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A Selective, Long-Lived Deep-Red Emissive Ruthenium (II) Polypyridine Complexes for the Detection of BSA

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Abstract

A selective, label free luminescence sensor for bovine serum albumin (BSA) is investigated using ruthenium (II) complexes over the other proteins. Interaction between BSA and ruthenium (II) complexes has been studied using absorption, emission, excited state lifetime and circular dichroism (CD) spectral techniques. The luminescence intensity of ruthenium (II) complexes (**I** and **II**), has enhanced at 602 and 613 nm with a large hypsochromic shift of 18 and 5 nm respectively upon addition of BSA. The mode of binding of ruthenium (II) complexes with BSA has analyzed using computational docking studies.

Keywords: Ruthenium (II) complex, luminescence, Bovine serum albumin (BSA)**,** biosensor, Docking

INTRODUCTION

Fluorescent probes are having wide range of applications in the field of biosensors and proteomics [1-3]. Protein detection is one of the important field, since proteins playing various roles like transporter, building blocks and pathogens in biology. Most of the molecular probes and sensors are fluorescent organic molecules. Their photophysical properties like emission intensity and excited state life time are used as sensing pathways. However, the use of such probes to reveal the local biological environment is scarcely addressed in the literature [4]. Even few reports are investigated on the mechanism of protein detection by light-up probes [4, 5] and these light-up probes are mainly used for molecular recognition such as DNA, proteins and as stains in gel electrophoresis [6-9].

The organic fluorophores have limitations, such as, lower excited state lifetime, poor photostability and lower wavelength absorption [10, 11]. The fluorescence of probe molecule should not overlap with background fluorescence from proteins. In this regard, the use of luminescent transition-metal complexes as sensors continues to attract considerable interest because of their long lifetimes compared to their purely organic counterparts and higher wavelength absorption [12-14]. Luminescent transition metal complexes, especially those with d^6 electronic configuration such as ruthenium(II), osmium(II) and rhenium(I), are receiving much interest in imaging and luminescent probe for biomolecules [15, 16]. Because of the high photostability, low-energy absorption, and relatively long lived luminescence of ruthenium(II)-polypyridine complexes[17], we envisage ruthenium(II)-polypyridine complexes as promising candidates for luminescent biological probes.

The most abundant protein of circulatory system is serum albumins. Serum proteins play an important role in the transportation and delivery of drug molecules in the blood [18]. Among the serum albumins, bovine serum albumin (BSA) has a wide range of physiological functions involving the binding, transportation and delivery of fatty acids, porphyrins, bilirubin, steroids, etc. It is also consumed as dietary proteins. This heart shaped protein, BSA has three homologous domains I-III and each domain is made up of two subdomains, A and B, having unique binding properties. BSA is a single-chain 582 amino acid protein with 17 cystine cross-linked residues [19-22]. BSA has two tryptophans, Trp-134 and Trp-212, embedded in the first sub-domain IB and sub-domain IIA, respectively [23, 24]. Several techniques such as electrochemistry [25], Rayleigh light scattering [26], two-photon excitation [27], seeded liquid beam desorption mass spectrometry [28], Raman scattering [29] and optical sensor [30] have been applied for the detection of BSA. Recently, many optical techniques have been utilized to investigate the interaction of proteins with ligands, because these methods are sensitive and relatively easy to use [31-33]. Among these, fluorescence technique is the most useful method to study the biomolecular interactions since it is sensitive to low concentrations and amicable with physiological environments [34].

Herein we report a label free biosensor using Ru(II) complexes for selective recognition of BSA in phosphate buffer solution (pH 7.4). This approach relies on the hydrophobic interaction with probe molecule using steady state emission signal change. The mode of binding is also analyzed using docking studies.

EXPERIMENTAL

Materials

The commercial samples of $RuCl_3.nH_2O$, 4,7-diphenyl-1,10-phenanthroline disulfonic acid (dpsphen), 4,7-diphenyl-1,10-phenanthroline (dpphen), ammonium hexaflurophosphate and other chemicals are procured from Sigma. Bovine serum albumin (BSA), human serum albumin (HSA), lysozyme, cytochrome c, myoglobin, thrombin, platelet derived growth factor (PDGF) are purchased from Sigma Aldrich and used as such. The stock, 1×10^{-3} M, solution of all the proteins were prepared using PBS buffer. Sodium chloride, potassium chloride, disodium phosphate, sodium phosphate and all the solvents are purchased from Merck and used as such. The luminophores $[Ru(dpphen)_3]^{2+}$ (**I**) and $[Ru(dpsphen)_3]^{4-}$ (**II**) (Chart I) are prepared using reported literature methods and characterised using ESI-MS spectroscopy (Figure S1 and S2) [35, 36].

Synthesis of Tris(4,7-diphenyl-1,10-phenanthroline)ruthenium(II) chloride (I)

The $[Ru(dpphen)_3]Cl_2$ is synthesized using literature method [35]. $RuCl_3.3H_2O$ (1 mmol) and 4,7-diphen-1,10-phenanthroline (3 mmol) is taken in ethylene glycol under nitrogen atmosphere and heated to reflux for 72 h and the crude product is chromatographed using silica gel. The solution on evaporation yielded orange red crystals. Yield = 85% ESI-MS (m/z) 548.6496 (M-$2Cl^-$ doubly charged species).

Synthesis of Tetrasodium tris[1,10-phenanthrolinediyl-4,7-di(benzenesulphanato) ruthenate(II) hexahydrate (II)

The $Na_4[Ru(dpsphen)_3]$ is synthesized using literature method [36]. The blue solution (0.5 mmol, 5 mL) is prepared by refluxing 1 g of $RuCl_3.3H_2O$ in 50% ethanol for 4-5 hours in a hot water

bath. This solution is transferred into a degassed solution of the ligand, 4,7-diphenylphenanthroline disulphonic acid (1 g, 2 mmol), in water (20 mL) and the mixture is heated at reflux under nitrogen for 12 h. The resultant red solution is filtered hot, and the red filtrate evaporated to dryness to give an orange-brown solid. This is recrystallized from water-ethanol mixture, and then dissolved in water (5 mL) and then chromatographed using water as eluent. The red band is collected and evaporated to dryness to give a brown solid. ESI-MS (m/z): 263.6496 (6^- charged species) [36].

Apparatus and Spectral Measurements

All the solutions of Ru(II) complexes and proteins are prepared using phosphate buffer at pH 7.4. The UV-vis absorption spectra are recorded at room temperature on Analtik Jena Specord S100 diode-array spectrophotometer - equipped with 1.0 cm path length quartz cells. The UV-vis absorption spectral studies are carried out in the wavelength range from 300 nm to 700 nm. During the spectral measurement the concentration of the complex is maintained constant and the concentration of BSA was varied. The emission spectra are recorded using JASCO FP6300 Spectrofluorimeter with 10 mm path length cuvette. Emission spectra are recorded in the range of 500 to 800 nm. Titrations are done manually via a micro-syringe and the fluorescence intensity of complex in the absence and in the presence of the BSA is measured after incubation for 5 min. Excited state lifetime of Ru(II) complexes are determined by time dependent luminescence decay measurements using a Czerny-Turner monochromator and analyzed by Hamamatsu R-928 photomultiplier tube. Circular dichroism (CD) measurements are performed on JASCO J810 spectropolarimeter at room tempreature over the wavelength 200-360 nm. Parameters are set as follows: path length, 50 mm; resolution, 0.5 nm; scan speed, 50 nm min^{-1};

band width, 1 nm; response 1 s. All the measurements are done at room temperature and repeated three times using freshly prepared samples and the results are reported as the average.

Determination of binding constant

Scatchard equation

The binding constants between the probe and the protein have been determined from the fluorescence intensity data using Scatchard plot. The Scatchard equation is shown in eq (1) [37, 38]

$$\upsilon/C_F = nK_a - \upsilon K_a \qquad (1)$$

In eq (1) υ – ratio of the concentration of bound ligand to total available binding sites. C_F – number of binding sites per protein molecule. n – binding stoichiometry. The plot of the data υ/C_F vs. υ is non-linear which shows the cooperative binding.

Hills equation

Since, the Scatchard plot is non-linear we have calculated the binding constant using Hills equation, eq (2) [39]

$$\log[\theta/(1-\theta)] = n \log [BSA] - \log K_d \qquad (2)$$

θ - number of binding sites (C_F in Scatchard equation) , K_d – dissociation constant(the inverse of K_a), n – Hills coefficient of cooperativity which gives the nature of cooperativity. $n > 1$ positive cooperative binding , $n < 1$ negative cooperative binding and $n = 0$ non cooperative binding.

Circular dichroism (CD)

The concentration of protein is fixed at 2×10^{-6} M and Ru(II) complexes are varied (2×10^{-6}, 5×10^{-6} and 10×10^{-6} M). The helicity of the proteins is calculated by equations (3) and (4)[40].

$$\text{Mean residual elipticity (MRE)} = \text{Observed CD}/ C_p n l \times 10 \qquad (3)$$

where, Cp – molar concentration of protein, n- amino acid residues, l – path length.

$$\alpha \text{ (helix \%)} = (-\text{MRE} - 4000)/ (33000-4000) \times 100 \qquad (4)$$

In eq. (4) 4000 is the MRE of the β–form and random coil conformation cross at the observed wavelength, and 33,000 is the MRE value of a pure α–helical at the observed wavelength.

Details of docking studies

The pdb (protein data bank) files of protein molecule has been downloaded from protein data bank (http://www.rcsb.org/pdb/home/home.do, file code: 1AO6)[41]. The final model chosen, is minimized using DISCOVER module of Insight II with 1000 rounds of steepest descent and 1000 rounds of conjugate minimization. The cavity of the protein model has been picked by using Computed Atlas of Surface Topography of Proteins (CASTp) [42]. The docking towards the luminophores of proteins such as tryptophan and tyrosine has been done using GOLD[43]. For viewing the docked solutions and to generate pictures UCSF Chimera candidate version 1.5.3 is used [44].

RESULTS AND DISCUSSION

The structures of the Ru(II) complexes, synthesized for the present study, are shown in Chart 1. The absorption and emission spectra of complex **I** in aqueous solution containing acetonitrile/ buffer (5% /95%) and **II** in buffer are shown in supporting information, Figure S3 and the spectral data of Ru(II) complexes are collected in Table 1. The absorption spectra of **I** and **II** show strong ligand centered absorption peaks at 278 nm and less intense metal to ligand charge transfer (MLCT) absorption on 464 nm [35]. The strong high energy absorption bands at 278 nm are assigned to ligand centered (LC) π–π* transition and low energy absorption at 464 nm to spin allowed MLCT transition from the Ru dπ- orbital to the π* orbital of the ligand (dπ Ru → π*). The ^{3}MLCT emission maximum of complexes **I** and **II** are observed at 620 nm and 618 nm

respectively. The lifetimes of these complexes are 2.9 and 4.0 μs and agree with the literature values [35, 36].

Absorption and luminescence titration of Ru(II) complex with proteins

Absorption spectral technique is a simple method for exploring the structural changes and to analyze the complex formation between the reactants [45]. The absorption spectra of Ru(II) complexes are recorded in the presence of various concentration of BSA. The results for complexes **I** and **II** are shown in Figure 1. The addition of BSA leads to a gradual decrease in the absorption intensity of Ru(II) complexes at 464 nm(**I** and **II**). The above results indicate the presence of hypsochromic effect in this system. Similar results are reported already with organic dyes [46].

The luminescence behavior of Ru(II) complexes in the presence of proteins have also been studied to get more details on the protein binding. The luminescence spectra of the complexes **I** and **II** at different concentrations of BSA are shown in Figure 2. The interaction between Ru(II) complexes (**I** and **II**) and BSA is found to produce dramatic enhancement in the emission intensity of Ru(II) complexes. In the absence of BSA, moderate emission of Ru(II) complexes (**I** and **II**) in aqueous buffer medium at 620 nm and 618 nm respectively are observed. The complex **I** shows an enormous enhancement accompanied by a blue shift of about 18 nm in the presence of BSA, whereas, the complex **II** shows comparatively lower enhancement along with 5 nm blue shift in the luminescence maximum. This different behavior can be attributed to the more hydrophobic nature of the complex **I** than the complex **II** (complex **II** is hydrophilic in nature). The neutral and hydrophobic nature of the probe molecule affects interaction with hydrophobic cavities of the protein (BSA) and such a shift in the luminescence maximum toward

blue end of the spectrum is a reflection of the sensitivity of the probe to changes in the polarity of its surrounding microenvironment [47, 48].

The observed blue shift in the luminescence maximum of complex **I** (from 620 nm to 602 nm) confirms the restriction on the solvent dipolar-dipolar relaxation imposed by the constrained environment of the probe in the hydrophobic region of the protein matrix. The increase in intensity further indicates that the progressive binding of the probe with protein makes nonradiative channels present in aqueous buffer medium is less operative [49]. The enhancement of luminescence intensity of Ru(II) complexes in protein solution can be rationalized in terms of binding of the probe, Ru(II) complexes, with the protein. The strength of the binding can be identified through the determination of the binding constant between the probe and the protein using Scatchard and Hills plot (Figure S4 and S5) are given in supporting information and the binding constants are 3.0×10^4 M^{-1} and 2.1×10^3 M^{-1} for complex **I** and **II**.

Excited state lifetime studies

The luminescence lifetime is a sensitive parameter to establish the local environment around a luminophore [50, 51]. Therefore the excited state lifetimes of the Ru(II) complexes are measured in the absence and in the presence of BSA. Figure 3 shows the luminescence decay curve of complexes **I** and **II** (1×10^{-6} M) in the absence and in the presence of BSA. In the absence of BSA, the lifetime values of Ru(II) complexes **I** and **II** are 2.9 μs and 4.0 μs respectively. In the presence of BSA, the lifetime of complex **I** is increased from 2.9 μs to 5.2 μs and that of complex **II** is increased from 4.0 μs to 4.9 μs. These results indicate that the Ru(II) complexes strongly bind with BSA and the increase in the luminescence intensity as well as the excited state lifetime are similar.

Selective binding with BSA

The selectivity of probes **I** and **II** is confirmed by binding of these complexes with a set of extracellular proteins such as HSA, BSA, myoglobin, lysozyme, thrombin, PDGF and cytochrome C are measured and their luminescence responses are compared (Figure 4). HSA and BSA are inducing luminescence intensity of Ru(II) complexes **I** and **II** dramatically. The careful observation of Figure 4 indicates that myoglobin and lysozyme interact moderately with the probes. The interactions of other extracellular proteins are negligible. The above results can be attributed for the hydrophobicity and the size of the proteins. HSA/BSA contains several hydrophobic binding sites for interaction with the metal complexes [52].

Myoglobin is a small monomeric form of heme protein. Its interior is hydrophobic and the surface is slightly hydrophilic in nature [53]. The interior and exterior of myoglobin are well-distinguished by hydrophobic and hydrophilic side groups. The Ru(II) complexes **I** and **II** bind *via* hydrophobic interaction into the interior of the myoglobin. However the lysozyme is highly hydrophilic in nature resulting in the weak interaction with the complex **I** but considerable luminescence enhancement is observed in the presence of complex **II** which is due to the hydrophilic interaction of lysozyme with complex **II** [54]. Though the myoglobin and BSA are hydrophobic in nature, BSA shows enormous enhancement than the myoglobin on the luminescence intensity of Ru(II) complexes **I** and **II**. This is due to the stronger hydrophobicity of the BSA than the myoglobin [55]. In addition the size of the protein is also in this order: BSA > myoglobin > lysozyme [56]. The detection limit as low as 3×10^{-7} M.

Conformational change of BSA in the presence of Ru(II)-complex

Probe binding with protein results in change in the secondary and tertiary structures[23]. The change in secondary and tertiary structure of BSA upon binding of complex I and II is studied using circular dishroism technique. The peaks at 208 and 218 nm are corresponds to the alpha and beta helical conformations of the protein [23, 57]. These peaks exhibit a decrease in intensity on the addition of Ru(II) complexes (Figure 5).

The α–helicity and molar elipticity are calculated using equation 3 and 4. Upon addition of Ru(II) complexes to BSA the extent of α-helicity of the protein decreases from 55% to 45 % and 53 % for complexes **I** and **II**, respectively. According to the literature, far-UV (200-260 nm) CD spectra is giving the quantitative information on the secondary structure and the near UV (260-300 nm) CD spectra is giving the information of the tertiary structure of BSA [58-60]. The CD spectra of BSA in the absence and in the presence of Ru(II) complex are observed to be similar in shape. There is no additional signal in the near UV region. The changes at 200-260 nm region indicates the conformational changes in the secondary structure of BSA in the presence of Ru(II) complexes. Therefore, the secondary structure of BSA is changed upon addition of Ru(II) complexes with decrease in α helicity. There is no change in tertiary structure of BSA upon binding with Ru(II) complex.

Docking studies

The results of spectroscopic studies presented above are supported by the docking studies using GOLD software, [43] to rationalize the possible binding site and nature of interaction between Ru(II)-complex and BSA. Since, BSA has sequence homology of 80 % over 578 residues with HSA (PDB entry 1AO6) as template and also the complexes under investigation are selectively sensing HSA, the crystal structure of HSA is used for docking studies. The crystal structure of

HSA comprises three domains. The three domains (I, II, III) have similar 3D structures and are highly asymmetric in assembly with 17 disulfide bridges. Each domain can be further divided into subdomains "A" and "B", which are composed of six and four α-helices, respectively. A long extended loop traverses the two subdomains to link those together [41]. Our model of the BSA shows that the three domains are identical to those in HSA, and the binding sites for the complexes **I** and **II** analyzed in this work have an equivalent binding site in HSA. The binding cavity of HSA has been identified from the online tool, Computed Atlas Surface Tropology of protein (CASTp) and we choose the large binding cavity (shown in Figure S6).

The hydrophobic binding area shown in surface view, in Figure S6, is available for Ru(II) complex docking. The larger binding cavity of binding volume 3587.5 $Å^3$ with six openings, is selected for docking among 76 binding cavities, since other cavities are smaller in volume to accommodate Ru(II) complexes and few openings for interactions. The experimental results indicate that the Ru(II) complex strongly binds with BSA through the non-covalent hydrophobic interaction. The possible interaction is mainly with the aromatic moiety of the protein such as Phe, Tyr and Trp. We have selected the Tyr138 as a targeting moiety which is available in this cavity, since it is situated deep inside of selected binding cavity. The most probable positions of binding of complexes **I** and **II** to HSA are shown in Figure 6.

The subdomain 1B having aromatic amino acid, rotated 90º away, accommodates the incoming ligands. Complex **I** preferably binds with HSA into the domain 1B moieties such as tyrosine and phenyl alanine *via* the hydrophobic interaction (Figure S7). The aromatic moiety of ligand interacts through π-π interaction with Tyr 136, Tyr 138, Phe 132 and Phe155. This rigidity of probe through the π-π interaction increases the emission intensity with the addition of BSA. The complex **II** also binds to the same domain *via* π-π interaction with Tyr 161 and Phe 157. The

hydrophilic SO_3^- group interacts with hydrophilic HSA surface (Figure S8). Hence the complex **I** has π-π interaction with Tyr 136, Tyr 138, Phe 132 and Phe155 simultaneously but the complex **II** binds with Tyr 161 and Phe 157. This binding site has 4 - 6 aromatic amino acids. The adjacent amino acids and the distances with the complex are shown in Table 2.

The complex **I** accumulate on the binding packet of HSA **IB** subdomain, and subdomain **IB** is in the inner hydrophobic pockets, and consequently it is expected to have high fluorescence intensity. On the other hand, the complex **II** also binds to IB domain, which is partially exposed to the solvent due the hydrophilicity of the SO_3^- moiety. Both complexes have stacked π-π interaction in the more hydrophobic site such as Try and Phe and the nearest groups are shown in Table 2. This also supports the strong binding of complex **I** with BSA and accounts for the enormous increase in the luminescence intensity compared to complex **II** [61].

CONCLUSION

We have demonstrated the selective BSA sensor system using the deep-red emissive Ru(II)-complexes with the simple technique. The Ru(II)-complexes show moderate luminescence in the absence of BSA. The luminescence intensity of probe enormously increases upon the addition of BSA due to the strong binding of the probe with BSA through the hydrophobic interaction. The α-helix content of BSA is found to decrease in the presence of probes (**I** and **II**). The binding constant values indicate that the complex **I** shows strong binding than the complex **II**, which is attributed to the hydrophobicity of complex **I.** The docking studies endorse the spectroscopic results. The selectivity of BSA over other extra cellular proteins is confirmed under steady state luminescence assay. Therefore, the complex I and II can be envisaged as efficient sensors for BSA.

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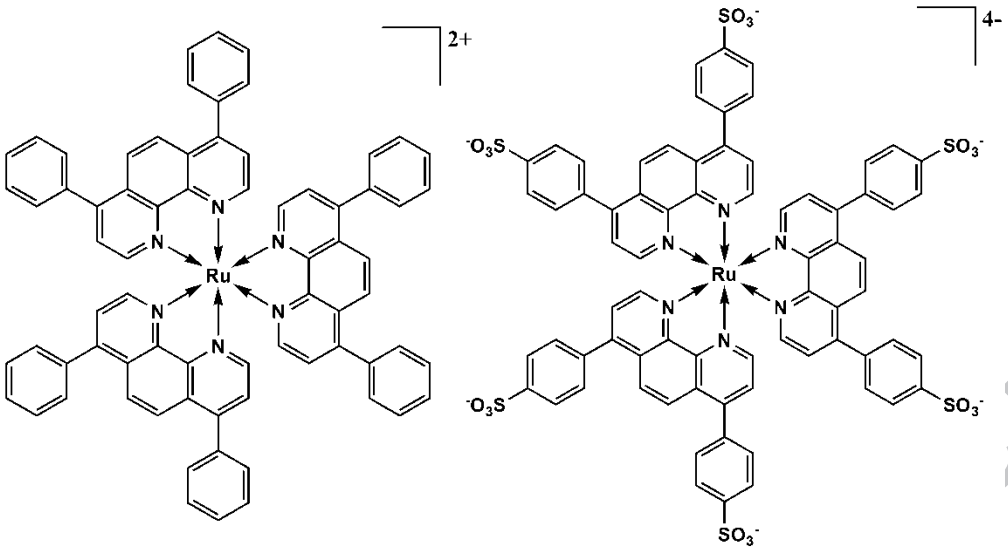

Chart 1. Structure of complexes **I** and **II**

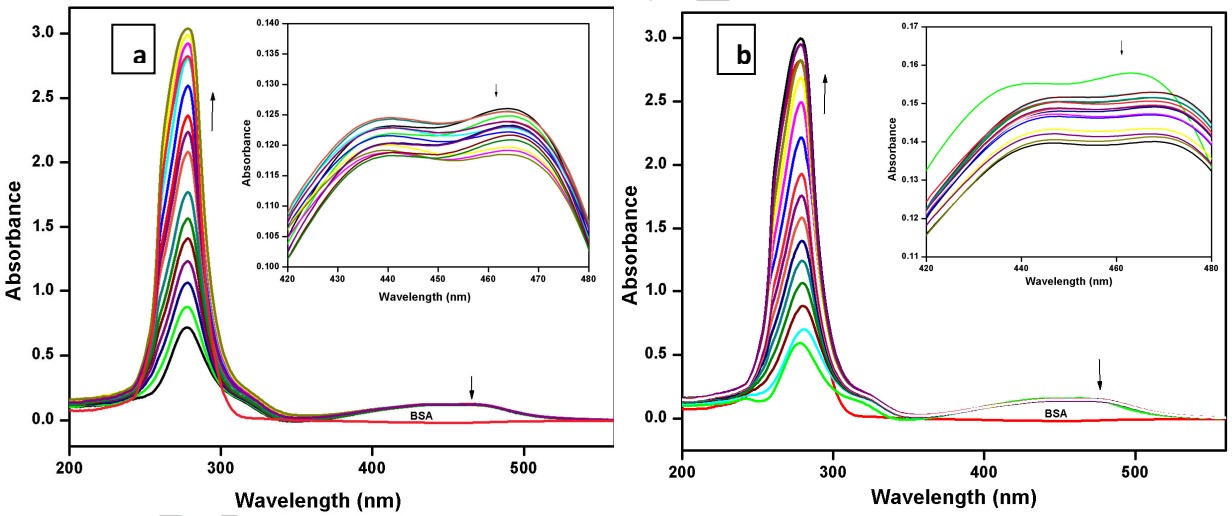

Figure 1. UV-vis absorption spectral changes of complex (A) **I** and (B) **II** in the presence of different concentration of BSA. The concentration of Ru(II) complex and BSA are 1×10^{-6} M, $0 \rightarrow 167$ μM respectively.

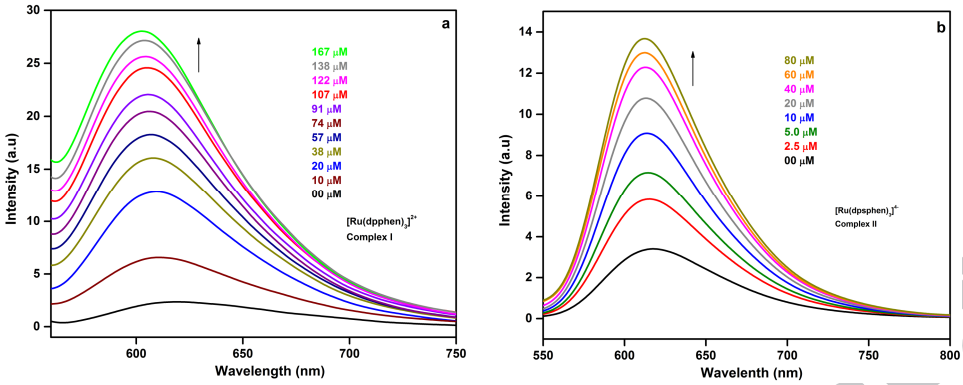

Figure 2. Luminescence spectra of complex (a) **I** and (b) **II** in the presence of various concentration of BSA. The concentration of Ru(II) complexes and BSA are 1×10^{-6} M, 0 → 167 μM respectively. Excitation maximum: 460 nm

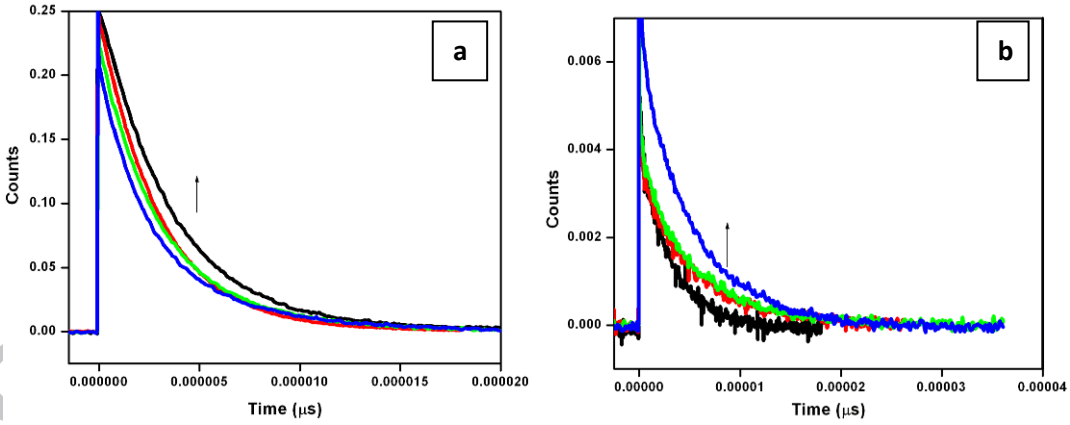

Figure 3. Llifetime decay of (a) complex **I** and (b) complex **II** in the presence of BSA.

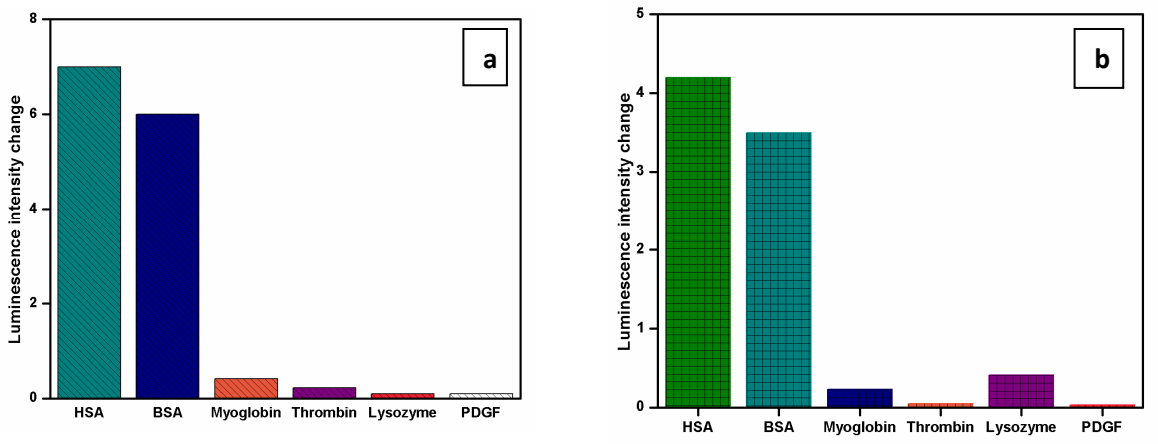

Figure 4. Selectivity of the sensor system towards BSA (a) complex **I** and (b) complex **II**. The concentration of each proteins are170 μM and the Ru(II) complexes are 1×10^{-6} M.

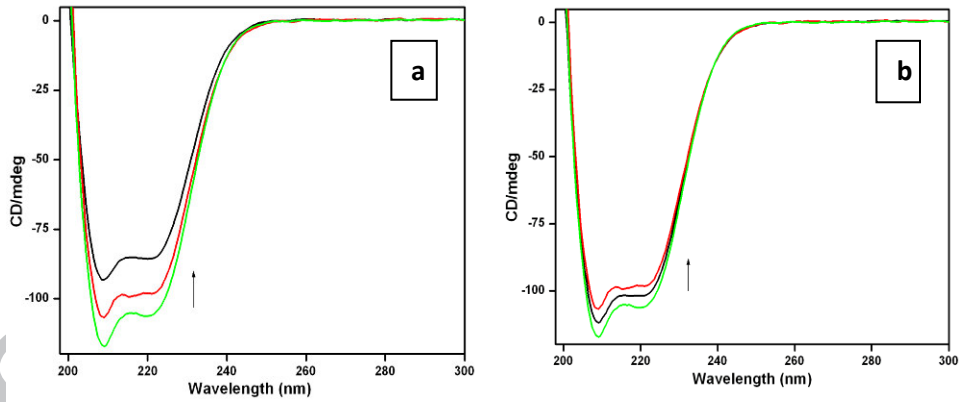

Figure 5. CD spectra of BSA in the presence of (a) complex **I** and (b) complex **II**. The concentration of BSA and Ru(II) complexes are $10x10^{-6}$, 1×10^{-6} M, 2×10^{-6} M, 5×10^{-6} M.

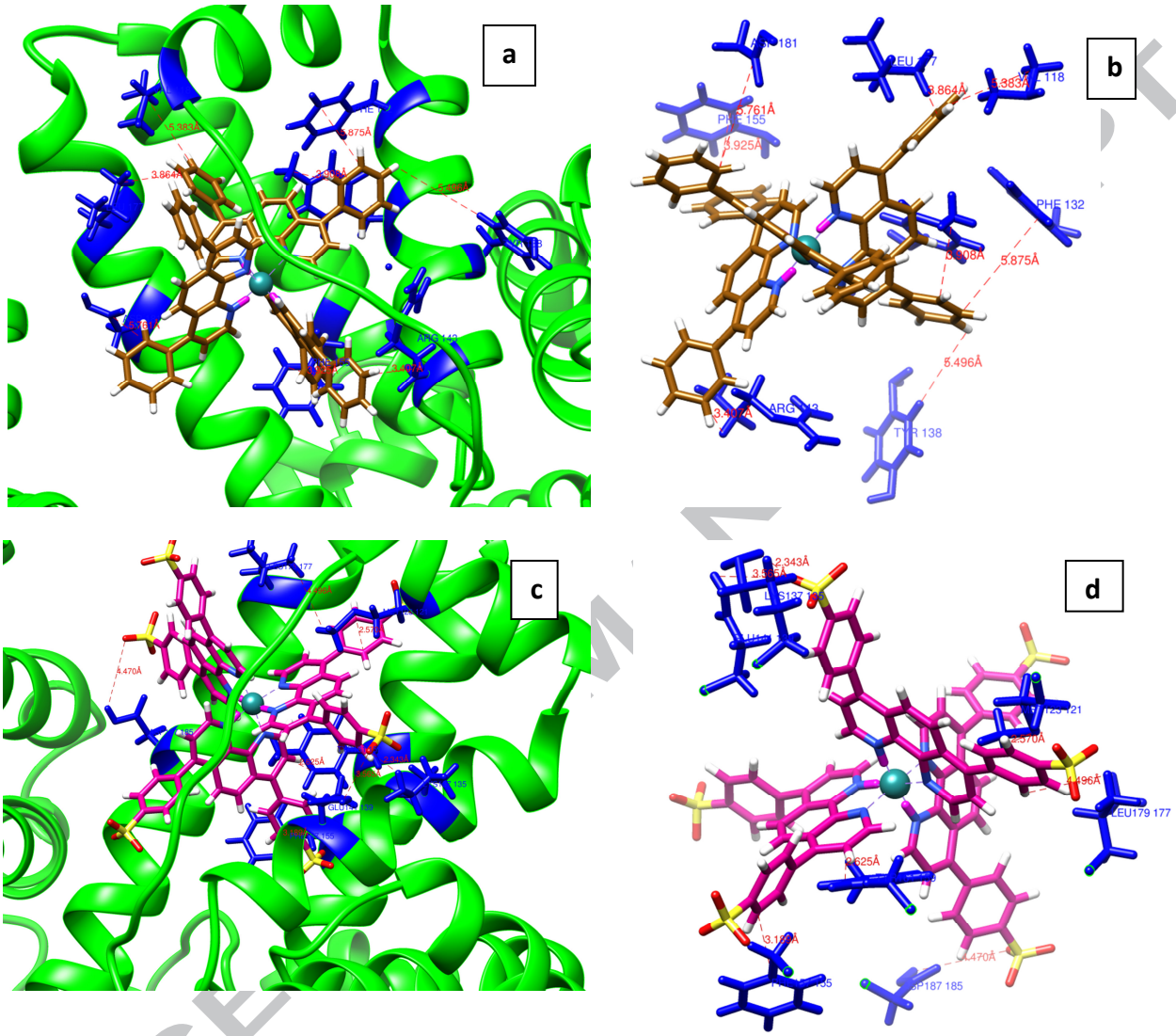

Figure 6. (a) Docking area of complex **I** (b) Interaction of complex **I** with amino acid residues in HSA (c) Docking area of complex **II** (d) Interaction of complex **II** with amino acid residues in HSA.

Table 1. Photophysical properties of complexes **I** and **II.**

Complex	$\lambda_{abs,}$ nm[a]	$\lambda_{abs,}$ nm[b]	$\lambda_{em,}$ nm[a]	$\lambda_{em,}$ nm[b]	τ, μs[a]	τ , μs[b]	K_a, M^{-1}	n
I	463	471	620	602	2.9±0.01	5.2±0.02	3.0×10^4	0.566
II	464	468	618	613	4.0±0.02	4.7±0.03	2.1×10^3	0.622

a - Complex alone in buffer; b - Complex with BSA

Table 2. The distance between the probe and amino acid residues.

Complex I		**Complex II**	
Amino acid	Distance (Å)	Amino acid	Distance (Å)
Tyr 136	3.9	Tyr 161	2.6
Phe 132	5.8	Phe 157	3.1
Val 118	5.3	Glu 141	4.2
Tyr 138	5.4	Asp 187	4.4
Phe 155	3.9	Leu 179	4.4
Arg 143	3.4	Met 123	2.5
Asp 181	5.7		

A Selective, Long-Lived Deep-Red Emissive Ru(II) Polypyridine Complexes for the Detection of BSA

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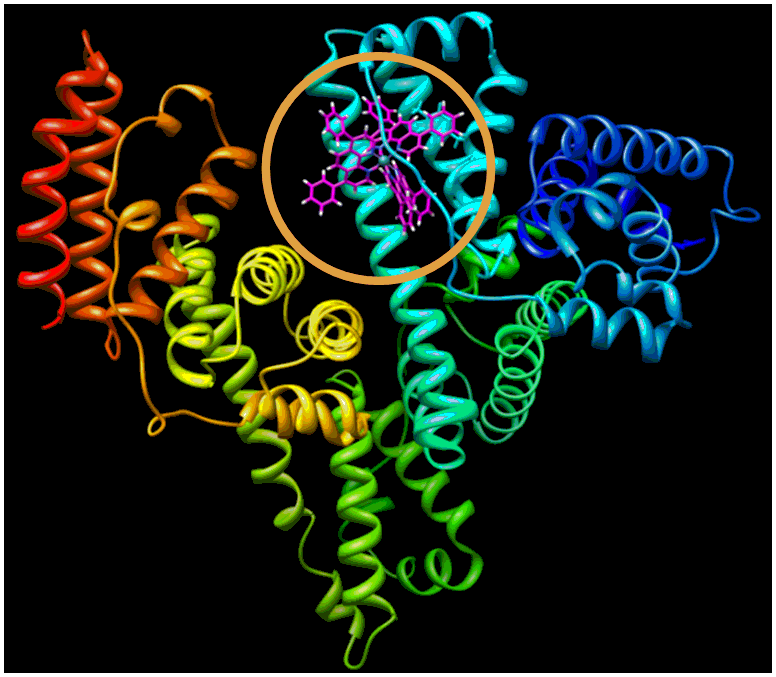
Graphical Abstract

A novel luminescence turn-on detection method for BSA have been developed using Ru(II) complexes, *via* non-covalent interactions.



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Research Highlights

- Binding of ruthenium(II) complexes is efficient with BSA.
- Sensing of ruthenium(II) complexes with BSA and HSA is higher than other proteins.
- The α helicity of BSA has decreased with the addition of ruthenium(II) complexes.
- Mode of binding is established by docking studies.