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New method to research the reaction of methyl green and bovine serum albumin

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ABSTRACT

Under simulated physiological conditions, we studied the reaction mechanism of Methyl Green with Bovine serum albumin at different temperature (293K, 303K, 310K) by utilizing fluorescence quenching method and a new method (synchronous fluorescence), respectively. The results indicate that Methyl Green could quench the intrinsic fluorescence of Bovine serum albumin strongly, and the quenching mechanism was a static quenching process. The hydrogen bonding and van der Waals force played an important role on the conjugation reaction between Methyl Green and Bovine serum albumin. The order of magnitude of binding constants (K_a) was 10^4, the number of binding site (n) in the binary system was approximately equal to 1 and the primary binding for Hydrogen bonding and van der Waals force were located at the structure domain II A of Bovine serum albumin. The results obtained by the two methods were consistent, which indicated synchronous fluorescence spectroscopy can replace traditional fluorescence quenching method to study reaction mechanism of dyes with proteins.

KEYWORDS

Fluorescence spectroscopy;
Synchronous fluorescence;
Methyl Green;
Bovine serum albumin;
Interaction.

INTRODUCTION

Serum albumin, the most abundant protein constituent in blood plasma, can be combined with a lot of endogenous and exogenous compounds and plays a fundamental role in the disposition and transportation of various molecules. Therefore, investigating the binding mechanism of endogenous or exogenous compounds and serum albumins has very significant implications for the life sciences, chemistry, pharmacy and clinical medicine. The methods of studying on the interactions of protein with small molecular ligands are mainly spectroscopy (UV-vis absorption spectroscopy, fluorescence spectroscopy, circular dichroism method, fourier transform infrared spectrometry, resonance light scattering method and chemiluminescence method), high performance liquid chromatography (HPLC), the equilibrium dialysis method, capillary electrophoresis method and electrochemical method. However, the fluorescence spectrometry is the most widely used one. The conventional fluorescence spectroscopy studies the reaction mechanism of small molecule material and proteins, which mainly by studying the change of fluorescence intensity of protein in the maximum emission wavelength

before and after adding the small molecule material, and then got the binding constants, binding sites and the donor-to-acceptor distance with other information between proteins and small molecule material[1-3]. Synchronous fluorescence spectrometry technology was first proposed by Lloyd[4], and the biggest difference between it and fluorescence measurement method is scanning the excitation and emission monochromators simultaneously. Comparing with conventional fluorescence spectroscopy, synchronous fluorescence method has some advantages such as good selectivity, high sensitivity, less interference etc[5], and it can be used for the simultaneous determination of multi-component mixture[6]. However, research of the binding constant and binding sites between the protein and the dye with this method has not been reported.

Methyl green (MG) is a basic triphenylmethane and dicationic dye usually used for staining of solutions in medicine and biology[7]. In this study, we study the binding mechanism of dyes and proteins by utilizing traditional fluorescence quenching method and synchronous fluorescence method, whose research object is Methyl Green, respectively. We can see the results of two methods are consistent. The new method is a useful supplement to the fluorescence spectroscopy's application about the research of combination mechanism of proteins and dyes.

EXPERIMENTAL

Apparatus and materials

All fluorescence spectra were recorded on a Shimadzu RF-5301PC spectro-fluorophotometer. All spectrophotometric measurements were made with a Shimadzu UV-265 spectrophotometer. All pH measurements were made with a pHS-3C precision acidity meter (Leici, Shanghai). All temperatures were controlled by a CS501 super-heated water bath (Nantong Science Instrument Factory).

Bovine serum albumin was purchased from Sigma(the purity grade inferior 99%) and stock solutions (1.0×10^{-5} mol L^{-1}) were prepared by doubly distilled water. Methyl Green(2.0×10^{-3} mol L^{-1}) were prepared by doubly distilled water, respectively. All the stock solutions were further diluted as working solutions prior to use. The Tris–HCl buffer (0.05 mol L^{-1}, pH=7.4) containing 0.15 mol L^{-1} NaCl was selected to keep the pH value constant and to maintain the ionic strength of the solution. All other reagents were of analytical reagent grade and double-distilled water was used during the experiment. And all the stock solutions were stored at 277 K.

The fluorescence intensities were corrected for the absorption of excitation light and re-absorption of emitted light to decrease the inner filter using the following relationship[8]:

$$F_{cor} = F_{obs} \times e^{(A_{ex}+A_{em})/2} \quad (1)$$

where F_{cor} and F_{obs} are the corrected and observed fluorescence intensities respectively. A_{ex} and A_{em} are the absorbance values of aspirin at excitation and emission wavelengths, respectively. The fluorescence intensity used in this paper was corrected.

Procedures

Fluorescence spectra and synchronous fluorescence spectra

In the experiment we use the 1.0 mL of pH 7.40 Tris-HCl, a certain amount of 1.0×10^{-5} mol L^{-1} BSA solution, and different concentrations of MG (1.0×10^{-3} mol L^{-1}) were added into 10 mL colorimetric tube sequentially. The samples were diluted to scaled volume with double-distilled water, mixed thoroughly by shaking, and kept static for 30 minutes. The fluorescence emission spectra were measured at 293, 303, and 310 K with the width of the excitation and emission slit adjusted at 5.0 and 5.0 nm, respectively. An excitation wavelength of 280 nm (or 295 nm) was chosen and the emission wavelength was recorded from 285 to 450 nm. The synchronous fluorescence spectra were scanning obtained by simultaneously the excitation and emission monochromators. It were recorded at $\Delta\lambda$=15 nm and 60 nm in the absence and presence of various amounts of MG over a wavelength range of 280-400 nm.

Determination of the binding sites

At 293K, different concentrations (1.0×10^{-5} mol L^{-1}) of site marker I (WF), II (IB), or III (DG) were added to the mixture of BSA-MG systems. After the method according to 2.2.1 operation.

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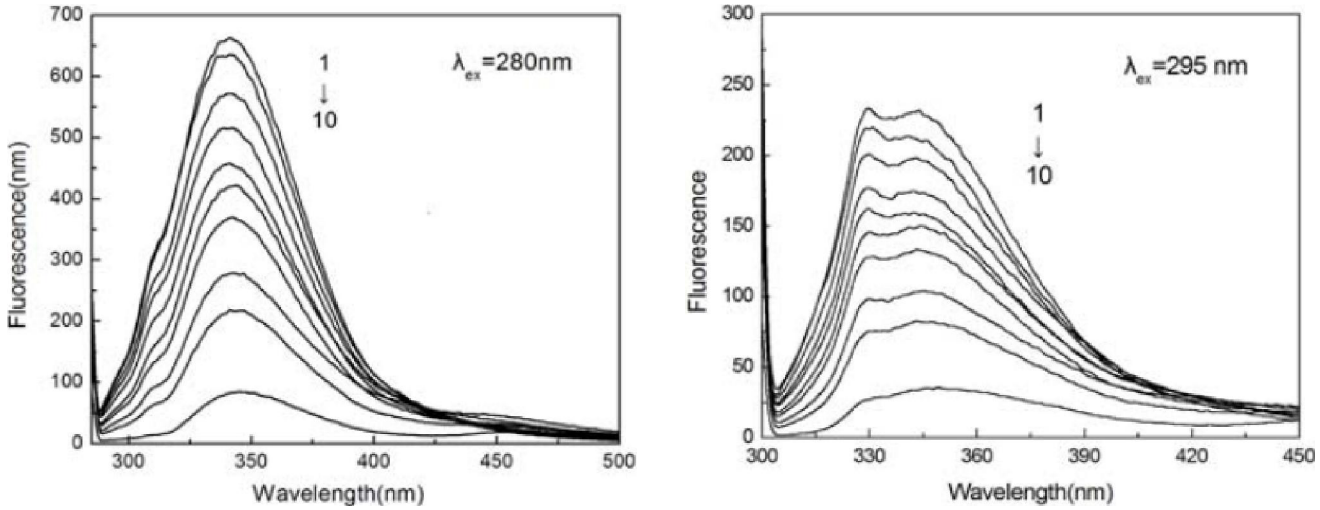

Figure 1 : Fluorescence emission spectra of BSA-MG (T=293K); C_{BSA}=3×10^{-7} mol L^{-1}, 1~10: C_{MG}=(0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.0, 1.5, 2.0, 4.0)×10^{-4} mol L^{-1}

TABLE 1 : Quenching reactive parameters of MG and BSA at different temperatures

System	λ_{ex}(nm)	T/(K)	K_q/(L mol^{-1}·s^{-1})	r_1	K_a/(L mol^{-1})	n	r_2
BSA-MG	280	293	9.80×10^{11}	0.9967	1.91×10^4	1.01	0.9938
		303	8.67×10^{11}	0.9989	1.68×10^4	1.04	0.9978
		310	8.09×10^{11}	0.9953	0.98×10^4	1.06	0.9922
	295	293	9.12×10^{11}	0.9944	2.04×10^4	1.05	0.9971
		303	8.83×10^{11}	0.9981	1.66×10^4	1.04	0.9984
		310	8.16×10^{11}	0.9968	1.03×10^4	1.03	0.9973

r_1 is the linear relative coefficient of F_0/F~[Q]; r_2 is the linear relative coefficient of $lg(F_0-F)/F$~lg[Q].

RESULT AND DISCUSSION

Fluorescence quenching spectra of BSA MG system

The intrinsic fluorescence of protein is a sensitive tool to study the conformation of protein when its environment or structure gets change. The quenching mechanism of fluorescence can be classified into static quenching and dynamic quenching[9]. Dynamic quenching is mainly caused by collisional encounters between the fluorophore and the quencher, static quenching is mainly resulted from the formation of stable compound between fluorophore and quencher[10]. Figure 1 shows the fluorescence emission spectra of BSA in the presence of various concentrations of MG at 293 K. The fluorescence emission intensity of BSA decreased regularly with the gradual addition of MG. This result indicates that MG can interact with BSA and quench its intrinsic fluorescence, changing the microenvironment of the fluorophores.

If it is assumed that the fluorescence quenching mechanism of BSA by MG is dynamic quenching process, fluorescence quenching can be described by Stern-Volmer equation[11].

$$F_0 / F = 1 + K_q \tau_0 [Q] = 1 + K_{sv}[Q] \quad (2)$$

Where F and F_0 are the relative fluorescence intensities in the presence and absence of quencher, respectively; [Q] is the concentration of quencher, K_{sv} is the Stern-Volmer quenching constant, which measures the efficiency of quenching. k_q is the quenching rate constant of the biomolecule, τ_0 is the average lifetime of the biomolecule in absence of quencher evaluated at about 10^{-8} s[12]. According to the Stern-Volmer plots of F_0/F versus quencher concentration at different temperatures (293, 303, and 310 K). The quenching rate constant K_q was obtained and listed in TABLE 1. It is obvious K_q decreases with rising temperatures, revealing that the quenching is initiated by static quenching process. Moreover, the values of K_q between BSA and or MG are all greater than 2×10^{10} L·mol^{-1}·s^{-1}. Therefore, MG

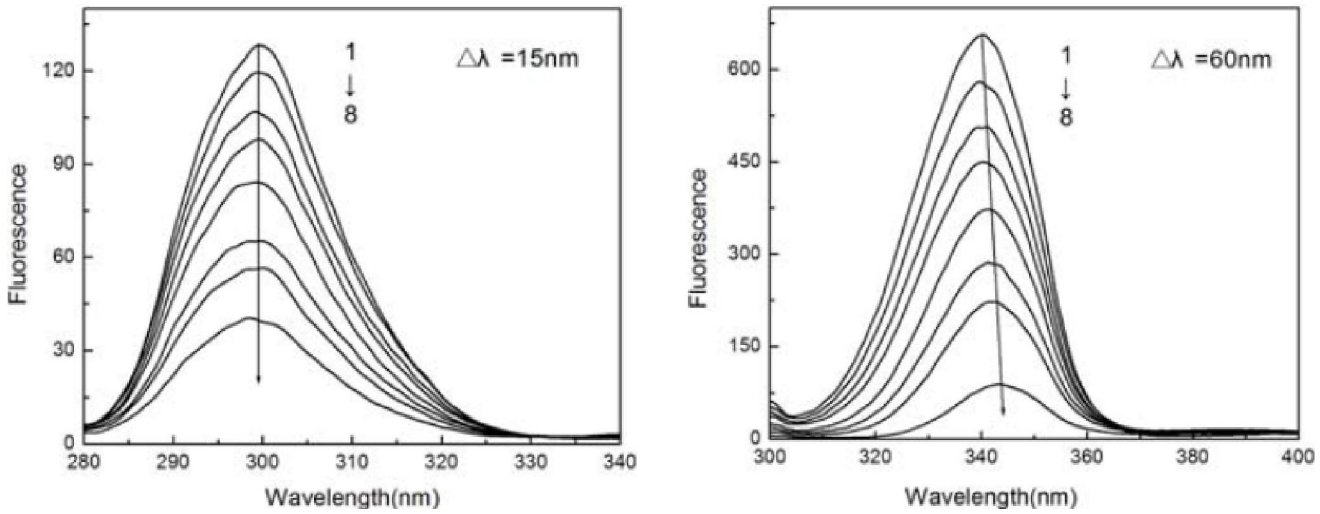

Figure 2 : Synchronous fluorescence spectra of BSA-MG system (T=293K); C_{BSA}=3.0×10^{-7} mol L^{-1} 1~8: C_{MG}=(0, 0.1, 0.2, 0.4, 0.6, 1.0, 1.5, 2.0)×10^{-4} mol L^{-1}

binding BSA was a static quenching process proved to be true[13].

For the static quenching interaction, under the assumption that there are similar and independent binding sites in the biomolecule, the binding constant and the number of binding sites can be derived from the double logarithm regression curve (Eq. (3))[14]

$$\lg[(F_0 - F)/F] = n\lg[Q] + \lg K_a \quad (3)$$

Where K_a is the binding constant, n is the number of binding sites. [Q] is the total concentrations of MG. The curve of log [(F_0-F)/F] versus log [Q] is drawn and fitted linearly, then the value of n and K_a can be obtained from the plot. And TABLE 1 gives the corresponding calculated results. The value of n almost equals to 1, indicating that there is one class of binding site for MG to BSA molecule. In other words, MG and BSA form a complex with molar ratio 1:1. According to the results shown in TABLE 1, the binding constants of the interaction between MG and BSA decreases with the rising temperature, further suggested that the quenching was a static process[15]. At 280 nm wavelength the Trp and Tyr residues in BSA are excited, whereas the 295 nm wavelength excites only Trp residues. From TABLE 1 shows, At the same temperature the binding constant K_a of BSA-MG system at 280 nm and 295 nm were basically the same. This phenomenon showed that Trp residues played an important role in the interaction between MG and BSA.

Synchronous fluorescence spectra

The synchronous fluorescence spectra can provide information on the molecular microenvironment, particularly in the vicinity of the fluorophore functional groups[16]. When Δλ is 15 nm, synchronous fluorescence detects characteristics of tyrosine(Tyr) residues, but when Δλ is 60 nm, characteristic information from tryptophan(Trp) residues is highlighted[17].

The synchronous fluorescence spectra of BSA-MG systems shown in Figure 2. As seen in Figure 2, when Δλ was fixed at 15nm, no shift of λ_{max} was apparent. But the λ_{max} had red shifted when Δλ=60nm. This suggested that the interaction of BSA with MG have a distinct effect on the conformation of the microenvironment around Trp residues, and did not changed the microenvironment of Tyr residues[18]. These were coincident with the result of Section 3.1.

High concentrations of dyes make protein molecules extend, reducing the energy transfer between the amino acid residues, and reducing their fluorescence intensity. For the quenching process, according to Eqs. (2) (3), the corresponding results are shown in TABLE 2. From TABLE 2 it can be seen the values of K_q decreased with the rising temperature in all systems, which indicated that the probable quenching mechanism of the interaction between BSA and MG was a static process. Obviously, the K_q value of protein quenching procedure initiated by MG was greater than 2×10^{10} L mol^{-1}·s^{-1}. This indicated that the quenching was not initiated from dynamic collision but from the formation of a complex. The n value approaches unity, suggesting that one molecule of MG combines with one molecule of BSA;

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TABLE 2 : Quenching reactive parameters of MG and BSA at different temperatures

System	Δλ(nm)	T/(K)	K_q/(L mol^{-1}·s^{-1})	r_3	K_a/(L mol^{-1})	n	r_4
BSA-MG	15	293	9.40×10^{11}	0.9951	1.75×10^{4}	1.08	0.9977
		303	8.72×10^{11}	0.9937	1.14×10^{4}	1.00	0.9925
		310	7.99×10^{11}	0.9976	0.93×10^{4}	1.05	0.9903
	60	293	9.89×10^{11}	0.9964	2.07×10^{4}	1.01	0.9956
		303	8.94×10^{11}	0.9975	1.59×10^{4}	1.06	0.9967
		310	8.13×10^{11}	0.9962	1.01×10^{4}	1.07	0.9959

r_3 is the linear relative coefficient of $F_0/F\sim Q$]; r_4 is the linear relative coefficient of $lg(F_0-F)/F\sim lg[Q]$.

the decreasing trend of K_a with the increasing temperature was in accordance with binding constants dependence on the temperature as mentioned above, which indicates that MG-BSA would be partly decomposed when the temperature rising, it also indicates it is a static quenching. The quenching mechanism obtained by synchronous fluorescence method was coincident with the one obtained by fluorescence method. Comparing TABLE 1 and TABLE 2, we can see that the quenching parameters obtained by two methods have the same order of magnitude.

Identification of the binding site

At 280 nm wavelength the Trp and Tyr residues in BSA are excited, whereas the 295 nm wavelength excites only Trp residues. In BSA sub-hydrophobic domain, IIIA (containing both Trp 212 and Tyr 263) and IIA (containing only Tyr: Tyr 401, Tyr411, Tyr 497) is the major binding site of small molecule ligands[19]. Based on the Stern-Volmer equation, comparing the fluorescence quenching of BSA excited at 280 nm and 295 nm allows to estimate the participation of Trp and Tyr groups in the system[20]. As seen in Figure 3, in the presence of MG, the quenching curves of BSA excited at 280 nm and 295 nm overlap approximately. This phenomenon showed that Trp residues played an important role in the interaction between MG and BSA. BSA has two tryptophan moieties (Trp 134 and Trp 212). Trp 134 is embedded in the first sub-domain IB and is more exposed to a hydrophilic environment, whereas Trp 212 is embedded in sub-domain IIA and deeply buries in the hydrophobic loop. So, it is considered that MG most likely binds to the hydrophobic pocket located in sub-domain IIA[21].

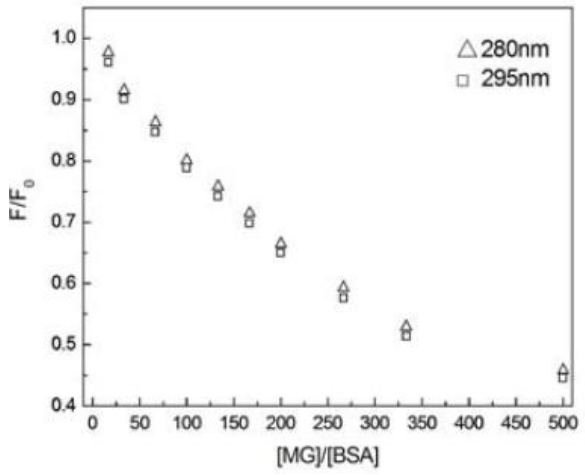

Figure 3 : Fluorescence emission spectra of BSA-MG (T =293 K); C_{BSA} = 3.0×10^{-7} mol L^{-1}, C_{MG}=$5.0\times10^{-6}\sim4.0\times10^{-4}$ mol L^{-1}

The crystal structure of BSA is a heart-shaped helical monomer composed of three homologous domains named I, II, and III, with each domain including two sub-domains called A and B to form a cylinder[22]. The principal ligand-binding regions of albumin are hydrophobic cavities in sub-domains IIA and IIIA, which have similar chemical properties. These two binding cavities are also referred to as sites I, II, and III (site I in sub-domain IIA, sites II and III in sub-domain IIIA). To identify the binding site on BSA, site marker competitive experiments were carried out, using the drug which specially binds to a known site or region on BSA. X-ray crystallography studies have shown that warfarin (WF) binds to sub-domain IIA whereas ibuprofen (IB) and digoxin (DG) are believed to bind to IIIA binder sites II and III, respectively[23]. Information about BSA-MG binding site can therefore be obtained by monitoring changes in the fluorescence of MG-bound BSA caused by binding by site I (WF), site II (IB), and site III (DG) markers. Binding constants determined on the basis of Eq. (3) show the effect of WF, IB, and DG on BSA-MG at 293 K. The results are presented in TABLE 3. It can be seen that the binding constant for

TABLE 3 : Binding constants and the rate of change of binding constants φ for BSA-MG systems with adding site markers regents (T=293K)

System		WF		IB		DG	
		K_a/(L mol^{-1})	φ /(%)	K_a/(L mol^{-1})	φ/(%)	K_a/(L mol^{-1})	φ/(%)
BSA-MG	λex=280 nm	5.26×10^3	-77.13	9.91×10^3	-56.91	9.64×10^3	-58.09
	Δλ=60nm	5.83×10^3	-69.48	1.05×10^3	-45.03	9.81×10^3	-48.64

φ=(K_a-K_a)/K_a (K_a is the binding constants of BSA-MG systems for the same condition)

TABLE 4 : The thermodynamic parameters of BSA-MG at different temperatures

System		T /(K)	K_a/(L·mol^{-1})	ΔH /(KJ·mol^{-1})	ΔS /(J·mol^{-1}·K^{-1})	ΔG /(KJ·mol^{-1})
BSA-MG	Δλ= 15nm	293	1.75×10^4			-23.77
		303	1.14×10^4	-28.37	-15.70	-23.61
		310	0.93×10^4			-23.50
	Δλ= 60nm	293	2.07×10^4			-24.31
		303	1.59×10^4	-30.87	-22.39	-24.08
		310	1.01×10^4			-23.93
	λex= 280nm	293	1.91×10^4			-24.19
		303	1.68×10^4	-28.02	-13.07	-24.06
		310	0.98×10^4			-23.97
	λex= 295nm	293	2.04×10^4			-24.31
		303	1.66×10^4	-29.14	-16.49	-24.14
		310	1.03×10^4			-24.03

the ternary system BSA-WF-MG was the most different, indicating that WF hinders the formation of BSA-MG and can compete for the same binding site in subdomain IIA (site I). The conclusions of the synchronous fluorescence method were consistent with fluorescence method.

Type of interaction force of BSA-MG systems

Basically, four main types of interactions, hydrogen bonds, electrostatic forces, van der Waals forces, and hydrophobic forces play critical roles in the interactions between small molecules and macromolecules[24]. In order to characterize the force between MG and BSA, thermodynamic parameters on the temperatures were analyzed. The thermodynamic parameters, free energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS) are important for confirming the binding mode. The thermodynamic parameters can be calculated using Eqs. (4) and (5)[25,26].

$$R\ln K = \Delta S - \Delta H / T \quad (4)$$

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

In the present case, K is analogous to the effective quenching constants K_a for the quencher-acceptor system at the corresponding temperature and R is gas constant. If it is assumed that the enthalpy change (ΔH) nearly had no change within the investigated temperature, there should be a good linear relationship between ln K and 1/T. The values of thermodynamic parameters (ΔH, ΔS and ΔG) were obtained and shown in TABLE 4. If ΔH<0 and ΔS<0, van der Waals' interactions and hydrogen bonds play major roles in the binding reaction. If ΔH>0 and ΔS>0, hydrophobic interactions are dominant. If ΔH<0 and ΔS>0, electrostatic forces are more important in the binding reaction[27].

The van der Waals interactions and hydrogen bonds play major roles in the binding process between MG and BSA (From TABLE 4 shows ΔH<0 and S<0). The negative ΔG in TABLE 4 supports the opinion that the binding interaction between BSA and MG were spontaneous. The conclusions of the synchronous fluorescence method were consistent with fluorescence method.

CONCLUSIONS

In this paper, the binding of MG to BSA under physiological conditions was studied by traditional fluorescence quenching method and synchronous fluores-

cence, and used the same equation for processing data, respectively. From all dates, we could see dates obtained by both methods were in the same order of magnitude and very close, quenching mechanism and type of interaction force were consistent, which indicated synchronous fluorescence spectroscopy could replace traditional fluorescence quenching method to study reaction mechanism of dyes with proteins. In addition synchronous fluorescence method has some advantages such as good selectivity, high sensitivity, less interference etc, which makes synchronous fluorescence method have more advantages than traditional fluorescence quenching method to study the reaction mechanism of dyes with proteins.

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