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Article

Rhenium(I) Polypyridine Complexes as Luminescence-Based Sensors for the BSA Protein

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The binding interaction of rhenium(I) complexes *fac*-$[Re(CO)_3(NN)(py)]^+$, py = pyridine and NN = 1,10-phenanthroline (phen), 4,7-diphenyl-1,10-phenanthroline (ph_2phen) or 4,7-dichloro-1,10-phenanthroline (Cl_2phen), and bovine serum albumin (BSA) was investigated at physiological pH using emission intensity variation and circular dichroism (CD) spectroscopy. The photophysical investigations showed that in the presence of BSA, the metal-to-ligand-charge transfer (^{3}MLCT) emission of the rhenium(I) complexes was quenched due to entrapment of the complex within the protein environment. Additionally, high Stern-Volmer (K_{SV}) and binding (K_b) constants were determined from luminescence data, revealing the occurrence of a strong interaction and/or association. The differences in K_{SV} values can be tentatively associated with an electron-withdrawing constant (σ) defined by Hammett equation. The CD results showed that the extent of α-helicity of the BSA decreased upon the addition of rhenium complexes, which provided further support for the interaction of rhenium(I) complexes and the protein.

Keywords: rhenium(I) polypyridine complexes, BSA, luminescence-based sensor

Introduction

The use of coordination compounds in the design of photosensors offers a vast range of applications from small molecule probes to biomolecule probes, such as proteins and DNA.[1-4] In particular, the emissive property of rhenium(I) polypyridyl complexes, *fac*-$[Re(CO)_3(NN)L]^n$ (n = 0 or +1), is generally ascribed to the metal-to-ligand charge transfer (^{3}MLCT) excited state. This excited state can be modulated by changing the polypyridine ligand, NN, as well as the spectator ligand, L, and can be conveniently employed in the development of luminescent sensors.[5-8]

The rhenium(I) complexes have several advantages over organic compounds employed for the same purpose:[3,6] long emission lifetimes, which enhance the detection sensitivity in time-resolved techniques; photostability; the nature of their emission-phosphorescence with a large Stokes shift, which can minimize self-quenching; environment-sensitive emission, among other benefits. Additionally, such complexes exhibit high membrane permeability, being stable under physiological conditions.[3,8-11] While there are several investigations on the photophysical behavior of these complexes in fluid, usually in acetonitrile and dichloromethane, rigid media,[12-17] and in the presence of various analytes,[18-21] studies in physiological conditions or in cellular media are very recent.

Serum albumins have been one of the most studied proteins with functions crucial to facilitating the disposition and transportation of various ligands such as metal ions, fatty acids, steroids, among others.[22] In particular, bovine serum albumin (BSA) is highly stable and its structure is similar to the human albumin (HAS), presenting a 76% sequence identity. One of the main differences between these two proteins is that BSA possesses two tryptophan residues, while HAS possesses only one. One tryptophan residue in BSA is buried into a hydrophobic pocket and is reported to be near the surface of the albumin molecule in the second α-helix of the first domain; the second tryptophan residue is located in the hydrophilic pocket of the protein. As there is evidence of conformational changes induced by the interaction between BSA and rhenium(I) metal complexes,[8,23-26] an intense effort has been dedicated to better understand the effect of the position and attachment of electron withdrawing/donating groups to coordinated ligands. Greater knowledge of ligand modification can contribute to a deeper comprehension of the interaction processes necessary to design spectroscopic probes.

In this study, the binding interactions of rhenium(I) complexes *fac*-$[Re(CO)_3(NN)(py)]^+$, NN = 1,10-phenanthroline (phen), 4,7-diphenyl-1,10-phenanthroline (ph_2phen) or 4,7-dichloro-1,10-

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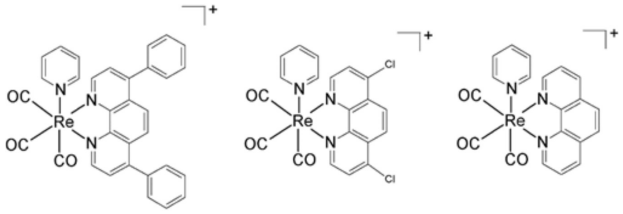
phenanthroline (Cl_2phen), py = pyridine, Scheme 1, and BSA were investigated at physiological pH using luminescence changes and circular dichroism (CD) spectroscopy. Interaction of these metal complexes with the well-studied BSA makes it possible to establish a baseline of behavior, making it possible to use them in more complicated situations. For instance, electron donating groups attached to polypyridyl ligands coordinated to Re^{I} promote destabilization of the $^3MLCT_{Re \rightarrow NN}$ excited state energy level at the same time reduces the energy of intraligand, $^3IL_{NN}$, excited state. On the other hand, electron withdrawing groups promote the ^{3}MLCT stabilization. The emissive properties of rhenium(I) complexes are, generally, dominated by a ^{3}MLCT character, although some ^{3}IL emission could also be evident to a greater or lesser degree, thus, the rationalization of the mechanism deactivation pathway after excitation in function of coordinated polypyridyl ligand is crucial to improve this system for designing probes. Therefore, the aim of this work was to provide more information to aid the use of this system as a probe in chemical recognition.



Scheme 1. Chemical structures of rhenium(I) polypyridyl compounds.

Experimental

Materials

All solvents, from Aldrich, Synth or Merck, were reagent grade, except for those used in the photophysical measurements, where high performance liquid chromatography (HPLC) grade solvents from Aldrich were employed. [$ClRe(CO)_5$], 1,10-phenanthroline (phen), 4,7-diphenyl-1,10-phenanthroline (ph_2phen), 4,7-dichloro-1,10-phenanthroline (Cl_2phen), trifluoromethanesulfonic acid (tfms), pyridine (py) and bovine serum albumin (BSA Mw = 68000) from Aldrich were used as received.

Syntheses of rhenium(I) complexes

fac-[$ClRe(CO)_3(NN)$] complexes (NN = phen, ph_2phen or Cl_2phen) were prepared according to the literature.[27-31] [$ClRe(CO)_5$] and an excess of the NN ligand were suspended in xylene and heated to reflux for several hours. The crude product was recrystallized from dichloromethane by the slow addition of *n*-pentane. These *fac*-[$ClRe(CO)_3(NN)$] complexes were converted to *fac*-[$(tfms)Re(CO)_3(NN)$] as previously described[27-29] by adding trifluoromethanesulfonic acid to *fac*-[$ClRe(CO)_3(NN)$] suspended in dichloromethane and precipitated by the addition of diethyl ether. *fac*-$[Re(CO)_3(NN)(py)]^+$ complexes were synthesized following the procedure previously described[28,29,31,32] by refluxing *fac*-[$(tfms)Re(CO)_3(NN)$] with an excess of py in methanol and precipitated with NH_4PF_6.

fac-$[Re(CO)_3(phen)(py)]PF_6$

^{1}H NMR (60 MHz, CD_3CN) δ 9.4 (dd, 2H), 8.6 (dd, 2H), 7.9 (m, 7H), 6.9 (m, 2H). Anal. calcd. for $C_{20}H_{13}N_3O_3F_6PRe \cdot ¼py$: C, 36.62%, H, 2.04%, N, 6.53%; found: C, 37.44%, H, 1.73%, N, 6.29%.

fac-$[Re(CO)_3(ph_2phen)(py)]PF_6$

^{1}H NMR (200 MHz, CD_3CN) δ 9.66 (d, 2H), 8.40 (d, 2H), 8.09 (s, 2H), 8.07 (d, 2H), 7.85 (t, 1H), 7.65 (m, 10H), 7.30 (d, 2H). Anal. calcd. for $C_{32}H_{21}N_3O_3F_6PRe$: C, 46.49%, H, 2.56%, N, 5.08%; found: C, 46.03%, H, 2.59%, N, 5.14%.

fac-$[Re(CO)_3(Cl_2phen)(py)]PF_6$

^{1}H NMR (200 MHz, CD_3CN) δ 9.55 (d, 2H), 8.25 (d, 2H), 8.50 (s, 2H), 8.38 (m, 2H), 7.78 (m, 1H), 7.23 (m, 2H). Anal. calcd. for $C_{20}H_{11}N_3O_3F_6PRe.H_2O$: C, 31.55%, N, 5.52%, H, 1.72%; found: C, 31.57%, N, 5.44%, H, 1.52%.

Methods

Stock solutions of the individual complexes (ca. 1×10^{-3} mol L^{-1}) were prepared in acetonitrile (HPLC) and the BSA stock solution (ca. 1×10^{-6} mol L^{-1}), based on its molecular weight of 68,000, was prepared in freshly buffer solution at physiological pH (7.4).

Absorption spectra were recorded on an Agilent 8453 spectrophotometer. Proton nuclear magnetic resonance spectra (^{1}H NMR) were obtained on a Bruker AC-200 (200 MHz) or 60 MHz spectrometer at 298 K using CD_3CN as a solvent. Residual CH_3CN signals were employed as an internal standard.

Circular dichroism (CD) measurements were performed on JASCO 720 spectropolarimeter at room temperature from 200-360 nm. Parameters are set as follows: path length, 10 mm; resolution, 1 nm; scan speed, 20 n ms^{-1}; response, 1 s; band width, 1 nm. Every CD spectrum was average three times. All measurements were made by keeping the BSA concentration as constant (1×10^{-6} mol L^{-1}) while varying the concentration of the rhenium(I) complex (1×10^{-6} and 1×10^{-5} mol L^{-1}).

The CD results can be expressed as mean residual elipticity (MRE) in deg cm^2 $dmol^{-1}$ using equation 1 as reported in the literature.[33,34]

$$MRE = \frac{obs}{10nlC_p} \quad (1)$$

where Θ_{obs} is the CD in millidegree, n is the number of amino acid residues (583), l is the path length of the cell and C_p is the mole fraction. The helical content is determined from the MRE values at 208 nm using equation 2.

$$- \quad (\quad) = \left[\frac{(-MRE_{208nm} - 4000)}{(33000 \quad 4000)} \right] \times \ 00 \quad (2)$$

Here, MRE_{208nm} is the observed MRE at 208 nm, 4000 is the MRE of the β-form and random coil conformation cross at 208 nm, and 33000 is the MRE value of the pure α-helical at 208 nm.

Emission spectra at room temperature were recorded with a Varian Cary Eclipse steady state spectrophotometer using a 1.00 cm optical length quartz cuvette. Titrations were done manually via micropipette by adding a degassed BSA solution into a quartz cuvette containing a degassed rhenium(I) complex solution.

The luminescence quenching measurements were performed at different BSA concentrations and the Stern-Volmer constant, K_{SV}, values were determined from the Stern-Volmer plot using equation 3:

$$I_0/I = 1 + K_{SV}[Q] \quad (3)$$

where I_0 and I are the luminescence intensities of the rhenium(I) complex in the absence and in the presence of BSA, respectively and [Q] is the concentration of BSA.

The binding constant, K_b, of the rhenium(I) complex with BSA can be determined using equation 4.[33]

$$\log\left[\frac{(I_0 \quad I)}{I}\right] = \log K_b + n\log[BSA] \quad (4)$$

where I_0 and I are the luminescence intensities of Re^I complex in the absence and in the presence of BSA, respectively, n is the number of binding sites and [BSA] is the concentration of BSA.

Results and Discussion

The electronic absorption spectra of *fac*-$[Re(CO)_3(NN)(py)]^+$ complexes in acetonitrile, Figure 1a, exhibit two main absorption bands: the higher energy band, which was assigned to IL, and the lower energy band, assigned to $MLCT_{Re \rightarrow NN}$ as reported in the literature.[12,31,35,36] The electronic spectra are similar, except for a small bathochromic shift of the low energy band caused by the MLCT stabilization promoted by the two electron withdrawing chloro or phenyl groups attached to the phen.[31]

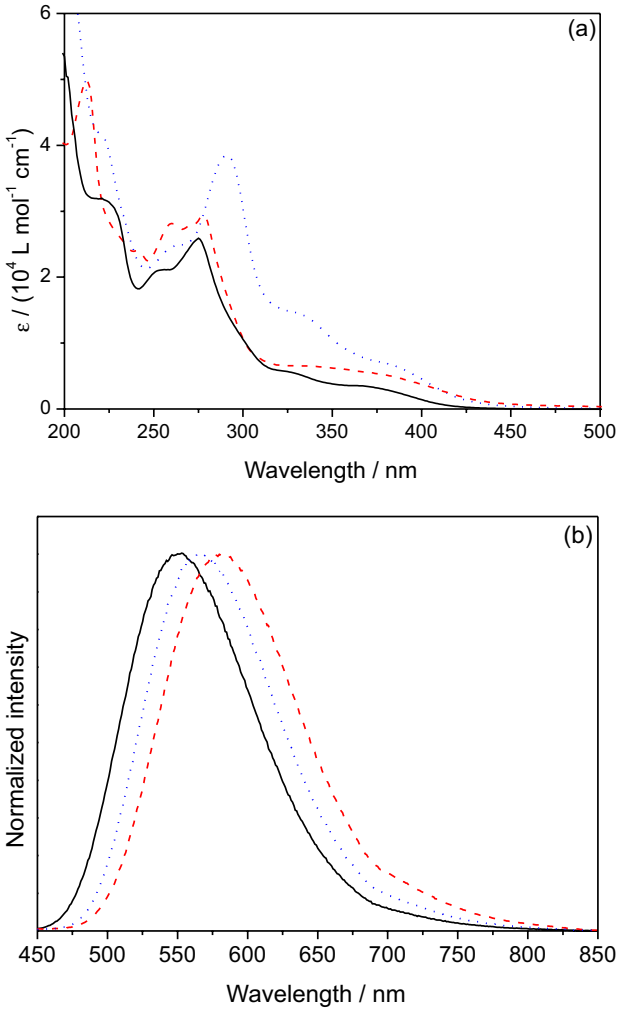


Figure 1. (a) Electronic spectra and (b) normalized emission spectra of *fac*-$[Re(CO)_3(NN)(py)]^+$, NN = phen (—), Cl_2phen (---) or ph_2phen (….) in acetonitrile.

All three complexes exhibit a characteristically broad and structureless emission band in acetonitrile, Figure 1b, arising from the $^3MLCT_{Re \rightarrow NN}$ excited state. Emission maxima are dependent on the NN ligand, which mainly affects the ^{3}MLCT excited state energy. As discussed in previous work,[31] the energy of the ^{3}MLCT excited state in *fac*-$[Re(CO)_3(Cl_2phen)(py)]^+$ is more stabilized, by the two electron-withdrawing chloro groups relative, to the other two parent rhenium complexes.

The addition of BSA to a solution of *fac*-$[Re(CO)_3(ph_2phen)(py)]^+$, Figure 2, leads to a decrease

in the luminescence intensity and a small hypsochromic shift (ca. 5 nm), which can be due to changes in local environment of the Re^{I} complexes promoted by the protein environment. Similar behavior is observed for the other two complexes, Supplementary Information (SI) section (Figures S1 and S2).

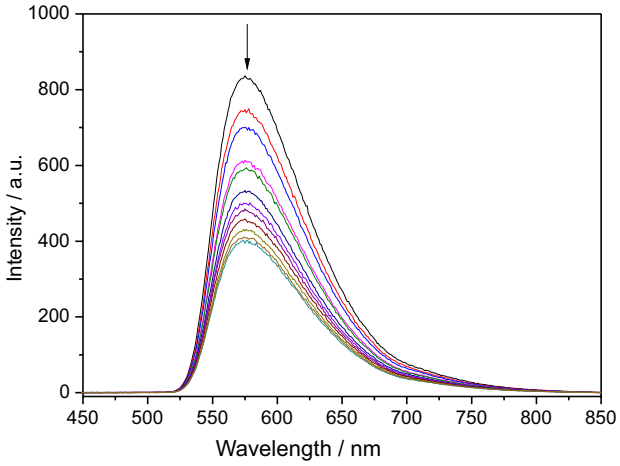


Figure 2. Changes in the emission spectra of *fac*-$[Re(CO)_3(ph_2phen)(py)]^+$ (2.5×10^{-5} mol L^{-1}) as a function of BSA addition ($0 \rightarrow 9$) 10^{-6} mol L^{-1}. λ_{exc} = 350 nm at room temperature.

The luminescence behavior of the rhenium(I) complex in the presence of BSA could be due to entrapment of the complex within the protein environment. As previously reported by Liu and co-workers,[33] the BSA crystal structure is in the shape of a heart composed of three homologous domains, called I-III. Within each domain are two subdomains, labeled A and B, forming a cylinder. There are two tryptophan residues (Trp134 and Trp212) in BSA: one located on the first subdomain IB (Trp134) that is exposed to a more hydrophilic environment, and another located in subdomain IIA deeply buried in the hydrophobic cavity. The authors concluded that hydrophobic cavities located in subdomains IIA and IIIA (called sites I and II) are the main areas where ligands interact with BSA. Rajagopal and co-workers,[37] reported docking studies that revealed the $[Ru(ph_2phen)_3]^{2+}$ complex strongly binds to BSA through a non-covalent hydrophobic interaction and noted that this interaction is mainly with the aromatic moiety of the protein such as phenylalanine, tyrosine and tryptophan in subdomain IB. As the data in reference 37 did not allow us to give the precise binding location of rhenium(I) complexes on BSA, we can only conclude that the trapping promotes an interaction/association between the complex and the BSA since a quenching of the Re^{I}-complex emission occurs.

The quenching process refers to the decrease in emission intensity of a given substance.[38] Several molecular interactions can result in quenching including excited state reactions, molecular rearrangements, energy transfer (trivial, long-range dipole dipole-Coulombic, or short-range electron exchange) and ground state complex formation. The biochemical applications of quenching are due to these molecular interactions and have been described as either static or dynamic quenching. Both types of quenching require molecular contact between the fluorophore and the quencher. In static quenching a non-fluorescent complex is formed between the fluorophore and the quencher. Dynamic quenching is due to the collision between the excited fluorophore and the quencher,[38,39] thus, the energy transfer must occur during the lifetime of the excited fluorophore. Stern-Volmer plots allow the calculation of quenching constants and generally can elucidate the mechanism of quenching process. The Stern-Volmer plot for *fac*-$[Re(CO)_3(ph_2phen)(py)]^+$ is shown in Figure 3 and in the SI section (Figures S3 and S4); values determined for all three complexes are presented in Table 1.

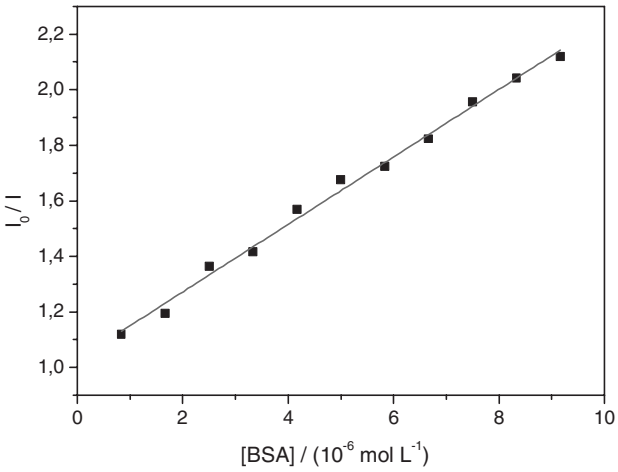


Figure 3. Stern-Volmer plot for the interaction of *fac*-$[Re(CO)_3(ph_2phen)(py)]^+$ with BSA.

Table 1. Calculated values for rhenium(I) complexes titrated with BSA at room temperature

fac-$[Re(CO)_3(NN)(py)]^+$	K_{SV} / (L mol^{-1})	K_b / (L mol^{-1})	n
phen	9.96×10^4	5.1×10^4	0.94
ph_2phen	1.24×10^5	1.3×10^5	0.93
Cl_2phen	4.20×10^3	4.8×10^3	0.83

K_{SV}: Stern-Volmer constant; K_b: binding constant; phen: 1,10-phenanthroline; ph_2phen: 4,7-diphenyl-1,10-phenanthroline; Cl_2phen: 4,7-dichloro-1,10-phenanthroline; n: number of binding sites.

Static and dynamic quenching can be distinguished by their differing dependence on temperature, viscosity or mainly by lifetime measurements. However, even without the lifetime measurements of the complex in the presence and absence of BSA, it is possible to note that the K_{SV}

value determined here for *fac*-[Re(CO)$_3$(ph$_2$phen)(py)]$^+$ (considering that its lifetime is 1-10 μs in CH_3CN solution)[35,40] is likely two orders of magnitude higher than the values found for *fac*-[Re(CO)$_3$(phen)(L)]$^+$, L = nicotinic acid or nicotinamide,[26] indicating a strong interaction between BSA and the Re^I complex. Additionally, considering this range in lifetime of rhenium(I) complexes, then the bimolecular quenching constant (k_q) between BSA and the Re^I complexes were estimated to be ca. 10^{10}-10^{11} L s^{-1} mol^{-1}, which is the same order or higher than the diffusion-limited quenching value indicating some binding interaction.[38] Quenching constant values higher than the diffusion controlled quenching value were reported for *fac*-[Re(CO)$_3$(dnbpy)(L)]$^+$, dnbpy = 4',4'-dinanoyl-2',2'-bipyridine, which were associated with the static quenching process via the formation of complex Re-BSA.[25] Therefore, by analogy we can conclude that the phosphorescence quenching by very low concentration of BSA is associated with a static quenching mechanism.

The comparison of non-substituted phen complex with the other two compounds shows that the electron-withdrawing ability plays an important role on the mechanistic events. There is a significant change in the K_{SV} values with the change of coordinated ligand in the Re^I complex, especially when comparing *fac*-[Re(CO)$_3$(ph$_2$phen)(py)]$^+$ and *fac*-[Re(CO)$_3$(Cl$_2$phen)(py)]$^+$. Looking at the substituents constants (σ) defined by Hammett equation[41] is possible to tentatively assign the lowest K_{SV} value determined for *fac*-[Re(CO)$_3$(Cl$_2$phen)(py)]$^+$ with a higher electron-withdrawing constant. And since the substituent constant of phenyl group is very close to the non-substituted phen constant, the K_{SV} values obtained for both complexes are very similar. This behavior could be also due to better accommodation of the phenyl substituents in the protein cavity as previously reported.[37,42] On the other hand, it seems that information concerning the mechanism of such process, particularly of biological activity, is usually extremely sketchy.

Higher values were also determined for the binding constant, K_b, obtained from the plot of log [($I_0 - I$)/I] *versus* log [BSA] for *fac*-[Re(CO)$_3$(ph$_2$phen)(py)]$^+$, Figure 4 (Figures S5 and S6 in the SI section for the other two complexes), and Table 1. The n value close to 1 suggests an one to one interaction for *fac*-[Re(CO)$_3$(ph$_2$phen)(py)]$^+$ and *fac*-[Re(CO)$_3$(phen)(py)]$^+$ complexes and the protein, and a negative cooperative binding between *fac*-[Re(CO)$_3$(Cl$_2$phen)(py)]$^+$ and the protein.

The emission titration experiments confirm the interaction between the Re^I complex and BSA. The intramolecular forces responsible for maintaining secondary and tertiary structures of the protein can be

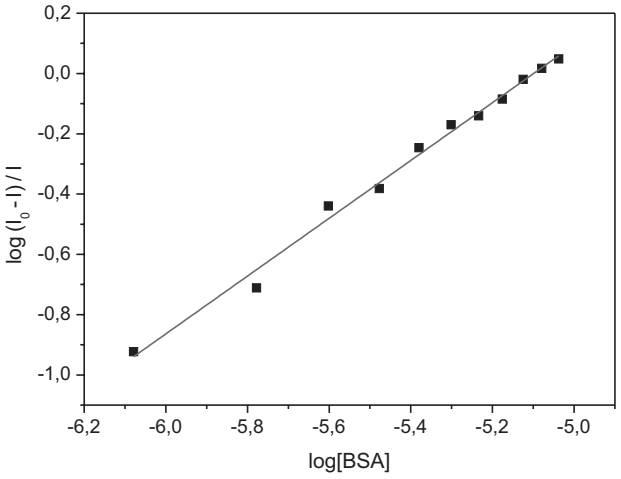


Figure 4. Plot of log [(I_0 – I)/I] *versus* log [BSA] of *fac*-[Re(CO)$_3$(ph$_2$phen)(py)]$^+$.

altered through this interaction, resulting in a protein conformational change. To determine more details about how the BSA structure is affected by the presence of the Re^I complex, circular dichroism spectra were obtained. The conformational changes of BSA in the presence of *fac*-[Re(CO)$_3$(ph$_2$phen)(py)]$^+$ are shown in Figure 5; CD spectra for the other two complexes are in the SI section (Figures S7 and S8). The data for all three complexes are summarized in Table 2.

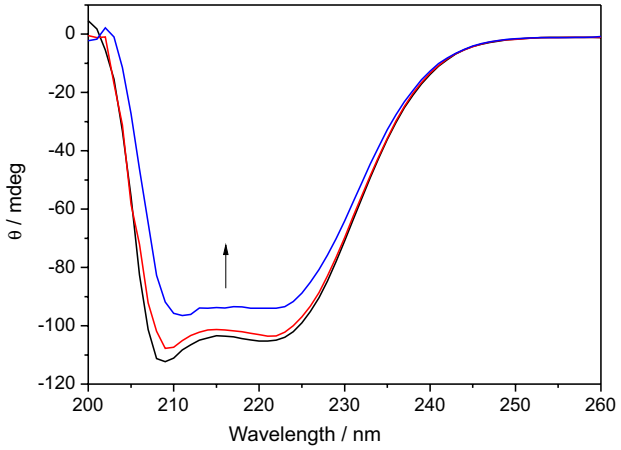


Figure 5. Circular dichroism spectra of BSA in the presence and the absence of *fac*-[Re(CO)$_3$(ph$_2$phen)(py)]$^+$. [BSA] = 1 × 10^{-6} mol L^{-1} and [complex] = 1 × 10^{-6} mol L^{-1} and 1 × 10^{-5} mol L^{-1}.

Table 2. The percentage of α-helix structure of BSA in the presence of *fac*-[Re(CO)$_3$(NN)(py)]$^+$ complexes (BSA:complex)

fac-[Re(CO)$_3$(NN)(py)]$^+$	1:1 / %	1:10 / %
phen	50	42
ph$_2$phen	48	38
Cl$_2$phen	49	40

phen: 1,10-phenanthroline; ph$_2$phen: 4,7-diphenyl-1,10-phenanthroline; Cl$_2$phen: 4,7-dichloro-1,10-phenanthroline.

It is observed that the BSA displays two negative bands at 208 and 222 nm, typical of proteins that have an α-helix structure, which are ascribed to n → π* transition of the carbonyl group of the peptide. The CD spectrum in the far UV region (200-260 nm) provides quantitative information of secondary structures and the near UV region (260-300 nm) provides information on the tertiary structures of BSA. Therefore, the changes observed at 200-260 nm indicates a conformational change in the secondary structures of BSA while in the presence of rhenium compounds. On the other hand, the spectra revealed no change in the region of 260-300 nm upon the addition of Re^{I} complexes, indicating that the tertiary structure of BSA remains the same upon binding with the Re complex.

A decrease of the residual ellipticity, θ, as a function of added complex can also be observed. In the pure form of this protein, 57% is of the α-helical structure. Furthermore, the BSA spectrum in the absence and presence of rhenium complexes are similar in shape, indicating that the α-helix structure is still dominant. However, the extent of α-helicity of the protein decreases upon the addition of rhenium complexes. Contrary to the observed in K_{SV} and K_b values, the magnitude of percentage of α-helical structure of BSA seems to be independent of the complex within the experimental error. The errors in CD spectroscopy are usually higher than the fluorescence technique. However, the CD method is very useful to ascertain the possible influence of the interaction process on the secondary structure of the proteins. Therefore, these changes observed in BSA secondary structures could be due to the association and/or interaction of the Re^{I} compound with the amino acid residue of the polypeptide chain of BSA, which diminishes the hydrogen bond within the protein.

Conclusions

The photophysical properties of rhenium(I) compounds, *fac*-$[Re(CO)_3(NN)(py)]^+$ where NN = phen, ph_2phen or Cl_2phen, and the circular dichroism spectra of BSA showed the interaction of Re-BSA. This interaction caused conformational changes in the protein BSA by a decrease in the α-helix stability. The observed quenching process of rhenium(I) complexes in the presence of BSA upon excitation was due to entrapment of the complex within the protein environment. The lowest K_{SV} value determined for *fac*-$[Re(CO)_3(Cl_2phen)(py)]^+$ can be tentatively associated with a higher electron-withdrawing constant, and the K_{SV} values obtained for the other two complexes are very similar since the substituent constant of phenyl group is very close to the non-substituted phen constant. Thus, these Re^{I} complexes could be very attractive as luminescence-based probes and sensors for macromolecules such as protein and DNA.

Supplementary Information

Supplementary data (emission spectra of *fac*-$[Re(CO)_3(NN)(py)]^+$, NN = phen or Cl_2phen, in the presence of various concentration of BSA, Stern-Volmer plot, plot of log $[(I_0 - I) / I]$ *versus* log [BSA] and circular dichroism spectra of BSA in the presence and the absence of *fac*-$[Re(CO)_3(NN)(py)]^+$) are available free of charge at http://jbcs.sbq.org.br as PDF file.

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