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Study on the interaction of 3,3-bis(4-hydroxy-1-naphthyl)-phthalide with bovine serum albumin by fluorescence spectroscopy

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Abstract

The interaction between 3,3-bis(4-hydroxy-1-naphthyl)-phthalide (NPP) and bovine serum albumin (BSA) have been studied by fluorescence spectroscopy. The binding of NPP quenches the BSA fluorescence. By the fluorescence quenching results, it was found that the binding constant $K = 5.30 \times 10^4$ L mol^{-1}, and number of binding sites $n = 0.9267$. In addition, according to the synchronous fluorescence spectra of BSA, the results showed that the fluorescence spectra of BSA mainly originate from the tryptophan residues. Finally, the distance between the acceptor NPP and BSA was estimated to be 1.94 nm using Föster's equation on the basis of fluorescence energy transfer. The interaction between NPP and BSA has been verified as consistent with the static quenching procedure and the quenching mechanism is related to the energy transfer.

Keywords: Fluorescence; 3,3-Bis(4-hydroxy-1-naphthyl)-phthalide; Bovine serum albumin

1. Introduction

Albumins, especially bovine (BSA) and human (HSA) sera albumins labeled with fluorescent probes are commonly used as the model systems for the investigations of surface-induced conformational changes in protein interfaces [1]. From biopharmaceutical point of view, one of the most important biological functions of albumins is their ability to carry drugs as well as endogenous and exogenous substances, and numerous experiments with the aim to characterize the binding capacity and sites of albumins have been carried out [1,2]. Analysis is based on monitoring the change of a physicochemical property of the protein–probe system upon binding either directly (direct technique) or after separation of the bound and free probe (indirect technique). Among the direct techniques, fluorometry is extensively used and is considered to be superior to the indirect techniques (equilibrium and dynamic dialysis, ultrafiltration, gel filtration) because, to a first approximation, they do not disturb the binding equilibrium upon separation [3].

The spectral changes observed on the binding of fluorophores with proteins are an important tool for the investigations of the topology of binding sites, conformational changes and characterization of substrate to ligand binding [1]. Besides, determination of protein quantity in biological liquids is of great importance in biology and medicine [2] and fluorescent probes are successfully applied for this approach [4], including ethidium bromide [5], eosin B and eosin Y [6], bromocresol purple [7], coomassie brilliant blue [8], nano orange and albumin blue [3]. In this study, we present the data showing that the fluorescence probe, 3,3-bis(4-hydroxy-1-naphthyl)-phthalide (NPP), the derivative of phthaleins, is a triphenylmethane dyes (Fig. 1). It has tarnishable effect for the development of printing and dyeing. It was used to prepare ink whose color on substrates disappeared after 30–50 min [9]. It is also useful in plasma sterilization [10]. The NPP have some advantages over other probes. It is well known that the most of dyes have toxicity which cause appreciable harm to the human [3,5–8]. Then, NPP is uncommon biological dye which shows innocuity. Moreover, the NPP exists in two

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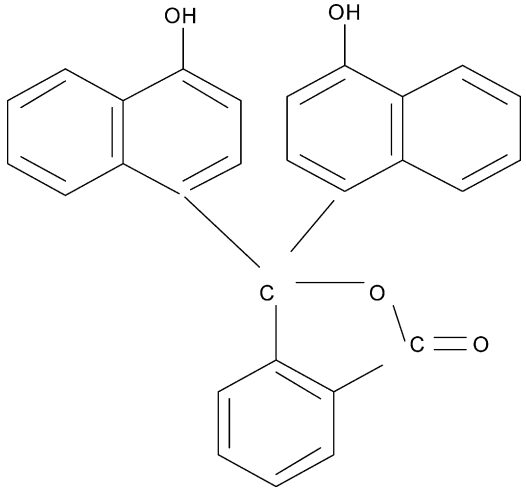

Fig. 1. The structure of 3,3-bis(4-hydroxy-1-naphthyl)-phthalide (NPP).

molecular forms in solutions, the acidic form and basic form [11], It is very sensitive to the change of pH value, the specific structure makes the NPP suitable for a variety of biomolecule applications. NPP displays a strong fluorescence emission with a peak at 365 nm on excitation at 286 nm. Its fluorescence intensity is increased in the presence of proteins. In the current work, bovine serum albumin (BSA) is selected as our protein model because of its lone-standing interest in the protein community [12]. Critical literature survey reveals that attempts have not been made so far to investigate the mechanism of interaction of NPP with BSA. This is the first attempt made to investigate the mode of interaction of NPP with BSA.

In this work, the binding reaction and the effect of the energy transfer between NPP and bovine serum albumin (BSA) was investigated by fluorescence spectra methods. It was demonstrated that the reaction constant and the number of binding sites could be explicitly derived. In addition, the conformational changes of BSA are discussed on the basis of synchronous fluorescence spectra.

2. Materials and methods

2.1. Materials

All starting materials were analytical reagent grade and double distilled water was used throughout. BSA (Chemical Reagent Ltd., Shanghai, China) was directly dissolved in double distilled water to prepare their stock solutions (1.0×10^{-4} mol L^{-1}) which were then stored at 0–4 °C. 1×10^{-3} mol L^{-1} NPP (Anderich) solution was obtained by dissolving it in 25 mL ethanol and 25 mL water, Britton–Robinson buffer solution of pH 5.02 was prepared.

2.2. Apparatus

All the fluorescence measurements were carried out on a F-4500 spectrofluorometer (Hitachi) equipped with a 150 W xenon lamp source and 1.0 cm quartz cell. A shimadzu UV-265 double-beam spectrophotometer (Tokyo, Japan) equipped with 1.0 cm quartz cells was used for scanning the UV spectrum. All pH measurements were made with a pHS-3 digital pH-meter (Shanghai Leici Device Works, China) with a combined glass electrode.

2.3. Procedure

An appropriate volume of the BSA solution (1.0×10^{-4} mol L^{-1}), 0.5 mL of NPP solution (1.0×10^{-4} mol L^{-1}), 1 mL of Britton–Robinson buffer solution (pH 5.02) were transferred into a 10 mL volumetric flask. The mixtures were diluted to the mark with double distilled water and then were shaken. Fluoresecnce spectra, absorption spectra and synchronous fluorescence spectra were obtained. Fluorescence quenching spectra were obtained at excitation and emission wavelength of 286 and 300–500 nm, respectively, and the range of synchronous scanning was: $\Delta\lambda = 15$ nm; $\Delta\lambda = 60$ nm. All measurements were performed at room temperature.

3. Results and discussion

3.1. Effect of pH and reaction time

The effect of pH on the interaction of NPP and BSA was investigated at pH 1.2–9.15 with Britton–Robinson buffer solution (Fig. 2). The maximum $F - F_0$ was occurred when pH was 5.02. So, pH 5.02 was selected as the suitable pH value in this assay. The influence of reaction time on the fluorescence intensity was also studied. The results showed

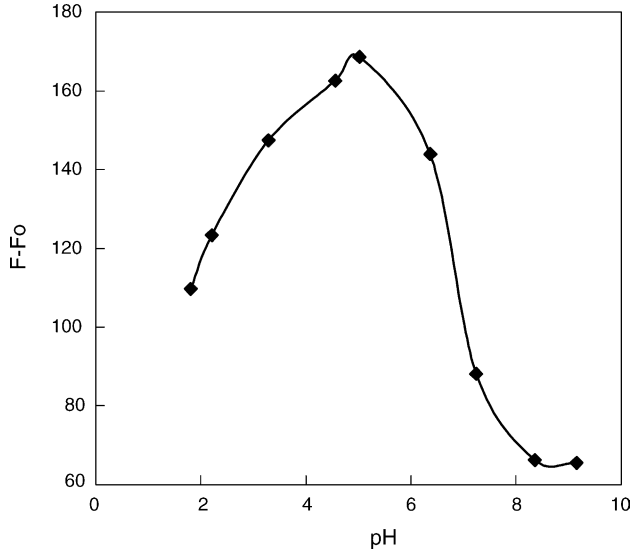

Fig. 2. Effect of pH on the interaction of NPP and BSA. $C_{BSA} = 1.0 \times 10^{-6}$ mol L^{-1}; $C_{NPP} = 1.0 \times 10^{-5}$ mol L^{-1}; F is the fluorescence intensity of NPP with BSA in the pH range of 1.2–9.15; F_0 is the fluorescence intensity of NPP in the pH range of 1.2–9.15.

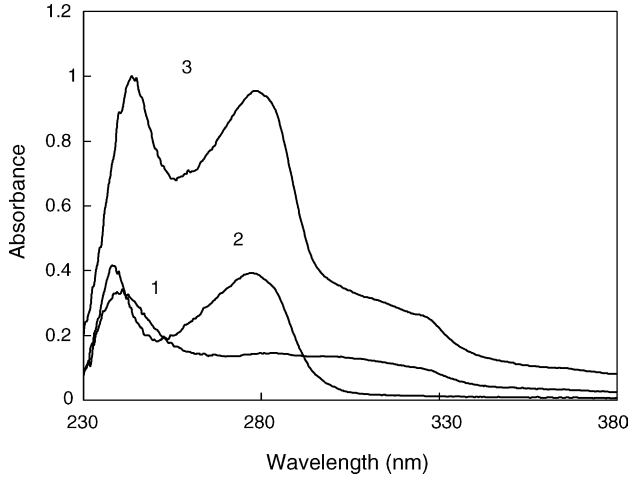

Fig. 3. The UV absorption spectrum of BSA in the presence of NPP: (1) the UV absorption spectrum of NPP, $C_{NPP} = 1.0 \times 10^{-5}$ mol L^{-1}; (2) the UV absorption spectrum of BSA, $C_{BSA} = 1.0 \times 10^{-5}$ mol L^{-1}; (3) the UV absorption spectrum of BSA–NPP complex, $C_{BSA} = 1.0 \times 10^{-5}$ mol L^{-1}, $C_{NPP} = 1.0 \times 10^{-5}$ mol L^{-1}.

that the maximum fluorescence intensity was reached when the mixed solutions had reacted in 25 min. The fluorescence remained for more than 1 h. So, 30 min was adopted in this assay.

3.2. Binding constants of BSA with NPP

Fig. 3 shows the absorption spectrum BSA in the presence of NPP at pH 5.02. It is suggested possibly that the complex was formed NPP–BSA.

In order to confirm the formation of this complex, the fluorescence quenching spectra of BSA with varying concentrations of NPP is shown in Fig. 4. The fluorescence intensity (F) of BSA was decreased regularly with increasing concentration of NPP. It is noted from Fig. 4 that a complex was formed between NPP and BSA, which is responsible to the quenching of the fluorescence of BSA.

The possible quenching mechanism can be interpreted by the fluorescence quenching spectra of the protein and the $F_0/F - C$ (Stern–Volmer) curves of BSA with NPP as shown in Fig. 5. The quenching equation is presented by

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

where F and F_0 are the fluorescence intensity with and without NPP, respectively, K_q the quenching rate constant of the biomolecule, K_{sv} the Stern–Volmer quenching constant, τ_0 the average lifetime of the biomolecule without NPP and $[Q]$ is the concentration of NPP. Obviously,

$$K_{sv} = K_q\tau_0 \quad (2)$$

Because the fluorescence lifetime of the BSA is 10^{-8} s^{-1} [13], K_{sv} is the slop of linear regressions of Fig. 5. According to Eq. (2), the quenching constant K_q can be obtained from the slope ($K_q = 4.867 \times 10^{13}$ L mol^{-1} s^{-1}, $r = 0.998$).

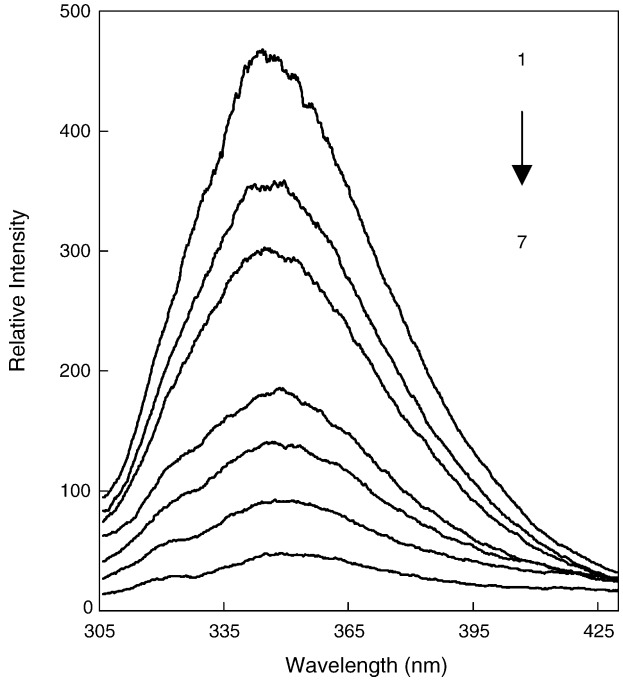

Fig. 4. The fluorescence quenching spectrum of bovine serum albumin at various concentration of NPP; $C_{BSA} = 1.0 \times 10^{-5}$ mol L^{-1}; C_{NPP} of 1–7: 0, 0.05×10^{-5}, 0.1×10^{-5}, 0.3×10^{-5}, 0.5×10^{-5}, 0.8×10^{-5}, 1×10^{-5} mol L^{-1}.

However, the maximum scatter collision quenching constant K_q of various quenchers with the biopolymer is 2×10^{10} L mol^{-1} S^{-1}[14]. Obviously, the rate constant of protein quenching procedure initiated by NPP is greater than the K_q of the scatter procedure. This means that the quenching is not initiated by dynamic collision but from the formation of a complex. For the static quenching interaction [15], if it is assumed that there are similar and independent binding sites in the biomolecule, the relationship between the fluorescence

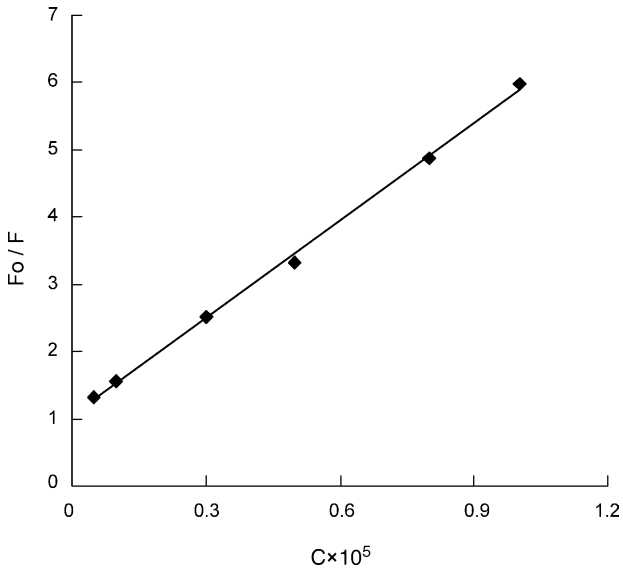

Fig. 5. The Stern–Volmer curves for quenching NPP with BSA.

intensity and the quenching medium can be deduced from the following formula:

$$nQ + B \rightarrow Q_n + B \quad (3)$$

where B is the biomolecule with a fluorophore, Q is the quenchable pharmaceutical molecule, $Q_n + B$ is the quenched biomolecule whose resultant constant is K_a. Here:

$$K_a = \frac{[Q_n + B]}{[Q]^n[B]} \quad (4)$$

If the overall amount of biomolecules (bound or unbound with the quenchable molecule) is B_0, then $[B_0] = [Q_n + B] + [B]$, here $[B]$ is the concentration of unbound biomolecule, then the relationship between fluorescence intensity and the unbound biomolecule as $[B]/[B_0] = F/F_0$ that is:

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

where K is the binding constant of NPP with BSA, which can be determined by the slope of the $\log[(F_0 - F)/F]$ versus $\log[Q]$ curves as shown in Fig. 6. Thus, we can obtain binding constant K and binding sites of NPP with BSA from the intercept and slope of Fig. 6 ($K = 5.30 \times 10^4$ L mol^{-1}, $n = 0.9267$).

3.3. Characteristics of synchronous fluorescence spectra

The synchronous fluorescence spectra were obtained by scanning simultaneously the excitation and emission monochromators. In the synchronous spectra [16], the sensitivity associated with fluorescence is maintained while offering several advantages: spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects. The synchronous fluorescence spectra only shows the tyrosine residues and the tryptophan residue of BSA when the wavelength interval ($\Delta\lambda$) is 15 nm and $\Delta\lambda$ is 60 nm, respectively [17].

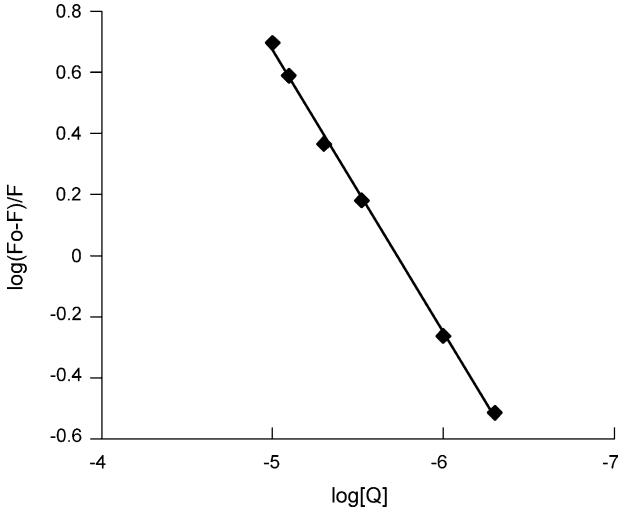

Fig. 6. Plots of $\log[(F_0 - F)/F]$ vs. log[Q].

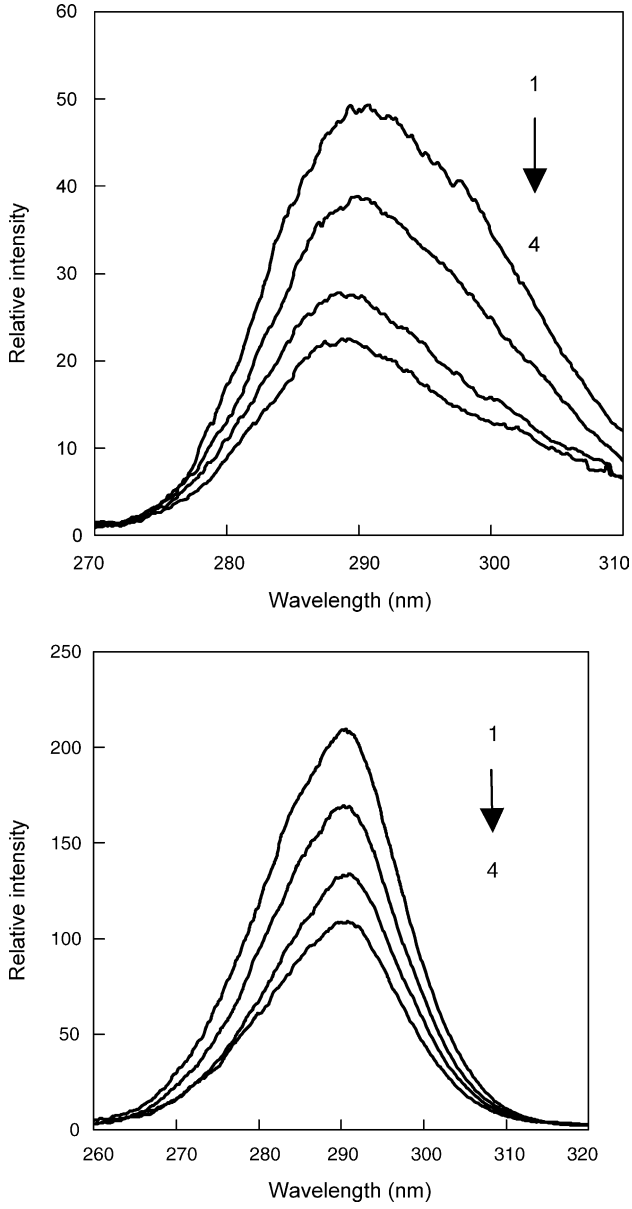

Fig. 7. The synchronous fluorescence spectra of bovine serum albumin at 15 nm wavelength interval (a) and at 60 nm wavelength interval (b), $C_{BSA} = 1.0 \times 10^{-5}$ mol L^{-1}; C_{NPP} of 1–4:0, 0.05×10^{-5}, 0.1×10^{-5}, 0.5×10^{-5} mol L^{-1}.

The maximum excitation and emission wavelengths of the residues are relative to the polarity of micro-environment, so we can judge the conformational change of protein from the variation of the excitation wavelength. The tyrosine residues and the tryptophan residues of the fluorescence spectra of BSA at various concentrations of NPP are shown in Fig. 7(a and b), respectively.

The emission wavelength of the tyrosine is blue-shifted with increasing concentration of NPP. Comparing the fluorescence emission wavelength of the typtophan, no significant change was observed. It is indicated that the interaction of NPP with BSA does not affect the conformation of tryptophan micro-region. Firstly, combined with tryptophan, NPP grad-

ually interacts with tyrosine and brings changes to BSA and results in blue-shifts of fluorescence wavelength and deviation from the curve of static quenching. The hypsochromic effects of BSA fluorescence emission are far more than those of tyrosine residues'. Besides the conformational changes of the protein, tyrosine is no longer fluorescent due to quenching, and the energy transfer process between tryptophan and tyrosine is also hindered. So fluorescence of tyrosine becomes the main luminescent species, leading to relatively larger blue-shifts.

3.4. Energy transfer between NPP and BSA

According to Föster's non-radiative energy transfer theory [18,19], the energy transfer effect is related not only to the distance between the acceptor and donor (r_0), but also to the critical energy transfer distance (R_0), that is:

$$E = \frac{R_0^6}{R_0^6 + r_0^6} \tag{6}$$

where R_0 is the critical distance when the transfer efficiency is 50%.

$$R_0^6 = 8.8 \times 10^{-25} K^2 N^{-4} \Phi J \tag{7}$$

where K^2 is the spatial orientation factor of the dipole, N the refractive index of the medium, Φ the fluorescence quantum yield of the donor, J the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. Therefore,

$$J = \sum F(\lambda)\varepsilon(\lambda)\lambda^4 \frac{\Delta\lambda}{\sum F}(\lambda)\Delta\lambda \tag{8}$$

where $F(\lambda)$ is the fluorescence intensity of the fluorescent donor at wavelength λ, $\varepsilon(\lambda)$ the molar absorptivity of the acceptor at wavelength λ, E is the energy transfer efficiency. Then, the energy transfer efficiency is given by

$$E = 1 - \left(\frac{F}{F_0}\right) \tag{9}$$

The overlap of the absorption spectrum of NPP and the fluorescence emission spectrum of BSA is shown in Fig. 8. J can be evaluated by integrating the spectra in Eq. (8). Under these experimental conditions, we found $R_0 = 1.76$ nm from Eq. (7) using $K^2 = 2/3$, $\Phi = 0.032$, $n = 1.3467$ [20], the energy transfer effect $E = 0.352$ from Eq. (7) and the distance between NPP and tryptophan residue in BSA is 1.94 nm. Obviously, they are lower than 7 nm after interaction between NPP and BSA. These accord with conditions of Föster's non-radiative energy transfer theory, indicating again the static quenching interaction between NPP and BSA. Furthermore, suggesting that the binding reaction of NPP to BSA is through energy transfer, which will quench the fluorescence of the protein. In addition, considering the overlap of the absorption spectrum of NPP and the fluorescence emission spectrum of BSA, The contribution of NPP to the overall absorption at excitation wavelength (286 nm) was further calculated using the absorbance of NPP, BSA, BSA–NPP complex at wavelength (286 nm), respectively. The value was found to be 36.5%.

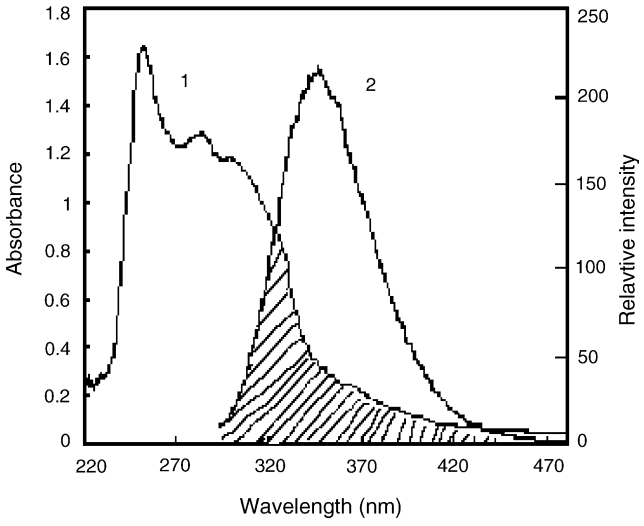

Fig. 8. The overlap of the UV absorption spectrum of NPP with the fluorescence emission spectrum of BSA: (a) the UV absorbance spectrum of NPP, $C_{NPP} = 1.0 \times 10^{-5}$ mol L^{-1} and (b) the fluorescence spectrum of BSA, $C_{BSA} = 1.0 \times 10^{-5}$ mol L^{-1}.

4. Conclusion

Fluorescence method is an important tool for the investigation of the interaction between dye and protein. The use of dyes for protein determination is well established, however, other parameters, such as mode of interactions, binding constant and number of binding sites are important. The binding characteristics of NPP and BSA can be identified by means of fluorescence spectrum. It was found that the equilibrium constant $K = 5.30 \times 10^4$ L mol^{-1}, and number of binding sites $n = 0.9267$. Based on the mechanism of Föster energy transference, the transfer efficiency of energy and distance between the acceptor BSA and was found to be 0.352 and 1.94 nm, respectively. We further investigated the synchronous fluorescence spectra of BSA. The results showed that the fluorescence spectra of BSA mainly originate from the tryptophan residues. The mechanism of the interaction between NPP and DNA is regarded as essential for use as a probe of the biomolecule, the interaction between NPP and DNA will be further study in future work.

Acknowledgements

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References

[1] F. Moreno, M. Cortijo, J. Gonzalez-Jimenez, Photochem. Photobiol. 70 (1999) 695–700.
[2] M. Kessler, O. Wolfbeis, Anal. Biochem. 200 (1992) 254–259.
[3] M.E. Georgiou, C.A. Georgiou, M.A. Kouppairs, Anal. Chem. 71 (1999) 2541–2550.
[4] R. Haughland, Handbook of Fluorescent Probes and Research Chemicals, Molecular Probes Inc., Eugene, OR, 1996.
[5] V. Nissinen, B. Riesmeier, K.H. Kroner, Biotechnol. Technol. 1 (1987) 9.
[6] A.A. Waheed, K.S. Rao, P.D. Gupta, Anal. Biochem. 287 (2000) 73–79.
[7] Y. Nakamaru, C. Sato, Biochim. Biophys. Acta 1480 (2000) 321–328.
[8] M.L. Silber, B.B. Davitt, Prep. Biochem. Biotechnol. 30 (2000) 209–229.
[9] K. Iwata, T. Takashima, K. Kawabata, Japan, Kokai Kokkyo Koho 79 62, 019(Cl.C09D11/16), 1979-5-18.
[10] S. Sudo, H. Sugiyama, Japan, Kokai Kokkyo Koho 303, 618(Cl.G01N31/22), 2002-10-18.
[11] S.Y. Tong, X. Jin, Chem. Online 8 (1988) 52–55.
[12] T. Peter, All Abount Albumin: Biochemistry, Genetics and Medical Applications, Academic Press, San Diego, CA, 1995.
[13] J.R. Lakowica, G. Weber, Biochemistry 12 (1973) 4161.
[14] W.R. Ware, J. Phys. Chem. 66 (1962) 455.
[15] M. Alain, B. Michel, D. Michel, J. Chem. Educ. 63 (1986) 365–366.
[16] G.Z. Chen, X.Z. Huang, J.G. Xu, Z.Z. Zheng, Z.B. Wang, Methods of Fluorescence Analysis, second ed., Science Press, Beijing, 1990.
[17] E.A. Brustein, N.S. Vedenkina, M.N. Irkova, Photochem. Photobiol. 18 (1973) 263.
[18] L.A. Sklar, B.S. Hudson, R.D. Simoni, Biochemistry 16 (1977) 5100.
[19] J.R. Lakowicz, Principles of Fluorescence Spectroscopy, Plenum Press, New York, 1983.
[20] L. Cyril, J.K. Earl, W.M. Sperry, Biochemists Handbook, E & F.N. Spon, London, 1961.