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Determination of proteins using fluorescence enhancement of Tb^{3+}–benzoylacetone–sodium dodecyl benzene sulfonate–protein system

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Abstract

A new system for the determination of proteins has been developed in this paper. Proteins can enhance the fluorescence intensity of the Tb–benzoylacetone (BA) system in the presence of sodium dodecyl benzene sulfonate (SDBS). Under optimum conditions, the enhanced fluorescence intensity is in proportion to the concentration of proteins in the range of 1.0×10^{-8} to 6.0×10^{-6} g mL^{-1} for bovine serum albumin (BSA), 3.0×10^{-8} to 6.0×10^{-6} g mL^{-1} for egg albumin (EA) and 1.0×10^{-8} to 5.0×10^{-6} g mL^{-1} for human serum albumin (HSA). Their detection limits (S/N = 3) are 3.9×10^{-9}, 4.0×10^{-9} and 8.5×10^{-9} g mL^{-1}, respectively. In addition, the interaction mechanism is also investigated.

Keywords: Fluorescence enhancement; Protein; Tb^{3+}; Benzoylacetone; SDBS

1. Introduction

The proteins are fundamental elements of life, and the increase or decrease of protein contents in serum can display the conditions of human health [1,2]. So quantitative analysis of proteins is important in many fields, such as biochemical analysis, food analysis and clinic analysis. The traditional spectrophotometry for protein determination using dyes as analytical reagents, such as the Lowry [3], Amaranth [4], coomassie brilliant blue [5,6], bromophenol blue [7], indigo carmine [8], and quercetin [9] methods, have some disadvantages. Such as that they have a limited sensitivity and are time-consuming or complicated to operate [10]. In addition, the intrinsic fluorescence of proteins is weak [11] for the analysis of proteins at low concentration. Therefore, many fluorometric methods [12–14] are developed, which are focusing on investigating sensitive [15], stabile and simple fluorescence probes for proteins determination [16–24]. Among these probes, rare earth chelates probes are widely applied because they have the luminescence characteristics of rare earth ions, such as narrow spectral width, long luminescence life-time, large stokes shift and also have higher sensitivity.

It is well known that β-diketone can chelate with lanthanide ions and sensitize fluorescence of rare earth ions (such as Eu^{3+}, Sm^{3+}, Tb^{3+}, Dy^{3+}) [22–24]. In this paper, BA (β-diketone) is selected as a ligand. The Tb^{3+}–BA–SDBS ion association system exhibits the characteristic fluorescence of Tb^{3+} at low level, whereas the fluorescence intensity of the system is considerably enhanced by proteins. Based on this phenomenon, the novel method for the determination of proteins is developed.

2. Experimental

2.1. Chemicals

Stock standard solution (1.00×10^{-2} M) of Tb^{3+} was prepared by dissolving Tb_4O_7 (Yuelong Chemical Co., Shanghai; 99.9%) in hydrochloric acid and heating until nearly dry, then diluting with doubly distilled water.

Benzoylacetone solution (BA 1.00×10^{-2} M) was prepared by dissolving 0.08100 g BA in 50 mL volumetric flask with 95% alcohol.

Proteins (BSA, HSA and EA) are purchased from Sigma Chemical Co., USA without further purification. And their stock solutions of 1.00×10^{-4} g mL^{-1} were prepared by dissolving protein in doubly distilled water, stored at 0–4 °C.

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Stock solution of sodium dodecyl benzene sulfonate (SDBS, 1.00×10^{-2} M) was prepared by dissolving 0.8712 g SDBS in 250 mL volumetric flask with water.

A 0.05 M Tris–HCl buffer solution was prepared by dissolving of 3.03 g Tris in 500 mL volumetric flask with water and adjusted the pH to 8.27 with 6 M HCl.

All the chemicals used are of analytical grade and doubly distilled water was used throughout.

2.2. Apparatus

Normal fluorescence measurements were recorded with a LS-55 spectrofluorimeter (PE). The RLS spectra were obtained by scanning simultaneously the excitation and emission monochromators (namely, $\Delta\lambda = 0$ nm) from 200 to 600 nm. The surface tension was measured on Processor Tensiometer-K12 (Krüss Corp.). The precise degree of the measurement is 0.01 mN m^{-1} by the Wilhelmy-plate. All pH measurements were made with a Delta 320-S acidity meter (Mettler Toledo, Shanghai). All absorption spectra were recorded with an UV-4100 spectrophotometer (Hitachi).

2.3. Analytical procedure

To a 25 mL test tube, solutions were added in the following order: Tb^{3+}, BA, SDBS, BSA and Tris–HCl buffer. The mixture was diluted to 10 mL with doubly distilled water. The fluorescence intensity was measured in a 1 cm quartz cell, the excitation and emission slits were both 10 nm with a scan speed of 500 nm/min. The enhanced fluorescence intensity of Tb^{3+}–BA–SDBS by proteins was represented as $\Delta I_f = I_f - I_0$. Here I_f and I_0 were the intensities of the systems with and without protein, respectively.

3. Result and discussion

3.1. Fluorescence spectra

Excitation and emission spectra of Tb–BA (1), Tb–BA–SDBS (2), Tb–BA–SDBS–EA (3), Tb–BA–SDBS–HSA (4), Tb–BA–SDBS–BSA (5) systems are shown in Fig. 1. From this figure, it can be seen that under the excitation of 350 nm, the fluorescence of Tb–BA system is very weak. When SDBS is added, the intrinsic fluorescence of Tb^{3+} is obviously enhanced, with the emission peaks of 490 and 545 nm, corresponding to the $^5D_4 \rightarrow {}^7F_6$ and $^5D_4 \rightarrow {}^7F_5$ transitions of Tb^{3+}, respectively. And the proteins could strongly enhance the fluorescence of the Tb–BA–SDBS system, which indicates that there are interaction among proteins, SDBS and Tb–BA. The fluorescence intensity at 545 nm is the strongest, so the 545 nm is chosen as the emission wavelength in further research.

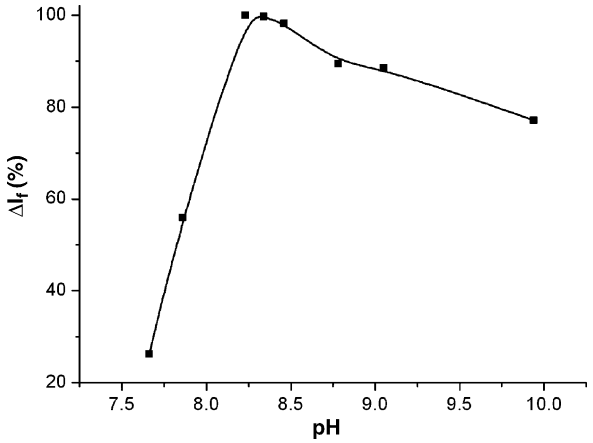

Fig. 2. Effect of pH on ΔI_f. Conditions: Tb^{3+}, 1.2×10^{-5} M; BA, 1.0×10^{-4} M; SDBS, 1.0×10^{-4} M; BSA, 5.0×10^{-6} g mL^{-1}.

3.2. Effect of pH and the choice of buffer solution

The effect of pH on the fluorescence intensity of the system is shown in Fig. 2. The experimental results indicate that in the pH range 8.20–8.40 the enhanced fluorescence intensity ΔI_f reaches a maximum. Under acidic conditions, the fluorescence intensity diminished, which may be that hydronium ion's competition with metal ion for BA counteracts the formation of Tb^{3+}–BA complex. Whereas at high pH, the metal ion will form hydrated hydroxides rather than chelate complexes [25]. So pH 8.27 is used for subsequent work. The effects of different buffers

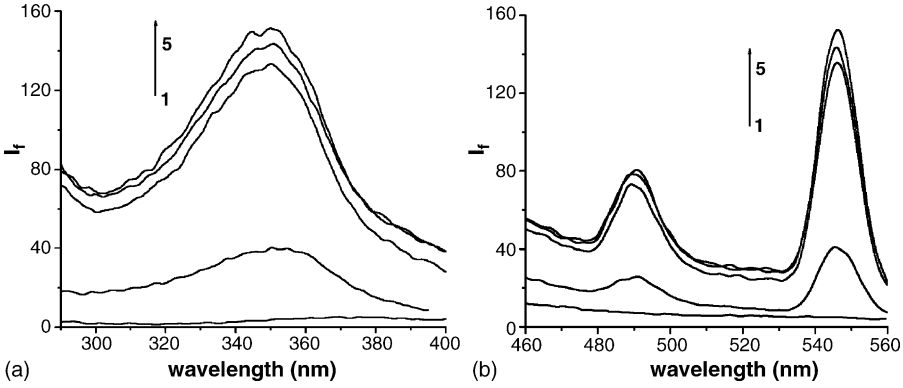

Fig. 1. Excitation and emission spectra: (a) excitation spectra and (b) emission spectra. (1) Tb–BA–Tris, (2) Tb–BA–SDBS–Tris, (3) Tb–BA–SDBS–EA–Tris, (4) Tb–BA–SDBS–HSA–Tris, (5) Tb–BA–SDBS–BSA–Tris. Conditions: Tb^{3+}, 1.2×10^{-5} M; BA, 1.0×10^{-4} M; SDBS, 1.0×10^{-4} M; Tris–HCl, 5.0×10^{-3} M (pH 8.27); BSA, 5.0×10^{-6} g mL^{-1}; HSA, 5.0×10^{-6} g mL^{-1}; EA, 5.0×10^{-6} g mL^{-1}.

on the ΔI_f of this system are also tested. The results indicate that among the buffers: Tris–HCl, $Na_2B_4O_7$–HCl, NH_4Cl–NH_3 and KH_2PO_4–NaOH, Tris–HCl is the most suitable buffer. Further research indicates that the optimal volume of Tris–HCl is 1.0 mL.

3.3. Effect of Tb^{3+} concentration

The effect of the concentration of Tb^{3+} is tested at pH 8.27, with the concentrations of BA 1.0×10^{-4} M, SDBS 1.0×10^{-4} M, BSA 5.0×10^{-6} g mL^{-1}. Experiments indicate that the enhancement of the fluorescence intensity of the system reaches a maximum when Tb^{3+} concentration is in range of 1.1×10^{-5} to 1.5×10^{-5} M. So 1.2×10^{-5} M of Tb^{3+} is chosen in the research.

3.4. Effect of BA concentration

The enhanced fluorescence intensity ΔI_f is further studied as a function of BA concentration at pH 8.27 when the concentrations of Tb^{3+}, SDBS and BSA are located at 1.2×10^{-5} M, 1.0×10^{-4} M and 5.0×10^{-6} g mL^{-1}, respectively. The results show that the fluorescence intensity enhancement of Tb–BA–SDBS–BSA system reaches a maximum when the concentration of BA is 1.0×10^{-4} M. So 1.0×10^{-4} M of BA is chosen in the research. Previous investigation [23] indicates that the composition molar ratio for BA and lanthanide ions is 3:1, but from our experiment data it can be seen that when the ratio is larger than 3:1, the ΔI_f still increase with the increase of BA concentration. It is obvious that BA is excessive for the formation of Tb–BA complex in this system.

3.5. Effect of surfactants

The effects of different surfactants are studied. The results indicate that the cation surfacants, such as cetyltrimethyl ammonium bromide (CTAB), cetylpyridine bromide (CPB), and nonion surfactant–Triton X-100 have little effect on the system, whereas anion surfactants SDBS, sodium dodecyl sulfonate (SDS), sodium laurel sulfate (SLS) could enhance fluorescence of the system. The increasing extent (ΔI_f, %) of them are 100, 2, 45, respectively. Comparing the ΔI_f of the systems in the presence of anion surfactants, the biggest ΔI_f occured in Tb–BA–SDBS–BSA system. Our previous investigations indicate that BSA can bind with anionic surfactant SDBS through "aromatic ring stacking" and form negative micelle-like cluster complexes with the "necklace and head" structure [26]. Among these three anionic surfactants, SDBS has much more enhancement effect than the other two surfactants, which may be as a result of the SDBS's aromatic ring. So SDBS is used in the system.

The effect of SDBS concentration is also tested as shown in Fig. 3a. When the concentration of SDBS is less than 1.0×10^{-4} M, the value of ΔI_f increases with the increase of SDBS concentration. After 1.0×10^{-4} M, the increasing extent of I_0 is larger than that of I_f, namely the ΔI_f decreases with the additon of SDBS. The results indicate that the fluorescence intensity enhancement of the system reaches the maximum when the concentration of SDBS is 1.0×10^{-4} M. So 1.0×10^{-4} M is chosen in the research.

From Fig. 3b, it can be seen that the surface tension of this system first decreases sharply with the increase of SDBS concentration, soon gets to a minimum and then remains constant. The concentration 4.0×10^{-4} M may be regarded as the apparent critical micelle concentration (CMC) of SDBS in this system. So it can be seen that the selected concentration of SDBS is below its CMC, which shows that SDBS exists as the pre-micelle or single molecule in the studied system.

3.6. Adding sequence and signal stability

Adding sequence and signal stability are investigated. The experiments indicate that the addition sequence of reagents affects the fluorescence intensity of the system, and the order of Tb^{3+}, BA, SDBS, BSA and Tris–HCl is the best. Under the optimum condition, the effect of time on the fluorescence intensity is studied. The results show that the fluorescence intensity reaches a maximum at 20 min after all the reagents added, and it remains stable for over 24 h. Therefore, this system exhibits good stability.

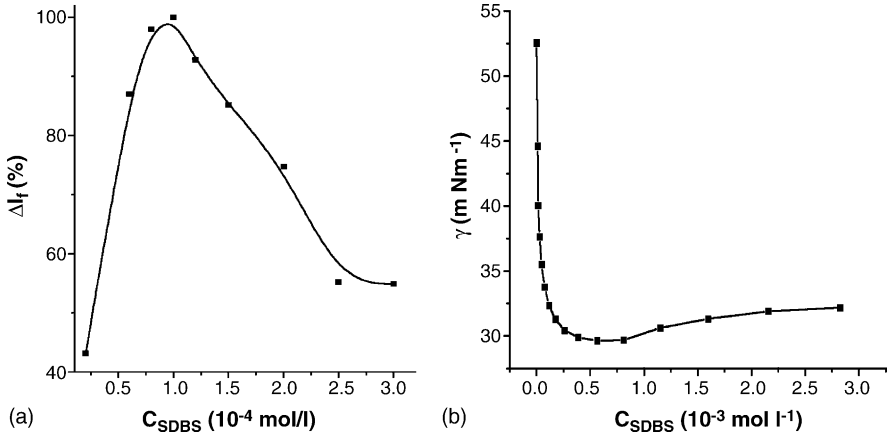

Fig. 3. (a) Effect of SDBS concentration on ΔI_f. (b) Surface tension of SDBS in the Tb^{3+}–BA–SDBS–BSA system. Conditions: Tb^{3+}, 1.2×10^{-5} M; BA, 1.0×10^{-4} M; Tris–HCl, 5.0×10^{-3} M (pH 8.27); BSA, 5.0×10^{-6} g mL^{-1}.

Table 1
Interference from foreign substances

Foreign substance	Concentration coexisting ($\times 10^{-6}$ M)	Change of I_f (%)
K^+, Cl^-	56	−4.5
Ca^{2+}, Cl^-	46	−4.1
NH_4^+, Cl^-	63	−4.5
Mn^{2+}, SO_4^{2-}	23	−5.1
Zn^{2+}, Cl^-	12	−4.7
Ba^{2+}, Cl^-	12	−4.6
Al^{3+}, NO_3^-	1.0	−5.3
Na^+, Cl^-	16	−4.7
Mg^{2+}, SO_4^{2-}	30	−4.5
Al^{3+}, Cl^-	0.9	−5.6
Na^+, CO_3^{2-}	21	−4.0
Fe^{3+}, Cl^-	0.02	−3.6
Fe^{3+}, SO_4^{2-}	0.04	−3.0
L-Asp	15	−4.5
DL-Glu	30	−2.3
L-His	30	−3.8
DL-Lys	60	−4.6
L-Phe	9	−5.2
Pro	22	−4.8
Cys	6.0	−4.6
DL-Thr	11	−5.0
ctDNA	0.70 g mL^{-1}	−4.3
fsDNA	0.80 g mL^{-1}	−5.2
yRNA	0.9 g mL^{-1}	−4.8

Conditions: Tb^{3+}, 1.2×10^{-5} M; BA, 1.0×10^{-4} M; SDBS, 1.0×10^{-4} M; Tris–HCl, 5.0×10^{-3} M (pH 8.27); BSA, 5.0×10^{-7} g mL^{-1}.

Table 2
Analytical parameters of this method

Proteins	Linear range (μg mL^{-1})	Linear regression equation, ΔI_f (μg mL^{-1})	r[a]	Limit of detection (ng mL^{-1})
BSA	0.010–6.0	$2.057 + 0.250C$	0.998	3.9
HSA	0.010–6.0	$4.551 + 0.202C$	0.993	4.0
EA	0.030–6.0	$4.275 + 0.209C$	0.999	8.5

[a] Correlation coefficient.

3.7. Effect of foreign substances

The interference of foreign substances is tested and shown in Table 1. It is found that most of amino acids and metal ions except Fe^{3+} and Al^{3+} has not or has little effect on the determination of BSA under the permission of ±5% relative error. As for Fe^{3+} and Al^{3+}, they have strong combination ability with O atom, so they can compete with Tb^{3+} to combine with BA, which will decreases the intensity of the system.

4. Analytical applications

4.1. The calibration graph and detection limits

Under the optimum condition defined, the calibration graphs for BSA, EA and HSA are obtained and shown in Table 2. It can be seen that there is a linear relationship between the fluorescence intensity of the system and the concentration of proteins in the range of 1.0×10^{-8} to 6.0×10^{-6} g mL^{-1} for BSA, 3.0×10^{-8} to 6.0×10^{-6} g mL^{-1} for EA and 1.0×10^{-8} to 5.0×10^{-6} g mL^{-1} for HSA, and their detection limits (S/N) are 3.9×10^{-9}, 8.5×10^{-9} and 4.0×10^{-9} g mL^{-1}, respectively.

4.2. Recovery test and determination of actual sample

The standard addition method is used for both recovery test and the determination of HSA in the people serum, EA in the egg serum. And the results are compared with the UV spectrophotometric method as shown in Table 3. From the results of both this method and spectrophotometric method and the recovery ratio (97–102% for HSA, 96–104% for EA), it can be seen that the precision and accuracy of this method are satisfactory.

5. Interaction mechanism of the system

5.1. Formation of Tb^{3+}–BA–SDBS–BSA complex

From Fig. 1, it can be seen that when SDBS and protein are together added to Tb^{3+}–BA system, the fluorescence of the system can be greatly enhanced, which indicates that there is the interaction among Tb^{3+}–BA, SDBS and protein.

The resonance light scattering (RLS) spectra of the systems are shown in Fig. 4. When BSA is added to Tb^{3+}–BA–SDBS system, the resonance light scattering intensities of the system is greatly enhanced, which indicates that there are interaction among them, and forms a large Tb^{3+}–BA–SDBS–BSA complex in this system.

In addition, when Tb^{3+} is added into BSA and SDBS systems, the resonance light scattering intensities of the two systems are enhanced, which indicates that Tb–BSA and Tb–SDBS complexes are formed through electrostatic force. The RLS of Tb^{3+}–SDBS–BSA is much larger than those of Tb^{3+}–SDBS and Tb^{3+}–BSA, which indicates that Tb^{3+}, SDBS, BSA can form a large complex by electrostatic force, just as our previous investigation [18]. That is also the reason why anionic surfactants have enhancement effect on the system compared with cationic and non-ionic surfactants. It can also be seen that the RLS shapes of the systems with or without BA are obviously

Table 3
The results of samples determination

Samples	Methods	Concentration (mg mL^{-1})	Average (mg mL^{-1})	R.S.D. (%)
EA	Proposed method	79.9, 78.4, 78.7, 80.2, 79.5	79.3	0.77
	UV method	81.6, 80.0, 80.9, 82.5, 81.8	81.4	0.95
HSA	Proposed method	73.2, 74.8, 72.8, 73.8, 73.4	73.6	0.76
	UV method	75.2, 76.7, 75.9, 76.0, 75.8	75.9	0.54

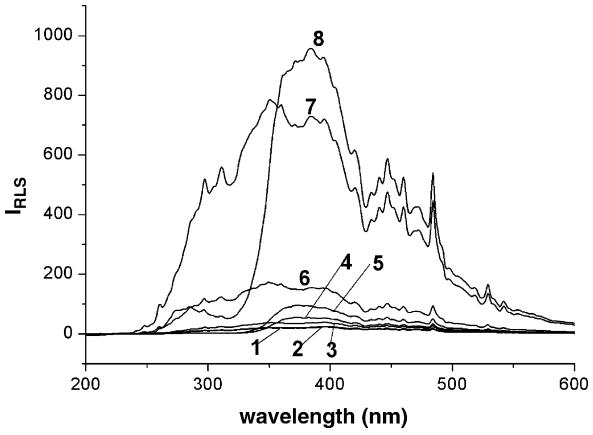

Fig. 4. Resonance light scattering spectra of the system. (1) SDBS, (2) BSA, (3) Tb^{3+}–BSA, (4) Tb^{3+}–BA, (5) Tb^{3+}–BA–SDBS, (6) Tb^{3+}–SDBS, (7) Tb^{3+}–SDBS–BSA, (8) Tb^{3+}–BA–SDBS–BSA. Conditions: Tb^{3+}, 1.2×10^{-5} M; BA, 1.0×10^{-4} M; SDBS, 1.0×10^{-4} M; Tris–HCl, 5.0×10^{-3} M (pH 8.27); BSA, 5.0×10^{-6} g mL^{-1}.

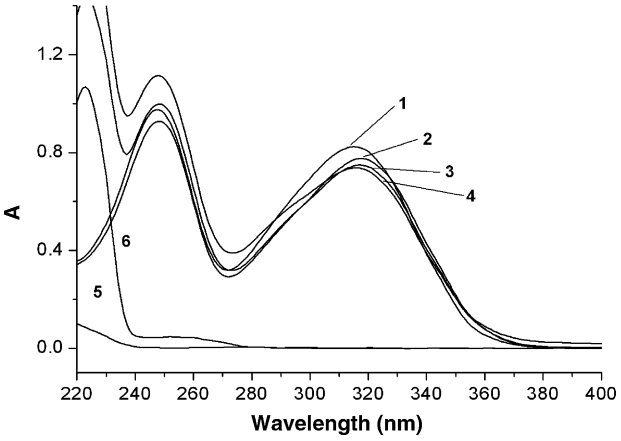

Fig. 6. Absorption spectra. (1) BA, (2) Tb^{3+}–BA, (3) Tb^{3+}–BA–SDBS, (4) Tb^{3+}–BA–SDBS–BSA, (5) BSA, (6) SDBS. Conditions: Tb^{3+}, 1.2×10^{-5} M; BA, 1.0×10^{-4} M; SDBS, 1.0×10^{-4} M; Tris–HCl, 5.0×10^{-3} M (pH 8.27); BSA, 5.0×10^{-6} g mL^{-1}.

different, which indicates the BA participate in the formation of the Tb^{3+}–BA–SDBS–BSA complex. So we deduced that the Tb^{3+} of Tb–BA complex could bind with SDBS and BSA through electrostatic force and then formed the aggregate.

It is well known that among all the amino acid residues in proteins, tryptophan and tyrosine residues can contribute to fluorescence spectra. The synchronous fluorescence spectra of BSA and Tb^{3+}–BA–SDBS–BSA systems are shown in Fig. 5. At small wavelength interval ($\Delta\lambda = 20$ nm), the synchronous fluorescence spectra are characteristic of tyrosine, whereas at large wavelength interval ($\Delta\lambda = 60$ nm), the spectra are attributed to that of tryptophan [27,28]. From Fig. 5, it can be seen that when Tb^{3+}, BA, SDBS, are added into BSA, the fluorescence of tryptophan and tyrosine residues of protein are obviously quenched. This indicates that there is the interaction between Tb^{3+}–BA–SDBS and both tryptophan and tyrosine residues in BSA.

5.2. *The fluorescence enhancement of Tb^{3+}–BA–SDBS–BSA system*

From Fig. 1 and Fig. 6, it can be seen that the fluorescence and absorption spectra of Tb^{3+}–BA–SDBS–BSA system are similar to those of Tb^{3+}–BA–SDBS (BA, Tb^{3+}–BA, Tb^{3+}–BA–SDBS) systems and the Tb^{3+}–BA–SDBS–BSA system has excitation band of 300–380 nm. From Fig. 6, it can be seen that in above wavelength range, BA has obvious absorption, whereas SDBS and BSA have not. The facts mentioned above indicate that the fluorescence of Tb^{3+}–BA–SDBS–BSA system is from the absorption of BA, rather than BSA or SDBS.

From Fig. 1, we can see that the fluorescence intensity of Tb^{3+}–BA can be greatly enhanced by proteins in the presence of SDBS. The coordination number of Tb^{3+} in its complex is 6–8. Previous investigation indicates that the compositions molar ratio for BA and lanthanide ions is 3:1. Therefore, the coordination number of Tb^{3+} cannot be satisfied, then Tb^{3+} will coordinate with H_2O in solution and form the Tb^{3+}–BA–$(H_2O)_n$ complex. It is considered that at pH 8.27, SDBS and BSA is negatively charged, then they can easily combine with Tb^{3+} in Tb^{3+}–BA–$(H_2O)_n$ complex through electrostatic force and the H_2O molecules in the complex are released. Thus the non-radiative energy loss through O–H vibration of H_2O molecules in Tb^{3+} complex will be decreased. In addition, SDBS and BSA provide a hydrophobic micro-environment for the Tb^{3+} complex, which can be proved by the polarity of the system.

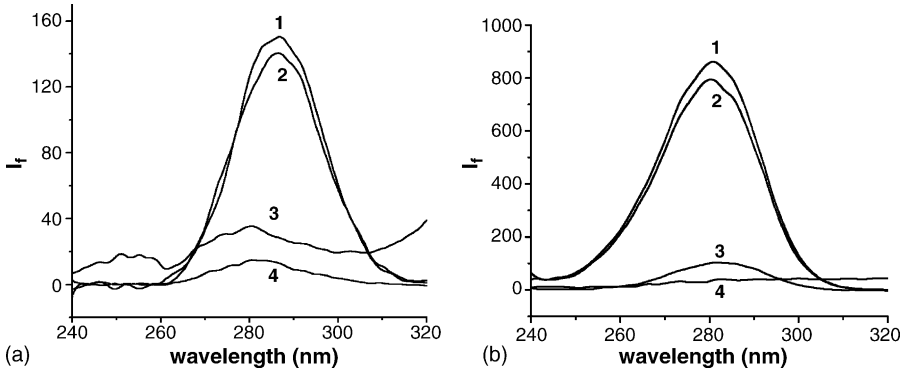

Fig. 5. Synchronous fluorescence spectra of BSA in different systems: (a) $\Delta\lambda = 20$ nm, (b) $\Delta\lambda = 60$ nm. (1) BSA, (2) Tb^{3+}–BSA, (3) Tb^{3+}–BA–BSA, (4) Tb^{3+}–BA–SDBS–BSA. Conditions: Tb^{3+}, 1.2×10^{-5} M; BA, 1.0×10^{-4} M; SDBS, 1.0×10^{-4} M; Tris–HCl, 5.0×10^{-3} M (pH 8.27); BSA, 5.0×10^{-6} g mL^{-1}.

Table 4
Comparison of the polarity of different systems

Medium	I_1/I_3
H_2O	1.725
BSA	1.585
SDBS	1.681
SDBS–BSA	1.465

The ratio of the first to the third fluorescence bands of pyrene monomer (I_1/I_3) is a well established parameter which reflects the polarity changes of a system experienced by the pyrene probe [29,30]. A low value reflects a lower polar environment than a high value. From Table 4, it is seen that the BSA–SDBS system provides a minimum polarity for Tb^{3+}–BA complex in comparison to SDBS and BSA, and the hydrophobic environment of BSA–SDBS system can also prevent the collision between complex and water and decrease the energy loss of the Tb^{3+}–BA–SDBS–BSA system. Therefore the fluorescence intensity of Tb^{3+}–BA complex be greatly enhanced.

In addition, we noticed that BA in this system is excessive for the formation of Tb–BA complex (described in Section 3.4). Experiment data shows that when the ratio is larger than 3:1, the ΔI_f still increase with the increase of BA concentration until the ratio is about 8:1. So we deduce that the abundant BA molecules are also solubilized into BSA–SDBS cluster by hydrophobic force, which can shorten the distance between the Tb^{3+}–BA complex and the excessive BA molecules. So the Tb^{3+} in the Tb^{3+}–BA can accept the energy from the excessive BA molecules through intermolecular energy transfer, which also enhance the fluorescence intensity of the system.

Based on the above facts, we consider that it is the synergic effect of hydrophobic microenvironment of BSA–SDBS and the intermolecular energy transfer for the fluorescence enhancement in this system.

6. Conclusions

The fluorescence of Tb^{3+}–BA can be enhanced greatly by the addition of protein in the presence of SDBS. Based on this, a novel fluorimetric method has been developed for the determination of proteins. This method is used for the determination of the HSA in human blood and the EA in egg and their results are satisfactory. The proposed method has high sensitivity, stability and reproducibility.

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