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Article

Rhenium(I) Polypyridine Biotin Isothiocyanate Complexes as the First Luminescent Biotinylation Reagents: Synthesis, Photophysical Properties, Biological Labeling, Cytotoxicity, and Imaging Studies

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We report here the design of the first class of luminescent biotinylation reagents derived from rhenium(I) polypyridine complexes. These complexes [Re(N–N)$(CO)_3$(py-biotin-NCS)](PF_6) (py-biotin-NCS = 3-isothiocyanato-5-(*N*-((2-biotinamido)ethyl)aminocarbonyl)pyridine; N–N = 1,10-phenanthroline (phen) (**1a**), 3,4,7,8-tetramethyl-1,10-phenanthroline (Me_4-phen) (**2a**), 4,7-diphenyl-1,10-phenanthroline (Ph_2-phen) (**3a**)), containing a biotin unit and an isothiocyanate moiety, have been synthesized from the precursor amine complexes [Re(N–N)$(CO)_3$(py-biotin-NH_2)]-(PF_6) (py-biotin-NH_2 = 3-amino-5-(*N*-((2-biotinamido)ethyl)aminocarbonyl)pyridine; N–N = phen (**1c**), Me_4-phen (**2c**), Ph_2-phen (**3c**)). To investigate the amine-specific reactivity of the isothiocyanate complexes **1a**–**3a**, they have been reacted with a model substrate ethylamine, resulting in the formation of the thiourea 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 (**1b**), Me_4-phen (**2b**), Ph_2-phen (**3b**)). All the rhenium(I) complexes have been characterized, and their photophysical properties have been studied. The avidin-binding properties of the thiourea complexes **1b**–**3b** have been examined by the 4′-hydroxyazobenzene-2-carboxylic acid (HABA) assay. Titration results indicated that the complexes exhibited emission enhancement by ca. 1.4–1.5-fold upon binding to avidin, and the lifetimes were elongated to ca. 0.8–2.0 μs. Additionally, we have biotinylated bovine serum albumin (BSA) with the isothiocyanate complexes. All the resultant rhenium–BSA bioconjugates displayed intense and long-lived orange-yellow to greenish-yellow emission upon irradiation in aqueous buffer under ambient conditions. The avidin-binding properties of the bioconjugates have been investigated using the HABA assay. Furthermore, the cytotoxicity of the thiourea complexes **1b**–**3b** toward the HeLa cells has been examined by the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. The IC_{50} values were determined to be ca. 17.5–28.5 μM, which are comparable to that of cisplatin (26.7 μM) under the same conditions. The cellular uptake of complex **3b** has been investigated by fluorescence microscopy, and the results showed that the complex was localized in the perinuclear region after interiorization.

Introduction

The avidin−biotin system is a useful tool in immunology, histochemistry, and in situ hybridization.[1] For example, antibodies multiply labeled with biotin can increase the sensitivity of heterogeneous bioassays by signal amplification; this is illustrated in Chart 1. Also, biotinylated biomolecules can be purified with affinity chromatography and detected by ELISA and blotting techniques. These applications rely on the facts that (1) avidin can be readily modified with fluorescent tags[2] and enzymes[3] without losing its affinity to biotin and (2) the biological characteristics and properties

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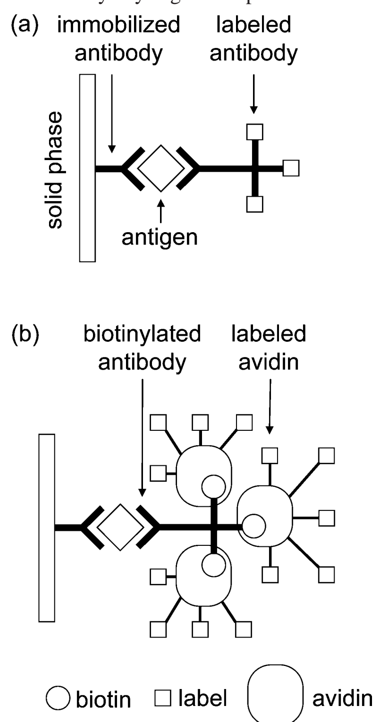
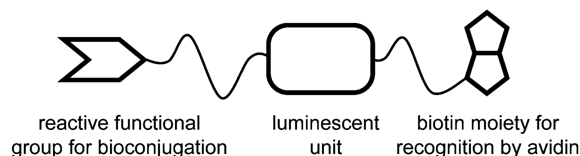
Chart 1. Use of the Biotin–Avidin System To Increase the Sensitivity of Heterogeneous Bioassays by Signal Amplification[a,b]



[a] Antigen recognized by immobilized and labeled antibodies. [b] Antigen recognized by immobilized and biotinlyated antibodies; the latter is detected by labeled avidin.

of common biomolecules are usually retained after biotinylation. A number of biotinylation reagents that are reactive toward different functional groups of biomolecules have been designed.[4] To optimize avidin–biotin assays and to ensure labeling reproducibility, the extent of biotinylation of the biomolecules involved must be known. This is routinely determined by the 4′-hydroxyazobenzene-2-carboxylic acid (HABA) assay,[5] which is based on the decrease of absorption due to the displacement of avidin-bound HABA molecules (λ_{abs} = 500 nm) by the biotinylated species. Additionally, two chromogenic biotin reagents, biotin-X 2,4-dinitrophenyl-X-L-lysine NHS ester[6a] (ϵ_{364nm} = 15 000 $dm^3\ mol^{-1}\ cm^{-1}$) and EZ-Link NHS-chromogenic-biotin[6b] (ϵ_{354nm} = 29 000 $dm^3\ mol^{-1}\ cm^{-1}$), have been used to label biomolecules, allowing spectrophotometric determination of the degree of biotinylation. A fluorometric assay involving the displacement of a quencher-substrate from a fluorescent avidin conjugate by the biotinylated species has also been developed.[6a] Although this assay offers higher sensitivity, it requires two reagents and the biotinylated species cannot be reused after analysis. To the very best of our knowledge, luminescent biotinylation reagents, which contain a reactive functional group for bioconjugation and a biotin moiety for recognition by avidin (Chart 2), have never been explored. There are three major reasons for the development of these reagents. First, they render the biotinylated proteins or DNA to possess luminescence properties that could lead to new in vitro and in vivo bioassay designs. Second, the extent of biotinylation of biomacromolecules can be directly determined by more sensitive spectrofluorometric methods. Finally, they can be employed to biotinylate small molecular substrates and allow the isolation and purification of the specific biological receptors, for example, by affinity chromatography. Also, biological uptake of the biotinylated compounds may be followed by luminescence spectroscopy and microscopy.

Chart 2. Components of a Luminescent Biotinylation Reagent



Isothiocyanate (–N=C=S) is a useful functional group for bioconjugation because it reacts readily with the ϵ-amine group of lysine residues and the N-terminal of proteins to form a stable thiourea moiety.[5b] We have reported (1) luminescent transition metal polypyridine isothiocyanate complexes as biological labeling reagents[7] and (2) luminescent biotin complexes as noncovalent probes for avidin.[8] In view of the rich photophysical properties of rhenium(I) polypyridine complexes,[7a,8a,b,f,h,9–28] we anticipate that a new class of specific luminescent biotinylation reagents could be achieved by functionalizing luminescent rhenium(I) poly-

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pyridine complexes with a reactive group and a biotin pendant. Herein, we describe a new series of rhenium(I) polypyridine biotin isothiocyanate complexes [Re(N−N)-$(CO)_3$(py-biotin-NCS)](PF_6) (py-biotin-NCS = 3-isothiocyanato-5-(*N*-((2-biotinamido)ethyl)aminocarbonyl)pyridine; N−N = 1,10-phenanthroline (phen) (**1a**), 3,4,7,8-tetramethyl-1,10-phenanthroline (Me_4-phen) (**2a**), 4,7-diphenyl-1,10-phenanthroline (Ph_2-phen) (**3a**)), which have been synthesized from the reaction of the precursor amine complexes [Re(N−N)$(CO)_3$(py-biotin-NH_2)](PF_6) (py-biotin-NH_2 = 3-amino-5-(*N*-((2-biotinamido)ethyl)aminocarbonyl)-pyridine; N−N = phen (**1c**), Me_4-phen (**2c**), Ph_2-phen (**3c**)) with thiophosgene in acetone at 298 K (Scheme 1). To investigate the amine-specific reactivity of the isothiocyanate complexes **1a**−**3a**, they have been reacted with a model substrate ethylamine, resulting in the formation of the thiourea 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 (**1b**), Me_4-phen (**2b**), Ph_2-phen (**3b**)) (Scheme 1). The avidin-binding properties of the thiourea complexes **1b**−**3b** have been investigated by the HABA assay and emission titrations. Additionally, the biotinylating ability of the isothiocyanate complexes **1a**−**3a** has been demonstrated using a model protein bovine serum albumin (BSA). We have also investigated the emission and avidin-binding properties of the resultant rhenium−BSA bioconjugates. Furthermore, the cytotoxicity of the thiourea complexes **1b**−**3b** toward the HeLa cells has been examined by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay. The cellular uptake of complex **3b** has also been investigated by fluorescence microscopy.

Experimental Section

Materials and Synthesis. All solvents were of analytical reagent grade. $Re(CO)_5Cl$ (Aldrich), phen (Acros), Me_4-phen (Acros), Ph_2-phen (Acros), 5-aminonicotinic acid (Acros), NHS (Acros), *N*,*N*′-dicyclohexylcarbodiimide (Acros), KPF_6 (Acros), biotin (Acros), ethylamine (Acros), thiophosgene (Aldrich), cisplatin (Acros), HABA (Sigma), avidin (Calbiochem), BSA (Calbiochem), pronase (Calbiochem), MTT (Sigma), and nocodazole (Acros) were used

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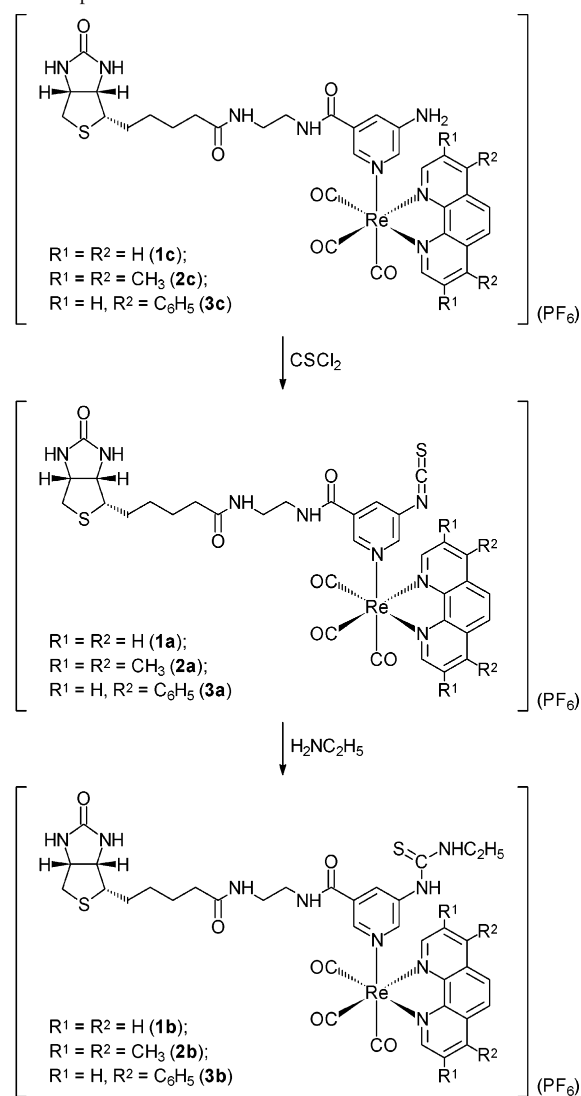
Scheme 1. Synthesis and Structures of the Rhenium(I) Polypyridine Biotin Complexes



without purification. Biotinylethylenediamine,[29] $[Re(N-N)(CO)_3(CH_3CN)](CF_3SO_3)$,[8f] $[Re(Me_4\text{-phen})(CO)_3(py\text{-NCS})](PF_6)$ (**2d**) (py-NCS = 3-isothiocyanatopyridine),[7a] and $[Re(Me_4\text{-phen})(CO)_3(py\text{-biotin})](PF_6)$ (**2e**) (py-biotin = 3-(*N*-((2-biotinamido)ethyl)-aminocarbonyl)pyridine)[8f] were prepared as described previously. All buffer components were of molecular biology grade. PD-10 columns and YM-30 centricons were purchased from Pharmacia and Amicon, respectively. Human cervix epithelioid carcinoma (HeLa) cells were obtained from American Type Culture Collection. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), trypsin−EDTA, and penicillin/streptomysin were purchased from Invitrogen. The growth medium for cell culture contained DMEM with 10% FBS and 1% penicillin/streptomysin.

5-Aminonicotinic Acid *N*-Hydroxysuccinimidyl Ester. 5-Aminonicotinic acid (409 mg, 2.96 mmol) was dissolved in hot DMF (40 mL) under an inert atmosphere of nitrogen. After the solution was cooled to room temperature, a DMF (10 mL) solution of NHS (450 mg, 3.91 mmol) and *N,N′*-dicyclohexylcarbodiimide (670 mg, 3.25 mmol) was added. A white solid appeared, and the suspension was stirred under nitrogen at room temperature for 12 h. The mixture was filtered, and the filtrate was evaporated to dryness under reduced pressure, resulting in a yellow solid. The solid was washed with 2-propanol and then air-dried. Yield: 317 mg (46%). Positive-ion ESI-MS ion cluster at m/z 236 $\{M + H^+\}^+$.

3-Amino-5-(*N*-((2-biotinamido)ethyl)aminocarbonyl)-pyridine, Py-biotin-NH_2. Biotinylethylenediamine (508 mg, 1.77 mmol) was dissolved in hot DMF (30 mL) under an inert atmosphere of nitrogen. After the solution was cooled to room temperature, 5-aminonicotinic acid *N*-hydroxysuccinimidyl ester (417 mg, 1.77 mmol) dissolved in DMF (15 mL) was added. The solution was stirred under nitrogen at room temperature for 12 h. It was then evaporated to dryness under reduced pressure to give a yellow solid. Recrystallization of the solid from MeOH/diethyl ether yielded py-biotin-NH_2 as a white solid. Yield: 580 mg (81%). ^{1}H NMR (300 MHz, DMSO-d_6, 298 K, TMS): δ 8.67 (s, 1 H, py−3-CON*H*), 8.13 (s, 1 H, C_2H_4−N*H*−biotin), 7.99 (s, 1 H, H2 of pyridine), 7.81 (s, 1 H, H6 of pyridine), 7.25 (s, 1 H, H4 of pyridine), 6.44 (s, 1 H, NH of biotin), 6.37 (s, 1 H, NH of biotin), 5.51 (s, 2 H, NH_2), 4.30−4.26 (m, 1 H, NCH of biotin), 4.12−4.08 (m, 1 H, NCH of biotin), 3.06 (d, 1 H, J = 10.8 Hz, SCH of biotin), 2.83−2.77 (m, 1 H, SCH of biotin), 2.55 (d, 1 H, J = 12.5 Hz, SCH of biotin), 2.04−2.03 (m, 2 H, $COCH_2C_3H_6$ of biotin), 1.55−1.26 (m, 6 H, $COCH_2C_3H_6$ of biotin). IR (KBr) ν/cm^{-1}: 3288 (br, NH), 1701 (s, C=O), 1648 (s, C=O). Positive-ion ESI-MS ion cluster at m/z 408 $\{M + H^+\}^+$.

$[Re(N-N)(CO)_3(py\text{-biotin-}NH_2)](PF_6)$ (N−N = Phen (1c), Me_4-phen (2c), Ph_2-phen (3c)). A mixture of $[Re(N-N)(CO)_3(CH_3CN)](CF_3SO_3)$[8f] (0.30 mmol) and py-biotin-NH_2 (122 mg, 0.30 mmol) in THF (30 mL) was refluxed under an inert atmosphere of nitrogen for 12 h. The yellow solution was then evaporated to dryness, resulting in a yellow solid. The complex was converted to the hexafluorophosphate salt by metathesis with KPF_6 in MeOH and then purified by column chromatography on alumina. The desired product was eluted with CH_3CN/MeOH (10:1, v/v). Upon recrystallization of the crude product from CH_2Cl_2/diethyl ether, the complex formed as yellow crystals. Complex **1c**. Yield: 161 mg (50%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ 9.90 (d, 2 H, J = 4.7 Hz, H2 and H9 of phen), 9.10 (d, 2 H, J = 8.2 Hz, H4 and H7 of phen), 8.38−8.33 (m, 4 H, H3, H5, H6 and H8 of phen), 8.14 (s, 1 H, H2 of pyridine), 8.07 (s, 1 H, py−3-CON*H*), 8.03 (s, 1 H, H6 of pyridine), 7.55 (s, 1 H, C_2H_4−N*H*−biotin), 7.41 (s, 1 H, H4 of pyridine), 5.90 (s, 1 H, NH of biotin), 5.70 (s, 1 H, NH of biotin), 5.57 (s, 2 H, NH_2), 4.50−4.46 (m, 1 H, NCH of biotin), 4.29−4.25 (m, 1 H, NCH of biotin), 3.44−3.36 (m, 4 H, C_2H_4−NH−biotin), 3.19−3.07 (m, 1 H, SCH of biotin), 2.66 (d, 1 H, J_{gem} = 12.6 Hz, SCH of biotin), 2.22 (t, 2 H, J = 7.3 Hz, $COCH_2C_3H_6$ of biotin), 1.70−1.30 (m, 6 H, $COCH_2C_3H_6$ of biotin). IR (KBr) ν/cm^{-1}: 3448 (br, NH), 2032 (s, C≡O), 1919 (s, C≡O), 1650 (m, C=O), 842 (s, PF_6^-). Positive-ion ESI-MS ion cluster at m/z 857 $\{M - PF_6\}^+$. Anal. Calcd for $C_{33}H_{34}N_8O_6SPF_6Re\cdot H_2O$: C, 38.86; H, 3.56; N, 10.99. Found: C, 39.11; H, 3.64; N, 12.20%. Complex **2c**. Yield: 178 mg (51%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ 9.66 (s, 2 H, H2 and H9 of Me_4-phen), 8.47 (s, 2 H, H5 and H6 of Me_4-phen), 8.22 (d, 1 H, J = 2.3 Hz, H2 of pyridine), 8.11 (s, 1 H, py−3-CON*H*), 8.07 (d, 1 H, J = 1.5 Hz, H6 of pyridine), 7.58 (s, 1 H, C_2H_4−N*H*−biotin), 7.45 (s, 1 H, H4 of pyridine), 5.88 (s, 1 H, NH of biotin), 5.75 (s, 1 H, NH of biotin), 5.54 (s, 2 H, NH_2), 4.50−4.45 (m, 1 H, NCH of biotin), 4.24−4.20 (m, 1 H, NCH of biotin), 3.45−3.31 (m, 4 H, C_2H_4−NH−biotin), 3.03−2.99 (m, 1 H, SCH of biotin), 2.94 (s, 6 H, CH_3 at C4 and C7 of Me_4-phen), 2.81 (s, 6 H, CH_3 at C3 and C8 of Me_4-phen), 2.67 (d, 1 H, J_{gem} = 12.6 Hz, SCH of biotin), 2.20 (t, 2 H, J = 6.7 Hz, $COCH_2C_3H_6$ of biotin), 1.61−1.21 (m, 6 H, $COCH_2C_3H_6$ of biotin). IR (KBr) ν/cm^{-1}: 3448 (br, NH), 2030 (s,

(29) Garlick, R. K.; Giese, R. W. *J. Biol. Chem.* **1988**, *263*, 210−215.

$C\equiv O$), 1919 (s, $C\equiv O$), 1655 (m, C=O), 844 (s, PF_6^-). Positive-ion ESI-MS ion cluster at m/z 914 {M − PF_6}$^+$. Anal. Calcd for $C_{37}H_{42}N_8O_6SPF_6Re\cdot H_2O$: C, 41.30; H, 4.12; N, 10.41. Found: C, 41.28; H, 4.42; N, 10.27%. Complex **3c**. Yield: 198 mg (64%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ 9.99−9.97 (dd, 2 H, J = 5.4 and 3.1 Hz, H2 and H9 of Ph_2-phen), 8.31 (dd, 2 H, J = 5.3 and 3.5 Hz, H3 and H8 of Ph_2-phen), 8.26 (s, 2 H, H5 and H6 of Ph_2-phen), 8.17 (s, 2 H, H2 of pyridine and py−3-CON*H*), 8.04 (s, 1 H, H6 of pyridine), 7.75−7.69 (m, 10 H, C_6H_5 of Ph_2-phen), 7.57 (s, 1 H, C_2H_4−N*H*−biotin), 7.48 (s, 1 H, H4 of pyridine), 5.92 (s, 1 H, NH of biotin), 5.73 (s, 1 H, NH of biotin), 5.62 (d, 2 H, J = 4.1 Hz, NH_2), 4.50−4.46 (m, 1 H, NCH of biotin), 4.28−4.24 (m, 1 H, NCH of biotin), 3.44−3.37 (m, 4 H, C_2H_4−NH−biotin), 3.09−3.06 (m, 1 H, SCH of biotin), 2.98−2.97 (m, 1 H, SCH of biotin), 2.65 (d, 1 H, J_{gem} = 12.6 Hz, SCH of biotin), 2.19 (t, 2 H, J = 6.9 Hz, $COCH_2C_3H_6$ of biotin), 1.63−1.26 (m, 6 H, $COCH_2C_3H_6$ of biotin). IR (KBr) ν/cm^{-1}: 3433 (br, NH), 2032 (s, $C\equiv O$), 1919 (s, $C\equiv O$), 1655 (m, C=O), 843 (s, PF_6^-). Positive-ion ESI-MS ion cluster at m/z 1009 {M − PF_6}$^+$. Anal. Calcd for $C_{45}H_{42}N_8O_6SPF_6Re\cdot H_2O$: C, 46.11; H, 3.78; N, 9.56. Found: C, 46.35; H, 3.99; N, 9.68%.

[Re(N−N)(CO)$_3$(py-biotin-NCS)](PF_6) (N−N = Phen (1a), Me_4-phen (2a), Ph_2-phen (3a)). Thiophosgene (26 μL, 0.34 mmol) was added to a mixture of [Re(N−N)(CO)$_3$(py-biotin-NH_2)](PF_6) (0.17 mmol) and finely crushed $CaCO_3$ (64 mg, 0.64 mmol) in acetone (10 mL) under an inert atmosphere of nitrogen. The suspension was stirred in the dark at room temperature for 4 h. The mixture was filtered, and the filtrate was evaporated to dryness to give a yellow solid. Subsequent recrystallization of the complex from acetone/diethyl ether resulted in the formation of the complex as yellow crystals. Complex **1a**. Yield: 109 mg (63%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ 9.99 (s, 2 H, H2 and H9 of phen), 9.53−9.50 (m, 1 H, NH of biotin), 9.10 (s, 2 H, H4 and H7 of phen), 9.00−8.95 (m, 1 H, NH of biotin), 8.81−8.77 (m, 2H, H2 and H6 of pyridine), 8.39−8.35 (m, 5H, H3, H5, H6 and H8 of phen and H4 of pyridine), 8.21 (s, 1 H, py−3-CON*H*), 7.57 (s, 1 H, C_2H_4−N*H*−biotin), 4.81 (s, 1H, NCH of biotin), 4.60 (s, 1 H, NCH of biotin), 3.41−3.31 (m, 4 H, C_2H_4−NH−biotin), 2.72−2.71 (m, 1 H, SCH of biotin), 2.27−2.25 (m, 2 H, $COCH_2C_3H_6$ of biotin), 1.64−1.29 (m, 6 H, $COCH_2C_3H_6$ of biotin). IR (KBr) ν/cm^{-1}: 3422 (br, NH), 2110 (m, N=C=S), 2035 (s, $C\equiv O$), 1919 (s, $C\equiv O$), 1655 (m, C=O), 843 (s, PF_6^-). Positive-ion ESI-MS ion cluster at m/z 899 {M − PF_6}$^+$. Anal. Calcd for $C_{34}H_{32}N_8O_6S_2PF_6Re\cdot H_2O$: C, 38.45; H, 3.23; N, 10.55. Found: C, 38.24; H, 3.15; N, 10.42%. Complex **2a**. Yield: 108 mg (61%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ 9.78 (s, 2 H, H2 and H9 of Me_4-phen), 9.69−9.65 (m, 1 H, NH of biotin), 9.21 (s, 1 H, NH of biotin), 8.85 (d, 2 H, J = 5.6 Hz, H2 and H6 of pyridine), 8.48 (s, 3 H, H5 and H6 of Me_4-phen and H4 of pyridine), 8.25 (s, 1 H, py−3-CON*H*), 8.03 (s, 1 H, C_2H_4−N*H*−biotin), 4.71−4.66 (m, 1 H, NCH of biotin), 4.49−4.45 (m, 1 H, NCH of biotin), 3.42−3.40 (m, 4 H, C_2H_4−NH−biotin), 3.24−3.14 (m, 2 H, SCH of biotin), 2.82 (s, 6 H, CH_3 at C3 and C8 of Me_4-phen), 2.76−2.72 (m, 1 H, SCH of biotin), 2.61 (s, 1 H, SCH of biotin), 2.28−2.26 (m, 2 H, $COCH_2C_3H_6$ of biotin), 1.64−1.30 (m, 6 H, $COCH_2C_3H_6$ of biotin). IR (KBr) ν/cm^{-1}: 3423 (br, NH), 2116 (m, N=C=S), 2035 (s, $C\equiv O$), 1919 (s, $C\equiv O$), 1655 (s, C=O), 843 (s, PF_6^-). Positive-ion ESI-MS ion cluster at m/z 955 {M − PF_6}$^+$. Anal. Calcd for $C_{38}H_{40}N_8O_6S_2PF_6Re\cdot H_2O$: C, 40.82; H, 3.79; N, 10.02. Found: C, 40.62; H, 4.02; N, 9.73%. Complex **3a**. Yield: 154 mg (76%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ 10.09 (d, 2 H, J = 5.4 Hz, H2 and H9 of Ph_2-phen), 10.00−9.93 (m, 1 H, NH of biotin), 8.93 (s, 1 H, H2 of pyridine), 8.89 (s, 1 H, H6 of pyridine), 8.33−8.24 (m, 6 H, H3, H5, H6 and H8 of Ph_2-phen, H4 of pyridine and py−3-CON*H*), 7.81−7.67 (m, 11 H, C_6H_5 of Ph_2-phen and C_2H_4−N*H*−biotin), 4.71−4.68 (m, 1 H, NCH of biotin), 4.50−4.42 (m, 1 H, NCH of biotin), 3.44−3.37 (m, 4 H, C_2H_4−NH−biotin), 2.97 (s, 1 H, SCH of biotin), 2.21−2.18 (m, 2 H, $COCH_2C_3H_6$ of biotin), 1.74−1.32 (m, 6 H, $COCH_2C_3H_6$ of biotin). IR (KBr) ν/cm^{-1}: 3423 (br, NH), 2121(m, N=C=S), 2034 (s, $C\equiv O$), 1919 (s, $C\equiv O$), 1655 (s, C=O), 842 (s, PF_6^-). Positive-ion ESI-MS ion cluster at m/z 1050 {M − PF_6}$^+$. Anal. Calcd for $C_{46}H_{40}N_8O_6S_2PF_6Re\cdot H_2O$: C, 45.50; H, 3.49; N, 9.23. Found: C, 45.35; H, 3.50; N, 9.52%.

[Re(N−N)(CO)$_3$(py-biotin-TU-Et)](PF_6) (N−N = Phen (1b), Me_4-phen (2b), Ph_2-phen (3b)). A mixture of [Re(N−N)(CO)$_3$(py-biotin-NCS)](PF_6) (0.17 mmol) and ethylamine (0.17 mmol) in acetone (30 mL) was stirred at room temperature under an inert atmosphere of nitrogen for 12 h. The solution was evaporated to dryness, forming an orange solid, which was then purified by column chromatography on alumina. The desired product was eluted with acetone/MeOH (10:1, v/v). Upon recrystallization of the crude product from CH_2Cl_2/diethyl ether, the complex formed as orange crystals. Complex **1b**. Yield: 122 mg (66%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ 9.96 (s, 1 H, NH of pyridine), 9.87 (d, 2 H, J = 3.8 Hz, H2 and H9 of phen), 9.73 (s, 1 H, H4 of pyridine), 9.09 (d, 2 H, J = 8.2 Hz, H4 and H7 of phen), 8.70 (s, 1 H, H2 of pyridine), 8.38−8.32 (m, 4 H, H3, H5, H6 and H8 of phen), 8.05 (s, 1 H, Et−N*H*), 7.95 (s, 1 H, py−3-CON*H*), 7.81 (s, 1 H, H6 of pyridine), 7.61 (s, 1 H, C_2H_4−N*H*−biotin), 6.89 (s, 1 H, NH of biotin), 6.18 (s, 1 H, NH of biotin), 4.61−4.58 (m, 1 H, NCH of biotin), 4.39−4.36 (m, 1 H, NCH of biotin), 3.79−3.74 (m, 2 H, CH_2 of Et), 3.42−3.39 (m, 4 H, C_2H_4−NH−biotin), 3.17 (s, 1 H, SCH of biotin), 2.68 (d, 1 H, J_{gem} = 12.6 Hz, SCH of biotin), 2.27 (s, 2 H, $COCH_2C_3H_6$ of biotin), 1.73−1.24 (m, 9 H, $COCH_2C_3H_6$ of biotin and CH_3 of Et). IR (KBr) ν/cm^{-1}: 3432 (br, NH), 2033 (s, $C\equiv O$), 1919 (s, $C\equiv O$), 1686 (m, C=O), 1234 (m, C=S), 846 (s, PF_6^-). Positive-ion ESI-MS ion cluster at m/z 943 {M − PF_6}$^+$. Anal. Calcd for $C_{36}H_{39}N_9O_6S_2PF_6Re\cdot H_2O$: C, 39.06; H, 3.73; N, 11.39. Found: C, 38.97; H, 3.97; N, 11.12%. Complex **2b**. Yield: 106 mg (51%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ 9.93 (s, 1 H, NH of pyridine), 9.78 (s, 1 H, H4 of pyridine), 9.62 (s, 2 H, H2 and H9 of Me_4-phen), 8.68 (s, 1 H, H2 of pyridine), 8.47 (s, 2 H, H5 and H6 of Me_4-phen), 8.06 (s, 1H, Et−N*H*), 7.97 (s, 1 H, py−3-CON*H*), 7.81 (s, 1 H, H6 of pyridine), 7.62 (s, 1 H, C_2H_4−N*H*−biotin), 6.82 (s, 1 H, NH of biotin), 6.17 (s, 1 H, NH of biotin), 4.59−4.56 (m, 1 H, NCH of biotin), 4.38−4.32 (m, 1 H, NCH of biotin), 3.80−3.71 (m, 2 H, CH_2 of Et), 3.44−3.39 (m, 4 H, C_2H_4−NH−biotin), 3.18−3.12 (m, 1 H, SCH of biotin), 2.68 (d, 1 H, J_{gem} = 12.9 Hz, SCH of biotin), 2.30−2.23 (m, 2 H, $COCH_2C_3H_6$ of biotin), 1.66−1.20 (m, 9 H, $COCH_2C_3H_6$ of biotin and CH_3 of Et). IR (KBr) ν/cm^{-1}: 3432 (br, NH), 2031 (s, $C\equiv O$), 1919 (s, $C\equiv O$), 1686 (m, C=O), 1245 (m, C=S), 846 (s, PF_6^-). Positive-ion ESI-MS ion cluster at m/z 1001 {M − PF_6]}$^+$. Anal. Calcd for $C_{40}H_{49}N_9O_6S_2PF_6Re\cdot H_2O$: C, 41.23; H, 4.41; N, 10.82. Found: C, 41.53; H, 4.65; N, 11.03%. Complex **3b**. Yield: 126 mg (85%). ^{1}H NMR (300 MHz, acetone-d_6, 298 K, TMS): δ 9.95 (s, 1 H, NH of pyridine), 9.90 (d, 2 H, J = 5.1 Hz, H2 and H9 of Ph_2-phen), 9.49 (s, 1 H, H4 of pyridine), 8.93 (s, 1 H, H2 of pyridine), 8.28−8.26 (m, 4 H, H3, H5, H6 and H8 of Ph_2-phen), 8.17 (s, 1 H, Et−N*H*), 7.95 (s, 1 H, py−3-CON*H*), 7.78−7.68 (m, 12 H, C_6H_5 of Ph_2-phen, H6 of pyridine and C_2H_4−N*H*−biotin), 6.84 (s, 1 H, NH of biotin), 6.19 (s, 1 H, NH of biotin), 4.60−4.55 (m, 1 H, NCH of biotin), 4.37−4.34 (m, 1 H, NCH of biotin), 3.74−3.65 (m, 2 H, CH_2 of Et), 3.44−3.37 (m, 4 H, C_2H_4−NH−biotin), 3.19−3.14 (m, 1 H, SCH of biotin), 2.66 (d, 1 H,

J_{gem} = 12.8 Hz, SCH of biotin), 2.28−2.25 (m, 2 H, $COCH_2C_3H_6$ of biotin), 1.70−1.20 (m, 9 H, $COCH_2C_3H_6$ of biotin and CH_3 of Et). IR (KBr) ν/cm^{-1}: 3426 (br, NH), 2032 (s, C≡O), 1919 (s, C≡O), 1686 (m, C=O), 1235 (m, C=S), 843 (s, PF_6^-). Positive-ion ESI-MS ion cluster at *m/z* 1097 {M − PF_6}$^+$. Anal. Calcd for $C_{48}H_{47}N_9O_6S_2PF_6Re{\cdot}H_2O$: C, 45.78; H, 3.92; N, 10.01. Found: C, 46.02; H, 4.14; N, 9.89%.

Physical Measurements and Instrumentation. The instruments used for the characterization and photophysical measurements have been described previously.[8b] Luminescence quantum yields were measured by the optically dilute method[30] using an aerated acetonitrile solution of $[Re(phen)(CO)_3(pyridine)](CF_3SO_3)$ (Φ = 0.18, excitation wavelength at 355 nm) as the standard solution.[15b] The methods by which the HABA assay, emission titrations, and determination of avidin-binding parameters of the thiourea complexes **1b**−**3b** were undertaken have also been previously described.[8f,31]

Biotinylation of BSA with the Isothiocyanate Complexes 1a−3a. The isothiocyanate complex (1.2 μmol) in anhydrous DMSO (50 μL) was added to BSA (1.23 mg, 18.6 nmol) in 50 mM carbonate buffer (450 μL) at pH 9.7. The suspension was stirred for 12 h in the dark at room temperature, and the solid residue was removed by centrifugation. The supernatant was then diluted to 1.0 mL with 50 mM potassium phosphate buffer at pH 7.4 and loaded onto a PD-10 column equilibrated in the same buffer. The first elution band with strong orange-yellow or greenish-yellow luminescence was collected. Finally, the bioconjugates **BSA-1b**−**BSA-3b** were washed successively with potassium phosphate buffer using a YM-30 centricon, concentrated to 1.5 mL and stored at 4 °C.

Digestion of the Bioconjugates BSA-1b−BSA-3b by Pronase. The bioconjugate in 50 mM potassium phosphate buffer at pH 7.4 (1.5 mL) was heated at 80 °C for 30 min. After the solution was cooled to room temperature, pronase (2 mg) in water (200 μL) was added. The mixture was maintained at 37 °C for 24 h prior for analysis by the HABA assay.

Cytotoxicity Assays. Cytotoxicity assays were conducted in 96-well, flat-bottomed microtiter plates. The supplemented culture medium (100 μL) with ca. 10 000 cells per well was incubated at 37 °C under a 5% CO_2 atmosphere for 24 h. The thiourea complexes **1b**−**3b** were dissolved in the culture medium with 1% DMSO and the solutions added to the wells. The concentrations of the complexes ranged from 2 to 23 μM. Supplemented media with 1% DMSO (100 μL) was used as a control. After the microtiter plate was incubated for 48 h, 10 μL of MTT in PBS (5 mg/mL) was added to each well. The microplate was incubated for another 3 h. Solubilization solution (100 μL) containing 10% SDS in 2-propanol/0.04 M hydrochloric acid (1:1, v/v) was added to each well, and the plate was incubated for 24 h. All the assays were run in parallel with a negative control (i.e., vehicle control) and 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.

Cellular Uptake Studies. 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 thiourea complex **3b** (10 μM). After incubation for 24 h, the medium was removed, and the cell layer was washed gently with PBS (1 mL × 3). The coverslip was mounted onto a glass slide, imaged using a Carl Zeiss Axioplan 2 imaging fluorescence microscope with the excitation wavelength in the range of 420−490 nm, and the emission measured using a 545 nm long-pass filter.

Results and Discussion

Synthesis. The design of the luminescent amine-specific biotinylation reagents, complexes **1a**−**3a**, is based on the use of a trifunctional compound, 5-aminonicotinic acid. The pyridine may then be coordinated to the rhenium(I) center, the carboxyl group functionalized with an amine−biotin derivative, and the amine group readily activated by thiophosgene to form the amine-specific isothiocyanate group. The resultant pyridine−biotin−isothiocyanate ligand, together with the use of various diimines, leads to the production of luminescent rhenium(I) complexes as biotinylation reagents with tunable emission colors. The isothiocyanate complexes **1a**−**3a** were prepared from the reaction of the precursor amine complexes **1c**−**3c** with thiophosgene in acetone at room temperature (Scheme 1). To examine the reactivity of the isothiocyanate complexes toward aliphatic amines, they were reacted with a model substrate, ethylamine, which resulted in the formation of the thiourea complexes **1b**−**3b** (Scheme 1). All the complexes were characterized by ^{1}H NMR, positive-ion ESI-MS, IR, and microanalyses.

Electronic Absorption and Emission Properties. The electronic absorption spectral data of the complexes are summarized in Table 1, and the electronic absorption spectrum of complex **1b** in CH_2Cl_2 at 298 K is shown in Figure 1. With reference to previous studies on related rhenium(I) polypyridine complexes,[7a,8a,b,f,h,9,10a,b,11a,c,12−14,15a,b,d−f,16,17a,c,d,18−20,21a,c,22b,c,23a,b,24−28] the intense absorption bands at ca. 248−300 nm with extinction coefficients on the order of 10^4 dm^3 mol^{-1} cm^{-1} have been assigned to spin-allowed intraligand (^{1}IL) ($\pi \rightarrow \pi^*$) (N−N and pyridine ligands) transitions. Additionally, the absorption shoulders at ca. 322−397 nm with smaller extinction coefficients have been assigned to spin-allowed metal-to-ligand charge-transfer (^{1}MLCT) (dπ(Re) → π^*(N−N)) transitions. The Ph_2-phen complexes **3a**−**3c** showed lower-energy ^{1}IL absorption bands due to the electron-withdrawing phenyl substituents of the diimine ligand.

All the complexes exhibited intense and long-lived orange-yellow to greenish-yellow luminescence in fluid solutions at 298 K upon photoexcitation. The emission spectrum of complex **1b** in CH_2Cl_2 is shown in Figure 1. The photophysical data are listed in Table 2. This emission has been attributed to a triplet metal-to-ligand charge-transfer (^{3}MLCT) (dπ(Re) → π^*(N−N)) excited state.[7a,8a,b,f,h,9,10a,b,11a,c,12−17,18a,b,d,19,20a,c,21,22,23a,b,d,24,25a,c,26−28] Supporting this assignment is the observation that the isothiocyanate complexes **1a**−**3a** emitted at slightly higher energy than their thiourea **1b**−**3b** and amine **1c**−**3c** counterparts (Table 2). We attributed this to the electron-withdrawing isothiocyanate moiety rendering the metal center less electron-rich and hence increasing the ^{3}MLCT emission energy.

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

complex	medium	λ_{abs}/nm (ϵ/dm^3 mol^{-1} cm^{-1})
1a	CH_2Cl_2	259 sh (16 040), 276 (19 700), 294 sh (12 660), 333 sh (3910), 374 sh (2515)
	CH_3CN	258 sh (17 305), 274 (20 170), 300 sh (11 415), 328 sh (6165), 370 sh (3185)
1b	CH_2Cl_2	256 sh (28 055), 274 sh (28 000), 298 sh (12 945), 332 sh (6870), 389 sh (2265)
	CH_3CN	255 sh (26 495), 271 (27 365), 297 sh (12 750), 330 sh (6675), 382 sh (2085)
	buffer[a]	328 sh (7690), 382 sh (2385)
1c	CH_2Cl_2	260 (30 425), 277 sh (27 050), 298 sh (11 990), 335 (7960), 389 sh (3100)
	CH_3CN	259 (26 530), 268 (26 875), 296 sh (13 655), 327 (9305), 375 sh (3675)
2a	CH_2Cl_2	250 (27 230), 281 (33 575), 327 sh (11 150), 376 sh (2948)
	CH_3CN	250 sh (30 755), 280 (38 545), 326 sh (12 105), 370 sh (3260)
2b	CH_2Cl_2	253 (41 670), 279 (41 635), 328 sh (13 790), 376 sh (3395)
	CH_3CN	249 (33 350), 278 (31 735), 325 sh (10 895), 372 sh (2545)
	buffer[a]	322 sh (11 540), 372 sh (2795)
2c	CH_2Cl_2	254 (29 595), 281 (29 705), 338 sh (9895), 371 sh (3790)
	CH_3CN	248 (37 245), 280 (35 875), 330 sh (12 945), 370 sh (3955)
3a	CH_2Cl_2	265 sh (20 370), 290 (30 140), 342 sh (9570), 394 sh (4805)
	CH_3CN	262 (28 895), 288 (46 170), 334 sh (15 500), 387 sh (6335)
3b	CH_2Cl_2	270 (41 035), 287 (45 580), 341 sh (17 920), 397 sh (6470)
	CH_3CN	261 (35 370), 287 (40 110), 335 sh (16 270), 389 sh (5580)
	buffer[a]	336 sh (12 920), 392 sh (4565)
3c	CH_2Cl_2	267 (28 895), 292 (33 545), 342 sh (15 300), 395 sh (6385)
	CH_3CN	263 (32 245), 292 (39 245), 337 sh (17 440), 387 sh (6755)

[a] 50 mM potassium phosphate at pH 7.4 containing 30% DMSO (DMSO was used to increase complex solubility).

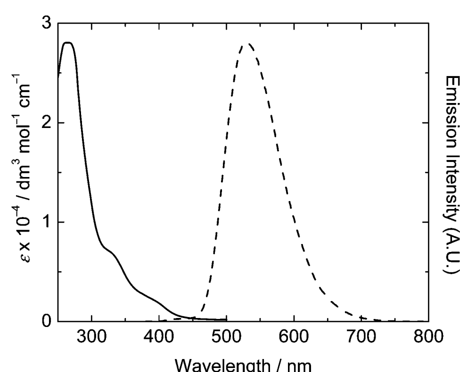

Figure 1. Electronic absorption (—) and emission (− − −) spectra of complex **1b** in CH_2Cl_2 at 298 K.

Nonetheless, the energy difference is relatively small because the electron density of the rhenium(I) center is only remotely controlled by the substituents on the pyridine ligand. The longer emission lifetimes of the Me_4-phen complexes in fluid solutions at room temperature (Table 2) suggest the presence of substantial triplet intraligand ^{3}IL ($\pi \rightarrow \pi^*$) (Me_4-phen) character in their emissive states.[8b,f,14a,15b,c,e,16a,b,c,22c,28b] This is also reflected by the very similar emission wavelengths of complexes **2a**−**2c** in different solvents. The emission of all the complexes in alcohol glass at 77 K showed a significant blue-shift owing to the rigidochromic effect (Table 2), which is commonly observed in luminescent rhenium(I) polypyridine complexes.[7e,8b,h,9,11a,12a,13b,14d,15a,d−f,16a−c,19b,21,28b−e]

HABA Assay. The thiourea complexes **1b**−**3b** can be considered as models for biomolecules biotinylated by the isothiocyanate complexes **1a**−**3a**. Thus, the avidin-binding properties of complexes **1b**−**3b** in buffer have been examined by the HABA assay.[5] The avidin−HABA adduct is known to display an intense absorption band at 500 nm. As the affinity of biotin to avidin (K_d = ca. 10^{-15} M) is much higher than that of HABA ($K_d = 6 \times 10^{-6}$ M), addition of biotin to a solution of the avidin−HABA adduct will displace the bound HABA molecules, leading to a decrease of absorbance at 500 nm. In this work, addition of complexes **1b**−**3b** to an avidin−HABA solution resulted in a decrease of absorbance, indicative of the specific binding of the complexes to avidin. Interestingly, the plots of $-\Delta A_{500\ nm}$ versus [Re]/[avidin] show that the equivalence points occurred at [Re]/[avidin] = ca. 4.5, 5.2, and 4.8, for complexes **1b**−**3b**, respectively. As avidin may only bind up to four biotin molecules, the occurrence of the equivalence points at [Re]/[avidin] > 4 suggests that the binding affinities of these complexes are not substantially higher than that of HABA.

Emission Titrations. The binding of the thiourea complexes **1b**−**3b** to avidin has been studied by emission titrations using the complexes as titrants. Two control experiments, in which (1) avidin was absent or (2) avidin was presaturated with excess biotin, were also performed. Similar to other luminescent transition metal biotin complexes we have reported,[8] the thiourea complexes **1b**−**3b** showed enhanced emission intensities (ca. 1.4−1.5-fold) in the presence of avidin (Table 3). The titration curves for complex **2b** are shown in Figure 2. Since no changes were observed in the control experiments, the emission enhancement must result from the specific binding of complexes **1b**−**3b** to avidin. The emission lifetimes of the complexes were elongated from ca. 0.4−1.1 to 0.8−2.0 μs upon the binding event (Table 3). These changes of photophysical properties were ascribed to the increase in the hydrophobicity and rigidity of the local environment of the complexes upon binding to avidin.[8] The first dissociation constants (K_d) of the avidin adducts of the thiourea complexes **1b**−**3b** were estimated to be 7.9×10^{-8}, 1.2×10^{-7}, and 5.6×10^{-7} M, respectively. These values are up to 3 orders of magnitude higher than those of other rhenium(I) polypyridine biotin complexes,[8a,b,f,h] probably due to the steric hindrance of the thiourea moiety and the lack of a long spacer-arm in the current complexes. The avidin-binding affinity of complex **1b** is higher than those of complexes **2b** and **3b**, which may

Table 2. Photophysical Data of the Rhenium(I) Polypyridine Biotin Complexes

complex	medium (*T*/K)	λ_{em}/nm	τ_o/μs	Φ
1a	CH_2Cl_2 (298)	527	2.92	0.28
	CH_3CN (298)	546	1.43	0.12
	glass (77)[a]	462 sh, 509	9.58	
1b	CH_2Cl_2 (298)	531	2.40	0.51
	CH_3CN (298)	546	1.11	0.10
	buffer (298)[b]	546	0.18	0.017
	glass (77)[a]	474 sh, 492	11.17	
1c	CH_2Cl_2 (298)	535	2.92	0.56
	CH_3CN (298)	548	1.15	0.16
	glass (77)[a]	490 sh, 509	10.03	
2a	CH_2Cl_2 (298)	485 sh, 512	11.13	0.44
	CH_3CN (298)	485 sh, 514	5.11	0.14
	glass (77)[a]	470 (max), 503, 542 sh	84.59 (49%), 16.79 (51%)	
2b	CH_2Cl_2 (298)	488 sh, 510	14.41	0.52
	CH_3CN (298)	482 sh, 513	7.59	0.39
	buffer (298)[b]	485 sh, 514	6.28	0.037
	glass (77)[a]	468 (max), 500, 537 sh	103.10 (56%), 21.21 (44%)	
2c	CH_2Cl_2 (298)	489 sh, 513	14.67	0.45
	CH_3CN (298)	484 sh, 515	13.50	0.20
	glass (77)[a]	470 (max), 501, 540 sh	111.69 (43%), 20.84 (57%)	
3a	CH_2Cl_2 (298)	542	8.75	0.29
	CH_3CN (298)	553	3.77	0.19
	glass (77)[a]	510, 536 sh	21.28	
3b	CH_2Cl_2 (298)	543	8.45	0.46
	CH_3CN (298)	556	2.60	0.14
	buffer (298)[b]	560	2.41	0.010
	glass (77)[a]	507, 546 sh	21.93	
3c	CH_2Cl_2 (298)	547	7.64	0.42
	CH_3CN (298)	558	3.85	0.24
	glass (77)[a]	509, 543 sh	21.52	

[a] In butyronitrile glass. [b] 50 mM potassium phosphate at pH 7.4 containing 5% DMSO. For quantum yield measurements, 50 mM potassium phosphate at pH 7.4 containing 30% DMSO was used.

Table 3. Relative Emission Intensities and Lifetimes of the Thiourea Complexes **1b**−**3b** in Aerated Buffer/DMSO (97:3, v/v) at 298 K

complex	*I* (τ/μs)[a]	*I* (τ/μs)[b]	*I* (τ/μs)[c]
1b	1.00 (0.44)	1.42 (0.77)	1.08 (0.44)
2b	1.00 (1.14)	1.46 (1.96)	1.03 (1.11)
3b	1.00 (0.90)	1.51 (1.96)	1.11 (0.87)

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

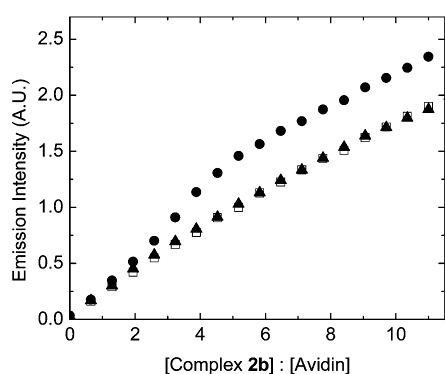

Figure 2. Luminescence titration curves for the titrations of (i) 3.8 μM avidin (●), (ii) 3.8 μM avidin and 380 μM biotin (▲), and (iii) a blank solution (□) with complex **2b**.

be the consequence of the steric demands of the Me_4-phen and Ph_2-phen ligands of the latter complexes.

Biotinylation of BSA with the Isothiocyanate Complexes 1a−3a. To evaluate the biotinylation properties of the isothiocyanate complexes **1a**−**3a**, we have used them to label a model protein, BSA. The resultant bioconjugates, denoted by **BSA-1b**−**BSA-3b** due to their structural similarity with the corresponding thiourea complexes **1b**−**3b**, were purified by size exclusion chromatography and ultrafiltration. Control experiments using the biotin-free isothiocyanate complex [Re(Me_4-phen)(CO)$_3$(py-NCS)](PF_6) (**2d**)[7a] and the isothiocyanate-free biotin complex [Re(Me_4-phen)(CO)$_3$(py-biotin)](PF_6) (**2e**)[8f] were also performed. Whereas BSA was successfully labeled with complex **2d**, no luminescent bioconjugate was produced when complex **2e** was used, confirming that the bioconjugation originates from the reaction of the isothiocyanate moiety of the complexes with BSA. The bioconjugates **BSA-1b**−**BSA-3b** displayed intense and long-lived orange-yellow to greenish-yellow ^{3}MLCT/^{3}IL emission upon irradiation in aqueous buffer under ambient conditions. The photophysical data are listed in Table 4. Additionally, the electronic absorption and emission spectra of the bioconjugate **BSA-1b** in phosphate buffer are shown in Figure 3. All the bioconjugates **BSA-1b**−**BSA-3b** showed biexponential emission decay, which is not uncommon for biomolecules labeled with luminescent transition metal complexes.[7,12a,b,28a,32] The average lifetimes are longer than

Table 4. Photophysical Data of the Bioconjugates **BSA-1b**−**BSA-3b** in Degassed 50 mM Phosphate Buffer at pH 7.4 at 298 K

conjugates	λ_{em}/nm	τ_o/μs	Φ
BSA-1b	543	0.89 (9%), 0.10 (91%)	0.011
BSA-2b	488 sh, 518	7.15 (39%), 1.02 (61%)	0.024
BSA-3b	552	2.91 (32%), 0.25 (68%)	0.010

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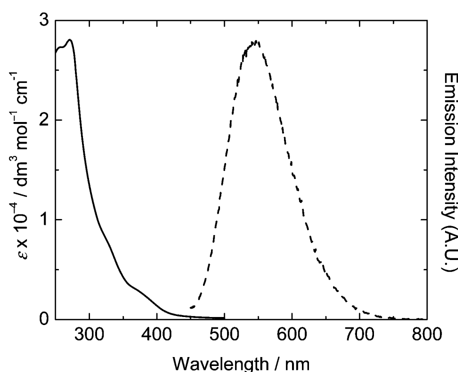

Figure 3. Electronic absorption (—) and emission (– – –) spectra of the bioconjugate **BSA-1b** in 50 mM phosphate buffer at pH 7.4 at 298 K.

those of the thiourea complexes **1b**−**3b** under the same experimental conditions, as a result of the more hydrophobic local environment associated with the protein molecules.[7,8,12a,b,28a,b,d,e,32] From a correlation of the luminescence intensities of the thiourea complexes to their concentrations, the biotin/BSA ratios of the bioconjugates **BSA-1b**−**BSA-3b** have been determined to be ca. 1.9, 2.5, and 1.8, respectively. Since fluorometric methods offer higher detection sensitivity and lower limits of detection compared with absorption methods, the current luminescent biotinylation reagents are particularly useful for the detection and quantitation of biotinylated molecules. Under the experimental conditions employed, the limits of detection for the thiourea complexes **1b**−**3b** were ca. 90, 10, and 120 nM, respectively. These concentrations are comparable with the fluorometric displacement assay (40−800 nM)[6a] and about 1−2 orders of magnitude lower than the micromolar range of both the HABA assay[5] and the absorption methods, which use the two chromogenic biotinylation reagents.[6]

The avidin-binding properties of the bioconjugates **BSA-1b**−**BSA-3b** have been studied by the HABA assay. Upon addition of the biotinylated proteins to a solution of avidin and HABA, only a small change in the absorbance at 500 nm was observed. This indicated that the avidin-bound HABA molecules were not displaced by the biotin moieties of the bioconjugates. It is likely that the biotin groups on the bioconjugates are sterically restricted by the protein matrix and inaccessible to the biotin-binding sites of the avidin molecules. Thus, we used the protease pronase to digest the bioconjugates (by hydrolysis of the peptide bonds) prior for analysis by the HABA assay. Addition of the digestion mixtures to an avidin−HABA solution resulted in a decrease of absorbance at 500 nm, indicating that the biotin groups of the bioconjugates bound to avidin. Interestingly, the biotin/BSA ratios were determined to be 2.2, 2.4, and 1.4 for **BSA-1b**−**BSA-3b**, respectively, which are in good agreement with the results from the emission measurements (ca. 1.9, 2.5, and 1.8, respectively).

Cytotoxicity Assays. Although relatively unexplored, cytotoxicity studies of tricarbonylrhenium(I) complexes are receiving increasing attention.[33] To understand the potential cytotoxicity of biomolecules biotinylated with the isothiocyanate complexes, the MTT assay was employed to examine the cytotoxicity of the thiourea complexes **1b**−**3b**, which can be considered as models for biomolecules biotinylated by complexes **1a**−**3a**, toward the cervical epithelioid carcinoma cell line (HeLa).[34] The IC_{50} values of complexes **1b**−**3b** have been determined to be 22.7, 17.5, and 28.5 μM, respectively. This is comparable to that of cisplatin (26.7 μM) and indicates potential anticancer properties. The cytotoxicity of complexes **1b**−**3b** is lower than the rhenium(I) diphosphine complexes [$Re(CO)_3$(diphosphine)Br][33a] but slightly higher than the related rhenium(I) diimine complex [$Re(CO)_3$(2-appt)Cl] (2-appt = 2-amino-4-phenylamino-6-(2-pyridyl)-1,3,5-triazine) (IC_{50} = ca. 50 μM), which has been identified as a minor-groove binder to double-stranded DNA.[33b]

Cellular Uptake Studies. The cellular uptake of rhenium(I) complexes has attracted much attention recently because of their potential diagnostic and therapeutic applications.[35] As noted in the Introduction, one of the reasons for the development of luminescent biotinylation reagents is that they can be used to biotinylate small molecules and biological uptake of the labeled compounds may then be examined by luminescence spectroscopy and microscopy. Thus, we have studied the cellular internalization properties of the thiourea complex **3b**, which acts as a model for biomolecules labeled by complex **3a**, and its possible use as a biological imaging reagent. Incubation of HeLa cells with the complex at 37 °C under a 5% CO_2 atmosphere for 24 h resulted in cellular uptake. Upon visible-light irradiation, the cytoplasm of the cells exhibited orange luminescence (Figure 4). Interestingly, their nuclei displayed much weaker emission, indicative of negligible nuclear uptake of the complex. The complex was not homogeneously distributed within the cytoplasm but localized in the perinuclear region (Figure 4). From the images, it appears that the complex binds to the Golgi apparatus,[36a,b] although it may also bind to other hydrophobic organelles such as endoplasmic reticulum and mitochondria.[36c] The internalization of the complex appears to occur via energy-requiring processes such as endocytosis since there was no evidence of uptake following incubation at 4 °C. When the cells were loaded with the cytoskeletal inhibitor nocodazole, the complex was more evenly distributed in the

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Figure 4. Bright-field (left), overlaid (middle), and fluorescence (right) microscopy images of HeLa cells incubated with complex **3b** (10 μM) at 37 °C for 24 h.

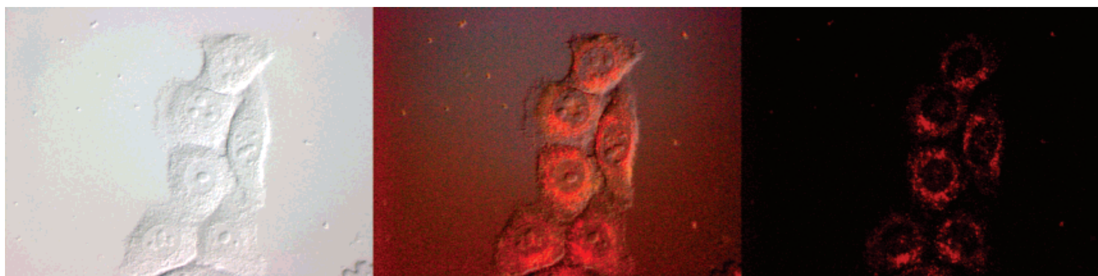

Figure 5. Bright-field (left), overlaid (middle), and fluorescence (right) microscopy of HeLa cells incubated with nocodazole (30 μM) at 37 °C for 90 min followed by complex **3b** (10 μM) at the same temperature for 24 h.

perinuclear region (Figure 5), highlighting the important role of cytoskeleton in the intracellular transportation of the complex.

Conclusion

We have designed a series of rhenium(I) biotin isothiocyanate complexes as the first class of luminescent biotinylation reagents. The photophysical properties of these complexes and their amine and thiourea counterparts have been studied. These isothiocyanate complexes provide the biotinylated biological molecules with rich luminescence properties allowing direct determination of the degree of biotinylation by sensitive spectrofluorometric methods. The cytotoxicity of the thiourea complexes toward HeLa cells has been examined, and the IC_{50} values are comparable to that of the anticancer drug cisplatin. The cellular uptake of the thiourea complex **3b** has also been examined. Interestingly, the internalized complex maintains its intense emission in the cytoplasm of the cell. It can be seen from the fluorescence images that the complex was localized in the perinuclear region, as a result of possible interactions with hydrophobic organelles such as the Golgi apparatus. These important results reveal that the isothiocyanate complexes not only serve as novel biotinylation reagents but may also contribute to the development of luminescent tracers for specific intracellular delivery of biomacromolecules and small molecular substrates including potential anticancer drugs.

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