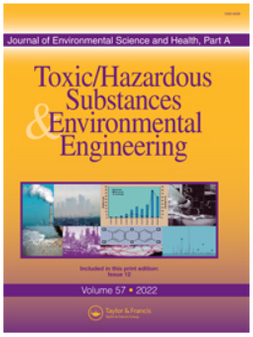

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Using fluorescence and circular dichroism (CD) spectroscopy to investigate the interaction between di-n-butyl phthalate and bovine serum albumin

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ABSTRACT

The interaction between di-n-butyl phthalate (DBP) and bovine serum albumin (BSA) in physiological Tris-HCl buffer at pH 7.4 was investigated by fluorescence quenching technique. By analyzing the fluorescence spectrum and intensity, it was observed that the DBP had a strong ability to quench the intrinsic fluorescence of BSA through a static quenching procedure. The binding constants K and the number of binding sites n of DBP with BSA were calculated to be 0.11×10^2 $L{\cdot}mol^{-1}$ and 0.52 at 298 K, respectively. The thermodynamic parameters of enthalpy change (ΔH) and entropy change (ΔS) were also calculated to be positive showing that hydrophobic forces might play a major role in the binding of DBP to BSA. The binding process was spontaneous in which Gibbs free energy change (ΔG) was negative. The distance (r) between the donor (BSA) and acceptor (DBP) was calculated to be 2.02 nm based on Forster's non-radiative energy transfer theory, which indicated that the energy transfer from BSA to DBP occurs with a high possibility. The synchronous fluorescence, three-dimensional fluorescence, and circular dichroism (CD) spectra showed that the binding of di-n-butyl phthalate to BSA induced conformational changes in BSA. The interaction between DBP and BSA can help researchers better understand the nature of poisons and serve people in the right way with first aid and detoxification.

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Bovine serum albumin (BSA); di-n-butyl phthalate (DBP); fluorescence spectra; binding process; toxicity

Introduction

Di-n-butyl phthalate (DBP), one of the most popular phthalate esters (PAEs) with low molecular weight, is easily detected in many products including flexible plastics, medical devices, and some cosmetic formulations.[1,2] DBP has received widespread concerns due to its high production volume, in millions of tons annually, and its negative effects on human health.[3,4] With the widespread manufacture and application of PAEs, they are inevitably released into the environment and also have been detected in human serum and plasma.[5–8] In recent years, PAEs have gained the increasing interest of a large number of scientists since they are known for their carcinogenic, endocrine-disrupting, and toxic effects on environmental quality and human health.[9,10] In light of this negative health potential, many countries have enacted environmental regulations to prevent the human intake of PAEs. These active developments have led to increasing interest and research efforts on the effects and control of PAEs.[10] Because the affinity between PAEs and serum albumin strongly influences PAEs distribution and determines the free fraction available for subsequent interactions with targeted receptors, it is an important factor in understanding the pharmacokinetics and pharmacodynamic properties of PAEs.[10,11]

Serum albumin, the most abundant protein constituent in blood plasma, plays a fundamental role in the disposition and transportation of various molecules and can react with many different ligands in vivo and in vitro.[12,13] As the biological functions of a protein depend on its structure, the resultant structural alternations due to its interaction with ligands can affect the transport, metabolism, and availability of serum albumin for other ligands.[13,14] Normally, pollutants will interact with serum albumin after they enter the bloodstream. Bovine serum albumin (BSA) was used as the model protein because of its water-solubility, its stability, as well as its sequence similarity to human serum albumin (HSA)[15] for evaluating the di-n-butyl phthalate toxicity to health.

This study aimed to investigate the interaction between di-n-butyl phthalate and BSA by fluorescence spectroscopy and the quenched mechanism of BSA by di-n-butyl phthalate. The binding constant, the number of binding sites, and the thermodynamic parameters were calculated. Besides, the binding forces, the energy transfer, and the conformation investigation were also discussed.

Materials and methods

Materials

BSA was purchased from Sigma Chemical Co. and used without purification. DBP was obtained from Accu Standard Inc. All solutions were prepared in Tris-HCl buffer at pH 7.4. BSA

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was made up of a stock solution of a concentration of 1.0×10^{-4} mol·L^{-1}. All other materials were of analytical reagent grade and double distilled water was used throughout.

Apparatus

Fluorescence spectra were measured by a spectrofluorimeter (Hitachi Model F-4600 spectrophotometer) equipped with a 150 W Xenon lamp and a slit width of 2.5 nm. A quartz cell of 1.00 cm was used for measurements. The absorption spectra were performed on a double beam U-3900 spectrophotometer (Hitachi, Japan) equipped with a 150 W Xenon lamp and a slit width of 10 nm. A quartz cell of 1.00 cm was used for measurements. Circular dichroism (CD) spectra were recorded by a MOS-450 spectropolarimeter (Bio-Logic, France).

Procedures

Fluorescence measurements

The BSA concentration was fixed at 1.0×10^{-5} mol·L^{-1}, and the DBP concentration varied from 0.5×10^{-5} mol·L^{-1} to 3.0×10^{-5} mol·L^{-1} for BSA. The fluorescence spectra were recorded at two temperatures (298 and 310 K) in the range of 300-500 nm upon excitation at 280 nm for BSA.

The synchronous fluorescence spectra of BSA in the absence and presence of the increasing amount of DBP were determined. The wavelength difference of 15 and 60 nm was maintained between excitation (E_x) and emission (E_m) wavelengths.

The three-dimensional fluorescence spectra were measured under the following conditions: the emission wavelength was recorded between 200 and 600 nm, the initial excitation wavelength was set at 200 nm with an increment of 10 nm, and the other scanning parameters were just the same as those of the fluorescence emission spectra.[16]

Energy transfer between DBP and BSA

The absorption spectrum of DBP (1.0×10^{-5} mol·L^{-1}) was recorded in the range of 300–500 nm. The emission spectrum of BSA (1.0×10^{-5} mol·L^{-1}) was also recorded in the range of 300–500 nm. Then, the overlap of the UV absorption spectrum of DBP with the fluorescence emission spectrum of BSA was used to calculate the energy transfer.

CD measurements

CD measurements were recorded on a MOS-450 (Bio-Logic, France) spectropolarimeter (200-250 nm and cell length path was 1 cm) by keeping the concentration of BSA constant (1.0×10^{-6} mol·L^{-1}) while varying the DBP concentration from 0 to 1.0×10^{-4} mol·L^{-1}.

Results and discussion

Binding property of di-n-butyl phthalate to bovine serum albumin

Generally, the fluorescence of protein is caused by three intrinsic fluorophores present in the protein, i.e. tryptophan, tyrosine, and phenylalanine residues.[17,18] Actually, tryptophan and tyrosine residues mainly contribute to the intrinsic fluorescence of many proteins, because the quantum yield of phenylalanine residue is very low.

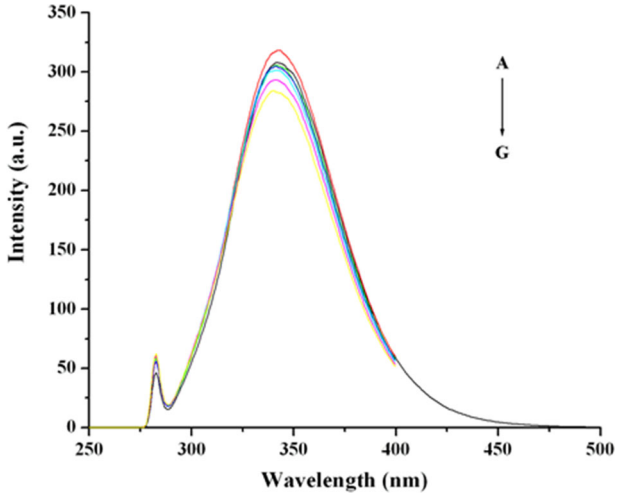

Figure 1. The changes of FL spectra of DBP-BSA intensity with DBP concentration (A = 0; B = 0.5×10^{-5}; C = 1.0×10^{-5}; D = 1.5×10^{-5}; E = 2.0×10^{-5}; F = 2.5×10^{-5}; G = 3.0×10^{-5} mol·L^{-1}).

Fluorescence quenching refers to any process that decreases the fluorescence intensity of a fluorophore due to a variety of molecular interactions. These include excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching.[17,19]

Figure 1 shows that with the addition of DBP, the fluorescence intensity of BSA decreased regularly. These phenomena indicated that DBP could quench BSA intrinsic fluorescence. Quenching can occur by different mechanisms, which are usually classified as dynamic quenching and static quenching. For dynamic quenching, the decrease in intensity is usually described by the well-known Stern-Volmer equation.[20,21]

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

where F_0 and F are the fluorescence intensities in the absence and presence of quencher (triphenyltin), respectively, K_q is the bimolecular quenching constant, τ_0 is the lifetime of the fluorophore in the absence of quencher, [Q] is the concentration of quencher and K_{sv} is the Stern-Volmer quenching constant. According to the Stern-Volmer equation and our experimental results, the bimolecular quenching constant was calculated to be 1.88×10^{11} L·mol^{-1} s^{-1} based on the average fluorescence lifetime of a biopolymer is about 10^{-8}s. Generally, the collisional quenching constant of various kinds of quenchers with a biopolymer is 2.0×10.[10][22, 23] However, the rate constant of the protein quenching procedure initiated by DBP is greater than the collisional quenching constant. It can be concluded that the quenching process is static quenching, which is similar to Bai's result.[16]

Binding constants and binding points

In the static quenching process, if assuming that there are similar and independent binding sites in the biomolecule,

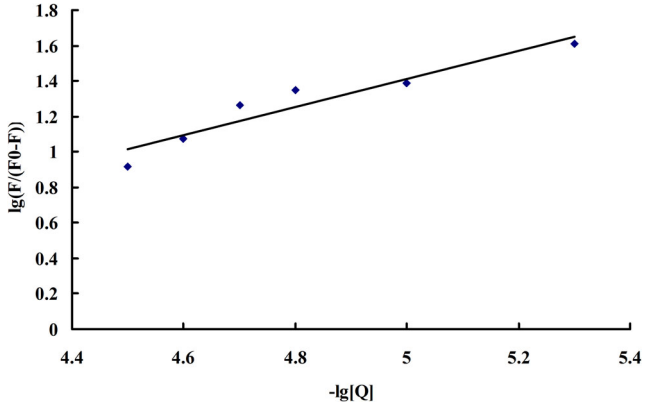

Figure 2. The number of binding sites n and equilibrium constant K of DBP-BSA.

the relationship between the fluorescence intensity and the quenching medium can be deducted from the following equation[24]:

$$nQ + B \rightarrow Q_nB \tag{2}$$

where B is the fluorophore, Q is the quencher, and Q_nB is the postulated complex between a fluorophore and n molecules of the quencher. The constant K is given by the following equation:

$$K = \frac{[Q_nB]}{[Q]^n[B]} \tag{3}$$

If the overall amount of biomolecules (bound or unbound with the quenchable molecule) is B_0, then $[B_0] = [Q_nB] + [B]$, where B is the concentration of unbound biomolecules. Since $[B]/[B_0] = F/F_0$, the relationship between the fluorescence intensity and the unbound biomolecules can be expressed as:

$$\lg\left(\frac{F_0 - F}{F}\right) = \lg K + n\lg[Q] \tag{4}$$

The Eq. (4) can be modified as follows:

$$\lg\left(\frac{F}{F_0 - F}\right) = n\lg[Q]^{-1} - \lg K \tag{5}$$

This implies that a plot of $\lg\left(\frac{F}{F_0-F}\right)$ versus –lg [Q] can be used to determine K and n. By making a plot shown in Figure 2, the value of K was found to be 0.11×10^2 and the value of n was noticed to be 0.52 at 298 K.

Types of the interaction force between BSA and DBP

The interaction forces between a compound and a biomolecule may involve hydrophobic force, electrostatic interaction, van der Waals interaction, hydrogen bonds, etc.[16] According to the data on enthalpy change (ΔH) and entropy change (ΔS), the model of interaction between a compound and a biomolecule can be concluded[25]:

1. $\Delta H > 0$ and $\Delta S > 0$, hydrophobic force,
2. $\Delta H < 0$ and $\Delta S < 0$, van der Waals interaction and hydrogen bonds,
3. $\Delta H < 0$ and $\Delta S > 0$, electrostatic interaction.

Table 1. Relative thermodynamic parameters of the system of DBP–BSA.

T (K)	ΔH (kJ mol^{-1})	ΔS (J K^{-1})	ΔG (kJ mol^{-1})
298	−11.52	18.72	−5.94
310		18.71	−5.72

In order to elucidate the interaction of DBP with BSA, the thermodynamic parameters were calculated from Eqs. (6–8). If the temperature does not vary significantly, the enthalpy change (ΔH) can be regarded as a constant. The free energy change (ΔG) can be estimated from the following equation, based on the binding constants at different temperatures:

$$\Delta G = -RT\ln K \tag{6}$$

where R is the gas constant, T is the experimental temperature, and K is the binding constant at the corresponding T. Then the enthalpy change (ΔH) and entropy change (ΔS) can be calculated from Eqs. (7) and (8):

$$\ln\left(\frac{K_2}{K_1}\right) = \left(\frac{1}{T_1} - \frac{1}{T_2}\right) \times \frac{\Delta H}{R} \tag{7}$$

where K_1 and K_2 are the binding constant at the experiment temperatures T_1 (298 K) and T_2 (310 K), respectively.

$$\Delta S = \frac{\Delta H - \Delta G}{T} \tag{8}$$

The results of thermodynamic parameters from the fluorescence quenching experiments are listed in Table 1. The negative value of free energy (ΔG) supports the assertion that the binding process is spontaneous. The positive enthalpy ($\Delta H > 0$) and entropy ($\Delta S > 0$) values of the interaction of DBP and BSA indicate that the hydrophobic force might play a major role in the binding between DBP and BSA.[26]

Energy transfer between DBP and BSA

Föster's theory (1948) of non-radiative energy transfer was used to determine the distances between the BSA residue and DBP bound to BSA. According to Föster's theory, the energy transfer effect is not only related to the distance between the donor (tryptophan residue) and acceptor (DBP) (see Figure 3), but also influenced by the critical energy transfer distance R_0. The value of E was calculated using Eqs. (9–11).

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{(R_0^6 + r^6)} \tag{9}$$

where F and F_0 are the fluorescence intensities of BSA in the presence and absence of DBP; r is the distance between acceptor and donor, and R_0 is the critical distance when the transfer efficiency is 50%.

$$R_0^6 = 8.8 \times 10^{-25} k^2 N^{-4} \Phi J \tag{10}$$

where k^2 is the spatial orientation factor of the dipole; N, is the refractive index of the medium; Φ, is the fluorescence quantum yield of the donor; and J is the overlap integral of the fluorescence emission spectrum of the donor and the

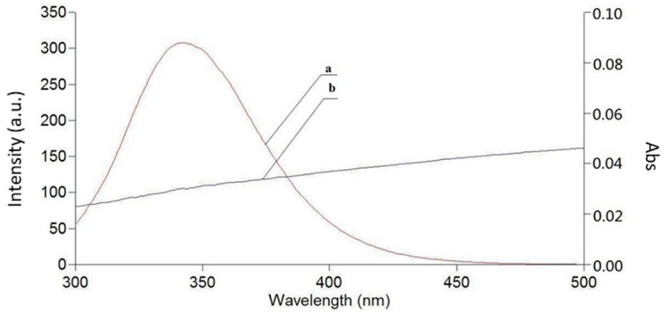

Figure 3. The fluorescence spectrum of BSA (a) and the absorption spectrum of DBP (b).

absorption spectrum of the acceptor J is given by:

$$J = \frac{\Sigma F(\lambda)\ \varepsilon(\lambda)\lambda^4 \Delta\lambda}{\Sigma F(\lambda)\Delta\lambda} \tag{11}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor of wavelength λ; and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ. In the present case, $k^2 = 2/3$, $N = 1.336$ and $\Phi = 0.118$. We could calculate that $J = 3.63 \times 10^{-17}$, $R_0^6 = 2.23 \times 10^{-42}$, $E = 3.22\%$ and $r = 2.02$ nm. The donor-to-acceptor distance ($r < 7$ nm) indicated that the energy transfer from BSA to DBP occurred with a high possibility.

Conformation investigation

Synchronous fluorescence spectroscopy

Synchronous fluorescence spectrometry (SFS) has become a standard tool to explore conformational changes in proteins due to the presence of other molecules or quenchers. The spectra are obtained through the simultaneous scanning of the excitation and the emission monochromators of a spectrofluorimeter, with a fixed wavelength difference ($\Delta\lambda$) between them.[27] It can provide information about the molecular environment in the vicinity of chromosphere molecules. By measuring the shift of the maximal emission wavelength, corresponding changes in the polarity around the fluorophores can be estimated.[27,28] When $\Delta\lambda$ between excitation wavelength and emission wavelength was fixed at 60 or 15 nm, the synchronous fluorescence provides the characteristic information of tryptophan residues or tyrosine residues. The synchronous fluorescence spectra of BSA upon the addition of DBP gained at $\Delta\lambda = 15$ and 60 nm are shown in Figure 4a and b. It is apparent from Figure 4a and b that the emission maximum has an evident shift of tyrosine residues and tryptophan residues upon the addition of DBP, which indicates that the conformation of BSA was changed.

Three-dimensional fluorescence spectroscopy

To further discuss the conformational change of BSA after the addition of DBP, the three-dimensional fluorescence spectra were measured (see Figure 5a and b). A huge peak1 was observed in Figure 5a, while peak1 decreased slightly after the addition of DBP in Figure 5b. In addition, the thin and long peak (peak a) was Rayleigh scattering ($\lambda_{ex}=\lambda_{em}$)

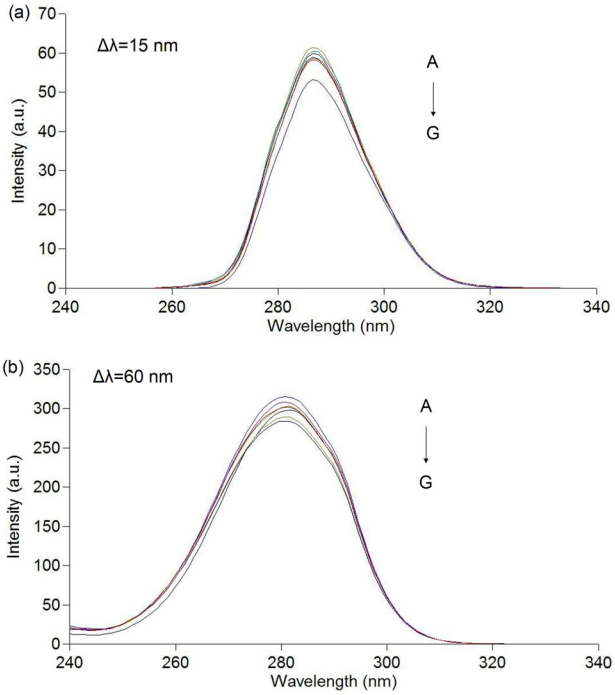

Figure 4. The changes of synchronous fluorescence intensity of DBP-BSA solutions with DBP concentration (4a, $\Delta\lambda = 15$ nm; 4 b, $\Delta\lambda = 60$ nm) (A = 0; B = 0.5×10^{-5}; C = 1.0×10^{-5}; D = 1.5×10^{-5}; E = 2.0×10^{-5}; F = 2.5×10^{-5}; G = 3.0×10^{-5} mol·L^{-1}).

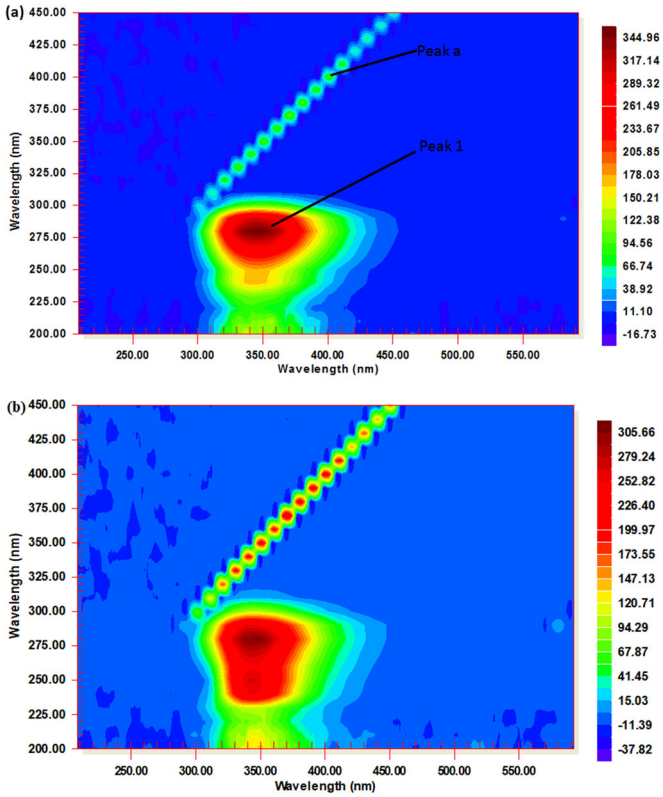

Figure 5. Three-dimensional fluorescence contour map of BSA (a) and DBP-BSA (b) system.

and it became stronger after adding DBP. The possible reason may be that a BSA–DBP complex came into being, which made the diameter of the macromolecule increased. As a result, the scattering effect was enhanced.[29] As the

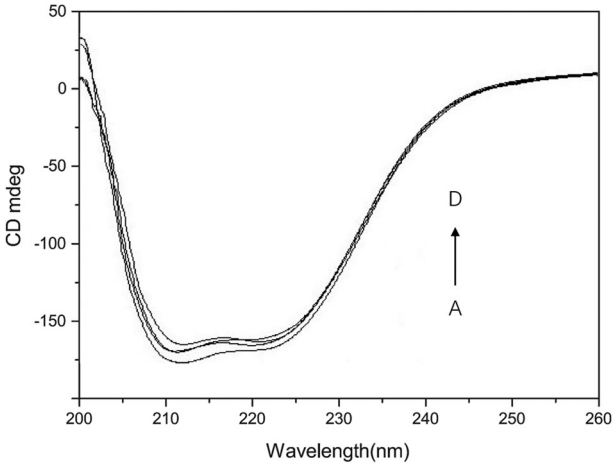

Figure 6. The change of CD spectra of DBP-BSA solution with pyrene concentration. The concentrations of DBP (A = 0; B = 1.0×10^{-6}; C = 1.0×10^{-5}; D = 1.0×10^{-4} mol·L^{-1}).

corresponding parameters are shown in Figures 5 and 6, peak1 decreased with the addition of DBP. That means the fluorescence peak of BSA was quenched by DBP. Peak1 mainly revealed the intrinsic fluorescence of tryptophan and tyrosine residues. It is known that the spatial structure of BSA consists of three domains and each domain consists of two subdomains to form a cylindrical structure. Almost all of the hydrophobic amino acid residues are embedded within the cylinder to form a hydrophobic cavity.[30] We guess the DBP molecule binding site might be in such a hydrophobic cavity. The introduction of the DBP molecule led to the change in the polarity of the hydrophobic microenvironment, which caused the conformational change of BSA.

CD spectroscopy

CD spectroscopy is an essential quantitative analytical technique used to analyze the conformational structure of proteins in an aqueous solution.[31,32] The high content of α-helices in BSA was revealed by the two minima around 208 and 222 nm.[33] The reasonable explanation is that the negative peaks between 208-209 nm and 222-223 nm are both contributed to an n→π^* transfer for the peptide bond within the α-helix.[32] The CD spectrum of BSA in the absence and presence of different DBP with varying concentrations is shown in Figure 6. As expected, the α-helices of BSA show a strong double minimum at 220 and 208 nm, which is the typical α-helix structure of BSA. The intensities of this double minimum reflect the amount of helicity of BSA. Upon the addition of DBP to BSA, the extent of BSA α-helices decreases. The decreased helicity suggests the binding of DBP with BSA induces a slight unfolding of the constitutive polypeptides of protein, which results in a conformational change of the protein that increases the exposure of some hydrophobic regions that were previously buried. This is in agreement with the synchronous fluorescence and three-dimensional fluorescence results.

Conclusions

This paper investigated the interaction between BSA and DBP in Tris-HCl buffer solution by use of the fluorescence technique. The results showed that the fluorescence of BSA was quenched by DBP through static quenching. The binding constant (K) and the binding points (n) were obtained to be 0.11×10^2 and 0.52 at 298 K, respectively. The acting forces between DBP and BSA were mainly hydrophobic forces. The average binding distance between the donor (BSA) and the acceptor (DBP) was 2.02 nm. These results of SFS indicate that DBP did not induce the conformational change of BSA.

This paper provided an approach for exploring the toxicity of chemical pollutants and serum albumin by fluorescence spectroscopy. The interaction between serum albumin and chemical pollutants can benefit researchers to understand the nature of poisons and guide people in the right way of first aid and detoxification. Hence, this study is greatly significant in the environmental toxicology and clinical medicine field.

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Data availability statement

The authors confirm that the original data supporting the findings of this study are available within the article or its supplementary materials.

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