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Binding interaction between serum albumins and perylene-3,4,9,10-tetracarboxylate – A spectroscopic investigation

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ABSTRACT

A comprehensive understanding of the transportation of perylene-3,4,9,10-tetracarboxylate (PTCA) derivatives in blood will be beneficial for the investigations in drug design and toxicology. Hence we hereby investigated the binding interaction of perylene-3,4,9,10-tetracarboxylate tetrapotassium salt (PTK) with serum albumin in physiological buffer solution (pH 7.4) at 298 K by fluorescence spectra, synchronous fluorescence spectra, and Circular Dichroism (CD) techniques. It was proved that PTK quenched the intrinsic fluorescence of Serum albumins by forming a complex between them which is confirmed from the fluorescence lifetime studies. The binding constant (K) were calculated using the modified Stern–Volmer equation which indicates that affinity of HSA is more than that of BSA towards PTK. Using Forster Resonance Energy Transfer theory, the distance (r_0) between Serum Albumins and PTK was calculated. In addition, the results obtained from the synchronous fluorescence and CD spectra suggested the change in the microenvironment and conformation of Serum Albumin during the binding reaction.

1. Introduction

For the *in vivo* treatment of diseases, drugs have to be disseminated to the targeted tissues through the circulatory system. For this, serum albumins serve as a transport carrier of drugs [1]. Serum Albumins (SA), the most abundant soluble protein in the systemic circulation comprising 52–60% in plasma, are capable of bind reversibly with a large variety of relatively insoluble endogeneous and exogeneous ligands even though their principal function is to transport metabolites such as nutrients, hormones, fatty acids and a variety of pharmaceuticals [2,3]. Apart from an important role in maintaining colloidal osmotic pressure in blood, they can play a dominant role in drug disposition and efficacy since it increases the apparent solubility of hydrophobic drugs in plasma [4]. The binding of a ligand to serum albumin affect their conformation changes which alters their secondary and tertiary structure of albumin [5]. Studies on the interaction of ligands with serum albumin are significant as these studies can furnish information on the structural features that determine the therapeutic effectiveness of drugs. Hence these binding studies become a vital and fascinating research field in chemistry, life sciences and clinical medicine [6]. Many researchers have studied the binding interaction between serum albumins and various organic molecules [5–8]. But the interaction of Perylene-3,4,9,10-tetracarboxylic acid (PTCA) derivatives with serum albumins has not been reported so far.

PTCA derivatives are thermoresponsive organic dyes with highly developed conjugated π-electronic systems. Studies on PTCA derivatives attract a greater interest since it is having various applications such as electrophotographic photoreceptors, optical switches, chemical sensors, light-emitting diodes, field-effect transistors, solar cells and colourful liquid crystal displays owing to their ease of fabrication of large area devices and their technological merits in producing photoconductive compositions such as high molar absorptivity, high quantum yields of fluorescence with excellent photochemical and thermal stabilities as well as electrochemical performance [9–13]. Furthermore, PTCA derivatives have been widely studied for biochemical and pharmacological purposes because they are useful as G-quadruplex telomere targeting agents, telomerase inhibitors, antibacterial agents, and potential anticancer agents [14–16]. However perylene moiety tend to have greater carcinogenic, chronic impact potential and phototoxic effect since it is a heavier polyaromatic hydrocarbon [17,18]. In this circumstance, a comprehensive understanding of the disposition and transportation of PTCA derivatives in blood would benefit from the investigation of the interaction of perylene-3,4,9,10-tetracarboxylate tetrapotassium salt (PTK) with serum albumin since all perylene derivatives are synthesized using PTK.

Though many methods are available to study their interactions, optical techniques play a crucial role. These techniques are

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advantageous due to its exceptional sensitiveness, rapidness, abundant theoretical foundations, selectiveness and relative ease [19,20] whereas the conventional approaches suffer from lack of sensitivity, long analysis time or both and use of bio-molecule concentration far in excess [21,22]. An incalculable amount of information can be acquired about the structural fluctuations and the microenvironment changes surrounding the fluorophore in the protein from the measurements and analyses of the fluorescence emission spectra, the fluorescence lifetime, fluorescence polarization, etc. Hence, fluorescence spectroscopy plays a pivotal role in the investigation of interactions between serum albumins and the drug molecule at low concentration under physiological conditions [23]. Circular Dichroism is an excellent rapid sensitive tool to determine the secondary structure, folding and binding properties of proteins quantitatively. It also gives a deeper insight on the stereochemistry of the protein–drug adduct, and so on the mechanism of binding and thus very useful for monitoring the conformational changes in the protein upon interaction with the drug molecule [7,24].

In this present work, the binding interaction of perylene-3,4,9,10-tetracarboxylate tetrapotassium salt (PTK) with serum albumins such as Human Serum Albumin (HSA) and Bovine Serum Albumin (BSA) were investigated in detail using the fluorescence spectroscopic and circular dichroism techniques. The binding mechanism between PTK and serum albumin regarding the binding parameters, the binding distances by means of the Föster energy transfer theory and the effect of PTK on the protein conformation were investigated in this work.

2. Experimental

2.1. Materials

Perylene-3,4,9,10-tetracarboxylic acid dianhydride, Serum albumin (BSA and HSA) were purchased from Sigma–Aldrich. Serum albumin solutions were prepared in pH 7.4 phosphate buffer solution and stored at 0–4 °C. All other reagents used were of analytical reagent grade. Water from a Milli Q apparatus (Millipore, USA) was used throughout the experiments. All the experiments were performed at 298 K.

2.2. Instrumentation

UV–Vis absorption spectra were recorded with a T90+ UV–vis spectrophotometer (PG Instruments, United Kingdom). Fluorescence measurements were performed on a RF-5301 PC spectrofluorophotometer (SCHIMADZU, Japan). The samples were degassed using pure nitrogen gas for 15 min prior to each experiment. Fluorescence lifetime measurements were carried out in a picosecond time correlated single photon counting (TCSPC) spectrometer with a tunable Ti-sapphire laser (TSUNAMI, Spectra physics, USA). The fluorescence decay curves were analyzed using the software provided by IBH (DAS-6). Circular dichroism (CD) spectra were recorded on a Jasco J-810 spectropolarimeter under constant nitrogen flush over a wavelength range of 190–270 nm at a scanning speed of 200 nm min^{-1}. Mass spectra (ESI-MS) were recorded on a Micromass QUATTRO 11 spectrometer coupled to a Hewlett Packard series 1100 degasser.

2.3. Procedures

2.3.1. Preparation of perylene-3,4,9,10-tetracarboxylate tetrapotassium salt (PTK)

PTK was prepared by the simple alkaline hydrolysis [25] of perylene-3,4,9,10-tetracarboxylic acid dianhydride (Scheme 1). Perylene-3,4,9,10-tetracarboxylic acid dianhydride (1.96 g, 5 mM) was dispersed to a 5% potassium hydroxide (22.4 g, 20 mM) solution and the mixture was stirred along with heating at 90 °C until no residues were found. The resulting solution was fluorescent green in colour. This solution was cooled and then filtered using membrane filter to remove the trace amount of unreacted Perylene-3,4,9,10-tetracarboxylic acid dianhydride. The filtered solution was dried at room temperature in a dark environment. This was characterized using ESI-Mass spectroscopy analysis. MS (ESI): calc. for $C_{24}H_8K_4O_8$: 579.88 (M^+); found: 580.38.

2.3.2. SA-PTK interaction studies

Serum albumin concentration was kept constant at 3 μM and the concentration of PTK has been varied from 0 to 4 μM. This mixture was sonicated in an ultrasonic bath for 1 min. The interaction between SA and PTK was monitored using fluorescence spectroscopic techniques. For Circular Dichroism technique, serum albumin concentration was kept constant at 3 μM and PTK concentrations were 0, 3, 6 μM.

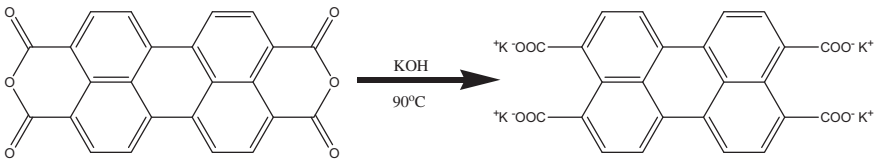
3. Results and discussion

3.1. Photophysical properties of PTK

Fig. 1 shows the absorption and fluorescence spectrum of aqueous solution of PTK. In the absorption spectrum, a shoulder and two absorbance peaks centered at 412, 438 and 466 nm were attributed to the electronic transitions of 0 → 2, 0 → 1 and 0 → 0, respectively, while two maximum emissions were observed at 481 (0 → 0) and 508 nm (0 → 1) in the fluorescence spectrum of PTK excited at 440 nm. The emission maximum at 481 (0 → 0 transition) is a mirror image relative to the 0 → 0 absorption, with the Stokes-shift of 15 nm [13,25,26].

3.2. Fluorescence characteristics of serum albumins with PTK

Crystal structure analyses have revealed that HSA contains 585 amino acid residues with 17 tyrosyl residues and only one tryptophan (Trp) located at position 214 along the chain whereas BSA contains 582 amino acid residues with 20 tyrosyl residues and two tryptophans located at positions 134 and 212 in which Trp-212 is in



perylene-3,4,9,10-tetracarboxylic acid dianhydride

perylene-3,4,9,10-tetracarboxylate tetrapotassium salt

Scheme 1. Synthesis of PTK.

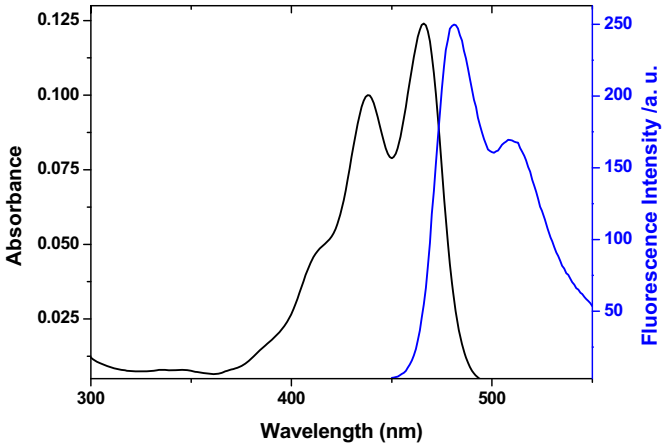


Fig. 1. Absorption and fluorescence spectrum of PTK. [PTK] = 3 μM.

chemical microenvironment similar to that Trp-214 in HSA and Trp-134 is located at the surface of the molecule [4,27]. When excited at 280 nm, the intrinsic fluorescence of serum albumins appears at 340 nm which is originating from tryptophan (Trp), tyrosine (Tyr), and phenylalanine (Phe) residues. Intrinsic fluorescence characteristics are very sensitive to its microenvironment and would be ultimately weakened if there is a slight change in their local surroundings, such as conformational transition, bio-molecular binding, and denaturation [28].

The fluorescence emission spectrum of serum albumins quenched by PTK was shown in Fig. 2. With the increasing concentration of PTK, the fluorescence intensities of serum albumins decreased remarkably with no alteration in the shape of the emission spectrum which indicates that the interaction between PTK and serum albumins occurs [1]. Furthermore, increase in PTK caused an accompanying enhancement around the emission wavelength at 486 nm and 512 nm suggesting that there was a strong association and non-radioactive energy transfer between PTK and serum albumin [29]. The isoacitinic point was found at 448 nm for both BSA and HSA which indicates that the quenching of serum albumins depends on the formation of complex between PTK and serum albumins. The normalized quenching effects of PTK on the fluorescence of serum albumins, i.e., F/F_0 ratio versus concentration of PTK, are presented in Fig. 3a. At 1: 10 M ratio of PTK/serum albumins, PTK quenches about 2.5% of HSA's fluorescence and about 4.5% of BSA's fluorescence whereas at 1:1 M ratio of PTK/serum albumins, PTK quench about 24% of HSA's fluorescence and about 23.5% of BSA's fluorescence. This indicates that PTK quenches the intrinsic fluorescence of BSA more than that of HSA at low concentration but at higher concentration, PTK quenches HSA more than that of BSA [1].

The fluorescence quenching is usually analysed by the well-known and most habitually used Stern–Volmer equation which is as follows:

$$\frac{F_0}{F} = 1 + K_q\tau_0[Q] = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F denotes the steady state fluorescence intensities in the absence and presence of the quencher Q respectively, K_q is the quenching rate constant of biomolecule, τ_0 is the average lifetime of molecule without quencher and its value for serum albumins is 10^{-8} s, [Q] is the quencher concentration, K_{SV} is the Stern–Volmer quenching constant. Stern–Volmer plots of serum albumins are shown in Fig. 3b. From the slopes of the linear plots, the Stern–Volmer quenching constants (K_{SV}) were calculated as 1.03×10^5 L mol^{-1} for HSA and 1.06×10^5 L mol^{-1} for BSA. The

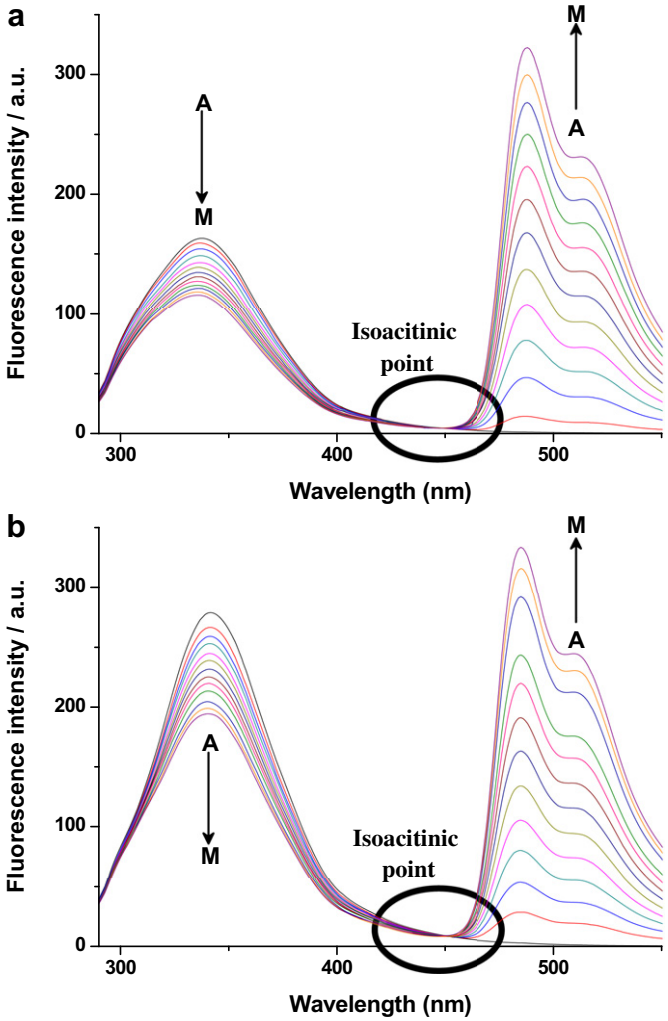


Fig. 2. Fluorescence spectra (λ_{ex} = 280 nm) of serum albumin (3 μM) [(a) HSA and (b) BSA] quenched by PTK in the concentration range of 0–4 μM. From A–M curve, PTK concentrations are 0, 0.33, 0.67, 1, 1.33, 1.67, 2, 2.33, 2.67, 3, 3.33, 3.67, and 4 μM.

bimolecular quenching rate constants, k_q, of HSA and BSA are found to be 1.03×10^{13} L mol^{-1} s^{-1} and 1.06×10^{13} L mol^{-1} s^{-1}, respectively, which are greater than that of scatter procedure (2×10^{10} L mol^{-1} s^{-1}) [1]. This shows that the quenching mentioned above is not initiated by dynamic collision but from the ground state complex formation of static quenching.

Static quenching can easily be distinguished from that of dynamic by monitoring their dependence on binding constants at different temperatures or preferably by lifetime measurements. In this study, we used the lifetime measurements to confirm the quenching mechanism as static. Fig. 4 shows the fluorescence decay of serum albumins (Fig. 4a: HSA; Fig. 4b: BSA) in the absence and presence of PTK. An increase in the concentration of PTK had no effect in the lifetime of serum albumins which reveals that the quenching follows static mechanism [1] i.e., PTK interacts with serum albumins by the formation of ground state complex.

3.3. Binding constants and the number of binding sites

For static quenching, the binding constant (K) to a site and the number of binding sites (n) between PTK and serum albumins can be calculated using the double logarithm formula of modified Stern–Volmer equation:

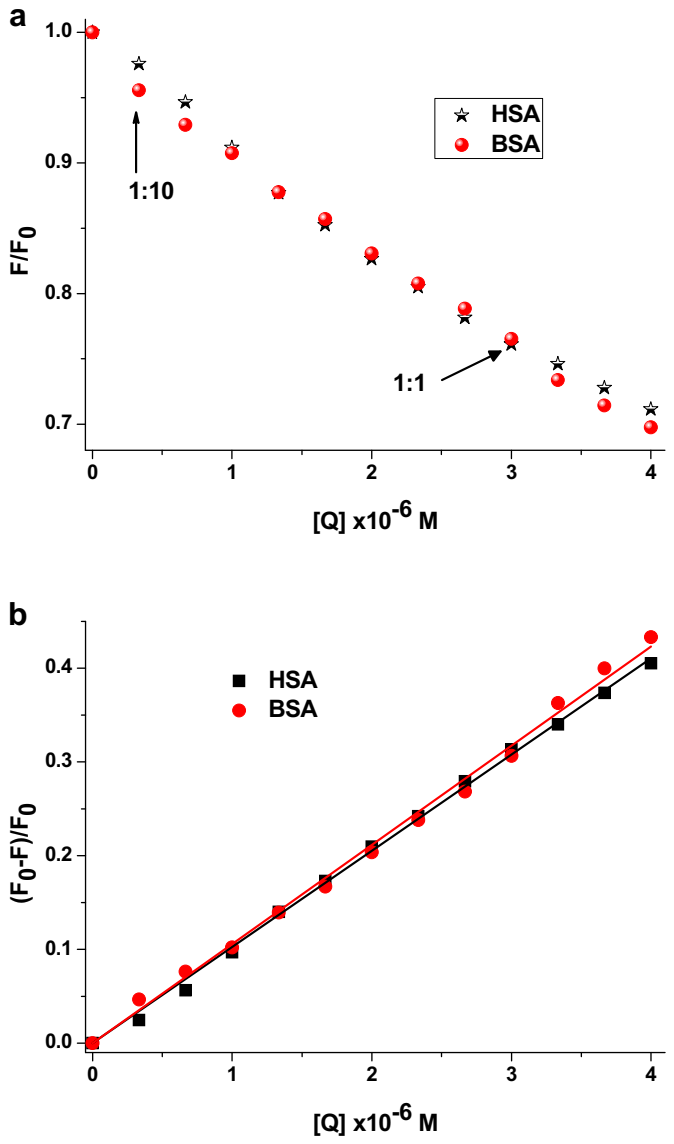


Fig. 3. Normalized quenching plot (a) and Stern–Volmer plot (b) for serum albumins in the presence of PTK.

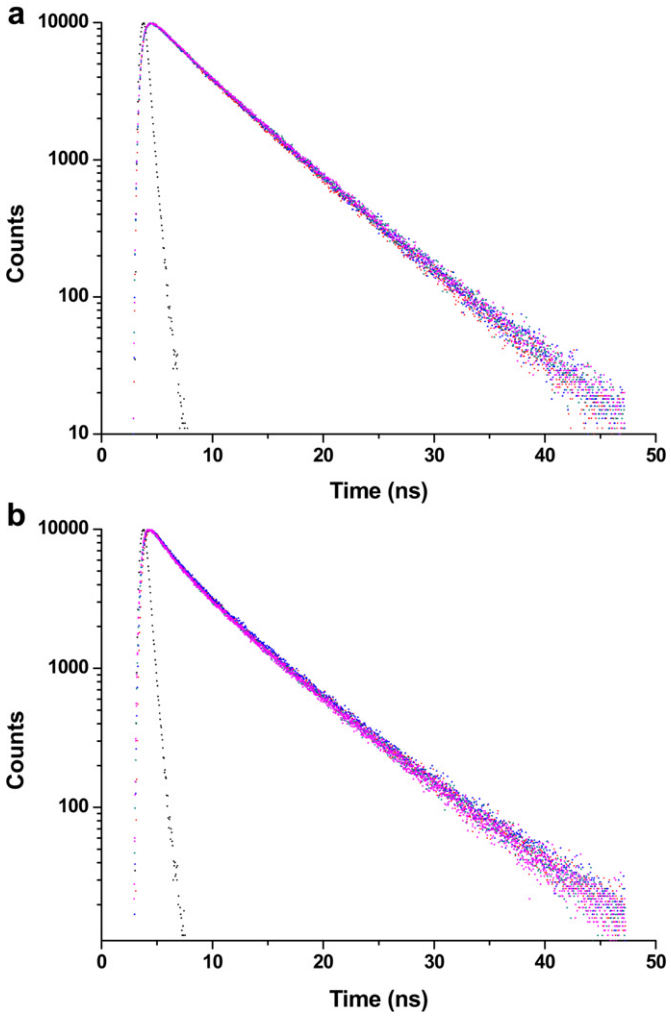


Fig. 4. Fluorescence decay curves (λ_{ex} = 280 nm) of serum albumin (3 μM) [(a) HSA and (b) BSA] in the presence of PTK in the concentration range of 0–4 μM. From A-E curve, PTK concentrations are 0, 1, 2, 3, and 4 μM. The monitored wavelength for HSA and BSA are 342 nm and 338 nm respectively.

$$\log\left[\frac{F_0 - F}{F}\right] = \log K + n\log[Q] \quad (2)$$

A plot of log $[(F_0 - F)/F]$ versus log [Q] (Fig. 5) gives a straight line where the values of n and K can be obtained from the slope and the intercept respectively. The values of K and n have been found out to be 4.94×10^5 L mol^{-1} and 1.12 for HSA and 3.72×10^4 L mol^{-1} and 0.92 for BSA. The binding constant of HSA with PTK is found to be about 13 times greater than that of BSA. This indicates that affinity of HSA is more than that of BSA towards PTK. From the number of binding sites, we can conclude that there was one independent class of binding site on serum albumins for PTK and it formed a 1:1 complex with the serum albumin molecule.

The free energy change (ΔG) of binding PTK with serum albumins can be estimated using the Van't Hoff equation:

$$\Delta G = -RT\ln K \quad (3)$$

where R is the gas constant. The value of ΔG was found to be −32.48 kJ mol^{-1} for HSA and −27.07 kJ mol^{-1} for BSA. This negative value of ΔG confirms that the process of binding between PTK and serum albumins is spontaneous [5].

3.4. Energy transfer from serum albumin to PTK

Energy transfer between small molecules bound to biomacromolecule has been widely used to study protein–drug interaction and changes in protein conformation upon binding to a target analyte. The fluorescence quenching of serum albumins after binding with PTK indicated the occurrence of energy transfer between PTK and serum albumins. According to Förster resonance energy transfer (FRET) theory, energy transfer, which occurs through direct electrodynamic interaction between the primarily excited molecules and their neighbours, is controlled by the following three aspects: (1) The donor should have strong fluorescence quantum yield, (2) more spectral overlap between the donor emission (serum albumin) and the acceptor absorption (PTK), (3) the distance (r_0) between the acceptor and the donor should be within 7 nm. This theory points out that the energy transfer efficiency 'E', in addition to its dependence on the distance (r_0) between the acceptor and the donor, depends upon the critical energy transfer distance, R_0 (Förster's distance; the distance at

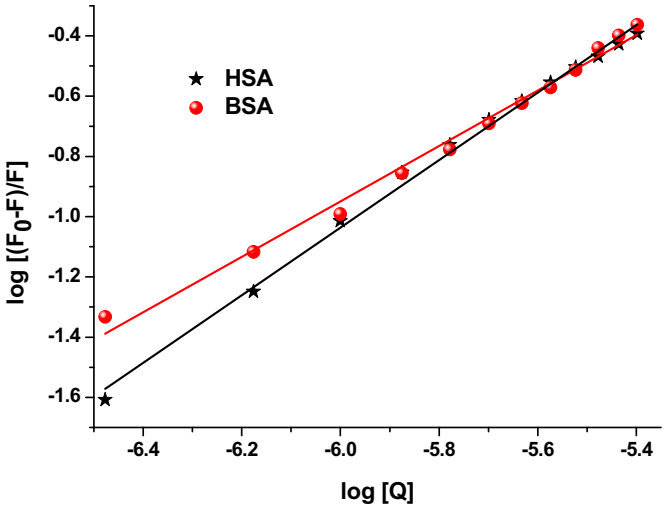


Fig. 5. Modified Stern–Volmer plot for Serum Albumins versus PTK.

which the efficiency of energy transfer is 50%), which is expressed by the following equation:

$$E = \frac{R_0^6}{R_0^6 + r_0^6} \tag{4}$$

The magnitude of R_0 is dependent on the spectral characteristics of the donor emission and acceptor absorption of the molecules. R_0 is expressed as follows

$$R_0^6 = 8.8 \times 10^{-25}\left[\kappa^2 N^{-4}\phi_D J\right] \tag{5}$$

where κ^2 is the spatial orientation factor related to the geometry of the donor and acceptor of dipoles, $\kappa^2 = 2/3$ for random orientation as in fluid solution, N is the refractive index of the medium (1.36 for BSA and 1.336 for HSA), ϕ_D is the fluorescence quantum yield of the donor (0.118 for BSA and 0.15 for HSA), J is the overlap integral of the fluorescence emission spectrum of the donor serum albumins and the absorption spectrum of the acceptor PTK (Fig. 6), which could be calculated by the equation

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}{\int_0^\infty F(\lambda)d\lambda} \tag{6}$$

where $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range from λ to $(\lambda + \Delta\lambda)$, with the total intensity normalized to unity and $\varepsilon(\lambda)$ is the molar extinction coefficient of the acceptor at λ. The efficiency of energy transfer also can be obtained using the equation

$$E = 1 - \frac{F}{F_0} \tag{7}$$

Using Equations (4)–(7), that the following values were obtained: $R_0 = 3.68$ nm, $E = 0.16$ and $r_0 = 4.85$ nm for HSA and $R_0 = 2.62$ nm, $E = 0.16$ and $r_0 = 3.43$ nm for HSA. This donor-to-acceptor distance r_0 obeys two criteria (i) $r_0 < 7$ nm and (ii) $0.5R_0 < r_0 < 1.5R_0$ which indicated that the energy transfer from serum albumins to PTK occurs with high probability [1]. This again supports the static quenching mechanism.

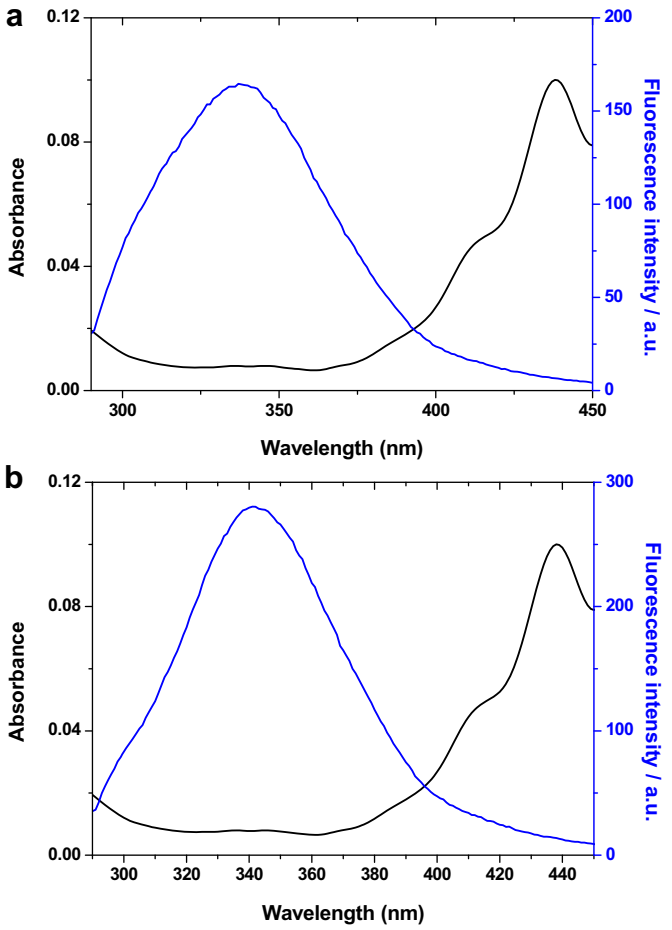


Fig. 6. Overlap plots of fluorescence spectra of serum albumins (a) HSA and (b) BSA with absorption spectra of PTK.

3.5. Conformational investigation

The synchronous fluorescence spectroscopy introduced by Lloyd [30] is the simple sensitive method to acquire information about the molecular environment in a vicinity of the chromosphere molecules in low concentration under physiological conditions. In Serum Albumins, the synchronous fluorescence spectra give the characteristic information of tyrosine residues or tryptophan residues when $\Delta\lambda$ between excitation wavelength (λ_{ex}) and emission wavelength (λ_{emi}) were stabilized at 15 or 60 nm [29]. The synchronous fluorescence spectra for interaction between Serum Albumins and PTK were presented in Fig. 7 ($\Delta\lambda = 15$ nm) & Fig. 8 ($\Delta\lambda = 60$ nm). The fluorescence intensity of serum albumin weakened feebly along with the addition of PTK in Fig. 7 ($\Delta\lambda = 15$ nm) and decreased evidently with the addition of PTK in Fig. 8 ($\Delta\lambda = 60$ nm). These results implied that PTK was bound to serum albumin and located in close proximity to the Tyr residues, which affected the polarity near the Tyr residues [29].

From Fig. 7, it was found that the maximum emission wavelength of serum albumins (HSA and BSA) slightly red-shifts when $\Delta\lambda$ was 15 nm. Such red-shift indicates that the microenvironments around tyrosine residue was disturbed and the hydrophilicity of Try residue increased in the presence of PTK, and the spreading of peptide strands increased [31]. Whereas when $\Delta\lambda = 60$ nm (Fig. 8), the maximum emission wavelength does not show any shift which indicates that the tryptophan moiety does not involve in the binding. The synchronous spectral studies indicate that the binding

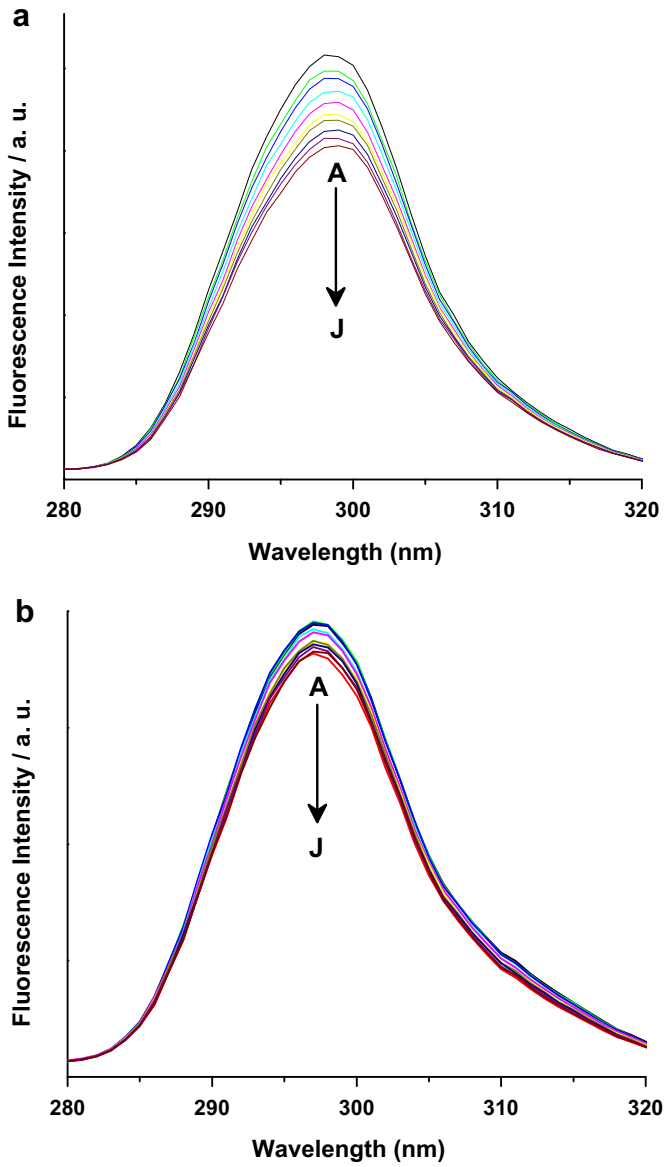


Fig. 7. Synchronous fluorescence spectra ($\Delta\lambda = 15$ nm) of serum albumins (3 μm) [(a) HSA and (b) BSA] quenched by PTK in the concentration range of 0–3 μM. From A–J curve, PTK concentrations are 0, 0.33, 0.67, 1, 1.33, 1.67, 2, 2.33, 2.67, and 3 μM.

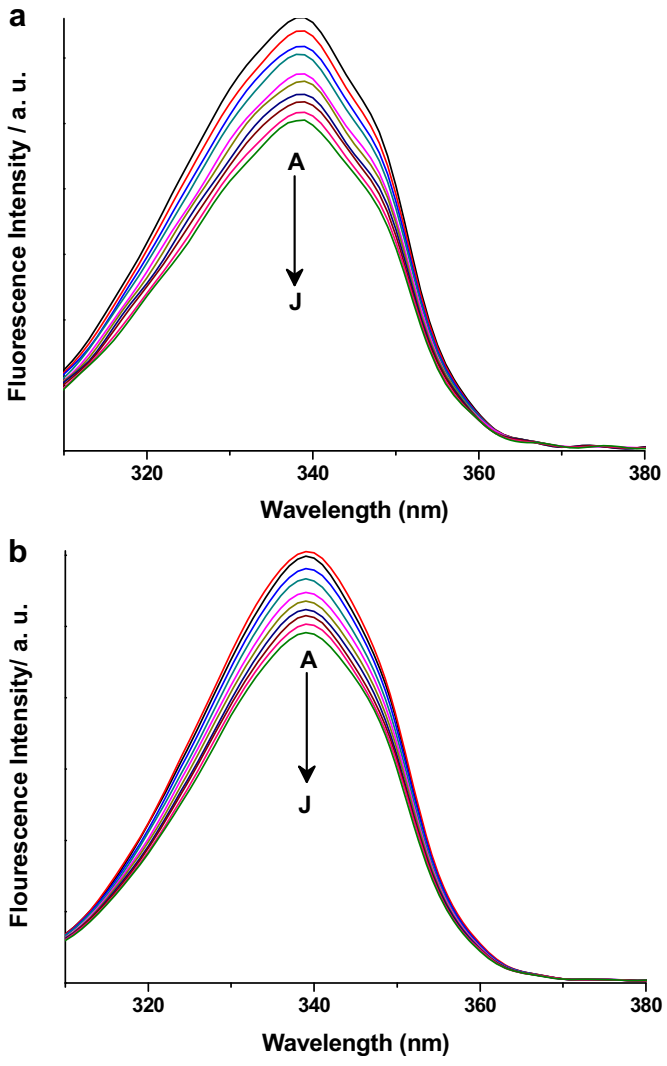


Fig. 8. Synchronous fluorescence spectra ($\Delta\lambda = 60$ nm) of serum albumins (3 μm) [(a) HSA and (b) BSA] quenched by PTK in the concentration range of 0–3 μM. From A–J curve, PTK concentrations are 0, 0.33, 0.67, 1, 1.33, 1.67, 2, 2.33, 2.67, and 3 μM.

site is near the tyrosine moiety. Compared with BSA, the Tyr and Trp residues of HSA were altered more deeply. From this point, the binding affinity between HSA and PTK should be higher than that of BSA and PTK [29].

To ascertain the possible influence of PTK binding on the secondary structure of serum albumins, CD measurement was also performed in the presence and absence of PTK. The CD spectra of PTK-SA system are shown in Fig. 9. The spectra were scanned in the wavelength region 190–300 nm to probe transition in backbone amide. As Fig. 9 showed, serum albumin exhibits two negative bands in the ultraviolet region at 208 and 222 nm, a characteristic of the typical α-helix structure as both contribute to the $n \rightarrow \pi^*$ transfer for the bond of the α-helix [5]. The CD result can be expressed as Mean Residue Ellipticity (MRE) in deg cm^2 $dmol^{-1}$, which is defined as

$$\mathrm{MRE} = \frac{\theta_{\mathrm{obs}}}{10nl[P]}$$

where θ_{obs} is the CD in millidegree, n is the number of aminoacid residues (585 for HSA and 582 for BSA), l is the path-length of the cell, and $[P]$ is the mole concentration of serum albumin. The helical content was calculated from the values of MRE at 208 nm using the following equation

$$\alpha - \mathrm{helix} = \left[\frac{\mathrm{MRE}_{208\ \mathrm{nm}} - 4000}{33000 - 4000}\right] \times 100\%$$

where 4000 is the MRE of the β-form and random coil conformation cross at 208 nm, and 33,000 is the MRE value of a pure α-helix at 208 nm [6]. The α-helix of serum albumins was observed to decrease, as seen from the decrease in band intensity at all wavelengths of the CD spectra without any significant shift of the peaks. It can be calculated that the native HSA solution has a 61.73% of α-helix, while α-helix content of HSA decreases to 59.92% and 58.09 with the addition of PTK in the concentration ratio of 1:1 and 1:2 respectively. Whereas the native BSA solution has a 65.13% of α-helix, while α-helix content of BSA decreases to 63.62% and 62.11 with addition of PTK in the concentration ratio of 1:1 and 1:2 respectively. The fact that α-helix content decreased indicated that the secondary structure of serum albumins had changed during the

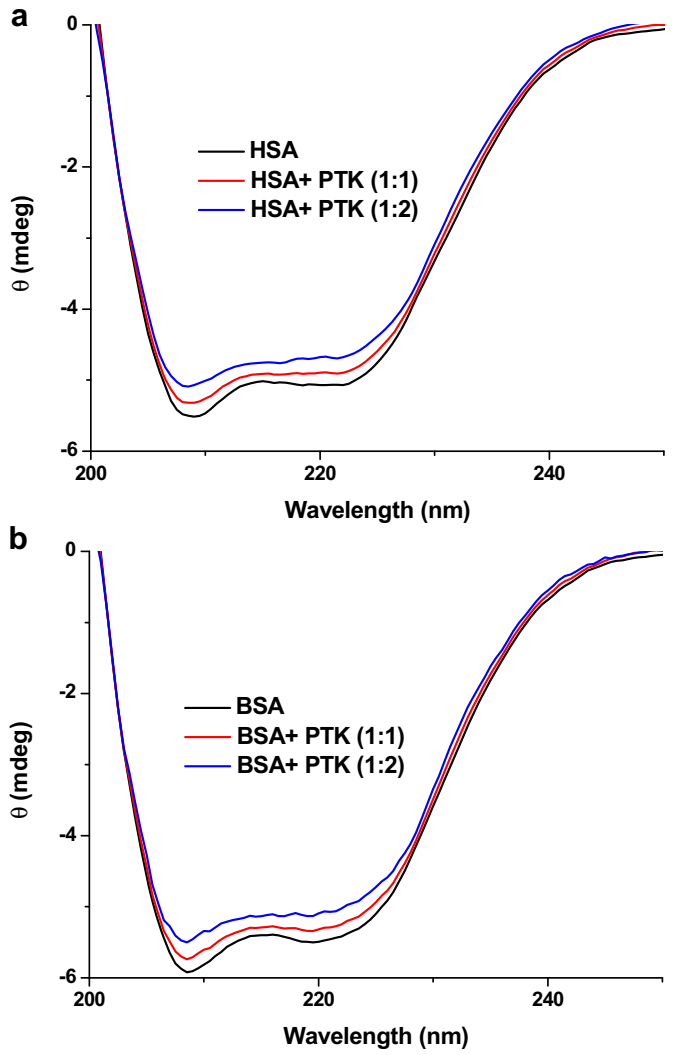


Fig. 9. CD spectra of serum albumins (3 μm) [(a) HSA and (b) BSA] in the presence of PTK in the concentration of 0 and 3 μM.

reaction between PTK and serum albumins which suggests that PTK bound with the amino acid residues of the main polypeptide chain of protein [5]. However, the CD spectra of serum albumins in the absence and presence of PTK are similar in shape without any conspicuous change, indicating that the structure of serum albumins after PTK binding to serum albumins is also predominantly α-helical [4,32]. So we can conclude that the binding of PTK to Serum Albumins induced some conformational changes.

4. Conclusion

The binding interaction of PTK and Serum Albumins in physiological buffer solution was investigated by fluorescence quenching and Circular Dichroism measurements. The Stern–Volmer quenching constant, K_{sv}, suggests that PTK quenched the intrinsic fluorescence of HSA than that of BSA. Lifetime studies confirmed the mechanism of binding as static mechanism. The value of ΔG indicated the spontaneity in the PTK-Serum Albumin binding reaction. The distance r between the donor (BSA) and acceptor (PTK), calculated according to Förster's energy transfer theory, suggests the occurrence of high probable non-radioactive energy transfer from serum albumins to PTK. Wavelength shifts in synchronous fluorescence spectra showed that the microenvironment and molecular conformation of Serum Albumin is changed in the presence of PTK which is further confirmed from the decrease of α-helical content in the CD spectra. All these experimental results revealed that PTK could bind to Serum Albumins and be effectively transported in the body, which could be a useful guideline for further investigations in drug design and toxicology.

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