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Short communication

Fluorometric investigation of the interaction between methylene blue and human serum albumin

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Abstract

The interaction between methylene blue (MB) and human serum albumin (HSA) was investigated by fluorescence spectroscopy and UV–vis absorbance spectroscopy. In the mechanism discussion, it was proved that the fluorescence quenching of HSA by MB is a result of the formation of MB–HSA complex and electrostatic interactions play a major role in stabilizing the complex. The Stern–Volmer quenching constant K_{SV} and corresponding thermodynamic parameters ΔH, ΔG and ΔS were calculated. Binding studies concerning the number of binding sites n and apparent binding constant K_b were performed by fluorescence quenching method. The distance r between the donor (HSA) and the acceptor (MB) was obtained according to fluorescence resonance energy transfer (FRET). Wavelength shifts in synchronous fluorescence spectra showed the conformation of HSA molecules is changed in the presence of MB.

Keywords: Methylene blue; Human serum albumin; Fluorescence quenching; Thermodynamic parameters; Fluorescence resonance energy transfer

1. Introduction

Methylene blue (formula: $C_{16}H_{18}N_3ClS$; CAS registry number: 61-73-4), is also named methylenum caeruleum, tetramethylthionine chloride or swiss blue. It is a coplanar polycyclic aromatic basic dye that belongs to the thiazine class (its structure shown in Fig. 1). It has long been used as a tool to dissect intracellular redox metabolism [1–3], as well as for biological staining and diagnosis of diseases including carcinoma [4–6], and can be used as an effective antidote [7]. Moreover, some researchers have reported that it was potentially effective for the treatment of chloroquine-resistant malaria caused by *Plasmodium falciparum* and had a promising application in the photodynamic therapy for anticancer treatment [8,9].

Human serum albumin (HSA) is the most abundant protein in the circulatory system. Its principal function is to transport fatty acids, while it is also capable of binding an extraordinarily broad range of drugs [10]. Much of the clinical and pharmaceutical interest in the protein derives from its effects in drug pharmacokinetics [11]. The crystallographic analysis of HSA revealed that the protein, a 585-amino acid residue monomer, contains three homologous α-helical domains (I–III) and a single tryptophan (Trp-214) [12]. Many drugs and other bioactive small molecules bind reversibly to albumin and other serum components [13–15], which implicates HSA's role as carriers. Serum albumin often increases the apparent solubility of hydrophobic drugs in plasma and modulates their delivery to cells in vivo and in vitro; they can play a dominant role in drug disposition and efficacy [16]. Accordingly, it is important to understand and predict ligand/drug displacement interactions for a variety of endogenous and exogenous ligands/drugs. However, detailed investigations of the interaction of HSA with MB have yet to be conducted.

Fluorescence spectroscopy is a powerful tool for the study of the reactivity of chemical and biological systems. For fluorescence quenching, the decrease in intensity is described by the well-known Stern–Volmer equation ($F_0/F = 1 + K_{SV}[Q]$)

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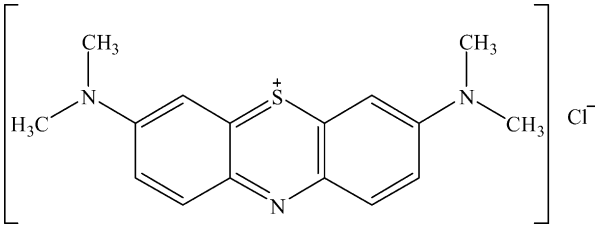

Fig. 1. Molecular structure of methylene blue.

[17]. The aim of our work was to determine the affinity of MB to HSA and to investigate the thermodynamics of their interaction. We also tried to find the stoichiometry of MB and HSA binding. In order to attain these objectives, we planned to carry out detailed investigation of MB–HSA association using fluorescence spectroscopy and UV–vis absorbance spectroscopy. Through fluorescence resonance energy transfer (FRET) and synchronous fluorescence spectra, we planned to further investigate the effect of energy transfer and the effect of MB binding on the environment adjacent to the tryptophan residue in HSA.

2. Materials and methods

2.1. Materials

HSA and MB were both purchased from Sigma (St. Louis, MO, USA). The buffer Tris had a purity of no less than 99.5% and NaCl, HCl, etc. were all of analytical purity (Shanghai Chemical Reagent Plant, China). The samples were dissolved in Tris–HCl buffer solution (0.05 mol L^{-1} Tris, 0.15 mol L^{-1} NaCl, pH 7.4 ± 0.1). The solutions of albumin were prepared 15 min before usage in each experiment. All solutions were prepared with doubly distilled water.

2.2. Equipments

The UV spectrum was recorded at room temperature on a TU-1901 spectrophotometer (Puxi Analytic Instrument Ltd. of Beijing, China) equipped with 1.0 cm quartz cells. All fluorescence spectra were recorded on F-2500 spectrofluorimeter (Hitachi, Japan) equipped with 1.0 cm quartz cells and a thermostat bath. Sample masses were accurately weighted on a microbalance (Sartorius, ME215S) with a resolution of 0.1 mg.

2.3. Spectroscopic measurements

The absorption spectroscopy of MB was performed at room temperature. The fluorescence measurements were performed at different temperatures (292, 298, 304 and 310 K). The widths of both the excitation slit and the emission slit were set to 2.5 nm. An excitation wavelength of 295 nm was chosen since it provides no excitation of tyrosine residues and therefore neither emission nor energy transfer to the lone indole side chain would be nonnegligible. Appropriate blanks corresponding to the buffer were subtracted to correct the fluorescence background.

3. Results and discussion

3.1. Fluorescence characteristics of HSA

Fluorescence quenching refers to any process which decreases the fluorescence intensity of a sample. A variety of molecular interactions can result in quenching, including excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching. The different mechanisms of quenching are usually classified as either dynamic quenching or static quenching. Dynamic and static quenching can be distinguished by their differing dependence on temperature and viscosity. Dynamic quenching depends upon diffusion. Since higher temperatures result in larger diffusion coefficients, the bimolecular quenching constants are expected to increase with increasing temperature. In contrast, increased temperature is likely to result in decreased stability of complexes, and thus lower values of the static quenching constants.

In order to discuss the results within the linear concentration range, we selected to carry out the experiment within the linear part of Stern–Volmer dependence (F_0/F against [Q]). In this experiment, the concentrations of HSA solution were stabilized at 1.0×10^{-5} mol L^{-1} to ensure sensitivity and avoid the inner filter effects [18]. Concentration of MB varied from 0 to 3.6×10^{-5} mol L^{-1} at increments of 0.4×10^{-5} mol L^{-1}. The effect of MB on HSA fluorescence intensity is shown in Fig. 2. As the data show, the fluorescence intensity of HSA decreased regularly with the increase in MB concentration. The inset in Fig. 2 shows that within

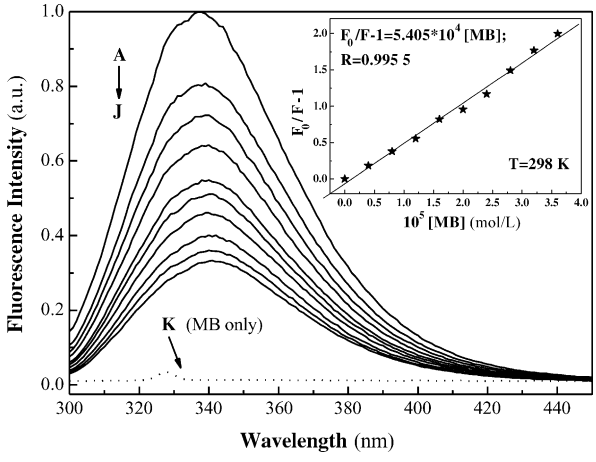

Fig. 2. Emission spectra of HSA in the presence of various concentrations of MB (T = 298 K, λ_{ex} = 295 nm). c(HSA) = 1.0×10^{-5} mol L^{-1}; c(MB)/(10^{-5} mol L^{-1}), A–J: 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6; curve K shows the emission spectrum of MB only. The insert corresponds to the Stern–Volmer plot.

Table 1
Stern–Volmer quenching constants of the system of MB–HSA at different temperatures

pH	T (K)	K_{SV} ($\times 10^{-4}$ L mol^{-1})	R[a]	S.D.[b]
7.4	292	5.598	0.9950	0.0594
	298	5.405	0.9955	0.0605
	304	5.279	0.9978	0.0375
	310	5.152	0.9966	0.0501

[a] R is the correlation coefficient.
[b] S.D. is the standard deviation for the K_{SV} values.

the investigated concentration range, the results agree with the Stern–Volmer equation.

In order to confirm the quenching mechanism, we analyzed the fluorescence quenching data with the assumption that dynamic quenching occurred. For dynamic quenching, the decrease in intensity is described by the well-known Stern–Volmer equation:

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

where, F_0 and F denote the steady-state fluorescence intensities in the absence and in the presence of quencher (MB), respectively, K_{SV} is the Stern–Volmer quenching constant, and [Q] is the concentration of quencher.

The results in Table 1 show that the Stern–Volmer quenching constant K_{SV} is inversely correlated with temperature, which indicates that the probable quenching mechanism of MB–HSA binding reaction is initiated by compound formation rather than by dynamic collision. Therefore, the quenching data were analyzed according to the modified Stern–Volmer equation [19]:

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K_a}\frac{1}{[Q]} + \frac{1}{f_a} \quad (2)$$

In the present case, ΔF is the difference in fluorescence in the absence and presence of the quencher at concentration [Q], f_a is the fraction of accessible fluorescence and K_a is the effective quenching constant for the accessible fluorophores.

The dependence of $F_0/\Delta F$ on the reciprocal value of the quencher concentration $[Q]^{-1}$ is linear with the slope equalling to the value of $(f_a K_a)^{-1}$. The value f_a^{-1} is fixed on the ordinate. The constant K_a is a quotient of the ordinate f_a^{-1} and the slope $(f_a K_a)^{-1}$. The corresponding results at different temperatures are shown in Table 2. The decreasing trend of K_a with increasing temperature was in accordance with K_{SV}'s dependence on temperature as mentioned above. It shows that the binding constant between MB and HSA is considerable and the effect of temperature is small. Thus, MB can be stored and carried by protein in the body.

3.2. The determination of the force acting between MB and HSA

Analysis of Stern–Volmer plots in this system yields equilibrium expressions for static quenching, K_a, which are analogous to associative binding constants for the quencher–acceptor system [20,21]. The interaction forces between drugs and biomolecules may include electrostatic interactions, multiple hydrogen bonds, van der Waals interactions, hydrophobic and steric contacts within the antibody-binding site, etc. [22]. In order to elucidate the interaction between MB and HSA, the thermodynamic parameters were calculated from the van't Hoff plots.

If the enthalpy change (ΔH) does not vary significantly in the temperature range studied, both the enthalpy change (ΔH) and entropy change (ΔS) can be evaluated from the van't Hoff equation [23]:

$$\ln K_a = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (3)$$

where K_a is analogous to the effective quenching constants K_a at the corresponding temperature and R is the gas constant. The temperatures used were 292, 298, 304 and 310 K. The enthalpy change (ΔH) is calculated from the slope of the van't Hoff relationship. The free energy change (ΔG) is then estimated from the following relationship [23]:

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

Fig. 3, by plotting the data in Table 2, shows that assumption of near constant ΔH is justified. Table 2 shows the values of ΔH and ΔS obtained for the binding site from the slopes and ordinates at the origin of the fitted lines.

The negative values of free energy (ΔG), seen in Table 2, supports the assertion that the binding process is spontaneous. The negative enthalpy (ΔH) and positive entropy (ΔS) values of the interaction of MB and HSA indicate that the electrostatic interactions played a major role in the binding reaction [24].

Table 2
Modified Stern–Volmer association constants K_a and relative thermodynamic parameters of the system of MB–HSA

T (K)	K_a ($\times 10^{-4}$ L mol^{-1})	S.D.[a]	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	S.D.[b]
292	4.269	0.038 2	−6.298	−25.880	67.062	0.0031
298	4.058	0.042 7		−26.282		
304	3.839	0.073 9		−26.684		
310	3.678	0.072 8		−27.087		

[a] S.D. is the standard deviation for the K_a values.
[b] S.D. is the standard deviation for thermodynamic parameters.

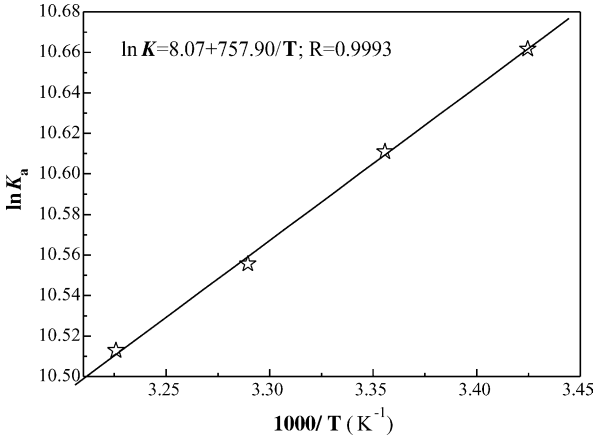

Fig. 3. van't Hoff plot, pH 7.40, c(HSA) = 1.0×10^{-5} mol L^{-1}.

3.3. Analysis of binding equilibria

When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between free and bound molecules is given by the equation [25]:

$$\log\left(\frac{F_0 - F}{F}\right) = \log K_b + n \log[Q] \quad (5)$$

where, in the present case, K_b is the binding constant to a site, and n is the number of binding sites per HSA.

The dependence of $\log(F_0/F-1)$ on the value of log[Q] is linear with slope equal to the value of n and the value $\log K_b$ is fixed on the ordinate. Table 3 gives the results for HSA at different temperatures analyzed in this fashion. The correlation coefficients are greater than 0.99, and the standard deviation is less than 0.05, indicating that the assumptions underlying the derivation of Eq. (5) are satisfactory. Table 3 shows the results of K_b and n decreased with the temperature rising, which may indicate forming an unstable compound MB–HSA in the binding reaction. The compound would possibly be partly decomposed when the temperature increases, therefore, the values of K_b and n decreased with the temperatures rising, which was in accordance with the trend of K_{SV} and K_a, as mentioned above.

3.4. Energy transfer from HSA to MB

FRET is a nondestructive spectroscopic method that can monitor the proximity and relative angular orientation of fluorophores, the donor and acceptor fluorophores can be entirely separate or attached to the same macromolecule. A transfer of energy could take place through direct electrodynamic interaction between the primarily excited molecule and its neighbors [26]. The "spectroscopic ruler" is suitable for distance measurement over several nanometers [27]. Using FRET, the distance r between MB and HSA (Trp-214 [11,12,28]) could be calculated by the equation [28,29]:

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

where E denotes the efficiency of transfer between the donor and the acceptor, and R_0 is the critical distance when the efficiency of transfer is 50%:

$$R_0^6 = 8.79 \times 10^{-25} K^2 n^{-4} \phi J \quad (7)$$

In Eq. (7), K^2 is the orientation factor related to the geometry of the donor and acceptor of dipoles and $K^2 = 2/3$ for random orientation as in fluid solution; n is the average refracted index of medium in the wavelength range where spectral overlap is significant; ϕ is the fluorescence quantum yield of the donor; J is the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor (Fig. 4), which could be calculated by the equation:

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 d\lambda}{\int_0^\infty F(\lambda) d\lambda} \quad (8)$$

where $F(\lambda)$ is the corrected fluorescence intensity of the donor in the wavelength range, from λ to $\lambda + \Delta\lambda$; $\varepsilon(\lambda)$ is the extinction coefficient of the acceptor at λ.

In the present case, $K^2 = 2/3$, $n = 1.36$ and $\phi = 0.074$ [30], according to the Eqs. (6)–(8), we could calculate that $J = 1.466 \times 10^{-14}$ cm^3 L mol^{-1}, $E = 0.306$, $R_0 = 2.389$ nm and $r = 2.738$ nm. The average distances between a donor fluorophore and acceptor fluorophore on the 2–to–8 nm scale [31] and $0.5R_0 < r < 1.5R_0$ [32], which indicates that the energy transfer from HSA to MB occurs with high probability.

Table 3
Binding constants K_b and binding sites n at different temperatures

pH	T (K)	K_b ($\times 10^{-5}$ L mol^{-1})	n	R^a	S.D.[b]
7.4	292	2.203	1.130	0.9972	0.0205
	298	1.675	1.110	0.9971	0.0204
	304	1.570	1.105	0.9984	0.0204
	310	1.265	1.091	0.9977	0.0243

[a] R is the correlation coefficient.
[b] S.D. is the standard deviation for K_b values.

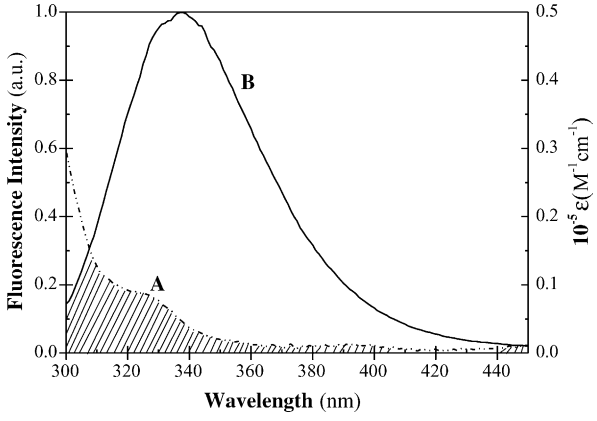

Fig. 4. Spectral overlap of MB absorption (A) with HSA fluorescence (B) (c(HSA) = c(MB) = 1.0×10^{-5} mol L^{-1}).

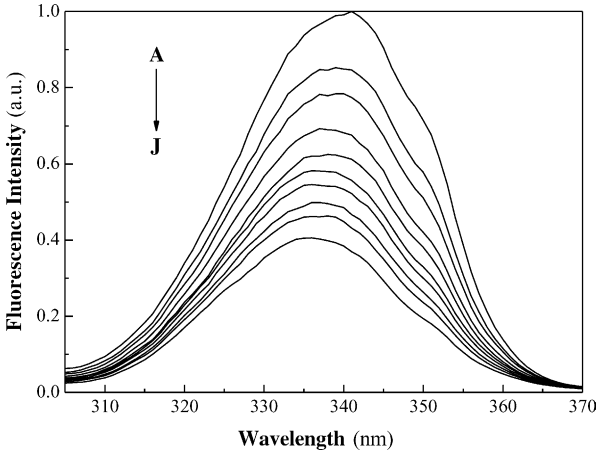

Fig. 5. Synchronous fluorescence spectrum of HSA (T = 298 K, $\Delta\lambda$ = 60 nm). c(HSA) = 1.0×10^{-5} mol L^{-1}; c(MB)/(10^{-5} mol L^{-1}); A–J: 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, 3.6.

3.5. Conformation investigation

Trp-214, conserved in mammalian albumins, plays an important structural role in the formation of HSA [12]. Spectroscopy is an ideal tool to observe conformational changes in proteins since it allows non-intrusive measurements of substances in low concentration under physiological conditions. It is advantageous to use intrinsic fluorophores for these investigations in order to avoid complicated labeling with an extrinsic dye [33].

The synchronous fluorescence spectra give information about the molecular environment in the vicinity of the chromosphere molecules. In the synchronous spectra, the sensitivity associated with fluorescence is maintained while offering several advantages: spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects. The authors [34] suggested a useful method to study the environment of amino acid residues and to measure the possible shift in wavelength emission maximum λ_{max}, that corresponds to the changes of polarity around the chromophore molecule. When the D-value ($\Delta\lambda$) between excitation wavelength and emission wavelength were stabilized at 60 nm, the synchronous fluorescence gives the characteristic information of tryptophan residues [35]. The effect of MB on HSA synchronous fluorescence spectroscopy is shown in Fig. 5.

It is apparent from Fig. 5 that the maximum emission wavelength blue-shifts (from 341 to 335.5 nm) at the investigated concentrations range when $\Delta\lambda$ = 60 nm. The blue-shift of the emission maximum suggests a less polar (or more hydrophobic) environment of Trp-214 residue [36].

4. Conclusions

In this paper, we investigated the nature and magnitude of the interaction of MB with HSA by spectroscopic methods including fluorescence spectroscopy and UV–vis absorption spectroscopy. The results of synchronous fluorescence spectra indicate that the conformation of HSA molecules is changed significantly in the presence of MB. Stern–Volmer plots and related calculations indicate the presence of static component in quenching mechanism, spontaneity in MB–HSA binding reaction and the electrostatic nature as a major factor in the interaction.

Acknowledgements

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