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ORIGINAL ARTICLE

OFF/ON Red-Emitting Fluorescent Probes for Casein Recognition and Quantification Based on Indolium Derivatives

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Abstract Five derivatives of 2, 3, 3-trimethyl-3H-indolium containing different electron donor groups (H1 - H5) were synthesized for the determination of proteins. H3, a sensitive red-emitting fluorescent probe, was found for the discrimination of hydrophobic proteins from hydrophilic. The OFF - ON fluorescence switch of H3 was caused by the formation of twisted intramolecular charge-transfer (TICT) state when it combined with hydrophobic proteins in aqueous buffer. There was a good linear relationship between the emission intensity of H3 and the casein concentration (r = 0.9989). Based on this, a novel casein quantitative assay method was developed, and the method was applied to determinate casein in milk powder samples. Successfully, the results were in good agreement with Biuret method. In addition, a simple and sensitive method was established to differentiate and quantify three casein components (α-, β-, and κ-casein) due to their much different binding constants to H3 probe.

Keywords Fluorescent probes · Red-emitting · Discrimination · Hydrophobic protein · Casein · Quantification

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Introduction

Protein biochemists are searching for various ways to detect proteins with high sensitivity and good binding linearity to facilitate both qualitative and quantitative analysis [1, 2]. There are several methods to detect proteins as follows: Biuret method [3], Lowry method [4], Bradford method [5], Absorption and fluorescence spectrometry. Fluorescence spectrometry is a conventional and highly sensitive analytical method, with low background noises and wide dynamic ranges [6–8]. Recently many fluorescent bioprobes based on solvatochromic fluorophores [9], disassembly-induced emission [10, 11] and aggregation induced emission enhancement (AIEE) [12, 13] have been developed for the detection of proteins. Generally, the fluorescence of the bioprobes can be enhanced and/or red/blue shifted by complexation with proteins, thus enabling visual observation of the biomacromolecular species. Most of the fluorescent probes for proteins are based on their specific reactions, by which a non-fluorescent substrate can be converted to a "turn-on" product [14–17]. However, the reported fluorescent probes usually suffer from several limitations, such as shorter emission wavelength which prevent it from in-vivo applications due to auto-fluorescence from biological systems. One prospect is the preparation of full-color fluorescent sensors that are excited by visible light irradiation and fluorescence become more visually perceptible and spectroscopically separated.

Cyanine-based probes have received immense attention and been widely used as NIR fluorescent labels for biological applications [18–20]. As a subgroup of cyanine dyes, hemicyanines were widely used as label probes for biomolecules to provide rational signals when they noncovalent interacting with biomolecules [21–23]. But their poor solubility in water limited their unique application in live systems. Indolium, such as derivatives of 2,3,3-trimethyl-3H-indolium

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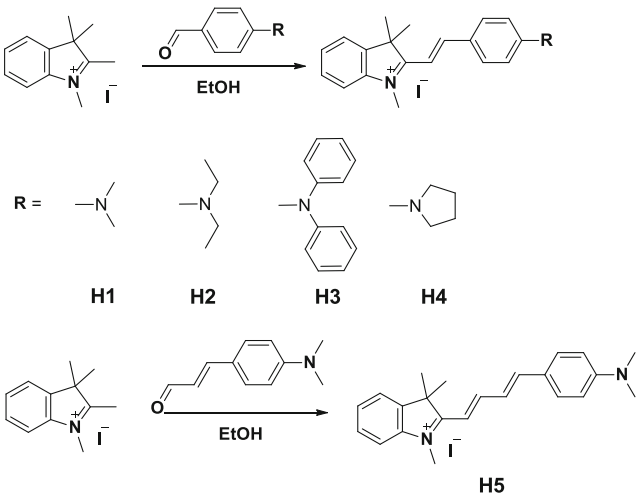
[24, 25], benzo[e]indoline [26] bearing strong electron-withdrawing feature, π-conjugation with electro donor groups were used as fluorescence probes for biomolecules because of their good water solubility and long wavelength emission. Pyridinium as 2-photon absorption fluorophore was also used to perform noninvasive membrane staining [27]. Three hemicyanine dyes containing N-(carboxyethyl)-2-methylbenzothiazolium group and bearing different substituent could bind to bovine serum albumin and possess sufficient high photostability [28]. Trans-4-[4-(dimethylamino)-styryl]-N-methylpyridinium iodide (HC) was used to gain more insight about reverse micelle interface properties [29]. Hemicyanine dye constructed with benzothiazolium groups and terminal anilino groups was used in the selective fluorescent staining of RNA in live HeLa, KB and V79 cells [30].

In this paper, five derivatives of 2, 3, 3-trimethyl-3H-indolium containing different electron donor groups (H1 - H5, Scheme 1) were synthesized, and their spectral properties as well as the changes of these properties upon the interaction with proteins were studied. It was found that H3 could be built as an efficient fluorescent probe for both discrimination of hydrophobic proteins from hydrophilic and differentiation of three components of casein (α-, β-, and κ-casein) by their much different binding constants. In addition, the rational quantity determination for total casein, as well as three components (α-, β-, and κ-casein) was constructed based on the sensitive fluorescence response of H3.

Experimental

Materials

4-(Dimethylamino)benzaldehyde, 4-(Diethylamino)benzaldehyde, 4-(Diphenylamino)benzaldehyde and 4-(1-Pyrrolidino)benzaldehyde were purchased from Aldrich, 4-Dimethylaminocinnamaldehyde was from TCI. All other starting materials were obtained commercially as analytical-grade and without further purification. Column chromatography was performed with silica gel (mesh 200–300). Human serum albumin (HSA), bovine serum albumin (BSA), egg albumin (EA), lysozyme (Lys), cytochrome *c* (Cyt *c*), myogolobin (Mb), hemoglobin (Hb), casein, α-, β-, and κ-casein were all purchased from Sigma. All these proteins were dissolved in phosphate buffered saline (PBS, pH 7.4, 0.02 M) to prepare stock solutions with concentrations of 0.1 mM and stored in a refrigerator before use. The stock solutions (5.0 mM) of compound H1 - H5 were prepared in CH_3CN.



Scheme 1 Preparation of hemicyanine dyes H1 - H5

Synthesis of five Hemicyanine Dyes

(E)-2-(4-(dimethylamino)styryl)-1,3,3-trimethyl-3H-indol-1-ium iodide (H1), (E)-2-(4-(diethylamino)styryl)-1,3,3-trimethyl-3H-indol-1-ium iodide (H2), (E)-2-(4-(diphenylamino)styryl)-1,3,3-trimethyl-3H-indol-1-ium iodide (H3), (E)-1,3,3-trimethyl-2-(4-(pyrrolidin-1-yl)styryl)-3H−indol-1-ium iodide (H4), 2-((1E,3E)-4-(4-(dimethylamino)phenyl)buta-1,3-dien-1-yl)-1,3,3-trimethyl-3H-indol-1-ium iodide (H5) were synthesized by a simple one-step reaction (Scheme 1). Synthesis details and characterization by ^{1}H NMR analyses were shown in supporting information (ESI). ^{1}H NMR spectra were recorded on Bruker 400 NMR spectrometers using $CDCl_3$ as solvent.

UV-Visible and Fluorescence Measurements

UV-vis spectra were recorded on a Shimadzu UV-2600 spectrophotometer, with a quartz cuvette (path length 1.0 cm). Fluorescence measurements were performed on a Hitachi F-4600 spectrofluorimeter with slit widths were set at 5.0 nm and 10.0 nm for excitation and emission. The samples were excited at appropriate wavelength, respectively.

Cyclic Voltammetry Measurements

Cyclic voltammetry was performed on a Autolab electrochemical work station (PGSTAT12). A three-electrode arrangement in a single cell was used for the measurement: a Pt wire as the auxiliary electrode, a 2.0 mm Au plate electrode as the working electrode, and a saturated calomel electrode (SCE) as the reference electrode. The sample solution contained 0.1 M Tetrabutylammonium Hexafluorophosphate ($TBAPF_6$) as a supporting electrolyte in the solvent.

The Quantification of Casein in Milk Powder Samples

Three milk powder samples were purchased from supermarket. Our pre-treatment procedures for milk powder samples

were based on the reference [31] and the experimental details are described as follows.

The pre-treatment for full milk powder: 1) 25 mg milk powder was dissolved in 5 ml distilled water; 2) Milk solution was centrifuged for 10 min at 5000 rpm and the upper layer of fatty acid was peeled off; 3) Acetic acid (2 M) was added to adjust pH to 4.7, casein would be deposited. The solution was centrifuged for 10 min at 10,000 rpm to get the precipitate; 4) The protein precipitate was rinsed with ethanol for 2 times to remove adhered fatty acid; 5) The precipitate was washed with distilled water for 2 times and resolved with PBS. A few drops of 0.1 M NaOH were added to help solving the casein precipitate and the final volume for sample solution was 5 ml. Finally, the pH was adjusted to pH 7.0 with 0.1 M acetic acid.

The pre-treatment for skim milk powder: The method is same to the whole milk powder processing without the step 2 and 5.

In this method, we mixed 30 μl prepared sample solution with 1.0 mL H3 (4.0 μM). The fluorescence intensity was recorded under the same experimental conditions. As a comparison, the evaluation based on Biuret method (Chinese GB/T21676–2008; NY/T 1678–2008) was obtained.

Results and Discussion

Spectroscopic Properties of H1 – H5 in Different Media

The basic structures of the chromophores and auxochromes determine the spectral properties of the functional dyes. The bioactive trimethyleneindolium is adopted in all five compounds, and different terminal aniline groups are selected because they create dramatic red shifts and possibly bring a significant structural deformation of the chromophore molecule when micro-surrounding change. The absorption and emission spectra of H1 - H5 (10.0 μM) in CH_2Cl_2 are shown in Fig. 1. All compounds exhibit a broad and structure-less lowest-energy absorption band with large molar extinction coefficients ranging from 46,000 to 82,000 $M^{-1}.cm^{-1}$. Considering the push - pull structures of these compounds, their absorption bands are assigned as intramolecular charge transfer (ICT) transitions. The very close values of λ_{abs} for H1 - H4 indicate their similar donor strengths, and the significant red-shift of H5 is owing to the expanding π-conjugation with stronger ICT. Except for H3, other four compounds emit rather strong fluorescence, and the emission maxima of H5 are

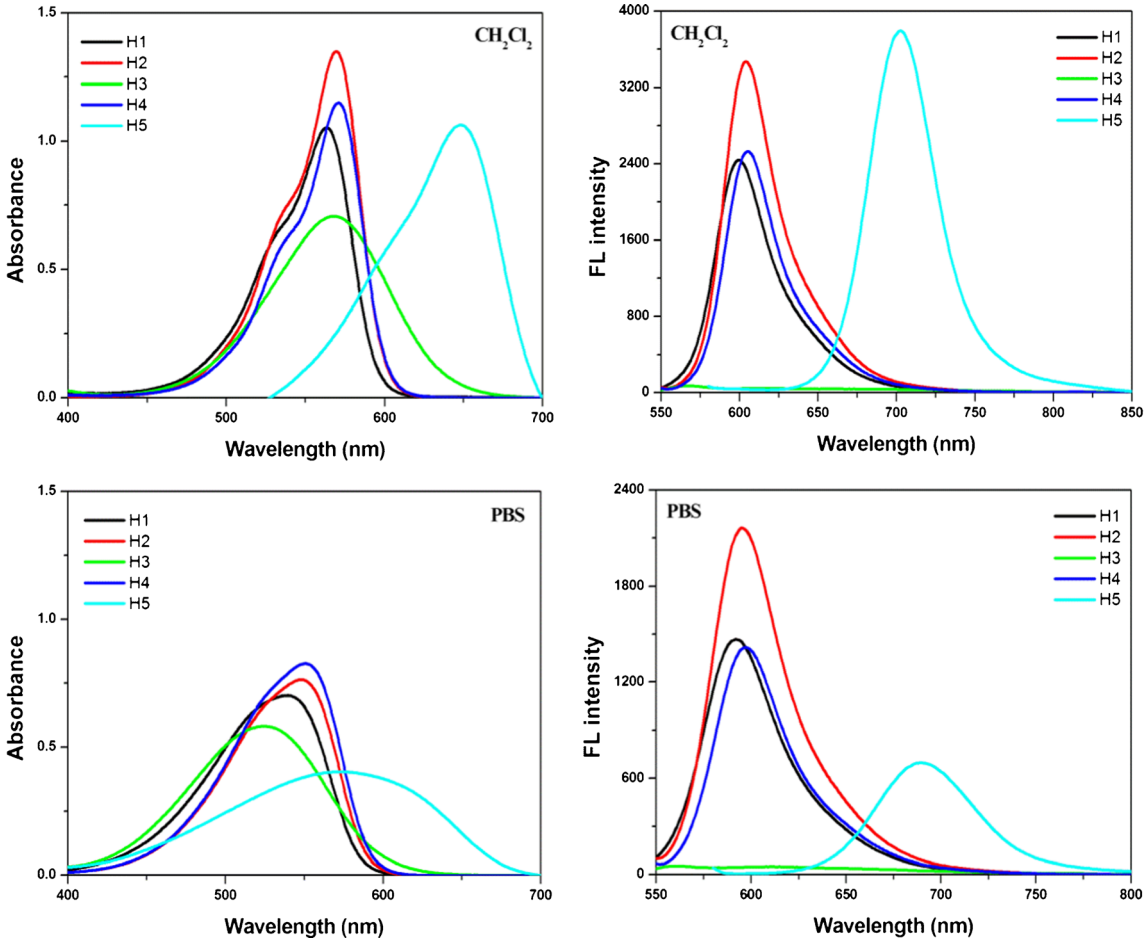


Fig. 1 UV-Vis absorption and fluorescence spectra of H1 - H5 in CH_2Cl_2 or PBS buffer (pH 7.2–7.4). c_H = 10.0 μM

almost 100 nm higher than H1, H2 and H4 as expected. The almost non-fluorescence of H3 indicates that the solvent relaxation in the excited singlet state of H3 is more significant than in other compounds.

The spectra measurements of H1 - H5 in other solvents and aqueous PBS buffer were conducted (Table S1). The λ_{abs} of these compounds is dependent on the solvent polarities. From CH_2Cl_2 to water, a negative shift is observed, 24 nm for H1, while 44 nm for H3. For H3, the positive shift in weak emissions can be found (from 612 nm in CH_2Cl_2 to 621 nm in buffer), and the stocks shift change from 1266 cm^{-1} to 2981 cm^{-1}. The weak emission and enormous stokes shift of H3 in polarity solution is probably due to the formation of the twisted intramolecular charge-transfer (TICT) state [32] and a rotational twist of the diphenylamino group relative to the trimethyleneindolium.

To understand the relationship between the optical properties and the electronic structures, the HOMOs and LUMOs of H1-H5 were obtained by DFT/B3LYP/6-311G (d, p) calculations on the software package GAMESS platform [33, 34]. The electron densities of the HOMO levels of luminogens are mainly localized at both ends of compounds, whereas those of the LUMO levels are predominantly distributed at connecting -C = C- and indolium group, showing explicit ICT feature (Fig. 2). Especially for H3, the HOMO-LUMO excitation move thoroughly the electron distribution from diphenyamino moiety to the central -C = C- and indolium group. Such cross-couplings result in strong transition dipole moments between states and the enhancement of TICT [35]. These calculation results further testify the structural and electronic effects, and highly consistent with the spectral characters.

Electrochemistry of H1 –H5

Cyclic voltammetry measurements were carried out to confirm the influence of different donors on the HOMO, LUMO levels and energy gaps. As shown in Fig. 3, almost all compounds exhibit three reversible single-electron redox couples in CH_2Cl_2, corresponding to the successive reversible oxidation of neutral to the radical trication. For H1, H2 and H3, the oxidation peaks locate at 0.347, 0.712 and 1.24 V successively, while the third oxidation peak of H4 and H5 shift

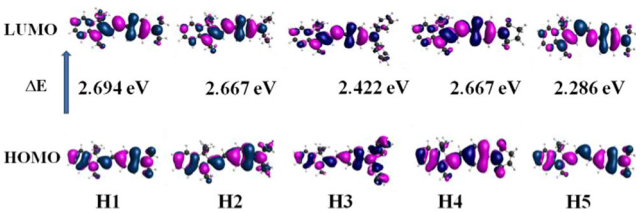


Fig. 2 DFT (B3LYP/6-311G(d, p))-calculated molecular frontier orbitals for H1 - H5. Surface isovalue: ± 0.02

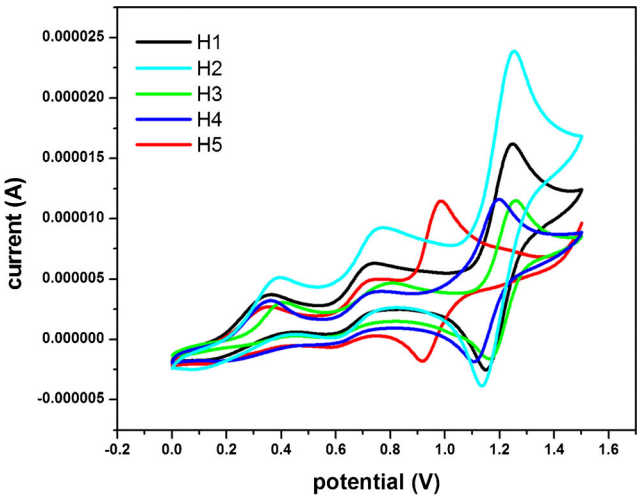


Fig. 3 Cyclic voltammograms of H1 - H5. Scan rate = 0.1 V/s; solvent = CH_2Cl_2, supporting electrolyte =0.1 M $TBAPF_6$, c_H = 1.0 mM

to 1.17 V, 0.987 V, respectively. It suggests that they should be more susceptible to the reaction with singlet oxygen and other oxidation species which contribute to the photo-fading of dyes. The higher HOMO and lower energy gap for H5 are well consistent with experimental results and show that the rising of HOMO energy levels is attribute to the expanding π-conjugation.

Responses of Fluorophores H1 – H5 to Proteins

To prove whether H1-H5 could act as various protein sensors, the fluorescence spectra of five compounds toward proteins (Table S2) were investigated. As seen from Figs. S6, S7, S8, S9 and S10, the responses of the fluorescence intensity value of H1 - H5 for various proteins are diverse and differentiable. For H1, H2, H4 and H5, there are no obvious emission enhancements with addition of various proteins except casein. Whereas, the quenched emission of H3 quickly recover in different degree with increasing proteins (casein, HAS, BSA, EA and Hb). Fluorescence response patterns of H1-H5 for various proteins were built. As shown in Fig. 4, 2.4 μM proteins elicits about a fluorescence enhancement of H3 (~63-fold for casein, ~45-fold for HSA, ~20-fold for BSA, ~9-fold for Hb, ~7-fold for EA, respectively.). Casein is a hydrophobic protein [31], BSA and HSA both have hydrophobic pockets, the hydrophobic interaction is possibly a main effect for the binding process. The marginal affinities of H3 for other bioanalytes (Mb, Lys, Cyt *c*) are because of the absence of suitable hydrophobic cleft on their surface. By contrast, casein induces less fluorescence enhancement (< 5-fold) for other compounds. The above results demonstrate that H3 is highly selective toward hydrophobic proteins through the OFF - ON fluorescence response.

As shown in Fig. 5, compound H3 exhibits a significant turn-on fluorescence signal with the addition of casein. To find

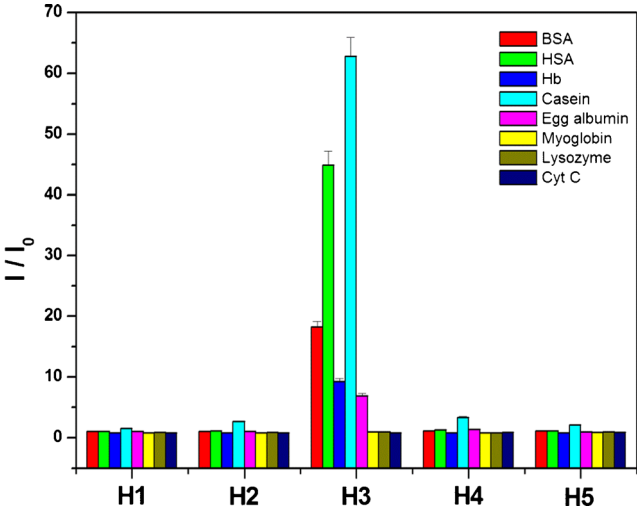


Fig. 4 Fluorescence response patterns of H1 - H5 on addition of proteins. The relative fluorescence intensity of H1 - H5 at their maximal emission wavelength in the presence of 2.4 μM various proteins, c_{H} = 4.0 μM. 0.02 μM PBS, pH 7.0

out whether the restriction of intramolecular rotation plays any role in the OFF - ON process, we studied the fluorescence behavior of H3 in Triton X-100 micelle (TX-100). It is observed the fluorescence emission of H3 enhances ~40-folds and the emission blue shift with increasing TX-100 (Fig. S11). As it is known that micelle system restricts the rotation and vibrational mode of the molecule and ultimately suppresses the TICT leading to fluorescence enhancement [36]. Casein exists as special micellar structure in aqueous solution with the assistance of hydrophobic interaction [31]. About 80 ~ 95 % of the casein in milk is in the form of colloidally dispersed particles, which are casein micelles [37]. Casein belongs to the rapidly growing family of unstructured proteins which has been attracted much of the interest recently [38]. Thus the fluorescence enhancement of H3 is caused by binding to the

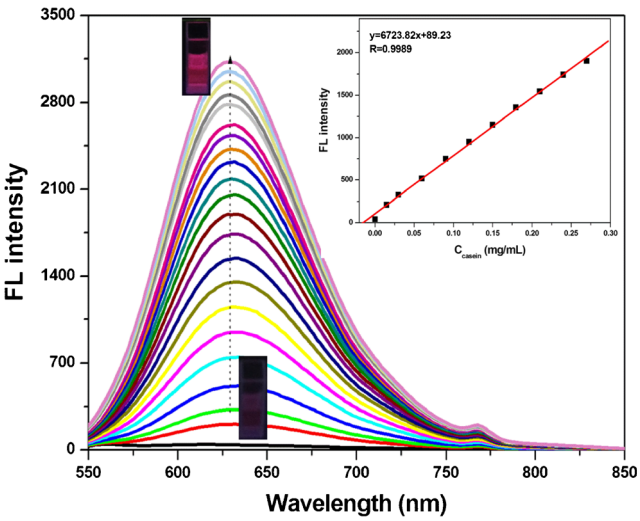


Fig. 5 Fluorescence spectra of H3 before and after the addition of casein from 0 to 8.0 μM (0 ~ 0.6 mg/mL)

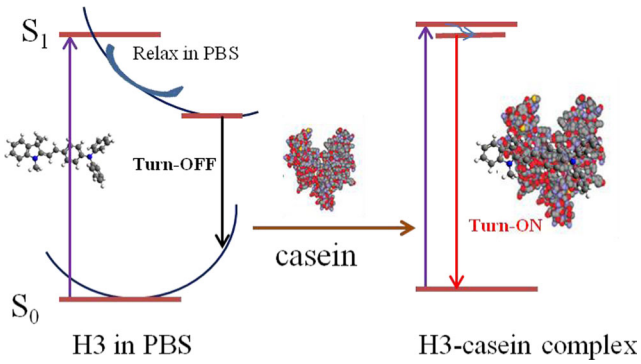
hydrophobic pocket of casein (Scheme 2). As a result, H3 fails to rotate freely and TICT gets restricted.

Quantitative Analysis for Casein by H3 Probe

The linearity of fluorescence intensity to proteins concentration is an important concern for proteins quantification when developing a new staining agent. The emission intensity of H3 is plotted as a function of the casein concentration, and a typical calibration graph of the response to casein is shown in inset of Fig. 5. This plot shows a good linear relationship between the emission intensity and the casein concentration (r = 0.9989) up to 600 μg/mL of casein. The detection limit is 0.38 μg/mL (signal-to-noise ratio was 3.0). The linear relationships between the emission intensity of H3 and other proteins concentration are also obtained to construct the protein-to-protein (P-P) variation (Table S2), which is obtained by the following equation: ratio = (slope of various proteins)/(slope of casein) [1]. The response for casein is normalized to 1.00. It is apparent that the P-P variations for H3 are much different toward hydrophobic proteins. The reason may be due to the differences of the hydrophobicity of proteins, as well as their relative molecular mass values. The proteins with high molecular mass are found to be higher P-P ratios. Thus, we present a sensitive fluorescence probe for qualitative and quantitative analysis to casein.

Application of Casein Determination in Milk Powder Samples

Rapid and direct determination of casein content in milk has been a long wish for breeding organizations and the dairy industry as well as for control laboratories [31]. We have tried to quantify casein in real milk. Firstly, we test the interference of common components in real milk. All the potential interferences are prepared with a similar or a little higher concentration (*w*/w) as in actual milk, respectively. The interfering substances include surfactants (hexadecyl trimethyl ammonium bromide (CTMAB),



Scheme 2 Turn - on mechanism of H3 in hydrophobic pockets of casein

Table 1 The results of casein quantification in different milk powder samples

Manufacturer	Samples	Casein in milk powder (g/100 g)	
		Biuret method	Proposed method
Mengniu	Full milk	7.23	6.61
Yili	Skimmed milk	17.87	18.67
Wandashan	Skimmed milk	16.93	16.78

sodium dodecyl sulfate (SDS)), salts (NaCl, 5 %; KCl, 5 %), nitrogenous substances (melamine, 0.3 %; urea, 5 %) and fat (lecithin, 5 %). The fluorescence intensity induce by the interferences are compared with that induces by casein solution (casein, 5 %) (Fig. S12). From the results, we can conclude that all the interferences could hardly turn on the fluorescence of H3 except for lecithin. Lecithin can form micelles in water, so it may result in the H3 probe embedding in the hydrophobic cavity of micelles.

With pre-treatment, we detected the samples and calculated values according to the standard curve (shown in inset of Fig. 5). The results are presented in Table 1. For three milk powder samples, with the same sample and same pre-treatment process, the absolute difference of the results of H3 method and Biuret test is both less than 10 % of corresponding arithmetic mean, indicating that the results of our experiment are acceptable, and the sensitivity, accuracy and precision of the fluorescence method are also satisfactory. Additionally, it is affordable for most laboratories because of the normal requirement, low equipment cost and easy operation.

Table 2 The binding constants K and thermodynamic parameters ΔG for H3 with different casein

Caseins	R^2	K (10^4 M^{-1})	ΔG (kJ/mol)
α-Casein	0.995	9.99	-28.5
β-Casein	0.999	1.46	-23.8
κ-Casein	0.998	7.04	-27.7

Comparasion of Binding of H3 to three Casein Components

Casein exists as micelles made of polypeptides known as α-, β-, and κ-casein [37], which are almost similar in size, molecular weight (24 kD for α, β, and 19 kD for κ-casein) and net negative charge but differ in their degree of unfoldedness [39–41]. As seen from Figs. S13, S14 and S15, the spectra of H3 exhibits a significant fluorescence enhancement with the addition of α-casein, and then κ-casein, whereas β-casein shows minimal impact. A quantitative estimate or the extent of binding of H3 to different casein components is obtained from an examination of the emission data on the Benesi-Hildebrand (B-H) relation [42, 43].

$$\frac{1}{I-I_0} = \frac{1}{I_1-I_0} + \frac{1}{(I_1-I_0)K[\text{casein}]} \quad (1)$$

In which I_0 and I are the emission intensities in the absence of and at a certain concentration of casein, respectively. As seen in Fig. 6, plots of $1/[I\text{-}I_0]$ vs 1/[caseins] produce a straight line. A quantitative estimate of the binding constant K and the free energy change ΔG for this process were determined and presented in Table 2. The obtained binding constant (K) for α-

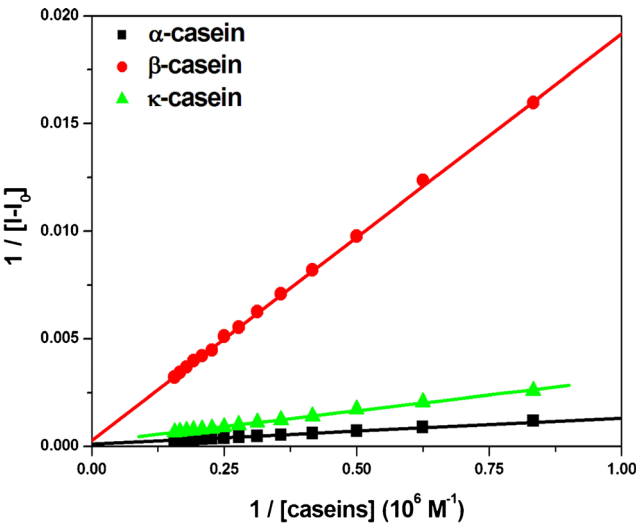


Fig. 6 Benesi-Hildebrand plot of $1/[I\text{-}I_0]$ vs 1/[caseins] for binding of H3 (4.0 μM) to α-, β-, and κ-caseins

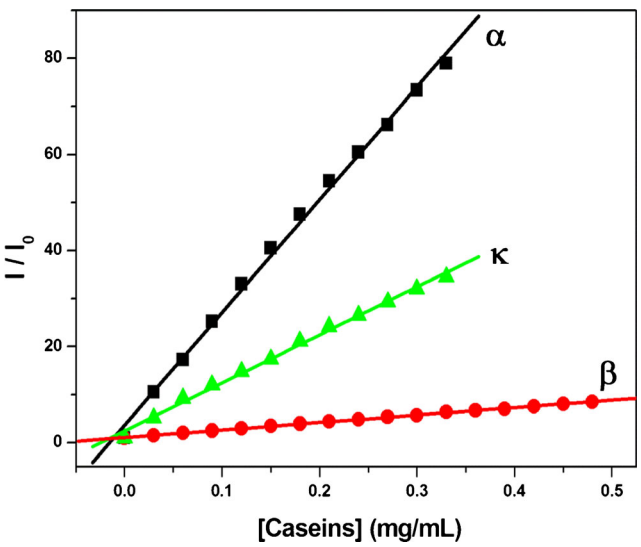


Fig. 7 The fluorescence intensity I/I_0 of H3 (4.0 μM) vs concentration of α-, β-, and κ-caseins

Table 3 The results of linear fitting of calibration curves and the limit of detection values for H3 with different casein

Caseins	Linear equation	R^2	Limit of Detection (μg/mL)		
			The proposed method	Ref 1 [47]	Ref 2 [48]
α-casein	$I/I_0 = 3.66 + 235$ [Casein]	0.997	2.29	9.6	18.8
β-casein	$I/I_0 = 1.11 + 15.5$[Casein]	0.999	29.8	16.8	15.0
κ-casein	$I/I_0 = 2.49 + 99.7$[Casein]	0.996	5.86	7.6	6.0

casein (9.99×10^4 M^{-1}) is almost 7 times of that for β-casein (1.46×10^4 M^{-1}).

The different fluorescence responses of H3 toward three casein components could be due to the structural discrepancy between the proteins. As is known to all, α-casein contains two tryptophan (Trp) residues (Trp-37, Trp-66 in α s1 -casein, and Trp-193, Trp-109 in α s2 -casein), while β- and κ-caseins have only one Trp (Trp-143 in β- and Trp-76 in κ-caseins) [44]. In addition, a large amount of proline residues in β-casein would disrupt the formation of α-helical and β-sheet in aqueous media [45].

The separation and quantification of individual casein in milk, cheese and other dairy products is a major task for several years, since it can provide valuable informations [46]. In our experiments, the quantitative analysis of α-, β-, and κ-caseins can be performed by calibration curves (Fig. 7). Good linear relationships are shown in a certain range for α-, β-, and κ-caseins, and the correlation coefficients are all above 0.995. According to the equation$D = 3\delta/slope$, the limit of detection (LOD) values are calculated and shown in Table 3. The detection limits are 2.29, 5.86 and 29.8 μg/mL for α-, κ-, and β-caseins, respectively. In compared with two casein quantification methods based on high-performance liquid chromatography (HPLC) (Table 3) [47, 48], the proposed method owns advantages in precise and accurate, and it is affordable for most laboratories because of low equipment cost and easy operation.

Conclusion

The photophysical properties of five red-emitting fluorescent probes (H1 - H5) were described in different media. The absorptions and emissions were assigned as intramolecular charge transfer (ICT) transitions. Whereas, the formation of twisted intramolecular charge-transfer (TICT) state of H3 resulted in weak emission and enormous stokes shift in polarity solution. The TICT restriction and fluorescence enhancement of H3 were caused by binding to hydrophobic proteins, and P-P variations for H3 were quite different for proteins. A good linear relationship between the emission intensity of H3 and concentration of casein was established for casein quantification in milk samples, and the results of H3 probe method was verified to be acceptable, selective and affordable as compared with Biuret method. Moreover, H3 could be used to differentiate and quantify three casein components (α-, β-, and κ-caseins) because of their much different binding constants.

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References

1. Suzuki Y, Yokoyama K (2005) Design and synthesis of intramolecular charge transfer-based fluorescent reagents for the highly-sensitive detection of proteins. J Am Chem Soc 127:17799–17802
2. Angel LU, Rout B, Ilani T, Eisenstein M, Motiei L, Margulies D (2015) Protein recognition by bivalent, 'turn-on' fluorescent molecular probes. Chem Sci 6:5419–5425
3. Escobedo JO, Rusin O, Wang W, Alpturk O, Kim KK, Xu X, Strongin RM (2006) Detection of biological thiols. In: Geddes CD, Lakowicz JR (eds) Reviews in fluorescence 2006, vol 3. Springer, US, pp. 139–162
4. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. J Biol Chem 193:265–275
5. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
6. De Silva AP, Gunaratne HQN, Gunnlaugsson T, Huxley AJM, McCoy CP, Rademacher JT, Rice TE (1997) Signaling recognition events with fluorescent sensors and switches. Chem Rev 97:1515–1566
7. Ambrose WP, Goodwin PM, Jett JH, Orden AV, Werner JH, Keller RA (1999) Single molecule fluorescence spectroscopy at ambient temperature. Chem Rev 99:2929–2956
8. Patton WF (2000) Making blind robots see: the synergy between fluorescent dyes and imaging devices in automated proteomics. BioTechniques 28:944–957
9. Loving G, Imperiali B (2008) A versatile amino acid analogue of the solvatochromic fluorophore 4-N,N-dimethylamino-1,8-naphthalimide: a powerful tool for the study of dynamic protein interactions. J Am Chem Soc 130:13630–13638
10. Hou TC, Wu YY, Chianga PY, Tan KT (2015) Near-infrared fluorescence activation probes based on disassembly-induced emission cyanine dye. Chem Sci 6:4643–4649
11. Savariar EN, Ghosh S, Gonzalez DC, Thayumanavan S (2008) Disassembly of noncovalent amphiphilic polymers with proteins and utility in pattern sensing. J Am Chem Soc 130:5416–5417
12. Tong H, Hong Y, Dong YQ, Häussler M, Z L, Lam JWY, Dong YP, Sung HHY, Williams ID, Tang BZ (2007) Protein detection and quantitation by tetraphenylethene-based fluorescent probes with aggregation-induced emission characteristics. J Phys Chem B 111:11817–11823

13. Tong H, Hong YN, Dong YQ, Häussler M, Lam JWY, Li Z, Guo ZF, Guo ZH, Tang BZ (2006) Fluorescent "light-up" bioprobes based on tetraphenylethylene derivatives with aggregation-induced emission characteristics. Chem Commun. doi:10.1039/B608425G
14. Tong H, Wang LX, Jing XB, Wang FS (2003) "Turn-on" conjugated polymer fluorescent chemosensor for fluoride ion. Macromolecules 36:2584–2586
15. Basak A, Bag SS, Basak A (2005) Design and synthesis of a novel enediynyl pentapeptide with predominantly β-turn structural motif and its potential as a fluorescence-based chemosensor. Bioorg Med Chem 13:4096–4102
16. Ma GY, Muller AM, Bardeen CJ, Cheng Q (2006) Self-assembly combined with photopolymerization for the fabrication of fluorescence "turn-on" vesicle sensors with reversible "on - off" switching properties. Adv Mater 18:55–60
17. Fan LJ, Jones WE (2006) Studies of photoinduced electron transfer and energy migration in a conjugated polymer system for fluorescence "turn-on" chemosensor applications. J Phys Chem B 110: 7777–7782
18. Guo ZQ, Nam SW, Park S, Yoon J (2012) A highly selective ratiometric near-infrared fluorescent cyanine sensor for cysteine with remarkable shift and its application in bioimaging. Chem Sci 3:2760–2765
19. Guo ZQ, Zhu WH, Zhu MM, Wu XM, Tian H (2010) Near-infrared cell-permeable Hg^{2+} selective ratiometric fluorescent chemodosimeters and fast indicator paper for $MeHg^{+}$ based on tricarbocyanines. Chem Eur J 16:14424–14432
20. Chen XQ, Nam SW, Kim GH, Song N, Jeong Y, Shin I, Kim SK, Kim J, Park S, Yoon J (2010) A near-infrared fluorescent sensor for detection of cyanide in aqueous solution and its application for bioimaging. Chem Commun 46:8953–8955
21. Li YH, Wang YJ, Yang S, Zhao YR, Yuan L, Zheng J, Yang RH (2015) Hemicyanine-based high resolution ratiometric near-infrared fluorescent probe for monitoring pH changes in vivo. Anal Chem 87:2495–2503
22. Song FL, Wang L, Qiao XQ, Wang BS, Sun SG, Fan JL, Zhang LH, Peng XJ (2010) Asymmetric trimethine 3H-indocyanine dyes: efficient synthesis and protein labeling. Org Biomol Chem 8:4249–4251
23. Liu XD, Sun R, Xu Y, Xu YJ, Ge JF, Lu JM (2013) A benzoxazine-hemicyanine based probe for the colorimetric and ratiometric detection of biothiols. Sensors Actuators B 178:525–531
24. Reja SI, Khan IA, Bhalla V, Kumar M (2016) A TICT based NIR-fluorescent probe for human serum albumin: a pre-clinical diagnosis in blood serum. Chem Commun 52:1182–1185
25. Kabatc J, Jureka K, Kostrzewskaa K, Orzeł Ł (2015) Hemicyanine dyes derived from 2,3,3-trimethyl-3H-indolium as candidates for non-covalent protein probes. J Pharm Biomed Anal 114:433–440
26. Sun Y, Fan SW, Zhao D, Duan L, Li RF (2013) A fluorescent turn-on probe based on benzo [E] indolium for cyanide ion in water with high selectivity. J Fluoresc 23:1255–1261
27. Kimura Y, Momotake A, Takahashi N, Kasai H, Arai T (2012) Polarity-dependent photophysical properties of hemicyanine dyes and their application in 2-photon microscopy biological imaging. Chem Lett 41:528–530
28. Kabatc J, Jurek K, Orzeł Ł (2015) New N-(carboxyethyl)-2-methylbenzothiazole-based hemicyanine dyes: synthesis, spectra, photostability and association with bovine serum albumin. J Mol Struct 1084:114–121
29. Quintana SS, Moyano F, Falcone RD, Silber JJ, Correa NM (2009) Characterization of multifunctional reverse micelles' interfaces using hemicyanines as molecular probes. II: effect of the surfactant. J Phys Chem B 113:6718–6724
30. Miao JT, Fan C, Sun R, Xu YJ, Ge JF (2014) Optical properties of hemicyanines with terminal amino groups and their applications in near-infrared fluorescent imaging of nucleoli. J Mater Chem B 2: 7065–7072
31. Liu YY, Wang Z, Zhang GX, Zhang W, Zhang DQ, Jiang XY (2012) Rapid casein quantification in milk powder with aggregation induced emission character of tetraphenylethene derivative. Analyst 137:4654–4657
32. Cao C, Liu XG, Qiao QL, Zhao M, Yin WT, Mao DQ, Zhang H, Xu ZC (2014) A twisted-intramolecular-charge-transfer (TICT) based ratiometric fluorescent thermometer with a mega-stokes shift and a positive temperature coefficient. Chem Commun 50:15811–15814
33. Schmidt MW, Baldridge KK, Boatz JA, Elbert ST, Gordon MS, Jensen JH, Koseki S, Matsunaga N, Nguyen KA, Su SJ, Windus TL, Dupuis M, Montgomery JA (1993) Parallel wersion (OpenMPI) running with 48 nodes. J Comput Chem 14:1347–1363
34. Lee C, Yang W, Parr RG (1988) Development of the colle-salvetti correlation-energy formula into a functional of the electron density. Phys Rev B 37:785–789
35. Huang YY, Cheng TR, Li FY, Luo CP, Huang CH (2002) Photophysical studies on the mono- and dichromophoric hemicyanine dyes II. Solvent effects and dynamic fluorescence spectra study in chloroform and in LB films. J Phys Chem B 106: 10031–10040
36. Ren MG, Deng BB, Kong XQ, Zhou K, Liu KY, Xu GP, Lin WY (2016) A TICT based fluorescent probe for rapid and specific detection of hydrogen sulfide and its bio-imaging applications. Chem Commun 52:6415–6418
37. Walstra P (1999) Casein sub-micelles: do they exist? Int Dairy J 9: 189–192
38. Phadungath C (2005) Casein micelle structure: a concise review. C J Sci Technol 27:201–212
39. Fox PF, McSweeny PLH (1998) Dairy chemistry and biochemistry. Blackie academic and professional. Springer, London, pp. 150–169
40. Swaisgood HE (2003) Chemistry of the caseins. In: Fox PF, McSweeney PLH (eds) Advanced dairy chemistry-1 proteins, vol 1. Springer, US, pp. 139–201
41. Bleck GT, Brown EM, Butler JE, Creamer LK, Hicks CL, Hollar CM, Ng-Kwai-Hang KF, Swais-Good HEJ (2004) Nomenclature of the proteins of cows' milk-sixth revision. Dairy Sci 87:1741–1674
42. Kim YH, Cho DW, Yoon M, Kim D (1996) Observation of hydrogen-bonding effects on twisted intramolecular charge transfer of p-(N,N-diethylamino)benzoic acid in aqueous cyclodextrin solutions. J Phys Chem 100:15670–15676
43. Benesi HA, Hildebrand JH (1949) A spectrophotometric investigation of the interaction of iodine with aromatic hydrocarbons. J Am Chem Soc 71:2703–2707
44. Kumosinski TF, Brown EM, Farell HM (1993) Three-dimensional molecular modeling of bovine caseins: an energy-minimized β-casein structure. Jr J Dairy Sci 76:931–945
45. Swaisgood HE (1992) Chemistry of caseins. In: Fox PF (ed) Advanced dairy chemistry-1 proteins, vol 1. Elsevier Science, Oxford, pp. 63–110
46. Molina E, Frutos M, Ramos M (2000) Capillary electrophoresis characterization of the casein fraction of cheeses made from cows', ewes' and goats' milks. J Dairy Res 67:209–216
47. Bramanti E, Sortino C, Onor M, Beni F, Raspi G (2003) Separation and determination of denatured α s1-, α s1-, β- and κ-caseins by hydrophobic interaction chromatography in cows', ewes' and goats' milk, milk mixtures and cheeses. J Chromatogr A 994:59–74
48. Velosoc ACA, Teixeirab N, Ferreira IMPLVO (2002) Separation and quantification of the major casein fractions by reverse-phase high-performance liquid chromatography and urea–polyacrylamide gel electrophoresis detection of milk adulterations. J Chromatogr A 967:209–218