

COMMUNICATIONS

Ruthenium(II)–Phenanthroline–Biotin Complexes: Synthesis and Luminescence Enhancement upon Binding to Avidin

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We report the synthesis, characterization, and avidin-binding properties of two novel ruthenium complexes, $[\text{Ru}(\text{bpy})_2(\text{phen-biotin})][\text{PF}_6]_2$ **1** and $[\text{Ru}(\text{phen})_2(\text{phen-biotin})][\text{PF}_6]_2$ **2** (bpy = 2,2'-bipyridine; phen = 1,10-phenanthroline, phen-biotin = 5-(10-amidobiotinyl)-1,10-phenanthroline). We demonstrate that both biotinylated compounds bind to avidin through their biotin moieties with high affinity and in a 4:1 ratio. The binding of compounds **1** and **2** to avidin results in an enhancement in luminescence intensity ($\sim 1.4\times$, $\sim 1.6\times$, respectively), relative to the unbound biotinylated ruthenium complexes. This behavior is markedly different from biotinylated organic dyes, whose fluorescence is quenched upon binding to avidin. Thus, ruthenium–biotin complexes **1** and **2** can form the basis of new, simplified biotin–avidin assays, which involve luminescence detection of the relevant biotinylated molecule through cross-linking with avidin.

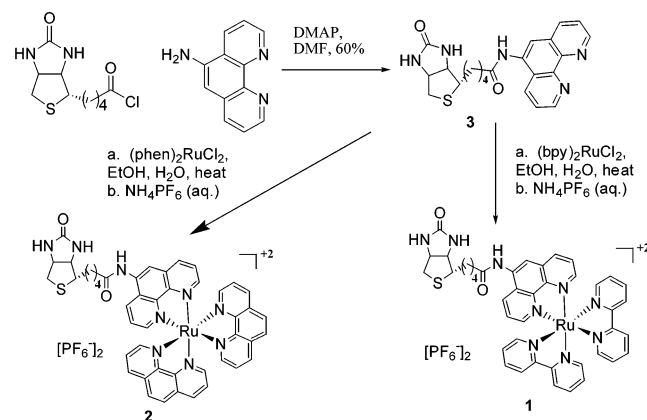
INTRODUCTION

The binding of biotin to (strept)avidin has found widespread applications in biomolecule detection, medical diagnostics, immunoassays, cytochemistry, and nanoscience (1–7). This is primarily due to the strength of the interaction between the ligand and the protein ($K_d = 1.3 \times 10^{-15}$ M) and to the presence of four binding sites for biotin on avidin, which in turn allows for the cross-linking of different biotinylated molecules (1). Bioanalytical applications based on the biotin/avidin interaction have typically used enzymatic reactions, radiolabeling techniques, and fluorescence for detection/visualization (8–12). Of these, fluorescence-based assays are some of the most straightforward and rapid detection techniques

(13–16). The simplest design for these biotin–avidin assays would be covalent attachment of the fluorescent dye to biotin, followed by fluorescence detection of the relevant biotinylated molecule through cross-linking with avidin. However, biotinylated organic dyes have been shown to lose their luminescence intensity upon binding to avidin, likely through resonance energy transfer mechanisms (17–22). To overcome this problem, long spacers, such as poly(ethylene glycol), have been used to maximize the distance between the biotin-conjugated organic dyes and the avidin binding site (17, 18). As a result of these difficulties, most fluorescence-based detection methods have relied on the more difficult conjugation of the organic dyes to the protein itself, rather than simple attachment to biotin (18, 19).

Among many inorganic chromophores, ruthenium(II) bipyridyl or phenanthroline complexes have recently

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Scheme 1. Synthesis of Ru(II)–Phenanthroline–Biotin Complexes 1 and 2

emerged as promising candidates for a variety of bioanalytical applications (13, 23–26). These complexes present some unique photophysical properties, which distinguish them from their organic counterparts, including their chemical inertness and photostability, the tunability of their photophysical characteristics, the relative insensitivity of their photophysical properties to environmental changes, their large Stokes shifts, and their long excited-state lifetimes (13). We here report the facile synthesis of ruthenium complexes **1** and **2**, where a $(\text{bpy})_2\text{Ru}(\text{phen})^{2+}$ or $(\text{phen})_3\text{Ru}^{2+}$ center is conjugated to a biotin moiety ($\text{bpy} = 2,2'$ -bipyridine, $\text{phen} = 1,10$ -phenanthroline). Unlike organic dyes, binding of these Ru(II)–biotin complexes to avidin results in an enhancement in their luminescence intensity, thus making them useful tools for fluorescence-based biotin–avidin assays.

The synthesis of complexes **1** or **2** was carried out by first coupling 5-amino-1,10-phenanthroline (27, 28) with the acyl chloride (29) derivative of biotin, to give the biotin–phenanthroline ligand **3**. The target complexes **1** and **2** were then obtained by refluxing ligand **3** with Ru(bpy)₂Cl₂ or Ru(phen)₂Cl₂ (30) in good yield (70–80%) and were isolated as their PF₆[−] salts (Scheme 1) (46).

The UV/vis absorbance spectrum of ruthenium–biotin complexes **1** and **2** in CH₂Cl₂ shows bands at 450 nm, assigned to Ru(II) $d\pi-\pi^*$ metal-to-ligand charge transfer (MLCT) transitions (Figure 1) (46). In addition, more intense bands at 250–300 nm were assigned to ligand-centered $\pi-\pi^*$ transitions. These absorbances and assignments are consistent with a number of related complexes in the literature (13, 30). Steady-state emission spectra were recorded for complexes **1** and **2** in dichloromethane and showed strong luminescence at 589 nm for **1** and 580 nm for **2**, upon excitation of the MLCT band

at 450 nm (Figure 1). The emission wavelength showed a slight increase with increasing solvent polarity, likely due to the stabilization of the MLCT state with polar solvents (46).

The binding properties of the Ru–biotin complexes **1** and **2** to avidin were first examined using the 4'-hydroxyazobenzene-2-carboxylic acid (HABA) assay (32, 46). HABA can bind to avidin at the same binding site as biotin, with an association constant of 10^7 M^{-1} and with the same stoichiometry as unmodified biotin (4:1). Upon binding to avidin, HABA displays a new peak in its absorption spectrum at 500 nm. When biotin or biotinylated molecules are added, the displacement of HABA from avidin results in the reduction of this HABA–avidin absorption at 500 nm. The HABA–avidin complex was thus generated and was then titrated with the Ru–biotin complex **1**. An immediate decrease in the absorption at 500 nm was detected, and a new peak at ca. 450 nm, corresponding to the ruthenium complex **1**, was observed. The titration required exactly 4 mol equiv of complex **1** to avidin, after which no further changes in the absorption peak at 500 nm were detected (46). These results are consistent with immediate displacement of HABA from avidin and concomitant binding of Ru–biotin complex **1** to the protein. Similar results were obtained when the HABA–avidin complex was titrated with complex **2** (46). Thus, complexes **1** and **2** bind to avidin in a 4:1 ratio and with greater affinities than the HABA–avidin interaction.

To investigate the luminescence changes of complexes **1** and **2** in the presence of avidin, an avidin solution (in buffer A, containing 100 mM NaCl, 50 mM NaH₂PO₄, 1 mM EDTA, pH adjusted to 7.5 using NaOH) was titrated with complex **1** (16 μM), and luminescence at 615 nm was monitored (Figure 2, curve II). In addition, a blank buffer solution was titrated with complex **1** (16 μM , Figure 2, curve I). These experiments were repeated using complex **2** (Figure 3). Comparison of the titration curves I and II revealed that the luminescence of the Ru–biotin complexes is *enhanced* upon binding to avidin (33).

The emission intensity of the Ru–biotin complex **1** fully bound to avidin (4:1) ratio was measured to be ca. 1.4 times greater than unbound complex **1**, and complex **2** showed a greater enhancement of 1.6 (Figure 4). As a control experiment, the titration of avidin with Ru(bpy)₃²⁺, which has similar structural and photophysical properties to complexes **1** and **2**, but lacks a biotin moiety, showed no detectable change in its luminescence intensity. Thus, complexes **1** and **2** bind to avidin through their biotin moiety in a 4:1 ratio and show luminescence enhancement as a result of this interaction. This is in marked contrast with the behavior of biotinylated organic

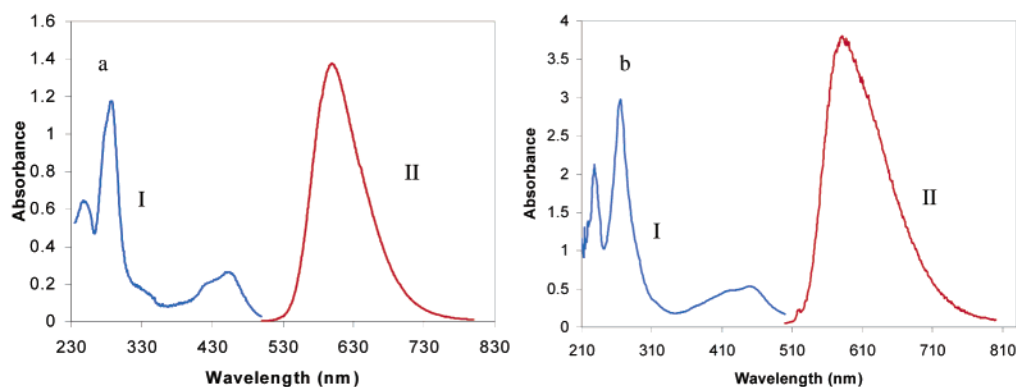


Figure 1. Electronic absorption (I) and emission (II) spectra of complexes **1** (a) and **2** (b) in CH₂Cl₂ at ambient temperature.

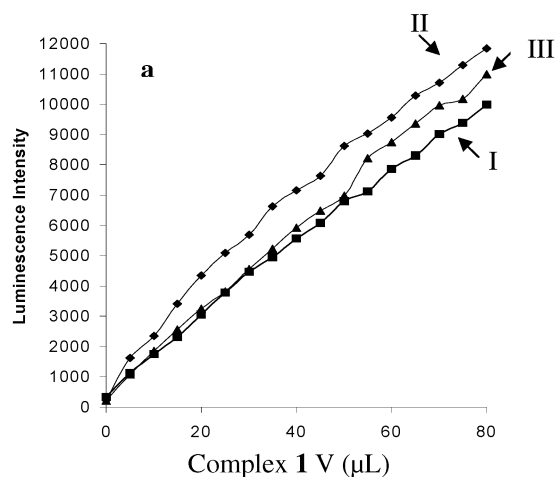


Figure 2. Luminescence titration curves for the titrations of (I) a blank buffer A solution with complex **1**, (II) an avidin solution (20 nmol) with complex **1**, (III) a preformed complex of avidin (20 nmol), and biotin (80 nmol) with complex **1** (16 M); the time delay between each addition was approximately 8 min.

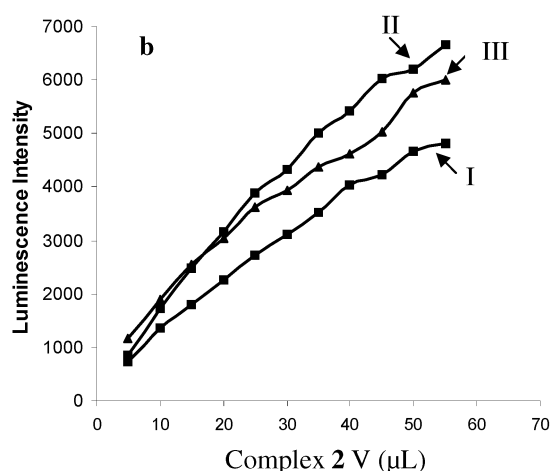


Figure 3. Luminescence titration curves for the titrations of (I) a blank buffer A solution with complex **2**, (II) an avidin solution (10 nmol) with complex **2**, (III) a preformed complex of avidin (10 nmol), and biotin (40 nmol) with complex **2** (16 M); the time delay between each addition was approximately 8 min.

fluorophores, whose fluorescence is quenched upon binding to avidin. The increase in luminescence intensity of the ruthenium complexes may be a result of the positioning of these chromophores in a more hydrophobic environment upon binding to avidin. Indeed, Ru(II) polypyridine complexes show a higher luminescence quantum

yield in organic solvents, compared to water (34–36). Interestingly, preliminary experiments performed on the interaction of complex **2** with streptavidin, which is more hydrophobic and less positive in charge than avidin, showed a greater luminescence enhancement of ~ 1.9 (9, 47). To our knowledge, there is only one previous report describing a rhenium–biotin complex, which binds to avidin with luminescence enhancement (37). The tris-chelated Ru-complexes **1** and **2** are expected to display greater stability than the rhenium complex, which possesses monodentate pyridine and carbonyl ligands (38). In addition, ruthenium (II) bipyridine complexes are redox active, and can be used for electrochemiluminescence experiments, with a significant amplification of their luminescence signals (41).

To compare the avidin binding affinity of the Ru–biotin complexes to that of biotin itself, a complex of avidin–biotin in a 1:4 ratio was generated. This sample was then titrated with Ru–biotin complexes **1** (or **2**) (16 μM), and the luminescence intensity at 615 nm was monitored (Figures 2 and 3, titration curves III). Complex **2** showed consistently higher luminescence intensity than unbound Ru–biotin (curve I in Figure 3). This suggests partial displacement of biotin from avidin by molecule **2** and concomitant binding of this complex to avidin, resulting in an increase in its luminescence intensity. Complex **1**, on the other hand, showed similar luminescence intensity to that of unbound Ru–biotin, which increased only when relatively high concentrations were attained (curve III in Figure 2). Thus, the binding constant of complex **2** to avidin is likely higher than that of complex **1**. A similar preliminary experiment was performed with streptavidin, where 4 equiv of molecule **2** were added to a preformed streptavidin:biotin complex. In this case, an enhancement of ~ 1.8 was noticed, consistent with an even greater binding affinity of complex **2** to streptavidin (47).

A final experiment was carried out, where a preformed complex of avidin–Ru–biotin in a 4:1 ratio was generated and then titrated with a solution of biotin (16 μM). The titration curves for both complexes **1** and **2** showed a steady decrease in luminescence intensity, until a ratio of biotin:avidin of approximately 4:1 was reached, upon which the luminescence intensity of the ruthenium complexes was unchanged with further biotin addition (46). This result is consistent with displacement of complexes **1** and **2** from avidin by biotin itself, suggesting that biotin binds to avidin with a higher affinity than complexes **1** and **2**. Thus, complexes **1** and **2** showed a relatively high binding affinity to avidin, albeit lower than biotin itself ($K_d = 10^{-15}$ M), with a 4:1 binding stoichiometry and concomitant luminescence enhance-

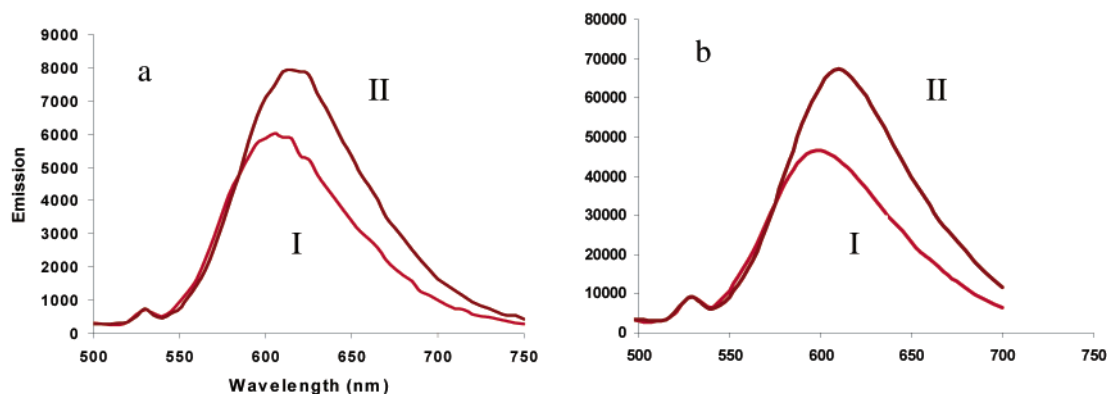


Figure 4. (a) Emission of complex **1** in buffer A (I) and in buffer A with avidin (II) (4:1 ratio). (b) Emission of complex **2** in buffer A (I) and in buffer A with avidin (II) (4:1 ratio).

ment. Interestingly, when a similar experiment was performed using streptavidin instead of avidin, the addition of 4 equiv of biotin to a preformed complex of streptavidin:molecule **2** did not cause any decrease in luminescence intensity, even after 12 h. This is consistent with little displacement of complex **2** from avidin by biotin and again suggests higher binding affinity of complex **2** to streptavidin, as compared to avidin (47).

In conclusion, we have generated a new class of ruthenium complexes, where (bpy)₂Ru(phen)²⁺ or (phen)₃Ru²⁺ centers are conjugated to a biotin moiety. These complexes bind to avidin and streptavidin through their biotin moiety with high affinity, in a 4:1 ratio, and with concomitant enhancement in their fluorescence intensity. This is in marked contrast with organic dyes, which lose their fluorescence upon binding to avidin. Thus, these new ruthenium–biotin complexes can form the basis of new, simplified biotin–avidin assays, which consist of cross-linking the unknown biotinylated molecule and the ruthenium–biotin complex to avidin, for luminescence detection. Ru(II) polypyridine complexes present other advantages over the currently used chromophores, including their photostability and resistance to photobleaching, and their large Stokes shifts, which allow many of these chromophores to be linked to the protein for signal amplification, without quenching their luminescence (19, 21, 39–45). Further work is in progress in our laboratory to extend the applications of these and related transition metal–biotin complexes to more selective bioassays.

ACKNOWLEDGMENT

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Supporting Information Available: Synthesis of the phenanthroline–biotin ligand. Synthesis of complex **1** [Ru(bpy)₂(phen-biotin)](PF₆)₂. Synthesis of complex **2** [Ru(phen)₂(phen-biotin)](PF₆)₂. Electronic absorption and emission data for complexes **1** and **2**. Binding specificity of complexes **1** and **2** toward avidin:HABA assays. Competitive binding assay: titration of Ru–biotin–avidin complexes **1** and **2** with biotin. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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- (47) We are carrying out further investigations on the interaction of complexes **1** and **2** with streptavidin, and these will be reported in a future contribution.

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