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A selective and sensitive fluorescent albumin probe for the determination of urinary albumin†

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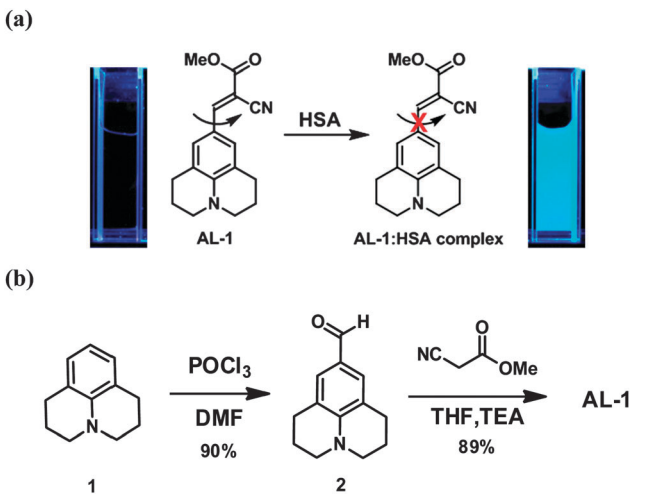
In this communication, we report a simple albumin probe based on a fluorescent molecular rotor for the detection of trace albumin levels in urine. In the presence of albumin, the probe exhibits remarkable 400-fold fluorescence enhancement with high selectivity and sensitivity. The probe was successfully applied in the quantitative detection of urinary albumin.

Human serum albumin (HSA), with a normal concentration range between 35 and 50 g L^{-1} in blood plasma, is the most abundant protein in the circulatory systems. In contrast, levels of albumin in urine are normally below 30 mg L^{-1}, as the kidneys can prevent useful substances such as albumin and other proteins from entering urine. However, when the filtering ability of the kidneys is damaged, elevated urinary albumin levels or albuminuria can occur.[1] Several studies have identified albuminuria as a key indicator for the intensified treatment of diabetes mellitus and for the early diagnosis of renal disease.[2] Furthermore, albuminuria is also one of the most important predictors of cardiovascular disease in non-diabetic individuals.[3] Therefore, the quantitative detection of albumin in biological fluids especially in urine, has gained much importance in diagnosis and preventive medicine during the past few decades.[4]

Although various methods have been utilised successfully for the detection of albumin, including immunoassays, capillary electrophoresis, colorimetric and fluorescent probes, these methods are inadequate for the practical routine analysis of urine samples to quantify low concentrations of albumin, particularly in the 30 mg L^{-1} region that covers the usual cutoff limits between normal and increased albumin excretion.[5] While immunoassays are sufficiently responsive and selective for detecting albuminuria, the high costs of antibodies and multistep operation procedures have limited their application for the routine screening of urine samples.[6] Although colorimetric dyes such as bromocresol purple have been used extensively in clinical laboratories to quantify albumin in blood plasma, they are not suitable to determine trace urinary albumin due to the low sensitivity and selectivity of the dyes.[7] On the other hand, fluorescent probes have several advantages over immunoassays and absorption techniques, particularly in the gain of sensitivity, lower cost of production and technical simplicity. Many fluorescent probes, especially those based on environment-sensitive fluorophores have been reported to detect HSA by binding to the HSA hydrophobic domain.[8] However, many of them either do not show high selectivity toward albumin or require long synthetic steps to prepare the fluorescent probes.



Scheme 1 (a) Schematic illustration of the fluorescent probe **AL-1** for HSA detection. In the absence of HSA, **AL-1** shows weak fluorescence due to unrestricted torsional rotation. Binding of **AL-1** to HSA imposes restricted bond rotation to the probe and lead to a dramatic fluorescence increase. The images show 2 μM **AL-1** in a cuvette before (left) and after addition of 20 μM HSA (right) under excitation using a UV lamp (365 nm). (b) Synthesis of **AL-1**.

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In this communication, we report on the use of a simple and novel fluorescent probe **AL-1** for the determination of albumin levels in urine samples (Scheme 1a). **AL-1** belongs to a class of fluorescent dyes called molecular rotors. Previously, fluorescent molecular rotors have been used to investigate the viscosity of blood plasma,[9] plasma membranes and cytoplasm of living cells[10] as well as for the rapid detection of biomolecular interactions.[11] These molecules are characterized by a charge-transfer-excited singlet state which can be rapidly deactivated through intramolecular rotation about the donor–acceptor bond. The fluorescence of molecular rotors is mainly sensitive to the viscosity of the environment and to a smaller extent, solvent polarity. Thus, in a highly constrained environment such as glycerol, a large fluorescence increase can be observed due to the restricted bond rotation (Fig. S1, ESI†). As HSA contains several hydrophobic binding sites for many hydrophobic drugs, we expect that the binding of hydrophobic **AL-1** to HSA can impose restricted bond rotation on the probe and lead to a dramatic fluorescence increase. **AL-1** can be prepared easily in two synthetic steps, requiring only one purification step with a total yield of 80% (Scheme 1b). **AL-1** is water-soluble and does not form aggregates in aqueous buffer at concentrations under 25 μM (Fig. S2, ESI†). The high solubility of the hydrophobic **AL-1** molecule in pH 7.4 PBS buffer may be due to the protonation of nitrogen at the bicyclic ring.

In aqueous buffer, **AL-1** shows extremely weak fluorescence (ε = 30 531 M^{-1} cm^{-1} and ϕ = 0.0022) as would be expected for the probe for completely unrestricted torsional rotation. In the presence of HSA, the fluorescence of **AL-1** increases significantly (ε = 52 342 M^{-1} cm^{-1} and ϕ = 0.5572) and can be observed directly under a handheld UV lamp (Scheme 1a). The binding of **AL-1** to HSA induces fluorescence enhancement of about 400-fold which is higher than most of the other HSA fluorescent probes reported to date (Fig. 1a). In comparison, a previously reported fluorescent albumin probe based on a different type of fluorescent molecular rotor exhibited only moderate fluorescence enhancement of around 36-fold in the presence of albumin.[12]

When HSA was added in increasing concentrations, the probe showed a concentration dependent fluorescence enhancement (Fig. 1a, inset). We also observed a slight blue-shift in the absorption and emission spectra of **AL-1** upon binding to HSA. In the absence of HSA, **AL-1** displays an absorption maximum at 464 nm and an emission maximum at 500 nm, as compared to a maximum at 456 nm and 490 nm, respectively, in the presence of HSA (Fig. S3, ESI†). The limit of detection (LOD) for **AL-1** to detect HSA was determined to be as low as 6 nM (0.4 mg L^{-1}) and the fluorescence response was linear in the range of 0–1 μM (Fig. S4, ESI†). In an attempt to optimize the fluorescence enhancement for the HSA detection, we synthesized several **AL-1** derivatives and found that the hydrophobic bicyclic ring and the ester moiety of **AL-1** are important to induce large fluorescence enhancement, as derivatives without these moieties generally gave lower fluorescence turn-on ratios in the presence of albumin (Fig. S5, ESI†). We believe that the ester functional group might also participate in the bonding with HSA, leading to

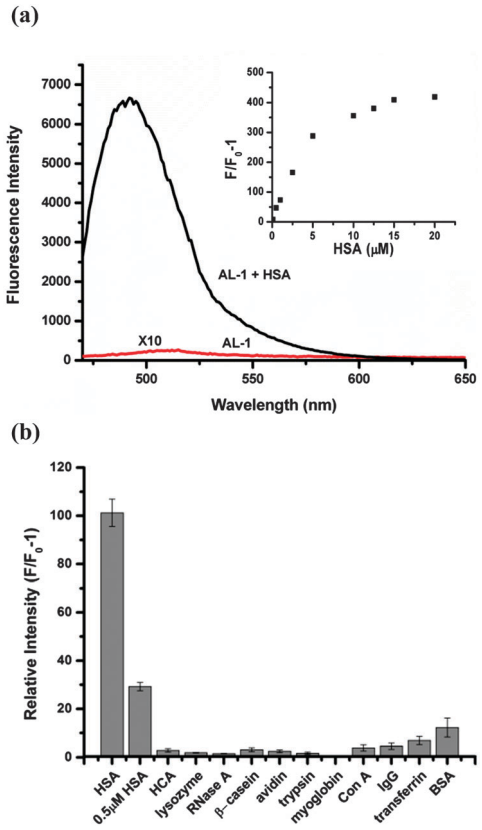

Fig. 1 (a) Fluorescence spectra of 2 μM **AL-1** in the presence of 12.5 μM HSA. The fluorescence spectrum of free **AL-1** was magnified 10-times. The inset shows the fluorescent titration curve of 2 μM **AL-1** with increasing HSA concentration. λ_{ex} = 440 nm, λ_{em} = 490 nm. (b) Relative fluorescence intensity of 2 μM **AL-1** with different proteins. The concentrations of HSA are at 2 μM and 0.5 μM while all others are at 20 μM except BSA which was tested at 2 μM. HCA = human carbonic anhydrase, RNase A = Ribonuclease A, ConA = Concanavalin.

increased hydrophobic interaction and better positioning of the probe in the binding pocket of HSA, hence contributing significantly to the high fluorescence increase.

Besides the superior sensitivity and an extremely high fluorescence activation ratio, **AL-1** also shows excellent selectivity toward HSA when tested against a collection of 11 different proteins (Fig. 1b). In the presence of 0.5 μM and 2 μM HSA, **AL-1** exhibits fluorescence enhancement of around 30- and 100-fold, respectively, while other proteins displayed only a marginal fluorescence increase even with a 10-fold increase in concentration at 20 μM. It is also remarkable to note that **AL-1** is highly selective between HSA and BSA, as the three-dimensional structures of albumins from different species are assumed to be very similar based on their highly homologous primary sequences.[13]

A Job's plot analysis was performed to determine the stoichiometry of the complex formed between **AL-1** and HSA (Fig. 2a). The fluorescence intensity of **AL-1** peaked at 1 : 1 mole fraction of **AL-1** to HSA which indicates that the probe binds mainly at one site of the protein. A relatively high HSA binding affinity with the dissociation constant (K_d) of 1.77 ± 0.65 μM

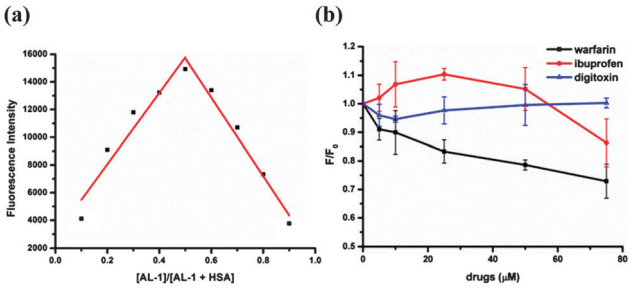

Fig. 2 (a) Job plot analysis. **AL-1** was mixed with HSA at different ratios in PBS buffer (pH = 7.4) while maintaining the total concentration at 10 μM. (b) Displacement of **AL-1** from the HSA–**AL-1** complex by the addition of site-specific drugs, warfarin, ibuprofen and digitoxin, respectively.

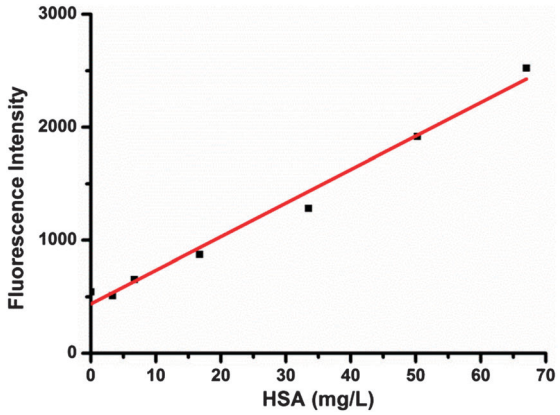

Fig. 3 Fluorescence response of 10 μM **AL-1** in a urine sample spiked with various concentrations of HSA (0.67 mg L^{-1} to 67 mg L^{-1}). $y = 29x + 429$, $R^2 = 0.98$. The albumin level was determined to be 14.7 mg L^{-1}.

was obtained, when a one-site binding model was fitted to the titration of **AL-1** with increasing concentrations of HSA. The characterization of the drug binding sites on HSA is an important step to understand its binding properties for predicting potential drug interactions, X-ray crystallography studies have identified several drug binding sites on HSA with subdomains IIA and IIIA being identified as its primary drug binding sites.[14] To verify the binding site of **AL-1**, a competitive assay to displace **AL-1** (2 μM) from HSA (5 μM) was conducted with three site-specific drugs including warfarin (domain IIA), ibuprofen (domain IIIA) and digitoxin (domain IIIA). Warfarin exhibited concentration dependent displacement of **AL-1** and about 30% of **AL-1** was displaced by 75 μM warfarin, while both ibuprofen and digitoxin showed almost no change in fluorescence (Fig. 2b). These results clearly indicate that the fluorescence turn-on response of **AL-1** is due to its specific binding to the domain IIA of HSA. Furthermore, we also determined the fluorescence lifetime of **AL-1** and found that the probe exhibits bi-exponential fluorescence decay, which is longer in the presence of HSA (Fig. S6, ESI†). In the absence of HSA, **AL-1** exhibits fluorescence lifetimes of $\tau_1 = 0.028$ ns and $\tau_2 = 0.89$ ns, while in the presence of HSA, the probe displays fluorescence lifetimes of $\tau_1 = 0.19$ ns and $\tau_2 = 1.19$ ns, respectively. The results are consistent with the nature of the fluorescent molecular rotors which show longer fluorescence lifetimes in a restricted environment.[15]

The direct quantification of albumin levels in urine is usually hampered by the interference of other biological substances and high background fluorescence of urine. Being highly selective and sensitive with high fluorescence turn-on upon binding to HSA, we believe that **AL-1** can be applied directly to determine the albumin concentration of urine. To validate the practicality of the application, three urine samples collected from three healthy male donors without medical history were spiked with various concentrations of HSA (from 10 nM to 1 μM; 0.67 mg L^{-1} to 67 mg L^{-1}) and the fluorescence response was measured in microtiter plates without further dilution. We observed an excellent linear correlation between the fluorescence increase of **AL-1** and the amount of HSA in the urine samples (Fig. 3). With the standard addition method, the urinary albumin levels of the male donors were determined to be 12.9 mg L^{-1}, 2.6 mg L^{-1}, and 14.7 mg L^{-1}, respectively. The urine samples were also tested by immunoassay for the validation of the albumin levels determined by **AL-1**, which were determined to be 17.3 mg L^{-1}, 3.2 mg L^{-1}, and 9.7 mg L^{-1}, respectively (Fig. S7, ESI†). As the background fluorescence emission spectrum of urine is similar to that of the HSA bound **AL-1**, this might potentially lead to errors in determining albumin levels. However, upon comparing the albumin levels obtained by using **AL-1** and immunoassay, we found that the albumin levels detected by our probe are in a close range to the values obtained by using immunoassay. Thus, we believe that our probe can provide precise albumin levels in urine.

In addition, we also compared our **AL-1** probe with another albumin binding dye, bromocresol purple (BCP), for the detection of urinary albumin. Another three urine samples collected from the same healthy male donors on different days were used for the comparison. As BCP has lower sensitivity for albumin, the urine samples were concentrated 10-fold before BCP measurement. Using the BCP dye, we determined the albumin concentration in the three samples to be 8.6 mg L^{-1}, 4.5 mg L^{-1}, and 14.9 mg L^{-1}, respectively (Fig. S8, ESI†), while the albumin levels of the same samples using **AL-1** were determined to be 3.2 mg L^{-1}, 20 mg L^{-1}, 24.8 mg L^{-1}, respectively. The results showed that the albumin levels detected by our fluorescent probe **AL-1** displayed some deviations from the BCP method. As compared to the BCP dye, we believe that our probe yielded more accurate results as it is more sensitive and does not require the concentration of the urine samples, which might have incurred the loss of some albumin during concentration steps. Thus, **AL-1** is a robust and rapid albumin probe which can be applied to routine urine screening for albuminuria. The albumin levels of the three samples show much different values because the urinary albumin concentration is dependent on the hydration status of the person. When the person is well hydrated, the urinary albumin concentration will be low because of dilution and *vice versa*.

In conclusion, we have developed a highly sensitive and selective fluorescent probe for albumin quantification in urine. Our fluorescent molecular rotor probe **AL-1** exhibits remarkable

fluorescence enhancement of 400-fold in the presence of HSA with a detection limit of around 6 nM. **AL-1** displayed high binding affinity with HSA and was found to bind specifically to the domain IIA of HSA by displacement assay. The probe was used in the quantitative detection of albumin level in urine samples from several healthy donors and the results were validated by immunoassay. As compared to the immunoassay, quantifying urinary albumin with **AL-1** is advantageous due to the low-cost, rapid detection time and a facile operation procedure, which can be completed within 30 minutes. Furthermore, **AL-1** is stable for long-term storage as no degradation was observed for **AL-1** in the DMSO stock solution for at least 6 months (Fig. S9, ESI†). Finally as **AL-1** is simple in structure, very easy to synthesize and stable for long-term storage, we believe that this fluorescent albumin probe presents a low-cost alternative to immunoassay for the practical application in the analysis of low level urinary albumin.

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