Synthesis and Structural Studies of an Organic Complex and its Association with BSA

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The self-assembly of one novel organic complex based on chlorogenic acid (HCA) and 2,2'-bipyridine (2,2'-bipy) has been synthesized and characterized. The complex achieved by hydrogen-bonding interactions, adopted a 1:1 stoichiometry in a solid state. The proton transfer occurred from the carboxyl oxygen to the aromatic nitrogen atom to form salts CA·(2,2'-Hbipy), the 2,2'-Hbipy molecule individually occupies the pseudo-tetragonum that is formed with CA. In this paper, the interactions of CA·(2,2'-Hbipy) with bovine serum albumin (BSA) were studied by fluorescence spectrometry. For CA·(2,2'-Hbipy), HCA and 2,2'-bipy, the average quenching constants for BSA were 2.4384×10^4 , 4.653×10^3 , and $3.059 \times 10^3 \text{ L} \cdot \text{mol}^{-1}$, respectively. The mechanism for protein fluorescence quenching is apparently governed by a static quenching process. The Stern-Volmer quenching constants and corresponding thermodynamic parameters ΔH , ΔG and ΔS were calculated. The binding constants and the number of binding sites were also investigated. The conformational changes of BSA were observed from synchronous fluorescence spectra.

Key Words : Bovine serum albumin, Organic complex, Chlorogenic acid, Fluorescence quenching, 2,2'-Bipyridine

Introduction

Pharmaceutical research involving an active pharmaceutical ingredient (API) often deals with various solid forms, such as polymorphs, host-guest complexes, network solids, co-crystals, solvates (including hydrates), and organic complex (salt).¹

Co-crystals are a well-known² but relatively unexplored class of compounds. They can be defined as multiple-component crystal systems which were usually based on hydrogen bonds or non-covalent interactions.³ The study of co-crystal is a topic of current interest⁴ because they are an important tool for improving the physical properties of active pharmaceutical ingredients, such as solubility, hygroscopicity, thermal stability, and processability in the pharmaceutical industry.⁵ Although the practical applications of crystal engineering and supramolecular synthesis in pharmaceutical sciences are still emerging areas of research, cultivating co-crystals is becoming an accepted approach for creating solid dosage forms.^{6,7}

Similar to co-crystals, organic salts are also a multi-component system that can have different physical and chemical properties. In salts, protons are transferred between the components. Salts make APIs more bio-available, but more than 30% of pharmaceutical compounds lack suitable functionality for salt formation.¹ So, to formulate good physical and chemical properties of pharmaceutical salt have been the focusing area for decades.

Serum albumin is known to act as a transport protein that binds to a wide variety of compounds, including many drugs, hormones, bilirubin, and fatty acids.^{8,9} In this paper, we choose BSA as the protein model for that human

albumins and BSA are homologous proteins, and its medical importance, stability, binding, and transport properties, as well as the fact that all other studies consistently confirm.

Previous studies have investigated the binding of BSA to small organic molecules¹⁰ and metal-organic complexes,¹¹ which show its interactions with drugs can have a strong influence on the free concentrations of drugs in plasma and the pharmacologic and pharmacokinetic properties of a drug.¹² The methods always involve fluorescence quenching, equilibrium dialysis, ultrafiltration, isothermal titration calorimetry, heteronuclear 2-D NMR, and reversed-phase liquid chromatography. These small molecules are stabilized when binding to BSA through a series of weak interactions, such as the π -stacking interactions, hydrogen-bonding, and van der Waals interactions.¹³

This report will use quenching measurements of albumin fluorescence to determine the interaction between organic complex and BSA, which should reveal the accessibility of quenchers to albumin's fluorophores, help to better understand albumin binding mechanisms and the nature of the binding phenomenon to organic complex [8(b)].

Chlorogenic acid, an important active component in traditional Chinese medicine, exists widely in numerous plant species¹⁴ and may interact covalently or non-covalently with BSA.^{15,16} To the best of our knowledge, few crystals of chlorogenic acid have been reported,^{17,18} and the interaction between an organic complex based on chlorogenic acid and biological macromolecules has not been reported until now.

To get more information about the binding force quality and the mechanism of the interactions between organic complex and the biological macromolecules, we synthesized a organic complex based on chlorogenic acid with 2,2'-bipy

Scheme 1. Molecular structures of chlorogenic acid and 2,2'-bipyridine.

(Scheme 1) and studied the interaction between the crystal and BSA. By analyzing the fluorescence parameters, we obtained much information about the interactive features and the structural changes in the bio-macromolecules.

Experimental

Materials. BSA was obtained from Shanghai Yuanju Biological Technology Co. Ltd. (China). The chlorogenic acid was extracted from the *Blumea riparia* (Bl.) DC.¹⁹ BSA stock solution, 1.0×10^{-4} mol L⁻¹, was prepared in 0.1 mol L⁻¹ Tris-HCl buffer solution (pH = 7.4) and then stored in refrigerator prior to use. Compounds stock solution, were prepared by dissolving the drugs (CA·(2,2'-Hbipy), HCA and 2,2'-bipy) in the same buffer. These solutions (1×10^{-3} mol L⁻¹) are stable when kept under 2-5 °C). All other chemicals were commercial materials of analytical grade and were used without purification, and double distilled water was used throughout.

Apparatus and Measurements. The X-ray diffraction intensity data for the crystal was collected on a Bruker SMART CCD diffractometer equipped with a graphite-monochromated MoK α radiation source ($\lambda = 0.71073$ Å) at 298(2) K. All absorption corrections were performed with SADABS program.²⁰ The structure was solved by direct methods and refined with full-matrix least-squares and expanded using Fourier techniques. Anisotropic thermal parameters were assigned to all non-hydrogen atoms. All the H atoms bonded to Carbon atoms were located at the ideal positions with the C-H=0.93 Å (aromatic), 0.97 Å (methylene) and the thermal factors being set 1.2 times (for aromatic and methylene) or 1.5 times (methyl) of their carrier atoms.

H atoms bonded to N and O atoms were found from the difference maps with the constraints of N-H=0.86(1) Å and O-H=0.82(1) Å and the thermal factors being set k times of their carrier atoms ($k = 1.2$ for N and 1.5 for O atoms, respectively). All calculations were performed using SHELXTL-97 program.²¹ The crystallographic data was summarized in Tables S1.

The fluorescence measurements were carried out by RF-5301PC model spectrofluorometer (Shimadzu, Japan) equipped with a xenon lamp source and a 1.0 cm cell. Fluorescence spectra were obtained at excitation wavelength of 295 nm, with the excitation and emission slit widths set at 3 nm. And the range of synchronous scanning was: $\Delta\lambda = 15$ nm; $\Delta\lambda = 60$ nm, with the excitation and emission slit widths at 3 nm and 3 nm. All pH measurements were made with a pHs-3C digital pH meter (Shanghai Leici Device Works, China) with a combined glass-calomel electrode. The temperature was controlled by a water-bath and temperatures were kept in a certain range ($T \pm 0.1$ °C) throughout the experiment.

Results and Discussion

Description of the Structure. The organic complex of CA·(2,2'-Hbipy) forms in the space group $P2_1(1)$ with the asymmetric unit. Single crystals of the CA·(2,2'-Hbipy) complex were obtained from an ethanol solution *via* slow evaporation of the solvent.²² The proton transfer occurred from the carboxyl oxygen to the aromatic nitrogen atom to form salts (Fig. 1). Extended left-handed helical chains were formed (Fig. 2) by hydrogen bonds [O7 $\cdots$ O4, H7o $\cdots$ O4, O7-H7o $\cdots$ O4 ($-x, y+1/2, -z+1$): 2.666(11), 1.88 Å, 160.1°; O6 $\cdots$ O4, H6o $\cdots$ O4 ($-x, y+1/2, -z+1$): 2.772(11), 1.99(9) Å, 159(8)°; O6 $\cdots$ O8, H6o $\cdots$ O8 ($-x, y+1/2, -z+1$): 2.894(11), 2.33(8) Å, 126(7)°]. The adjacent chains were connected and aligned along the b -axis *via* O-H $\cdots$ O hydrogen bonding [O3 $\cdots$ O5, H3o $\cdots$ O5, O3-H3o $\cdots$ O5 ($x+1, y, z$): 2.772(11), 1.97 Å, 165.1°; O9 $\cdots$ O4, H9o $\cdots$ O4, O9-H6o $\cdots$ O4 ($x+1, y, z$): 2.715(11), 2.45 Å, 100.1°] interactions, forming a two-dimensional supra-molecular sheet, as shown in Figure 3(a). An interesting structural feature of the crystal was that each

Figure 1. Atomic numbering scheme for CA·(2,2'-Hbipy) with displacement ellipsoids at the 50% probability level. The highlighted region depicts the proton transfer region in the molecular complex.

Figure 2. (a) View of the 1D supramolecular helical chain formed by multiple O-H...O hydrogen bonds in the crystal structure of CA·(2,2'-Hbipy); (b) Space-filling representation of the helices.

Figure 3. (a) Supramolecular sheet formed by inter-chain O-H...O hydrogen bonding between the CA; (b) 2,2'-Hbipy fill of the pseudo-tetragonum of the 2D sheet.

pseudo-tetragonum in the 2D sheet was filled by a 2,2'-Hbipy molecule (Fig. 3(b)), each 2,2'-Hbipy molecule was fixed by only one N-H...O [N2...O5, H2N...O5, N2-H2N...O5 ($x+1, y, z+1$): 2.750(12), 2.13 Å, 128.5°] hydrogen bond between the carboxyl oxygen and the 2,2'-bipy nitrogen atom to form 3D architecture.

Fluorescence Quenching Spectra Study. Proteins are considered to have intrinsic fluorescence due to the presence

Figure 4. The quenching effect of CA·(2,2'-Hbipy) (a); HCA (b) and 2,2'-bipy (c) on BSA fluorescence intensity. $\lambda_{\text{ex}} = 295$ nm; BSA, 5.00×10^{-6} mol·L $^{-1}$; CA·2,2'-Hbipy, a-j: 0.00, 0.50, 1.00, 1.50, 2.00, 2.50 3.00, 3.50 4.00 and 4.50×10^{-5} mol·L $^{-1}$; HCA, a-i: 0.00, 2.50, 5.00, 7.50, 10.00, 12.50, 15.00 and 17.5×10^{-5} mol·L $^{-1}$; 2,2'-bipy, a-i: 0.00, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50 and 4.00×10^{-5} mol·L $^{-1}$.

of amino acids, mainly tryptophan, tyrosine and phenylalanine.²³ BSA solutions excited at 295 nm emit fluorescence attributable mainly to tryptophan residues. Figure 4 shows the fluorescence quenching spectra of solutions containing a BSA fixed concentration and different concentrations of each of the studied compounds (CA·(2,2'-Hbipy), HCA, and 2,2'-bipy). The fluorescence intensity of BSA decreased regularly with an increase in the concentration of the com-

plexes, which indicates that the studied compounds can bind to BSA. An obviously red shifts for the combination of CA·(2,2'-Hbipy) and CA with BSA could be deduced that conformational changes induced by the interaction lead to the polarity around the Trp residues was increased and the hydrophobicity was decreased, which agrees with a recent study that the tertiary structure of proteins changed after binding with the other compounds.²⁴ The effect of 2,2'-bipy on the fluorescence spectra of BSA, there was weak blue shift of λ_{em} , this means that the molecular conformation of the protein was effected, which shown that the tertiary structure of proteins changed after binding with the 2,2'-bipy, while the secondary structure remained intact.

Fluorescence quenching is described by the Stern-Volmer equation²⁵:

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

$$K_q = K_{SV} / \tau_0$$

where F_0 and F are the fluorescence intensities before and after the addition of the quencher, respectively. K_q , K_{SV} , τ_0 and $[Q]$ are the quenching rate constant of the bio-molecule, the Stern-Volmer dynamic quenching constant, the average life-time of the bio-molecule without the quencher ($\tau_0 = 10^{-8}$ s²⁶), and the concentration of the quencher. Fluorescence quenching could proceed *via* different mechanisms, usually classified as dynamic quenching and static quenching. For dynamic quenching, the maximum scatter collision quenching constant of various quenchers with the biopolymer is $2.0 \times 10^{10} \text{ L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$. If the K_{SV} is much greater than $2.0 \times 10^{10} \text{ L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$, it can be concluded that the quenching is not initiated by dynamic quenching, but probably partly by static quenching resulted from the formation of drug-BSA complex.

For the complex-BSA systems, the Stern-Volmer graphs are presented in Figure 5 and the values of K_{SV} and K_q ($=K_{SV}/\tau_0$) obtained from the plots at 24 °C are shown in Table 1. The values of K_q are larger than $2.0 \times 10^{10} \text{ L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1}$ for a variety of quenchers with biopolymers,²⁷ which indicated that the probable quenching mechanism of fluorescence of BSA by complex was not initiated by

Figure 5. The Stern-Volmer curves of fluorescence quenching of BSA by CA·(2,2'-Hbipy), HCA and 2,2'-bipy at 24 °C.

Table 1. Stern-Volmer quenching constants for the interaction of compounds with BSA at 24 °C

Complex	CA·(2,2'-Hbipy)	HCA	2,2'-bipy
$K_{SV}(\text{L} \cdot \text{mol}^{-1})$	2.4384×10^4	4.653×10^3	3.059×10^3
$K_q(\text{L} \cdot \text{mol}^{-1} \cdot \text{s}^{-1})$	2.4384×10^{12}	4.653×10^{11}	3.059×10^{11}
r^2	0.9922	0.9936	0.9900

dynamic collision but complex-BSA formation. In other word, the fluorescence quenching of BSA mainly result from complexes formation and governed by a static quenching mechanism, while from dynamic collision could be

Figure 6. Double-log plot of CA·(2,2'-Hbipy) (a); HCA(b) and 2,2'-bipy (c) quenching effect on BSA fluorescence at 24 °C, 30 °C, 35 °C.

negligible in the concentration range studied.²⁸

Calculation of Binding Constant. For static quenching, the relationship between fluorescence quenching intensity and the concentration of quenchers can be described by the modified form of the Stern-Volmer equation.²⁹

$$\log(F_0 - F)/F = \log K_b + n \log[Q] \quad (2)$$

Thus, fluorescence intensity data can also be used to obtain the binding constant (K_b) and the number of binding sites (n), which can be determined by the slope and the intercept of double logarithm regression curve of $\log(F_0 - F)/F$ versus $\log[Q]$ based on the Eq. (2).

The values of K_b and n at 24 °C, 30 °C and 35 °C were obtained from the double logarithm regression curve (Fig. 6).

Table 2 summarizes the values of K_b and n at each temperature studied. The n values are nearly 1, which indicate the existence of a single binding site in BSA for compounds. The binding constant inversely correlated with temperatures for interactions of HCA with BSA, which may indicate forming an unstable compound,³⁰ this means that higher temperatures should weaken the binding and the reaction is exothermic. However, for the interaction of 2,2'-bipy with BSA, the increasing trend of binding constant with increasing temperature, which suggest that the compound was stable and the reaction is endothermic. For CA-(2,2'-Hbipy) with BSA, the binding constants at 24 °C is greater than they are in the 30 °C and 35 °C, which suggest that maybe the 24 °C is the most proper temperature for the compound-BSA combining.

Binding Modes. The acting compound between small organic molecule and bio-molecule includes hydrogen bond, van der Waals force, electrostatic and hydrophobic interaction.³¹ The signs and magnitudes of thermodynamic parameters (ΔH and ΔS) for protein reactions can account for the main forces contributing to protein stability. Both the ΔH and ΔS are positive imply a hydrophobic interaction; contrary, both the negative parameters of ΔH and ΔS show the van der Waals forces or hydrogen bond formation; when $\Delta S > 0$ and $\Delta H \approx 0$ indicate an electrostatic force.³² For this reason, the temperature dependence on the binding constant

was studied at three different temperatures (24, 30 and 35 °C). The thermodynamic parameters could be calculated on the basis of the van't Hoff equation:

$$\ln k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (4)$$

K is the binding constant at temperature T and R is gas constant. The value of ΔH and ΔS were obtained from linear van't Hoff plot. The free energy change (ΔG) can be estimated from the following relationship:

$$\Delta G = \Delta H - T \Delta S \quad (5)$$

The results are presented in Table 3. The negative ΔG revealed that the binding processes are spontaneous for the three compounds. For the formation of HCA-BSA coordination compounds, the negative ΔH and ΔS values showed that both hydrogen bond and van der Waals forces play a role in the binding process.³³ For the formation of (2,2'-bipy)-BSA complex, the fact that ΔH and ΔS are both positive suggest a strong contribution of the hydrophobic effect. From the negative values of ΔH (−96.698 kJ·mol^{−1}) and the positive ΔS observed in the formation of CA-(2,2'-Hbipy)-BSA, it can be concluded that it cannot be mainly attributed to electrostatic interactions since for electrostatic interactions ΔH is very small, almost zero. Negative ΔH value is observed whenever there is hydrogen bonding in the binding. So we can infer that both the electrostatic interactions and hydrogen bonding play a role in the binding process.³⁴

Conformation Investigation. The synchronous fluorescence spectra can provide very important information to study the microenvironment of amino acid residues by measuring the emission wavelength shift. The spectra were obtained through the simultaneous scanning of the excitation and the emission monochromators of a spectrofluorimeter, with a fixed wavelength difference ($\Delta\lambda$) between them.³⁵ When the $\Delta\lambda$ between excitation wavelength and emission wavelength was stabilized at 15 or 60 nm, the synchronous fluorescence gave the characteristic information of tyrosine residues or tryptophan residues.³⁶

In this work, the synchronous fluorescence spectra of BSA

Table 2. Binding constants and binding sites at three temperatures for the complex-BSA system

Complex	24 °C		30 °C		35 °C	
	K_b (mol·L ^{−1})	n	K_b (mol·L ^{−1})	n	K_b (mol·L ^{−1})	n
CA-(2,2'-Hbipy)	4.114×10^4	1.0579	4.723×10^3	0.8412	7.278×10^3	0.8809
HCA	3.314×10^5	1.44	1.692×10^5	1.3677	1.044×10^5	1.3219
2,2'-bipy	9.17×10^2	0.855	1.260×10^3	0.8837	2.757×10^3	0.9737

Table 3. Thermodynamic parameters of the benzodiazepines-BSA system

Drug	ΔG	ΔG	ΔG	ΔH (KJ·mol ^{−1})	ΔS (KJ ^{−1} ·mol ^{−1})
	24 °C (KJ·mol ^{−1})	30 °C (KJ·mol ^{−1})	35 °C (KJ·mol ^{−1})		
CA-(2,2'-Hbipy)	−25.296	−22.862	−21.645	−96.698	234.56
HCA	−11.169	−7.333	−3.498	−239.859	−767.03
2,2'-bipy	−16.434	−18.707	−19.843	50.187	227.26

Figure 7. Synchronous fluorescence spectra of BSA: (a) $\Delta\lambda = 60$ nm; (b) $\Delta\lambda = 15$ nm. [BSA] = 1.00×10^{-5} mol·L $^{-1}$; [CA·(2,2'-Hbipy)]: 0.00, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00, 3.50, 4.00 and 4.50×10^{-5} mol·L $^{-1}$; [HCA]: 0, 2.50, 5.00, 7.50, 10.00, 12.50, 15.00 and 17.5 (1.00×10^{-5} mol·L $^{-1}$); [2,2'-bipy]: 0.00, 0.50, 1.00, 1.50, 2.00, 2.50, 3.00 and 3.50×10^{-5} mol·L $^{-1}$.

at various complexes concentrations when $\Delta\lambda = 60$ nm and $\Delta\lambda = 15$ nm are shown in Figure 7. When $\Delta\lambda = 60$ nm [Fig. 7(a)], the addition of the complexes leads to a decrease in the synchronous fluorescence intensity with the red-shifts of the spectral peak. This indicates that the tryptophan residues increases, and it places in a less hydrophobic environment

but more exposed to the solvent molecules during the binding process.³⁷ Meanwhile, when the synchronous fluorescence spectra for $\Delta\lambda = 15$ nm (Fig. 7(b)), the emission peaks show essentially no shift over the investigated concentration range, which indicates that complexes have little effect on the micro-environment of tyrosine residues in the BSA.

Conclusions

In summary, we have determined the crystal structure of the complexes formed by HCA and 2,2'-bipy with a 1:1 stoichiometry. The complex unit contains one 2,2'-Hbipy molecule and one CA molecule, the CA molecules are connected to each other through hydrogen bonds to form a 2D supra-molecular sheet. The 2,2'-Hbipy is adjunctive to the 2D sheet through the hydrogen bonds formed by carboxyl oxygen and a 2,2'-bipy nitrogen atom.

The results indicated that the fluorescence quenching mechanism of BSA by CA-(2,2'-Hbipy), HCA and (2,2'-bipy) were consistent with the static model, and the binding reaction was spontaneous. Hydrogen bonds, hydrophobic forces and van der Waals forces played a major role in the complex-BSA interaction. Synchronous fluorescence spectra showed that the secondary structure of the BSA molecules was affected significantly in the presence of the complexes.

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Appendix A. Supplementary Data. CCDC 818753 contains the supplementary crystallographic data for CA-(2,2'-Hbipy). The data can be obtained free of charge from The Cambridge Crystallographic Data Centre: <http://www.ccdc.cam.ac.uk/conts/retrieving.html>.

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- Synthesis CA-(2,2'-Hbipy): To an ethanol aqueous solution (5 mL) of chlorogenic acid HCA (35.4 mg, 0.1 mmol) was added an ethanol solution (10 mL) of 2,2'-bipyridine (2,2'-bipy) (19.82 mg, 0.1 mmol), the mixture was left for evaporating at room temperature to obtain yellow block crystal after several days. Anal. Calcd for C₂₆H₂₆N₂O₉ (%): C, 61.17; H, 5.13; N, 5.49. Found: C, 61.23; H, 5.20; N, 5.40.
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