

# Spectroscopic Identification of Interactions of Pb<sup>2+</sup> with Bovine Serum Albumin

Yihong Liu · Lijun Zhang · Rutao Liu · Pengjun Zhang

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**Abstract** The effect of Pb<sup>2+</sup> targeted to bovine serum albumin (BSA) in vitro was investigated by fluorescence, synchronous fluorescence, UV absorption and circular dichroism (CD) spectrophotometry. The characteristic fluorescence of BSA was quenched, which indicated that Pb<sup>2+</sup> changed the skeleton of BSA and caused the gradual exposure of aromatic amino acid residues (Trp, Tyr, Phe) in the internal hydrophobic region of BSA. When the concentration of Pb<sup>2+</sup> was higher than  $1 \times 10^{-4}$  mol/L, the BSA was completely denatured. The excess lead ion interacted with the aromatic amino acid residues of BSA exposed to the solution, which decreased the fluorescence of BSA further. According to the experiment results, we found that a lead-BSA complex was formed following static quenching and the binding site was calculated approximately equal to 1. This work reflected the interaction mechanism of BSA and Pb<sup>2+</sup> from the perspective of spectroscopy.

**Keywords** Bovine serum albumin · Pb<sup>2+</sup> · Spectroscopy · Interaction

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Yihong Liu and Lijun Zhang make equal contribution to this research work and share the first author.

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Y. Liu · L. Zhang · R. Liu · P. Zhang  
Shandong Key Laboratory of Water Pollution Control and Resource Reuse, School of Environmental Science and Engineering, Shandong University, China-America CRC for Environment & Health,  
Shandong Province, 27# Shanda South Road,  
Jinan 250100, People's Republic of China

R. Liu (✉)  
School of Environmental Science and Engineering,  
Shandong University,  
Jinan 250100, People's Republic of China  
e-mail: rutaoliu@sdu.edu.cn

## Introduction

Proteins are important functional biological macromolecules. The main reason of many diseases (such as cataracts, mad cow disease and diabetes) is protein denaturation [1, 2]. Recent research shows that small molecular contaminants in the environment could induce protein denaturation [3–5], so it is of practical significance to investigate the toxic interaction mechanism between environmental contaminants and proteins.

Many heavy metals are essential trace elements, but they are also widely available as small molecular contaminants in the environment. Their presence in the body above a certain concentration could result in heavy metal poisoning [6–8]. Lead is a common heavy metal contaminant in the environment, arising from mining smelting processes, the use of lead products, automobile exhaust emissions and so on. Lead could infiltrate into organisms through respiratory tract, digestive tract and skin, and then interact with biological macromolecules (proteins, DNA and so on) by multiple forces, including hydrogen bonding, hydrophobic interactions and electrostatic forces [9, 10]. The interaction may induce denaturation, and even induce cells to become cancerous [11, 12]. Currently, some research on the interaction between lead and functional proteins in vitro has been reported [13, 14], but little work has focused on the interaction mechanisms between lead and protein at the molecular level, especially their interaction modes and binding sites.

Because of its similarity to the conformation of human serum albumin (HSA), bovine serum albumin (BSA) was selected as our model protein for experiments under simulative physiological conditions. We investigated the effect of Pb<sup>2+</sup> targeted to bovine serum albumin (BSA) by fluorescence, synchronous fluorescence, UV absorption and circular dichroism (CD) spectrophotometry. This research provides a new approach for the evaluation of lead's effects on functional protein molecules at the molecular level.

## Experimental

### Apparatus

The fluorescence spectra and synchronous fluorescence spectra were made with a HITACHI-850 fluorescence spectrometer (Hitachi, Japan). The fluorescence life time was determined on a FLS920 fluorescence spectrometer (Edinburgh Instruments Ltd., Britain). The UV absorption spectra were measured on a SHIMADZU UV-vis-2450 spectrophotometer (SHIMADZU, Kyoto, Japan). The CD spectra were performed on a JASCO-810 Circular Dichroism Spectrometer. All the pH measurements were made with a pHs-3C acidity meter (Pengshun, Shanghai, China).

### Reagents

A stock solution of BSA ( $1 \times 10^{-5}$  mol/L) was prepared by dissolving 0.0733 g of BSA in 100 ml of water. Denatured BSA was prepared by heating solution in water bath. Tryptophan (Trp) (China National Pharmaceutical Group Corp, CP) was prepared as a  $1 \times 10^{-2}$  mol/L stock solution.

Lead (II) acetate was made up as a stock solution of concentration  $1 \times 10^{-2}$  mol/L. HAC-NaAc buffer solutions were prepared from 0.2 mol/L HAC (Tianjin Standard Technology Co., Ltd., AP) and 0.2 mol/L NaAc (Shandong Laiyang, Jingxi Chemical Plant, AP).

The solutions was preserved at 0–4 °C and shaken gently as needed to redissolve the contents.

All the chemicals used were analytical-reagent grade and ultrapure water was used throughout.

### Experimental Procedure

#### Fluorescence and Synchronous Fluorescence Spectra

In order to ensure proper experimental conditions, a 3-D scanning experiment was applied. The results show that the optimal excitation wavelength ( $\lambda_{ex}$ ) is 275 nm. The excitation and emission slit widths were set at 5.0 nm. The voltage was 600 V.

To prepare the test solutions, one milliliter of BSA solution and 1.0 ml of HAC-NaAc buffer solution (pH 5.5) were placed in a 10 ml standard volumetric flask. Different volume of  $Pb^{2+}$  solution was added and the mixture was diluted to the volume with water and mixed. After 10 min stabilization, the fluorescence emission spectra were measured on the HITACHI-850 fluorescence spectrometer from 284 nm to 420 nm at scanning speed of 300 nm/min ( $\lambda_{ex}=275$  nm, excitation/emission slit width=5 nm).

The synchronous fluorescence spectra of BSA in the presence of  $Pb^{2+}$  were measured with fixed  $\Delta\lambda$  of 20 nm and 70 nm.

#### Fluorescence Lifetime Measurement

Time-resolved fluorescence measurements were carried out on a FLS920 Combined Fluorescence Lifetime and Steady State Spectrometer (Edinburgh, U.K.). BSA was excited at 275 nm.

The samples were prepared as in section [Fluorescence and synchronous fluorescence spectra](#).

#### Absorption Spectrum

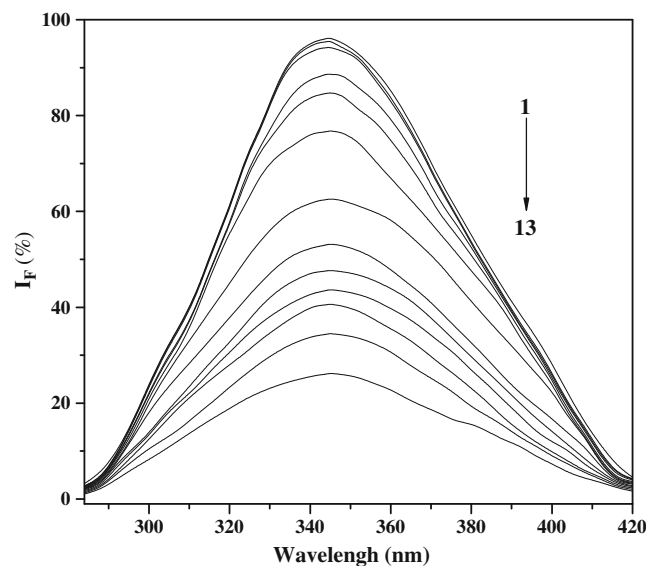
Absorption spectra were measured on SHIMADZU UV-vis-2450 spectrophotometer. Because the mixed solutions of  $Pb^{2+}$  and buffer solution have higher absorption intensity in the ultraviolet region (especially from 200 nm to 220 nm), all absorption spectra used mixed solutions of  $Pb^{2+}$  (same concentration with corresponding sample) and buffer solution as reference solutions.

The samples were prepared as in section [Fluorescence and synchronous fluorescence spectra](#).

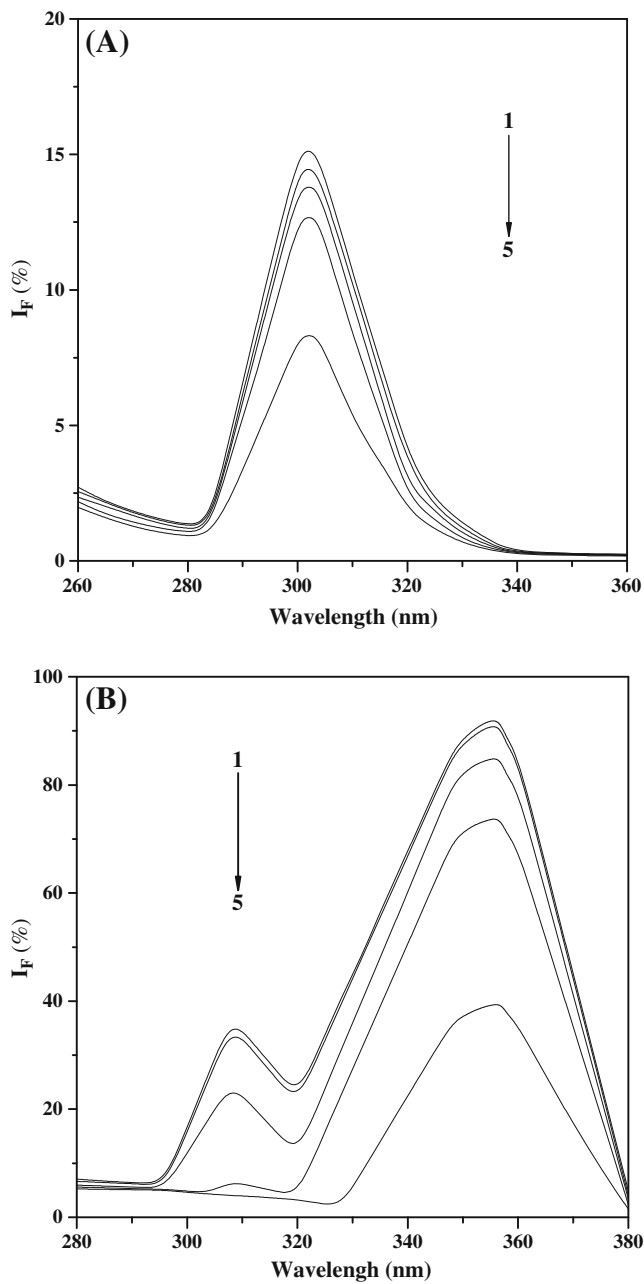
#### CD Spectrum

The CD spectrum was carried out on JASCO-810 Circular Dichroism Spectrometer from 190 nm to 250 nm. The HAC-NaAc buffer solution was chosen as blank solution.

The samples were prepared as in section [Fluorescence and synchronous fluorescence spectra](#).



**Fig. 1** Effect of  $Pb^{2+}$  on the fluorescence spectrum of BSA. **Conditions:** ( $\lambda_{ex}=275$  nm) BSA:  $1.0 \times 10^{-6}$  mol/L Buffer: HAC-NaAc, pH 5.5, CPb<sup>2+</sup>(1–13): 0,  $1.0 \times 10^{-5}$ ,  $5.0 \times 10^{-5}$ ,  $1.0 \times 10^{-4}$ ,  $5.0 \times 10^{-4}$ ,  $1.0 \times 10^{-3}$ ,  $3.0 \times 10^{-3}$ ,  $5.0 \times 10^{-3}$ ,  $7.0 \times 10^{-3}$ ,  $9.0 \times 10^{-3}$ ,  $1.0 \times 10^{-2}$ ,  $1.5 \times 10^{-2}$ ,  $2.5 \times 10^{-2}$  mol/L

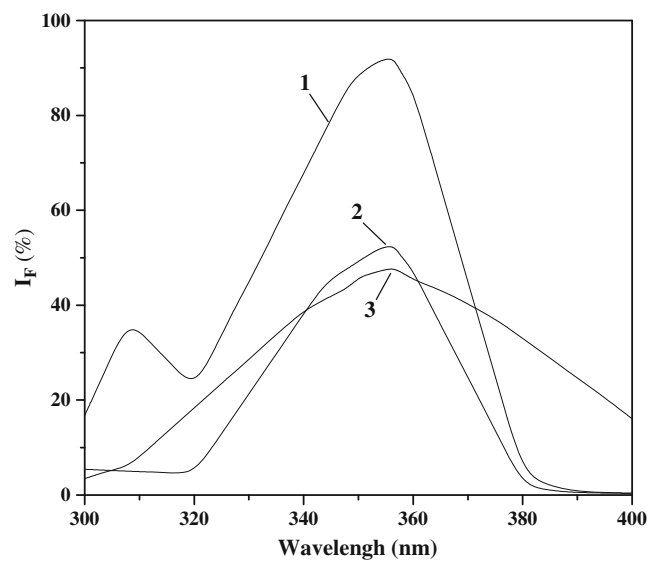


**Fig. 2** Effect of  $Pb^{2+}$  on the synchronous fluorescence spectra of BSA. **a**  $\Delta\lambda=20$  nm, **b**  $\Delta\lambda=70$  nm. **Conditions:** BSA:  $1.0 \times 10^{-6}$  mol/L Buffer: HAc-NaAc, pH=5.5.  $CPb^{2+}(1-5)$ : 0,  $1.0 \times 10^{-5}$ ,  $1.0 \times 10^{-4}$ ,  $1.0 \times 10^{-3}$ ,  $1.0 \times 10^{-2}$  mol/L

### Results and Discussion

#### The Influence of Lead Concentration on the Fluorescence Intensity of BSA

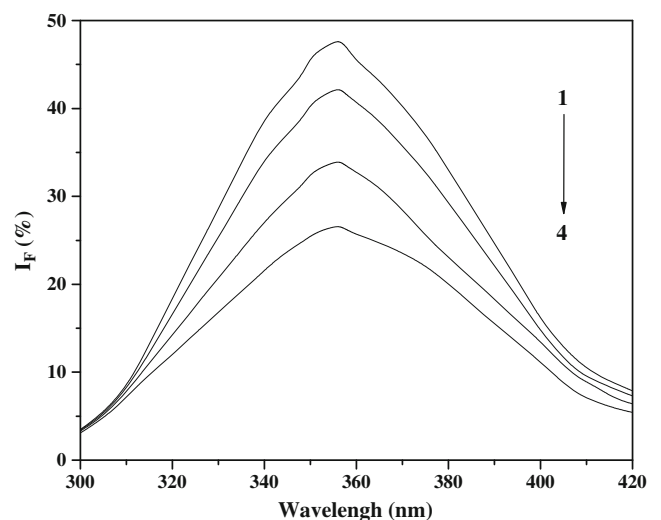
The intrinsic fluorescence of BSA is contributed by its Trp (tryptophan), Tyr (tyrosine) and Phe (phenylalanine) residues. Among the three fluorescent groups, the Trp residues play the master role, the Tyr residues are the next, while the



**Fig. 3** Comparison of the synchronous fluorescence spectra of native BSA ( $\lambda_{ex}=275$  nm) (1) with denatured BSA ( $\lambda_{ex}=275$  nm) (2) and the fluorescence spectrum of free Trp ( $\Delta\lambda=70$  nm) (3). **Conditions:** BSA:  $1.0 \times 10^{-6}$  mol/L Trp:  $2.0 \times 10^{-6}$  mol/L Buffer: HAc-NaAc, pH=5.5

fluorescence of Phe residues is very weak and can be neglected [15, 16]. One BSA molecule has two Trp residues, which are located at positions 134 and 212. Trp212 is located in the internal hydrophobic region and Trp134 is located near the surface.

From Fig. 1, it can be seen that the fluorescence peak of BSA is around 345 nm, and the intrinsic fluorescence of BSA decreased regularly as the  $Pb^{2+}$  concentration increased. These results indicated that there were interactions between  $Pb^{2+}$  and BSA. Lead could increase the exposure of Trp-212 to the medium and therefore quench the fluorescence of BSA.



**Fig. 4** Effect of  $Pb^{2+}$  on the fluorescence Spectra of Trp. **Conditions:** ( $\lambda_{ex}=275$  nm) Trp:  $2.0 \times 10^{-6}$  mol/L Buffer: HAc-NaAc, pH 5.5,  $CPb^{2+}(1-4)$ : 0,  $1.0 \times 10^{-3}$ ,  $5.0 \times 10^{-3}$ ,  $1.0 \times 10^{-2}$  mol/L

## Synchronous Fluorescence Spectroscopy

To further demonstrate the interaction between BSA and lead, we used synchronous fluorescence spectroscopy which can separate the different fluorescent groups of BSA based on different D-values between the excitation and emission wavelengths ( $\Delta\lambda$ ). When the D-value ( $\Delta\lambda$ ) is set at 20 or 70 nm, synchronous fluorescence gives the characteristic information of tyrosine or tryptophan residues, respectively [17].

Figure 2a is the spectrum of the Tyr residues ( $\Delta\lambda=20$  nm) and Fig. 2b shows the Trp residues fluorescence ( $\Delta\lambda=70$  nm). The fluorescence of the Trp residues in BSA is much stronger than that of the Tyr residues, and the  $\text{Pb}^{2+}$ -induced fluorescence quenching of the Trp residues is stronger than that of the Tyr residues.

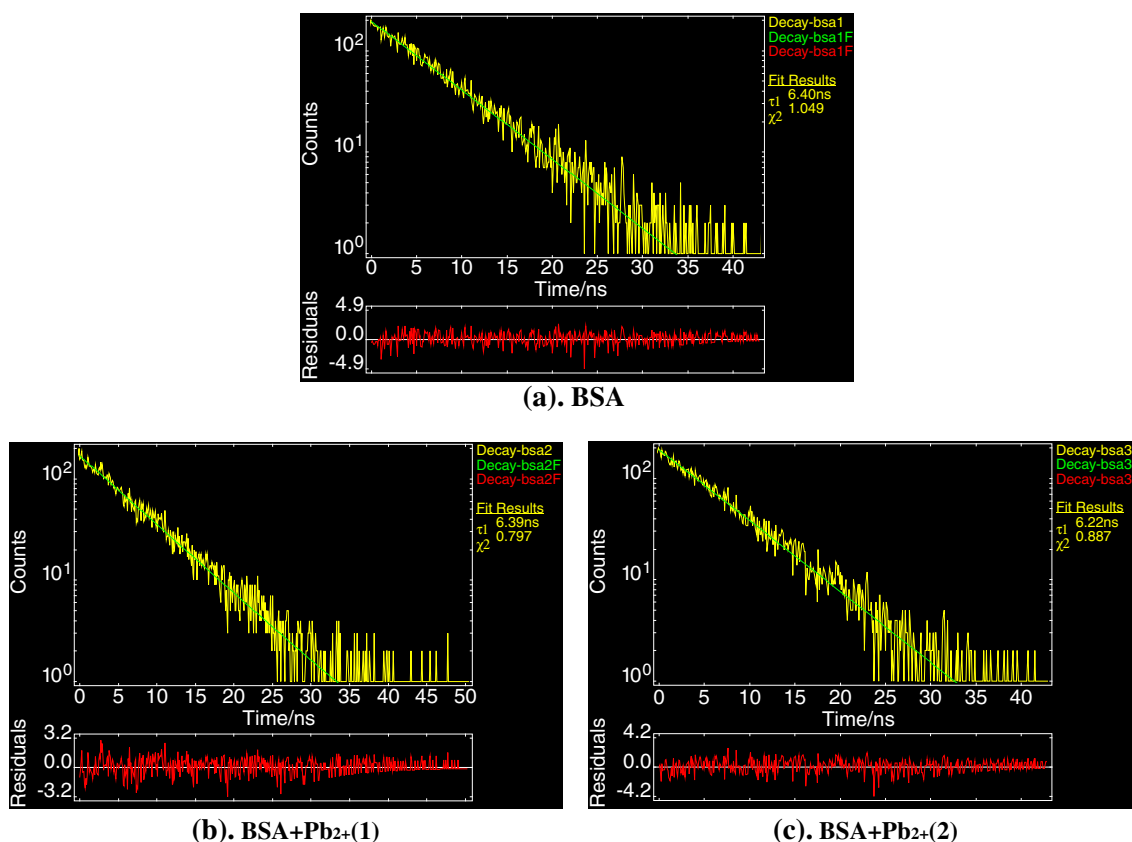
## Interaction Between Trp and $\text{Pb}^{2+}$

The intrinsic fluorescence of BSA is almost all contributed by the tryptophan residue. In order to analyze the effect of  $\text{Pb}^{2+}$  on BSA, we compared the fluorescence spectra of Trp in solution with that of Trp residues in BSA. Because a BSA molecule has two Trp residues, the molar concen-

trations of Trp samples were twice as high as BSA. The synchronous fluorescence of BSA ( $\Delta\lambda=70$  nm) was used to compare with the fluorescence of Trp in solution.

As Fig. 3 shows, the  $I_{F_{\max}}$  of Trp solution is 52% of native BSA but is not far off the thermally denatured BSA. Analogous to the mechanism of micelle-sensitized fluorimetry [18], the fluorescence intensity of Trp residues in internal hydrophobic region of BSA is expected to be higher than that of free Trp in water. When BSA was denatured, the Trp residues were exposed to water and their fluorescence intensity decreased. These results indicate that lead disassembled the skeleton structure of BSA, and therefore increased the exposure of Trp residues in the internal hydrophobic region to the medium, leading to fluorescence quenching (Fig. 1). The conclusion is consistent with some related research [13].

Moreover,  $\text{Pb}^{2+}$  can cause fluorescence quenching of Trp itself, as shown in Fig. 4. The quenching may be caused by the combination of  $\text{Pb}^{2+}$  to the N in the indole ring of Trp. We infer that there were two steps in the reaction. First,  $\text{Pb}^{2+}$  disassembled the skeleton structure of BSA, and increased the exposure of Trp-212 to the medium, which partially decreased the fluorescence intensity of BSA. Through this process, BSA was denatured. Second,  $\text{Pb}^{2+}$  interacts with the exposed Trp residues, leading to the further fluorescence quenching.



**Fig. 5** Fluorescence lifetimes of BSA and BSA- $\text{Pb}^{2+}$ . **Conditions:** ( $\lambda_{\text{ex}}=275$  nm,  $\lambda_{\text{em}}=345$  nm) BSA:  $1.0 \times 10^{-6}$  mol/L Buffer: HAc-NaAc, pH 5.5,  $\text{CPb}^{2+}$ : (1):  $1.0 \times 10^{-4}$  mol/L, (2):  $1.0 \times 10^{-3}$  mol/L

## The Fluorescence Quenching Mechanism

### Mode of Fluorescence Quenching

The different modes of fluorescence quenching are normally divided into dynamic quenching and static quenching. Molecular diffusion or collision between quenchers and fluorescent substances lead to dynamic quenching. Static quenching occurs when quenchers and fluorescent substances form non-fluorescent substances. Usually dynamic quenching greatly reduces the fluorescence life time, but static quenching does not.

Fluorescence lifetime is an effective element to determine which mode the system is quenched by [19]. As shown in Fig. 5, The change of BSA fluorescence lifetime in the presence of Pb<sup>2+</sup> was determined on a FLS920 fluorescence spectrometer (λ<sub>ex</sub>=275 nm, λ<sub>em</sub>=345 nm).

The fluorescence lifetime of native BSA is 6.40 ns. It changes to 6.39 ns (Fig. 5b) and 6.22 ns (Fig. 5c) when Pb<sup>2+</sup> is added to the BSA. The fluorescence lifetime is changed indistinctly, therefore the fluorescence quenching of BSA by Pb<sup>2+</sup> can be attributed to static quenching.

### Numbers of Binding Sites

For a static quenching process, the following Eq. 1 can be employed to calculate the binding constant and binding sites [16, 20].

$$\lg \frac{F_0 - F}{F} = \lg K_A + n \lg Q \tag{1}$$

F<sub>0</sub> and F are the fluorescence intensities of BSA in the absence and presence of lead. Q is the concentration of quencher (Pb<sup>2+</sup>), K<sub>A</sub> is the binding constant, and n is the number of binding sites. As Fig. 6 shows, a straight line can be plotted for lg[(F<sub>0</sub>-F)/F] vs. lgQ. From the slope and the intercept, we could obtain that the binding constant is 73.63 and the number of binding sites is 0.8039. The results indicate that the binding ratio between BSA and Pb<sup>2+</sup> is approximately equal to 1:1.

### Conformational Investigation

To further investigate the conformational changes of the BSA, UV-vis absorption and CD were used in this work.

### Absorption Spectroscopy of BSA in the Presence of Pb<sup>2+</sup>

The effect of Pb<sup>2+</sup> on BSA structure was evaluated by UV absorption spectra (Fig. 7).

BSA has two main absorption bands in the ultraviolet region, which represents the skeleton of BSA from 200 nm to 230 nm and the aromatic amino acid residues (Trp, Tyr, Phe) 260 nm to 300 nm, respectively [21]. It can be seen in Fig. 7 (A) that the skeleton absorption peak intensity of BSA at 220 nm continuously decreases and red shifts as the Pb<sup>2+</sup> concentration increases. At the same time, in Fig. 7 (B) absorption peak intensity of BSA at 280 nm increases slightly. The result further indicates that Pb<sup>2+</sup> interacted with the groups which maintain the skeleton structure of BSA (such as hydroxy, amino, sulfhydryl). Pb<sup>2+</sup> loosens the protein skeleton and promotes the unfolding process, exposing Trp and other aromatic amino acid residues in the internal hydrophobic region to the medium.

### Circular Dichroism Spectra of BSA in the Presence of Pb<sup>2+</sup>

CD spectroscopy is an effective method to investigate the conformational changes of proteins [22]. In order to analyze the toxicity effects of Pb<sup>2+</sup> on BSA, CD spectra of BSA were carried out from 190 nm to 250 nm (Fig. 8). The negative peaks at 208 and 219 nm represent the α-helix structure of BSA [23]. The intensity of the two negative peaks decreased with the increasing concentration of Pb<sup>2+</sup>. When the concentration of Pb<sup>2+</sup> increased to 1.5 × 10<sup>-4</sup> mol/L, the negative peaks disappeared and the shape of the curve completely changed.

The proportion of α-helix in the BSA secondary structure can be calculated by Eqs. 2 and 3 [17, 24, 25]:

$$MRE = \frac{\text{ObservedCD}(m \text{ deg})}{C_{pnl} \times 10} \tag{2}$$

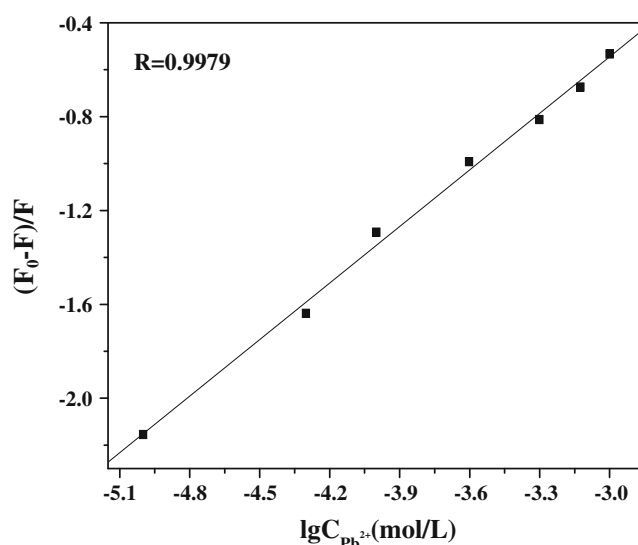
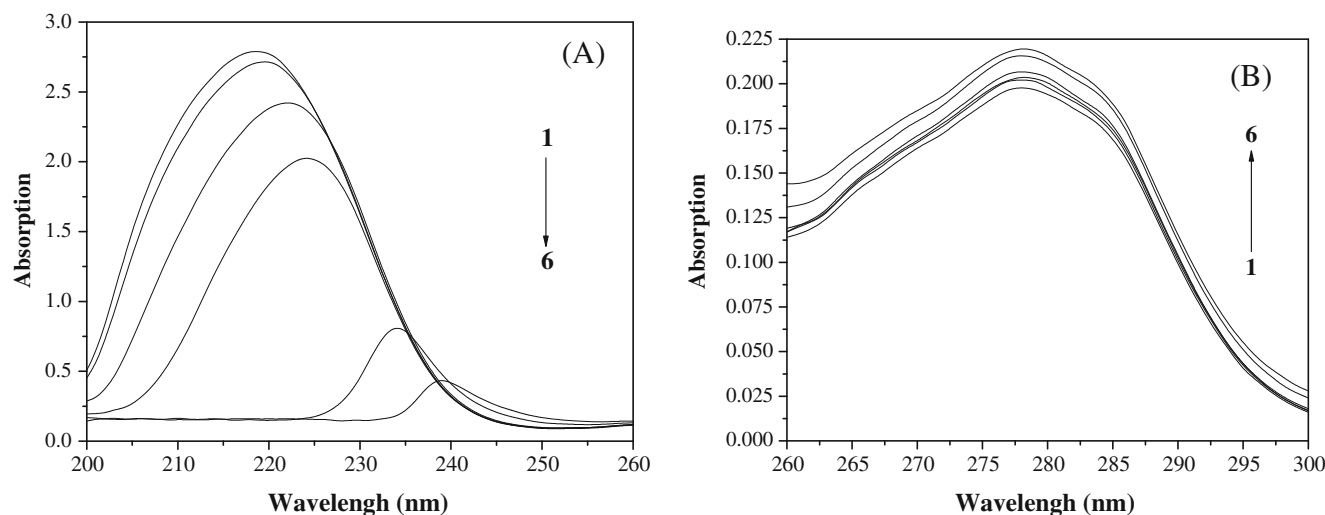


Fig. 6 Double-reciprocal curve of the fluorescence intensity. Conditions: BSA: 1.0 × 10<sup>-6</sup> mol/L Buffer: HAc-NaAc, pH 5.5

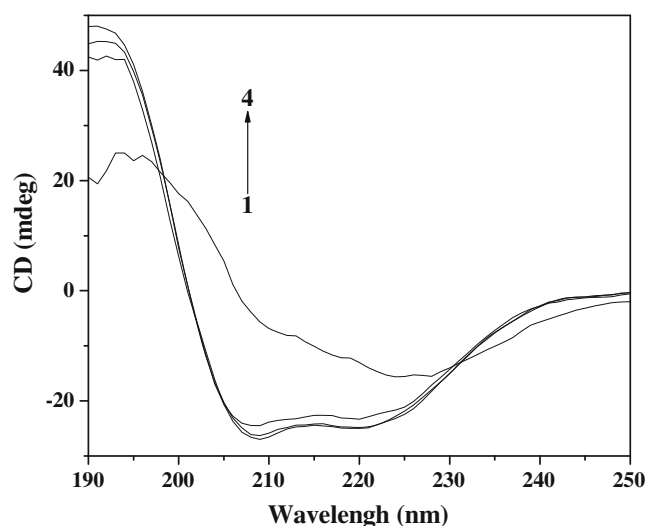


**Fig. 7** Effect of  $\text{Pb}^{2+}$  on the absorption spectra of BSA. **Conditions:** BSA:  $5.0 \times 10^{-6}$  mol/L Buffer: HAc-NaAc, pH 5.5,  $\text{CPb}^{2+}$  (1–6): 0,  $1.0 \times 10^{-5}$ ,  $5.0 \times 10^{-5}$ ,  $1.0 \times 10^{-4}$ ,  $5.0 \times 10^{-4}$ ,  $1.0 \times 10^{-3}$  mol/L

where  $C_p$  is the molar concentration of the protein,  $n$  is the number of amino acid residues and  $l$  is the path length. The  $\alpha$ -helix contents of BSA were calculated from the MRE value at 208 nm, using Eq. 3.

$$\alpha - \text{Helix}(\%) = \frac{-\text{MRE}_{208} - 4000}{33000 - 4000} \times 100 \quad (3)$$

where  $\text{MRE}_{208}$  is the observed MRE value at 208 nm, 4000 is the MRE of the  $\beta$ -form and random coil conformation cross at 208 nm, and 33000 is the MRE value of a pure  $\alpha$ -helix at 208 nm. From the above equations, the  $\alpha$ -helix content of BSA was calculated quantitatively, as shown in Table 1.



**Fig. 8** Effect of  $\text{Pb}^{2+}$  on the CD spectrum of BSA. **Conditions:** BSA:  $2.0 \times 10^{-7}$  mol/L Buffer: HAc-NaAc, pH 5.5,  $\text{CPb}^{2+}$  (1–4): 0,  $1.0 \times 10^{-6}$ ,  $5.0 \times 10^{-5}$ ,  $1.5 \times 10^{-4}$  mol/L

The proportion of  $\alpha$ -helix declined from 61.7% to 55.5%, which indicates that the skeleton structure was loosened in the presence of  $\text{Pb}^{2+}$  and the Trp residues of BSA in the internal hydrophobic region was exposed to medium. The data also shows that lead has an obvious deleterious effect on BSA (consistent with the results of fluorescence spectroscopy and UV absorption spectroscopy).

## Conclusions

The biotoxicity mechanism of  $\text{Pb}^{2+}$  is investigated by the effects of  $\text{Pb}^{2+}$  on the bovine serum albumin (BSA). The characteristic fluorescence of BSA decreases regularly as the concentration of  $\text{Pb}^{2+}$  increases. In addition, through the comparison of the fluorescence spectra of BSA and free Trp, we found that the interaction between BSA and  $\text{Pb}^{2+}$  includes two processes. When the concentration is low,  $\text{Pb}^{2+}$  reacts with the outer groups of BSA (such as hydroxy, amino, sulfhydryl) and alters the skeleton structure. Hence the protein begins to unfold and Trp212 is exposed, which lead to a decrease of the fluorescence intensity of BSA. At higher concentrations,  $\text{Pb}^{2+}$  continues to interact with the exposed Trp residues, leading to a further quenching of the fluorescence intensity. Employing Eq. 1, the binding ratio between BSA and  $\text{Pb}^{2+}$  was calculated to be nearly 1:1. The

**Table 1** The influence of  $\text{Pb}^{2+}$  on the  $\alpha$ -helix content of BSA in Fig. 8

	Curve 1	Curve 2	Curve 3
$\alpha$ -Helix	61.7%	60.2%	55.5%



results of UV absorption spectra and CD spectra are consistent with these results.

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