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Spectroscopic studies on the interaction between methylene blue and bovine serum albumin

Yan-Jun Hu [a], Yi Liu [a,b,*], Ru-Ming Zhao [a], Jia-Xin Dong [a], Song-Sheng Qu [a]

[a] College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, PR China
[b] State Key Laboratory of Virology, Wuhan University, Wuhan 430072, PR China

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Abstract

The interaction between methylene blue (MB) and bovine serum albumin (BSA) was investigated by fluorescence and UV–vis absorbance spectroscopy. In the mechanism discussion, it was proved that the fluorescence quenching of BSA by MB is mainly a result of the formation of MB–BSA complex and electrostatic interactions play an important role to stabilize the complex. The Stern–Volmer quenching constant K_{SV} and corresponding thermodynamic parameters ΔH, ΔG, and ΔS were calculated. The distance r between donor (BSA) and acceptor (MB) was obtained according to fluorescence resonance energy transfer (FRET). The effect of MB on the conformation of BSA has been analyzed by means of UV–vis absorbance spectra and synchronous fluorescence spectroscopy.
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Keywords: Methylene blue; Bovine serum albumin; Fluorescence quenching; Thermodynamic parameters; Fluorescence resonance energy transfer

1. Introduction

Protein–drug binding greatly influences absorption, distribution, metabolism, and excretion properties of typical drugs [1]. Serum albumins are the most abundant proteins in the circulatory system of a wide variety of organisms, being the major macromolecule contributing to the osmotic blood pressure [2]. In addition to blood plasma, serum albumins are also found in tissues and bodily secretions throughout the body; the extravascular protein comprises 60% of the total albumin [3]. In this work, bovine serum albumin (BSA) is selected as our protein model because of its medically important, low cost, ready availability, unusual ligand-binding properties [4], and the results of all the studies are consistent with the fact that human and bovine serum albumins are homologous proteins [4–6].

This paper investigates the association of BSA with methylene blue (MB); MB (Formula: $C_{16}H_{18}N_3ClS$, CAS Registry Number: 61-73-4), is also named methylenum caeruleum, tetramethylthionine chloride or Swiss blue. As its structure shown in Fig. 1, it is a coplanar, polycyclic and aromatic basic dye that belongs to the thiazine class. It has long been used as a tool to dissect intracellular redox metabolism [7–9], as well as for biological staining and diagnosis of diseases including carcinoma [10,11], and can be used as an effective antidote [12]. 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 [13,14]. It is widely accepted in the pharmaceutical industry that the overall distribution, metabolism, and efficacy of many drugs can be altered based on their affinity to serum albumin. In addition, many promising new drugs are rendered ineffective because of their unusually high affinity for this abundant protein. Obviously, binding studies of drugs with BSA are useful for the understanding of the reaction mechanism, as well as for providing guidance for the application and design of new drugs. 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 BSA with MB have yet to be conducted.

Fluorescence quenching is the decrease of quantum yield of fluorescence from a fluorophore induced by a variety of molecular interactions with quencher molecule. Application of the spectral methods can reveal the reactivity of chemical and

* Corresponding author. Tel.: +86 27 87218284; fax: +86 27 68754067.
E-mail addresses: yjhu@263.net, prof.liuyi@263.net (Y. Liu).

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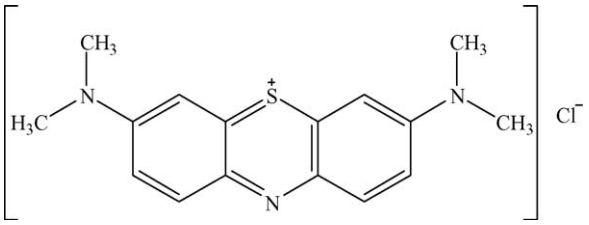

Fig. 1. Molecular structure of methylene blue.

biological systems in low concentration under physiological conditions, and there have been several studies on fluorescence quenching of albumin induced by drugs or other bioactive small molecules [15–18]. In this paper, the quenching of the intrinsic tryptophan fluorescence of BSA has been used as a tool to study the interaction of MB with this transport protein in an attempt to characterize the chemical association taking place.

2. Materials and methods

2.1. Materials

BSA and MB, being electrophoresis grade reagents, 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, 0.10 mol L^{-1} NaCl, pH 7.4 ± 0.1). All solutions were prepared with doubly distilled water. Sample masses were accurately weighted on a microbalance (Sartorius, ME215S) with a resolution of 0.1 mg.

2.2. Equipments and spectral measurements

The UV spectrum was recorded at room temperature on a TU-1901 spectrophotometer (Puxi Analytic Instrument Ltd., Beijing, China) equipped with 1.0 cm quartz cells. The fluorescence spectra at different temperatures (298, 302, 306 and 310 K) were recorded on F-2500 Spectrofluorimeter (Hitachi, Japan) equipped with 1.0 cm quartz cells and a thermostat bath. Excitation wavelength was 280 nm. The widths of both the excitation slit and the emission slit were set to 2.5 nm. Appropriate blanks corresponding to the buffer were subtracted to correct the fluorescence background.

2.3. Principles of fluorescence quenching

Fluorescence quenching 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 the quencher. Hence, Eq. (1) was applied to determine K_{SV} by linear regression of a plot of F_0/F against $[Q]$.

In many instances, the fluorophore can be quenched both by collision and by complex formation with the same quencher. When in this case, the Stern–Volmer plot exhibits an upward curvature, concave towards the y-axis at high $[Q]$, and F_0/F is related to $[Q]$ by the following form of the Stern–Volmer equation [19,20]:

$$\frac{F_0}{F} = (1 + K_D[Q])(1 + K_S[Q]) \quad (2)$$

where K_D and K_S are the dynamic and static quenching constants, respectively. The first factor on the right-hand side in Eq. (2) describes the "dynamic" quenching, resulting from encounters of quencher and fluorophore during the excited state and the second factor describes the "static" quenching, that is quenching by formation of a complex between quencher and fluorophore predating the excitation. It will be noticed that if the quenching effect of either of these two kinds is much greater than the other, the quenching follows the Stern–Volmer linear dependence of F_0/F upon $[Q]$ (Eq. (1)). If there is a departure from linearity, due to the term in $[Q]^2$ being appreciable, it is certain that both ground-state complexes and excited-state interactions contribute to the quenching, which accounts for the upward curvature observed at high $[Q]$ when both static and dynamic quenching occur for the same fluorophore.

3. Results and discussions

3.1. Effect of MB on BSA spectra

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 dynamic quenching, static quenching or static and dynamic quenching participates in it simultaneously. 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 this experiment, the concentrations of BSA were stabilized at 1.0×10^{-5} mol L^{-1}, and the 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 BSA fluorescence intensity is shown in Fig. 2. As can be seen from Fig. 2, addition of increasing concentrations of MB caused a progressive reduction of the fluorescence intensity. The inset in Fig. 2 shows that within the investigated concentrations range, the results agree with the Stern–Volmer Eq. (1). The Stern–Volmer plot does not show deviation towards the y-axis obviously at the experimental concentration range, which is an indication that either dynamic quenching or static quenching is predominant.

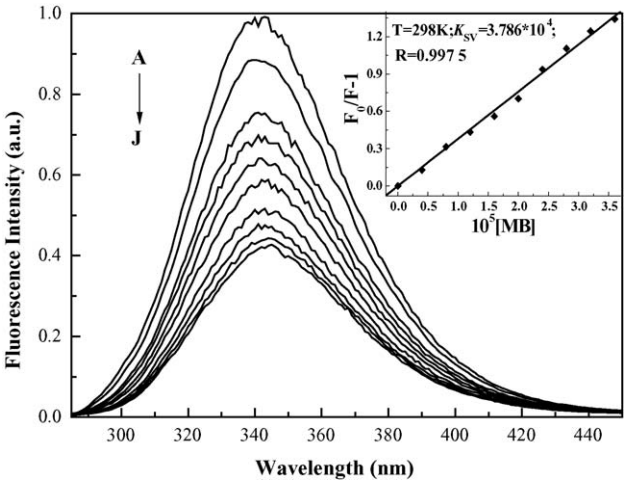

Fig. 2. Effect of MB on fluorescence spectrum of BSA (T = 298 K). c (BSA) = 1.0×10^{-5} mol L^{-1}; c (MB)/(10^{-5} mol L^{-1}), A–J: 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4, 2.8, 3.2, and 3.6, respectively. The inset corresponds to the Stern–Volmer plot.

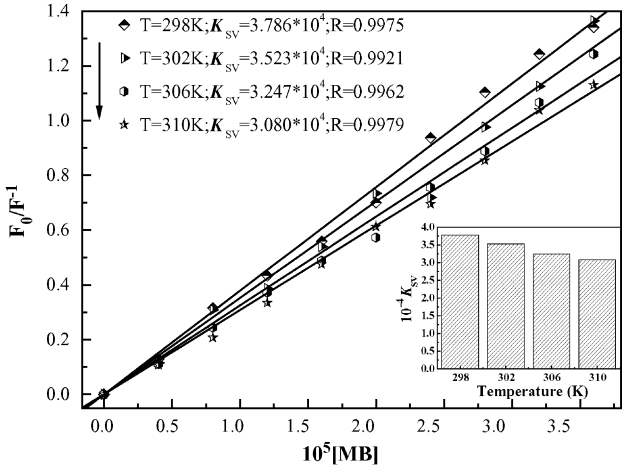

Fig. 3. Stern–Volmer plots for the quenching of BSA by MB at different temperatures. The inset shows the relationship of the Stern–Volmer quenching constants K_{SV} and temperature T.

Fig. 3 displays the Stern–Volmer plots of the quenching of BSA tryptophan residues fluorescence by MB at different temperatures. Table 1 summarizes the calculated K_{SV} at each temperature studied, the results show that the Stern–Volmer quenching constant K_{SV} is inversely correlated with temperature, which indicate that the probable quenching mechanism of MB–BSA binding reaction is initiated by compound formation rather than by dynamic collision [19]. In other words, the fluorescence quenching of BSA results from complex formation is predominant, while from dynamic collision could be negligible. For reconfirming the probable quenching mechanism of fluorescence of BSA by MB is mainly initiated by ground-state complex formation, we used the difference absorption spectroscopy to obtain spectra, the UV–vis absorption spectrum of BSA and the difference absorption spectrum between BSA–MB and MB at the same concentration could not be superposed within experimental error (Fig. 4), this result reconfirm that the probable quenching mechanism of fluorescence of BSA by MB is mainly a static quenching procedure [19]. Therefore, the quenching data were analyzed according to the modified Stern–Volmer equation [21]:

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

pH	T (K)	$10^{-4}K_{SV}$ (L mol^{-1})	R[a]	S.D.[b]
7.40	298	3.786	0.9975	0.0346
	302	3.523	0.9921	0.0589
	306	3.247	0.9962	0.0379
	310	3.080	0.9979	0.0269

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

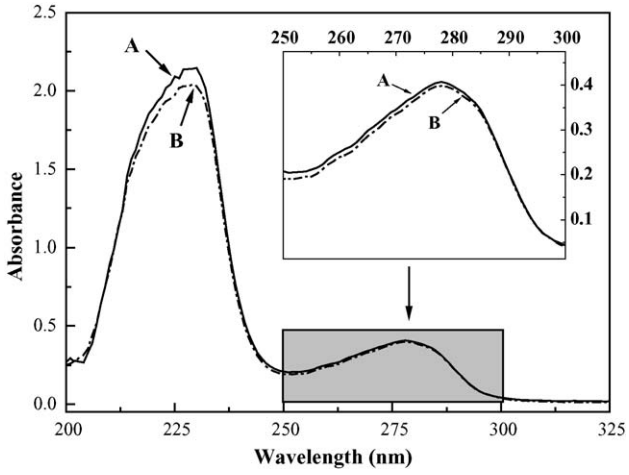

Fig. 4. UV–vis spectra of BSA in the presence of MB. (A) The absorption spectrum of BSA only; (B) the difference absorption spectrum between MB–BSA and MB at the same concentration; c (BSA) = c (MB) = 1.0×10^{-5} mol L^{-1}.

$$\frac{F_0}{\Delta F} = \frac{1}{f_a K_a [Q]} + \frac{1}{f_a} \quad (3)$$

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, which are analogous to associative binding constants for the quencher–acceptor system [22].

The dependence of $F_0/\Delta F$ on the reciprocal value of the quencher concentration $[Q]^{-1}$ is linear with slope equal 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 an ordinate $f_a{}^{-1}$ and 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 BSA is moderate 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 BSA

The interactions forces between drugs and biomolecules may include electrostatic interactions, multiple hydrogen bonds, van

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

T (K)	$10^{-3}K_a$ (L mol^{-1})	R[a]	S.D.[b]	ΔH (kJ mol^{-1})	ΔG (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
298	2.449	0.9943	0.1655	−10.309	−19.326	30.263
302	2.302	0.9917	0.2424		−19.448	
306	2.185	0.9989	0.1192		−19.569	
310	2.084	0.9993	0.1079		−19.670	

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

der Waals interactions, hydrophobic and steric contacts within the antibody-binding site, etc. In order to elucidate the interaction of MB with BSA, the thermodynamic parameters were calculated from the van't Hoff plots.

If the enthalpy change (ΔH) does not vary significantly over the temperature range studied, then its value and that of entropy change (ΔS) can be determined from the van't Hoff equation:

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

where associative binding constants K are analogous to the effective quenching constants K_a at the corresponding temperature and R is the gas constant. The temperatures used were 298, 302, 306 and 310 K. The enthalpy change (ΔH) is calculated from the slope of the van't Hoff relationship. The free energy change (ΔG) is estimated from the following relationship:

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

Fig. 5, by fitting the data of 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.

From Table 2, it can be seen that the negative sign for free energy (ΔG) means that the binding process is spontaneous. The negative enthalpy (ΔH) and positive entropy (ΔS) values of the interaction of MB and BSA indicate that the specific electrostatic interactions played major role in the reaction [23]. BSA is characterized by two tryptophan residues: Trp-134 is thought to be located on the surface (sub-domain IA) and Trp-212 is considered to be located within a hydrophobic pocket [24], which is in a well-characterized binding cavity (sub-domain IIA) for small charged aromatic molecules [2,3,25]. The study with proteolytic fragments of BSA suggests that sub-domain IIA constitute the essential region for bilirubin and several dyes binding [26,27]; the crystallographic analysis of serum albumin also revealed that the major ligand binding sites are identified within this region [28]; it is also known that the binding activity of the sub-domain IIA affects conformational changes [29], which agrees with the conformation investigation below. Thus, combining this analysis with the structure of MB, we can infer that the binding site for MB on BSA is mainly located in sub-domain IIA. Furthermore, the specific electrostatic interactions are characterized by a concomitant increased thermal stability of the protein conformation.

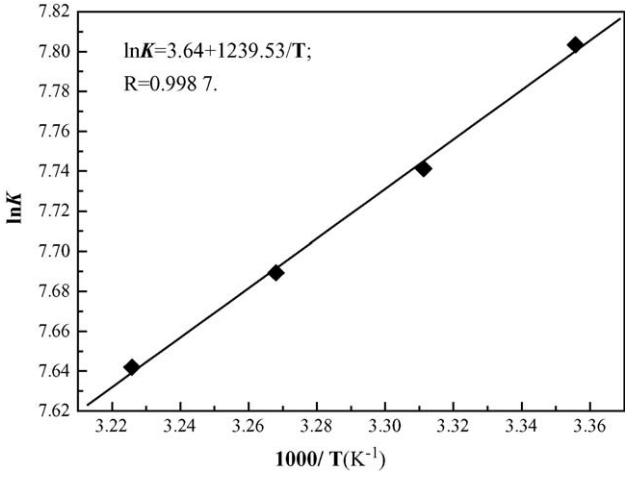

Fig. 5. van't Hoff plot, pH 7.40, c (BSA) = 1.0×10^{-5} mol L^{-1}.

3.3. Energy transfer from BSA to MB

FRET is a non-destructive 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 [30], which will happen under conditions: (i) the donor can produce fuorescence light; (ii) fuorescence emission spectrum of the donor and UV–vis absorbance spectrum of the acceptor have more overlap; and (iii) the distance between the donor and the acceptor is approach and <8 nm [31]. Using FRET, the transfer efficiency (E) could be calculated by the equation:

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

where E could be determined experimentally from the donor emission in the absence (F_0) and presence of the acceptor (F), normalized to the same donor concentration [32]; r is the actual distance between the donor (BSA) and the acceptor (MB); R_0 is the critical distance when the efficiency of transfer is 50%, which depends on the quantum yield of the donor, the extinction coefficient of the acceptor, the overlap of donor emission and acceptor excitation spectra, and the mutual orientation of the chromophores.

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

In Eq. (7), K^2 is the space factor of orientation; n the refracted index of medium; ϕ the fluorescence quantum yield of the donor;

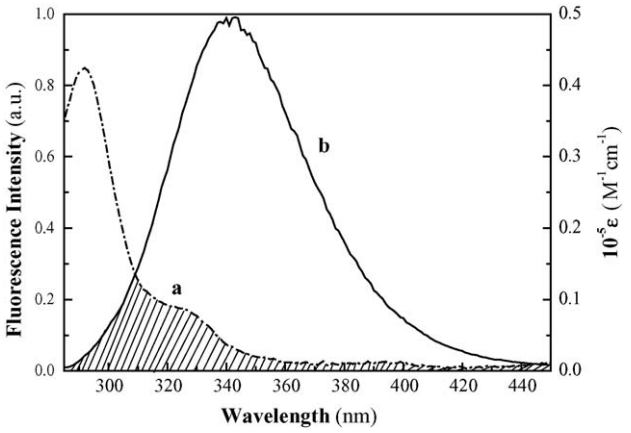

Fig. 6. Spectral overlap of MB absorption (a) with BSA fluorescence (b). c (BSA) = c (MB) = 1.0×10^{-5} mol L^{-1}.

J is the effect of the spectral overlap between the emission spectrum of the donor and the absorption spectrum of the acceptor (Fig. 6), 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 λ to $\lambda + \Delta\lambda$ and $\varepsilon(\lambda)$ is the extinction coefficient of the acceptor at λ.

In the present case, $K^2 = 2/3$, $n = 1.36$, $\phi = 0.15$ [33], according to the Eqs. (6)–(8), we could calculate that $J = 1.42 \times 10^{-14}$ cm^3 L mol^{-1}, $E = 0.27$, $R_0 = 2.67$ nm, and $r = 3.15$ nm. The average distance $r < 8$ nm, which indicates that the energy transfer from BSA to MB occurs with high probability.

3.4. Conformation investigation

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. Tryptophan is highly sensitive to the local environment and also displays a substantial spectral shift. As a result, the position of the spectra maximum (λ_{max}) depends on the properties of the environment of the tryptophanyl residues [34], and the fluorescence spectra depends upon the degree of exposure of the tryptophanyl side chain to the polar, aqueous solvent and upon its proximity to specific quenching groups [6]. Hence, it could be used as an ideal tool to study protein structure and conformation.

To explore the structural change of BSA by addition of MB, we measured UV–vis absorbance spectra (Fig. 7) and synchronous fluorescence spectra (Fig. 8) of BSA with various amounts of MB. Fig. 7 displays the UV–vis absorbance spectra of BSA at different contents of MB. It is clear from the figure that the baselines of the UV–vis absorbance spectra at 300–250 nm are raised and the absorption spectra maximum red shift (from

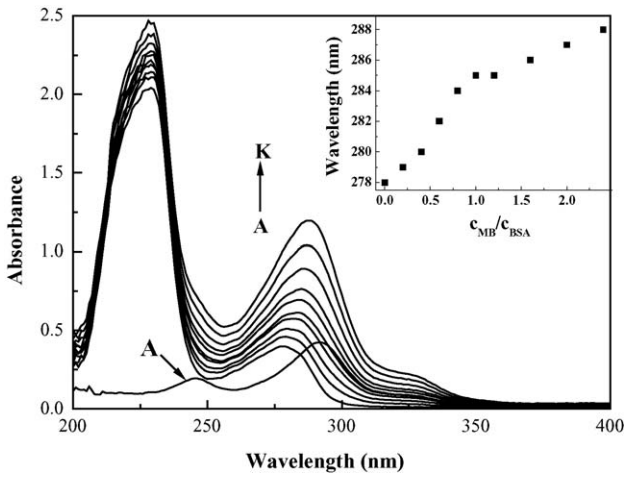

Fig. 7. UV–vis absorbance spectra of BSA in the presence of MB. Curve A: c (BSA) = 0, c (MB) = 1.0×10^{-5} mol L^{-1}; B–K, c (BSA) = 1.0×10^{-5} mol L^{-1}, c (MB)/(10^{-5} mol L^{-1}): 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, 2.0, 2.4. The inset shows the relationship of the absorption spectra maximum wavelength at about 280 nm and the mole ratio between MB and BSA.

278 to 288 nm), indicating that the changes in the environment of the tryptophanyl residues of BSA are occurring and the peptides strand extended less, the hydrophobicity was increased. The absence of water in the molecular interior increases the conformational stability, the rigidity, mechanical strength, and the contributions of electrostatic interactions.

Synchronous fluorescence spectroscopy introduced by Llody [35,36] has been used to characterize complex mixtures providing fingerprints of complex samples [37]. It involves the simultaneous scanning of excitation and the fluorescence monochromators of a fluorimeter, while maintaining a fixed wavelength difference ($\Delta\lambda$) between them. The synchronous fluorescence spectra give information about the molecular environment in a vicinity of the chromospheres 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

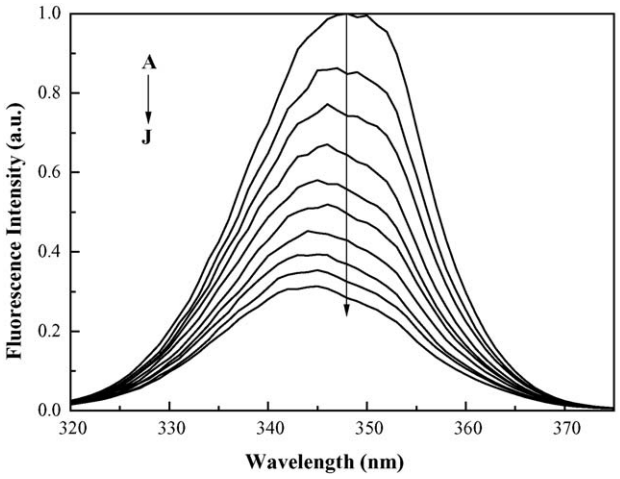

Fig. 8. Synchronous fluorescence spectrum of BSA: (T = 298 K, $\Delta\lambda$ = 60 nm), c (BSA) = 1.0×10^{-5} mol L^{-1}; c (MB)/(10^{-5} mol L^{-1}); A–J: 0, 0.4, 0.8, 1.2, 1.6, 2.0, 2.4,.2.8, 3.2, and 3.6, respectively.

effect. The authors [38] suggested a useful method to study the environment of amino acid residues was by measuring the possible shift in wavelength emission maximum λ_{max}, the shift in position of emission maximum corresponding to the changes of the 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 [39]. The effect of MB on BSA synchronous fluorescence spectroscopy is shown in Fig. 8.

It is apparent from Fig. 8 that the maximum emission wavelength blue-shifts (from 348 to 345 nm) at the investigated concentrations range when $\Delta\lambda = 60$ nm. The blue-shifts effect express the conformation of BSA was changed. It is also indicated that the polarity around the tryptophan residues was decreased and the hydrophobicity was increased. The conclusion agrees with the result of conformational changes by UV–vis absorbance spectra.

4. Conclusions

In this paper, the interaction of MB with BSA was studied by spectroscopic methods including fluorescence spectroscopy and UV–vis absorption spectroscopy. The results of synchronous fluorescence spectra and UV–vis spectra indicate that the secondary structure of BSA molecules is changed dramatically in the presence of MB. The experimental results also indicate that the probable quenching mechanism of fluorescence of BSA by MB is mainly a static quenching procedure, and the binding reaction is spontaneous and electrostatic interactions played major role in the reaction.

Acknowledgements

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