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Spectroscopic investigation on the interaction of J-aggregate with human serum albumin

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Abstract

The interactions of three cyanine dyes, which exhibit different *meso* substituent in polymethine chain, with human serum albumin (HSA) have been investigated by the means of absorption, fluorescence and circular dichroism (CD) spectra. In phosphate buffer solution (PBS), the mentioned dyes exist not as isolated monomers but rather in the formation of J-aggregation. In the presence of HSA, the absorption and fluorescence emission spectra indicated that the J-aggregation was decomposed to monomer because of the strong affinity between dye molecules and HSA. Besides the association of cyanine dyes with HSA, binding to HSA gave rise to the J-aggregation CD signals. The *meso* substituent in the polymethine plays an important role in the interaction of HSA and the J-aggregation. Spectral studies showed that the dye bound with HSA in a 1:1 formation. The apparent constant (K_a) value was roughly identified by analysis of the corresponding fluorescence data at various HSA concentrations. The higher affinity of the molecule with *meso* phenyl towards HSA with respect to molecules with *meso* ethyl or methyl can be attributed to the arrangement of molecules in J-aggregation and the hydrophobic force between the molecules and HSA.
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Keywords: Human serum albumin; J-aggregation; Interaction; Induced CD; Apparent constant

1. Introduction

Human serum albumin (HSA) is the most abundant protein in blood plasma, which exhibits an exceptional ability to reversibly bind a wide range of endogenous and exogenous substances [1,2]. This unique property enables HSA to fulfill a fundamental biological role as a universal carrier and reservoir in body tissues [3]. The structure and properties as well as its interactions with other molecules have been investigated in order to understand how they affect the functionality of the molecules. It is generally accepted that HSA is a globular protein consisting of a single peptide chain of 585 amino acids. It is composed of three homologous domains, I, II, and III, which have similar 3D structures [1]. Despite very high stability, HSA is a flexible protein with the structure susceptible to environmental factors such as pH, ionic strength, etc. [1]. This structural flexibility may be an important factor in the ligand–protein interaction. Because of its clinical and pharmaceutical importance, much attention has therefore been paid to investigating the complex of HSA with a number of natural and synthetic ligands [4].

Among the ligands, polymethine (cyanine) dyes and their derivates have attracted considerable attention due to their novel properties and wide applications [5]. Until now, reported applications for cyanine dyes included noncovalent label [6], diagnostic probe [7], drug displacement [8], cell protector [9] and DNA sequences applications [10]. This is based on their novel photophysical and photochemical properties, which strongly depend on the nature of the surrounding environment [11,12]. Compared to other molecules, the cyanine dyes exhibit numerous advantageous properties [13]. First, most cyanine dyes exhibit maximum absorption and fluorescence in visible and near infrared regions (400–1100 nm), while few biomolecules possess intrinsic fluorescence in these regions. Second, in

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many cases, cyanine dyes exist not as isolated monomers but rather as an aggregation of multiple chromophores [14], which can be utilized for studying complex biomolecules [15]. It was reported that the aggregation (Fig. 1) results in the formation of different structures depending on the angle of slippage α and is usually described as H-aggregation (hypsochromic) and J-aggregation (bathochromic) [16]. As was detailed, large molecular slippage ($\alpha < \sim 32°$) results in a bathochromic shift while small slippage ($\alpha > \sim 32°$) results in a hypsochromic shift [17,18]. The type of aggregation is dependent on several factors such as dye structure, dye concentration, solvent polarity, solution pH, ionic strength, temperature and so on [19,20]. Recently, the interactions of monomeric and H-aggregation of cyanine dyes with HSA have been investigated in a number of works [6,8,18,21]. The J-aggregation has been the subject of keen interest and proved to be useful in numerous fields [22,23]. While, less attention has been paid on studying the interaction between HSA and the J-aggregation [24].

In this paper, we describe the spectroscopic investigation on the interaction of HSA with the J-aggregation by using absorption, fluorescence and circular dichroism (CD) spectra. J-aggregation assembled by the cyanine dyes that exhibits the same molecular backbone but different *meso* substituents in the polymethine chain was chosen (Chart 1): 3,3′-di(3-sulfopropyl)-4,5,4′,5′-dibenzo-9-phenyl-thiacarbocyanine triethylammonium salt (PTC); 3,3′-di(3-sulfopropyl)-4,5,4′,5′-dibenzo-9-ethyl-thiacarbocyanine triethylammonium salt (ETC) and 3,3′-di(3-sulfopropyl)-4,5,4′,5′-dibenzo-9-methyl-thiacarbocyanine triethylammonium salt (MTC).

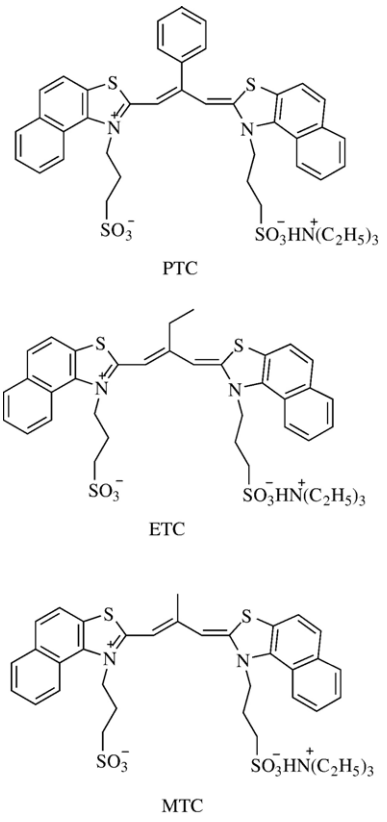

Chart 1. Chemical structures of the cyanine dyes.

2. Experimental

HSA was purchased from Sigma and used without further purification. The cyanine dyes were synthesized according to the methods suggested by previous literatures [25,26]. Their identity was verified by mass spectrometry (MS) and nuclear magnetic resonance (NMR). Other chemicals were commercially available. To prepare the phosphate buffer solution (PBS, pH 7.12, containing 10 mM phosphate saline and 10 mM NaCl), analytical grade NaH_2PO_4, Na_2HPO_4 and NaCl were used as received and double distilled water was used throughout the experiments.

HSA stock solution was prepared by dissolving a certain amount of corresponding sample directly into PBS. However, the stock solutions of cyanine dyes were prepared by dissolving the materials into a small amount of methanol and then diluted with PBS; the percent of methanol is less than 5%. All of the stock solutions were kept in a refrigerator in the dark. The measured sample was prepared by mixing a quantity of dye solution with HSA solution, and then diluted by PBS. The sample solutions were kept in darkness overnight before measurement in order to realize the full association of dye molecule with the proteins.

Solution pH values were measured with a PHS-3C instrument calibrated using standard buffer solutions. The absorption and fluorescence spectra were obtained from a UV-1601PC spectrophotometer and a Hitachi F4500 fluorimeter, respectively. The CD measurements were carried out on a Jasco-810 automatic recording spectropolarimeter at room temperature. Spectra data were collected with scan speed of 500 nm/min and response time of 1 s. Each spectrum was the average of four scans and corrected by the PBS solution. 1 cm

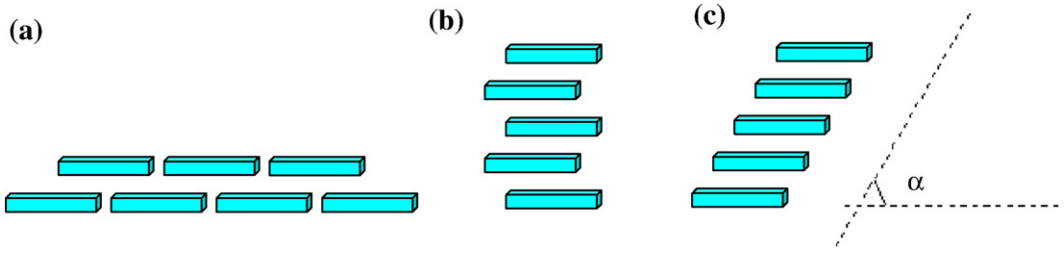

Fig. 1. Representative models of the aggregation in solution: dye aggregation with brickwork **(a)**, ladder **(b)**, and staircase **(c)** molecular arrays. In the figure, the cube represents dye molecule and α is the angle of slippage.

quartz cells were used throughout the absorption, fluorescence and CD experiments.

3. Results and discussion

3.1. Absorption spectra

The used cyanine dyes, PTC, ETC and MTC, are all composed of two hydrophobic heteroaromatic fragments linked by a polymethine chain as well as a different substitute group in the *meso* position of the polymethine chain. The extensive conjugation leads them to show long-wavelength maxima and large molar absorptivities [14]. As other cyanine dyes with similar structure, their photophysical and photochemical properties strongly depend on the nature of the surrounding medium.

The absorption spectra of PTC, ETC and MTC in methanol solvent each shows one broad band located at 600, 580 and 572 nm, respectively, as illustrated in Fig. 2a. However, different optical behaviors are observed in PBS solution. Each of the dye reveals a predominant absorption band (Fig. 2b), which is red-shifted and significantly narrowed with respect to the monomeric band. It is well known that self-assembly in polar solutions is a common property of cyanine dyes, which contain extended planar π-electron conjugated systems [27]. Based on previous details [24], the observed sharp band can be attributed to the J-aggregation, which is constructed by numerous dye molecules in a self-assembly model due to the salt effect in solution [19]. Although PTC, ETC and MTC have a strong tendency to form corresponding J-aggregation, their aggregation behaviors are different to some extent. Compared to the J-aggregation formed by MTC and by ETC, the J-aggregation of PTC shows a 26 nm bathochromic shift in absorption spectra, which can be attributed to the extension of the conjugated system due to the effects of *meso* phenyl in the polymethine chain [28].

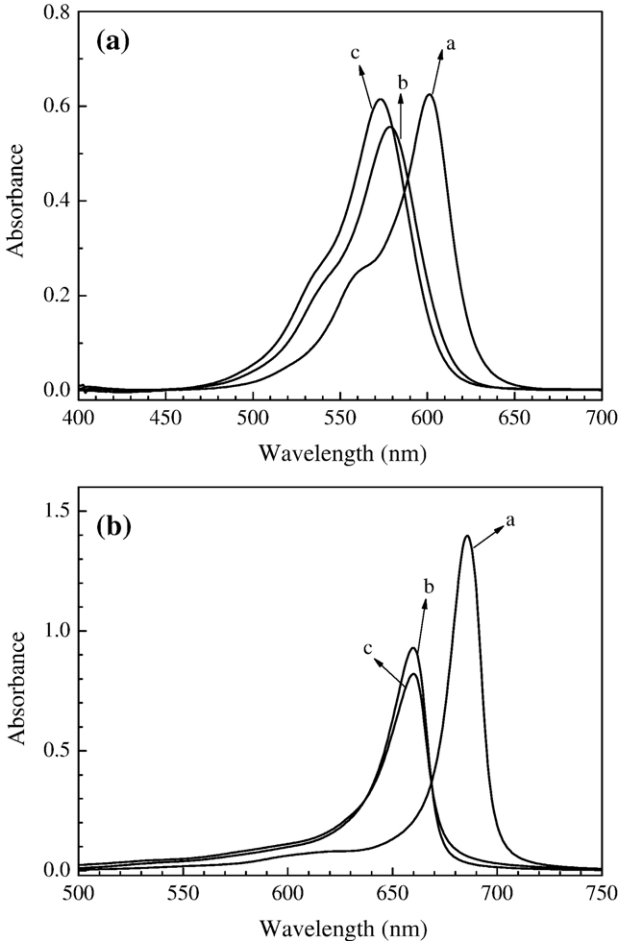

Fig. 2. **(a)** The absorption spectra of cyanine dyes in methanol: [PTC]=4.8 μM (a), [ETC]=5.0 μM (b), and [MTC]=6.0 μM (c). **(b)** The same concentration of cyanine dyes in PBS: PTC (a), ETC (b), and MTC (c).

As reported [24], the stepwise titration of HSA to J-aggregation of PTC results in an absolute decomposition of the aggregation. The addition of HSA to the solution of J-aggregation self-assembled by ETC resulted in substantial spectral changes. In the presence of HSA, the absorbance of J-aggregation was reduced and accompanied with the appearance of a new broad peak located at 610 nm, which could be assigned to monomeric ETC (Fig. 3a). The existence of an isobestic point (625 nm) in the absorption spectra indicated that there exists the equilibrium between the J-aggregation and the monomer. Because the monomer appeared only when HSA was present, they were bound to HSA. Further titration of HSA into the solution, the band belonging to J-aggregation decreased but did not disappear completely, indicating an incomplete transformation of the J-aggregation to monomer even at the circumstance of high concentration of HSA. The transition from J-aggregation to monomer could be explained in terms of the complexation of HSA with ETC molecule. Considering that the J-aggregation is assembled through intermolecular van der Waals forces between the adjacent molecules and binding of dye molecules to HSA through electrostatic force and hydrophobic interaction, as a result, the decomposition of the J-aggregation by HSA indicates a stronger affinity in the latter case [21].

Similar results were observed when HSA was gradually added to the solution of J-aggregation formed by MTC. As can be seen from Fig. 3b, the J-aggregation of MTC has a very weak tendency to switch to a corresponding monomer when the HSA solution was introduced. MTC existed mostly as J-aggregation even if the concentration of HSA was greatly higher than that of MTC, suggesting the fact that the intermolecular forces between the molecules in the J-aggregation are stronger than the electrostatic and hydrophobic forces between the cyanine dye molecules and HSA. With respect to the J-aggregation formed by ETC, J-aggregation of MTC interacted with HSA in a rather weaker way.

It is noteworthy that the J-aggregation assembled by PTC was decomposed by HSA completely [24], however, that of ETC and of MTC was only partly decomposed. That could be attributed to the dye molecular structure. It is well known that, in aqueous solution, the size and shape of *meso* substituents in the polymethine of cyanine dyes play important roles in determining the arrangement of dye molecules in aggregation [29]. Compared to ethyl and methyl, the bulky phenyl may

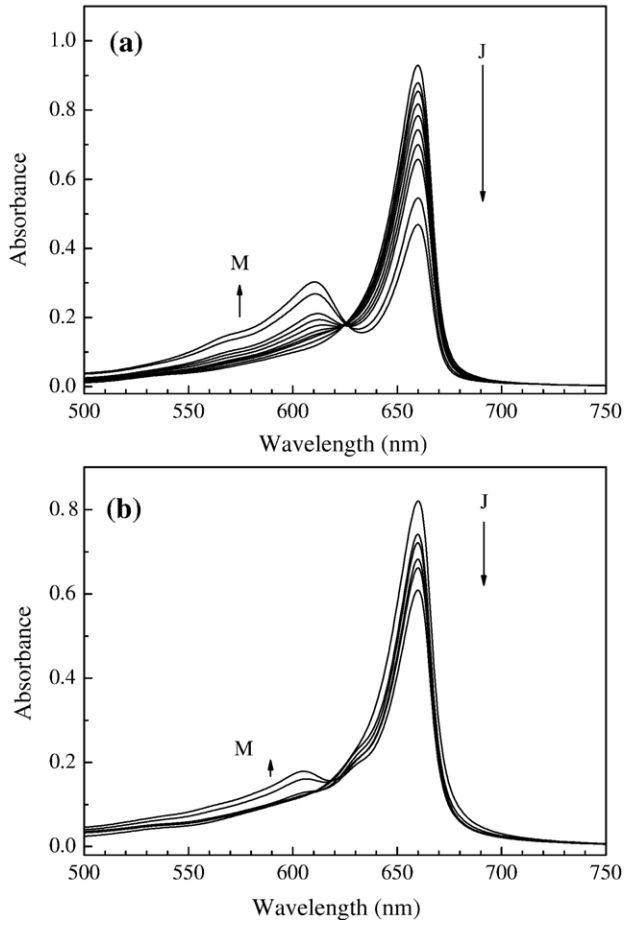

Fig. 3. **(a)** The absorption spectra of ETC (5.0 μM) in PBS in the presence of HSA. Curves from top to bottom correspond to 0.0, 1.3, 2.5, 3.8, 5.0, 6.3, 7.5, 8.8, 10.0, and 15.0 μM HSA, respectively. **(b)** The absorption spectra of MTC (6.0 μM) with HSA concentration from top to bottom is 0.0, 3.0, 6.0, 9.0, 12.0, 18.0 μM.

result in a stronger steric hindrance which enlarges the distance between adjacent molecules in the J-aggregation and reduces the intermolecular force to some extent. As a result, J-aggregation formed by PTC is not so stable as the J-aggregation that formed by ETC and by MTC to some extent. On the other hand, the existence of the *meso* phenyl in the polymethine chain enhances the hydrophobicity of the dye molecule, making PTC to emerge into the hydrophobic cavity of HSA easier than ETC and MTC.

3.2. Fluorescence spectra

In methanol solvent, the fluorescence spectrum of PTC (ETC or MTC) has only one emission band at 622 nm (608 nm or 602 nm) upon excitation at 560 nm. While using the excitation wavelength at 630 nm, no J-aggregation emission band was observed for each of the dyes. The results suggest that the cyanine dyes exist as monomer in methanol, which is consistent with that of the absorption spectra.

The fluorescence spectrum of ETC in the absence and presence of HSA upon excitation at 580 nm is shown in Fig. 4. When HSA is absent in solution, there is only one weak sharp emission band at 666 nm with excitation at 590 nm and 630 nm in the fluorescence spectrum. As ETC exists mostly in the form of J-aggregation rather than monomer under this condition, the observed emission band could be attributed to J-aggregation. No monomer band was observed, indicating the cyanine dye existed in J-aggregation in the absence of HSA. However, in the presence of HSA, it can be seen that one broad fluorescence emission band located at 620 nm as well as a band at 666 nm is observed, which belong to the monomer and J-aggregation of ETC, respectively. When HSA was added into the J-aggregation solution step by step, there is a strong and constant enhancement of the fluorescence intensity of the monomer, which agrees with the absorption spectrum. The fluorescence intensity of J-aggregation enhances a little due to the resonance energy transfer between the monomer and J-aggregation. It is inferred that the observed phenomenon could be explained in terms of the appearance of the monomer of the cyanine dye, which is said to exhibit a higher fluorescence quantum yield than the J-aggregation in PBS solution. Simultaneously, we also noted that there is a gradual red shift of the monomer's fluorescence emission band when HSA is titrated into the J-aggregation solution, suggesting the association of ETC and HSA. Similar fluorescence results were obtained when HSA was gradually added to the solution of the J-aggregation assembled by MTC and by PTC, respectively, which indicated that the corresponding J-aggregation was decomposed by HSA.

HSA has two primary hydrophobic binding sites commonly referred to as site I and site II, located at domains IIA and IIIA, respectively [1]. Once the ligands were inserted into the binding sites, they exhibited different fluorescence behavior due to the changed microenvironment [30]. It was reported that the fluorescence intensity of the cyanine dye increased upon the complexation with HSA, due to shielding effects [31]. The red shift in the fluorescence spectrum suggests that the dye molecule has been brought into a more hydrophobic environment, resulting in a decrease of energy gap between the ground and triplet states, when it interacted with the HSA [30].

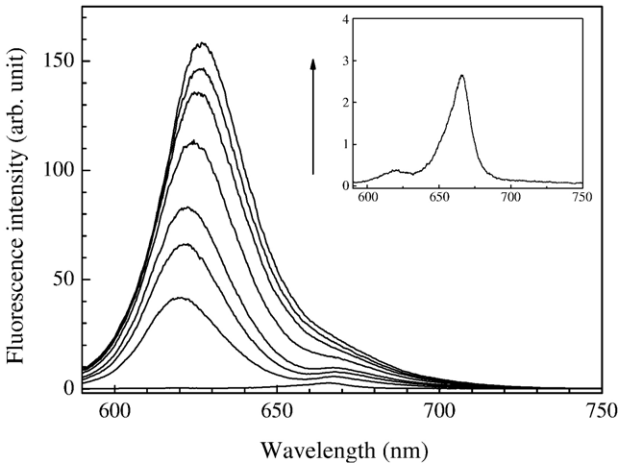

Fig. 4. Fluorescence emission spectra of ETC (5.0 μM) as a function of HSA concentration. Curves from top to bottom correspond to 0.0, 1.3, 2.5, 3.8, 5.0, 6.3, 7.5, 8.8, and 10.0 μM HSA, respectively. Inset gives the fluorescence emission spectrum of the J-aggregation of ETC in the absence of HSA.

3.3. CD measurements

In general, the J-aggregation is induced to present chirality when dye molecules self-assemble on the unsymmetrical or chiral surface of template, such as polypeptide or DNA [32,33]. Based on our previous details [24], HSA could not only complex with the PTC molecule but induce the J-aggregation to exhibit strong CD signals. Furthermore, the feature of the CD signal could transform from positive phase to negative phase when changing the concentration of HSA or the solution's pH value. It is clarified that the chiral transformation of J-aggregation could be assigned to the α-helix and random-coil content in HSA, both of which exhibit their own functions [3].

Consistent with the results obtained from PTC, no CD signal was observed of the J-aggregation spontaneously assembled by ETC and by MTC in the absence of HSA. However, when HSA was present, the J-aggregation of ETC and MTC both showed only a weak bisignate CD signal (Fig. 5a and b). The observation of the induced CD originated from the conformational changes because local distortion was caused when the J-aggregation and HSA interacted. We noted that the feature of the induced CD signal kept stable when increasing the concentration of HSA, which suggested that only one content of HSA was accessible to the J-aggregation. Judging from the feature of the CD signal, it can be inferred that J-aggregation of ETC and MTC was only induced by the random coil content of HSA [34]. Though α-helix is the most abundant content of HSA [3], J-aggregation prefers to bind to the random-coil of HSA indicating that there exists a higher repulsive force between the neighboring dye molecules when they were induced by the α-helix.

The intensity of induced CD depends on the relative concentration ratio of HSA and the dye greatly. Considering that HSA decomposed and induced the J-aggregation, it was distinct that the intensity of CD signal changed with increasing concentration of HSA. During the titration of HSA into J-aggregation solution, J-aggregation was in excess at the beginning; the added HSA served mostly for the center of chirality and thus enhanced the CD intensity of J-aggregation. Whereas, when HSA was in excess, the CD intensity of J-aggregation decreased because J-aggregation was decomposed to monomer by HSA. As a matter of fact, the CD intensity of the J-aggregation assembled by ETC increased and then decreased with the increase in the ratio of HSA to ETC (Fig. 5a). On the other hand, since J-aggregation of MTC interacted with HSA in a rather weak way, the CD intensity increased even under a high ratio of HSA to MTC (Fig. 5b).

As mentioned above, J-aggregation of ETC was partly transformed to monomer in the presence of HSA. As was reported, the monomer of ETC should have exhibited weak negative CD signal when it bound to HSA. No CD signal belonging to a bound monomer was observed in Figs. 5a and b, which seems conflicted with a previous report [21]; however, it was noteworthy that the induced J-aggregation exhibited a biphasic CD feature, which covered a wide region from about 615 nm to 690 nm. The CD signal of monomer could be affected by that of the J-aggregation, thus, it is reasonable that the CD signal of monomer is absent.

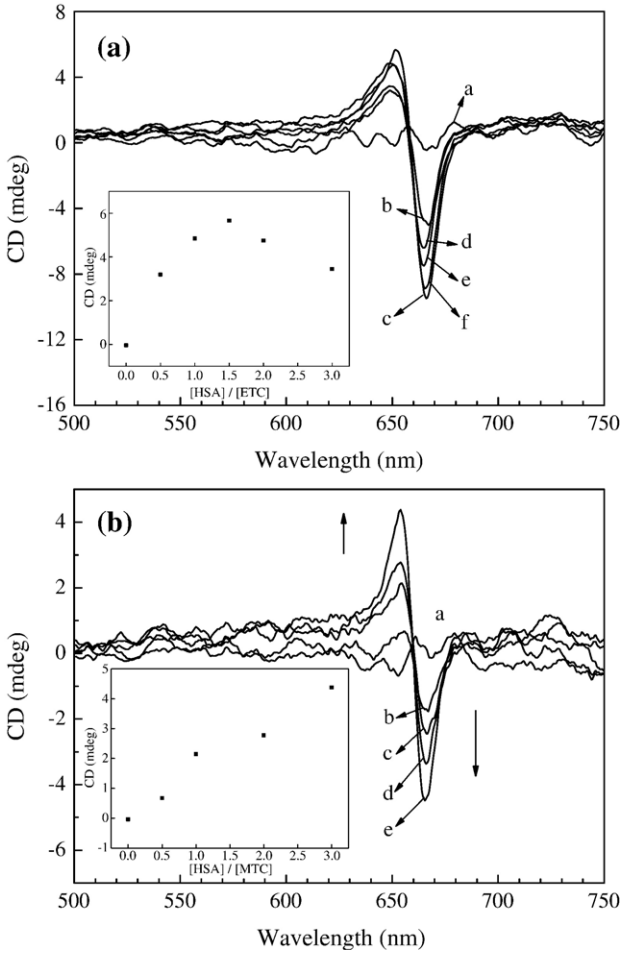

Fig. 5. **(a)** CD spectra of 5.0 μM ETC with [HSA]=0.0 μM (a); 2.5 μM (b); 5.0 μM (c); 7.5 μM (d); 10.0 μM (e); 15.0 μM (f). Inset gives the CD values at 650 nm as a function of the ratio of HSA to ETC. **(b)** CD spectra of 6.0 μM MTC with [HSA]=0.0 μM (a); 3.0 μM (b); 6.0 μM (c); 12.0 μM (d); 18.0 μM (e). Inset gives the CD values at 654 nm as a function of the ratio of HSA to MTC.

The different optical phenomena can be attributed to the *meso* substituent in the polymethine bridge. As mentioned above, the size of *meso* substituent plays important roles in determining the arrangement of the dye molecules in aggregation, such as the distance and angle of slippage between the adjacent molecules [29]. As methyl group is smaller in size than ethyl and phenyl, the J-aggregation formed by MTC is tighter and more rigid than those formed by ETC and PTC. Thereby, it is hard for the J-aggregation of MTC to be decomposed and induced by HSA.

3.4. Data analysis

From the viewpoint of future biophysical and biochemical applications, it seems interesting to study the interaction of J-aggregation in the presence of HSA. Among most of the important parameters for the understanding of such an interaction are the determination of the binding affinity, binding site, binding constant and location of the molecule in the microenvironment. Although it is challengeable for us to identify the exact location of the dye molecule in the complex protein environment, the number of binding sites (n) and binding constant studies have thrown light

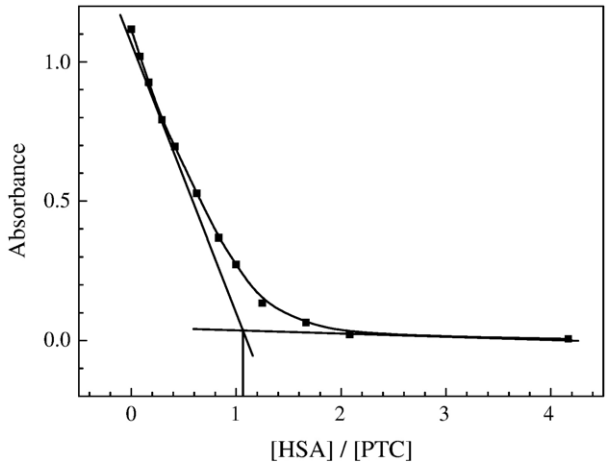

Fig. 6. The Job plot of absorbance of J-aggregation against the relative ratio of HSA and PTC.

on this aspect. According to absorption, fluorescence and CD results, the interaction between cyanine dyes J-aggregation and HSA involves the following processes.

$$J + \text{mHSA} \xrightarrow{K} m(\text{dye} - \text{HSA}) \quad (1)$$

$$J + \text{HSA} \xrightarrow{K'} J - \text{HSA} \quad (2)$$

where *J*, *HSA*, *dye–HSA*, *J–HSA*, and *m* represent the J-aggregation, HSA, dye–HSA complex, the J-aggregation that binds to HSA and the aggregation number of the J-aggregate. While *K* and *K′* is the binding constant of HSA to monomeric dye and J-aggregation, respectively. From a look at the absorption and CD spectra of ETC in the presence of HSA, it is troublesome to identify the equilibrium constant between HSA and J-aggregation because of the concomitant processes of decomposing and inducing J-aggregation. However, it appears possible to determine the apparent constant (K_a) from the enhancement of the fluorescence of the dye due to the association between the dye and HSA.

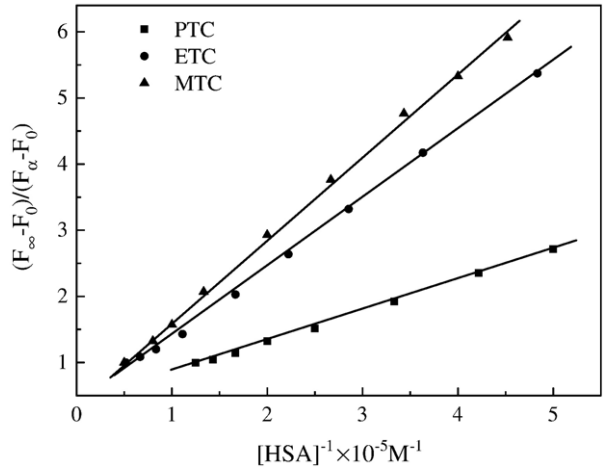

Fig. 7. The plot of $(F_\infty - F_0)/(F_x - F_0)$ against $[\text{HSA}]^{-1}$. For details see text.

To simply confirm the number of binding sites (*n*), we studied the optical behaviors of the J-aggregation as a function of the concentration ratio of HSA to the dye. According to the method suggested by Walwick and coworkers [35], a plot of the absorbance of the J-aggregation versus the ratio of HSA to PTC in buffer solution was performed (Fig. 6). The Job curves intercrossed at about 1.08 indicating that the number of binding sites (*n*) is 1. By using this method, it was identified that ETC and MTC also associated with HSA in 1:1, which is similar with the results obtained from other cyanine dyes [21].

Although it is of importance to identify the equilibrium constant between the J-aggregation and HSA, however, the complex processes prevented our effort to obtain it accurately. J-aggregation was said to be composed of a large number of dye molecules, thus, the concentration of J-aggregation is much lower than the monomer in solution. As a result, HSA that associated with J-aggregation could be ignored compared to that associated with the monomer. The apparent constant (K_a) of association of HSA and the dye could be roughly estimated. In order to further evaluate the binding interaction between HSA and monomeric dye, we calculated the K_a values according to the fluorescence data by using the modified equation [36]:

$$\frac{1}{F_x - F_0} = \frac{1}{F_\infty - F_0} + \frac{1}{K_a[P]} \cdot \frac{1}{F_\infty - F_0} \quad (3)$$

where F_0, F_x, and F_∞ represent the fluorescence intensities of monomeric dye molecules in the absence of HSA, in the presence of an intermediate HSA concentration, and at a concentration of complete interaction, respectively; and [*P*] is the concentration of HSA. Rearranging Eq. (3), the following equation can be obtained:

$$\frac{F_\infty - F_0}{F_x - F_0} = 1 + \frac{1}{K_a[P]} \quad (4)$$

Plots of $(F_\infty - F_0)/(F_x - F_0)$ against [*P*] for the three dyes showed remarkable linear dependence (Fig. 7), justifying the validity of the used equation and hence confirming the one-to-one association between HSA and the dyes. The binding constant values (K_a) could be determined from the slope of the corresponding plots. The data for the binding phenomena are tabulated in Table 1.

The calculated K_a values located at a normal range published previous of similar molecules associated with HSA [21]. From a look at the relative values of K_a, the values of K_a increase in the order: K_a (PTC) > K_a (ETC) > K_a (MTC), following the same order of hydrophobicity of the *meso* substituent. It was evident that PTC bound in a stronger way with HSA as compared to

Table 1
The apparent constants for dye–protein interaction at 298 K

HSA	Apparent constant (K_a) M^{-1}
PTC	2.04×10^5
ETC	1.00×10^5
MTC	0.77×10^5

ETC and MTC with HSA, which is consistent with the absorption and CD spectra.

4. Conclusions

In the present work, we studied the interaction between HSA and the J-aggregation that was self-assembled by several cyanine dyes by using several spectral approaches. It was demonstrated that J-aggregation transformed to a corresponding monomer when HSA is present. This could be explained in terms of the high affinity between HSA and the dye molecules. Furthermore, interaction of the J-aggregation with HSA resulted in the appearance and a step rise of dye monomer fluorescence and, as a consequence, gradual red shift of the fluorescence emission band. Binding to HSA gave rise to the J-aggregation of the dye CD signals during the addition of HSA, which suggested that J-aggregation may be distorted by the chiral contents of HSA (α-helix and random coil). It was identified that the *meso* substituent in the polymethine chain influenced the interaction dramatically. Absorption spectral studies indicate that the number of binding sites (n) is 1. By analyzing the fluorescence data, the apparent constants have been identified. The values of K_a increase in the order: K_a (PTC)>K_a (ETC)>K_a (MTC). The cyanine dye with *meso* phenyl showed much stronger affinity with HSA in contrast to the dyes with *meso* ethyl or *meso* methyl, probably due to its hydrophobicity.

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