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Fluorescence enhancement effect for the determination of proteins with morin–Al^{3+}–cetyltrimethylammonium bromide

Fei Wang, Jinghe Yang *, Xia Wu, Changxia Sun, Shufang Liu, Changying Guo, Feng Wang

Key Laboratory of Colloid and Interface Chemistry (Shandong University), Ministry of Education, School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, PR China

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Abstract

It is found that the fluorescence intensity of morin–Al^{3+} complex can be greatly enhanced by proteins in the presence of cetyltrimethylammonium bromide (CTAB). It is considered that protein and CTAB provide a hydrophobic environment with low polarity and large viscosity, resulting in the fluorescence enhancement of morin–Al^{3+} complex. The experiments indicate that under optimum conditions, the enhanced intensity of fluorescence is in proportion to the concentration of proteins (such as bovine serum albumin (BSA), human serum albumin (HSA) and egg albumin (EA)) in the wide range, and their detection limits (S/N = 3) are 2.6×10^{-9}, 1.4×10^{-8} and 1.6×10^{-8} g ml^{-1}, respectively. This method has satisfactorily been used for the determination of protein in actual sample. In comparison with most of fluorimetric methods reported, this method is quick and simple, and has high sensitivity, wide linear range and good stability.

Keywords: Fluorescence enhancement; Morin–Al^{3+} complex; Protein; Determination

1. Introduction

The quantitative determination of proteins is very important in a number of areas, such as biochemistry, immunodiagnostics and biotechnology. Unfortunately, the traditional methods for the determination of proteins have some disadvantages. For example, the Lowry [1] assay, based on a colored complex formed with tyrosine or tryptophan, can be used only for protein concentrations >10 mg ml^{-1}. The Bradford [2] assay, based on the binding of Coomassie blue to protein, has the drawback of contaminating vessels. The silver staining method [3] is complicated as multiple steps are involved. Furthermore, current methods have a limited sensitivity because they are based on absorption measurements [4,5]. To overcome these limitations, the fluorimetric methods using covalent and noncovalent probes have been widely used for the investigation and determination of proteins, because they possess high sensitivity and selectivity, and provide more information, and their advances for the determination of protein are summarized in some reviews [6–8]. The covalent fluorescent probes suitable for labeling the N-terminus or another functioned group of proteins have been successfully used because of their high sensitivity. However, many problems arising from inefficient chemistry, multiple derivatives and reaction conditions must be overcome through further studies. In comparison with covalent fluorescent labeling, noncovalent fluorescent labeling is very simple, fast and cheap. Most of the compounds serving as noncovalent fluorescent probes of protein are anionic dyes and their detection limits are in the range of 0.1–0.01 μg ml^{-1}. But there are few complexes as noncovalent fluorescent probe of protein [9,10]. In this study, the morin–Al^{3+} complex is acted as a noncovalent fluorescent probe for the study and determination of protein.

The importance of the polyhydroxyflavones as antitumor drugs has been widely recognized [11,12]. Morin (3,5,7,2′,4′-pentahydroxyflavone) is one of the polyhydroxyflavones and most frequently used as analytical reagent.

* Corresponding author. Tel.: +86 5318365459; fax: +86 5318564464.
E-mail address: yjh@sdu.edu.cn (J. Yang).

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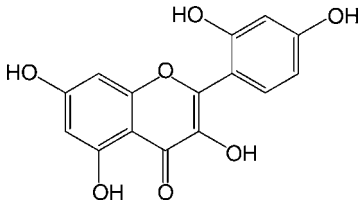
And it also has the conjugated and planar aromatic structure as is shown in the following:



It is well known that morin–Al^{3+} can emit intrinsic fluorescence of morin and is used for the determination of Al^{3+}. In this paper, it is found that the fluorescence intensity of morin–Al^{3+} complex is greatly enhanced by proteins (such as BSA, HSA and EA). The experimental results indicate that the enhanced fluorescence intensity is proportional to the concentration of proteins over a wide range and their detection limits are at nanogram level. In comparison with most of fluorescent probes [9,10,13–21], this probe has the advantages of high sensitivity, short reaction time and good stability for the determination of proteins. In addition, the interaction mechanism of system is also studied.

2. Materials and methods

2.1. Materials

Unless otherwise noted, all reagents and solvents used in this study were analytical grade. Proteins (BSA, HSA and EA) were 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 deionized water, stored at 0–4 °C. The morin was acquired from Beijing Chemical Company. Stock standard solution (1.0×10^{-3}mol l^{-1}) of morin was made by dissolving 0.0338 g of morin in ethanol and diluting to 100 ml with deionized water. A stock solution of $AlCl_3$ purchased from Chemical Co. of China, Beijing was prepared (1.0×10^{-3} mol l^{-1}) with deionized water. A 10% hexamethylenetetramine (HMTA)–HCl buffer solution was prepared by dissolving 10.0 g of HMTA in 90 ml deionized water, and adjusting the pH to 7.3 with HCl using an acidity meter. A stock solution of CTAB from Chemical Co. of China, Shanghai was prepared (1.0×10^{-3} mol l^{-1}) with deionized water. And a stock solution of pyrene from Sigma Chemical Co. was prepared (1.0×10^{-4} mol l^{-1}) with methanol.

2.2. Apparatus and methods

Normal fluorescence measurements were recorded with a LS-55 spectrofluorimeter (PE) in a 1 cm quartz cuvette and the excitation and emission slits were, respectively, 10 and 6 nm, with a scan speed of 500 nm/min. Fluorescence polarization measurements were made using a FLWINLab software fluorescence polarization analyzer.

The measurements of the actual samples with UV method were performed with a UV-240 spectrophotometer (Shimadzu) equipped with 1 cm pathlength quartz cells. Protein chemists typically use the near UV (aromatic region around 280 nm) to quantify protein concentration. So the standard addition method was used for measuring the absorbance of protein at 280 nm and the concentration of protein was calculated.

The surface tension was measured on Processor Tensiometer-K12 (Krüss Corp) with the precise degree of the measurement 0.01 mN m^{-1} by the Wilhelmyplate.

All pH measurements were made with a Delta 320-S acidity meter (Mettler Toledo, Shanghai).

2.3. Procedure

To a 25 ml test tube, solutions were added in the following order: HMTA–HCl, morin, BSA, Al^{3+} and CTAB and the mixture was diluted to 10 ml with water. The fluorescence intensity at 525 nm with an excitation wavelength of 425 nm was recorded and applied to the quantitative analysis of proteins.

3. Result and discussion

3.1. Fluorescence spectra

The excitation and emission spectra of morin (1), morin–Al^{3+} (2), morin–Al^{3+}–CTAB (3), morin–Al^{3+}–BSA (4), morin–Al^{3+}–CTAB–EA (5), morin–Al^{3+}–CTAB–HSA (6), morin–Al^{3+}–CTAB–BSA (7) systems are shown in Fig. 1. It can be seen that the fluorescence of morin–Al^{3+} is weak. However, the fluorescence intensity of the system is greatly enhanced when proteins (such as BSA, HSA and EA) are added to the morin–Al^{3+} system, especially in the presence of CTAB. This is a newly found fluorescence system. The excitation peak is at 425 nm, and the emission peak is at 525 nm, which is the characteristic fluorescence peak of morin and corresponds to π, π^* state. The phenomenon indicates that there are interactions among protein, morin–Al^{3+} and CTAB.

3.2. Effect of pH and the choice of buffer solution

The effect of solution pH on the fluorescence intensity of the system is shown in Fig. 2. It is seen that the solution pH can not only influence the intensity (I_f) of morin–Al^{3+}–CTAB–proteins system, but also influence the intensity (I_f^0) of morin–Al^{3+}–CTAB system, whereas the less effect on the ratio of I_f and I_f^0 (I_f/I_f^0) is obtained when pH is in the range of 7.3–7.6. The effect of different buffers (such as HMTA–HCl, Tris–HCl, BR, NaH_2PO_4–citric acid and NaH_2PO_4–Na_2HPO_4) on the I_f/I_f^0 of this system is also tested, and the results indicate that the buffer also has a large

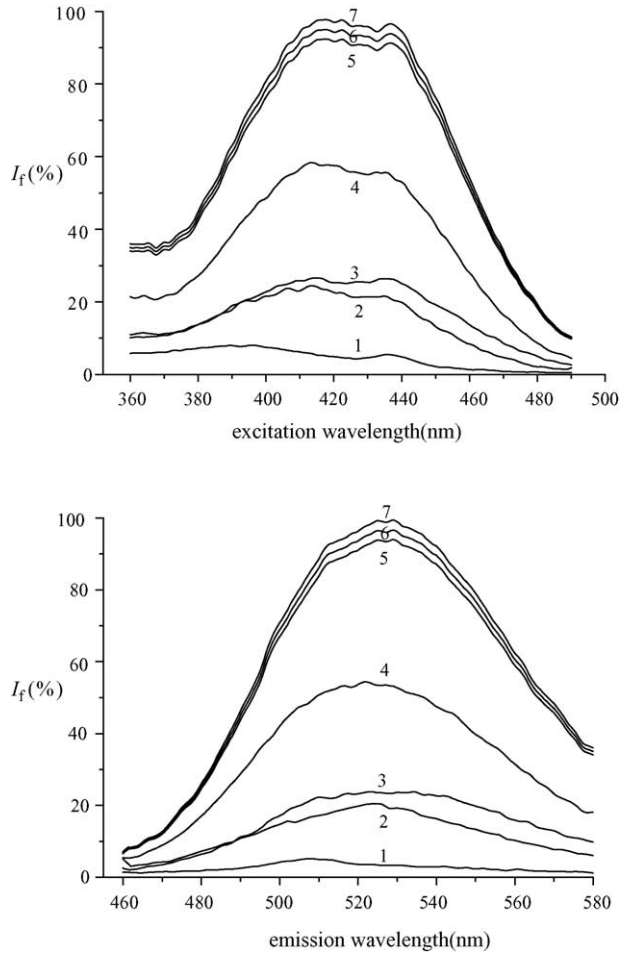


Fig. 1. Excitation and emission spectra: (1) morin; (2) morin–Al^{3+}; (3) morin–Al^{3+}–CTAB; (4) morin–Al^{3+}–BSA; (5) morin–Al^{3+}–CTAB–EA; (6) morin–Al^{3+}–CTAB–HSA; (7) morin–Al^{3+}–CTAB–BSA. Conditions—morin: 1.0×10^{-6} mol l^{-1}; CTAB: 1.0×10^{-4} mol l^{-1}; Al^{3+}: 1.76×10^{-5} mol l^{-1}; HMTA: 0.8% (pH 7.3); BSA: 5.0 μg ml^{-1}; HSA: 5.0 μg ml^{-1}; EA: 5.0 μg ml^{-1}.

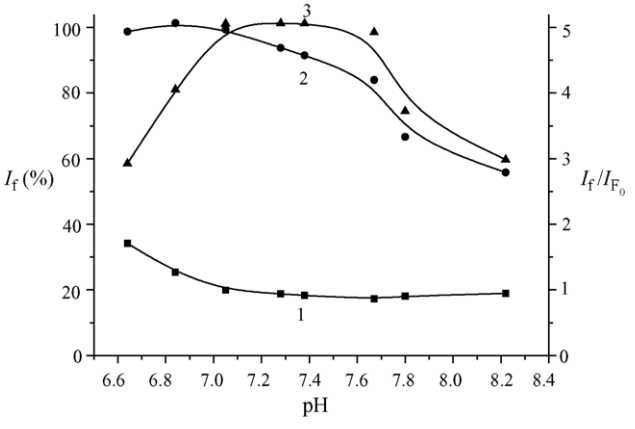


Fig. 2. Effect of pH. (1) I_f^0 (morin–Al^{3+}–CTAB); (2) I_f (morin–Al^{3+}–CTAB–BSA); (3) I_f/I_f^0. Conditions—morin: 1.0×10^{-6} mol l^{-1}; CTAB: 1.0×10^{-4} mol l^{-1}; Al^{3+}: 1.76×10^{-5} mol l^{-1}; HMTA: 0.8%; BSA: 5.0 μg ml^{-1}.

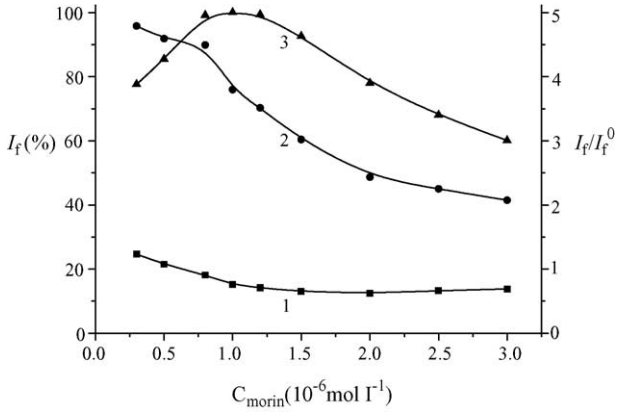


Fig. 3. Effect of morin concentration. (1) I_f^0 (morin–Al^{3+}–CTAB); (2) I_f (morin–Al^{3+}–CTAB–BSA); (3) I_f/I_f^0. Conditions—CTAB: 1.0×10^{-4} mol l^{-1}; Al^{3+}: 1.76×10^{-5} mol l^{-1}; HMTA: 0.8% (pH 7.3); BSA: 5.0 μg ml^{-1}.

effect on the I_f/I_f^0 of the system, and 0.8 ml HMTA–HCl is the most suitable buffer.

In addition, influence of ionic strength on the fluorescence intensity of this system is tested. Experiments indicate that the fluorescence intensity of the system has not obvious change when NaCl (<0.01 mol l^{-1}) is added into morin–Al^{3+}–CTAB–BSA system, and the effect is less than 10% even if the concentration of NaCl is higher 0.01 mol l^{-1}. Here, the influence of ionic strength on the reaction is ignored.

3.3. Effect of morin concentration

The effect of the concentration of morin is tested as shown in Fig. 3. It can be seen that morin concentration has large effect on I_f, little effect on I_f^0. But the I_f/I_f^0 of the system reaches the maximum and remains constant when the concentration of morin is 8.0×10^{-7} to 1.2×10^{-6} mol l^{-1}. So 1.0×10^{-6} mol l^{-1} is chosen in the research.

3.4. Effect of Al^{3+} concentration

The influence of Al^{3+} concentration on the fluorescence intensity system is shown in Fig. 4. It can be seen that Al^{3+} concentration has obvious effect on both I_f and I_f^0, but the I_f/I_f^0 of system has a maximum when the concentration of Al^{3+} is 1.1×10^{-5} to 2.2×10^{-5} mol l^{-1}. So 1.76×10^{-5} mol l^{-1} is chosen in the research.

3.5. Effect of surfactants and the choice of surfactant solution

The effect of surfactants (such as SDS, SLS, SDBS, CPB, OP and CTAB) on the system is studied. The experimental results show that there is not obvious rule between the fluorescence intensity and both the size and charges of surfactants, but the surfactants with aromatic ring (such as SDBS, CPB

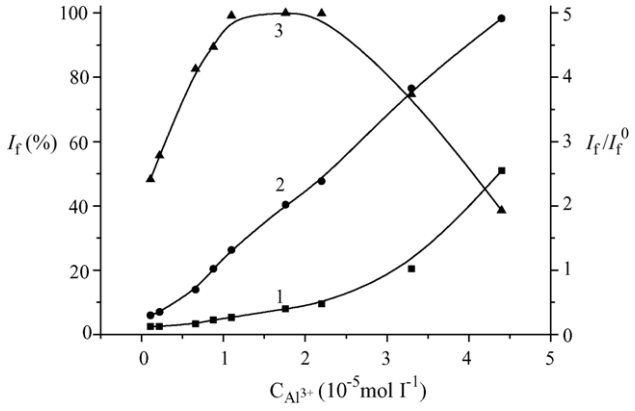


Fig. 4. Effect of Al^{3+} concentration. (1) I_f^0 (morin–Al^{3+}–CTAB); (2) I_f (morin–Al^{3+}–CTAB–BSA); (3) I_f/I_f^0. Conditions—morin: 1.0×10^{-6} mol l^{-1}; CTAB: 1.0×10^{-4} mol l^{-1}; BSA: 5.0 μg ml^{-1}; HMTA: 0.8% pH 7.3.

and OP) can decrease the intensity of the system, whereas the surfactants without aromatic ring have the enhancement effect, of them, cationic surfactant CTAB is the best. The effect of different concentrations of CTAB on the I_f/I_f^0 of this system is also tested in Fig. 5(a), and the results indicate that the I_f/I_f^0 of the system reaches the maximum and remains constant when the concentration of CTAB is 8.0×10^{-5} to 2.0×10^{-4} mol l^{-1}. So 1.0×10^{-4} mol l^{-1} is chosen in the research.

From Fig. 5(b), it can be seen that the surface tension of this system first decreases sharply with the increase of CTAB concentration, soon gets to a minimum and then remains constant. The concentration 3.9×10^{-4}mol l^{-1} may be regarded as the apparent critical micelle concentration (CMC) of CTAB in this system. So it can be seen that the selected concentration of CTAB is below its CMC, which shows that CTAB exists in the pre-micelle or single molecule in the studied system.

3.6. The addition order and stability of this system

Experiments show that the addition order of the reagents has an effect on the fluorescence intensity. On the basis of these results, we select HMTA–HCl, morin, BSA, Al^{3+} and CTAB as the best order for this assay. Under the optimum condition, the effect of time on the fluorescence intensity is studied. The result shows that the fluorescence intensity immediately reaches a maximum after all the reagents are added and remains stable for over 6 h. Therefore, this system exhibits rapid reaction and good stability.

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 Fe^{2+} have not or have little effect on the determination of BSA, under the permission of ±5% relative error.

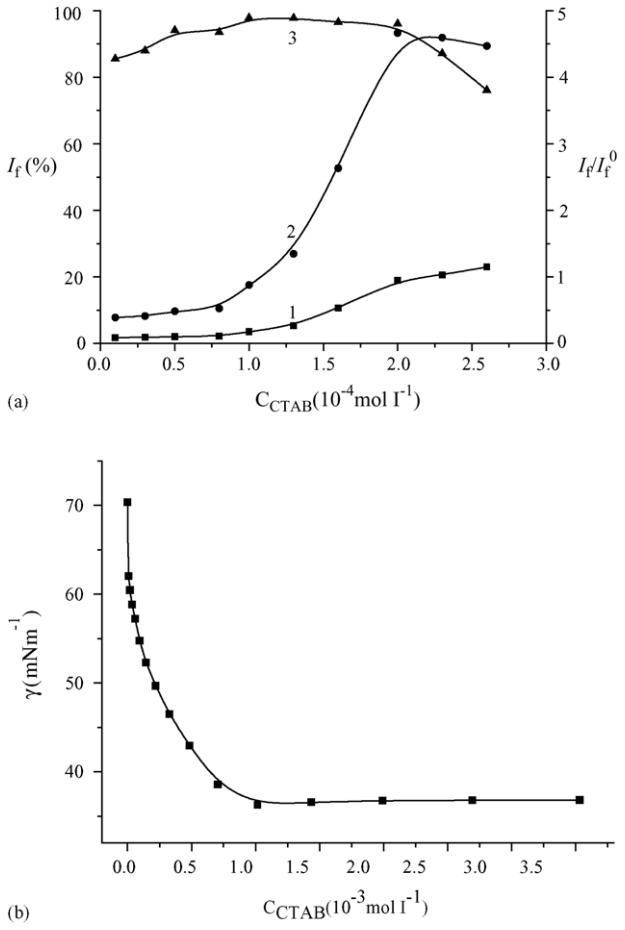


Fig. 5. (a) Effect of CTAB concentration, (b) surface tension of CTAB in the morin–Al^{3+}–CTAB–BSA system. (1) I_f^0 (morin–Al^{3+}–CTAB); (2) I_f (morin–Al^{3+}–CTAB–BSA); (3) I_f/I_f^0. Conditions—morin: 1.0×10^{-6} mol l^{-1}; Al^{3+}: 1.76×10^{-5} mol l^{-1}; HMTA: 0.8% (pH 7.3); BSA: 5.0 μg ml^{-1}.

4. Analytical applications

4.1. The calibration graph and detection limits

Under the optimum conditions 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 fluorescent enhancement of the system and the concentration in the range of 5.0×10^{-9} to 2.0×10^{-5} g ml^{-1} for BSA, 4.0×10^{-8} to 1.3×10^{-5} g ml^{-1} for EA and 2.0×10^{-8} to 1.5×10^{-5} g ml^{-1} for HSA, and their detection limits are 2.6×10^{-9}, 1.6×10^{-8} and 1.4×10^{-8} g ml^{-1}, respectively.

A comparison between this method with other fluorimetric methods for protein in sensitivity and linear range is summarized up in Table 3. It can be seen that this method has a higher sensitivity and a wider linear range than most of other

Table 1
Interference from foreign substances

Foreign substance	Concentration coexisting ($\times 10^{-6}$ mol l^{-1})	Change of I_f (%)
K^+, Cl^-	12	−4.2
Ca^{2+}, Cl^-	35	−5.0
NH_4^+, Cl^-	4.0	−4.7
Mn^{2+}, SO_4^{2-}	2.0	−3.6
Na^+, SO_4^{2-}	10	−4.4
Zn^{2+}, Cl^-	3.0	−4.8
Ba^{2+}, Cl^-	6.0	−4.2
Al^{3+}, NO_3^-	1.0	4.7
Na^+, Cl^-	5.0	−4.9
Mg^{2+}, SO_4^{2-}	6.0	−4.2
Al^{3+}, Cl^-	2.0	−4.7
Na^+, CO_3^{2-}	10	−5.2
Fe^{3+}, Cl^-	0.01	5.0
Fe^{2+}, SO_4^{2-}	0.01	−4.2
L-Asp	10	−5.0
DL-Glu	33	−5.1
L-His	15	−4.7
DL-Lys	8.0	−4.1
L-Phe	6.0	−4.7
Pro	13	−5.1
Cys	2.0	−5.2
DL-Thr	16	−4.5
ctDNA	120 μg ml^{-1}	−4.4
fsDNA	100 μg ml^{-1}	−4.1
yRNA	60 μg ml^{-1}	−4.7

Conditions—morin: 1.0×10^{-6} mol l^{-1}; CTAB: 1.0×10^{-4} mol l^{-1}; Al^{3+}: 1.76×10^{-5} mol l^{-1}; HMTA: 0.8% (pH = 7.3); BSA: 0.1 μg ml^{-1}.

Table 2
Analytical parameters of this method

Proteins	Linear range (μg ml^{-1})	Linear regression equation (μg ml^{-1})	r[a]	Limit of detection (ng ml^{-1})
BSA	0.0050–20.0	$I_f = 30.551 + 14.189C$	0.995	2.6
HAS	0.020–15.0	$I_f = 28.917 + 13.598C$	0.995	14
EA	0.040–13.0	$I_f = 32.886 + 11.906C$	0.999	16

[a] Correlation coefficient.

Table 4
The results of samples determination

Samples	Methods	Concentration (mg ml^{-1})	Average (mg ml^{-1})	R.S.D. (%)
EA	Proposed method	70.2, 69.7, 70.8, 71.5, 69.5	70.3	0.82
	UV method	71.2, 70.3, 71.8, 69.7, 70.6	70.7	0.83
HAS	Proposed method	58.7, 57.9, 58.1, 58.5, 59.3	58.5	0.55
	UV method	58.7, 59.8, 57.8, 58.7, 59.2	58.9	0.74

fluorimetric methods. In addition, this method is quick and has good stability.

4.2. Determination of actual sample

The standard addition method is used for the determination of HSA in the people serum and of EA in the egg serum, compared with the UV spectrophotometric method. The results are shown in Table 4, and it can be seen that the accuracy and precision of the method are satisfactory.

5. Interaction mechanism of the system

5.1. Formation of morin–Al^{3+}–CTAB–BSA complex

The resonance light scattering (RLS) spectra of morin (1), morin–Al^{3+}–CTAB (2), morin–Al^{3+} (3), morin–Al^{3+}–BSA (4), morin–Al^{3+}–CTAB–BSA (5) systems are shown in Fig. 6. When Al^{3+}, BSA and CTAB are added to morin system, the resonance light scattering intensities of the systems are enhanced. Especially, the resonance light scattering intensity of morin–Al^{3+}–CTAB–BSA system is higher than that of other four systems, which indicates that there is interaction among them, and forms a large morin–Al^{3+}–CTAB–BSA complex in this system.

Table 3
Compared with other fluorometric methods in sensitivity

Fluorigenic reagent	LOD (μg ml^{-1})	Linear range (μg ml^{-1})	Ref.
Doxycycline–europium	0.064 (HSA)	0–9.2 (HSA)	[9]
	0.115 (HSA)	9.2–34.5 (HSA)	
Eu (III)–chlorotetracycline	0.0089 (BSA)	0.20–10.0 (BSA)	[10]
	0.033 (HSA)	0.80–10.0 (HSA)	
Erythrosine	–	1.36–20.4	[13]
Magdala Red	0.1	0.1–4.0	[14]
Functionalized CdS nanoparticles	0.01 (BSA)	0.1–3.0 (BSA)	[15]
	0.019 (HSA)	0.1–1.4 (HSA)	
	0.08 (BSA)	0.1–3.2 (BSA)	[16]
Organic nanoparticles	0.062 (HSA)	0.1–4.5 (HSA)	[17]
	0.036 (BSA)	0.2–3.5 (BSA)	
5-(4-Carboxyphenylazo)-8-(4-methoxy)-benzylideneaminoquinoline	0.028 (HSA)	0.1–4.5 (HSA)	[18]
Nile Blue-SDBS	0.020 (HSA)	0–16 (HSA)	[19]
Calix [8] arenas and Ce (III)	2.83×10^{-3} (BSA)	1.1–11.4 (BSA)	[20]
Acridine Orange dimmer	0.08 (BSA)	0.66–39.8 (BSA)	[21]
This method	0.0026 (BSA)	0.0050–20.0 (BSA)	
	0.014 (BSA)	0.020–15.0 (HAS)	

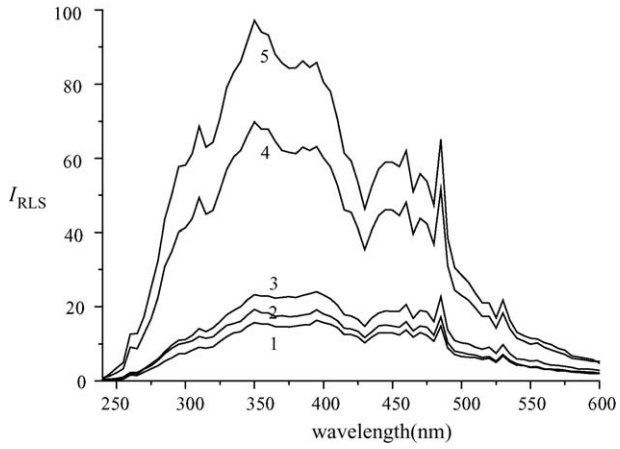


Fig. 6. Resonance light scattering spectra: (1) morin; (2) morin–Al^{3+}–CTAB; (3) morin–Al^{3+}; (4) morin–Al^{3+}–BSA; (5) morin–Al^{3+}–CTAB–BSA. Conditions—morin: 1.0×10^{-6} mol l^{-1}; CTAB: 1.0×10^{-4} mol l^{-1}; Al^{3+}: 1.76×10^{-5} mol l^{-1}; HMTA: 0.8% (pH 7.3); BSA: 5.0 μg ml^{-1}.

It is well known that morin can bind with Al^{3+} and form morin–Al^{3+} complex with the positive charge. And the isoelectric point of BSA is 4.7, when pH of the solution is 7.3, BSA is negatively charged. So under this condition, BSA can bind with morin–Al^{3+} through electrostatic attraction and form the morin–Al^{3+}–BSA complex. In addition, CTAB is a kind of cationic surfactant, which can also bind with negative BSA through electrostatic force. Therefore, it is possible that both morin–Al^{3+} and CTAB can bind to BSA through electrostatic attraction and form a large morin–Al^{3+}–CTAB–BSA complex, resulting in very strong RLS.

5.2. The fluorescence enhancement of morin–Al^{3+}–CTAB–BSA system

From Fig. 1, it is apparent that BSA can greatly enhance the fluorescence of morin–Al^{3+}, especially in the presence of CTAB. Furthermore, it can be seen that the emission peak of morin–Al^{3+} is moved to shorter wavelength, which comes from the change in microenvironment of the system. This change can also be proved by the variation in polarity and fluorescence polarization of the system in Table 5.

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 [22,23]. A low value reflects a lower polar environment than a high value. From Table 5, it is seen that the polarity of morin–Al^{3+} system decreases with adding CTAB and BSA, which makes the fluorescence intensity of morin–Al^{3+} complex be the strongest because the lowest excited state of singlet morin belongs to π, π^* state.

Table 5
Comparison of the polarity and microviscosity of the systems

Medium	H_2O	CTAB	CTAB–BSA
I_1/I_3	1.741	1.726	1.712
η (cP)	2.017	2.510	3.055

The microviscosity (η) of morin–Al^{3+} in different media is calculated using the equation developed by Shinitzky and Barenholz [24]:

$$\eta = 2P/(0.46 - P)$$

In the above equation, P refers to the observed fluorescence polarization of the probe (morin–Al^{3+}). From Table 5, it is seen that BSA–CTAB mixture provides a maximum microviscosity for morin–Al^{3+} complex, resulting in maximum fluorescence intensity of morin–Al^{3+} complex.

Based on the above facts, it is considered that BSA–CTAB system provides an optimum hydrophobic environment with low polarity and large viscosity for morin–Al^{3+} complex, which makes the fluorescence intensity of the latter be enhanced. In addition, the hydrophobic environment of BSA–CTAB system can also prevent the collision between complex and water and decrease the energy loss of the morin–Al^{3+}–CTAB–BSA system. Thus, the fluorescence quantum yield is improved and the fluorescence intensity of morin–Al^{3+} system is significantly enhanced.

6. Conclusions

In this paper, a new fluorimetric method for the determination of protein has been reported. Under optimum conditions, the enhanced intensity of fluorescence is in proportion to the concentration of proteins in the range of 5.0×10^{-9} to 2.0×10^{-5} g ml^{-1} for BSA, 4.0×10^{-8} to 1.3×10^{-5} g ml^{-1} for EA and 2.0×10^{-8} to 1.5×10^{-5} g ml^{-1} for HSA. Their detection limits (S/N = 3) are 2.6×10^{-9}, 1.6×10^{-8} and 1.4×10^{-8} g ml^{-1}, respectively. In comparison with most of fluorimetric methods reported, this method is quick and simple, and has high sensitivity, wide linear range and good stability. The interaction mechanism is also studied, which indicates that protein and CTAB provide a hydrophobic environment with low polarity and large viscosity, resulting in the fluorescence enhancement of morin–Al^{3+} complex.

Acknowledgements

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