Fluorescence probes for thiol-containing amino acids and peptides in aqueous solution†

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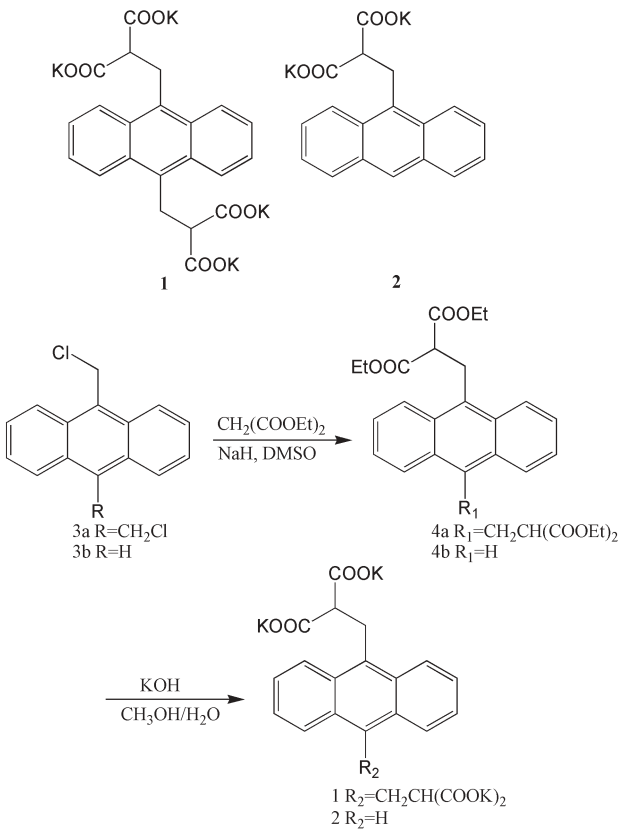
A new fluorescence probe is described using the "chemosensing ensemble" method. The probe shows high selectivity and sensitivity for thiol-containing amino acids and peptides, and can detect cysteine and homocysteine from healthy to abnormal levels under physiologically-relevant conditions.

Thiol-containing amino acids and peptides play many crucial roles in biological systems. For example, the level of homocysteine in plasma is an indicator for disorders including cardiovascular and Alzheimer's disease;[1] a deficiency of cysteine causing several health problems.[2] The level of thiol-containing amino acids and peptides in plasma has also been linked to AIDS.[3] Due to their important roles in biological systems, great attention has been paid to the detection of thiol-containing amino acids and peptides.[4] In order to replace the traditional detection methods, which require high performance liquid chromatography or capillary electrophoresis separations and toxic reagents, new methods are being developed. Most of these can be classified in two ways. One way is through covalent interaction between the receptor and the analyte, either *via* thiol groups alone or *via* both thiol and amine groups of the analyte, causing changes to its absorptional or emissional properties.[5] The other way is through non-covalent interaction between the receptor and the analyte, the so-called "chemosensing ensemble" approach.[6] In this method, the fluorescent indicator is bound to the receptor through non-covalent interactions (such as coordination), and the fluorescence of the indicator is either quenched or enhanced by the receptor. When the analyte displaces the indicator, the solution's fluorescence recovers.[7] Recently, another kind of homocysteine probe was reported using the redox chemistry of the thiol, based on absorption spectra.[8]

We are interested in exploring further the use of the "chemosensing ensemble" approach to selectively detecting thiol-containing amino acids and peptides under physiologically-relevant conditions. Our goal is to use simple, water soluble and sensitive fluorescence indicators to selectively detect thiol-containing amino acids and peptides.

Water soluble compound **1** was chosen as the indicator, its synthesis being shown in Scheme 1. Cu^{2+} was used as the receptor for quenching the fluorescence of the indicator. In **1**, the anthracene substructure was used as a fluorescence signalling unit and the carboxylate groups acted as binding sites. Since carboxylate groups are good ligands for Cu^{2+}, and **1** is multidentate, so only amino acids and peptides that have another coordinating site on the side chain can displace the indicator from the "chemosensing ensemble" probe, **1*Cu**. Due to the strong affinity of thiol groups for Cu^{2+}, we reason that the thiol-containing amino acids and peptides will complex preferentially to Cu^{2+} compared to **1**; that is, thiol-containing amino acids and peptides will recover the fluorescence from **1*Cu**. Additionally, in order to better understand how to apply the "chemosensing ensemble" method, a control indicator **2** was also synthesized, which had a different binding constant to Cu^{2+} compared to **1**.

As expected, upon addition of Cu^{2+}, the fluorescence of **1** and **2** was quenched. However, the absorption responses of **1** and **2** were different. A new peak was observed in the UV-Vis spectrum when **1** was titrated with Cu^{2+}, this peak being assigned to the complex of **1** and Cu^{2+}. However, the absorption spectrum showed little change after large excess of Cu^{2+} was added to **2**. The fluorescence titration profiles indicated that a 1 : 1 adduct was formed between



Scheme 1 Syntheses and structures of compounds **1** and **2**.

† Electronic Supplementary Information (ESI) available: Experimental details for the syntheses of **1** and **2**, fluorescence spectra of **1*Cu** and **2*Cu**, as well as the UV-Vis spectra. See http://www.rsc.org/suppdata/cc/b5/b503772g/

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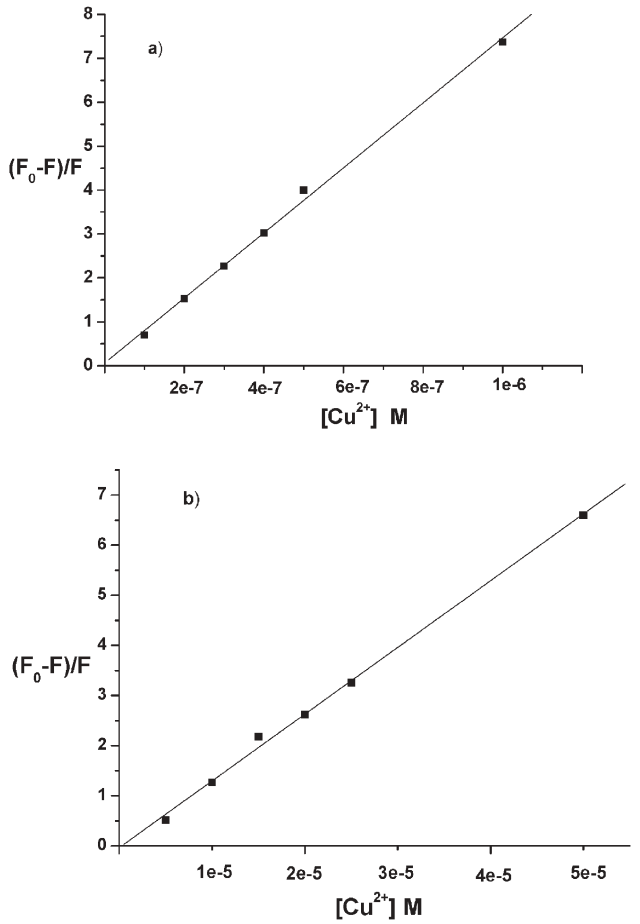

Fig. 1 Fluorescence titrations of **1** and **2** with Cu^{2+}: (a) **1** (5×10^{-7} M). (b) **2** (5×10^{-6} M). F_0 is the fluorescence intensity of the solution without Cu^{2+}.

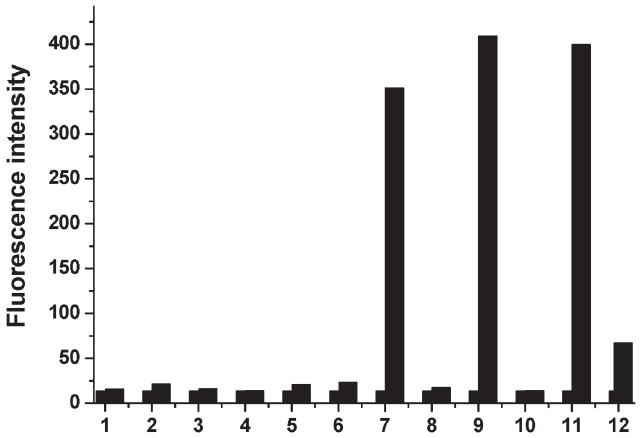

Fig. 2 Fluorescence intensity changes of **1*Cu** (5×10^{-7} M) before and after the addition of 10 equiv. of amino acids and peptides: 1. glycine, 2. glutamic acid, 3. methionine, 4. arginine, 5. tyrosine, 6. tryptophan, 7. glutathione, 8. serine, 9. cysteine, 10. ornithine, 11. homocysteine, 12. histidine.

2 and Cu^{2+}, with a binding constant of 1.5×10^5 (Fig. 1). Since **1** has two binding sites, so 1 : 1 and 1 : 2 adducts may be formed. From Fig. 1 we can conclude that when the ratio of Cu^{2+} : **1** is below 2, the 1 : 1 adduct dominates in solution. The binding constant for this adduct is $\sim 7.9 \times 10^6$, around 50 times larger than that of **2*Cu**.

The selectivity of "chemosensing ensemble" probe **1*Cu** for amino acids and peptides was proved in aqueous solution (Fig. 2). The non-fluorescence "chemosensing ensemble" probe was formed *in situ* by dissolving **1** and Cu^{2+} in de-gassed, de-ionized water (the molar ratio of **1** : Cu^{2+} was 1 : 2 to quench the fluorescence completely). As expected, when 10 equiv. of common amino acids and peptides with typical side-chain functional groups were added, only L-cysteine, homocysteine and L-glutathione, the thiol-containing amino acids and peptides, induced almost total fluorescence recovery of the solution. Other amino acids, namely L-glycine, L-glutamic acid, methionine, L-arginine, L-serine, L-ornithine, L-histidine, L-tyrosine and L-tryptophan, slightly increased the fluorescence intensity of the solution (histidine recovers $\sim 12\%$ fluorescence).

We believe it is the coordination of thiol-containing amino acids and peptides to Cu^{2+} that leads to the recovery of the solution's fluorescence. When probe **1*Cu** was titrated with cysteine, the absorption peak of free **1** was observed in the UV-Vis spectrum. This indicates, (i) there was no chemical reaction between **1** and cysteine and (ii) the fluorescence recovery was due to the free **1** in solution. In fluorescence spectra, after addition of thiol-containing amino acids, the fluorescence intensity of the solutions was time-dependent. This excludes the possibility that the redox chemistry between thiols and Cu^{2+} caused the recovery of fluorescence.[9]

Probe **1*Cu** was titrated with cysteine and homocysteine respectively in aqueous solution. The apparent binding constants for the interaction of **1*Cu** with cysteine and homocysteine at pH 7 were calculated from the competitive titration profiles, equating to 1.8×10^5 and 3.3×10^5 respectively.[10] Probe **1*Cu** was then studied under physiologically-relevant conditions (0.1 M NaCl, 0.05 M HEPES) (Fig. 3). From Fig. 3 it is apparent that probe **1*Cu** can detect both cysteine and homocysteine at both healthy and abnormal levels.

Compound **2**, the control indicator, was also studied as a potential "chemosensing ensemble" probe. However, owing to its low binding constant to Cu^{2+}, the control probe **2*Cu** did not show any remarkable selectivity for thiol-containing amino acids and peptides in aqueous solution. From this observation, we conclude that the binding constant of the indicator to the receptor is an important factor in successfully applying the "chemosensing ensemble" method.

In conclusion, a simple, water soluble "chemosensing ensemble" probe for thiol-containing amino acids and peptides was described. The probe, **1*Cu**, containing the low toxicity metal ion Cu^{2+} as the receptor was generated *in situ*. Using fluorescence monitoring as the detection method, the probe showed high selectivity for thiol-containing amino acids and peptides, detecting cysteine and homocysteine at healthy and abnormal levels under physiologically-relevant conditions. From this study, "chemosensing ensemble" probes have been shown to have great advantages in the detection of amino acids, peptides and other biomolecules. For example, there is the capability of detecting amino acids selectively by fine-tuning the binding constants of the indicator and receptor

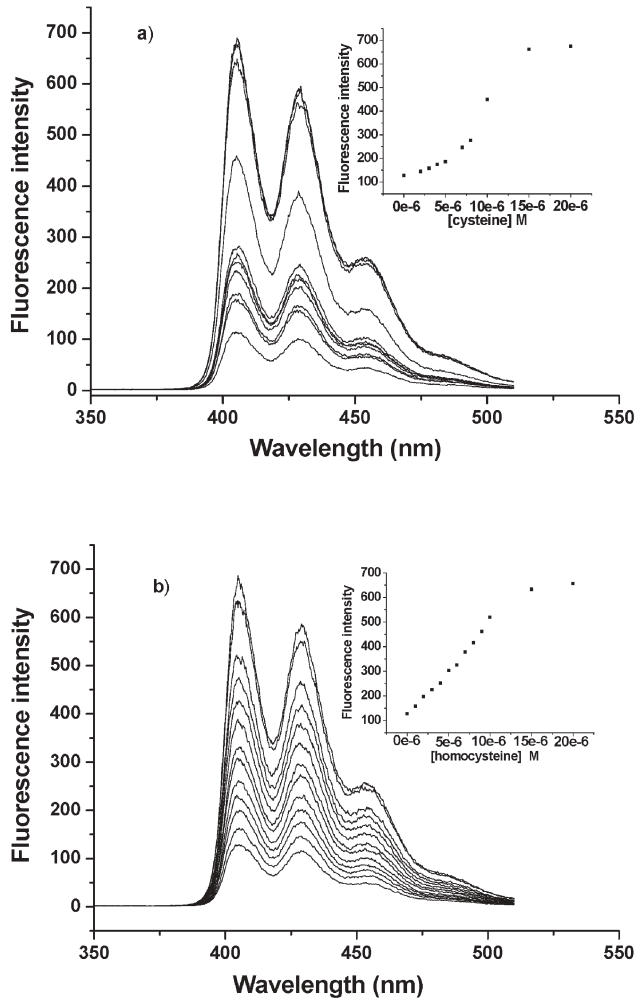

Fig. 3 Titration of **1*Cu** (10^{-6} M) with thiol-containing amino acids (a) cysteine and (b) homocysteine in a buffer solution (0.1 M NaCl, 0.05 M HEPES). Insets: Fluorescence intensity changes with the addition of analytes.

by changing the receptor (metal ion) or the binding site of the indicator. Work is now in progress in our lab to use "chemosensing ensemble" probes to detect various other amino acids and biomolecules.‡

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Notes and references

‡ Spectral data of compound **1**: δ_H NMR (300 Hz, D_2O) 8.31 (4 H, m), 7.47 (4 H, m), 4.00 (4 H, d), 3.41 (2 H, t). HRMS (FAB^-) Found: m/z = 522.9600 [M^--H]. $C_{22}H_{14}O_8K_3$ requires 522.9605. Spectral data of compound **2**: δ_H (300 Hz, D_2O) 8.32 (1 H, s), 8.23 (2 H, d), 7.94 (2 H, d), 7.36–7.46 (4 H, m), 3.95 (2 H, d), 3.38 (1 H, t). HRMS (FAB^-) Found: m/z = 331.0376 [M^--H]. $C_{18}H_{12}O_4K$ requires 331.0378.

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9 Cysteine can be oxidised by oxygen in the air at pH 7 (ref. 4). Free cysteine in the solution is oxidized gradually by oxygen. Free Cu^{2+} is liberated from the complex of [Cu*Cys] with the concentration of free cysteine decreasing in solution. The free Cu^{2+} then quenches the fluorescence of the solution again, causing the fluorescence intensity of the solution to be time-dependent.
10 The titration of **1*Cu** with cysteine and homocysteine was carried out until a constant value I_{max} was reached. The apparent binding constant, K, is defined by the equation: $K = [Cu^*S]_{tot}/[1^*Cu]_{tot}[S]_{tot}$, where $[Cu^*S]_{tot}$, $[1^*Cu]_{tot}$ and $[S]_{tot}$ (substrate: amino acid) are the total concentration of **Cu*S**, **1*Cu** and **S** respectively, all in their protonated and deprotonated forms. A constant pH in aqeuous solution and 1 : 1, probe : substrate interaction are assumed.