Photophysical properties and dynamics simulation of the interaction between human serum albumin and hydroxy polybrominated diphenyl ether

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ABSTRACT

The absorption, distribution and metabolism of various ligands in organisms are particularly important for understanding biological systems and processes. Herein, this work presents the detail in the interaction between 3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether and human serum albumin. Small molecule of ligand cause the endogenous fluorescence of human serum albumin to be quenched, and the quenching mechanism includes static quenching, dynamic quenching and non-radiative energy transfer. Synchronous fluorescence and site competition experiments demonstrate that 3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether has one binding site with human serum albumin (site I). In addition, similar conclusions are obtained from the molecular docking experiment. Molecular dynamics confirms that human serum albumin bound with 3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether has more compact structure and increased hydrophobicity. Circular dichroism spectra exhibits that the $\alpha$-helix of the complex are reduced by 21.2% relative to free human serum albumin. More importantly, thermodynamic reveals that hydrophobic interaction is identified as the primary driving force for the formation of the complex. This work provides valuable reference for the mechanism of interaction between organic pollutants and human serum albumin by multiple-spectra and theoretical simulation.

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Introduction

Human serum albumin (HSA) is a globular single chain polypeptide containing 585 amino acids with the molecular weight of 66.8 kDa.\[1\] The whole structure is composed of three homologous domains (I, II and III), each could divide into two sub-domains (A and B).\[2,3\] It mainly exists in the human blood circulatory system, and plays critical functions in the maintenance of plasma pressure, nutrition balance, binding and transportation for different chemicals.\[4-6\] As a transporter, HSA is used as a model for the research of protein-ligand interactions.\[7-10\] The interaction between ligand and protein provides important information of pharmacological and toxicological through binding site, thermodynamic function and secondary structure transformation.\[11\]

Over the past decades, polybrominated diphenyl ethers (PBDEs) are widely used as flame retardants for fire protection equipment and electronic products due to their excellent flame retardant properties.\[12\] Unfortunately, abundant PBDEs enter and contaminate the environment through the elimination and incineration of electronic equipment. Besides, PBDEs will accumulate in soil and organisms due to their high chemical stability and good fat solubility. Many researchers report that PBDEs have neurotoxic, developmentally toxic and highly carcinogenic.\[13,14\] Humans and animals that long-term exposure the environment of PBDEs can cause serious health risks. More importantly, PBDEs will produce hydroxy polybrominated diphenyl ethers (OH-PBDEs) in the liver cells of animals. According to reports, OH-PBDEs have higher cytotoxicity and higher affinity than their parent.\[15\] Therefore, it is especially important to understand the toxicological effects of OH-PBDEs.
and their absorption, transport and metabolism in human body.

The toxicity of PBDEs decreases as the number of Br atoms increases.\(^{[16–18]}\) Therefore, 2,2',4,4'-tetrabromodiphenyl ether (BDE-47), which is more toxic than its homologues. However, 3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether (3-OH-BDE-47) is a metabolite of BDE-47 and has higher toxicity than it.\(^{[19,20]}\) Herein, we report the multispectral method combined with computational simulation to investigate the interaction mechanism of HSA and 3-OH-BDE-47. Our works were divided into three parts: (1) The binding site was studied by synchronous fluorescence, site competition experiment and molecular docking simulation. (2) The secondary structure of 3-OH-BDE-47 binding to HSA was determined by circular dichroism (CD) spectra and molecular dynamics (MD) simulation. (3) The thermodynamic behavior between 3-OH-BDE-47 and HSA was investigated by molecular mechanics/Poisson-Boltzmann surface area (MM-PBSA).\(^{[21]}\)

**Materials and methods**

**Reagents**

HSA (purity >98%), Warfarin (purity ≥98%) and Ibuprofen (purity ≥99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-OH-BDE-47 (>98%, 50 μg/mL) was purchased from AccuStandard, Inc. (New Haven, U.S.A.). Other reagents were of analytical-reagent grade, and double-distilled water was used throughout the experiments. The concentration of Tris(hydroxymethyl)methyl aminomethane-Hydrochloric acid (Tris-HCl) buffer (pH = 7.40, and 0.9% NaCl was added to maintain the ionic strength) was 1.0 × 10\(^{-3}\) M, and kept in the dark at 4 °C. The concentrations of HSA and 3-OH-BDE-47 were 1.0 × 10\(^{-5}\) M and 1.0 × 10\(^{-6}\) M, respectively.

**Fluorescence measurements**

All fluorescent spectra measurements were taken on a F-7000 fluorescence spectrophotometer (Hitachi, Japan) quipped with a xenon lamp source and 1.0 cm quartz cell. Temperature was controlled at 3 different temperatures (293 K, 298 K, and 303 K) using a water bath temperature controller instrument with a rubber tube and stirrer. The excitation wavelength was set at 295 nm, and the range of emission wavelength was recorded at 290–500 nm, ignoring the inner filter effect of fluorescence.\(^{[22]}\) The excitation and emission slit widths were set at 2.5 nm and 5 nm, respectively. The fluorescence measurements were obtained at pH 7.4 using a fixed concentration of HSA (1.0 × 10\(^{-5}\) M) in the presence of different concentrations of 3-OH-BDE-47 solution (0, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 × 10\(^{-6}\) M).

The synchronous fluorescence spectrometry of HSA in the absence 3-OH-BDE-47 and the presence of the gradually increasing amount of 3-OH-BDE-47 was examined with constant differences of Δλ = 60 nm (Trp) and 15 nm (Tyr) between the excitation and emission monochromators, respectively. Site competitive experiments were carried out using warfarin and ibuprofen as fluorescent probes with fluorescence titration methods. Warfarin has a strong binding ability at HSA site I, while ibuprofen mainly binds at HSA site II. Prepared 1.0 × 10\(^{-6}\) M HSA-warfarin/ibuprofen solution. Then gradually added 3-OH-BDE-47 to the HSA-warfarin and HSA-ibuprofen solutions. Chose an excitation wavelength of 285 nm, set the excitation and emission slit widths to 5 nm, and measured the fluorescence intensity of the system.

**Ultraviolet-visible absorption spectrum**

The OH-BDE-47 investigated by UV–Vis spectrophotometer (TU-1901, Puxi Analytical Instruments Ltd., Beijing China) under the reference of 1.0 × 10\(^{-5}\) M Tris-HCl buffer solution. A quartz cell with an optical path of 1 cm was used to record the absorption spectrum in the wavelength range of 300–450 nm at 298 K.

**Fluorescence lifetime measurements**

Fluorescence lifetimes were measured by time-correlated single-photon counting system (TCSPC) from FL3P spectrometer (HORIBA Jobin Yvon CO, France) and with λ\(_{\text{ex}}\) = 295 nm. The decay curves were fitted to a biexponential function by DAS-6 decay analysis software using reduced \(\chi^2\) criterion and visual inspection of the
residuals as parameters. The instrument response function (IRF) was gauged at the excitation wavelength. The time-resolved fluorescence intensity decays were analyzed by deconvoluting the observed decays with the IRF and manifested as a sum of two exponentials through Eq. (1).

Average fluorescence lifetimes ($\tau$) for two exponential iterative fittings were calculated from the decay times and the normalized preexponential factors using the following Eqs. (1) and (2): \[ I(t) = \sum_{i=1}^{2} f_i \exp \left( -t/\tau_i \right) \] \[ \langle \tau \rangle = a_1 \tau_1 + a_2 \tau_2 \]

where $I(t)$ is the fluorescence intensity at time $t$, and $f_i$ is the amplitude associated with the fluorescence lifetime $\tau_i$. $a_1$, $a_2$ are the pre-exponential factor of first and second component, respectively.

Circular dichroism spectra

Circular dichroism (CD) spectra were recorded by a quartz cell with a path length of 1 cm with a Jasco-810 spectropolarimeter (Jasco, Tokyo, Japan) at room temperature. Measurements were taken at wavelengths from 195 to 250 nm with a scanning speed of 100 nm/min and changes in its secondary structure were measured. Each spectrum was corrected by subtracting the CD spectra of the corresponding buffer solution. All observed results at 208 nm are expressed as the mean residue ellipticity (MRE), which is defined by the following equation:

\[ MRE_{208} = \theta_{obs}/(10nC_{HSA}), \]

where $\theta_{obs}$ is the CD in millidegrees at 208 nm, $n$ is the number of amino acid residues, $l$ is the path length of the cell, and $C_{HSA}$ is the molar concentration of HSA. The percentage of $\alpha$-helix content was calculated from the MRE$_{208}$ using the following equation:

\[ \alpha - \text{helix} \, (\%) = \left( \frac{4000 + MRE_{208}}{33,000 - 4000} \right) \times 100 \]

Computational simulations

The crystal structure of HSA (PDB ID: 1AO6, resolution 2.5 Å) was downloaded from the Brookhaven Protein Data Bank (http://www.rcsb.org/pdb/). 1AO6 was constructed of A and B two chains without any crystallized ligand;\cite{27} the A chain and water molecules were removed, and B chain was used in this work. The 3-OH-BDE-47 ligand for molecular docking was obtained from Chemdraw software and optimized by the DFT method under the B3LYP(6-31G) combinatorial basis set in Gaussian 09 software. The binding location of 3-OH-BDE-47 in HSA was investigated using the docking software AutoDock 4.2.\cite{29} The Lamarckian genetic algorithm (GA) of AutoDock software was used to produce 10 blind dockings for 3-OH-BDE-47 and HSA, and analyze different conformations.\cite{30}

Molecular dynamics simulation of HSA and its complexes was carried out from Amber 99SB force field under the GROMACS (ver.4.6.5) simulation package.\cite{27} All titratable residues of HSA were protonated in the PDB2PQR server (http://nbcr222.ucsd.edu/pdb2pqr_2.0.0/),\cite{28} which automated pipeline for the setup, execution, and analysis of Poisson–Boltzmann electrostatics calculations. The ligand of 3-OH-BDE-47 topology file (CID: 10885611) was generated from the Antechamber module in Amber Tools 16. Special protonation states were modified for titratable residues (Asp, Glu, His, Lys, and Arg) at pH 7.4 manually. Next, the complex was immersed in a cubic box of extended single point charge (SPC) water model. Added 16 Na$^+$ into the simulation to neutralize solvated system. Execute steepest descent algorithm to ensure the system energy was minimized for release conflicting contacts. Subsequently, under 1.0 bar pressure and 300 K, the position restraint procedure was performed along with canonical ensemble (NVT) and isothermal-isobaric ensemble (NPT) ensembles for 1 ns. The Particle–Mesh–Ewald method was used to calculate electrostatic interactions. The visual analysis of HSA secondary structure was carried out by the VMD (ver.1.9.1) software package.

Results and discussion

Fluorescence quenching mechanism

The intrinsic fluorescence of HSA is almost entirely due to the Trp-214 residue alone.\cite{31} Fig. 1a shows the effect of 3-OH-BDE-47 on the
fluorescence emission spectra of HSA. At 298 K, with the increase of 3-OH-BDE-47, the fluorescence of HSA is regularly quenched. The fluorescence quenching mechanism is mainly divided into static quenching, dynamic quenching and non-radiative energy transfer.[32] The Stern–Volmer equations can be used for calculations.[33]

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

(5)

where \(F_0\) and \(F\) indicate the fluorescence emission intensities of HSA in the presence and absence of the quencher, respectively; \(K_{sv}\) is the fluorescence quenching rate constant, \(K_q\) is the bimolecular quenching rate constant, and the \(\tau_0\) is the endogenous fluorescence lifetime of the protein molecule before addition of quencher, approximately \(10^{-8}\) s; [\(Q\)] is the concentration of ligand small molecules.

Table 1 presents the quenching rate constants \(K_{sv}\) of 3-OH-BDE-47 with HSA at three temperatures. \(K_{sv}\) gradually increases as the temperature rises, and exhibits a good linear relationship (Fig. 1b). This indicates that the fluorescence quenching mechanism of 3-OH-BDE-47 and HSA is dynamic quenching. However, the values of \(K_q\)
are larger than 2.0 × 10^{10} \text{M}^{-1} \text{s}^{-1} (maximum collision rate constant) and the effect of temperature on \(K_{sv}\) is not obvious, indicating that the quenching mechanism of 3-OH-BDE-47 and HSA is combination of static and dynamic quenching mechanism (mixed quenching mechanism).

Therefore, the modified Stern–Volmer equation can be used for further calculations.\(^{35-37}\)

\[
F_0/(F_0 - F) = 1/f_a + 1/(f_a K_a(Q)) \quad (6)
\]

\[
\log [(F_0 - F)/F] = \log K_b + n\log[Q] \quad (7)
\]

where \(K_a\) and \(f_a\) are the effective quenching constant for the available fluorophores and the fraction of the nearby fluorophores, respectively; \(K_b\) is the binding constant of 3-OH-BDE-47 with HSA; The \(n\) is the number of binding sites.

The plot of \(F_0/(F_0 - F)\) vs \(1/(Q)\) yields \(1/f_a\) and \(1/(f_a K_a)\) as the intercept and the slope of the plot, respectively (Fig. 1c). The \(K_a\) value increases with the increase of temperature (Table 2), so it can further indicate that there is dynamic quenching between 3-OH-BDE-47 and HSA. Fig. 1d exhibits plotting \(\log [(F_0 - F)/F] vs \log[Q]\). It can be seen from Table 3 that the \(K_b\) value also increases with the increase of temperature, and the \(K_b\) value is larger than 10^{7}, indicating that 3-OH-BDE-47 has a strong binding force with HSA. Besides, the combined complex of 3-OH-BDE-47 and HSA becomes more stable as the temperature rises. The value of \(n\) is approximately 1, showing that there is only one binding site between 3-OH-BDE-47 and HSA.

### Table 1. The fluorescence quenching constants of 3-OH-BDE-47 with HSA at different temperatures.

| Compound      | \(T (K)\) | \(K_a (M^{-1})\) | \(K_b (M^{-1} \cdot S^{-1})\) | \(R^2\) |
|---------------|-----------|------------------|-----------------------------|---------|
| 3-OH-BDE-47   | 293       | 3.29 × 10^{10}   | 3.29 × 10^{14}              | 0.9871  |
| 291           | 5.02 × 10^{10} | 5.02 × 10^{14} | 0.9888                      |
| 303           | 6.80 × 10^{10} | 6.80 × 10^{14} | 0.9877                      |

**HSA**: Human serum albumin; 3-OH-BDE-47: 3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether; \(T\): temperature (Kelvin); \(K_a\): dynamic quenching constant; \(K_b\): the bimolecular quenching rate constant; \(R^2\): linear correlation coefficient; \(M\): mole.

### Table 2. Modified Stern–Volmer association constant (\(K_a\)) for 3-OH-BDE-47 with HSA and the relative thermodynamic parameters at three different temperatures.

| Compound      | \(T (K)\) | \(K_a (M^{-1})\) | \(\Delta H (kJ \cdot mol^{-1})\) | \(\Delta G (kJ \cdot mol^{-1})\) | \(\Delta S (J \cdot mol^{-1} \cdot K^{-1})\) |
|---------------|-----------|------------------|-------------------------------|-------------------------------|----------------------------------|
| 3-OH-BDE-47   | 293       | 1.29 × 10^{12}   | 170.12                        | −39.89                        | 714.43                           |
| 291           | 1.86 × 10^{12} | 0.9971           | −41.47                        | −47.01                        |
| 303           | 1.27 × 10^{12} | 0.9999           |                                |                                |

**HSA**: Human serum albumin; 3-OH-BDE-47: 3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether; \(T\): thermodynamic temperature; \(K\): temperature unit (Kelvin); \(K_a\): effective quenching constant for the available fluorophores; \(M\): mole; \(R^2\): linear correlation coefficient; \(\Delta H\): enthalpy change; \(\Delta G\): Gibbs free Energy; \(\Delta S\): entropy change.

### Time-resolved fluorescence spectra

The fluorescence lifetimes are very sensitive to the micro polarity of the adjacent environment of the fluorophore residue and to the excited state interactions between ligand and protein.\(^{38}\) Similarly, the fluorescence lifetime measurement is the most classic method to distinguish static and dynamic quenching.\(^{39}\)

Fig. 2a demonstrates time-resolved fluorescence decay profile of HSA in the presence and absence of 3-OH-BDE-47. The lifetime of Trp-214 in HSA gradually decreases upon the addition of 3-OH-BDE-47. The average lifetime of HSA decreases marginally from 4.814 ns to 4.330 ns (Table 4). This result exhibits that quenching process of HSA and 3-OH-BDE-47 is dynamic quenching, which was consistent with the conclusion of intrinsic fluorescence conclusion of intrinsic fluorescence.

### Non-radiative energy transfer

Generally, fluorescence resonance energy transfer (FRET) occurs whenever the emission spectrum of a fluorophore overlaps with the absorption spectrum of another molecule. In order to further confirm FRET happened between HSA and 3-OH-BDE-47. The Förster dipole-dipole non-radiative energy transfer formulas were used for calculated.\(^{40,41}\)

\[
E = 1 - F/F_0 = R_0^6 / (R_0^6 + r^6) \quad (7)
\]

\[
R_0^6 = 8.79 \times 10^{23} K^2 N^{-4} \phi J \quad (8)
\]

\[
J = [\Sigma F(\lambda)\varepsilon(\lambda) \lambda^4 \Delta \lambda] / [\Sigma F(\lambda) \lambda^4] \quad (9)
\]

where \(E\) is the efficiency of energy transfer, \(r\) is the distance between a donor and an acceptor, \(R_0\) is the critical distance at which transfer efficiency equals to 50%; \(K^2\) is the orientation factor related to the geometry of the donor-acceptor dipole (\(K^2 = 1.5\)); \(N\) is the refractive index of medium (\(N = 1.36\)), \(\Phi\) is the fluorescence quantum yield.
of the donor ($\Phi = 1.36$); $J$ expresses the degree of spectral overlap between the donor emission and the acceptor absorption. $F(\lambda)$ is the fluorescence intensity of the donor at wavelength range $\lambda$, and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength $\lambda$.

Table 3. Binding constants and the number of binding sites $n$ for the interaction of 3-OH-BDE-47 with HSA at three different temperatures.

| Compound    | $T$ (K) | $K_b$ (M$^{-1}$) | $n$  | $R^2$  |
|-------------|---------|------------------|------|--------|
| 3-OH-BDE-47 | 293 K   | $1.47 \times 10^2$ | 1.08 | 0.9933 |
|             | 298 K   | $2.22 \times 10^2$ | 1.08 | 0.9934 |
|             | 303 K   | $1.89 \times 10^2$ | 1.17 | 0.9967 |

HSA: Human serum albumin; 3-OH-BDE-47: 3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether; $T$: thermodynamic temperature; $K_b$: the binding constants; $n$: number of binding sites; $K$: temperature unit (Kelvin); $M$: mole; $R$: linear correlation coefficient.

Fig. 2b presents the overlap between the fluorescence spectrum of HSA and the UV absorption spectrum of 3-OH-BDE-47. Calculated by the formulas (7–9), the overlap integral $J$ of the two spectral overlap regions is $9.74 \times 10^{-14}$ M$^{-1}$ cm$^3$; Energy transfer efficiency $E$ is 30%; The critical distance $R_0$ equal 3.73 nm; The distance ($r$) between Trp-214 residue and 3-OH-BDE-47 is 4.28 nm. In addition, $r < 7$ nm and $0.5 R_0 \leq r \leq 1.5 R_0$.

Figure 2. (a) The fluorescence decay curve of HSA (1.0 $\times$ 10$^{-5}$ M) fitting graph of 3-OH-BDE-47 (0–2.0 $\times$ 10$^{-6}$ M, with regularly increments of 5.0 $\times$ 10$^{-7}$ M). (b) Spectral overlap between the fluorescence emission spectrum of HSA (1.0 $\times$ 10$^{-6}$ M) and the absorption spectrum of 3-OH-BDE-47 (1.0 $\times$ 10$^{-6}$ M), the absorption spectrum in the wavelength range of 300–500 nm as obtained in Tris-HCl buffer (1.0 $\times$ 10$^{-3}$ M, pH 7.4) at 298 K. (c) The synchronous fluorescence spectra of HSA in the absence and presence of 3-OH-BDE-47, $\Delta \lambda = 15$ nm; The concentration of albumin was 1.0 $\times$ 10$^{-5}$ M while the concentrations of 3-OH-BDE-47 (0–1.0 $\times$ 10$^{-8}$ M, with regularly increments of 1.0 $\times$ 10$^{-9}$ M). (d) The synchronous fluorescence spectra of HSA in the absence and presence of 3-OH-BDE-47, $\Delta \lambda = 60$ nm; The concentration of albumin was 1.0 $\times$ 10$^{-6}$ M while the concentrations of 3-OH-BDE-47 (0–1.0 $\times$ 10$^{-8}$ M, with regularly increments of 1.0 $\times$ 10$^{-9}$ M). HSA: human serum albumin; 3-OH-BDE-47: 3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether; nm: nanometer; M: mole; K: temperature (Kelvin); IRF: instrument response function; $\lambda$: wavelength; $F_0$: HSA fluorescence signal without 3-OH-BDE-47; $F$: HSA fluorescence signal in the presence of 3-OH-BDE-47.
indicating the non-radiative energy transfer occurs when 3-OH-BDE-47 is binding to HSA.\[30\]

**Synchronous fluorescence spectra**

Synchronous fluorescence spectroscopy is a technique to explore the change in the molecular environment of fluorophore residues.\[42\] In addition, synchronous fluorescence has the advantages like high sensitivity, good selectivity and less interference.\[43\] The difference $\Delta \lambda$ between the excitation and emission wavelengths of the fluorescence spectra of Tyr (tyrosine) and Trp (tryptophan) residues is 15 and 60 nm, respectively. Fig. 2c and d is synchronous fluorescence spectroscopy of 3-OH-BDE-47-HSA complex at the difference between excitation and emission wavelength of $\Delta \lambda = 15 \text{ nm}$ and $\Delta \lambda = 60 \text{ nm}$, respectively. As the content of small molecules increases, the fluorescence of HSA is quenched, and the maximum emission wavelength is blue-shifted. More importantly, the fluorescence quenching amplitude of Trp residues is more obvious than that of Tyr residues. This indicates that 3-OH-BDE-47 enters the cavity I of HSA, causing the microenvironment inside HSA to change.

**Binding site competition experiment**

Competition experiment is an effective and quick method to judge the binding area and binding site of small drug molecule and protein.\[44\] In this work, warfarin and ibuprofen were used as fluorescent probes for the binding site, respectively. Warfarin can specifically bind to HSA site I, while ibuprofen mainly binds in the cavity of HSA site II.\[45\] Added an amount of 3-OH-BDE-47 solution (0–1.0 $\times 10^{-8}$ M, with regularly increments of 2.0 $\times 10^{-9}$ M) to 1.0 $\times 10^{-6}$ M HSA-warfarin/ibuprofen solution. Then calculate the binding constant of the competition experiment according to the modified Stern–Volmer Eq. (6). The results obtained are shown in Table 5. When 3-OH-BDE-47 was added to the HSA-ibuprofen solution, the binding constant was sharp reduced. This indicated that 3-OH-BDE-47 occupies the warfarin site (site I). The results obtained were consistent with the results of synchronous fluorescence.

**Thermodynamic parameters**

The types of interactions between proteins and ligands include van der Waals forces, electrostatic interactions, hydrophobic interaction forces and hydrogen bonding forces.\[46\] According to Ross et al.,\[47\] it was shown that when $\Delta H > 0$, $\Delta S > 0$, it showed hydrophobic force; when $\Delta H < 0$, $\Delta S < 0$, it mainly showed hydrogen bond and van der Waals force; when $\Delta H < 0$, $\Delta S > 0$ is based on electrostatic attraction. The values of $\Delta G$, $\Delta H$, and $\Delta S$ for each system were calculated by thermodynamic formulas (10–12).

\[
\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (10)
\]

\[
\Delta G = -RT \ln K_a \quad (11)
\]

\[
\Delta G = \Delta H - T \Delta S \quad (12)
\]

where $R$ is the gas constant, $T$ is the experimental temperature, and $K$ is analogous to the effective quenching constants $K_a$ at the corresponding temperature.

Table 2 presents the thermodynamic parameters of 3-OH-BDE-47 and HSA at different

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**Table 4.** Lifetime of fluorescence decay of HSA at various concentrations of 3-OH-BDE-47.

| Protein | $[\text{C}_{3-\text{OH-BDE-47}}] \times 10^{-6}$ M | $\tau_1$ | $\tau_2$ | $\alpha_1$ | $\alpha_2$ | $<\tau>$ | $\chi^2$ |
|---------|---------------------------------|-----|-----|-----|-----|-----|-----|
| HSA     | 0.0                             | 4.053 | 13.294 | 0.9176 | 0.0824 | 4.814 | 1.247 |
|         | 1.0                             | 3.954 | 6.790 | 0.7087 | 0.2913 | 4.780 | 1.044 |
|         | 1.5                             | 3.463 | 5.510 | 0.4581 | 0.5419 | 4.572 | 1.012 |
|         | 2.0                             | 3.205 | 3.178 | 0.4298 | 0.5702 | 4.330 | 1.071 |

HSA: Human serum albumin; 3-OH-BDE-47: 3-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether; $\tau_1$, $\tau_2$: double exponential kinetic parameters, corresponding decay times; $\alpha_1$, $\alpha_2$ are the pre-exponential factor of first and second component, respectively; $<\tau>$: Average fluorescence lifetime of HSA protein; $\chi^2$ is the goodness-of-fit.

**Table 5.** Competitive binding constant of 3-OH-BDE-47-HSA complex at 298 K.

| Compound           | $T$ (K) | $K_a$ (M$^{-1}$) | Warfarin (M$^{-1}$) | Ibuprofen (M$^{-1}$) |
|--------------------|---------|-----------------|---------------------|----------------------|
| 3-OH-BDE-47 HSA    | 298 K   | 1.86 $\times 10^7$ | 3.96 $\times 10^6$  | 1.66 $\times 10^6$   |

HSA: Human serum albumin; 3-OH-BDE-47: 3-hydroxy-2,2′,4,4′-tetrabromodiphenyl ether; $T$: temperature unit (Kelvin); $M$: mole.
temperature. The value of $\Delta G$ at three temperature of 3-OH-BDE-47 and HSA is less than 0. This indicate that the process of binding the two substances can be spontaneous. However, the $\Delta S$ and $\Delta H$ are always larger than 0. Therefore, according to the rules,[47] the main force of binding of 3-OH-BDE-47 to HSA is the hydrophobic interaction force.

**Molecular docking**

AutoDock software was investigated the docking posture and residue for the interaction between 3-OH-BDE-47 and HSA. Fig. 3a and b exhibits 3-OH-BDE-47 inserted into the hydrophobic cavity of subdomain IIA in the molecular surface diagram of HSA by polarity (hydrophobic or hydrophilic). The results show the docking of 3-OH-BDE-47 with HSA nearby amino acid residue (Fig. 3c). 3-OH-BDE-47 was surrounded by Trp-214, Tyr-150, Lys-199, Glu-153, Ser-192, Lys-195, Gln-198, Arg-222, Leu-238, His-242, Arg-257, Ala-291 and Glu-292. Among them, the Arg-222 residue could form intermolecular hydrogen bonds with H and the Ala-291 residue also formed a hydrogen bond with O. The hydrogen bond distances were 1.825 Å and 1.998 Å, respectively. Thus, hydrogen bonding is an important factor in HSA–3-OH-BDE-47 complex stability in addition to the vdW forces. At the same time, the results shown by molecular docking provide a reliable theoretical basis for

**Figure 3.** (a) 3-OH-BDE-47 binds in the cavity of site I of HSA. The blue area in the figure represents the hydrophilic area, and the orange represents the hydrophobic area; (b) The secondary structure diagram of 3-OH-BDE-47 binding in the cavity of HSA at site I. Different colors in the protein secondary structure represent different regions of HSA; (c) 3-OH-BDE-47 interacts with the amino acid residues in the cavity of HSA site I. The green short line represents the hydrogen bond between 3-OH-BDE-47 and Arg222 and Ala291, and 1.825 Å and 1.998 Å represent the hydrogen bond distance. HSA: human serum albumin; 3-OH-BDE-47: 3-hydroxy-2,2',4,4'-tetrabromodiphenyl ether.
synchronous fluorescence experiment and competition experiment.

**Molecular dynamics simulation**

Fig. 4a demonstrates the root mean square deviation (RMSD) of HSA and HSA-3-OH-BDE-47 complex. The backbone of two system becoming steady after 20 ns during the simulation. The composite system increases about 0.2 nm (from 0.38 nm to 0.58 nm) compared to the free HSA system from simulation of the last 20 nm. The radius of gyration (Rg) can represent the compactness of the protein structure. Two systems reach equilibrium after 30 nm, and the Rg values of complex are smaller than free HSA (Fig. S1, Supporting Information). This indicates that 3-OH-BDE-47 enters the protein cavity to shrink the structure of HSA, which in turn changes the HSA secondary structure.

Root mean square fluctuation (RMSF) is an important parameter to research protein residue fluctuation and mobility. The box within the range of residues for subdomains IIA presents that subdomains IIB and IIB of HSA have the highest fluctuations, while subdomains IIA present less fluctuation (Fig. 4b). Furthermore, according to the fluctuation characteristics of the residues in the IIA subdomain, the RMSF values of Arg-222, His-242, Arg-257, His-288, Ala-291, and Glu-292, respectively (Fig. 4c). The RMSF of amino acid residues in the IIA subdomain changed. The results show that 3-OH-BDE-47 binds within this subdomain and validates the results of molecular docking. In order to verify the accuracy and reliability of the results, we performed repeated simulation experiments (Figs S2 and S3).

Solvent accessible surface area (SASA) is an important parameter to measure the hydrophilicity/hydrophobicity of a protein. Fig. 5(a) shows the SASA of free HSA and HSA-3-OH-BDE-47 complex two systems. The two systems reached equilibrium after 37 nm, and the SASA value of the complex is less than the free HSA. This result demonstrates that with the addition of ligand, the hydrophobicity of the complex system increases and becomes more and more stable.
Secondary structure

CD spectrum is an important method for measuring secondary and tertiary structure of the protein.\(^{48,49}\) The CD spectra of HSA exhibited two negative ellipticities corresponding to the \(\pi-\pi^*\) and \(n-\pi^*\) transition at 208 nm and 222 nm, respectively.\(^{50}\) Fig. 5b presents the CD spectra of HSA and HSA-3-OH-BDE-47 complexes. The \(\alpha\)-helical content of HSA was calculated from the MRE value using the CDPro software in the CDSSTR program.\(^{25}\) With the increase of 3-OH-BDE-47, the CD spectra curves of the system have changed. The \(\alpha\)-helix contents of HSA were decreased from 63.7% to 42.5% upon addition of 3-OH-BDE-47 at a molar concentration ratio of 1:10. According to the CD spectra and previous experiments, it can be proved that 3-OH-BDE-47 enters and binds to the cavity at HSA site I, resulting in the content of \(\alpha\)-helical in the HSA secondary decreasing and content of unordered structure increasing.

The \textit{do_dssp} program for molecular dynamics simulation was determined the secondary structure transition process of HSA (Table 6). There was some deviation in the structure of HSA-3-OH-BDE-47 complex and free HSA. The percentage of \(\alpha\)-helix decreased from 70% to 69%. In addition, there are significant changes in the annular area of the helical connection, such as coils (from 15% to 14%) or turns (from 7% to 8%).

Binding free-energy analysis

MM-PBSA method is usually used to calculate the binding free-energy of complex. The average data was analyzed and extracted from the simulated trajectories of 30–50 ns (Fig. 6a). Herein, \(\Delta G_{\text{np}}\) (non-polar solvation energy) and \(\Delta G_{\text{vdW}}\) (van der Waals energy) were less than 0, and \(\Delta G_{\text{nonpolar}}\) (nonpolar interaction) is equal to \(-128.06 \pm 9.91\) kJ/mol in the complex, indicating that there is a favorable hydrophobic interaction during the binding process. Similarly, the electrostatic interaction between 3-OH-BDE-47 and HSA due to \(\Delta G_{\text{elect}}\) (electrostatic energy) < 0. However, \(\Delta G_{\text{PB}}\) (electrostatic solvation energy) > 0 and \(\Delta G_{\text{polar}}\) (polar interaction) equal to \(36.27 \pm 17.17\) kJ/mol means that the electrostatic interaction is unfavorable for complex formation.\(^{51,52}\) Finally, the total binding free-energy \(\Delta G_{\text{bind}}\) for the complex was estimated to be \(-91.79 \pm 27.08\) kJ/mol. The first iteration of the simulated data is in good agreement with the original results (Fig. S4), and the experimental data obtained is also controlled within the error range. Although the second repeated experiment showed a big difference, similar conclusions were obtained by analyzing the experimental data (Fig. S5). Furthermore, the binding free-energy contributions of each residue are identified (Fig. 6b). Quantitatively evaluated from residues to indicate favorable (Ile-290, Leu-238, Tyr-150, and Ala-291) and unfavorable (Ser-192, His-242, Arg-218, Lys-195, Arg-257, and Glu-153). Remarkably, these
residues are mainly from the subdomain II of HSA (site I). More specifically, not all the residues are favorable for complex formation, which is important for the regulation of binding interactions.

Conclusions

In this work, the interaction between 3-OH-BDE-47 and HSA is studied by multiple spectroscopy techniques combined with theoretical simulation calculation. Firstly, steady-state fluorescence and time-resolved fluorescence indicated that the ligand causes HSA fluorescence to be quenched, and the quenching mechanism is mixed quenching. Secondly, The UV-Vis spectrum illustrates that non-radiative energy transfer occurs when the ligand binds to HSA. Subsequently, synchronous fluorescence and molecular docking shows that the ligand bound binds at the site I of HSA. Importantly, Site competition experiments show that 3-OH-BDE-47 has one binding site with HSA. Additionally, CD spectra provide structural information about the evolution of complexes and gave a possibility to explain the position of 3-OH-BDE-47 inside the hydrophobic pocket of HSA. The successful binding of 3-OH-BDE-47 to HSA is verified by MD simulations. Meanwhile, MM-PBSA analysis conclude that hydrophobic interaction is identified as the main driving force. Finally, even though our data do not correlate directly with in vivo bioassays, it lends significant insight into the interactions of HSA with toxic molecules and, further, these data may be useful for the development of pharmacologic and toxicologic research.

Disclosure statement

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