*Volume 1 Issue 1*

BIOCHEMISTRY
&
MOLECULAR BIOLOGY *Letters*

*Full Paper*

**BCAMBL, 1(1), 2015 [029-036]**

# Comparative studies on the interaction between metronidazole and lysozyme by fluorescence quenching spectroscopy and synchronous fluorescence spectroscopy

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## ABSTRACT

Under simulative physiological conditions, the interactions between lysozyme(LYSO) and metronidazole(MET) at different temperatures (298, 310 and 318 K) were studied using fluorescence quenching and synchronous fluorescence spectroscopy respectively. The results indicated that MET quenched the intrinsic fluorescence of LYSO via a static quenching procedure. The binding constants obtained from above method were of the same order of magnitude and very similar; the number of binding site in the interaction was closed to 1. The negative enthalpy changes and positive entropy changes implied that electrostatic interaction might play a main role in the interaction between MET and LYSO. Onthe basis of the Förster theory of the resonance energy transfer, the binding distance between LYSO and MET was less than 7 nm. In addition, the conclusions obtained from two methods using the same equation were consistent. It indicated that the synchronous fluorescence spectrometry could be used to study the binding mechanism between drug and protein, and was a useful supplementto the fluorescence quenching method. 

## KEYWORDS

Lysozyme;
Metronidazole;
Fluorescence quenching spectroscopy;
Synchronous fluorescence spectroscopy;
Interaction;
Quench.

## INTRODUCTION

Lysozyme (LYSO) is a 14.6 KDa single chain protein and composed of 129 amino acid residues, containing 6 tryptophanes (Trp), 4 disulfide bonds and 3 tyrosines (Tyr)[1]. Among the six Trp residues, three are located at the substrate binding site, two in the hydrophobic matrix box, while one is separated from the others. Trp108 and Trp62 are the most dominant fluorophores, both being located at the substrate binding sites[2]. It has many physiological and pharmaceutical functions, such as anti-inflammatory, antiviral, immune modulatory, anti-histaminic and anti-tumor activities. So it is extensively used in the pharmaceutical and food ûelds. Another important function of LYSO is its ability to carry drugs. It can cure some illness via the binding with active compounds. The effectiveness of drugs depends on their binding ability[3]. Therefore, studies on the interaction between drug and LYSO are of importance in view of realizing disposi-

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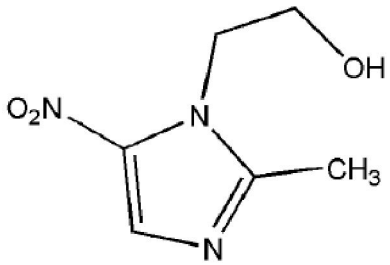



**Figure 1 : Chemical structure of metronidazole**

tion, transportat ion and metabolism of drug.

Metronidazole[4] (MET) (the structure shown in Figure 1) belongs to the nitroimidazole group. It is employed in both human and veterinary medicine to treat diseases caused by anaerobic bacteria (Bacteroides, Fusobacterium, Campylobacter, Clostridium) and protozoa (Trichomonas, Treponema, Histomonas).

Fluorescence quenching spectroscopy[5] is used to study the reaction mechanism for drugs and proteins, mainly by analysising emission spectrum of proteinwhen the excitation wavelength are 280nm and 295nm. The binding constants, binding sites, type of interaction force and binding distance between drugs andproteins are obtained from that method.

Synchronous fluorescence spectroscopy introduced by Lloyd has been used to characterize complex mixtures providing fingerprints of complex samples[6]. It involves simultaneous scanning of the excitation and emission monochromators while maintaining a constant wavelength interval between them, which is the biggest difference from fluorescence quenching method. The main advantages of synchronous fluorescence spectra are simpliûed spectra, narrowed bandwidth, high selectivity and sensitivity[7]. Synchronous fluorescence is often clearly illustrated microenvironment information of Tyr and Trp. However, using this method to study the binding constant and binding sites between the protein and the drug has not been reported. In our previous report, fluorescence quenching and synchronous fluorescence spectroscopy were used to study the binding constants, the number of binding sites, binding forces and energy-transfer parameters of LYSO with MET. The datas were obtained and analyzed. The results show that synchronous fluorescence spectroscopy is a beneficial method to investigate the binding mechanism between drug and protein.

## EXPERIMENTAL

### Apparatus

All fuorescence spectra were recorded with a Shimadzu RF-5301PC spectroûuorophotometer. Absorption was measured with an UV/vis recording spectrophotometer (UV-265, Shimadzu,Japan). All pH measurements were carried out with a pHS-3C precision acidity meter (Leici, Shanghai, China). All temperatures were controlled by a CS501 superheated water bath (Nantong Science Instrument Factory).

### Materials

Metronidazole(MET) was purchased from Yabao Pharmaceutical Group Co.,Ltd. Monitor. Lysozyme(LYSO) was purchased from Sigma Co (the purity grade inferior 99%). Stock solutions of LYSO ($2.0\times10^{-5}$M) and MET ($1.0\times10^{-3}$M) were prepared. All the stock solutions were further diluted as working solutions prior to use. Tris–HCl buffer solution containing NaCl (0.15 M) was used to keep the pH of the solution at 7.4. NaCl solution was used to maintain the ionic strength of the solution. All other reagents were of analytical grade, and all aqueous solutions were prepared with newly double-distilled water and stored at 277K.

In order to eliminate the inner ûlter effects of protein and ligand, absorbance measurements were performed at excitation and emission wavelengths of the fluorescence measurements. The fluorescence intensity was corrected using the following equation[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 MET at excitation and emission wavelengths, respectively. The fluorescence intensity used in this article was corrected.

### Procedures

### Fluorescence measurements.

In a typical fluorescence measurement, 1.0mL of

pH = 7.40 Tris-HCl, 0.2 mL LYSO solution($2.0\times10^{-5}$M)and different concentrations of MET were added into 10 mL colorimetric tube successively. The samples were diluted to scaled volume with water, mixed thoroughly by shaking, and kept static at different temperatures (298, 310and 318 K). The excitation wavelength for LYSO was 280nm (or295nm) with the excitation and emission slit widths set at 5nm. The solution was subsequently scanned on the fluorophotometer and determined the fluorescent intensity at 341nm.

**Synchronous fluorescence measurements.**

Solution preparation was as detailed above; we recorded the fluorescence spectra of the LYSO-MET system when the $\Delta\lambda$ value between the excitation and emission wavelengths was stabilized at 15 and 60 nm, respectively.

## RESULT AND DISCUSSION

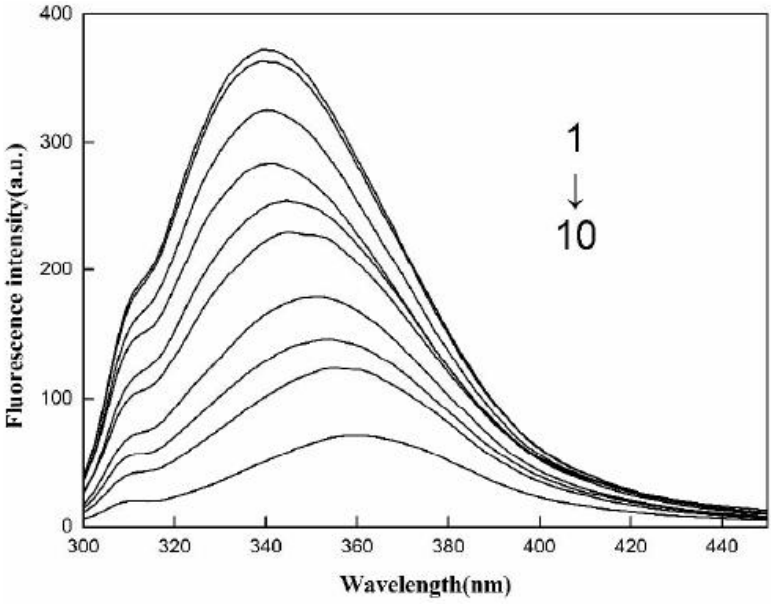
**Fluorescence quenching spectra of LYSO–MET system**

Proteins are considered to have intrinsic fluorescence due to the presence of amino acids, mainly Trp and Tyr. When the excitation wavelengths were at 280 nm and 295 nm, the emission peaks for LYSO were both located at 341 nm. Figure 2 shows the fluorescence emission spectra of LYSO in the presence of different concentrations of MET. It can be seen from Figure 2 that addition of MET leads to a significant reduction in the fluorescence intensity of LYSO (similar to 295 nm). Meanwhile, a slight red shift of emission wavelength from 339 to 360nm was observed. This result showed that MET could quench the intrinsic fluorescence of LYSO strongly and the quenching of LYSO fluorescence by MET was due to the formation of the LYSO–MET ground-state complex[9].

In order to confirm the quenching mechanism, the fluorescence quenching data were analyzed by the Stern–Volmer equation[10]

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

Where $F_0$ and F are the fluorescence intensities in the absence and presence of quencher, respectively. $\tau_0$ is the average lifetime of fluorescence without quencher and is $10^{-8}$ s. $K_{sv}$ is the Stern–Volmer quenching constant. $K_q$ is the quenching rate constant of biomolecule, and [L] is the concentration of the quencher. Based on the linear fit plot of $F_0/F$ versus [L], the $K_q$ values can be obtained. The calculated results were shown in TABLE 1. The quenching mechanism[11] is generally classified as either dynamic or static. Dynamic and static quenching can be distinguished by their different dependence on temperature. Quenching rate constants decrease with increasing temperature for the static quenching, but the reverse




$C_{LYSO}=4.0\times10^{-7}$mol/L 1~10: $C_{MET}$= (0, 0.2, 1.0, 2.0, 4.0 6.0, 8.0, 10, 13, 18) $\times10^{-5}$ mol/L

**Figure 2 Fluorescence emission spectra of LYSO-MET(T=298K,λex=280)**

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**TABLE 1 : Quenching reactive parameters of MET and LYSO at different temperatures**

| $\lambda_{ex}$(nm) | T/(K) | $K_q$/(L·mol$^{-1}$·s$^{-1}$) | $K_{sv}$/(L·mol$^{-1}$) | $r_1$ | $K_a$/((L·mol$^{-1}$) | n | $r_2$ |
|---|---|---|---|---|---|---|---|
| 280 | 298 | $2.01\times10^{12}$ | $2.01\times10^{4}$ | 0.9942 | $1.77\times10^{4}$ | 1.06 | 0.9961 |
| | 310 | $1.96\times10^{12}$ | $1.96\times10^{4}$ | 0.9956 | $1.70\times10^{4}$ | 1.02 | 0.9973 |
| | 318 | $1.93\times10^{12}$ | $1.93\times10^{4}$ | 0.9947 | $1.35\times10^{4}$ | 0.99 | 0.9915 |
| 295 | 298 | $1.98\times10^{12}$ | $1.98\times10^{4}$ | 0.9973 | $1.76\times10^{4}$ | 1.03 | 0.9923 |
| | 310 | $1.95\times10^{12}$ | $1.95\times10^{4}$ | 0.9934 | $1.70\times10^{4}$ | 1.01 | 0.9915 |
| | 318 | $1.91\times10^{12}$ | $1.91\times10^{4}$ | 0.9965 | $1.33\times10^{4}$ | 0.96 | 0.9957 |

***$K_q$* is the quenching rate constant; *$K_a$* is the binding constant; *n* is the number of binding site;*$r_1$* is the linear relative coefficient of $F_0/F$~[L];*$r_2$* is the linear relative coefficient of $\log(F_0-F)/F\sim\log\{[D_t]-n[B_t](F_0-F)/F_0\}$**

effect is observed for dynamic quenching. As shown in TABLE 1, The results revealed that the values of $K_{sv}$ and $K_q$ decreased with increasing temperature, and the $K_q$ were much greater than the maximum scatter collision quenching constant of various quenchers ($2\times10^{10}$ L·mol$^{-1}$·s$^{-1}$). Those all suggested that the quenching mechanism was due to static quenching in each case. In other words, the quenching of the LYSO fluorescence by MET was due to specific complex formation[12].

For static quenching process, the relationship between the fluorescence intensity and the concentration of quencher could be usually described by equation (3) to obtain the binding constant ($K_a$) and the number of binging sites (n) in most of paper[13]:

$$\log\left(\frac{F_0-F}{F}\right)=n\log K_a+n\log\left\{[D_t]-n\frac{F_0-F}{F_0}[B_t]\right\} \qquad (3)$$

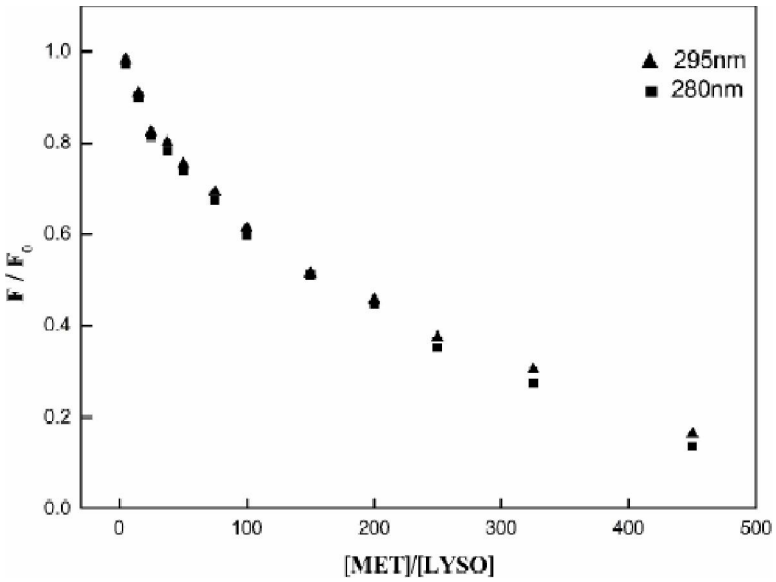
where [Dt] and [Bt] are the total concentrations of MET and LYSO, respectively. On the assumption that n in the bracket is equal to 1, the curve of $\log(F_0-F)/F$ versus $\log\{[D_t]-[B_t](F_0-F)/F_0\}$is drawn and fitted linearly, then the value of n can be obtained from the slope of the plot. If the n value obtained is not equal to 1, then it is substituted into the bracket and the curve of $\log(F_0-F)/F$ versus $\log\{[D_t]-[B_t](F_0-F)/F_0\}$ is drawn again. The above process is repeated again and again till n obtained is only a single value or a circulating value. Based on the n obtained, the binding constant $K_a$ can be also obtained. In the work, a calculation program was developed. The calculation process can be finished with calculator based on the simple program, and the calculating results can be obtained by inputting F, $[D_t]$ and $[B_t]$. The calculated results were shown in TABLE 1. As seen in TABLE 1, the values of n were approximately equal to 1 at different temperatures, indicating there is one binding site of MET to LYSO. Meanwhile, the binding constants $K_a$ decreased with increasing temperature implied the complex of MET–LYSO became less stable at higher temperature, which further evidenced that the fluorescence quenching was a static quenching process[14].

## The participation of amino acid residue studies in LYSO-MET system

At 280 nm wavelength, the Trp and Tyr residues in LYSO are excited, whereas the 295 nm wavelength excites only Trp residues[15]. Based on the Stern–Volmer equation, comparing the fluorescence quenching of LYSO excited at 280 nm and 295 nm allows to estimate the participation of Trp and Tyr groups in the system[16]. As seen in Figure 3, in the presence of MET, the quenching curves of LYSO excited at 280 nm and 295 nm overlap. This showed that only Trp residue played an important role in the interaction of MET with LYSO. From TABLE 1, at the same temperature, $K_a$ values at excitation wavelengths of 280 nm and 295 nm are of the same order of magnitude and similar, which also suggests that only Trp residues played an important part in the LYSO-MET system.

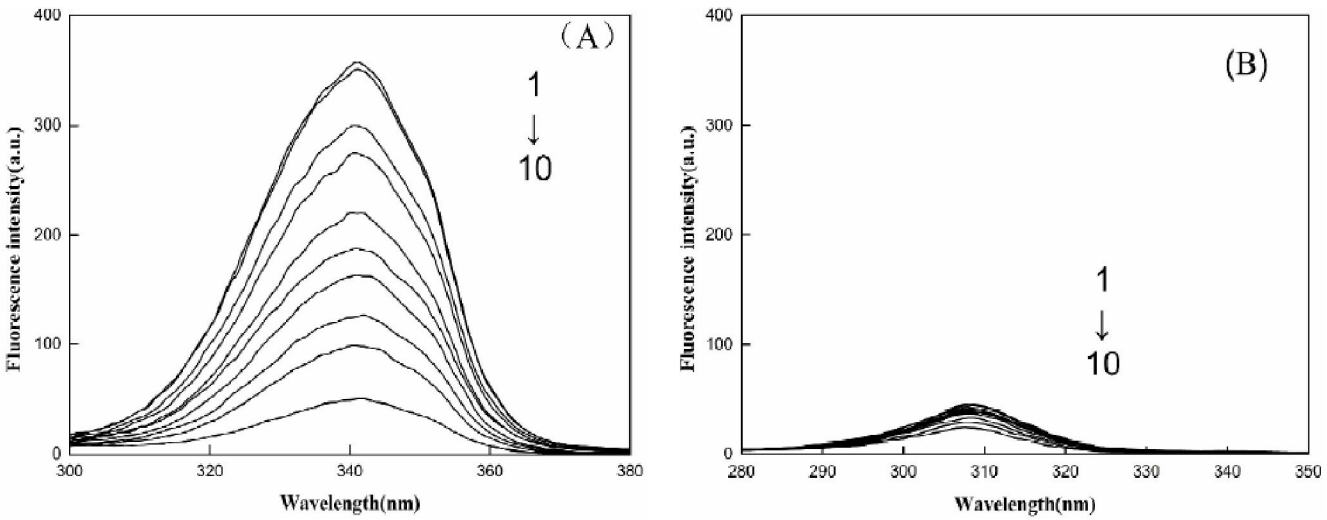
## Synchronous fluorescence spectra studies in LYSO-MET systems

As it is well known that, synchronous fluorescence spectra can provide the information on the molecular microenvironment, particularly in the vicinity of the fluorophore functional groups[17]. When $\Delta\lambda$ between the excitation and emission wavelengths is stabilized at either 15 or 60 nm, the synchronous ûuorescence spectrum can give the characteristics of Tyr residues or Try residues[18]. It can be seen from Figure 4 that the maxi-




$C_{LYSO}$ = 4.0×10$^{-7}$ mol/L, $C_{MET}$=2.0×10$^{-6}$~ 1.8×10$^{-4}$ mol/L

**Figure 3 : Fluorescence emission spectra of LYSO-MET (T =298K)**




$C_{LYSO}$ = 4.0×10$^{-7}$ mol/L; 1~10 $C_{MET}$ = (0, 0.2, 1.0, 2.0, 4.0, 6.0, 8.0, 10, 13, 18)×10$^{-5}$ mol/L

**Figure 4 : Fluorescence spectrum of LYSO-MET system(T = 298 K) (A)Δλ=60nm;(B)Δλ=15nm**

mum emission wavelength kept the position with increasing concentration of MET when Δλ was 60 and 15nm. It implies that the interaction of MET with LYSO does not signiûcantly affect the polarity around Trp and Tyr residues microregions. It is possible that the binding of MET to LYSO does not causes apparent change in conformation of LYSO. In addition, when Δλ was 60 nm, the fluorescence intensities of LYSO–MET decreased regularly, but the ûuorescence intensities in LYSO-MET showed no obvious decrease when Δλ was 15 nm. It indicated that only Try residues played an important role in the LYSO-CPS system, which was coincident with"only Trp residues played an important part in the LYSO-MET system".

The corresponding results for Δλ =60 nm, according to equations (2) and (3), are shown in TABLE 2. From TABLE 2, it can be seen that the values of $K_{SV}$ decreased with increasing temperature for LYSO-MET systems, which indicated that the probable quenching mechanism of the interaction between LYSO and MET was a static process. The value of $K_q$ were much greater than 2×10$^{10}$L·mol$^{-1}$·s$^{-1}$, which also suggested that the quenching was not initiated by dynamic collision but from the formation of a complex. The n value approached unity, indicating that MET bound with LYSO moderately through a 1: 1 binding

mode. In addition, The $K_a$ values decreased regularly with increasing temperature, which also indicated the process was static quenching. The quenching mechanism obtained by synchronous fluorescence method was coincident with that obtained by fluorescence method. Comparing TABLES 1 and 2 shows that the quenching parameters obtained using the two methods are of the same order of magnitude.

## Type of interaction force in LYSO–MET systems

Generally, the interaction forces between the small drug molecule and biological macromolecule include hydrogen bond, Van der Waal's force, electrostatic interactions and hydrophobic force[19]. For a binding interaction, the nature of interaction forces between quencher and biomacromolecule can be learned from the thermodynamic parameters. The thermodynamic parameters can be calculated on the basis of the following equation[20] :

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

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

When temperature varies in a small range, the ΔH could be considered as a constant[21]. According to the binding constants $K_a$ of MET to LYSO at different temperatures above (TABLE 1 and TABLE 2), the thermodynamic parameters were obtained conveniently. The calculated results were shown in TABLE 3. The negative values of ΔG indicated that the binding processes occurred spontaneously in all studied cases. Negative ΔH and positive ΔS indicate electrostatic interaction plays a major role in the binding reaction. Positive ΔH and ΔS are generally considered as the evidence for typical hydrophobic interactions. In addition, Van der Waal's force and hydrogen bonding formation in low dielectric media are characterized by negative values of ΔH and ΔS[22]. Therefore,the negative value of ΔH and positive value of ΔS showed that electrostatic attraction played a main role in the binding of MET to LYSO. The conclusions obtained from the synchronous fluorescence method were consistent with the fluorescence quenching method.

## Energy transfer between LYSO and MET

According to the Förster nonradioactive resonance energy transfer theory[23], the effective energy transfer from donor to acceptor will occur when two molecules meet the following preconditions: (1) the donor is a fuorophore; (2) the overlap is sufûcient between the ûuorescence emission spectrum of the donor and UV/vis absorption spectrum of the acceptor; and (3) the distance between the donor and the acceptor should be less than 7 nm. The overlap spectrum of the UV-vis absorption spectrum of quercetin and the fluorescence emission spectra of LYSO is shown in Figure 5. The energy transfer effect is not only related to the distance between the donor and acceptor, but also to the critical energy transfer distance $R_0$, that is[24]

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

**TABLE 2 : Quenching reactive parameters of MET and LYSO at different temperatures**

| T/(K) | $K_q$ /(L/mol·s) | $K_{sv}$ / (L/mol) | $r_3$ | $K_a$ /(L/mol) | n | $r_4$ |
|---|---|---|---|---|---|---|
| 298 | $2.07\times10^{12}$ | $2.07\times10^{4}$ | 0.9954 | $1.82\times10^{4}$ | 1.13 | 0.9983 |
| 310 | $1.99\times10^{12}$ | $1.99\times10^{4}$ | 0.9996 | $1.71\times10^{4}$ | 1.05 | 0.9975 |
| 318 | $1.94\times10^{12}$ | $1.94\times10^{4}$ | 0.9936 | $1.43\times10^{4}$ | 0.99 | 0.9920 |

$r_3$ is the linear relative coefficient of $F_0/F$~$[L]$;$r_4$ is the linear relative coefficient of $\log(F_0\text{-}F)/F$~$\log\{[D_t]\text{-}n[B_t](F_0\text{-}F)/F_0\}$

**TABLE 3 : The thermodynamic parameters of MET-LYSO at different temperatures in two ways**

| | T /(K) | $K_a$ /(L·mol$^{-1}$) | ΔH /(KJ·mol$^{-1}$) | ΔS/(J·mol$^{-1}$·K$^{-1}$) | ΔG /(KJ·mol$^{-1}$) |
|---|---|---|---|---|---|
| Δλ= 60nm | 298 | $1.82\times10^{4}$ | -8.98 | 51.42 | -24.30 |
| | 310 | $1.71\times10^{4}$ | | 52.06 | -25.12 |
| | 318 | $1.43\times10^{4}$ | | 51.30 | -25.29 |
| $\lambda_{ex}$= 280nm | 298 | $1.77\times10^{4}$ | -9.92 | 48.03 | -24.23 |
| | 310 | $1.70\times10^{4}$ | | 48.98 | -25.10 |
| | 318 | $1.35\times10^{4}$ | | 48.87 | -25.14 |

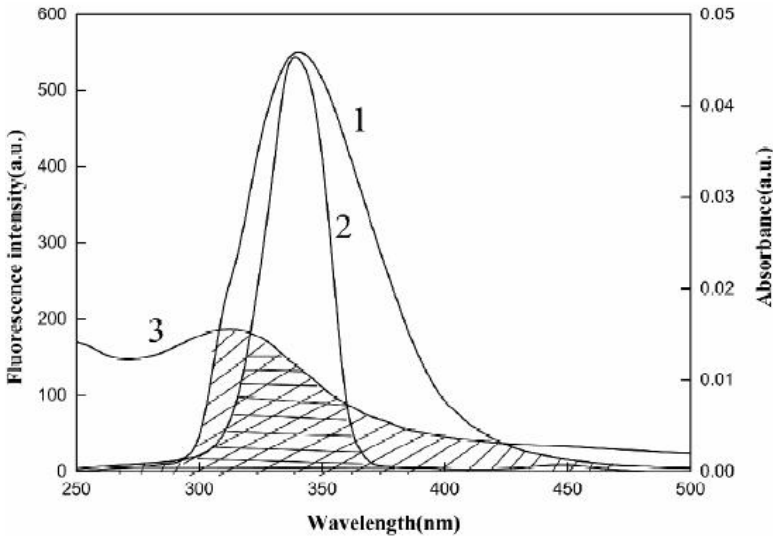



**Figure 5 : Overlap of the fluorescence emission spectrum of LYSO(λex=280) (1)and Δλ=60 nm(2) with the absorption spectrum of MET(3) (T =298K); $C_{MET}$ = $C_{LYSO}$ =8.0×10$^{-7}$ mol/L**

**TABLE 4 : Parameters of E,J, r, $R_0$ between MET and LYSO at different temperature**

| Method | T/(K) | E/(%) | J/(cm$^3$·L·mol$^{-1}$) | $R_0$/(nm) | r/(nm) |
|---|---|---|---|---|---|
| Fluorescence quenching spectroscopy | 298 | 3.98 | 6.57×10$^{-15}$ | 2.38 | 4.07 |
| | 310 | 3.28 | 6.46×10$^{-15}$ | 2.37 | 4.19 |
| | 318 | 2.66 | 6.23×10$^{-15}$ | 2.35 | 4.32 |
| Synchronous fluorescence spectroscopy | 298 | 4.12 | 6.01×10$^{-15}$ | 2.34 | 3.96 |
| | 310 | 3.50 | 5.95×10$^{-15}$ | 2.34 | 4.09 |
| | 318 | 2.78 | 5.85×10$^{-15}$ | 2.33 | 4.24 |

where E denotes the efficiency of transfer between the donor and the acceptor, r is the average distances between acceptor and donor and $R_0$ is the critical distance when the transfer efficiency is 50%. The value of $R_0$ is calculated using the following equation:

$$R_0^6 = 8.78 \times 10^{-25} K^2 \Phi N^{-4} J \quad (7)$$

where $K^2$ is the spatial orientation factor of the dipole, N is the refractive index of medium, Φ is the fluorescence quantum yield of donor and J is the spectral overlap integral between the ûuorescence emission spectrum of the donor and the absorption spectrum of the acceptor,which is given by:

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda / \sum F(\lambda)\Delta\lambda \quad (8)$$

where F(λ) is the fluorescence intensity of the donor when the wavelength is λ and ε(λ) is the molar absorption coefficient of the acceptor at same wavelength. Under these experimental conditions, it has been reported for LYSO that $K^2$= 2/3, N = 1.336, and Φ = 0.15[25]. Thus, J, E, $R_0$, and r were calculated as shown in TABLE 4. The distance r of donor to acceptor were less than 7nm, indicated the non-radiative energy transfer coming into being between LYSO and MET[26]. Furthermore, with the increase of concentration of MET, the values of r increased. These results indicated again a static quenching between the MET and LYSO because of the formation of LYSO–MET complexes[27]. In addition, the conclusions obtained using the two methods were basically consistent.

## CONCLUSIONS

The interaction of MET with LYSO has been investigated by using fluorescence quenching and synchronous fluorescence spectroscopy under simulated physiological conditions in this work, which used the same equation for processing the data. The binding constants $K_a$ obtained from above method were of the same order of magnitude and very similar. The quenching mechanism, number of binding sites, type of interaction force were consistent, which indicated syn-

chronous fluorescence spectrometry studied the binding mechanism between drug and protein was reasonable. In addition, synchronous fluorescence spectrometry has some advantages such as good selectivity, high sensitivity and less interference, which makes it more useful than fluorescence quenching method in studying the reaction mechanism between drugs and proteins.

## ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial support of National Science Foundation of China (Grant no. 21375032) and Hebei Provincial Key Basic Research Program (Grant no. 10967126D).

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