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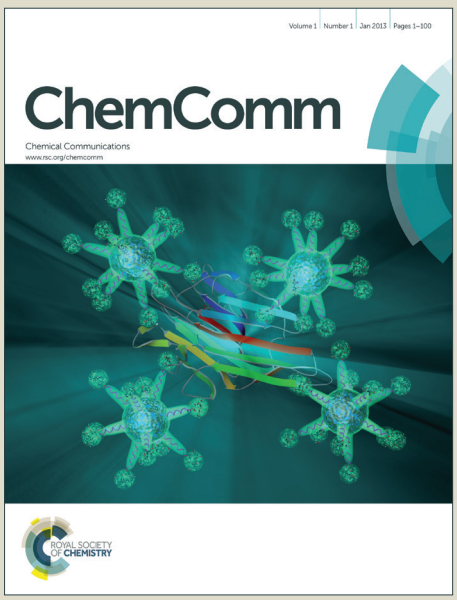

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ARTICLE TYPE

A Fluorescent Probe for the Site I Binding and Sensitive Discrimination of HSA from BSA

Jiangli Fan,*[a] Wen Sun,[a] Zhenkuan Wang,[a] Xiaojun Peng, [a] Yueqing Li*[b] and Jianfang Cao[a]

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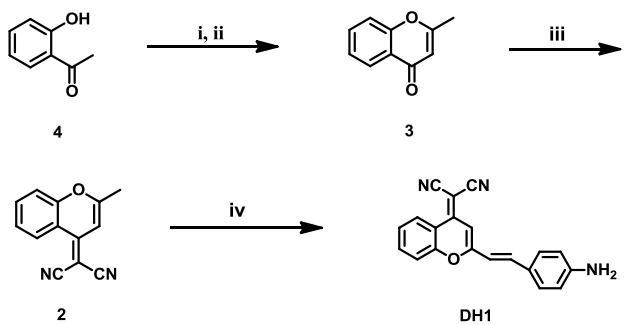
A fluorescent probe DH1 has successfully developed to detect HSA via Site I non-covalent bonding. DH1 shows dramatic fluorescence enhancement to HSA without interference from other proteins. Molecular docking method, for the first time, was utilized to show the deep insight into the sensing mechanism of the probe. Moreover, probe DH1 was successfully used to detect trace HSA in healthy human urine.

Human serum albumin (HSA) is the major constituent of blood plasma which plays an important role in maintaining the osmotic pressure of the blood compartment.[1] The pioneering work performed by Sudlow and co–workers three decades ago revealed that there are two major and structurally selective binding sites in HSA, namely, site I and site II.[2] Since a broad range of ligands can bind to HSA, many researchers have studied the structure and properties of this protein as well as its interactions with other molecules including various proteins, glycolipid and drugs.[3] The concentrations of serum albumin in body fluids could be considered as a reliable healthy indicator.[4] HSA in normal urine are less than 30 mg/L, while in serum, the normal range for albumin concentrations is approximately 35-55 g/L.[5] The presence of an excess amount of HSA in urine is called microalbuminuria, which is an early marker for cardiovascular disease and kidney disease in diabetes mellitus and hypertension.[6] On the other hand, a low level of HSA in the blood plasma, named hypoproteinemia, would be a sign for liver cirrhosis, failure, and chronic hepatitis.[7] Therefore, the accurate detection of HSA with high selectivity and low limitation in body fluids has a great clinical importance.

HSA, also available for therapeutic uses, is often used to replace lost fluid and help restore blood volume in trauma, burns and surgery patients. However, it is of great urgent to distinguish HSA and BSA in such a case. Because BSA is low cost and widely utilized as a replacement of HSA in many biochemical and pharmacological applications.[8] But actually, BSA only has 75.8% biological functions of HSA thus cannot replace HSA in many applications.[9] Misuses of the two proteins are likely to lead to fatal damage to patients. Therefore the distinction of HSA from BSA is a challenge and of great importance.

Fluorescence–based assays have found widespread application in the fluorescence imaging of various analytes because of the rapid, nondestructive, selective, and sensitive advantages of emission signals.[10] Although several fluorescent probes for serum albumin detection have been reported, we found that almost all probes had quite low excitation (< 500 nm) and emission wavelengths (< 600 nm) which could not avoid the interference from biological autofluorescence. Furthermore, most of fluorescent probes showed poor selectivity for HSA from BSA or their detection limits is not low enough (> 30 mg/L) to make them applicable in real practical fields.[11] In the clinical labs, radioimmunoassay (RIA), the commonly used method, has a good detection limit of 16 ng/L,[5] but it requires labeling proteins with radioactive isotopes. Therefore, it is of great importance to develop a sensitive probe for HSA determination in healthy human urine.

Dicyanomethylene–4H–chromene as well as its derivatives is a kind of important dye which is often utilized in many research fields such as electroluminescence (EL), nonlinear optical material (NLOM) and dye–sensitized solar cells.[12] Since this fluorophore has a long emission wavelength, often longer than 600 nm, its application in designing chemical molecular sensors has attracted an increasing attention.[13] Therefore, bearing these in mind, herein we present a fluorescent turn–on HSA probe **DH1** by introducing aromatic amine into dicyanomethylene –4H–chromene framework via double bonds to form a conjugated π–electron system. We found that **DH1** showed high selectivity and sensitivity for HSA over other biomolecules. Besides, the fluorescence responses of **DH1** towards HSA and BSA were quite different and this difference can help us easily discriminate the two species in different practical fields. Molecular docking method was firstly used to explain the interaction mechanism of **DH1** with site I of HSA.



Scheme 1 The structure and synthesis of **DH1** i) Na, $CH_3COOC_2H_5$, rt, 4 h, 53%; ii) AcOH, H_2SO_4, 120 °C, 30 min, 76.9%; iii) malononitrile, AcOH, H_2SO_4, 140 °C, 14 h, 32.5%; iv) toluene, piperidine, AcOH, 115 °C, 3h, 42.9%.

As depicted in **Scheme 1**, the reaction of 1–(2–hydroxyphenyl) ethanone with ethyl acetate followed by annulation reaction in CH_3COOH/H_2SO_4 for about 0.5 h to afford compound 3. This intermediate further reacted with malononitrile and then with N-(4-formyl-phenyl)acetamide to obtain the target molecules **DH1** which were well characterized by ^1H NMR, ^{13}C NMR, and HPLC–MS together with other important intermediates (Fig. S9–S12, ESI).

As is known, stable spectroscopic properties over a biologically relevant pH range and good photostability are necessary in complex biological environment. Since **DH1** was nonfluorescent in pure water solution, we utilized PBS buffer containing organic component (0.2 M, pH 7.4, 50% DMSO) as the test system which was just to guarantee the probe a certain level of fluorescence intensity. Acid/base titration revealed that probe **DH1** reached almost a constant value at pH 2.5–10, demonstrating that **DH1** can work in a wide pH range without influence for HSA detection (Fig. S1a). Under irradiation from a 500 W *I–W* lamp for more than 4 h, the maximal fluorescence intensity of **DH1** remained nearly constant showing its excellent photostability (Fig. S1b).

Next we examined the HSA–sensing performance of **DH1** in pure PBS buffer (0.2 M, pH 7.4). When excited at 520 nm, the probe itself was non-fluorescent, while the emission intensity at 620 nm increased dramatically with continuous addition of HSA to the test solution. When the amount of added HSA reached to 0.17 mg/mL, the fluorescence was found to reach a plateau, and a 70–fold fluorescence enhancement was obtained (Fig. 1a). The fluorescence intensity increased linearly with the concentration of HSA from 0 to 11.9×10^{-3} mg/mL (Fig. 1b). Thus, the detection limit (3σ/slope) was as low as 22.0 μg/L which was quite lower than that of other reported probes.[10]

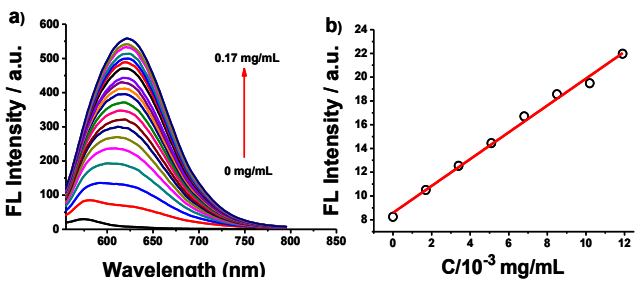


Fig. 1 a) Fluorescence emission spectra of **DH1** (5 μM) upon the addition of increasing concentrations of HSA (0–0.17 mg/mL) in PBS buffer (pH 7.4, 0.2 M). The arrow indicates the change in the emission intensity with the increased HSA; b) The fluorescence intensity at 620 nm of probe **DH1** (5 μM) was linearly related to the concentrations of HSA (0–0.012 mg/mL), Y = 8.5854 + 1.1292 * X, R= 0.994. Conditions: PBS buffer (pH 7.4, 0.2 M), λex = 520 nm.

The selectivity experiment of **DH1** was first performed with common ions which are found in environmental and biological settings. Fig. S2 and Fig. S3 showed that no fluorescence response occurred when these common ions were added into the solution of **DH1** (5 μM). Other proteins and biomolecules (0.17 mg/mL) including chymotrysin, protease, collagen, lysozyme, chymotrypsinogen A, haemoglobin, histone, L–Glutathione, DL–Homocysteine and L–cysteine exhibited almost no changes in fluorescence behaviour except HSA and BSA (Fig. 2). Interestingly, the fluorescence response of **DH1** toward HSA and BSA are quite different both in wavelength and fluorescence intensity. From the normalized fluorescence spectrum (Fig. S4), the emission wavelength of **DH1** after response to HSA and BSA are 620 nm and 645 nm respectively and the fluorescence-enhancement are 70–fold for HSA (620 nm) and 9–fold for BSA (645 nm) respectively. Such large differences allow easy distinguish of the two proteins. To the best of our knowledge, this is the first probe that can distinguish HSA and BSA through the difference of wavelength.

Since **DH1** constructs a strong 'pull-push' system (with an amino group as the electron donor and cyano group as the electron acceptor), it is sensitive to changes in the external environment. As seen from Fig. S5, **DH1** was expected to be polarity sensitive in different proportional 1,4-dioxane in water, and the fluorescent intensity increases as the environment becomes hydrophobic. We therefore proposed that the probe could interact with the protein's binding sites via noncovalent bonding and the polarity changes then caused the great changes of the fluorescence spectrum.

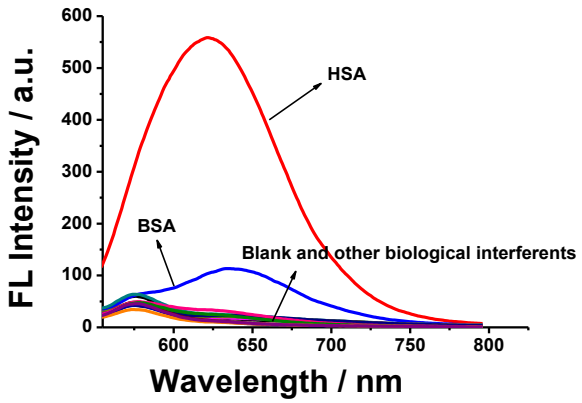


Fig. 2 Fluorescence responses of probe **DH1** (5 μM) to HSA，BSA and other biological interferents in PBS buffer (pH 7.4, 0.2 M). Data are shown for 0.17 mg/mL of HSA, BSA, chymotrysin, protease, collagen, lysozyme, chymotrypsinogen A, haemoglobin, histone, L–Glutathione, DL–Homocysteine and L–cysteine. λex = 520 nm.

The significantly different fluorescence response of **DH1** toward HSA and BSA interests us to find the internal cause. In an effort to identify the difference in 3D structures, the X–ray crystal structures of HSA compounded with phenylbutazone (PDB code 2BXP), HSA compounded with ibuprofen (PDB code 2BXG) and BSA (PDB code 4F5S) were collected from the PDB database (http://www.rcsb.org/pdb). Structural analysis demonstrated that the site II of BSA was similar to that of HSA (Fig. 3a). However, the site I of BSA was occupied by the Leu237 residue compared to the hollow site I of HSA (Fig. 3b and 3c). Therefore we speculated that the probe **DH1** selective binding to the site I of HSA.

To confirm that the additional Leu237 in the site I of BSA hampered insertion of the probe, **DH1**was docked with the drug binding site I in 4F5S, using LigandFit module in Discovery Studio 2.5. The docking simulation result showed that the **DH1** could not bind into the site I of BSA, while **DH1** could dock into

the site I of HSA (Fig S6b), in which hydrogen bonds play an important role in the protein-ligand interactions and make a great contribution to the binding affinity. In the crystal structure of HSA (2BXP), there was one hydrogen bond between carbonyl group of phenylbutazone and the guanidine group of Arg218 (Fig. S6a). However the cyano group and oxygen atom of **DH1** formed two hydrogen bonds with the guanidine groups of Arg218 and Arg257, separately (Fig. S6b). These docking results demonstrated that **DH1** selective bind to the site I of HSA which resulted in the difference of the fluorescent response from BSA.

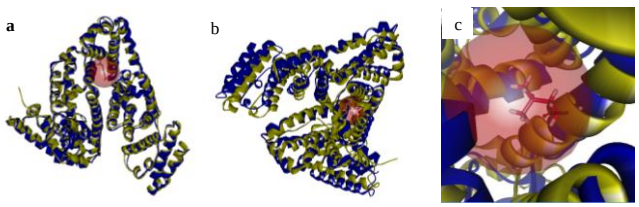


Fig. 3 A stereo view of the aligned crystal structures of serum albumins. a) HSA, blue (PDB code 2BXP); BSA, yellow (PDB code 4F5S); The binding site II with ibuprofen was shown by the transparent red ball; b) HSA, blue (PDB code 2BXG); BSA, yellow (PDB code 4F5S); the binding site I with phenylbutazone was shown by the transparent red ball; c) Detail view of the aligned binding site I showed that the Leu237 residue of BSA occupied the site I of BSA.

The favorable fluorescence properties of **DH1** for HSA prompted us to further establish its utility for the determination of HSA in biosystems. As HSA concentration in healthy urine is less than 30 mg/L and most dye-binding methods for HSA are not applicable to healthy subjects or appropriate for early diagnosis.[14] In our experiment, upon addition HSA (0–0.19 mg/mL) to 5 μM **DH1** urine (50% PBS buffer, pH 7.4, 0.2 M), the fluorescence intensity increased with the added concentration of HSA and got saturated when the amount of HSA reached to 0.19 mg/mL (Fig. S7). Human urine containing HSA at different concentrations was also prepared. These urine solutions were then incubated with 5 μM probe **DH1**. Fluorescence signals were measured and a good linear relationship was obtained in this solution with a detection limit (3σ/slope) of 5.51 mg/L (Fig. S8). Compared with RIA, the commonly used method in clinical labs, our proposed method is easy of operation and the detection limit is sufficient for HSA detection in human urine. The quantification experiment further confirmed that **DH1** was accurate and effective for quantitative detection of trance HSA in human urine (Tab. S1).

In summary, we have reported a new dicyanomethylene -4H-chromene based probe **DH1** that showed great sensing properties for HSA. **DH1** exhibited an obvious HSA induced large fluorescence enhancement in emission spectra without interference from different ions and other biomolecules that are commonly found in the environment or biosystems. Different fluorescence responses to HSA and BSA may provide us a sample method to selective discriminate the two similar proteins. Molecular docking method was firstly used to display the interactions between **DH1** and binding site I of HSA and different sensing process towards HSA and BSA. The practical applications showed that **DH1** can detect trace HSA in human urine. We expect this new probe to be useful in more chemical and medical applications.

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Notes and references

[a] *State Key Laboratory of Fine Chemicals, Dalian University of Technology, 2 Linggong Road, Dalian 116024, P.R. China.*
E-mail: fanjl@dlut.edu.cn
[b] *School of Pharmaceutical Science and Technology, Dalian University of Technology, 2 Linggong Road, Dalian 116024, P.R. China.*
E-mail: yueqingli@dlut.edu.cn

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