iridium(III) MLCT emitters.[5a,b,d,6b,c,e,8a,9b,10,11b−e,12c,14a,15a,d,16,17,20a,c,d,f] In view of the rich vibronic structures of the emission bands (Figures 1 and 2) and long excited-state lifetimes (Table 2), the involvement of ^{3}IL (π → π^*) (N−N) character is also possible.

Table 2. Photophysical Data for the Iridium(III) Diimine Bis(biotin) Complexes

complex	medium (*T*/K)	λ_{em}/nm	τ_o/μs	Φ
1a	CH_2Cl_2 (298)	523	2.24	0.70
	CH_3CN (298)	545	1.94	0.50
	buffer[a] (298)	531	2.09	0.44
	glass[b] (77)	479 (max), 515, 553 sh	7.03	
1b	CH_2Cl_2 (298)	525	1.94	0.63
	CH_3CN (298)	546	1.63	0.39
	buffer[a] (298)	518	3.13	0.38
	glass[b] (77)	479 (max), 515, 555 sh	6.47	
2a	CH_2Cl_2 (298)	576	1.12	0.49
	CH_3CN (298)	598	0.71	0.19
	buffer[a] (298)	595	0.38	0.13
	glass[b] (77)	523, 544 sh	6.42	
2b	CH_2Cl_2 (298)	576	0.94	0.40
	CH_3CN (298)	600	0.54	0.17
	buffer[a] (298)	581	0.53	0.15
	glass[b] (77)	525, 546 sh	5.98	

[a] 25% DMSO in 50 mM potassium phosphate buffer pH 7.4. [b] EtOH/MeOH (4:1 v/v).

Electrochemical Properties. The electrochemical properties of the complexes have been studied by cyclic voltammetry and the data are reported in Table 3. The cyclic voltammograms of all the complexes featured a quasi-reversible oxidation couple at ca. +1.3 V vs SCE, attributable to metal-centered iridium(IV/III) oxidation process.[5a,b,6−8,9b,10a,11b−e,14a,b,15b,16,18a,19b,c,20] In addition, the complexes also displayed an irreversible wave at ca. +1.0 V, which has been assigned to oxidation of the

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Table 3. Electrochemical Data for the Iridium(III) Diimine Bis(biotin) Complexes in CH_3CN (0.1 M TBAP) at 298 K (Glassy Carbon Working Electrode, Sweep Rate = 100 mV s^{-1}, All Potentials vs SCE)

complex	oxidn, $E_{1/2}$ or E_a/V	redn, $E_{1/2}$ or E_c/V
1a	+1.00,[a] +1.30[b]	−1.38,[a] −1.61,[b] −2.09,[a] −2.23[a]
1b	+1.02,[a] +1.29[b]	−1.36,[a] −1.61,[b] −2.07,[a] −2.25[a]
2a	+1.03,[a] +1.33[b]	−1.24,[a] −1.33,[b] −2.00,[a] −2.10[a]
2b	+1.01,[a] +1.34[b]	−1.25,[a] −1.33,[b] −1.96,[a] −2.12[a]

[a] Irreversible waves. [b] Quasi-reversible couples.

secondary amines of the cyclometalating ligands because no such oxidation waves are observed for common cyclometalated iridium(III) polypyridine complexes, including the precursor complexes $[Ir(pba)_2(N{-}N)](PF_6)$ in this work.[5a,b,6−8,9b,10a,11b−e,14a,b,15b,16,18a,19b,c,20] Also, similar oxidation waves are observed for the amine-containing complexes $[Ir(N{-}C)_2(bpy\text{-}CH_2NHC_2NH\text{-}biotin)](PF_6)$ (bpy-CH_2NHC_2NH-biotin = 4-(*N*-((2-biotinamido)ethyl)aminomethyl)-4′-methyl-2,2′-bipyridine).[20d] The reduced reversibility of the iridium(IV/III) couples may be a consequence of the prior irreversible oxidation of these amine moieties. The Me_4-phen and Ph_2-phen complexes exhibited first reduction waves at ca. −1.37 and −1.25 V vs SCE, respectively (Table 3). Related studies indicate that these waves may be assigned to the reduction of diimine ligands.[5a,b,6−8,9b,10a,11b−e,14a,b,15b,16a,18a,20] This is in accordance with complexes **1a**,**b**, which contain the electron-donating methyl substituents of Me_4-phen, undergoing reduction at more negative potential than complexes **2a**,**b**, which contain electron-withdrawing phenyl substituents on the Ph_2-phen ligand.

HABA Assays. The binding of the complexes to avidin has been studied by HABA assays. The assays are based on the competition between biotin and HABA on binding to the substrate-binding sites of avidin. The binding of HABA to avidin is associated with absorption features at ca. 500 nm.[2b] Since the binding of HABA to avidin ($K_d = 6 \times 10^{-6}$ M) is much weaker than that of biotin (K_d = ca. 10^{-15} M),[2b,29] addition of biotin replaces the bound HABA molecules, leading to a decrease of the absorbance at 500 nm. Additions of the complexes to a mixture of avidin and HABA resulted in a decrease in absorbance at 500 nm, indicating that the biotin moieties of the iridium(III) diimine bis(biotin) complexes are binding to avidin. The absorption titration curves for avidin−$HABA_4$ with unmodified biotin and complex **1a**, respectively, are shown in Figure 3. Importantly, the plots of $-\Delta A_{500\ nm}$ vs [Ir]:[avidin] for the four iridium(III) diimine bis(biotin) complexes showed that the equivalence points occurred at [Ir]:[avidin] = 2 indicating that the two biotin moieties of the same iridium(III) diimine bis(biotin) complex are capable of binding to avidin (intra- or intermolecularly, or both, with respect to the protein) and that the binding is substantially stronger than that of HABA.

Emission Titrations. The avidin-binding properties of the complexes have been investigated by emission titrations using the iridium(III) diimine bis(biotin) complexes as titrants.[20d−f,21b,d−f,h] The titration results were compared to two control experiments in which (i) avidin was absent and (ii) avidin was presaturated with excess unmodified biotin. The emission titration curves for complexes **1a** and **2a** are illustrated in Figures 4 and 5, respectively. Similar to our previous studies on luminescent transition metal biotin complexes,[20d−f,21b,d−f,h] all the complexes in this work displayed higher emission intensities and longer excited-state lifetimes in the presence of avidin (Table 4). The emission intensity and lifetime amplification factors of the complexes are 2.3 to 1.2 and 2.1 to 1.1, respectively, at the equivalence points. These changes of photophysical properties are ascribed to the binding of the biotin moieties of the

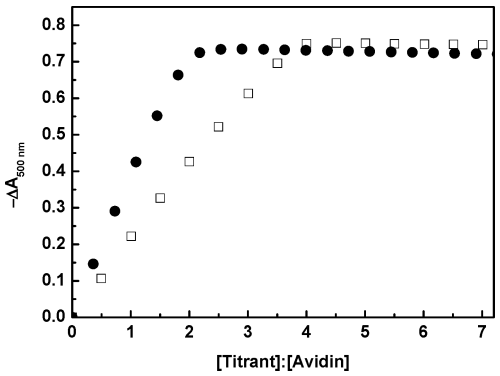


Figure 3. Absorption titration curves for the titrations of the avidin−$HABA_4$ adduct with complex **1a** (●) and unmodified biotin (□).

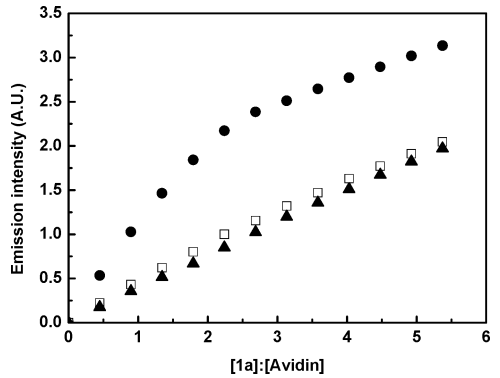


Figure 4. Emission titration curves for the titrations of (i) 0.67 μM avidin (●), (ii) 0.67 μM avidin and 67 μM unmodified biotin (▲), and (iii) a blank phosphate buffer solution (□) with complex **1a**.

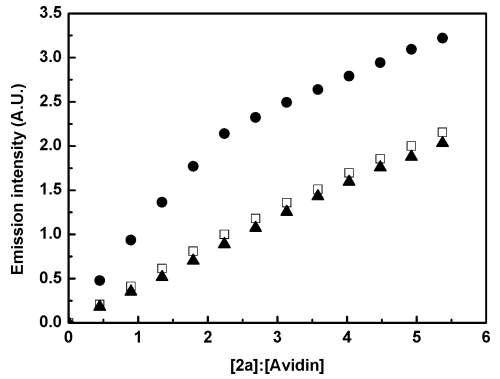


Figure 5. Emission titration curves for the titrations of (i) 0.67 μM avidin (●), (ii) 0.67 μM avidin and 67 μM unmodified biotin (▲), and (iii) a blank phosphate buffer solution (□) with complex **2a**.

(29) Hermanson, G. T. *Bioconjugate Techniques*; Academic Press: San Diego, CA, 1996; p 591.

Table 4. Relative Emission Intensities and Emission Lifetimes of the Iridium(III) Diimine Bis(biotin) Complexes in the Absence and Presence of Avidin (and Excess Biotin) in Aerated 50 mM Potassium Phosphate Buffer pH 7.4 at 298 K[a]

complex	I (τ/μs)[b]	I (τ/μs)[c]	I (τ/μs)[d]
1a	1.00 (0.73)	2.27 (1.54)	0.85 (0.70)
1b	1.00 (0.75)	1.58 (1.16)	0.94 (0.74)
2a	1.00 (0.24)	2.18 (0.47)	0.89 (0.24)
2b	1.00 (0.39)	1.20 (0.43)	0.97 (0.38)

[a] [Ir] = 1.34 μM. [b] [avidin] = 0 M, [biotin] = 0 M. [c] [avidin] = 0.67 μM, [biotin] = 0 M. [d] [avidin] = 0.67 μM, [biotin] = 67 μM.

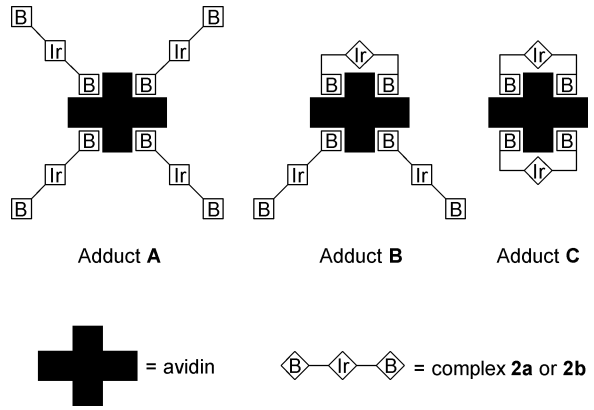
Table 5. First Dissociation Constants for the Iridium(III)−Avidin Adducts in 50 mM Potassium Phosphate Buffer pH 7.4 at 298 K and Results of Competitive Association and Dissociation Assays

complex	K_d/M	association assay/% avidin bound	dissociation assay/% avidin bound
1a	4.3×10^{-9}	82	98
1b	3.9×10^{-9}	80	92
2a	4.8×10^{-8}	53	83
2b	3.1×10^{-8}	44	83

complexes to the biotin-binding sites of avidin and evidenced by the control experiments. The observed emission enhancement and lifetime elongation are due to the increase in the hydrophobicity and rigidity of the local environment of the iridium(III) diimine bis(biotin) complexes once bound to avidin. Complexes **1b** and **2b**, which have longer spacer arms, exhibited smaller emission intensity enhancement and lifetime extension (Table 4), the reason being that these complexes remain more exposed to the polar buffer upon binding to the protein[21e,f,h] and that the more flexible spacer arms provide a more efficient nonradiative decay pathway. It is important to note that the equivalence points of the titrations appeared at [Ir]:[avidin] = 2 for all the complexes (Figures 4 and 5), in accordance with the HABA titration results. This reveals that the two biotin moieties of the same iridium(III) diimine bis(biotin) complex are able to bind to avidin. These emission titration results and the HABA assay results do not provide information on whether the binding is intra- or intermolecular in nature with respect to avidin.

First Dissociation Constants and Competition Assays. The first dissociation constants K_d of the complexes have been determined from the on- and off-rate constants of the iridium−avidin adducts from kinetics experiments.[30] The K_d values of the complexes range from 3.9×10^{-9} to 4.8×10^{-8} M (Table 5), which are about 6 or 7 orders of magnitude larger than that of native biotin (K_d = ca. 10^{-15} M).[2b] The K_d values of Ph_2-phen complexes are larger than that of Me_4-phen complexes, suggesting that the binding of the Ph_2-phen complexes to avidin is hindered by the bulky phenyl substituents. Competitive association and dissociation assays have been performed to study the competitive binding of iridium(III) diimine bis(biotin) complexes and native biotin to avidin.[21f] In the association assays, the iridium(III) complexes competed with native biotin on binding to avidin, whereas, in the dissociation assays, the complexes bound to avidin were challenged by native biotin. The percentage of the avidin molecules with bound iridium(III) complexes was

Chart 1. Possible Adducts Obtained from the Rapid Addition of Excess Complex **2a** or **2b** to Avidin



determined by emission intensity measurements in comparison with control experiments in which native biotin was absent. The results of the association and dissociation assays are summarized in Table 5. The Me_4-phen complexes showed a higher percentage of iridium-bound avidin molecules in the competitive assays compared with the Ph_2-phen complexes. This, again, reflects the effect of the bulky phenyl substituents of the Ph_2-phen ligand upon binding. The proportion of complex-bound avidin for each complex should be similar in the association and dissociation assays. However, all the complexes showed increased binding in the dissociation assays than in the association assays (Table 5). We conclude that the equilibrium had not been attained during the incubation period in the dissociation assays. The qualitative nature of the trends of these assays is emphasized nonetheless, and these experiments can serve as simple and convenient assays to preliminarily evaluate the avidin-binding properties of the complexes.

RET-Based Emission Quenching Assays. To study the possibility that these complexes may possess avidin cross-linking properties, RET-based emission quenching assays involving the adducts **2a**−avidin and **2b**−avidin and the conjugate QSY−avidin have been designed. In the preparation of luminescent avidin molecules, excess complex **2a** or **2b** was added rapidly to an avidin solution. Theoretically, only three types of adducts **A**−**C** can be formed under these conditions (Chart 1) because avidin is a tetrameric molecule that can bind up to four biotins. The HABA assay and emission titration results indicate that the two biotin units of the same iridium(III) complex can bind to avidin. Thus, the biotin pendants of adducts **A** and **B** should be capable of binding to the quencher-labeled conjugate QSY−avidin. Since the absorption band of QSY-7 (560 nm) overlaps significantly with the emission bands of complexes **2a** (595 nm) and **2b** (581 nm) in phosphate buffer, the emission of **2a**−avidin and **2b**−avidin is expected to be quenched via RET if the adducts bind to QSY−avidin (Förster distances = 35.5 and 36.9 Å, for complexes **2a**,**b** respectively).[31] The emission spectrum of the adduct **2b**−avidin in degassed buffer is shown in Figure 6. In the presence of QSY−avidin,

(30) Marek, M.; Kaiser, K.; Gruber, H. J. *Bioconjugate Chem.* **1997**, *8*, 560.

(31) (a) Selvin, P. R.; Rana, T. M.; Hearst, J. E. *J. Am. Chem. Soc.* **1994**, *116*, 6029. (b) Wu, P.; Brand, L. *Anal. Biochem.* **1994**, *218*, 1.

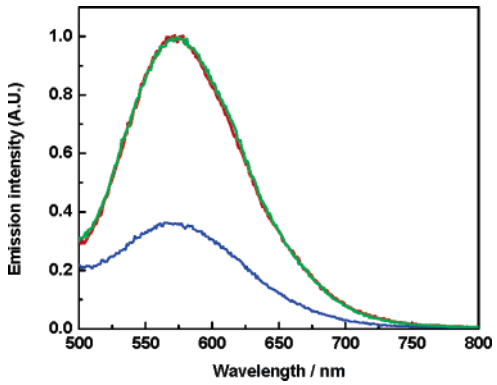


Figure 6. Emission spectra of the adduct **2b**−avidin in degassed phosphate buffer (red) and in the presence of QSY−avidin (blue) or biotin-blocked QSY−avidin (green).

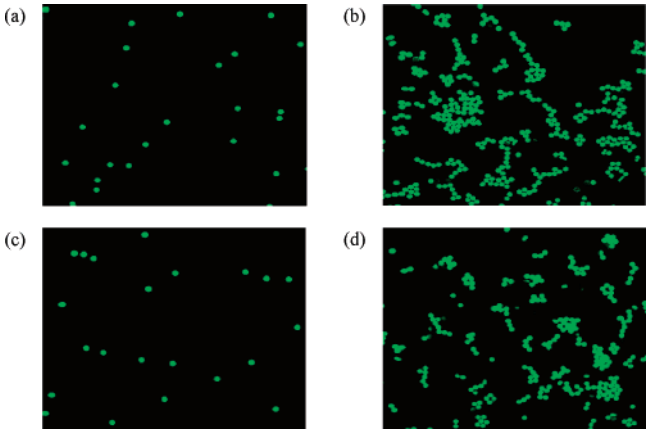


Figure 7. Confocal microscopy images of microsphere sample solutions containing complexes **1a** (a), **1b** (b), **2a** (c), and **2b** (d).

2b−avidin exhibited emission quenching (I/I_o = 0.36), suggestive of the occurrence of RET. No emission quenching occurred when biotin-blocked QSY−avidin was used instead (Figure 6). These findings indicate that complex **2b** can function as a cross-linker for avidin. However, in the case of the adduct **2a**−avidin, the presence of QSY−avidin did not cause any emission quenching. Therefore, it is likely that, under the experimental conditions used, addition of excess complex **2a** to avidin gave adduct **C** (Chart 1) as an exclusive or a major product since the longer spacer arms of complex **2b** enable this complex to have a higher potential to function as a cross-linker for avidin whereas the binding of complex **2a** to avidin is essentially intramolecular in nature.

Microscopy Studies. The cross-linking properties of the iridium(III) diimine bis(biotin) complexes have been studied using avidin-conjugated green-fluorescent microspheres (diameter = 10 μm). The confocal microscopy images of the fluorescent avidin-conjugated microsphere sample solutions containing complexes **1a**,**b** and **2a**,**b** are shown in Figure 7a−d, respectively. The observed emission originates from the dye embedded in the microspheres rather than the complexes because excitation of the complexes at 488 nm does not lead to noticeable emission, as evidenced by their excitation spectra. Aggregation of avidin-modified microspheres occurred in the sample solutions that contained complexes **1b** and **2b**, which have longer spacer arms (Figure 7b,d). We attribute this observation to the cross-linking of

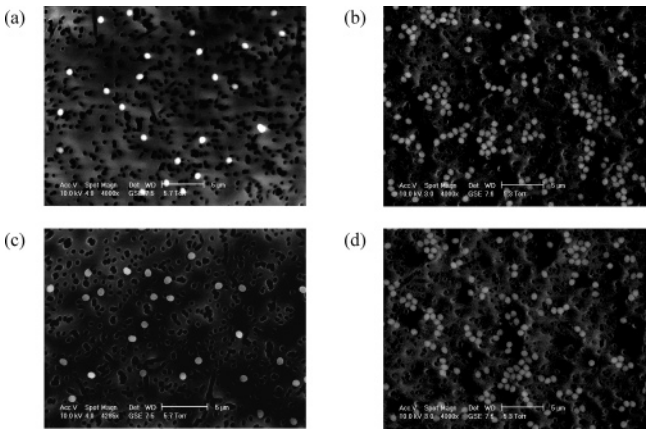


Figure 8. Scanning electron microscopy images of microsphere sample solutions containing complexes **1a** (a), **1b** (b), **2a** (c), and **2b** (d).

the avidin molecules on different microspheres by the complexes because similar aggregation did not occur when excess biotin was present in the reaction mixture from the outset. It is further supported by the finding that no aggregation was observed when a related monobiotin complex $[Ir(mppy)_2(bpy\text{-}CH_2NHC_2NH\text{-}biotin)]^+$ (Hmppy = 2-(4-methylphenyl)pyridine)[20d] was used instead of the current bis(biotin) complexes **1b** and **2b**. Importantly, under identical experimental conditions, complexes **1a** and **2a**, both of which are functionalized with shorter spacer arms, did not cause any aggregation of avidin-modified microspheres (Figure 7a,c). Interestingly, both biotin units on each of the four complexes can bind to avidin, as demonstrated by the HABA assays and emission titrations. Therefore, the lack of aggregation of microspheres in the cases of complexes **1a** and **2a** strongly indicates that these complexes bind to avidin preferentially in an intramolecular fashion, giving the [Ir_2•Av] adduct. These results clearly reflect the importance of the spacer arms on the cross-linking properties of the complexes. We have also modified microspheres of a much smaller size (diameter = 0.78 μm) with avidin and performed similar experiments to investigate if agglomeration of these smaller microsphere particles occurs. Again, incubation of these microsphere samples with complexes **1a** and **2a** only gave isolated microspheres in the scanning electron microscopy images (Figure 8a,c), while dimeric, trimeric, and other oligomeric microspheres were observed for complexes **1b** and **2b** (Figure 8b,d). These results are in accordance with the confocal microscopy studies and suggest that the longer spacer arms of complexes **1b** and **2b** enable them to function as a cross-linker for avidin.

HPLC Analysis. To further understand the avidin cross-linking properties of the complexes, size-exclusion HPLC experiments have been performed. Chromatograms of an equimolar mixture of avidin and the complexes are shown in Figure 9a−d. Under the chromatographic conditions used, avidin was eluted at 12.3 min (Figure 9e). Addition of complex **1a** or **2a** to a solution of avidin resulted in the appearance of a band of very low intensity at ca. 10.9 min ($I_{12.3\ min}$:$I_{10.9\ min}$ = 32.5 and 34.7 for complexes **1a** and **2a**, respectively) (Figure 9a,c). We have assigned this band to dimeric avidin molecules, by comparison with previous

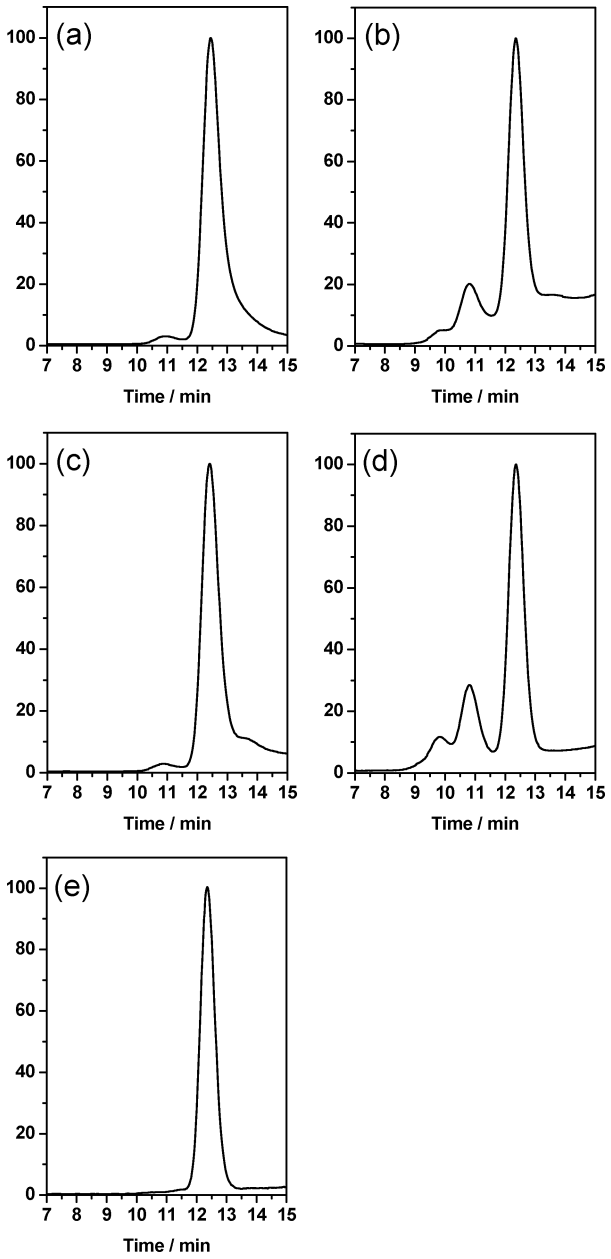


Figure 9. Chromatograms of an equimolar mixture of avidin (206 μM) and complexes **1a** (a), **1b** (b), **2a** (c), and **2b** (d), respectively, in phosphate buffer. The chromatogram of an avidin solution is shown in (e).

chromatographic work of oligomeric streptavidin molecules.[3a] The relatively low product yields reflect the inefficient cross-linking properties of the complexes with shorter spacer arms. Interestingly, when complexes **1b** and **2b** were used, the bands corresponding to avidin dimers were much higher in intensity (Figure 9b,d). An additional band at ca. 9.8 min, which is likely to originate from avidin trimers,[3a] was also observed ($I_{12.3\ min}$:$I_{10.9\ min}$:$I_{9.8\ min}$ = ca. 5.0:1:0.3 and 3.5:1:0.4 for complexes **1b** and **2b**, respectively).[32] No polymeric avidin species was detected under any of the conditions used.[33] These results show that complexes **1a** and **2a** are not efficient avidin cross-linkers, whereas complexes **1b** and **2b** can cross-link avidin molecules. Although no oligomeric or polymeric avidin molecules were produced, the identification of dimeric and trimeric avidin molecules is precisely in accordance with the findings of the RET-based emission quenching experiments and the microscopy studies described above.

Previous investigations on organic bis(biotin) molecules indicated that a linker length r (between the two amide carbon atoms, i.e., the original carboxyl carbon of biotin) shorter than 15 Å does not allow the second biotin molecule to project far enough from the surface of avidin to reach the biotin-binding site on another avidin molecule.[2a] Linkers of 15−17.5 Å lead to weak binding of the second biotin to another avidin molecule, and a linker of 18.4 Å could cross-link avidin tightly. However, linkers longer than 20 Å result in binding of a second biotin to the same avidin molecule.[2a] The intramolecular binding of complexes **1a** and **2a** (r = ca. 23 Å)[34] to avidin observed in the current work is in agreement with these conclusions. It is also in accordance with the observation that linear biotin dimers with r varying from 13 to 49 Å do not cross-link avidin effectively.[3a] In another example, two of the three biotin units of a 1,3,5-tris(biotin)benzene molecule (r = ca. 31 Å) bind to the same streptavidin molecule, whereas the third biotin binds to another streptavidin, resulting in efficient polymerization of the protein.[3a] Interestingly, this linker length is comparable to those of the spacer arms of complexes **1b** and **2b** (r = ca. 34 Å).[34] Therefore, the lack of avidin polymerization by these two complexes can be attributed to the smaller number of biotin moieties. Also, it may be expected that complexes **1b** and **2b** form predominantly intramolecular adducts with avidin.[3a] However, cross-linking of avidin to form dimers and trimers did occur, as indicated by the RET-based emission quenching experiments, microscopy studies, and HPLC analysis. Complete understanding of these interesting cross-linking properties requires further work. The contribution of the directional constraints associated with the rigid [Ir(N−C)$_2$] framework to the observed intermolecular avidin binding could provide a possible explanation.

Conclusions

This paper describes the synthesis, characterization, and photophysical and electrochemical properties of four luminescent cyclometalated iridium(III) diimine bis(biotin) complexes. The interactions of these complexes with avidin have been studied by HABA assays and emission titrations. Similar to other luminescent transition metal biotin complexes we reported previously, these iridium(III) diimine bis(biotin) complexes showed emission intensity enhancement and excited-state lifetime extension upon binding to the

(32) Sample solutions of various [Ir]:[avidin] ratios from 0.1 to 10 have been prepared and analyzed. We found that equimolar solutions of the complex and protein gave the strongest signals for the dimers and trimers. When higher metal concentrations were used, the chromatograms were complicated by erroneous baseline elevation and significant tailing of signals, which are probably the consequence of adsorption of the bioconjugates.

(33) Analysis of a sample of avidin polymerized by glutaraldehyde revealed that the avidin polymers were eluted at ca.7.0 min.

(34) The linker lengths were taken as double the distance between the iridium center and the amide carbons. These distances were determined from the computer program ChemDraw3D (Cambridge Soft Corp., Cambridge, MA) after structural and energy minimization of the fully extended conformation of the complexes.

protein. The possession of two biotin moieties was expected to enable the complexes to act as potential cross-linkers for avidin. Indeed, RET-based emission quenching experiments, microscopy studies using avidin-conjugated microspheres, and HPLC analysis demonstrated that both complexes **1b** and **2b** can cross-link avidin to give dimers and trimers. Interestingly, the spacer arms not only influence the photophysical properties of the complexes but also play a key role in the avidin cross-linking properties. While avidin cross-linkers have been reported, complexes **1b** and **2b** are the first luminescent cross-linking reagents for this useful protein. Utilization of these complexes and other related luminescent multibiotin transition metal complexes as bioanalytical reagents and building blocks for macromolecular assemblies is now underway.

Acknowledgment. We thank City University of Hong Kong (Project No. 7001632) for financial support. J.S.-Y.L. acknowledges the receipt of a Postgraduate Studentship and a Research Tuition Scholarship, both administered by City University of Hong Kong. We thank Dr. Charles Harford-Cross for reading this manuscript.

IC0612202