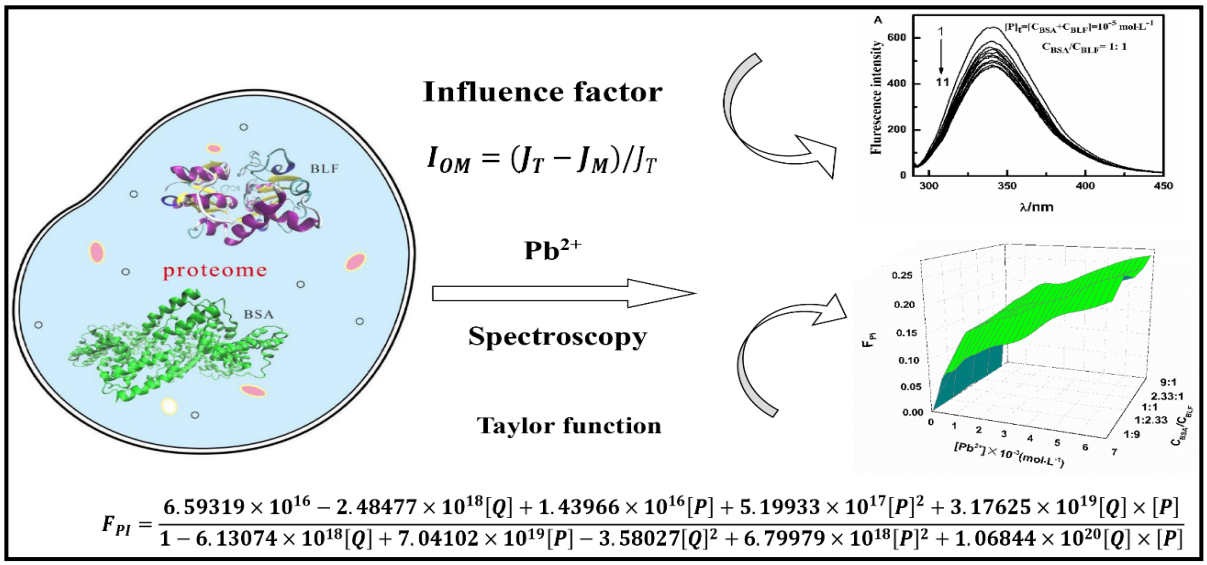
Imaging Serum Proteome Behavior in Process of Lead Transportation *in Vivo*: A Fluorescence Spectroscopic Analysis Insight

Luwei Tian, Ming Guo*, Xingtao Xu and Linfang Shi**
College of Chemistry and Materials Engineering, Zhejiang Agriculture & Forestry University, Hangzhou, Zhejiang 311300, China.
guoming@zafu.edu.cn*; lfshi2003@163.com**

(Received on 22nd November 2021, accepted in revised form 14th February 2022)

Summary: As a common heavy metal, Pb (II) can react with biological macromolecules in the human body and have an impact on human health, but there are few studies on its synergistic interaction with a variety of proteins of different abundance. Pb (II) binding with a synthetic model protein system (bovine serum albumin (BSA) and bovine lactoferrin (BLF)) was characterized using fluorescence spectroscopy and was described using a quantitative model. Pb (II) quenched the fluorescence of BSA-BLF, indicating that Pb (II) interacted with the BSA-BLF protein system, and was affected by single protein, mixed proteins and the solution microenvironment. A model was constructed and an indicator of the interaction (F_{PI}) was derived to quantify the interactions. There was a high correlation (R^2=0.9182, p<0.001) between the F_{PI} and the Pb (II) concentration when the interaction models were analyzed with a Taylor function. The effects of the solvent microenvironment on BSA, BLF and BSA-BLF were evaluated using the I_{OM} (overall microenvironmental influence factor). BLF was more easily affected by the solution microenvironment with increasing concentrations than BSA, while BSA-BLF was less affected.

Keywords: Pb (II), Mixed protein system, Molecular interaction, Fluorescence, Transport mechanism.



Introduction

Metal ions are essential to mammalian life in trace elements, as they play a key role in various biological processes [1]. However, heavy metals have become an increasing environmental pollutant due to industrial development. Therefore, there is an increasing interest in the interactions between heavy metal ions and proteins, especially with the correlation of some metal ions and degenerative cognitive diseases such as Alzheimer's and Parkinson's [2]. Exposure to Pb (II) poses potential risks to human health and ecosystem [3,4]. Pb (II) is stable in water and can accumulate, creating a hazardous environment. The influence of Pb (II) in water is a cumulative process of "dose-effect" on the human body [5]. Blood lead content increases with extended exposure, where it migrates within the body and gradually damages the brain, kidney and red blood cells. Chronic poisoning may result in mental impairment and anemia when the concentration of lead in blood exceeds 100 $lg{\cdot}L^{-1}$ [6,7]. Lead residues are a wide-scale problem, and have been detected in human

*To whom all correspondence should be addressed.

sera and blood globally [8,9].

Proteins are involved in various metabolic and regulatory pathways that are vital for the survival of biological cells, and these functional proteins are highly susceptible to contaminants. In recent years, the interactions between biomacromolecules and toxic pollutants have become a topic of great interest [10]. The biological transport of heavy metal ions in body fluid can involve an array of proteins [11]. Proteomics can be used to study the effects of proteins in different environments. However, an accurate and rapid risk assessment of hazardous pollutants is urgently needed. To date, most research on the interactions between protein and heavy metal ions has focused on high-abundance serum albumin in blood, rather than low-abundance proteins [12]. Much research has focused on single protein interactions with Pb (II) [13,14], and no research on the interactions between Pb (II) with two or more proteins [15].

Of the blood proteins, serum albumins are the most abundant. Serum albumins are the main soluble protein constituents of the circulatory system and can transport various compounds. Albumin is a vehicle for important biological ligands and a regulator of metabolic processes and has an affinity for a wide variety of substances [16, 17]. Lactoferrin is relatively low-abundance blood protein [13], but also has critical physiological functions. Lactoferrin transfers iron into the cells and controls the level of free iron in the blood and external secretions. It is a key component of the immune system and participates in host defense, including immune response, cell transcriptional activation and antibacterial activity [18, 19]. BSA is a 66 kDa protein that is organized into three homologous domains that are divided into nine loops. These loops are linked by 17 cysteine disulfide bonds [20, 21] that retain macromolecular rigidity in response to external influences. BLF is a single polypeptide chain comprised of 690 amino acids residues that fold into two globular lobes - each containing one iron-binding site. Bovine serum albumin (BSA) and bovine lactoferrin (BLF) are well characterized and are suitable macromolecules to represent a simple binary protein system for a lead interaction study [22].

Albumins from different mammalian species share many similarities in physicochemical properties [23]. The interaction of lead with these serum proteins could reveal binding and transport mechanisms and further elucidate the toxicology of metal ions.

Chromatographic methods have been widely used to assess interactions between small molecules of metal and proteins [24,25], while spectroscopic methods such as fluorescence and UV spectroscopy have been applied in recent years because they are rapid and relatively easy to use [26,27]. Most previous studies report the interaction of small molecules with a single biological macromolecule [28-30], while significantly fewer articles have been published on the use of spectroscopic methods to study the interaction of metal ions with binary protein systems. To better understand the interaction between heavy metal ions and multiple serum proteins, it is necessary to quantify changes of protein conformation [31]. A simple and effective way to quantitatively describe the interaction between lead ions and biological macromolecules is necessary to establish the binding mechanism between biological macromolecules and heavy metal ions. The investigation of the interaction between a heavy metal and binary protein systems composed of high and low abundance proteins in blood is a prospective topic in the field of small molecule-protein interaction studies. To our knowledge, the binding between Pb (II) and BSA-BLF has not been reported. In this research, bovine serum albumin and bovine lactoferrin were used as representatives of high and low-abundance proteins in blood and the interactions between Pb (II) and BSA-BLF were investigated using fluorescence spectroscopy [32].

Experimental

Reagents and chemicals

Bovine serum albumin and bovine lactoferrin with a purity of 98% were purchased from Sino-American Biotechnology (Shanghai, China). Stock solutions of BSA and BLF (1.0×10^{-5} mol·L^{-1} (M)) and Pb (II) (1.0×10^{-3} M) were prepared in Tris-HCl buffer (0.1 M Tris, 0.1 M NaCl, pH 7.4), and BSA/BLF (from 0.1 to 1.0×10^{-5} M) and Pb (II) (from 0.1 to 1.0×10^{-3} M) were used as controls. Lead nitrate and all other reagents were analytical grade. All aqueous solutions were made using deionized water.

Fluorescence spectroscopy

All fluorescence spectra were measured using a 1 cm quartz cell using a F-7000 spectrofluorometer (Hitachi, Co., Ltd., Tokyo, Japan). The fluorescence emission spectra were recorded from 250 nm to 500 nm with an excitation wavelength of 280 nm at 298 K, and the excitation and emission slits were set at 2.5 nm.

Correction of the internal filtration effect

The fluorescence inner filter effect refers to the fact that the emission and absorption spectra of a fluorescent substance overlap, which leads to reduced intensity of the emission fluorescence [33]. The background fluorescence of the buffer is subtracted, and the inner-filter effect is eliminated according to the following equation:

$$F_e = F_m e^{(A_1 + A_2)/2} \quad (1)$$

where F_e and F_m are the corrected and measured fluorescence, respectively; A_1 and A_2 are the sum of protein and ligand absorption at the excitation and max emission wavelengths, respectively. For Pb (II)-BLF, Pb (II)-BSA, Pb (II)-BSA-BLF fluorescence spectra no absorption was observed at the excitation wavelength of 280 nm. Therefore, inner filter effects are not considered in this study.

Binding parameters analysis

To explore the bond strength of BSA-BLF and Pb (II) complexes, the number of ligands binding with BSA-BLF was determined using Lineweaver-Burk and double logarithmic equations. Changes in protein conformation and the interaction between the protein mixture and Pb (II) were determined by curve fitting analyses using different binding models. The term I_{OM} (overall microenvironmental influence factor) was proposed to depict the extent to which the protein was affected by the microenvironment.

Development of the analytical model

To study the binding mechanism of the BSA-BLF-Pb (II) system, we proposed the parameter F_{PI}. Existing parameters describing the interaction between a protein and proteins have been confined to single protein interactions. We developed a different theoretical-based perspective of the Pb (II) complexation by constructing three-dimensional fluorescence diagrams. The Taylor level real rational function data was obtained from the three-dimensional models.

Results and Discussion

Interactions between Pb (II) and BSA-BLF

In vitro experiments are preferred as they can be easily repeated and are less affected by the environment. Nevertheless, *in vivo* experiments are greatly influenced by the environment and exhibits low repeatability. However, *in vivo* experiments will be further investigated in our follow-up study, as far as the current job being concerned in this paper, it was involved the *in vitro* experiments, and the preliminary results were provided.

It was shown in Fig. 1 that the emission spectra of BSA and BLF have maxima at 343 nm and 333 nm respectively. Titrating Pb (II) into a BSA and BLF solution gradually quenched fluorescence, indicating that Pb (II) interacted with the proteins. Quenching was dependent on the concentration of Pb (II) [34]. There was a small change in the microenvironment – as indicated by a slight red-shift in maxima emission wavelengths. BSA shifted from 343 nm to 345 nm and BLF shifted from 333 nm to 338 nm as shown in Fig. 2. Similarly, concentration-dependent quenching of BSA-BLF fluorescence was observed with higher Pb (II) concentrations. This phenomenon also demonstrated that Pb (II) interacted with BSA-BLF. A small red-shift of the maximum emission wavelength also indicated a minor change in the microenvironment of BSA-BLF.

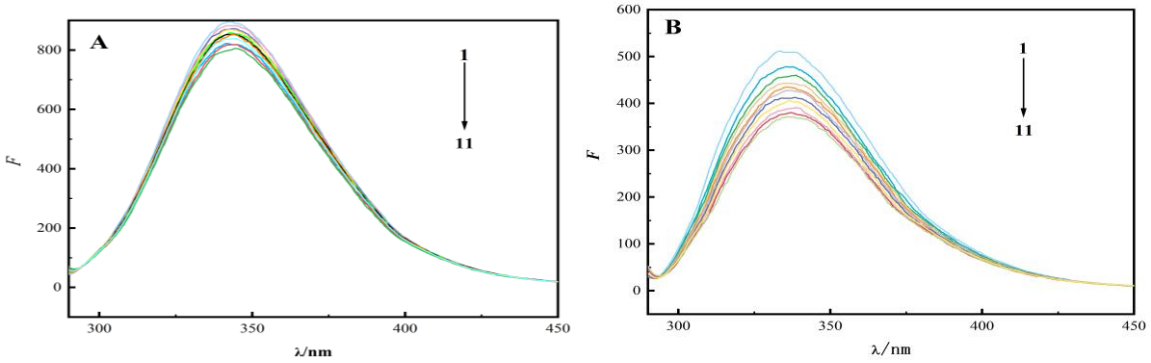


Fig. 1: The fluorescence emission spectra of BSA and BLF at different concentrations of Pb (II). A: The fluorescence emission spectra of BSA at different concentrations of Pb (II). B: The fluorescence emission spectra of BLF at different concentrations of Pb (II). (λ_{ex}=282 nm, λ_{em}=250-500 nm. c_{BSA}=c_{BLF}=1.0×10^{-5} M, $c_{Pb\ (II)}$ (×10^{-5} M), 1 to 11: 0, 0.25, 0.50, 1.00, 1.50, 2.00, 3.00, 4.00, 5.00, 6.00, and 7.00).

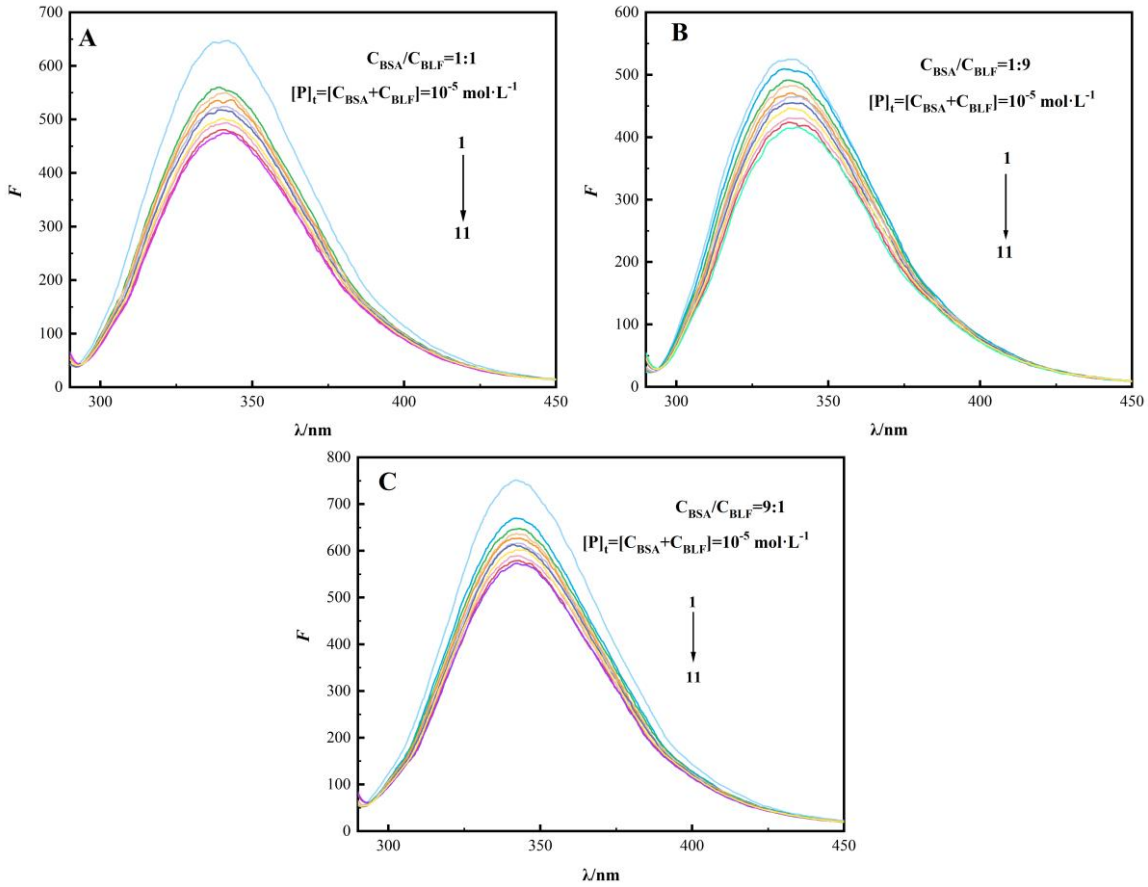


Fig. 2: The fluorescence emission spectra of the BSA-BLF binary protein mixture system at different concentrations of Pb (II). A: Fluorescence emission spectrum of BSA-BLF binary protein mixture system when the concentration of BSA/BLF is 1:1. B: Fluorescence emission spectrum of BSA-BLF binary protein mixture system when the concentration of BSA/BLF is 1:9. C: Fluorescence emission spectrum of BSA-BLF binary protein mixture system when the concentration of BSA/BLF is 9:1. (λ_{ex}=282 nm, $c_{BSA}+c_{BLF}=1.0\times10^{-5}$ M, $c_{Pb(II)}$ ($\times10^{-5}$ M), from 1 to 11: 0, 0.25, 0.50, 1.00, 1.50, 2.00, 3.00, 4.00, 5.00, 6.00, and 7.00).

According to the linear additive principle of fluorescence intensity, the theoretical fluorescence intensity (*F*) of the Pb (II)-BSA-BLF system should equal the sum of the *F* for Pb (II)-BSA and Pb (II)-BLF. When assessed as a single protein or binary protein mixture system (Fig. 1 and Fig. 2), $F_{(Pb\ (II)\text{-}BSA\text{-}BLF)}$ did not equal the sum of $F_{(Pb\ (II)\text{-}BSA)}$ and $F_{(Pb\ (II)\text{-}BLF)}$. This indicated that $F_{(BSA\text{-}BLF)}$ did not follow the linear additive principle. This phenomenon may be attributed to the interaction between Pb (II) and BSA-BLF, and is influenced by the interaction of mixed proteins (BSA-BSA/BLF-BLF) and other proteins. By comparing the theoretical *F* and measured *F* of Pb (II)-BSA-BLF, it can be concluded that BSA-BSA, BLF-BLF, BSA-BLF interactions had a significant influence on Pb (II)-BSA-BLF. As BSA-BLF did not follow the linear additive principle of fluorescence, the interactions required further analyses to quantify and verify the interaction between protein and Pb (II).

Binding parameters of Pb (II) to BSA-BLF

To analyze the interactions of BSA-BSA, BLF-BLF and BSA-BLF, the binding parameters were calculated for Pb (II)-BSA-BLF. Binding of Pb (II) to BSA-BLF was further characterized by the fluorescence spectroscopy. There are few established theoretical equations for the quantifying a binary protein system. We assumed that the BSA-BLF binary protein mixture system bound with an approximate intermolecular force (a number of peptide chains shaped the supramolecular system through reversible binding and entanglement effect). Therefore, results are described using the double-reciprocal plot and double-logarithmic plot,

which are appropriate for single-protein systems [35]. The following formula was used to further describe binding parameters:

$$(F_0 - F)^{-1} = F_0^{-1} + K^{-1}F_0^{-1}[Q]^{-1} \qquad (2)$$

where F_0 and F are the fluorescence intensities before and after the addition of Pb (II), respectively, $[Q]$ is the concentration of Pb (II) and K is the binding constant. It was shown in Fig. 3 that formula was used to determine K by linear regression of a plot of $(F_0\text{-}F)^{-1}$ against $[Q]^{-1}$. The values for the binding parameters of BSA-Pb (II), BLF-Pb (II) and BSA-BLF-Pb (II) are presented in Table-1.

.

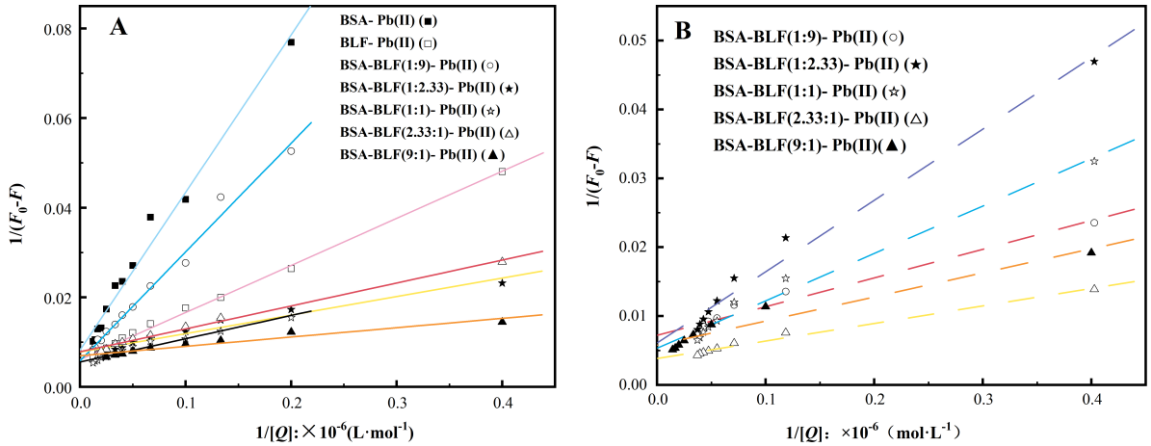


Fig. 3: Lineweaver-Burk plot for fluorescence quenching of BSA/BLF/BSA-BLF by Pb (II). A: Lineweaver-Burk plot for fluorescence quenching of BSA/BLF/BSA-BLF by Pb (II). B: Theoretical Lineweaver-Burk plot for the fluorescence quenching of BSA-BLF by Pb (II). (pH=7.40, λ_{ex}=282 nm, $c_{BSA/BLF/BSA-BLF}$=1.0×10^{-5} M.

Table-1: The binding parameters of Pb (II) to BSA/BLF/BSA-BLF.

Solution System	K (L·mol^{-1})	R^2	n	R^2
BSA-Pb (II)	2.34×10^4	0.991	0.77	0.995
BLF-Pb (II)	5.77×10^4	0.998	0.61	0.997
BSA-BLF (1:9)- Pb (II)	2.40×10^4	0.994	0.41	0.994
BSA-BLF (1:2.33)- Pb (II)	1.88×10^5	0.978	0.45	0.999
BSA-BLF (1:1)-Pb (II)	1.05×10^5	0.982	0.51	0.995
BSA-BLF (2.33:1)-Pb (II)	1.53×10^5	0.996	0.36	0.992
BSA-BLF (9:1)- Pb (II)	3.35×10^5	0.9645	0.35	0.9987

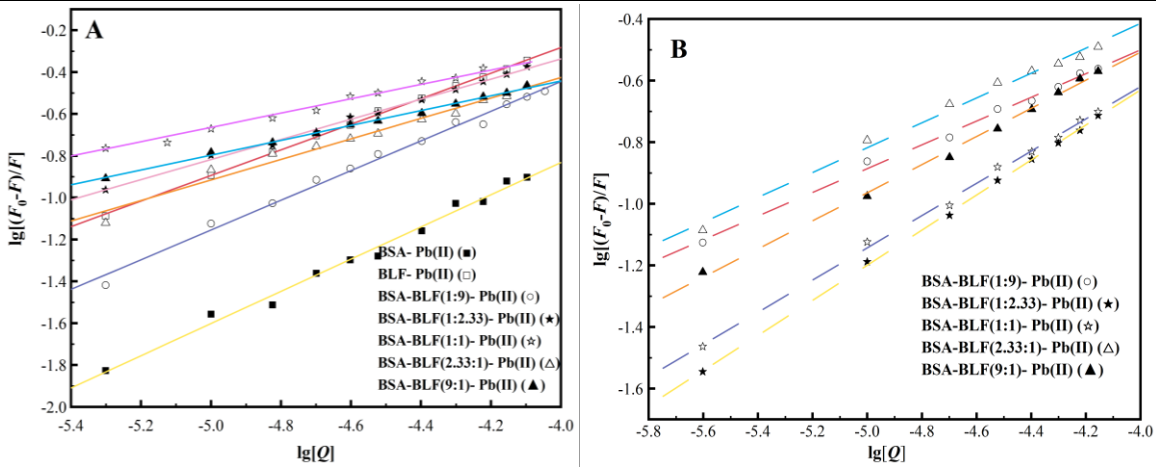


Fig. 4: lg[$(F_0\text{-}F)/F$]-lg[Q] double logarithmic plot of BSA/BLF/BSA-BLF by Pb (II). A: lg[$(F_0\text{-}F)/F$]-lg[Q] double logarithmic plot of BSA/BLF/BSA-BLF by Pb (II). B: Theoretical lg[$(F_0\text{-}F)/F$]-lg[Q] double logarithmic plot of BSA/BLF/BSA-BLF by Pb (II). (pH=7.40, λ_{ex}=282 nm, $c_{BSA/BLF/BSA-BLF}$=1.0×10^{-5} M).

Based on the aforementioned assumption, the value of *n* (derived from a double logarithmic regression) is presented in formula 3:

$$\lg[(F_0 - F)/F] = \lg K + n\lg[Q] \quad (3)$$

where F_0, F and K are the same parameters as in formula 2 and [Q] is the concentration of Pb (II). The value of n can be determined from the slope of the double logarithmic regression of lg [(F_0-F)/F] *versus* lg [Q], based on formula 3 (Fig. 4).

The spectra indicated that Pb (II) did interact with BSA-BLF, and was affected by BSA-BSA, BLF-BLF and BSA-BLF interactions. The obtained K values from the interaction between Pb (II) and BSA, BLF and BSA-BLF have an order of magnitude of 10^4 L·mol^{-1}, which means that the binging strength is relatively high, indicating strong binding affinities. This is further evidence that the binding interaction between Pb (II) and BSA, BLF, and BSA-BLF occurs [36,37]. $K_{(Pb\ (II)\text{-}BLF)}$ is slightly greater than $K_{(Pb\ (II)\text{-}BSA)}$ according to the fluorescence data, indicating that BLF is more susceptible to the microenvironment than BSA. In other words, the extent of aggregation, and the spatial structure of BLF, is more affected by the solution properties [38]. This suggests that Pb (II) interacted more strongly with BLF. BLF is more "flexible", while BSA is more "rigid". The following discussion is based on these conclusions. Also, $K_{(Pb\ (II)\text{-}BSA\text{-}BLF)}$ is larger than $K_{(BSA\text{-}Pb\ (II))}$ and $K_{(BLF\text{-}Pb\ (II))}$, except when there was a high BLF content relative to BSA (i.e., Pb (II)-BSA-BLF at a BSA:BLF ratio of 1:9), as shown in Table-1. With respect to Pb (II)-BSA-BLF (1:9), the concentration of BLF is higher than that of BSA, therefore theoretical $K_{(Pb\ (II)\text{-}BSA\text{-}BLF)}$ values should be closer to $K_{(Pb\ (II)\text{-}BLF)}$. However, values were closer to $K_{(BSA\text{-}Pb\ (II))}$. This may be due to Pb (II) more easily interacting with the better-dispersed protein (BSA) as a higher proportion of BLF molecules were likely to aggregate [21]. However, at greater BSA concentrations aggregation with more soluble or flexible BLF may have occurred. Therefore, interactions between BSA and BLF weaken the interaction of Pb (II)-BSA-BLF. As a result, the BSA-BLF (1:1) system had the lowest binding constants. Moreover, "rigid" BSA whose conformation is not easy to change has a lower tendency to aggregate and is more likely to bind with Pb (II), leading to an increase of $K_{(Pb\ (II)\text{-}BSA\text{-}BLF)}$ with increased BSA proportion (BSA/BLF= 2.33:1, 9:1). With respect to the binding site, $n_{(Pb\ (II)\text{-}BSA\text{-}BLF)}$ is smaller than $n_{(BSA\text{-}Pb\ (II))}$ and $n_{(BLF\text{-}Pb\ (II))}$ overall, which may be due to the titration of Pb (II) causing the peptide chains of BSA and BLF to be more flexible and the stability of the BSA-BLF system to decrease. In addition, with BSA-BLF (1:1) as a reference, n first increased and then decreased. BLF aggregates together, thus, it is difficult for Pb (II) to combine with high concentrations of BLF. In contrast, BSA dispersed with high concentrations of flexible BLF, enabling Pb (II) to easily react with BLF. BSA and BLF also accumulate because of partial structural domain overlap. It is difficult for Pb (II) to enter the overlapped domain, causing $n_{(Pb\ (II)\text{-}BSA\text{-}BLF)}$ to decrease once again. This phenomenon is explored in further detail below.

Table-2: The theoretical binding parameters of Pb (II) to BSA-BLF complex.

Solution System	K (L·mol^{-1})	R^2	n	R^2
BSA-BLF (1:9)-Pb (II)	**2.07×10^5**	**0.982**	**0.35**	**0.994**
BSA-BLF (1:2.33)-Pb (II)	**1.97×10^5**	**0.978**	**0.40**	**0.999**
BSA-BLF (1:1)-Pb (II)	**1.08×10^5**	**0.989**	**0.49**	**0.999**
BSA-BLF (2.33:1)-Pb (II)	**1.46×10^5**	**0.992**	**0.42**	**0.995**
BSA-BLF (9:1)-Pb (II)	**1.66×10^5**	**0.970**	**0.46**	**0.998**

As the BSA-BLF in Table-2 is mixed in a certain ratio, the respective BSA/BLF concentrations are calculated theoretically. For example, when BSA-BLF=1:9, the calculation shows that the concentration of BSA is 1×10^{-6} M and the concentration of BLF is 9×10^{-6} M. The corresponding concentrations of BSA and BLF solutions were prepared, and the fluorescence emission spectra were recorded by adding Pb(II) at a concentration of 1×10^{-5} M dropwise between 250 nm and 500 nm. The experimentally obtained fluorescence intensities were summed and brought into the equation to calculate the theoretical data in Table-2.

The theoretical data in Table-2 is calculated without considering the interaction of BSA-BSA, BLF-BLF and BSA-BLF and other factors. Table-1 is calculated directly from the experimental data. For the BSA-BLF-Pb (II) ternary system (1:9, 1:2.33 and 1:1), the K values in Table-1 are less than those in Table-2, while the other two groups (BSA: BLF=2.33:1 and 9:1) exhibit the opposite relationship. The reasons for this phenomenon are as follows: firstly, BSA plays a "depolymerization" role for the BLF which is at high concentrations of aggregation. As a result, the higher the proportion of BLF, the more dispersed BLF would be in the solution, thereby enhancing fluorescence quenching by Pb (II). The condition facilitates the interaction between Pb (II) and BSA-BLF. Secondly, BLF can't play a role of "depolymerization" role for BSA which is at high concentration of aggregation, resulting in the formation of overlapping domain between BSA and BLF, which makes it difficult for Pb (II) to enter it. As a result, the higher the proportion of BSA, the more overlapping domains there are, the more difficult it is for Pb (II) to enter. If the Pb (II) enters the overlapping domains, it binds very tightly with BSA-BLF.

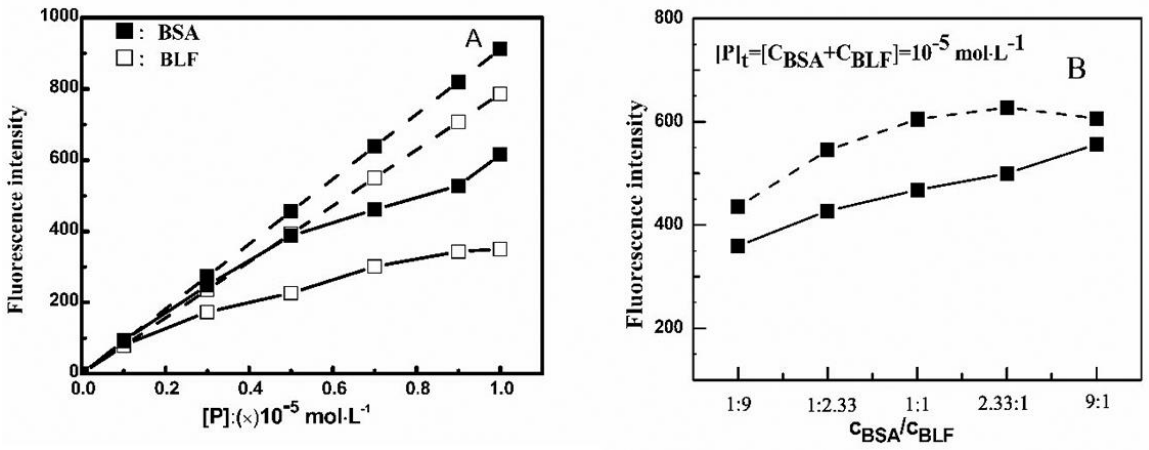


Fig. 5: Theoretical (dashed line) and measured (solid line) fluorescence intensity of different concentrations of BSA/BLF/BSA-BLF in the absence of Pb (II). A: The theoretical (dashed lines) and measured (solid lines) fluorescence intensity of different concentrations of BSA and BLF in the absence of Pb (II). B: The theoretical and real fluorescence intensity of different concentrations of the BSA-BLF binary protein mixture system in the absence of Pb (II).

Table-1 and Table-2 show that the actual number of binding sites is higher than the theoretical number. Addionally, the experimental binding constant is less than the theoretical value for the BSA-BLF binary protein mixture system (1:9, 1:2.33 and 1:1), while the other two groups (BSA-BLF=2.33:1 and 9:1) exhibit the opposite relationship. Thus, with increased BSA concentrations (BSA-BLF=2.33:1 and 9:1), BSA would have a dispersive effect on BLF [3]. This phenomenon, which affects the interactions between BSA and BLF, would affect the state of BLF in the Pb (II)-BSA-BLF. As a result, BLF would be more dispersed in the solution, thereby enhancing fluorescence quenching by Pb (II) [21]. This condition may facilitate the interaction of Pb (II) with BSA-BLF. In addition, with an increased proportion of BSA in the binary protein mixture system, it is difficult for Pb (II) to enter the part of the domain where the BSA and BLF molecules overlap. Therefore, the actual number of binding sites of Pb (II)-BSA-BLF is less than that for Pb (II)-BSA and Pb (II)-BLF. In contrast, with increased BLF (BSA-BLF=1:9, 1:2.33 and 1:1), BSA has a slight aggregation, whereas BLF does not have the same dispersive effect as BSA at high concentrations. The actual binding constant of Pb (II)-BSA-BLF is larger than the theoretical values because Pb (II) binds better with BSA-BLF. The data in Table-1 and Table-2 indicate that the theoretical binding parameters of the Pb (II)-BSA-BLF system differ greatly from the actual binding parameters because of the influence of the solution environment, which involves the interaction of mixed proteins with different abundance. These findings suggest that the complex interactions of the BSA-BLF, BSA-BSA and BLF-BLF system are important when analyzing the Pb (II)-BSA-BLF interaction.

Analysis of the interaction of single protein in BSA-BLF

Generally, fluorescence intensity measurements of various proteins are affected by the interaction between proteins in the system. To further study the interactions between BSA and BLF in the Pb (II)-BSA-BLF system. Similarly, the fluorescence value of BSA-BLF was used to plot the concentration (c_{BSA}/c_{BLF}), as shown in Fig. 5B.

Measured fluorescence of a protein is not linearly related to its concentration (Fig. 5). BSA-BLF are joined by hydrogen bonds or attracted by electrostatic force [39,40]. Protein aggregation lowered the fluorescence. Additionally, the BSA fluorescence value was much closer to the theoretical value than that of BLF, indicating that BLF is more susceptible to aggregation by other solutes than BSA. This result also confirmed our previous conclusion that BLF was more flexible than BSA. As the protein concentration gradually increases, BLF is likely to aggregate and cause the experimental fluorescence value to deviate from the theoretical value. In contrast, the deviation of BSA is small because BSA is more “rigid” and has a greater solubility in water. In addition, for the BSA-BLF mixed protein system, the experimental *F* is smaller than the theoretical sum because it is influenced by BSA-BSA, BLF-BLF and

BSA-BLF interactions. As shown in Fig. 5, the experimental *F* of BSA-BLF deviated further from the ideal state; the solution environment had a greater influence on the proteins. Therefore, to quantitatively depict the influence of the microenvironment on the fluorescence effect of proteins, we calculated parameter I_{OM} (overall microenvironmental influence factor) [41] as follows:

$$I_{\mathrm{OM}} = \left(J_T - J_M\right)/J_T \qquad (4)$$

where J_T is the integral of the value of the ideal fluorescence and J_M is the integral of the value of the experimental fluorescence.

The results revealed that $I_{OM(BSA-BLF)}$ =0.1937<$I_{OM(BSA)}$=0.2467<$I_{OM(BLF)}$=0.4369, this finding was consistent with the previous conclusion that BLF was more flexible. The $I_{OM(BSA-BLF)}$ is less than $I_{OM(BSA)}$ and $I_{OM(BLF)}$, suggesting that BSA-BLF is less easily affected by the solution environment than BSA and BLF. Conversely, BLF is easily affected by the microenvironment, as indicated by a higher $I_{OM(BLF)}$. All data indicated that BSA-BLF was less affected by the microenvironment than BSA and BLF. This condition also contributed to the BSA-BLF system having such complex interactions, including BSA-BLF, BSA-BSA and BLF-BLF. The results of the fluorescence spectrometry showed that Pb (II) quenched the fluorescence of BSA-BLF, indicating that Pb (II) interacted with the BSA-BLF protein system, and was affected by single protein, mixed proteins and the solution microenvironment. The same results were obtained for CD and ITC [42-44].

CD and fluorescence spectroscopy are both spectroscopic tests, while ITC is an isothermal titration calorimetric method. CD and ITC are only side-effects of fluorescence spectroscopy, while our study focuses on fluorescence spectroscopy, which can determine excitation spectra, emission spectra, etc., and can infer conformational changes of protein molecules in the solution environment and changes in the solution microenvironment. The fluorescence spectroscopy is sensitive compared to other spectroscopic and isothermal titration calorimetric methods. In the following study, we will carry out additional instrumental tests to gain a more comprehensive understanding of the binding mechanism of the Pb (II)-BSA-BLF ternary system.

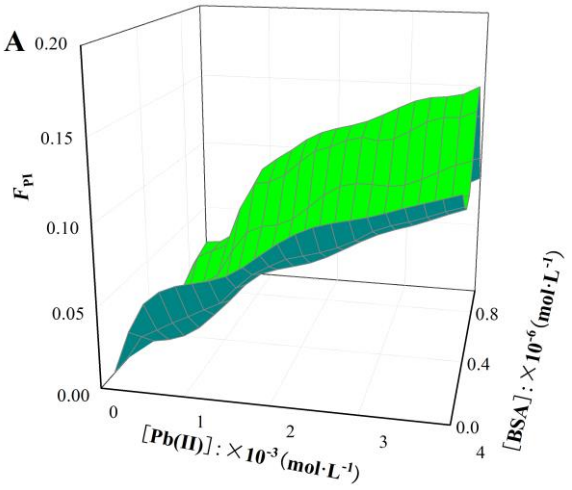
In conclusion, interactions of the mixed proteins with different abundance are influenced by the microenvironment. The BSA-BLF system is not only affected by the interaction between BSA and BLF but also by BSA and BLF themselves.

Fluorescence binding mechanism of Pb (II) and BSA-BLF

Studies of the interactions between single protein and ligands are being increasingly reported [45]. When Pb (II) binds to a single protein, it goes directly to the binding site of BSA or BLF. The higher the protein concentration, the greater the fluorescence burst per unit concentration of Pb (II) for a single protein. When Pb (II) binds to the BSA-BLF binary protein system, the Pb (II)-BSA-BLF ternary system interaction is not the sum of the Pb (II)-BSA and Pb (II)-BLF binary system interactions. There are interactions between BSA-BSA, BLF-BLF and BSA-BLF, and partial structural domain overlap of BSA-BLF. It is difficult for Pb (II) to enter the overlapped domain. If the Pb (II) enters the overlapping domains, it binds very tightly with BSA-BLF. However, here experiments were based on a binary protein and ligand interaction, which is a notably more complex system. Therefore, to study the interaction between BSA-BLF and Pb (II) at various concentrations, we proposed parameter F_{PI} as an indicative factor of the extent of the interaction.

$$F_{\mathrm{PI}} = \ln\left(F/F_0\right) \qquad (5)$$

where *F* is the relative intensity of interaction between the molecule and protein and F_0 is the relative intensity of interaction between molecules, a three-dimensional fluorescence diagram between F_{PI} and $c_{Pb(II)}$ is presented (Fig. 6).



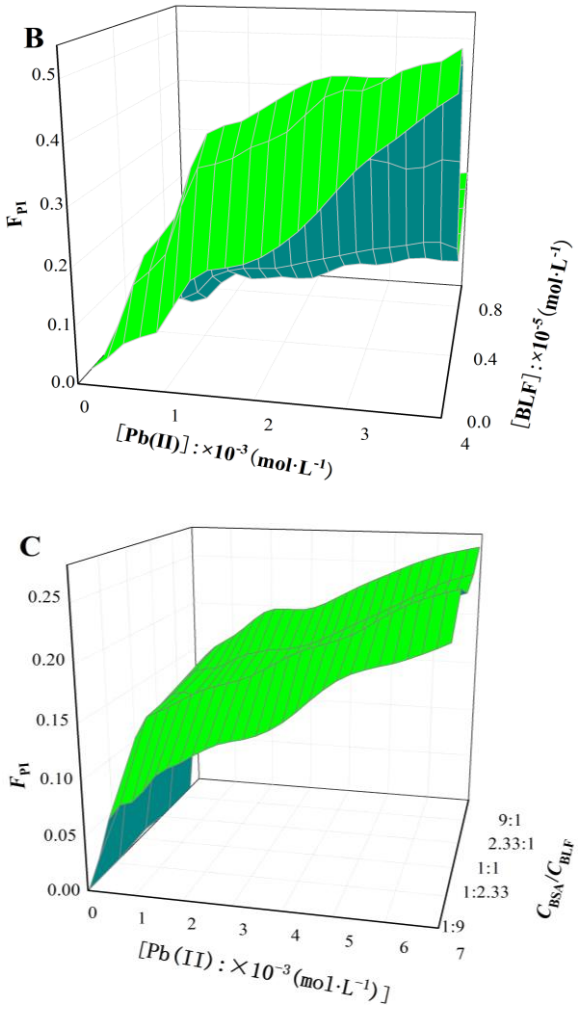


Fig. 6: A relationship diagram between F_{PI} and $c_{Pb(II)}$-c_{BSA}, $c_{Pb(II)}$-c_{BLF} and $c_{Pb(II)}$-c_{BSA}/c_{BLF}. A: A relationship diagram between F_{PI} and $c_{Pb(II)}$-c_{BSA}. B: A relationship diagram between F_{PI} and and $c_{Pb(II)}$-c_{BLF}. C: A relationship diagram between F_{PI} and $c_{Pb(II)}$-c_{BSA}/c_{BLF}. (The concentration of protein ranges from 0.1×10^{-5} M to 1×10^{-5} M, the concentration of Pb (II) range from 1×10^{-5} M to 11×10^{-5} M).

To investigate fluorescence as a measurement of the interaction between Pb (II) and BSA-BLF, we constructed a three-dimensional relationship of quenching (Fig. 6). The intrinsic fluorescence of BSA and BLF was influenced by the concentrations of Pb (II) and the individual proteins. The degree of fluorescence quenching of the proteins increased with increasing Pb (II) concentration. However, the value of F_{PI} did not increase linearly with increasing BSA/BLF concentration. As shown in Fig. 6A and Fig. 6B, of $F_{PI(BSA)}$ increase changed slightly, while the variation of $F_{PI(BLF)}$ increased significantly, once again indicating that BLF is more susceptible to the microenvironment than BSA. Further confirming its fluorescence intensity, the Pb (II)-BSA-BLF system was affected by the interaction and microenvironmental change of the proteins (BSA-BSA/BLF-BLF). As shown in Fig. 6, fluorescence quenching with BSA-BLF increased with stronger Pb (II) concentrations. However, $F_{PI(BSA-BLF)}$ did not change significantly with the ratio of two proteins - indicating that the BSA-BLF was less affected by the microenvironment (Fig. 6C). This finding was consistent with I_{OM} data.

Moreover, the three-dimensional relationship of fluorescence quenching was quantitatively depicted based on different concentrations of BSA/BLF, and different ratios of BSA-BLF. After several iterations of data fitting, we found a high correlation between F_{PI} and the concentration parameter estimation of Pb (II). A review of the literature reveals that Taylor function is applied to the analysis of fractional linear hybrid systems [46-48]. In this manuscript, the interaction between Pb (II) and BSA-BLF binary protein systems was studied by means of fluorescence spectroscopy. The F_{PI} parameter was proposed. Based on three-dimensional fluorescence spectroscopy, a three-dimensional fluorescence burst model with Pb (II) concentration as the X-axis, BSA/BLF/BSA-BLF concentration as the Y-axis and F_{PI} as the Z-axis was established. It is obvious that the relationship between F_{PI} and Pb (II) concentration, BSA/BLF/BSA-BLF concentration is no linear, so this paper establishes the link by Taylor functions, which provides a fast and effective way to study the interaction between heavy metal ions and binary protein mixed systems. The binary protein mixture system was analyzed with a Taylor series of one real rational function, using the following formula:

$$F_{PI}=\frac{0.01135+0.03387[Q]-0.03369[P]+0.02255[P]^2-0.03008[Q]\times[P]}{1+0.12344[Q]-2.13284[P]+0.00403[Q]^2+1.32353[P]^2-0.13307[Q]\times[P]}$$

(Taylor level real rational function of BSA and Pb (II), correlation coefficient R^2=0.8646, where [Q] is the concentration of Pb (II) at 1.0×10^{-3} M and [P] is the concentration of BSA at 1.0×10^{-5} M).

$$F_{PI} = \frac{0.16473 - 0.07264[Q] - 0.92313[P] + 2.24224[P]^2 + 0.7289[Q]\times[P]}{1 - 0.10907[Q] - 13.69931[P] - 4.44568\times10^{-6}[Q]^2 + 37.21382[P]^2 + 1.09042[Q]\times[P]}$$

(Taylor level real rational function of BLF and Pb (II), correlation coefficient R^2=0.8874, where [Q] is the concentration of Pb (II) at 1.0×10^{-3} M and [P] is the concentration of BLF at 1.0×10^{-5} M).

$$F_{PI} = \frac{6.59319\times10^{16} - 2.48477\times10^{18}[Q] + 1.43966\times10^{16}[P] + 5.19933\times10^{17}[P]^2 + 3.17625\times10^{19}[Q]\times[P]}{1 - 6.13074\times10^{18}[Q] + 7.04102\times10^{19}[P] - 3.58027[Q]^2 + 6.79979\times10^{18}[P]^2 + 1.06844\times10^{20}[Q]\times[P]}$$

(Taylor level real rational function of BSA-BLF and Pb (II), correlation coefficient R^2=0.9182, where [Q] is the concentration of Pb (II) at 1.0×10^{-3} M and [P] is the concentration of BSA-BLF at 1.0×10^{-5} M).

From these formulas, the interaction between BSA-BLF binary protein mixture system and Pb (II) can be quantified using mathematic formulas. However, the equation used to describe the interaction is highly complex. Once the concentrations of the small molecule and the protein are determined, the corresponding F_{PI} can be found, and the relative intensity between the small molecule and the protein can also be calculated. A larger F_{PI} indicates a smaller interaction between the molecules. For example, if the concentrations of BSA-BLF and Pb (II) are known, the relative fluorescence intensity F of the two binding interactions can be found, and the F_{PI} of BSA-BLF-Pb (II) can also be found by bringing it into the BSA-BLF-Pb (II) Taylor function. The higher F_0 indicates that BSA-BLF-Pb (II) is influenced by BSA-BSA, BLF-BLF and BSA-BLF interactions, which means that the Taylor function obtained from the simulations can be used to calculate the effect of intermolecular interactions on the test. In general, the fluorescence value is affected by the concentration of the protein, quencher and the microenvironment of the solution. In addition, the BSA-BLF system involves different concentration ratios, and the model is useful for identifying mechanisms of the interaction between ligands and the binary protein mixture system.

Conclusions

This study proposes a new approach for quantifying the interaction between heavy metal ions and binary protein mixture system using fluorescence spectroscopy. Here, the interaction between Pb (II) and BSA-BLF was investigated. Spectroscopic data was analyzed using Lineweaver-Burk and double logarithmic equations to obtain the binding constant and binding sites. A strong binding force was observed between Pb (II) and BSA-BLF. In addition to conventional methods, a new method with corresponding definitions, formulas and a preliminarily theoretical model to describe the interaction between BSA-BLF and Pb (II) using fluorescence. The challenge of this study was to quantify the effect of the microenvironment on fluorescence at different protein concentrations. We proposed a definition of I_{OM} to quantitatively explain how microenvironmental change affect fluorescence. I_{OM} indirectly indicates conformational properties. In addition, the theoretical binding parameters and actual parameters of BSA-BLF-Pb (II) were compared. This was to identify the areas of interaction between BSA and BLF that were influenced by the microenvironment, and that directly competed with BSA-BLF's interaction with Pb (II). The effects of BSA-BLF on conformational changes and microenvironmental factors were discussed, the interaction between Pb (II) and BSA-BLF based on the distribution, free concentration and metabolic mechanisms of Pb (II), and the spatial conformation of BSA-BLF. A three-dimensional model was established to describe the fluorescence resulting from interactions between BSA-BLF and Pb (II). There was a strong correlation (R^2=0.9182, p<0.001) between F_{PI} and the concentration parameter estimation of Pb (II). The interaction between BSA-BLF and Pb (II) was also reasonable for the Taylor series of one real rational function. These innovative methods provide a fast and efficient manner to study interactions between heavy metal ions and binary protein mixture system.

Acknowledgments

This work was supported by the Zhejiang Public Welfare Technology Application Research Project (LGN20B070001, LGN21B070001). The corresponding author and all co-authors severally declare no conflict of interest involved in this submission.

Author Contribution Statement

Luwei Tian and Ming Guo designed the research study. Luwei Tian and Xingtao Xu performed the research. Luwei Tian, Xingtao Xu and Linfang Shi analyzed the data. Luwei Tian and Ming Guo wrote the paper. Ming Guo and Linfang Shi reviewed the manuscript.

References

1. R. Wang, Y. Bai, Z. H. Liang, Y. Liu, L. L. Huang and W. J. Zheng, Interaction between selenomethionine and copper ions, *Acta Phys. Chim. Sin.*, **26**, 3225–3229 (2010).
2. A. Ms, B. Rdua and C. Gjsca, Shared perturbations in the metallome and metabolome of Alzheimer's, Parkinson's, Huntington's, and dementia with Lewy bodies: A systematic review, *Ageing Res Re*, **63**, 101152 (2020).
3. C. L. Huang, L. J. Bao, P. Luo, Z. Y. Wang, S. Li M and E. Y. Zeng, Potential health risk for residents around a typical e-waste recycling zone via inhalation of size-fractionated particle-bound heavy metals, *J. Hazard. Mater.*, **317**, 449-456 (2016).
4. K. M. Towle, L. C. Garnick and A. D. Monnot, A human health risk assessment of lead (Pb) ingestion among adult wine consumers, *Int. J. Food*, **4**, 7 (2017).
5. Z. Di, L. Jie, L. Chao, A. L. Juhasz, K. G. Scheckel, J. Luo, H. B. Li and L. Q. Ma, Lead relative bioavailability in lip products and their potential health risk to women, *Environ. Sci. Technol*, **50**, 6036-6043 (2016).
6. J. D. Holz, T. J. Sheu, H. Drissi, M. Matsuzawa, M. J. Zuscik and J. E. Puzas, Environmental agents affect skeletal growth and development, *Birth Defects Res. C Embryo Today*, **81**, 1–50 (2007).
7. A. Sharifi-Rad, J. Mehrzad, M. Darroudi, M. R. Saberi and J. Chamani, Oil-in-water nanoemulsions comprising berberine in olive oil: biological activities, binding mechanisms to human serum albumin or holo-transferrin and QMMD simulations, *J. Biomol. Struct. Dyn.*, **39**, 1029-1043 (2021).
8. P. Andraej, B. Anna, K. Pawel, J. S. Jaroslaw, N. Jarosław and Z. Wojciech, Serum concentrations of selected heavy metals in patients with alcoholic liver cirrhosis from the Lublin region in eastern Poland, *Int. J. Environ. Res. Public Health*, **13**, 582 (2016).
9. A. Moradi, N. Honarjoo, M. Etemadifar and J. Fallahzade, Bio-accumulation of some heavy metals in blood serum of residents in Isfahan and Shiraz, Iran, *Environ. Monit. Assess*, **188**, 269 (2016).
10. H. Mohammadzadeh-Aghdash, N. D. J. Ezzati, P. Dehghan, V. Panahi-Azar and A. Barzegar, Multi-spectroscopic and molecular modeling studies of bovine serum albumin interaction with sodium acetate food additive, *Food Chem*, **228 (AUG.1)**, 265-269 (2017).
11. I. A. Sergeeva, E. A. Shirshin, N. G. Zhdanova, V. V. Gibizova, G. P. Petrova, S. A. Kurguzenkov and V. V. Fadeev, The effect of lead cations on the fluorescence characteristics of bovine serum albumin in aqueous solution, *Opt. Spectrosc*, **115**, 42-45 (2013).
12. Y. Priyanka, S. Bhawana, S. Chiranjeev, S. Preeti and K. A. Satish. Interaction between the antimalarial drug dispiro-tetraoxanes and human serum albumin: a combined study with spectroscopic methods and computational studies, *ACS Omega*, **5**, 6472-6480 (2020).
13. M. Guo, L. He, P. J. Strong and H. L. Wang, Binding between lead ions and the high-abundance serum proteins, *Chemosphere*, **112**, 472-480 (2014).
14. J. Bai, Y. Chao, Y. Chen, S. Wang and R. Qiu, The effect of interaction between bacillus subtilis DBM and soil minerals on Cu (II) and Pb (II) adsorption, *J. Environ. Sci*, **78**, 328-337 (2018).
15. M. Manjushree andH. D. Revanasiddappa, Detailed investigation of effects of Zn^{2+}, Fe^{2+} and Cu^{2+} metal ions on the binding interaction between eletriptan hydrochloride an anti-migraine headache drug and bovine serum albumin by various spectrometric techniques and molecular docking studies, *J. Mol. Liq.*, **237**, 23-37 (2017).
16. N. C. Nina, A. Iuliana, G. G. Leontina, A. P. Brînduşa, E. Elena, R. Gabriela, E. B. Gabriela and S. Nicoleta, Fluorescence spectroscopy and molecular modeling of anthocyanins binding to bovine lactoferrin peptides, *Food Chem*, **318**, 126508 (2020).
17. J. Laxmi, M. Manjunath, N. Sharanappa and C. Shivamurti, Evaluation of the binding interaction between bovine serum albumin and dimethyl fumarate, an anti-inflammatory drug by multispectroscopic methods, *Spectrochimica Acta A*, **156**, 164-171 (2016).
18. M. E. Dragoserrano, R. Camposrodriguez, J. C. Carrero and M. L. G. De, Lactoferrin and peptide-derivatives: antimicrobial agents with potential use in nonspecific immunity modulation, *Curr. Pharm. Design*, **24**, 1067-1078 (2018).
19. R. Jiang and B. Lönnerdal, Cloning and characterization of the human lactoferrin receptor

gene promoter, *Biometals*, **31**, 357-368 (2018).
20. A. T. M. Karl, M. Yassene, L. R. Elena, V. P. Evgeniy, V. F-Nora, M. Chris, S. Albert and H. B. Christoph, Improving identification of in-organello protein-protein interactions using an affinity-enrichable, isotopically coded, and mass spectrometry-cleavable chemical crosslinker, *Mol. Cell. Proteomics*, **19**, 624-639 (2020).
21. B. Ahmed, H. Surat, C. Robert and T. R. Heidar-Ali, Locating the binding sites of Pb (II) ion with human and bovine serum albumins. *PloS. ONE*, **7**, e36723 (2012).
22. D. M. Manjunath, S. B. Kirthi, T. N. Sharanappa and A. C. Shivamurti, Multi-spectral characterization & effect of metal ions on the binding of bovine serum albumin upon interaction with a lincosamide antibiotic drug, clindamycin phosphate, *J. Photochem Photobiol B*, **138**, 324-330 (2014).
23. D. C. Carter and J. X. Ho, Structure of serum albumin, *Adv. Protein Chem.*, **45**, 153–203 (1994).
24. M. R. Housaindokht, J. Chamani, A. A. Saboury, A. A. Moosavi-Movahedi and M. Bahrololoom, Three binding sets analysis of α-lactalbumin by interaction of tetradecyl trimethyl ammonium bromide, *Bull. Korean Chem. Soc.*, **22**, 145-148 (2001).
25. W. C. Lee and K. H. Lee, Applications of affinity chromatography in proteomics, *Anal. Biochem.*, **324**, 1–10 (2004).
26. N. Zare-Feizabadi, Z. Amiri-Tehranizadeh, A. Sharifi-Rad, P. Mokaberi, N. Nosrati, F. Hashemzadeh, H. R. Rahimi, M. R. Saberi and J. Chamani, Determining the interaction behavior of calf thymus DNA with anastrozole in the presence of histone H1: spectroscopies and cell viability of MCF-7 cell line investigations, *DNA Cell Biol.*, **40**, 1039-1051 (2021).
27. F. Sadeghzadeh, A. A. Entezari, K. Behzadian, K. Habibi, Z. Amiri-Tehranizadeh, A. Asoodeh, M. R. Saberi and J. Chamani, Characterizing the binding of angiotensin converting enzyme i inhibitory peptide to human hemoglobin: influence of electromagnetic fields, *Protein Pept. Lett.*, **40**, 1007-1021 (2020).
28. J. Chamani, A. A. Moosavi-Movahedi, O. Rajabi, M. Gharanfoli, M. Momen-Heravi, G. H. Hakimelahi, A. Neamati-Baghsiah and A. R. Varasteh, Cooperative α-helix formation of *β*-lactoglobulin induced by sodium *n*-alkyl sulfates, *J. Colloid Interf. Sci.*, **293**, 52–60 (2006).
29. J. Chamani, A. A. Moosavi-Movahedi, A. A. Saboury, M. Gharanfoli and G. H. Hakimelahi, Calorimetric indication of the molten globule-like state of cytochrome *c* induced by *n*-alkyl sulfates at low concentrations, *J. Chem. Thermodynamics*, **35**, 199–207 (2003).
30. P. Mokaberi, F. Babayan-Mashhadi, Z. A. T. Zadeh, M. R. Saberi and J. Chamani, Analysis of the interaction behavior between Nano-Curcumin and two human serum proteins: combining spectroscopy and molecular stimulation to understand protein-protein interaction, *J. Biomol. Struct. Dyn.*, **39**, 3358-3377 (2021).
31. J. Wang, J. Wang, L. Zhu, H. Xie, B. Shao and X. Hou, The enzyme toxicity and genotoxicity of chlorpyrifos and its toxic metabolite TCP to zebrafish Danio rerio. *Ecotoxicology*, **23**, 1858-1869 (2014).
32. S. R. Atanu, K. D. Amit, C. Susmitnarayan and D. Swagata, Binding of antioxidant flavonol morin to the native state of bovine serum albumin: Effects of urea and metal ions on the binding, *J. Lumin.*, **145**, 741-751 (2014).
33. M. Dareini, Z. A. Tehranizadeh, N. Marjani, R. Taheri, S. Aslani-Firoozabadi, A. Talebi, N. N. Z. Eidgahi, M. R. Saberi and J. Chamani, A novel view of the separate and simultaneous binding effects of docetaxel and anastrozole with calf thymus DNA: Experimental and in silico approaches, *Spect. Rochim. Acta.*, **228**, 117528 (2019).
34. S. L. Zhuang, H. Wang, K. Ding, J. Wang, L. Pan, Y. Lu, Q. Liu and C. Zhang, Interactions of benzotriazole UV stabilizers with human serum albumin: Atomic insights revealed by biosensors, spectroscopies and molecular dynamics simulations, *Chemosphere*, **144(FEB.)**, 1050-1059 (2016).
35. Z. J. Hu, Analysis strategy of protein-protein interaction networks, *Methods in molecular biology (Clifton, N.J.)*, **939**, 141-181 (2013).
36. J.Chamani and A. A. Moosavi-Movahedi, Effect of *n*-alkyl trimethylammonium bromides on folding and stability of alkaline and acid-denatured cytochrome c: A spectroscopic approach, *J. Colloid Interf. Sci.*, **297**, 561-569 (2006).
37. S. Beigoli, A. Sharifi Rad, A. Askari, R. A. Darban and J. Chamani, Isothermal titration calorimetry and stopped flow circular dichroism investigations of the interaction between lomefloxacin and human serum albumin in the presence of amino acids, *J. Biomol. Struct. Dyn.*, **37**, 2265-2282 (2019).
38. M. Joanna, Ż. Krzysztof, W. Dariusz, U. Dorota, W. Małgorzata, W. Wiesław and C. Lech, Binding of Cu (II) ions to peptides studied by fluorescence spectroscopy and isothermal titration calorimetry. *Spectrochim. Acta A*, **153**, 451-456 (2016).
39. A. Kathiravan, S. Anandan and R. Renganathan,

Interaction of colloidal TiO_2 with human serum albumin: A fluorescence quenching study, *Colloid. Surfaces A*, **333**, 91-95 (2009).

40. F. Zhang, J. Zhang and S. L. Zhuang, Molecular interactions of benzophenone UV filters with human serum albumin revealed by spectroscopic techniques and molecular modeling, *J. Hazard. Mater.*, **263(pt.2)**, 618-626 (2013).
41. M. Guo, Y. Wang and X. T. Xu, The interaction between BLF and BSA and impact in interaction of RT-BSA-BLF system, *Acta. Pharm. Sin.*, **52**, 271-278 (2017).
42. M. Taif, M. A. Alam, M. E. Ullah, M. A. Ashraf and A. Jahan, Prediction of possible effects of arsenic and cadmium in human health using chemical-protein and protein-protein interaction network, *IEEE ACM T. Comput. Bi.*, **5**, 45-52 (2017).
43. M. Kirberger and J. J. Yang, Structural differences between Pb^{2+}- and Ca^{2+}-binding sites in proteins: Implications with respect to toxicity, *J. Inorg. Biochem*., **102**, 1901-1909 (2008).
44. A. Belatik, S. Hotchandani, R. Carpentier, H. A. Tajmir-Riahi and R. Subramanyam, Locating the Binding Sites of Pb(II) Ion with Human and Bovine Serum Albumins, *PLoS ONE*, **7**, e36723 (2012).
45. T. Zhang, H. Zhang, G. Liu and R. Liu, Interaction of Cu^{2+}, Pb^{2+}, Zn^{2+} with trypsin: what is the key factor of their toxicity, *J. Fluoresc.*, **24**, 1803-1810 (2014).
46. J. M. Worseck, A. Grossmann, M. Weimann, A. Hegele and U. Stelzl, A stringent yeast two-hybrid matrix screening approach for protein–protein interaction discovery, *Methods Mol. Biol.*, **812**, 63–87 (2011).
47. C. Xin, Reachability analysis of non-linear hybrid systems using Taylor Models, 2015.
48. P. Collins, M. Niqui and N. Revol, A Taylor function calculus for hybrid system analysis: validation in Coq. Federated Logic Conference (FLoC 2010), 2010..