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Solvatochromic fluorescent probes for recognition of human serum albumin in aqueous solution: Insights into structure-property relationship

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Abstract

Human serum albumin (HSA) as the most abundant protein in human blood plasma, serves many physiological functions. The dysregulation of HSA in serum or in urine is associated with various diseases, such as cirrhosis of liver, multiple myeloma, and cardiovascular disease. Therefore, to quantify HSA in body fluids with high selectivity and sensitivity is of great significance for disease diagnosis and preventive medicine. We herein developed a series of amide-functionalized flavonoids probes, **1–3**, for recognition of human serum albumin. All flavonoids could be easily prepared by a Claisen-Schmidt condensation and Algar-Flynn-Oyamada reaction, and showed positive solvatochromism on their dual emissions. The chemical structure of flavonoids played an important role on their HSA-sensing abilities. Among three probes, the compound **1** showed the highest sensitivity, the remarkable selectivity, and the quantitative response for HSA in aqueous solution. Together with its high tolerance of environmental pH, anti-interference properties, and time-insensitivity, thus it provides a promising sensing method for HSA.

Keywords

Fluorescent probes; Human serum albumin; Flavonoid; Solvatochromism; Selectivity

1. Introduction

Human serum albumin (HSA), as the most abundant protein in human blood plasma, serves many physiological functions, including disposition of exogenous ligands [1], maintaining the osmotic pressure of the blood compartment [2], and transport of various biological important species (*e.g.* fatty acids and drugs) [3]. The normal concentration for HSA in serum is around 35–50 g/L, while in urine is less than 30 mg/L. A significant decrease of HSA in serum is indicative of severe medical conditions, such as cirrhosis of liver, multiple

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.snb.2016.06.056>.

myeloma, and mortality [4]. Moreover, an excess amount of HSA in urine, such as microalbuminuria, is an early signal of cardiovascular disease and kidney filtering disorder [5]. In view of its indispensable factor on human health, to quantify HSA with the high selectivity and sensitivity in body fluids has gained great importance in diagnosis and preventive medicine.

Several analytical methods have been developed for detection of HSA. One widely used method is the colorimetric technique, such as bromocresol green (BCG method) [6] or bromocresol purple [7]. However, this method suffers highly time-sensitivity of bromocresol dyes towards HSA. The dynamic interaction between dyes and other proteins will lead to an overestimated albumin concentration after a short incubation time (30 s), which makes it difficult to measure massive albumin samples in parallel [8]. Besides, the immunoassay exhibits extraordinary selective and time-insensitive towards HSA [9,10], however, the high cost of antibodies and time-consuming preparation procedures of immunoassays severely limited its wider application. Alternatively, fluorescence spectroscopy recently has become an upsurge of interest for practical protein analysis due to its non-invasion, real-time signal readout, high sensitivity and selectivity [11–13]. Up to now, several fluorescent probes for HSA in aqueous media have been reported, for example, aggregation induced emission (AIE) dyes [14,15], squaraine dyes [16–22], and other fluorescent dyes [23–31]. Most of these HSA probes, however, suffer from complicated fabrication, poor antijamming capability, or time-sensitive, which limit their practical application in situ screening. Therefore, it remains a challenge to explore novel, simple and portable fluorescent probes for recognition of HSA.

As a broad class of natural products, flavonoids have been extensively studied for their antioxidant properties and anticancer activities in the health and medical sciences [32,33]. Recent study suggested that during the transportation of flavonoid drugs in blood serum, the interaction between HSA and flavonoids was responsible for the pharmacokinetic and pharmacodynamic properties of their antioxidative activity [34]. Further study revealed that the specific affinity between chromone ring of flavonoids and central hydrophobic cavity (Sudlow site I of domain IIA) of HSA could lead to a strong binding of flavonoids to HSA [35–37]. However, most of natural flavonoids and commercial flavonoid drugs, such as baicalein and naringenin, show a decrease of fluorescence upon binding with HSA [38], which are not suitable to quantify HSA.

In order to obtain a practical HSA probe, as shown in Scheme 1, our basic design principles include: (1) Because of that a large number of hydrogen bonds on chromone (ring A) can increase the binding sites between flavonoids and albumins, thus increase the binding constants (K_{SV}) [39]. The chromone skeleton of flavonoid will be modified by amide group to enhance the hydrogen bond interaction between the probe and HSA; (2) The introduction of donor- π -acceptor (D- π -A) structure into the fluorophore (ring B) will provide intramolecular charge transfer (ICT) characteristics[40]. The fluorescent dyes undergoing ICT normally show representative solvatochromism, that is, the dyes may emit weak fluorescence in aqueous media, while exhibiting a dramatic fluorescence enhancement once binding with hydrophobic pocket in HSA. Bearing these in mind, we herein disclose a series of flavonoid-based D- π -A fluorescent dyes **1–3** with different electron donors. We found

that all three dyes exhibit stable fluorescence, and remarkable positive solvatochromism of dual emissions. Among three dyes, compound **1** showed the highest sensitivity, linearly fluorescence response, high selectivity for HSA over other proteins and biomolecules, thereby providing a promising method for quantification of HSA.

2. Experimental

2.1. Synthesis

2.1.1. *N*-(2-(4-(Dimethylamino)phenyl)-3-hydroxy-4-oxo-4H-chromen-6-yl)butyramide (1)—*N*-(3-Acetyl-4-hydroxyphenyl)butyramide (50 mmol) was added to a solution of the 4-(dimethylamino)benzaldehyde (50 mmol) in ethanol (100 mL), then 30 mL of aqueous NaOH (16 g, 400 mmol) solution was added slowly. The mixture was stirred at 60°C for 3 h, and then cooled to room temperature for another 12 h. H₂O₂ solution (20 mL of 30%) was slowly added into the reaction solution, which was placed in an ice-water bath. After stirring at room temperature for 48 h, the mixture was poured into ice water and then placed into the refrigerator overnight. The precipitate was collected *via* filtration, and washed with ethanol. The product was purified by recrystallization from Hexane/Ethanol (v/v = 1/1). Yield = 21%. ¹H NMR (*d*₆-DMSO, 500 MHz): δ = 10.13 (s, 1H, *-OH*), 9.07 (s, 1H, *-NH*), 8.40 (d, 1H, *J* = 2.5 Hz), 8.11 (d, 2H, *J* = 9.0 Hz), 7.89 (dd, 1H, *J*₁ = 9.0 Hz, *J*₂ = 2.5 Hz), 7.67 (d, 1H, *J* = 9.0 Hz), 6.85 (d, 2H, *J* = 9.0 Hz), 3.00 (s, 6H), 2.34 (t, 2H), 1.66 (m, 2H), 0.95 (t, 3H). ¹³C NMR (*d*₆-DMSO, 125 MHz): *d* = 157.43, 157.02, 139.20, 138.55, 135.40, 126.91, 125.79, 119.78, 116.29, 113.27, 110.68, 110.24, 105.65, 104.43, 41.42, 40.27, 22.88, 18.58. MS, calcd for [C₂₁H₂₂N₂O₄ + H]⁺ *m/z*: 367.2; found: 367.2

2.1.2. *N*-(2-(4-(Diethylamino)phenyl)-3-hydroxy-4-oxo-4H-chromen-6-yl)butyramide (2)—40 mmol of *N*-(3-acetyl-4-hydroxyphenyl)butyramide and 40 mmol of 4-(dimethylamino)benzaldehyde were added to 150 mL of ethanol, then 40 mL of aqueous NaOH (15 g, 375 mmol) solution was added slowly. The mixture was stirred at 70°C for 1 h, and then cooled to room temperature for another 24 h. 20 mL of H₂O₂ solution (30%) was slowly added into the reaction solution, which was placed in an ice-water bath. After stirring at room temperature for 12 h, the mixture was poured into ice water and then placed into the refrigerator overnight. The precipitate was collected *via* filtration, and washed with ethanol. The product was purified by recrystallization from Hexane/Ethanol (v/v = 3/1). Yield = 16%. ¹H NMR (*d*₆-DMSO, 500 MHz): δ = 10.15 (s, 1H, *-OH*), 9.02 (s, 1H, *-NH*), 8.39 (d, 1H, *J* = 2.5 Hz), 8.08 (d, 2H, *J* = 9.0 Hz), 7.88 (m, 1H), 7.65 (d, 1H, *J* = 9.0 Hz), 6.80 (d, 2H, *J* = 9.0 Hz), 3.44 (m, 4H), 2.33 (t, 2H), 1.66 (m, 2H), 1.15 (t, 6H), 0.93 (t, 3H). ¹³C NMR (*d*₆-DMSO, 125 MHz): *d* = 172.07, 171.76, 150.68, 148.89, 147.32, 137.25, 136.16, 132.29, 129.68, 125.31, 121.95, 118.92, 117.52, 113.26, 111.25, 44.17, 38.77, 18.98, 14.08, 12.91. MS, calcd for [C₂₃H₂₆N₂O₄ + H]⁺ *m/z*: 395.2; found: 395.2.

2.1.3. *N*-(2-(4-(Diphenylamino)phenyl)-3-hydroxy-4-oxo-4H-chromen-6-yl)butyramide (3)—*N*-(3-Acetyl-4-hydroxyphenyl)butyramide (10 mmol) was added to a solution of the 4-(diphenylamino)benzaldehyde (10 mmol) in ethanol (50 mL), then 50 mL of aqueous NaOH (4 g, 100 mmol) solution was added slowly. The mixture was stirred at 80°C for 6 h, and then cooled to room temperature for another 48 h. H₂O₂ solution (10 mL

of 30%) was slowly added into the reaction solution, which was placed in an ice-water bath. After stirring at room temperature for 96 h, the mixture was poured into ice water and then placed into the refrigerator overnight. The precipitate was collected *via* filtration, and washed with cold ethanol. The product was purified by recrystallization from ethanol for three times. Yield = 17%. ^1H NMR (*d6*-DMSO, 500 MHz): δ = 10.15 (s, 1H, $-\text{OH}$), 9.35 (s, 1H, $-\text{NH}$), 8.43 (d, 1H, J = 3.0 Hz), 8.09 (d, 2H, J = 9.0 Hz), 7.90 (dd, 1H, J_1 = 9.0 Hz, J_2 = 2.5 Hz), 7.66 (d, 1H, J = 9.0 Hz), 7.38 (t, 4H), 7.16 (m, 6H), 7.04 (d, 2H, J = 9.0 Hz), 2.37 (t, 2H), 1.65 (m, 2H), 0.95 (t, 3H). ^{13}C NMR (*d6*-DMSO, 125 MHz): δ = 172.76, 171.80, 150.88, 149.06, 146.85, 145.92, 138.44, 136.32, 130.23, 129.39, 125.80, 124.65, 124.50, 121.90, 121.19, 119.13, 113.23, 38.77, 18.96, 14.07. MS, calcd for $[\text{C}_{31}\text{H}_{26}\text{N}_2\text{O}_4 + \text{Na}]^+$ m/z : 513.2; found: 513.2

2.2. Polarity parameters, $E_T(30)$

$E_T(30)$ values are based on the negatively solvatochromic pyridinium *N*-phenolate betaine dye as probe molecule [41], and they are simply defined, in analogy to Kosower's Z values [42,43], as the molar electronic transition energies (E_T) of dissolved dye, measured in kilocalories per mole (kcal/mol) at room temperature and normal pressure (1 bar), according to following equation:

$$E_T(30)(\text{kcal/mol}) = 28591/\lambda_{\text{max}}(\text{nm}) [44]$$

Where λ_{max} is the wavelength of the maximum of absorption band of indicator dye.

2.3. Detection methods

The fluorescent probes were previously dissolved in little amount of DMSO (1 mM) as the stock probe solution. For general protein detection experiments, 10 μL of stock probe solution (1 mM probe in DMSO) was added into 1 mL protein aqueous sample (neutralized with 1 mM of HEPES). After 30 s of mixing, fluorescent emission was collected. For protein titration experiments, 10 μL of stock probe solution (1 mM probe in DMSO) was added into 1 mL of HEPES buffer (1 mM, pH=7) as testing buffer. Then, the high concentrated protein (0.1–10 mM in water) was titrated into 1 mL of testing buffer. After 30 s of mixing, fluorescent emission was collected.

3. Results and discussion

3.1. Synthesis and characterization

To better understand the structure-property relationship, three flavonoid-based D- π -A dyes, **1–3**, were synthesized. As shown in Fig. 1, the flavonoids were synthesized by reaction of arylaldehyde and *N*-(3-acetyl-4-hydroxyphenyl) butyramide in two classic steps by a Claisen-Schmidt condensation and Algar-Flynn-Oyamada reaction [45]. All these three compounds were consisting of electron withdrawing chromone moiety as electron acceptor, while the donor part was represented by the *N,N*-disubstituted benzylamine. The full synthetic routes and characterization were shown in the experimental section and Fig. S1–S6 in supporting information (SI).

3.2. Optical properties

As shown in Fig. 2a, the absorption and fluorescence spectra of flavonoid dyes were investigated in THF. Compound **1–3** showed the similar absorption maximum at 405 nm, 412 nm, and 404 nm, respectively. No obvious red-shifted absorption of compound **3** with respect to **1** is probably due to that the non-planar triphenylamine could not provide enlarged π -electron donating. Upon 400 nm excitation, the fluorescent spectra of compound **1** showed the well divided dual emissions with the maximum at 484 nm (Normal type, N^*) and 572 nm (Tautomer, T^*). The large Stokes shift ($N^* > 80$ nm, $T^* > 160$ nm) of flavonoid dyes could efficiently reduce self-absorption, as well as determine the high-resolution and low-detection limits [46]. The compound **2** and **3** also showed similar dual emissions as compound **1**, of which the ratios were different due to different proton transfer efficiency.

As shown in Fig. 3a–c, the fluorescence spectra of flavonoid dyes **1–3** in different solvents were studied. In aprotic solvents, all three dyes exhibited two well-separated emission bands locating at around 470 nm and 580 nm, corresponding to N^* and T^* emission, respectively. In MeOH and water solution, only one emission band (N^*) was found, and the T^* emission was completely quenched (Table S1 in SI) due to that the highly dipolar molecule could form the strong H-bond with ketone group of chromophore thus shutting down the proton transfer [47]. As shown in Fig. 3d–f, we found a very good correlation between the position of the emission maximum (both N^* and T^*) and the empirical polarity parameter $E_T(30)$ [44] for all three dyes in aprotic solvents, thus showing classical positive solvatochromism. It is because that upon electronic excitation, an ICT along D- π -A structure of flavonoid will greatly increase the dipole moment of dye molecule. An increase in the solvent polarity could decrease the energy of excitation state, resulting in a red shift in the emission spectra.

3.3. Fluorescence response to HSA

To obtain the fluorescence response to HSA, the fluorescent spectra of flavonoids **1–3** were measured in the presence of a collection of 9 different proteins, including HSA, lipase, casein, collagen, gelatin, fibrinogen, lysozyme, and trypsin (Fig. 4). Upon addition of 1 equiv. of HSA, the compound **1** exhibited a significant fluorescence enhancement of around 320-fold. Upon addition of other proteins, there were no obvious fluorescence changes, indicating that the compound **1** showed excellent selectivity toward HSA among all proteins. For compound **2**, 1 equiv. of HSA led to a 75-fold fluorescence enhancement, while most other proteins displayed a marginal fluorescence enhancement except for casein with a 20-fold increase. The fluorescence intensities of **1** and **2** in water in presence of HSA could reach to the similar level as in low-polar solvents (Fig. 3), suggesting the flavonoids successfully bound with hydrophobic pocket of HSA, which provided a low polarity microenvironment. Nevertheless, the compound **3** exhibited insensitive to all proteins, shown in Fig. 4c. It may be ascribed to steric hindrance of its triphenylamine group blocking the entrance of HSA cavity. These results suggested that the chemical structures of probe were directly responsible for their binding properties with HSA.

The HSA titration experiment was further investigated based on compound **1** and **2** 1 mM HEPES buffer containing 1% DMSO. As shown in Fig. 5, upon addition of HSA, the fluorescence of **1** and **2** were found to be remarkably enhanced, and around 320-fold and 75-

fold fluorescence enhancement was observed when 1.0 equiv. of HSA was added, respectively. Based on $3\sigma/k$ rule, the corresponding detection limits basen on compound **1** and **2** were as low as 94 nM and 380 nM, respectively. Both of detection limits could meet the requirements of traditional HSA assay in serum or in urine. Moreover, the detection limit of compound **1** containing amide group was much lower than its flavone derivate 2-(4-(dimethylamino)phenyl)-3-hydroxy-4*H*-chromen-4-one (DL = 78 $\mu\text{g/mL}$ 1.16 μM , $3\sigma/k$ rule) [28], indicating that amide group provided additional hydrogen bonding sites between flavonoids and albumins to increase the binding constant.

Generally, the correlation between fluorescence signal and the concentration of targets was considered as the prerequisite of the quantitative detection ability. From the inserts of Fig. 5, it was found that the fluorecence intensity of compound **1** at emission maxima showed an excellent linear correlation ($R^2 = 0.99$) to the amount of HSA from 0 to 10 μM , while the relationship between compound **2** and HSA was not a good linearity ($R^2 = 0.93$) especially in low concentration of HSA (from 0 to 3 μM). The result demonstrated that the compound **1** had the potential for quantitative detection of HSA.

3.4. Practical HSA sensing properties of compound **1**

Due to the excellent selectivity, low detection limit, and linear response of compound **1**, we focused on exploring its other practical sensing properties, such as tolerance of environmental pH, anti-interference properties, and time-insensitivity. Generally, pH value is an important factor that may influence HSA sensing. Especially in urine sample, uric acid could predominantly cause the acidification of sample. As shown in Fig. S7 (SI), the fluorescence intensity of **1** was relatively stable in acidic environment. The fluorescence change was below 10% over the pH range from 4 to 8, suggesting a high tolerance of compound **1** in acidic environment.

As shown in Fig. 6, the anti-interference capacity of compound **1** for HSA was then examined in the presence of various biological species probably existing in testing samples, including metal ions (Na^+ , K^+ , Mg^{2+} , Zn^{2+} , Cu^{2+} , Fe^{3+}), urea, potassium urate, and common biomolecules (PO_4^{3-} , cysteine, tryptophan, lysine), respectively. The result showed that most of biological species did not influence the fluorescence response for HSA. Only the addition of Cu^{2+} severely decreased the fluorescence of compound **1**, due to that the Cu^{2+} could bind with hydroxyl group of flavonoid leading to the fluorescence quenching. In consideration of the low concentration of Cu^{2+} in testing sample, it normally did not influence the detection of HSA.

The time-insensitive manner is also critical for high-throughput measurement of HSA samples in parallel. As shown in Fig. 7, our method based on compound **1** was compared with widely used BCG method. After 30 s incubation with HSA, the apparent concentration of HSA using BCG methods gradually increased, and the BCG method could overestimate the HSA concentration over 8% within 10 min. In contrast, the fluorescence signal of compound **1** was relatively stable. Therefore, due to its time-insensitivity, this method could provide a greater range of time points to accurately measure HSA concentrations.

4. Conclusion

In summary, we developed a series of amide-functionalized flavonoids probes **1–3** for recognition of human serum albumin in aqueous solution. All flavonoids could be easily prepared by a Claisen-Schmidt condensation and Algar-Flynn-Oyamada reaction, and showed positive solvatochromism on their dual emissions. Among three probes, the compound **1** showed the high sensitivity, the high selectivity and a quantitative response for HSA in aqueous solution, together with high tolerance of environmental pH, anti-interference properties, and time-insensitivity, suggesting a promising sensing method for HSA. Moreover, the structure-property relationship between flavonoid dyes and HSA-sensing abilities were also discussed. This new HSA-sensing method is believed good prospects in practical measurement of HSA in aqueous samples.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.
Synthesis routes of compound **1–3**.

Fig. 2.
The UV-vis absorption (a) and normalized fluorescence spectra (b) of 10 μM of compound 1–3 in THF. Excitation wavelength: 400 nm.

Fig. 3.

The solvatochromism of compound 1-3. (a-c) The fluorescence spectra of flavonoids in different solvents; (d-f) Dependence of the emission maxima of flavonoids on polarity parameters, $E_T(30)$. Excitation wavelength: 400 nm.

Fig. 4.

Fluorescence spectra of 10 μ M of (a) compound **1**, (b) compound **2**, and (c) compound **3** in the presence of different kinds of proteins (10 μ M), respectively. (d) Fluorescence response (F/F_0) of compound **1** (black bar), **2** (red bar), and **3** (blue bar) in the presence of 10 μ M protein, respectively. F : The fluorescence intensity of dye in the presence of proteins. F_0 : The fluorescence intensity of dye (for interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 5.

(a) Fluorescence spectra of 10 μM compound **1** upon addition of different concentration of HSA (0–10 μM). Insert: Plot of the fluorescence enhancement ($F/F_0 - 1$) at 508 nm to HSA concentrations. (b) Fluorescence spectra of 10 μM compound **2** upon addition of different concentration of HSA (0–10 μM). Insert: Plot of the fluorescence enhancement ($F/F_0 - 1$) at 517 nm to HSA concentrations. All experiments were performed in 1 mM HEPES buffer containing 1% DMSO, pH = 7.4. F : The fluorescence intensity of dye in the presence of HSA. F_0 : The fluorescence intensity of dye in the absence of HSA.

Fig. 6.

Fluorescence response (F/F_0 , $\lambda = 508$ nm) of compound **1** ($10 \mu\text{M}$) in the presence of $10 \mu\text{M}$ metal ions (Na^+ , K^+ , Mg^{2+} , Zn^{2+} , Cu^{2+} , Fe^{3+}), urea, potassium urate, and common biomolecules (PO_4^{3-} , cysteine, tryptophan, lysine) (black bar), followed by the addition of $10 \mu\text{M}$ HSA (red bar), respectively. F : The fluorescence intensity of dye in the presence of biomolecules. F_0 : The fluorescence intensity of dye. The red bar of first column was not added the second portion of HSA. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Fig. 7. Time-dependence of HSA detection method with bromocresol green (BCG) and our method based on compound **1**. The signal data at 30 s were normalized to 1.

Scheme 1.

The chemical structure of 3-hydroxyflavone and design principles of probe **1–3**.