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**Synthesis of 2-aminonaphthalene-1-sulfonic acid Schiff bases and their interactions with human serum albumin**

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**Abstract**

**Objectives:** Human serum albumin (HSA) can bind and transport many substances to cells to meet various needs of the organism. The binding efficacy of HSA to these substances directly affects their functions. In this paper two Schiff base compounds were synthesized to explore the interaction between HSA and both compounds.

**Methods:** Fluorescence spectra and an AutoDock model were utilized to investigate the interaction mechanism and binding model between proteins and Schiff base products. The conformation change of HSA was detected by resonance light scattering and circular dichroism spectra.

**Results:** The two compounds bound easily with HSA, with binding constants of $10^4$. The binding sites for both compounds in HSA were within an appropriate distance for long-range interactions. Both compounds are accommodated in hydrophobic domains of HSA. However, electrostatic interactions and other supermolecular forces coexist between the compounds and protein. Binding of these compounds disturbed the protein secondary structure and caused a certain degree of destabilization.

**Conclusions:** The two Schiff base compounds can interact with HSA with high efficacy, which is helpful for explore the application of this type of Schiff base in biomedical research.

**Keywords:** conformation; human serum albumin; interaction; resonance light scattering; Schiff base compounds.

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**Introduction**

Human serum albumin (HSA) is a protein that exists in human blood, contributing nearly 80% of the osmotic pressure of blood [1]. It is also responsible for the maintenance of blood pH [2]. A main function of HSA is that it can bind many endogenous and exogenous substances and transport them to cells in vivo and in vitro to meet various needs of the organism [3]. The exogenous substances include drugs [4, 5], Schiff bases [6, 7], surfactants [8, 9], metal ions, and ionic liquids [10, 11]. HSA is composed of 585 amino acid residues and has a molecular weight of 66 kD. There are three domains, I, II, and III, and each is further divided into subdomains A and B. Two major specific binding sites were reported: site I in subdomain IIA and site II in subdomain IIIA [12]. The two specific sites could be discerned by a site I-specific probe (DA) and a site II-specific probe (DSS) [13]. In addition, subdomain IA was also reported to play an important role in drug binding [12]. The binding efficacy of HSA to drugs directly affects their functions of absorbing, distributing, and metabolizing in blood [14–16]. The binding efficacy of HSA to substances in return affects the conformation and further biological function of the protein [8, 9, 17]. Therefore, many studies on the binding capacity, binding site, and nature of the interactions between serum albumin and these substances have been conducted to elucidate the mechanism of action from the perspective of molecular theory.

Schiff bases have attracted considerable attention because they are widely used in medicine, catalysis, analytical chemistry, corrosion, and photochromic fields. Schiff bases have gained importance in medicinal and pharmaceutical fields due to their broad spectrum of biological activities, including antimicrobial [18], antifungal [19], antibacterial [20], anticancer [21], and antitumour [22] activities. Herein, two salicylic aldehyde derivative Schiff base compounds were synthesized, and the interactions between these compounds and HSA were explored by fluorescence spectra and the AutoDock model to evaluate the mechanism of action.
Experimental and methods

Reagents

Human serum albumin and 2-aminonaphthalene-1-sulfonic acid were obtained from Sigma-Aldrich (USA). Salicylaldehyde and vanillin were obtained from Pharmaceutical Group Chemical Reagent Co. LTD of China.

Methods

Synthesis

COMPOUND 1 (SAA): 2-Aminonaphthalene-1-sulfonic acid (0.4465 g) dissolved in a sodium hydroxide solution and a salicylaldehyde (0.3043 g) solution were mixed in a flask with a reflux condenser on a magnetic stirrer. The mixture was heated in a water bath and stirred for 3 h at 338 K. Then, the mixture was cooled, filtered, washed with ethanol a few times and heated dryly (yellow solid product) (Figure 1A).

Anal. calcd (%) for C_{17}H_{13}O_{4}NS (%): C, 62.37; H, 4.00; N, 4.28. Found (%): C, 62.56; H, 3.98; N, 4.11. MS (m/z): 327.00. Calcd 327.32. Mass spectrum, IR, ¹H NMR, and ¹³CNMR spectrum of SAA were shown in supplementary material (Figures S1, S3, S5, S7).

COMPOUND 2 (XAA): The procedure described above was repeated for vanillin (0.6050 g) instead of salicylaldehyde, and a yellow solid product was obtained (Figure 1B).

Anal. calcd (%) for C_{18}H_{15}O_{5}NS (%): C, 60.49; H, 4.23; N, 3.92. Found (%): C, 60.07; H, 4.12; N, 4.05. MS (m/z): 357.00. Calcd 357.38, Found 357.63. Mass spectrum, IR, ¹H NMR, and ¹³CNMR spectrum of XAA were shown in supplementary material (Figures S2, S4, S6, S8).

Spectra measurement

Fluorescence emission spectra were obtained on an F-4500 fluorescence spectrometer attached to a thermostatic circulating water bath set at the specified temperature. The excitation wavelength was 280 nm. The slit widths for excitation and emission were 5 nm. To eliminate the inner filter effect, which can reduce some emission of the fluorophore when the absorbing compound is introduced, the fluorescence intensity was corrected according to Eq. (1):

\[
F_{\text{corr}} = F_{\text{obsd}}10^{(A_1+A_2)/2}
\]

where \(F_{\text{corr}}\) is the corrected fluorescence intensity and \(F_{\text{obsd}}\) is the observed fluorescence intensity. \(A_1\) and \(A_2\) represent the absorbance of SAA (XAA) at the excitation wavelength and at emission wavelength, respectively [23, 24].

The effect of adding SAA (XAA) on HSA emission was measured after incubating the corresponding concentration of SAA (XAA) with HSA for 10 min to achieve equilibration.

The UV–vis absorption of SAA and XAA was measured by a 2250 spectrophotometer.

AutoDock simulating experiment

The structure of HSA (code: 1BJ5) was obtained from the Protein Data Bank. Docking was performed by using AutoDock 4.2 software. ChemDraw 18.0 was used to sketch the ligands, and energy minimization was performed using the MM2 force field. One hundred independent conformations of SAA (XAA) were obtained, and the results of the conformational clusters were exported according to the root-mean-square deviation (RMSD) criterion.

The most numerous conformations of the cluster were identified, in which 10 conformations for SAA and 12 for XAA were included. The docked conformation of the lowest energy was chosen from the cluster [25]. The MGLTools package and PyMOL viewer were used to examine the location of SAA (XAA) in HSA.

Circular dichroism measurements

CD spectra were recorded by a Bio-Logic CD spectrometer, model MOS-450 (BioLogic, France), using a 1 cm path length quartz cell. A wavelength range between 200 and 260 nm was scanned with a step size of 0.2 nm and a band width of 1 nm. Dry nitrogen gas was used to purge the machine before the measurements. The samples were prepared with fixed concentrations of HSA (5 µM). The effect of SAA or XAA (50 µM) on the ellipticity was recorded after incubating SAA (XAA) with HSA for 10 min prior to the CD spectra measurement.

SELCON3, CONTIN, and CDSSTR were used to analyse and estimate the secondary structure changes based on changes in the content of \(\alpha\)-helices, \(\beta\)-sheets, turns, and random coils.
Resonance light scattering

For the resonance light scattering (RLS) measurements, the excitation and emission were scanned simultaneously with $\Delta \lambda = 0$ nm from 220 to 600 nm with the same instrument used for the fluorescence measurements. The proteins were prepared with a fixed concentration of 5 µM. The solution containing a mixture of SAA or XAA (5 µM) with HSA was analysed after incubation for 10 min.

Results

Interaction of SAA (XAA) with HSA

Fluorescence quenching of HSA by SAA (XAA)

The change in intrinsic fluorescence of HSA upon addition of SAA was inspected to evaluate the interaction between HSA and SAA. The intrinsic fluorescence of HSA near 340 nm gradually decreased accompanied by a small blueshift upon gradual addition of SAA, and this was accompanied by fluorescence enhancement at 400 nm. An iso-fluorescence point at 380 nm was found. Similar results were observed for XAA (Figure 2A,B). HSA is a globular protein with a single tryptophan residue and 18 tyrosine residues, which are responsible for the intrinsic fluorescence. Because of the sensitivity of tryptophan to its microenvironment, the wavelength shift should be attributed to SAA (XAA) binding to HSA and altering the local surroundings of the tryptophan [5, 11]. A blueshift indicates that the tryptophan residue is shifted to a more hydrophobic environment. SAA and XAA, with a naphthalene ring, are predominantly hydrophobic species. Complex formation appears to increase the hydrophobicity in the microenvironment of tryptophan residues [26].

Two types of quenching mechanisms are often involved in the decrease in the emission of proteins. This quenching can be either static by forming a ground state complex or dynamic by a collision process in the excited state [27]. The effect of the static or dynamic quenching mechanism on the observed $K_{sv}$ constants in the Stern-Volmer Eq. (2) can be used to further differentiate ligand binding.

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

In this equation, $F_0$ and $F$ are the fluorescence intensities in the absence and presence of ligands, respectively, $[Q]$ is the concentration of ligand, $k_q$ is the bimolecular quenching constant, and $\tau_0$ is the average life time of the biomolecule [28]. The $k_q$ for the known maximum collision rate constant is $2 \times 10^{10}$ L mol$^{-1}$ s at a $\tau_0$ of approximately $10^{-8}$ s. When the calculated $k_q$ is higher than the known maximum value of the collision rate constant, it can be concluded that the quenching mechanism results in static quenching by ground state complex formation. We calculated the Stern–Volmer constant $K_{sv}$ for both ligands (Table 1). Obviously, the experimental values are not characteristic of collision or diffusion quenching for both compounds but the result of static quenching by formation of a ground state complex.

Figure 2: Fluorescence spectra change of HSA at different concentrations of SAA (A) and XAA (B) at 293 K and pH 7.4. $c$(BSA) = 10 µM, $c$(ligand) = 0, 2.5, 5.0, 7.5, 10, 12.5 µM. The corresponding concentration of SAA (XAA) with HSA was incubated for 10 min to achieve equilibration. Inset in Figure 2A (B) is the fluorescence emission of SAA (XAA).
Once the complex forms and the distance between the fluorophore of HSA and compound is the range within 8 nm > r > 2 nm, absorption overlaps of the compound will decrease the fluorescence emission of HSA by non-radiative energy transfer [28]. The more energy transferred, the lower the fluorescence emission of protein. This aspect will be discussed later.

The binding constant and sites

The binding constant and the number of binding sites were calculated [11, 27] according to Eq. (3).

$$\log \left( \frac{F_0 - F}{F} \right) = \log K + n \log Q$$  \hspace{1cm} (3)

where $K$ is the binding constant of HSA to SAA (XAA) and $n$ is the number of binding sites. $K$ was inferred from the intercept, and $n$ was deduced from the slope by plotting $\log \left( \frac{F_0 - F}{F} \right)$ versus $\log Q$ (Figure 3). The values of $n$ and $K$ are listed in Table 1. $K$ values greater than $10^4$ indicate that a stronger interaction occurred between HSA and the two compounds. The binding of XAA to HSA is slightly stronger than that of SAA, which could be from the slightly higher hydrophobicity of vanillin than salicylaldehyde.

Fluorescence resonance energy transfer

On the basis of Förster’s theory of non-radiative energy transfer, the energy transfer effect is determined by the distance between the donor and acceptor and by the critical energy transfer distance $R_0$, as given by Eq. (4) [28]:

$$E = \frac{1}{1 + (r/R_0)^6}$$  \hspace{1cm} (4)

In Eq. (4), the critical energy transfer distance $R_0$ indicates that 50% of the excitation energy is transferred to the acceptor when the distance between the donor and acceptor is $R_0$. $r$ is the real distance between the acceptor and the donor. $R_0$ can be determined by Eq. (5).

$$R_0^6 = 8.79 \times 10^{-4} k^2 Q_{Trp} n^{-4} J(\lambda)$$  \hspace{1cm} (5)

where the orientation factor of $k^2$ is decided by the geometry of the dipole and a value of 2/3 is often adopted. The $n$ value of the refractive index of the medium is 1.336. The value of the fluorescence quantum yield $Q_{Trp}$ is chosen as 0.15 [29] for HSA. The overlap integral $J$ is obtained according to Eq. (6) from the intersection between the donor emission spectrum and the acceptor absorbance spectrum.

$$J(\lambda) = \frac{\int F(\lambda) \varepsilon(\lambda) \lambda^3 d\lambda}{\int F(\lambda) d\lambda}$$  \hspace{1cm} (6)

$F(\lambda)$ is the fluorescence intensity of the donor at $\lambda$, and $\varepsilon(\lambda)$ is the molar absorbance coefficient of the acceptor at the same $\lambda$. The spectral overlaps are presented in Figure 4A,B. The results are shown in Table 2. The distances from both SAA and XAA to tryptophan are within long-range forces (8 nm > r > 2 nm), and energy transfer from the Trp residues to the compound is probable. The results strongly support formation of the HSA-SAA (XAA) complex and the occurrence of energy transfer [26]. Compared with SAA, XAA is located on HSA closer to tryptophan, which is in accordance with the stronger fluorescence quenching of XAA than of SAA.

The interactions between HSA and SAA (XAA)

The interactions between extraneous molecules and proteins include hydrophobic forces, electrostatic forces,
hydrogen bonds and van der Waal’s forces. The thermodynamic parameters of enthalpy change ($\Delta H^\theta$) and entropy change ($\Delta S^\theta$) can be used to discern binding modes [30]. From a thermodynamic point of view, $\Delta S^\theta > 0$ serves as a major indication of hydrophobic interactions in which ligands replace water molecules to result in greater disorder; $\Delta H^\theta < 0$ reflects the presence of van der Waal’s forces, hydrogen bond formation and electrostatic interactions between the protein and ligand [31, 32]. Based on the van’t Hoff Eq. (7) and the free energy change Eq. (8), thermodynamic parameters related to the reaction between SAA (XAA) and HSA are calculated, including Gibbs free energy change $\Delta G^\theta$, enthalpy changes $\Delta H^\theta$ and entropy changes $\Delta S^\theta$.

$$\Delta G^\theta = \Delta H^\theta - T \Delta S^\theta$$  
(7)

$$\Delta G^\theta = -RT \ln K^\theta$$  
(8)

In Eqs. (7) and (8), $K^\theta$ is the association constant of the reaction at the corresponding temperature ($T$) and $R$ is the perfect gas constant. By plotting $\Delta G^\theta$ vs. $T$, the $\Delta H^\theta$ and $\Delta S^\theta$ values can be obtained from the intercept and slope of the fitting line. The calculated $\Delta G^\theta$ (Table 3) for both reactions is negative, indicating the spontaneity of the process of binding of HSA to both molecules. For HSA-SAA (XAA), the positive $\Delta S^\theta$ and negative $\Delta H^\theta$ indicate that a single intermolecular force model cannot account for the binding. Both hydrophobic forces and electrostatic forces or (and) other interactions should contribute to the interaction between HSA and small molecules. Since both compounds with sulfonate groups are anionic, electrostatic interactions between HSA and approximately 80 positively charged groups at neutral pH are possible, which was observed in the binding of glipizide with HSA and of isoxsuprine hydrochloride with HSA in the presence of folic acid [5, 24]. The values of $\Delta H^\theta$ and $T \Delta S^\theta$ are similar, implying that there is a noticeable contribution of electrostatic force (and other interactions) to the spontaneous reaction between HSA and SAA (XAA).

**Molecular modelling of SAA (XAA) binding to HSA**

The binding model of HSA to SAA (XAA) was also evaluated by molecular modelling. AutoDock 4.2 was applied. Three similar homologous domains exist in HSA: domain I consists of residues 1–195, domain II includes residues 196–383, and domain III is composed of residues 384–585. The only tryptophan residue, Trp214, is in subdomain IIA. The binding site for both molecules is located in domain I and is in the range of the Förster radius from Trp214 to the ligand. The binding constants were calculated to be on the order of $10^4$, which is inconsistent with the experimental values. The binding free energies ($-5.7$ kcal for SAA and 7.1 kcal for XAA) were also obtained from the highlighted cluster from all conformations, with values similar to those obtained from the experiments.

There are 12 amino acid residues around SAA in the binding models that are within 4 Å from HSA to the ligand (Figure 5A), and all are hydrophobic residues (Leu115, Phe134, Leu135, Leu182, Ile 142, Tyr 138, Tyr161, Phe134, Phe165, Met123) except Arg114, and Arg117. Moreover,

![Figure 4](image-url): The overlap of fluorescence emission from HSA and the absorption from SAA (A), and from XAA (B).

**Table 2: Energy transfer parameters of HSA-SAA (XAA).**

<table>
<thead>
<tr>
<th>Acceptors</th>
<th>J/(mol/L) (nm)$^\ast$</th>
<th>$R_{0}/nm$</th>
<th>$E$</th>
<th>$r/nm$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAA</td>
<td>$3.08 \times 10^4$</td>
<td>20.97</td>
<td>0.21</td>
<td>2.62</td>
</tr>
<tr>
<td>XAA</td>
<td>$4.18 \times 10^4$</td>
<td>22.02</td>
<td>0.35</td>
<td>2.44</td>
</tr>
</tbody>
</table>
Tyr138 (Tyr161) forms π–π interactions with the naphthyl ring of SAA. Arg114 and the benzene ring of SAA form cation-aromatic interaction. Hydrogen bond exists between Arg117 and the oxygen of OH group.

Around XAA (Figure 5B), the hydrophobic residues Leu135, Tyr138, Leu139, Tyr161, Leu182, and Ile142 are the main residues among nine amino acid residues. The other three residues are Arg117, Arg186, and Ala158, which participate in electrostatic interactions and van der Waal’s forces between XAA and HSA. In addition, Arg186 (Arg117) and the naphthyl ring form cation-aromatic interactions. Tyr138 and Tyr161 form aromatic-aromatic interactions with the benzene ring. Hydrogen bond is found between Arg117 and the oxygen of the sulfonic acid group of XAA.

It has been reported that a number of biomolecules can be accommodated in domain I by exploiting hydrophobic contacts [12]; the binding site of HSA for SAA (XAA) is an example. Since the surface of SAA (XAA) is partly hydrophilic, electrostatic forces and other supermolecule forces

![Figure 5: (A) SAA binding site in HSA in stick structure (Left), and in 2D diagram (Right). (B) XAA binding site in HSA in stick structure (Left), and in 2D diagram (Right).](image-url)
also play a role when SAA (XAA) is closer to the hydrophobic domain of the protein.

**The binding effect on the conformation of HSA**

**Characteristics of the RLS spectra**

Resonance light scattering (RLS) can also be utilized to inspect the change in size of macromolecules. When proteins aggregate, the conformation of molecules changes, the molecular hydrophobicity increases, resonance energy transfer occurs between absorption and scattering, and clear RLS signal enhancement will be observed [33, 34]. The RLS spectra of HSA in the presence or absence of SAA (XAA) are shown in Figure 6. It can be seen that the RLS intensity of HSA were relatively weak. Upon adding an equivalent of SAA (XAA) to the HSA solution, an apparent increase in RLS was shown. Since RLS intensity is correlated with the molecular volume increase, the formation of the HSA-SAA (XAA) complex and the increase in particle size of the protein could be concluded.

**CD and conformational analysis**

CD spectra were also recorded to inspect the conformational changes of HSA in the presence of SAA (XAA), as shown in Figure 7. The contents of α-helices, β-sheets, turns, and random coils are listed in Table 4.

According to Figure 7, two negative bands at 208 and 222 nm, characteristic of the α-helical structure, increase upon addition of SAA (XAA). The calculated results in Table 4 show that the percentage of α-helix content in HSA decreased upon binding SAA (XAA). The β-sheet content increased in both HSA-ligand systems. This implied that binding of SAA (XAA) caused the secondary structural change of HSA. By increasing the unordered coil content, a decrease in the secondary structure of the protein was indicated, and destabilization occurred to a certain extent. XAA affects the secondary structure of HSA slightly more than SAA, which may be XAA slightly closer to the hydrophobic cavity or due to its greater binding within the hydrophobic cavity than SAA.

**Conclusions**

Two Schiff base compounds were synthesized, and their interactions with HSA were inspected. The binding of HSA to SAA (XAA) occurs at a ratio of 1:1. The distance between SAA (XAA) and Trp214 in HSA is 2.44 (2.62) nm, over which non-radiative energy transfer could occur. Both compounds are accommodated in hydrophobic domains of HSA. However, electrostatic interactions and other supermolecular forces coexist between the
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