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Spectrochimica Acta Part A 70 (2008) 1127–1133
SPECTROCHIMICA ACTA PART A
www.elsevier.com/locate/saa

2,5-di-[2-(3,5-bis(2-pyridylmethyl)amine -4-hydroxy-phenyl) ethylene] pyrazine zinc complex as fluorescent probe for labeling proteins

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Received 10 August 2007; accepted 24 October 2007

Abstract

The binding characteristics between 2,5-di-[2-(3,5-bis(2-pyridylmethyl)amine -4-hydroxy-phenyl) ethylene] pyrazine (**1**) or its complex (**1-Zn**) and serum albumins were studied by fluorescence spectroscopy in pH 7.4 aqueous solution. **1-Zn** emitted weak fluorescence at 580 nm in a pH 7.4 Tris–HCl buffer solution when excited at 435 nm, however, the fluorescence intensity increased upon addition of serum albumins with the blue shift of emission peak to 524 nm. The binding constants were estimated as 8.40×10^7 and 3.03×10^6 mol^{-1} L for bovine serum albumin (BSA) and human serum albumin (HSA) respectively, and the number of binding sites was 1 for each. The quenching mechanism of fluorescence of serum albumins by **1-Zn** was considered as a static quenching process. The binding distance between **1-Zn** and serum albumins and the energy transfer efficiency were obtained based on the theory of Förester spectroscopy energy transfer. The effect of **1-Zn** on the conformation of serum albumins was further analyzed using synchronous fluorescence spectrometry. The experiment results clearly showed that **1-Zn** is a highly sensitive protein sensor.

Keywords: 2,5-di-[2-(3,5-bis(2-pyridylmethyl)amine -4-hydroxy-phenyl) ethylene] pyrazine; Zinc complex; Fluorescent probe; Serum albumins

1. Introduction

As the major protein constituent of blood plasma, serum albumins facilitate the transport of many compounds such as fatty acids, hormones, bilirubin and many drugs [1]. They aroused common interests among bio-scientists, chemists and therapeutists [2]. It is known that fluorescence assay provides many advantages such as high sensitivity, high selectivity, on site detection, easy operation and low cost, thus it has been widely applied to detect proteins [3–6]. The search for good fluorescent probes for biosystem has attracted much attention. In order to avoid fluorescence bleaching and tissue burning, fluorescent compounds possessing high quantum yield, long wavelength of excitation and emission were favored. Recently, several fluorescent reagents have been developed for the detection of proteins in solution such as fluorescamine [7], cyanine dyes [8] and in sodium dodecyl sulfate (SDS)-polyacrylamide gels, such as SYPRO Ruby [9]. Some metal–organic complexes have many merits such as high stability, good solubility and high quantum yield [4,10]. We previously synthesized compound 2,5-di-[2-(3,5-bis(2-pyridylmethyl)amine-4-hydroxy-phenyl) ethylene] pyrazine which served as a sensor for lead ion [11]. In this further study, we found a strong green fluorescence at 524 nm of **1-Zn** (Scheme 1) in the presence of proteins when excited at 435 nm. Therefore, **1-Zn** is an ideal fluorophore because its excitation and emission wavelengths belong to the visible region, and it formed stable complex with proteins. **1-Zn** showed stronger binding affinity with proteins than **1** did. The theory of energy transfer was employed to investigate the binding mechanism of proteins with **1** or **1-Zn**. The binding mode, binding constants as well as binding distance have been displayed.

2. Experimental

2.1. Apparatus

All fluorescence measurements were carried out on a F-4500 spectrofluorimeter (Hitachi, Japan) equipped with a xenon

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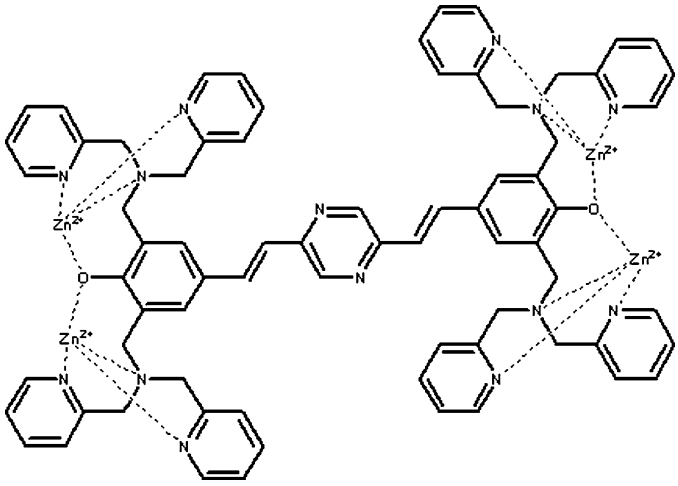
doi:10.1016/j.saa.2007.10.028



Scheme 1. Structure of sensor (**1-Zn**).

lamp source and a 1.0 cm quartz cell, and the scan speed was 240 nm min^{-1}. Absorption spectra were recorded on a Shimadzu-2501 UV–vis spectrophotometer (Japan) using a 1.0 cm quartz cell. All pH measurements were made with a pHS-3 digital pH-meter (Shanghai REX instrument factory, Shanghai, China) with a combined glass-calomel electrode.

2.2. Reagents

The synthesis and characterization of **1** can be referred to the literature [11].

The stock solution of **1** and $Zn(ClO_4)_2$ were prepared in dimethyl sulfoxide (DMSO) and the concentrations were 1.0×10^{-4} and 4.0×10^{-4} mol L^{-1} respectively. **1-Zn** was prepared by mixing **1** and zinc solution in a ratio of 1:1 (v/v). They should be freshly prepared and ultrasonicated for 5 min before usage.

HSA (Zhejiang Kangsheng Biological Products Institute) was prepared into 2.50×10^{-4} mol L^{-1} stock solution by dissolving 1.7000 g in 10 ml water and diluting to 100 mL with the 0.01 mol L^{-1} Tris–HCl buffer of pH 7.4 containing 0.15 mol L^{-1} NaCl. The stock solution of BSA (Wuhan Yafa Biological Technology Company) having the same concentration was prepared likewise. They were stored at 0–4 °C. All the chemicals used were of analytical grade and doubly distilled water was used throughout unless otherwise noted.

2.3. Procedure

To a 10 ml colorimetric tube, 0.5 ml BSA solution and **1-Zn** or **1** were added. The mixture was diluted to the mark with 0.01 mol L^{-1} Tris–HCl buffer. The concentration of DMSO was kept at 5% (v/v). The solution was fully mixed. Fluorescence quenching spectra were obtained at excitation wavelength of 280 nm. Fluorescence enhancing spectra were obtained at excitation wavelength of 435 nm. The fluorescence intensity around 550 nm was recorded and applied to the quantitative analysis of proteins.

The synchronous fluorescence spectra of solutions prepared in the similar way like the previous ones were recorded with $\Delta\lambda$ set as 15 and 60 nm respectively. The slit widths for all fluorescence measurements were set as 5 nm.

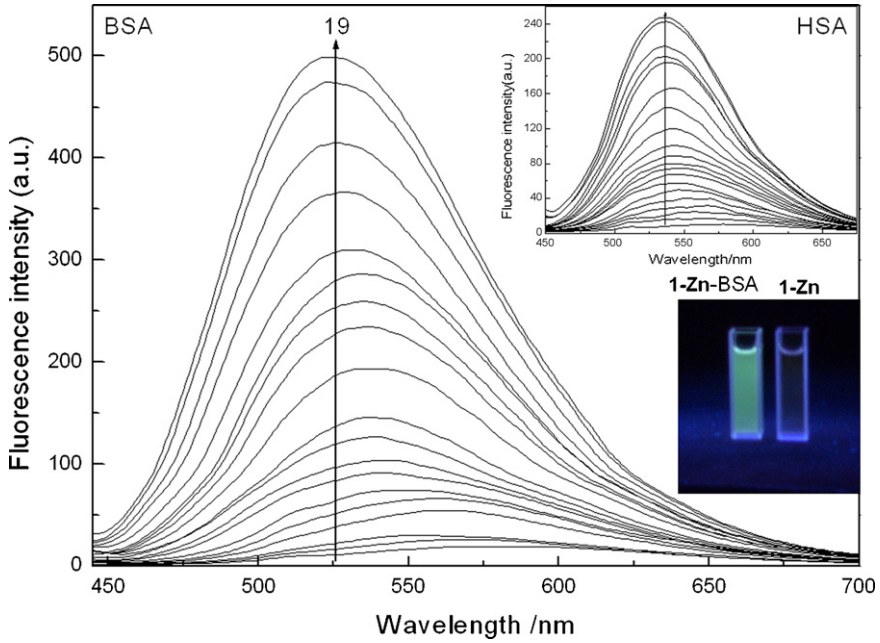


Fig. 1. Fluorescence titration of sensor **1-Zn** (1.0×10^{-6} mol L^{-1}) with BSA. The excitation wavelength was set at 435 nm. The arrows indicated the increase of protein's concentration. The concentrations of BSA were 0, 0.2, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.5, 5.5, 7.0, 8.5, 10.0, 20.0, 30.0, 40.0, 50.0 and 70.0×10^{-7} mol L^{-1}, respectively. Inset 1 was the fluorescence titration of the sensor with HSA. The concentrations of HSA were 0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.5, 5, 6.0, 7.0, 8.0, 9.0, 10.0, 20.0, 30.0, 40.0 and 60.0×10^{-7} mol L^{-1}, respectively. Inset 2 was the photograph of sensor **1-Zn** in the presence and absence of BSA.

3. Results and discussion

3.1. Fluorescence titrations of *1-Zn* with BSA and HSA

The fluorescence titrations of **1-Zn** with BSA and HSA were presented in Fig. 1. Upon addition of BSA, the maximum emission wavelength of **1-Zn** was remarkably blue shifted from 580 to 524 nm along with a dramatic enhancement of fluorescence intensity. The solution emitted green fluorescence and was easily detected via naked eyes (Seen in the inset of Fig. 1). At the same time a good linear response of fluorescence intensity as a function of BSA concentration was obtained. The linear equation is $\Delta F = -3.558 + 3.720 \times 10^6 C_{BSA}$ ($n = 9$, $R = 0.996$) while the concentration of BSA ranged from 1.17×10^{-7} to 4.5×10^{-6} mol L^{-1} and the detection limit was 1.17×10^{-8} mol L^{-1}. Similar results were obtained for HSA as follows, $\Delta F = -3.420 + 1.691 \times 10^6 C_{HSA}$ ($n = 9$, $R = 0.996$) and detection limit as 2.86×10^{-8} mol L^{-1}. It should be pointed out that the mixture emitted strong fluorescence in the visible region and the excitation wavelength was in the visible region as well. Thus, **1-Zn** is a good fluorescent probe for proteins.

Under identical condition, fluorescent titrations of **1** with BSA and HSA were also studied and presented in Fig. 2. For **1**, the trends of spectral change were similar to those of **1-Zn**, which were the blue shift of emission peak and intensity enhancement. But the sensitivity is lower than that of **1-Zn** and emission was difficult to observe by naked eyes. It was reasonable because **1-Zn** possessed higher quantum yield than **1** [11].

The effect of foreign compounds on the determination of BSA was studied. Upon addition of 2.0×10^{-6} mol L^{-1} BSA into 1.0×10^{-6} mol L^{-1} **1-Zn** solution, the existence of following amounts of foreign ions and compounds did not interfere with the determination of BSA while the error was less than ±10%: 1000-fold Na^+ and K^+, 50-fold Mg^{2+} and urea, 25-fold Ba^{2+}, Al^{3+}, Ag^+ and cystine, 22-fold glucose and methionine, 20-fold cysteine, 10-fold Ca^{2+}, Pb^{2+}, Ni^{2+}, Cd^{2+}, Fe^{3+}, tryptophan, tyrosine, glycine and 2-fold Hg^{2+}.

3.2. Fluorescence quenching mechanism

In order to illustrate the interaction between **1-Zn** and proteins, increasing amounts of **1-Zn** was added into protein solution and the spectral changes were shown in Fig. 3. The fluorescence intensity of BSA at 335 nm decreased. The similar result was obtained for HSA. The quenching was caused by intermolecular energy transfer between **1-Zn** and proteins under the excitation at 280 nm. The same experiments were carried out on **1** and results were presented in Table 1. We assume these phenomena were caused by dynamic quenching. For the dynamic quenching, the decrease in intensity is described by the well-known Stern–Volmer equation:

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

where F_0 and F are the steady-state fluorescence intensities in the absence and presence of **1-Zn** respectively, K_q is the quenching rate constant of biomolecule, τ_0 is the average lifetime of molecule without the quencher and its value is 10^{-8} s [12], K_{sv} is the Stern–Volmer quenching constant, $[Q]$ is the concentration of quencher.

From the Stern–Volmer plot, K_{sv} can be obtained as the slope, thus K_q was estimated and the results were shown in Table 1. Usually, the maximum diffusion collision quenching rate constant of various quenchers for the biomacromolecule is about

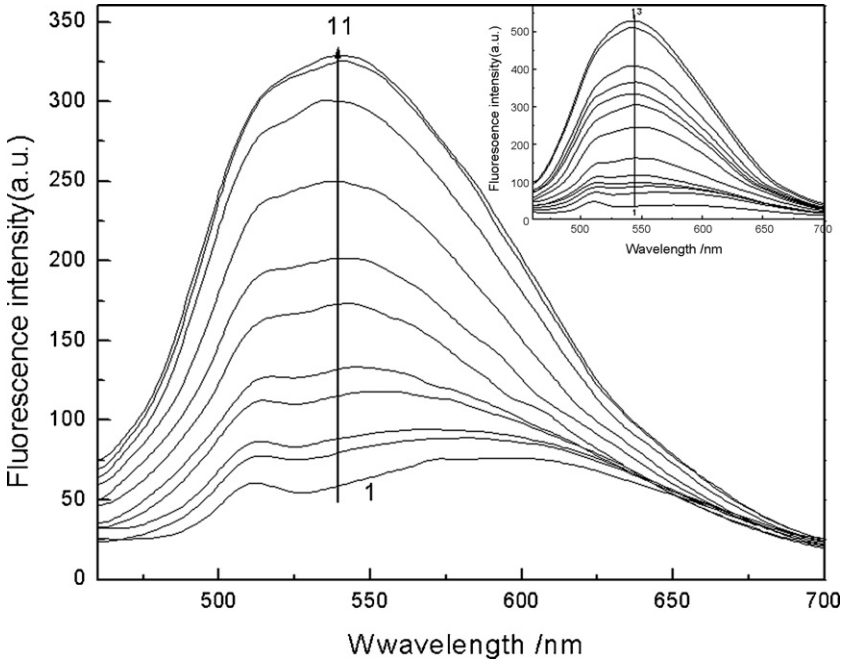


Fig. 2. Fluorescence titration of **1**(1.0×10^{-6} mol L^{-1}) with BSA. The arrows indicated the increase of protein's concentration. The concentrations of BSA were 0, 0.2, 0.5, 1.0, 2.0, 3.0, 5.0, 7.0, 8.0, 9.0 and 10.0×10^{-6} mol L^{-1} respectively. The inset was the fluorescence titration of **1**(1.0×10^{-6} mol L^{-1}) with HSA. The concentrations of HSA were 0, 0.5, 0.8, 1.0, 1.5, 2.5, 3.5, 4.5, 5.5, 6.0, 6.5, 7.5 and 8.5×10^{-6} mol L^{-1}, respectively. The excitation wavelength was set at 435 nm.

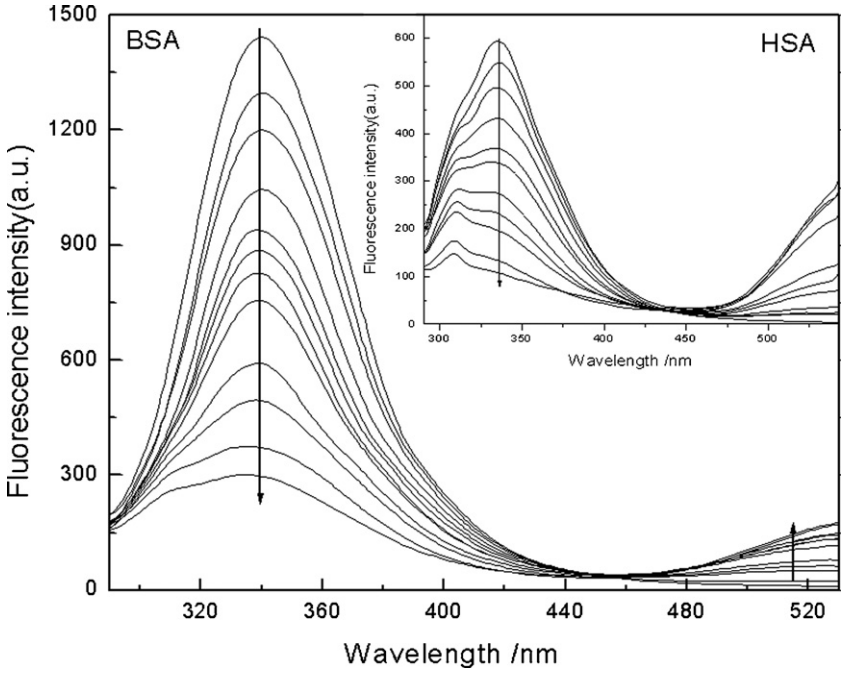


Fig. 3. Fluorescence spectra of BSA (1.0×10^{-6} mol L^{-1}) with increasing concentration of **1-Zn**. The arrows indicated the increase of **1-Zn**'s concentration. The concentrations of **1-Zn** were 0, 0.2, 0.5, 0.8, 1.0, 1.2, 1.5, 1.8, 2.0, 2.5, 3.0 and 3.5×10^{-6} mol L^{-1} respectively. The inset was the fluorescence spectra of HSA (1.0×10^{-6} mol L^{-1}) in the presence of **1-Zn**. The concentrations of **1-Zn** were 0, 0.2, 0.5, 1.0, 1.5, 2.0, 2.7, 3.5, 4.2, 5.2 and 6.7×10^{-6} mol L^{-1} respectively. The excitation wavelength was set at 280 nm.

2.0×10^{10} mol^{-1} L s^{-1} [13]. Obviously, the derived quenching rate constants were larger than the K_q of the diffusion course by three orders of magnitude for sensor **1-Zn** or two orders of magnitude for **1**. Hereby, the fluorescence quenching was the consequence of the static quenching instead of the dynamic collision quenching. Apparently, sensor **1-Zn** was more sensitive to proteins than **1** was.

3.3. Binding constants and number of binding sites

Binding constant displays the interaction between the quencher and the protein. It was easily estimated from the following equation [14] and the parameters for static quenching is described as follows:

$$lg\frac{F_0 - F}{F} = lg\, K_b + n\, lg[Q] \quad (2)$$

where F_0 and F are the steady-state fluorescence intensities in the absence and presence of the quencher respectively, K_b is the binding constant to a site, n is the number of binding sites per HSA or BSA, $[Q]$ is the concentration of the quencher.

From the plot, the binding constants and the number of binding sites could be obtained and were presented in Table 2. The results displayed that the binding constants between sensor **1**-Zn and proteins were lager than those of **1** and proteins by about two orders of magnitudes.

3.4. Energy transfer between 1-Zn and BSA or HSA

We can evaluate the distance and relative angular orientation between the protein and **1-Zn** using Fluorescence resonance energy transfer (FRET) method. An energy transfer could occur through direct electrodynamic interaction between the primarily excited molecule and its neighbors. The spectroscopic method is suitable for measuring distance over several nanometers [15]. Using FRET, the distance r of binding between **1-Zn** and the protein could be calculated by the equation [16]:

$$E = 1 - \frac{F}{F_0} = \frac{R_0^6}{R_0^6 + r^6} \quad (3)$$

where E is the efficiency of energy transfer between the donor and the acceptor, F and F_0 are the fluorescence intensities in

Table 1
Quenching rate constants for the interaction between receptors and proteins

Receptor	Linear equation	K_{sv}/mol^{-1} L	K_q/mol^{-1} L s^{-1}	R
1-Zn	BSA: $F/F_0 = 0.986 + 5.16 \times 10^5$ [**1-Zn**]	5.23×10^5	5.23×10^{13}	0.996
	HSA: $F/F_0 = 0.937 + 6.46 \times 10^5$ [**1-Zn**]	6.89×10^5	6.89×10^{13}	0.996
1	BSA: $F_0/F = 0.982 + 7.94 \times 10^4$ [**1**]	8.09×10^4	8.09×10^{12}	0.994
	HSA: $F_0/F = 1.05 + 9.94 \times 10^4$ [**1**]	9.47×10^4	9.47×10^{12}	0.999

Table 2
Binding constants and number of binding sites

	Linear equation	Binding constants K_b	Binding sites	R
1-Zn	BSA: $\lg(F_0-F)/F = 7.92 + 1.361\lg$ [**1**-Zn]	8.40×10^7 mol^{-1} L	1.36	0.995
	HSA: $\lg(F_0-F)/F = 6.48 + 1.15\lg$ [**1**-Zn]	3.03×10^6 mol^{-1} L	1.15	0.995
1	BSA: $\lg(F_0-F)/F = 5.30 + 1.07\lg$ [**1**]	2.02×10^5 mol^{-1} L	1.07	0.998
	HSA: $\lg(F_0-F)/F = 4.78 + 0.948\lg$ [**1**]	5.98×10^4 mol^{-1} L	0.95	0.999

the presence and absence of **1-Zn**, respectively, R_0 is the critical distance when the efficiency of transfer is 50%, which can be calculated by

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

where K^2 is the spatial factor of orientation; n is the refractive index of the medium, ϕ is the fluorescence quantum yield of the donor.

It was shown in Fig. 4 that there were overlaps between the absorption spectra of **1-Zn** and the emission spectra of BSA or HSA. From Fig. 4, we can calculate the overlap integral J.

$$J = \frac{\int_0^\infty F(\lambda)\varepsilon(\lambda)\lambda^4 \, d\lambda}{\int_0^\infty F(\lambda) \, d\lambda} \quad (5)$$

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

In the present case, $K^2 = 2/3$, $n = 1.36$, $\phi = 0.15$ [17]. We could calculate J, R_0, E and r. All these parameters were displayed in Table 3. The energy transfer will most probably take place when the average distance between a donor fluorophore and acceptor fluorophore is on the 2–8 nm scale [18] and $0.5R_0 < r < 1.5R_0$ [19]. The results indicated that the energy transfer from BSA to **1-Zn** occurred with high possibility. The parameters for receptor **1** were obtained according to the same method. It was obvious that **1-Zn** bound proteins more strongly because r was smaller than that of **1**-protein.

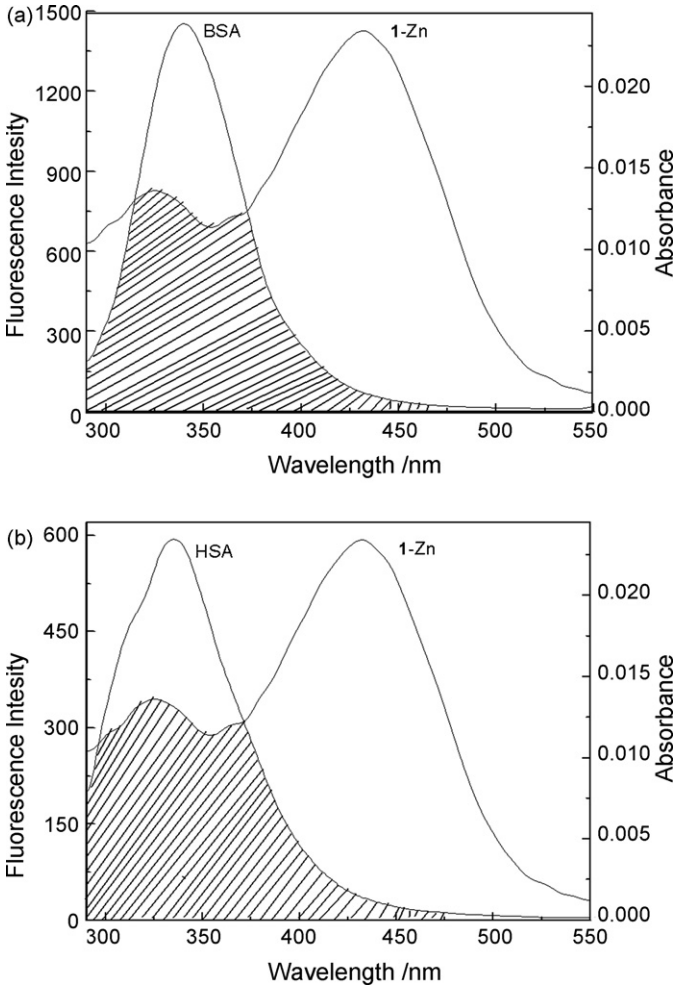


Fig. 4. Overlap of the fluorescence spectra of BSA (a) and HSA (b) with the absorption spectra of **1-Zn**. The concentrations of the protein and **1-Zn** were both 1.0×10^{-6} mol L^{-1}.

3.5. Conformation investigation

The synchronous fluorescence spectroscopy is a common method to provide information about the conformational changes of protein. They have several advantages like spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects. The spectrum characteristic of tyrosine and tryptophan residues in protein was observed when $\Delta\lambda$ was set as 15 and 60 nm respectively. Synchronous fluorescence spectral changes of BSA and HSA upon addition of **1-Zn** or **1** with varied concentration were displayed in Fig. 5. The maximum emission of BSA or HSA slightly change when $\Delta\lambda = 15$ nm or $\Delta\lambda = 60$ nm. However, the emission intensity underwent dramatic change. It has been shown in Table 4 that the slopes were similar for HSA and BSA when $\Delta\lambda$ was the same. However, the slope was higher when $\Delta\lambda$ was 60 nm than 15 nm indicating sensor **1-Zn** or **1** was closer to tryptophan residues than to tyrosine residues. The conformations of proteins were changed while binding **1** or **1-Zn** complex.

Table 3
Energy transfer parameters between receptors and protein

Receptor	Protein	J cm^3 (mol L)$^{-1}$	R_0 (nm)	E	R(nm)
1-Zn	BSA	1.080×10^{-14}	2.55	0.350	2.83
	HSA	1.056×10^{-14}	2.54	0.273	2.99
1	BSA	2.434×10^{-14}	2.94	0.073	4.49
	HSA	2.351×10^{-14}	2.95	0.108	4.20

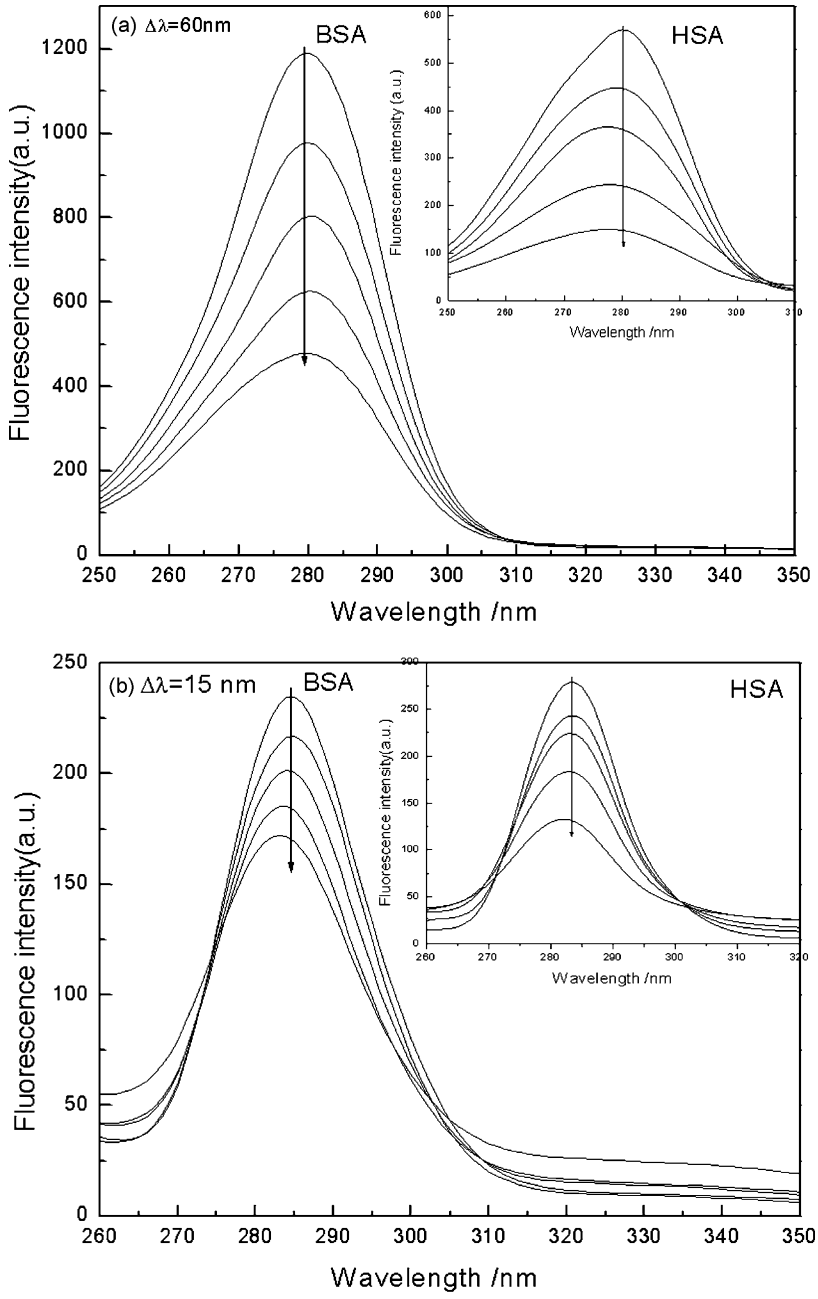


Fig. 5. Synchronous fluorescence spectra of BSA (1.0×10^{-6} mol L^{-1}) upon addition of **1-Zn**. The arrows indicated the increase of protein's concentration. (a) $\Delta\lambda = 15$ nm, (b) $\Delta\lambda = 60$ nm. The insets were the synchronous fluorescence spectra of HSA (1.0×10^{-6} mol L^{-1}) upon addition of **1-Zn**. The concentrations of **1-Zn** were 0, 0.1, 1.0, 1.5 and 2.0×10^{-6} mol L^{-1} for BSA and 0, 0.5, 2.0, 3.5 and 5.0×10^{-6} mol L^{-1} for HSA respectively.

Table 4
Linear relationships for the quenching synchronous spectra of proteins

	Protein	$\Delta\lambda$	Slope	R
1-Zn	BSA	15	-3.18×10^7	0.999
	BSA	60	-3.59×10^8	0.998
	HSA	15	-2.90×10^7	0.998
	HSA	60	-8.41×10^7	0.993
1	BSA	15	-6.07×10^6	0.993
	BSA	60	-6.26×10^7	0.995
	HSA	15	-8.26×10^6	0.992
	HSA	60	-5.03×10^7	0.991

4. Conclusions

A novel fluorescent complex (**1-Zn**) was used for sensing proteins. Its excitation wavelength and emission wavelength were at 435 and 528 nm, respectively. Therefore **1-Zn** is a good candidate to serve as fluorescent probe in biochemical system. Especially, emission of **1-Zn**-protein was easy to detect by naked eyes. **1-Zn** is a strong quencher for proteins' fluorescence and binds to BSA or HSA with high affinity. FRET studies indicated the energy transfer from proteins to **1-Zn**. Synchronous fluorescence spectroscopy revealed that the secondary structure of BSA or HSA molecules changed in the presence of **1-Zn**.

Acknowledgements

The authors gratefully acknowledge the financial support of this study by the State Key Laboratory of Chemo/Biosensing and Chemometrics of Hunan University (No.2005018), Jiangxi Province Education Ministry Foundation (No.2005-38), Jiangxi Province Natural Science Foundation (JXNSF No.0420041).

References

[1] A. Mallick, B. Haldar, N. Chattopadhyay, J. Phys. Chem. B 109 (2005) 14683.
[2] W.M. Pardridge, Am. J. Physiol. 252 (1987) 157.
[3] J.A. Mackintosh, D.A. Veal, P. Karuso, Proteomics 5 (2005) 4673.
[4] F. Wang, J. Yang, X. Wu, C. Sun, S.F. Liu, C. Guo, Z. Jia. Chem. Phys. Lett. 409 (2005) 14.
[5] B.K. Hoefelschweiger, A. Duerkop, O.S. Wolfbeis, Anal. Biochem. 344 (2005) 122.
[6] Y. Suzuki, K. Yokoyama, J. Am. Chem. Soc. 127 (2005) 17799.
[7] L.J. Jones, R.P. Haugland, V.L. Singer, Bio. Tech. 34 (2003) 850.
[8] A.S. Bhown, T.W. Cornelius, J.E. Volanakis, J.C. Bennett, Anal. Biochem. 131 (1983) 337.
[9] K. Berggren, T.H. Steinberg, W.M. Lauber, J.A. Carroll, M.F. Lopez, E. Chernokalskaya, L. Zieske, Z. Diwu, R.P. Haugland, W.F. Patton, Anal. Biochem. 276 (1999) 129.
[10] E.J. Jun, J.A. Kim, K.M.K. Swamy, S. Park, J. Yoon, Tetrahedron Lett. 47 (2006) 1051.
[11] F.Y. Wu, S.W. Bae, J.I. Hong, Tetrahedron Lett. 47 (2006) 8851.
[12] J.R. Lakowicz, G. Weber, Biochemistry 12 (1973) 4161.
[13] W.R. Ware, J. Phys. Chem. 66 (1962) 455.
[14] Y.-J. Hu, Y. Liu, X.-S. Shen, X.-Y. Fang, S.-S. Qu, J. Mole. Struct. 738 (2005) 143.
[15] A.P. Silva, H.Q.N. Gunaratne, T. Gunnlaugsson, A.J.M. Huxley, C.P. McCoy, J.T. Rademacher, T.E. Rice, Chem. Rev. 97 (1997) 1515.
[16] Y.V. Il'ichev, J.L. Perry, J.D. Simon, J. Phys. Chem. 106 (2002) 452.
[17] L. Cyril, J.K. Earl, W.M. Sperry, Biochemists' Handbook, E. F.N. Spon., London, 1961, p. 84.
[18] B. Valeur, J.C. Brochon, New Trends in Fluorescence Spectroscopy, Springer, Berlin, 2001, p. 25.
[19] B. Valeur, Molecular Fluorescence: Principles and Applications, Wiley, New York, 2001.