Interaction between Pirennoxine and Bovine Serum Albumin in Aqueous Solution

Zhixi Liao\textsuperscript{a}, Xianyong Yu\textsuperscript{a, b, c\ast}, Qing Yao\textsuperscript{a}, Pinggui Yi\textsuperscript{a\ast}

\textsuperscript{a} Key Laboratory of Theoretical Chemistry and Molecular Simulation of Ministry of Education, Hunan Province College Key Laboratory of QSAR/QSPR, School of Chemistry and Chemical Engineering, Hunan University of Science and Technology, Xiangtan 411201, People's Republic of China

\textsuperscript{b} State Key Laboratory of Physical Chemistry of Solid Surfaces, Xiamen University, Xiamen 361005, China

\textsuperscript{c} Key Laboratory of Computational Physical Sciences (Fudan University), Ministry of Education, Shanghai 200433, P.R. China

Abstract

This work concerns the interaction of pirennoxine sodium (PRX) and bovine serum albumin (BSA), which was conducted by spectroscopic means: fluorescence spectra, ultraviolet–visible spectra (UV–vis) and circular dichroism spectra (CD spectra) in physiological conditions. The results revealed the PRX can quench the fluorescence of BSA remarkably in aqueous solution. The quench mechanism has been obtained after corrected the fluorescence intensities for inner filter effects. The binding constants ($K_a$) were calculated according to the relevant fluorescence data at different temperatures. Moreover, from a series of analyses, we have obtained the binding sites, the binding distance and binding force. The effect of PRX on the conformation of BSA has been analyzed using synchronous fluorescence under experimental conditions. In addition, the CD spectra proved that the secondary structure of BSA changed in the presence of PRX in aqueous solution.

Key words: Bovine serum albumin; Prenoxine; Interaction; Spectroscopic means; Circular dichroism spectra

\textsuperscript{\ast} Corresponding author. Tel.: +86-731-58290187; Fax: +86-731-58290509.
E-mail address: yu_xianyong@163.com (X. Yu); pgyi@hnust.cn (P. Yi)
1. Introduction

Serum albumin, the major soluble protein in blood circulation, contributing many physiological functions of which most important are serving as a depot and a transport protein for many endogenous and exogenous drugs reversibly, has been one of the most extensively studied proteins. In biomedical fields, seeking biocompatible materials is one of the big challenges that researchers are faced with. In our work, bovine serum albumin (BSA) is selected as our protein model because it is well suited to these initial studied due to its functions has been known widely and structures has been extensively characterized [1, 2].

Pirenoxine (PRX), 1-Hydroxy-5-oxo-5H-pyrido[3,2] phenoxazine -3-carboxylic acid (shown in scheme. 1), was first introduced in 1958 in Japan and now is widely used clinically in Asia as an anti-cataractogenic agent [3, 4]. Pirenoxine is a medication used in the possible treatment and prevention of cataracts. A report in the journal of Inorganic Chemistry showed that in liquid solutions PRX could cause decreased cloudiness of a crystallin solution produced to mimic the environment of the eye [5]. Its chemical structure is very similar to the insect eye pigment, to prevent the lens with a water-soluble protein denaturation [6].

The binding ability of a drug to serum albumin has influence on the pharmacokinetics of a drug. In the present study, the mutual interaction of anti-cataractogenic drug prenoxine sodium with bovine serum albumin (BSA) was investigated by fluorescence, UV-vis spectroscopy and CD spectra, for the techniques are very sensitive, rapid and easily. Changes in the intrinsic fluorescence intensities of BSA-drug complex could provide considerable information regarding the binding characteristics and the therapeutic effectiveness of drugs [7]. Knowledge of the interactions is not only fundamental in theoretical, but also practical in therapeutic applications. Because the adsorbed proteins determine the route of internalization, organ disposition and rate of clearance from bloodstream, the protein-PRX interactions play an important role for understanding biodistribution, biocompatibility and therapeutic efficacy of PRX. Currently, the mechanism of protein binding to PRX is not well characterized. So what we have studied is meaningful.

![Scheme 1. The molecular structure of prenoxine sodium](image-url)
2. Material and method

2.1. Apparatus

Fluorescence spectra were recorded on a Shimadzu RF-5301 fluorescence spectrophotometer (Tokyo, Japan) with a SB-11 water bath (Eyela) and 1.0 cm quartz cells. The emission and excitation slits were 10 and 10 nm, respectively. Fluorescence quenching spectra were measured in the range of 280–500 nm with the excitation wavelength of 280 nm at three temperatures (294, 302, and 310 K). The synchronous fluorescence spectra were obtained by setting the excitation and emission wavelength interval (Δλ) at 15 and 60 nm. The absorption spectra were obtained from a Shimadzu UV-2501 spectrophotometer (Tokyo, Japan). The pH measurement was made with a Leici pHS-2 digital pH-meter (Shanghai, China) with a combinational glass calomel electrode. The CD spectra were recorded on Chirascan (London, UK)) using a 1 cm cell with 3 scans averaged for each CD spectra at room temperature.

2.2. Reagents

BSA (≥99%) was obtained from Huamei Bioengineering Co. (Shanghai, China) and was dissolved in a Tris–HCl (0.05 mol·L⁻¹, pH =7.40) buffer to form the BSA solution with a concentration of 1.0×10⁻⁵ mol·L⁻¹. A Tris–HCl buffer (0.05 mol·L⁻¹, pH =7.40) containing 0.1 mol·L⁻¹ NaCl was selected to keep the pH value constant and to maintain the ionic strength of the solution. Pirenoxine sodium was obtained from China Grand Pharmaceutical Co., Ltd and its stock solution (1.66×10⁻³ mol·L⁻¹) was prepared in double-distilled water. All other reagents were of analytical reagent grade and double–distilled water was used during the experiment.

2.3. Procedures

A 2.5 mL solution containing 1.0×10⁻⁵ mol·L⁻¹ BSA was titrated by successive additions of 1.66×10⁻³ mol·L⁻¹ pirenoxine sodium solution and the concentration of PRX varied from 0 to 2.99×10⁻⁶ mol·L⁻¹. Titrations were done manually by using micro-injector. Fluorescence spectra were measured in the range of 280–500 nm at the excitation wavelength of 280 nm. The fluorescence spectra were performed at three temperatures (294, 302, and 310 K).

The UV–vis absorption spectra of pirenoxine sodium solution with the concentration of
$1.0 \times 10^{-5}$ mol·L$^{-1}$ was measured in the range of 200–500 nm at 310 K.

The CD measurements of BSA in the presence and absence of PRX were recorded in the range of 180-260 nm at 302 K to investigate the secondary structure change of BSA.

### 3. Result and discussion

#### 3.1 Fluorescence quenching studies of BSA

Fluorescence spectroscopy is a suitable technique to study the interaction between these two substances because BSA has two tryptophan residues at position 134 and 212 [8] while PRX does neither contain any tryptophan nor other aromatic amino acids [9]. When 280 nm is used as the excitation wavelength, BSA can emit strong fluorescence at the wavelength of 340 nm [10]. From the results of fluorescence spectra and intensity, we get the Fig. 1. It shows that the fluorescence emission spectra ($\lambda$ at about 340 nm) obtained for BSA at pH 7.40 with the addition of PRX had a strong quenching. The drug can be bound to serum albumins with a high affinity.

![Fluorescence Quenching Spectra](image)

**Fig. 1** The fluorescence quenching spectra of BSA by PRX at different temperatures. $\lambda_{ex} = 280$ nm. [BSA] = $1.00 \times 10^{-5}$ mol·L$^{-1}$; [PRX] (a-j): 0, 3.32, 6.64, 9.96, 13.28, 16.60, 19.92, 23.24, 26.56, 29.88$ \times 10^{-6}$ mol·L$^{-1}$.

#### 3.2 Quenching mechanism analysis
In addition to static and dynamic quenching, another cause of the decrease in fluorescence intensity can be inner–filter effect [11]. Steiner [12] et al. proposed that if the absolute absorption value is not more than 0.3, the inner filter effect can be corrected following formula:

\[
F_{\text{corr}} = F_{\text{obs}} \times e^{\frac{A_{\text{ex}}+A_{\text{em}}}{2}}
\]  

(1)

where \(F_{\text{corr}}\) and \(F_{\text{obs}}\) represent the corrected and the observed fluorescence intensity respectively. \(A_{\text{ex}}\) and \(A_{\text{em}}\) are the absorbance of drug in the excitation and emission wavelength [13].

The ultraviolet absorption spectra in Fig. 2 were achieved by successive adding of PRX. BSA alone has two absorption peaks at about 200 and 280 nm, which stands for the strong absorption of peptide bond and aromatic residues respectively.

![Absorption spectra](image)

**Fig. 2** Absorption spectra of \(c_{\text{BSA}} = 1.0 \times 10^{-5}\) mol·L\(^{-1}\) in the presence of PRX solution: \(c_{\text{PRX}}\) (a–j): 0, 3.32, 6.64, 9.96, 13.28, 16.60, 19.92, 23.24, 26.56, 29.88×10\(^{-6}\) mol·L\(^{-1}\).

Quenching can occur by different mechanisms, which usually classified as dynamic quenching and static quenching. Dynamic and static quenching can be distinguished by their differing dependence on the temperature and viscosity. Dynamic quenching depends upon diffusion. Since higher temperatures result in larger diffusion coefficients, the bimolecular quenching constants are expected to increase with increasing temperature [14]. In contrast, increased temperature is likely to result in lower values of the static quenching constants.

It can be seen that there exists very significant linear relationship in the plot of \((F–F_0)/F–\) against \([Q]\) from Fig. 3 The regression equation was found as follows [15] :

\[
\frac{F_0}{F} = 1 + K_{sv}[Q] = 1 + K_q\tau_0[Q]
\]  

(2)
where $F_0$ is the fluorescence intensity of BSA without PRX added in; $F$ is the fluorescence intensity of BSA with different concentration of PRX; $[Q]$ is the concentration of PRX; $K_{SV}$ is the apparent interaction constant.

Table 1 The quenching constants of BSA by PRX at three temperatures.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$K_{SV}$ (L·mol$^{-1}$)</th>
<th>$K_q$ (×10$^{12}$ L·mol$^{-1}$·s$^{-1}$)</th>
<th>$R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>294 K</td>
<td>47579</td>
<td>4.76</td>
<td>0.9919</td>
</tr>
<tr>
<td>302 K</td>
<td>40910</td>
<td>4.09</td>
<td>0.9875</td>
</tr>
<tr>
<td>310 K</td>
<td>33547</td>
<td>3.35</td>
<td>0.9801</td>
</tr>
</tbody>
</table>

Table 1 shows the calculated values of $K_{SV}$ and $K_q$ (taking as fluorescence lifetime ($\tau_0$) of BSA at around 10$^{-8}$ s) for the interaction of the PRX with BSA. Usually, the maximum quenching rate constant of diffusion collision of various quenchers for the biomacromolecule is about 2.0×10$^{10}$ L·mol$^{-1}$·s$^{-1}$ [16]. Obviously, the derived quenching rate constants (which were about 4×10$^{12}$ L·mol$^{-1}$·s$^{-1}$) were higher than the $K_q$ of the diffusion course by two orders of magnitude for complexes. This shows that the quenching is not initiated by dynamic quenching, but by compound formation [17, 18]. Coupled that the quenching constants were decreasing with the increasing of temperatures, so that is static quenching actually occurred in the quenching process.

![Stern–Volmer plots for the PRX-BSA system at 310 K, 302 K and 294 K with pH 7.40.](image)

Fig. 3 Stern–Volmer plots for the PRX-BSA system at 310 K, 302 K and 294 K with pH 7.40.

### 3.3 Binding constant and the binding site of PRX-BSA compounds

The above results indicate that PRX acts as a quencher in its interaction with BSA. When a small quencher binds independently to a set of equivalent sites on a fluorescent macromolecule, the
relationship between the extent of fluorescence quenching of the macromolecule and the quencher concentration can be described by Eq. 1 [19] as follows:

$$\log \left( \frac{F_0 - F}{F} \right) = n \log K_a - n \log \left\{ \frac{1}{[D_t] - (F_0 - F)[P_t]/F_0} \right\}$$

where the $F_0$ and $F$ in equation are the fluorescence intensities in the absence and presence of quencher, $[P_t]$ is the total concentration of protein and $[D_t]$ is the total concentration of quencher. $K_a$ is the binding constant or the apparent association constant for drug–protein interaction, $n$ is the number of binding sites. Values of $K_a$ and $n$ can thereby be determined from the intercept and slope by the double–logarithm algorithm curve (Fig. 4) [20]. The Table 2 below shows the binding parameters and binding sites at three temperatures. From this table, it can be seen clearly that the binding parameter (binding capacity) decreases with increasing temperature. The value of $n$ is helpful to know the number of binding sites and to locate the binding site in BSA for the drug, which was noticed to be almost unity, indicating that there was one independent class of binding site on BSA for complex.

![Logarithmic plots of the fluorescence quenching of BSA with different concentrations of PRX.](image)

**Fig. 4** Logarithmic plots of the fluorescence quenching of BSA with different concentrations of PRX.

**Table 2** The binding constants and the number of binding sites of PRX to BSA

<table>
<thead>
<tr>
<th>$T$ (K)</th>
<th>$K_a$ (L·mol$^{-1}$)</th>
<th>$n$</th>
<th>$R$</th>
</tr>
</thead>
<tbody>
<tr>
<td>294</td>
<td>52323</td>
<td>0.95</td>
<td>0.9930</td>
</tr>
<tr>
<td>302</td>
<td>16826</td>
<td>1.09</td>
<td>0.9939</td>
</tr>
<tr>
<td>310</td>
<td>11748</td>
<td>1.14</td>
<td>0.9890</td>
</tr>
</tbody>
</table>
3.4. Thermodynamic parameters and nature of the binding forces

The intermolecular forces between PRX and BSA include hydrogen bonds, van der Waals forces, electrostatic interactions and hydrophobic interaction, etc. If the temperature changes little, the reaction enthalpy change is regarded as a constant. In order to explore the interaction of PRX with BSA, the thermodynamic parameters were calculated from the Van’t Hoff equation and corresponding thermodynamical functions based on the temperature effect [21-23].

\[
\ln \frac{K_{a1}}{K_{a2}} = \left( \frac{1}{T_1} - \frac{1}{T_2} \right) \frac{\Delta H}{R}
\]

The enthalpy change \( \Delta H \) and entropy change \( \Delta S \) for a binding reaction can be derived from the Van’t Hoff equations:

\[
\ln K_a = \frac{\Delta H}{RT} + \frac{\Delta S}{R}
\]

where \( K_a \) is analogous to the binding constant at the corresponding temperature, \( R \) is gas constant [22]. The free energy change can be obtained from the following relationship:

\[
\Delta G = -RT \ln K_a = \Delta H - T\Delta S
\]

Using the above three equations the values of \( \Delta G, \Delta H, \Delta S \) were obtained and shown in Table 3. The binding process was always spontaneous as demonstrated by the negative value of \( \Delta G \). Positive \( \Delta H \) and \( \Delta S \) values are frequently taken as typical evidence of hydrophobic interactions, while negative enthalpy and entropy changes arise from van der Waals and hydrogen bonding formation [24, 25]. Therefore, from these results, the binding of PRX complex to BSA is mainly hydrophobic interactions as shown by the positive value of \( \Delta S \) and \( \Delta H \).

<table>
<thead>
<tr>
<th>( T ) (K)</th>
<th>( \Delta H ) (kJ·mol(^{-1}))</th>
<th>( \Delta G ) (kJ·mol(^{-1}))</th>
<th>( \Delta S ) (J·mol(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>294</td>
<td>-26.56</td>
<td></td>
<td>330.9</td>
</tr>
<tr>
<td>302</td>
<td>7.07</td>
<td>-24.43</td>
<td>315.1</td>
</tr>
<tr>
<td>310</td>
<td>-24.15</td>
<td></td>
<td>306.1</td>
</tr>
</tbody>
</table>

3.5. Energy transfer

According to the Förster non-radio active energy transfer theory [26], the energy transfer efficiency \( E \) is not only related to the distance between the acceptor and donor (\( r \)), but also to the critical energy transfer distance (\( R_0 \)). The actual distance and the critical distance for 50% energy
transfer efficiency $R_0$ have the following relationship with $E$:

$$ E = 1 - \frac{F}{F_0} = \frac{R_0^6}{(R_0^6 + r^6)} $$  \hspace{1cm} (7)

$R_0$ was calculated by Eq. (7)

$$ R_0^6 = 8.78 \times 10^{-25} \kappa^2 N^{-4} \Phi J $$  \hspace{1cm} (8)

where $\kappa^2$ is the spatial orientation factor of the dipole, $N$ is the refractive index of the medium, $F$ is the fluorescence quantum yield of the donor, and $J$ is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. Therefore,

$$ J = \frac{\sum F(\lambda) e(\lambda) \lambda^4 \Delta \lambda}{\sum F(\lambda) \Delta \lambda} $$  \hspace{1cm} (9)

where $F(\lambda)$ is the fluorescence intensity of the donor and $E(\lambda)$ is the molar absorptivity of the acceptor when the wavelength is $\lambda$. The fluorescence emission spectrum of BSA and the UV-vis spectrum of the PRX complex are shown in Fig. 5, which reveals that they have some overlap [27, 28].

![Fluorescence spectra for BSA and UV absorbance spectra for PRX](image)

**Fig. 5** Fluorescence spectra for BSA and UV absorbance spectra for PRX; [BSA] = [PRX] = 1.00×10^{-5} mol·L^{-1}, $T$=302 K.

The value of $J$ is 7.27×10^{-16}, the orientation factor, $\kappa^2$, taken as 2/3 and the refractive index, $n$, was taken as 1.36. The quantum yield, $\Phi_{\text{Trp}}$, was determined in the study as 0.15 [29]. With use of the values of $J$, $\kappa^2$, $n$ and $\Phi_{\text{Trp}}$, $R_0$ value was calculated as 1.54 nm for the PRX complex. The $E$ was 0.29 for the complex. The actual distance $r$, between the binding site of complexes binding in BSA molecule was 1.79 nm. The average distance of less than 7 nm between a donor and acceptor indicated that the energy transfer from BSA to complexes occurred with high probability [30].
3.6. Conformation investigation

3.6.1 Synchronous fluorescence spectroscopy

Synchronous fluorescence is an effective and simple means to measure fluorescence quenching and the possible shift of the maximum emission wavelength $\lambda_{\text{max}}$. It’s relative to the alteration of the polarity around the chromophore microenvironment [31]. The conformational changes of BSA were evaluated by the measurement of synchronous fluorescence intensity of amino acid residues before and after the addition of PRX. The value of difference between excitation and emission wavelengths, represented by $\Delta\lambda$, is an important operating parameter. In this work, the synchronous fluorescence characteristics of BSA were studied at different scanning intervals ($\Delta\lambda$). As $\Delta\lambda$ is 15 nm, synchronous fluorescence indicates the characteristics of tyrosine residues; whereas, when $\Delta\lambda$ is 60 nm, it provides characteristic information on the tryptophan residues [32].

It is obvious from Fig. 6 that the shift of the $\lambda_{\text{max}}$ from 305 to 300.5 nm when $\Delta\lambda$=15 nm and the $\Delta\lambda$ from 342 to 345 nm when $\Delta\lambda$=60 nm, which is consistent with the fact that the conformation of BSA was changed. It is also indicated that the polarity around the tyrosine residues was decreased and the hydrophobicity was increased, but the polarity around the tryptophan residues was increased and the hydrophobicity was increased [33].

![Fig. 6 The synchronous fluorescence spectroscopy of BSA](image)

(a): $\Delta\lambda = 15$ nm, (b): $\Delta\lambda = 60$ nm; $c_{\text{BSA}} = 1.0 \times 10^{-5}$ mol·L$^{-1}$; $T = 302$ K; $c_{\text{PRX}}$ (a–j): 0, 3.32, 6.64, 9.96, 13.28, 16.60, 19.92, 23.24, 26.56, 29.88 (×10$^{-6}$ mol·L$^{-1}$).

3.6.2 CD spectra

Circular dichroism (CD) has been increasingly recognised as a valuable technique for examining the proteins structure in solution [34]. Further evidence for conformational changes in BSA was obtained by circular dichroism (CD) spectroscopic studies in the absence and presence of
PRX as shown in Fig. 7. PRX is a molecule which does not yield signals in CD spectra at the probe conditions. However, it tends to show a significant CD signal upon formation of the aggregate in the presence of the protein (Fig. 7 indicating an asymmetrical perturbation in the fluorophore) [35].

The information has been revealed that the CD spectra of BSA exhibited two negative bands at 208 and 222 nm, which is characteristic of the α-helix of proteins [36]. The α-helix of BSA in the absence and presence of PRX were calculated from Eq. (10) and Eq. (11) [37].

\[
MRE = \frac{CD(\text{mdeg})}{C_p n l \times 10}
\]  

\[\alpha\text{-Helix(\%)} = \frac{-MRE_{208} - 4000}{33000 - 4000} \times 100\] (11)

**Table 4** The comparison of the α-helix of BSA in the absence and presence of PRX.

<table>
<thead>
<tr>
<th>System</th>
<th>α-Helix content</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA(a)</td>
<td>56.8%</td>
</tr>
<tr>
<td>BSA+PRX(b)</td>
<td>54.9%</td>
</tr>
<tr>
<td>BSA+PRX(c)</td>
<td>52.7%</td>
</tr>
<tr>
<td>BSA+PRX(d)</td>
<td>49.5%</td>
</tr>
<tr>
<td>BSA+PRX(e)</td>
<td>48.4%</td>
</tr>
</tbody>
</table>

Fig. 7 CD spectra of BSA without and with PRX, \(c_{PRX}\) (a-e): 0, 13.28, 26.56, 39.84, 53.12 (×10\(^6\) mol·L\(^{-1}\)).

Fig. 7 showed the CD spectra of BSA in the absence and presence of PRX, and we can figure out that the α-helix of BSA decreased with increasing PRX dose from Table 4. The percentage of protein α-helix structure decreased indicated that PRX bound with the amino acid residue of the main polypeptide chain of BSA and destroyed their hydrogen bonding networks, which indicated
that PRX has changed the secondary structure of BSA [38].

4. Conclusions

In the paper, the binding properties of prenoxine to BSA were characterized by measuring the fluorescence spectra, UV-vis spectra and circular dichroism spectra. According to the fluorescence quenching calculation, the fluorescence quenching mechanism of BSA initiated by PRX we obtained is a static quenching. Hydrophobic interaction force plays a major role in stabilizing the complex. Otherwise, according to the Förster’s non-radioactive energy transfer theory, the binding distance \( r \) is 1.79 nm (far less than 7 nm), which showed that the energy transfer from BSA to PRX with high possibility. The measured synchronous fluorescence spectroscopy indicates that the conformation of BSA was changed in the presence of PRX. Further evidence of conformational changes of BSA upon addition of PRX was provided by CD technique, which implied that the \( \alpha \)-helix of BSA decreased with increasing PRX dose.

Acknowledgments

This work was supported by Scientific Research Fund of Hunan Provincial Education Department (12K101), Hunan Provincial Natural Science Foundation of China (14JJ7049 and 11JJ2007), National Natural Science Foundation of China (21172066), The Opening Project of State Key Laboratory of Physical Chemistry of Solid Surfaces (Xiamen University, No. 201309), Aid Program for Science and Technology Innovative Research Team in Higher Educational Institutions of Hunan Province.

References


The interaction between prenoxine sodium (PRX) and bovine serum albumin (BSA) was studied by fluorescence, circular dichroism (CD) and UV-vis spectroscopy. The quenching mechanism, binding constants, and binding distance were determined. Conformation change of BSA was also observed.
Highlights

► We explored the interaction between BSA and PRX by spectroscopic methods.

► The mainly binding forces is hydrophobic interactions.

► The fluorescence quenching mechanism is static quenching.

► The binding constants and binding sites were calculated.

► The conformation of BSA was changed due to the impact of PRX.