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A fluorescent probe for site I binding and sensitive discrimination of HSA from BSA†

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A fluorescent probe DH1 has been successfully developed to detect HSA *via* site I non-covalent bonding. DH1 shows a dramatic fluorescence enhancement towards HSA without interference from other proteins. The molecular docking method, for the first time, was utilized to provide 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 research work 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, glycolipids and drugs.[3] The concentrations of serum albumin in body fluids could be considered as a reliable healthy indicator.[4] HSA concentration in normal urine is less than 30 mg L^{-1}, while in serum, the normal range for albumin concentration is approximately 35–55 g L^{-1}.[5] The presence of an excess amount of HSA in urine results in microalbuminuria, which is an early marker of 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 of liver cirrhosis, failure, and chronic hepatitis.[7] Therefore, the accurate detection of HSA with high selectivity and a low detection limit in body fluids have a great clinical importance.

HSA, also available for therapeutic use, is often used to replace lost fluid and help restore blood volume in trauma, burns and surgery patients. It is of great urgency to distinguish HSA and BSA in such a case because BSA is of low cost and widely utilized as a replacement of HSA in many biochemical and pharmacological applications.[8] But actually, BSA has only 75.8% of the biological functions of HSA and thus cannot replace HSA in many applications.[9] Misuse of the two proteins is 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 measurement 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 the fluorescent probes showed poor selectivity for HSA over BSA or their detection limits is not low enough (>30 mg L^{-1}) to make them applicable in real practical fields.[11] In clinical labs, radioimmunoassay (RIA), the commonly used method, has a good detection limit of 16 ng L^{-1},[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-4*H*-chromene as well as its derivatives are important dyes which are 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 increasing attention.[13] Therefore, bearing these in mind, herein we present a fluorescent turn-on HSA probe **DH1** by introducing aromatic amine into the dicyanomethylene-4*H*-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. The molecular docking

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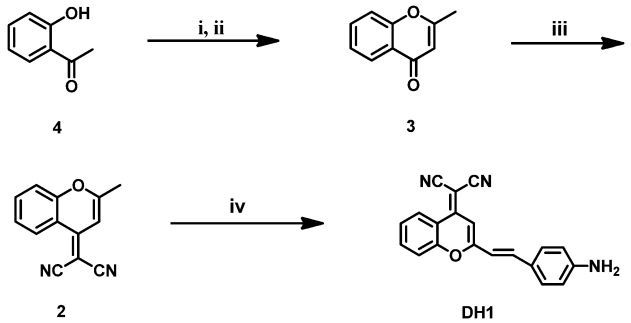

Scheme 1 The structure and synthesis of **DH1** (i) Na, $CH_3COOC_2H_5$, rt, 4 h, 53%; (ii) AcOH, H_2SO_4, 120 °C, 30 min, 76.9%; (iii) malononitrile, AcOH, H_2SO_4, 140 °C, 14 h, 32.5%; and (iv) toluene, piperidine, AcOH, 115 °C, 3 h, 42.9%.

method was for the first time used to explain the interaction mechanism of **DH1** with site I of HSA.

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 afforded compound **3**. This intermediate further reacted with malononitrile and then with *N*-(4-formyl-phenyl)acetamide to yield the target molecule **DH1**, which was well characterized by ^{1}H 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 a complex biological environment. Since **DH1** is nonfluorescent in pure water solution, we utilized a 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 over a wide pH range without any influence on HSA detection (Fig. S1a, ESI†). Upon irradiation with 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, ESI†).

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 0.17 mg mL^{-1}, 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 $\times$ 10^{-3} mg mL^{-1} (Fig. 1b). Thus, the detection limit (3σ/slope) was as low as 22.0 μg L^{-1}, which was quite lower than that of other reported probes.[10]

The selectivity experiment of **DH1** was first performed using common ions, which are found in environmental and biological settings. Fig. S2 and S3 (ESI†) show 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^{-1}) including chymotrypsin, protease, collagen, lysozyme, chymotrypsinogen A, haemoglobin, histone, L-glutathione, DL-homocysteine and L-cysteine exhibited almost no changes

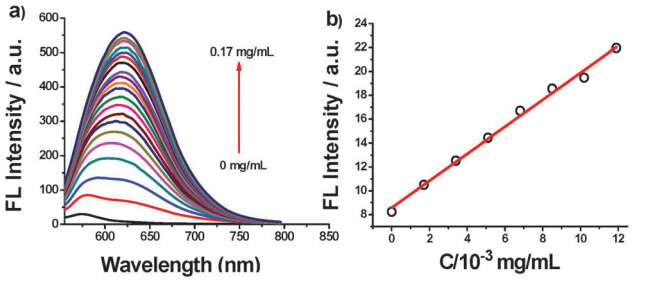

Fig. 1 (a) Fluorescence emission spectra of **DH1** (5 μM) upon addition of increasing concentrations of HSA (0–0.17 mg mL^{-1}) in PBS buffer (pH 7.4 and 0.2 M). The arrow indicates the change in the emission intensity with the increased HSA; (b) the fluorescence intensity at 620 nm of probe **DH1**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

in fluorescence behaviour except HSA and BSA (Fig. 2). Interestingly, the fluorescence responses of **DH1** toward HSA and BSA are quite different both in wavelength and fluorescence intensity. From the normalized fluorescence spectrum (Fig. S4, ESI†), the emission wavelength of **DH1** after response to HSA and BSA is 620 nm and 645 nm, respectively, and the fluorescence-enhancement is 70-fold for HSA (620 nm) and 9-fold for BSA (645 nm) respectively. Such large differences allow us to easily distinguish the two proteins. To the best of our knowledge, this is the first probe that can distinguish HSA and BSA through the difference in wavelengths.

Since **DH1** constructs a strong 'pull–push' system (with an amino group as the electron donor and a cyano group as the electron acceptor), it is sensitive to changes in the external environment. As seen from Fig. S5 (ESI†), **DH1** was expected to be polarity sensitive in different proportional 1,4-dioxanes 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* non-covalent bonding and the polarity changes then caused great changes in the fluorescence spectrum.

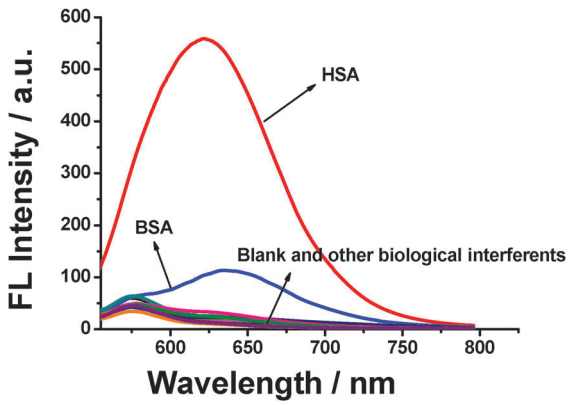

Fig. 2 Fluorescence responses of probe **DH1** (5 μM) to HSA, BSA and other biological interferents in PBS buffer (pH 7.4 and 0.2 M). Data are shown for 0.17 mg mL^{-1} of HSA, BSA, chymotrypsin, protease, collagen, lysozyme, chymotrypsinogen A, haemoglobin, histone, L-glutathione, DL-homocysteine and L-cysteine. λ_{ex} = 520 nm.

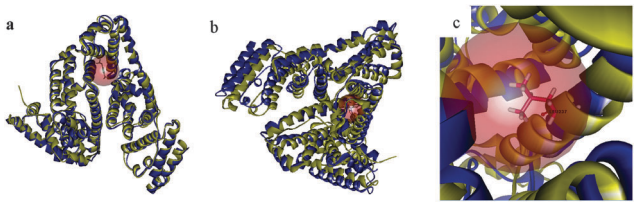

Fig. 3 A stereoview of the aligned crystal structures of serum albumins. (a) HSA, blue (PDB code 2BXP); BSA, yellow (PDB code 4F5S); binding site II with ibuprofen is shown by the transparent red ball; (b) HSA, blue (PDB code 2BXG); BSA, yellow (PDB code 4F5S); binding site I with phenylbutazone is shown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

The significantly different fluorescence responses of **DH1** toward HSA and BSA motivated 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) and 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 site II of BSA was similar to that of HSA (Fig. 3a). However, site I of BSA was occupied by the Leu237 residue compared to the hollow site I of HSA (Fig. 3b and c). Therefore we speculated that probe **DH1** selectively binds to site I of HSA.

To confirm that the additional Leu237 in site I of BSA hampered the insertion of the probe, **DH1** was docked with the drug binding site I in 4F5S, using the LigandFit module in Discovery Studio 2.5. The docking simulation results showed that **DH1** could not bind to site I of BSA, while **DH1** could dock into site I of HSA (Fig. S6b, ESI†), 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 the carbonyl group of phenylbutazone and the guanidine group of Arg218 (Fig. S6a, ESI†). However the cyano group and oxygen atom of **DH1** formed two hydrogen bonds with the guanidine groups of Arg218 and Arg257 separately (Fig. S6b, ESI†). These docking results demonstrated that **DH1** selectively binds to site I of HSA, which resulted in the difference in the fluorescence response from BSA.

The favorable fluorescence properties of **DH1** for HSA detection prompted us to further establish its utility for the determination of HSA in biosystems, as the HSA concentration in healthy urine is less than 30 mg L^{-1} and most dye-binding methods for HSA are not applicable to healthy subjects or appropriate for early diagnosis.[14] In our experiment, upon addition of HSA (0–0.19 mg mL^{-1}) to 5 μM **DH1** urine (50% PBS buffer, pH 7.4, and 0.2 M), the fluorescence intensity increased with the added concentration of HSA and got saturated when the amount of HSA reached 0.19 mg mL^{-1} (Fig. S7, ESI†). 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^{-1} (Fig. S8, ESI†). Compared with RIA, the commonly used method in clinical labs, our proposed method is easy to operate 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 trace HSA in human urine (Table S1, ESI†).

In summary, we have reported a new dicyanomethylene-4*H*-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 simple method to selectively discriminate the two similar proteins. The molecular docking method was for the first time used to display the interactions between **DH1** and binding site I of HSA and different sensing processes 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 for more chemical and medical applications.

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