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Colorimetric sensors with different reactivity for the quantitative determination of cysteine, homocysteine and glutathione in a mixture†

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We report a facile method to the quantitative detection of cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) in the mixture. BODIPY-based sensors 1 and 2 are synthesized, and the diverse reactivities of 1 and 2 in the presence of a mixture of biothiols lead to distinct absorption spectra difference at a specified period of time. Combining with a spectral analysis, we are capable of distinguishing three biothiols and achieving the quantitative determination of Cys, Hcy and GSH in a mixture.

Biological thiols play essential roles for maintaining the appropriate redox status in physiological and pathological processes.[1] Cysteine deficiency is involved in many diseases, such as slow growth in children, hair depigmentation, liver damage, skin lesions, etc.[2] An excess of homocysteine in blood plasma can lead to an increased risk of coronary heart diseases, stroke and Alzheimer's.[3] Glutathione, which is the most abundant intracellular thiol, serves as a pivotal indicator for maintenance of xenobiotic metabolism, intracellular signal transduction and gene regulation.[4] Given their different roles in biological systems, the quantification of individual thiols is of great importance.

Some sophisticated analytical techniques, including electrochemical detection and high performance liquid chromatography (HPLC) methods, have been applied for biothiols detection.[5] However, these techniques often require expensive instruments and complicated sample preparation. As a simple and inexpensive method, optical sensor has drawn significant attentions for the detection of biothiols.[6] Although a large number of optical sensors have been developed to distinguish biothiols from other amino acids and determine the total thiols in live cells or in blood plasma,[7] the discrimination of Cys, Hcy and GSH is still very difficult, and thus received most of the attentions in recent years. Strongin and co-workers reported the cyclization of Cys/Hcy with aldehydes[8] or acrylates[9] for the selective detection of Cys/Hcy, which inspired the development of optical sensors-based approaches to detect Cys or Hcy.[10] Several examples for the detection of GSH over Cys/Hcy were also achieved,[11] albeit in relatively less extent. Very recently, the attractive strategy was reported to simultaneously detect Cys/Hcy and GSH from different emission channels.[12] However, developing an effective and convenient optical sensor-based method, which can quantitatively detect each individual biothiol in a mixture, still remains a big challenge.

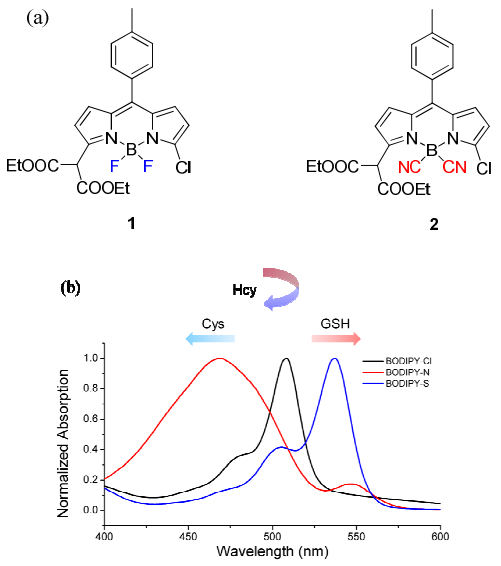

Fig. 1 (a) Chemical structures of **1** and **2**. (b) The absorption spectra of **1** with Cys/Hcy/GSH.

Herein we innovatively report a facile discrimination method that can quantitatively detect Cys, Hcy and GSH in a mixture based on monochlorinated boron dipyrromethene (BODIPY) derivatives **1** and **2** (Fig. 1a). The two sensors react with biothiols at different reaction rate, thus resulting in distinct spectra at a specified period of time. By taking advantage of the different reactivity of **1** and **2** for biothiols, combining with a spectral analysis based on "three linear equations with three unknowns" model, we are capable of distinguishing three biothiols and achieving the simultaneous detection of Cys, Hcy and GSH in a mixture.

In our previous work, we have developed a fluorescent /colorimetric sensor for the highly selective detection of GSH over Cys and Hcy based on monochlorinated BODIPY.[11a] Free thiol displaces the chlorine by thiol-halogen nucleophilic substitution, resulting in sulfur-subsitituted BODIPY which leads a significant red-shift in visible spectrum. The amino groups of Cys/Hcy but not GSH further displace the sulfur to form amino-substituted BODIPY, which exhibits blue-shifted absorption spectra.

Other than GSH determination, a notable and important feature of the sensor should be emphasized: the intramolecular rearrangement for Hcy takes an extended time than Cys due to the formation of less favourable 6-membered cyclic transition state. This difference in their reaction rates may provide a distinguishing capability for Cys and Hcy. Thus, at a specified period of time, the spectral changes of **1** for Cys, Hcy and GSH are expected to be distinct from each other. As shown in Fig. 1b, the absorption band at 520-570 nm is attributed to the exist of sulfur-substitution BODIPY produced by the reaction with thiols; meanwhile, the absorption band at 400-500 nm could be attributed to the subsequent formation of amino-substitution products by the reaction with Cys and Hcy to different extent.[11a] According to Beer–Lambert law, the absorbance is proportional to the concentration of substances. That is, theoretically, once the absorbance is measured, the concentrations of the N- or S-substitutend BODIPY, which is related to the presence of different biothiols, can be deduced. However, **1** still suffered from a low reactivity, especially with Hcy, which would limit its application on the biothiols determination. More importantly, after reacting with **1**, the similar spectra shift for Hcy and GSH at the early stage obviously brings misjudgement on the discrimination. Thus, a comparable sensor with higher reactivity is required.

In this work, we utilize an alternative molecule-design strategy of displacing the fluorine atoms (**1**) by cyano groups (**2**) (see the ESI,† Scheme S1) in order to further increase the reactivity. The stronger electron-withdrawing ability of cyano group makes the BODIPY core more electron deficient, that is, a more active nucleophilic substitution site.

We initially measured the time-dependent absorption response of **1** and **2** in the presence of GSH (Fig. 2a). For **1**, it took almost 40 min to reach the absorption maximum of 468 nm; in contrast, the reaction for **2** was complete within 5 min. The pseudo-1st order reaction rate constants were 0.076 min^{-1} ($t_{1/2}$ = 9.1 min) and 1.26 min^{-1} ($t_{1/2}$ = 0.5 min), respectively. It revealed that the reactivity of monochlorinated BODIPY was significantly elevated.

To understand the different activity of **1** and **2** at molecular and electronic levels, *ab* initio calculations were carried out using *Gaussian* program (Fig. 2b). The lower LUMO energy of **2** compared to **1** gives a clear idea that B-CN is more reducible, indicating it is more electron-acceptable from a nucleophile.

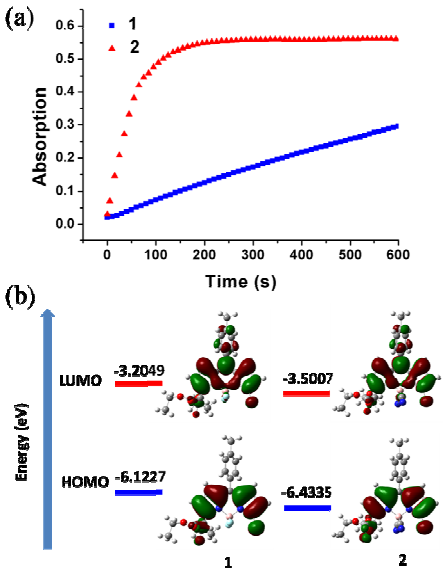

Fig. 2 (a) Time course of the absorption response of of **1** and **2** (10 μM) at 468 nm to 100 equiv of GSH in acetonitrile / HEPES buffer (1:3, v/v, 20 mM, pH 7.4) at 25 °C. (b) Energies and electron distribution patterns in the HOMO and LUMO states of molecules **1** and **2**.

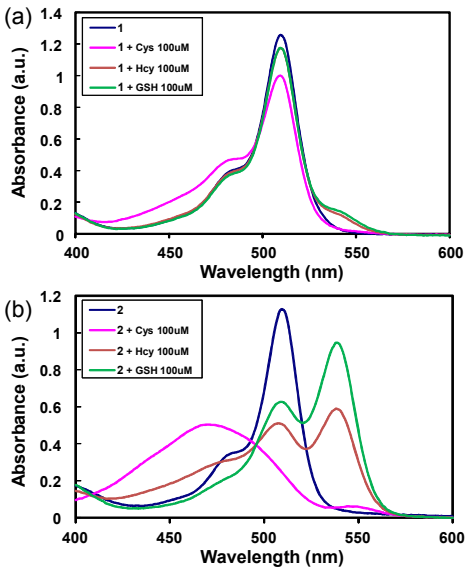

Fig. 3 The absorption spectra of (a) **1** (20 μM) and (b) **2** (20 μM) after 1 h addition of 100 μM Cys, Hcy or GSH in acetonitrile/HEPES buffer (v/v = 2:3, 20 mM, pH=7.4) at 25 °C.

Fig. 3 shows the absorption spectra of **1** and **2** after 1 h reaction with biothiols. **2** presented obviously higher reactivity than **1**. As mentioned, the two absorption bands at 520-570 nm and 400-500 nm are according to the S- and N-substituted BODIPY, which are related to the presence of different biothiols. By using artificial neural networks or partial least squares chemometric approaches, the component by component analysis in the mixture might be realized by employing sensor **2** only**.** However, these chemometric methods generally suffer from the trivial modelling and complicated calculation. Moreover, expensive spectra scanning unit is required in

order to obtain the full spectra data. To avoid these obstacles, we decide to combine **1** and **2**, and propose that **1** and **2** with different reaction rates would give different absorption at certain wavelength, and the concentrations of three biothiols in a mixture could be theoretically deduced. In other words, the concentrations of three biothiols, as three unknowns, could be resolved through three linear equations according to the additivity of absorbance.

Three representative wavelengths were taken into account to resolve three unknown biothiols, as well for the design convenience for home-made prototype device, which usually utilizes filter glasses instead of spectrum scanning unit. For the absorption band at 520-570 nm, we picked up 540 nm of **1** as one evaluated wavelength, which showed relatively larger absorption for sulfur-subsitituted BODIPY derivatives. For the absorption band at 400-500 nm, we chose a middle wavelength (~450 nm) of **1** as second evaluated wavelength, which avoided the specific wavelength of the chlorinated BODIPY. Meanwhile, since BODIPY sensor **2** has the different reaction rate with **1**, the third uncorrelated wavelength was then selected at 530 nm of sensor **2**.

With the aim of determining the individual biothiol from a mixture, (1) the additivity and (2) the linearity of absorbance are two essential factors. Firstly, in a mixture, three individual biothiol reacts with BODIPY sensors competitively. In order to simultaneously detect all three biothiols, the BODIPY sensors should be excessive enough to not only completely react with biothiols, but also cover their own concentration changes after the reaction. The reaction mechanism with excessive BODIPY sensor is proposed (see the ESI,† Scheme S2). 1 h was selected as an evaluation time since it is quite difficult to reach equilibrium for all three biothiols at low concentrations (see the ESI,† Fig. S1). As deducted, in the presence of largely excessive sensor, the reaction rate of each single biothiol in the mixture is not influenced by other co-existing thiols, thus the additivity is valid (see the ESI,† Scheme S3). When the individual concentration of biothiols was set as 80 μM, 500 μM was optimized as the final concentration of sensors. Under this condition, the experimental spectra showed good agreement with the speculated ones (see the ESI,† Fig. S2), thus ensured the additivity.

Other than the additivity, the linear relationship between the individual biothiol concentration and the spectrum absorbance is the other important factor. Different from equilibrium-based reactions, our system carried multistep non-equilibrium reactions and thus showed different chemical reaction kinetics. Based on the rate equations of reactions, the final concentration of each product could be deducted (see the ESI, † Scheme S4). As shown in the deductions, each product concentration is proposed to be linearly proportional to the biothiol's initial concentration. It indicates that after 1h reaction, the variation in absorbance is linearly proportional to biothiol's initial concentration.

Based on these deductions, we thus collected the absorbance data for three individual biothiol at various concentrations (5-80 μM) (see the ESI,† Fig. S3) and obtained good linear relationships between three biothiols concentrations and spectral absorbance change at three representative evaluated wavelengths. As shown in Fig. 4,the absorbance change at 450 nm for GSH of **1** is almost zero, and thus its contribution to entire absorbance change could be omitted.

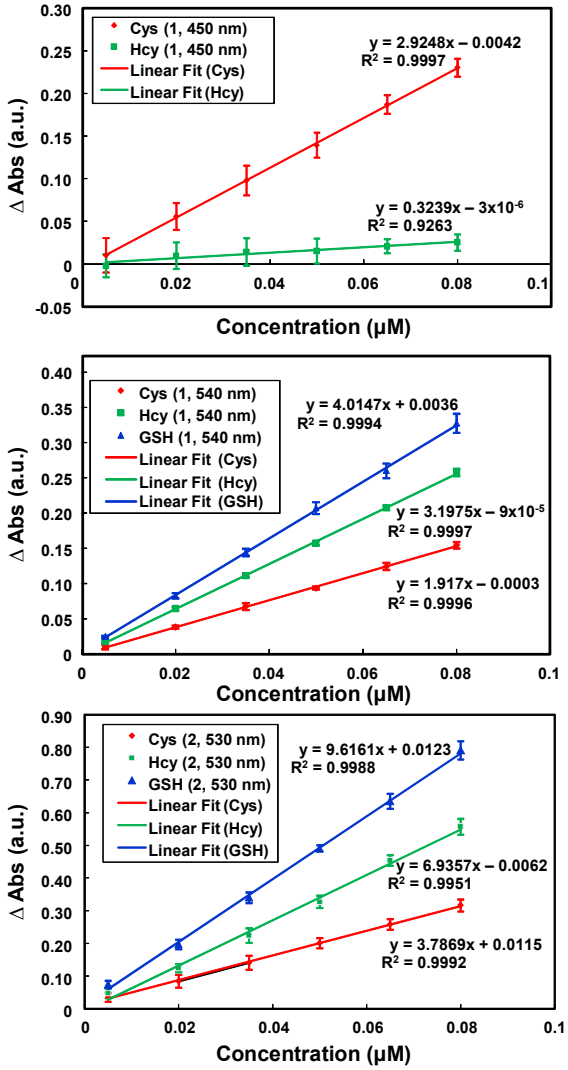

Fig. 4 The linear relationship between Cys/Hcy/GSH and the absorbance change at three selected wavelengths after 1 h reaction with BODIPY sensors.

From these fitting linear equations, three uncorrelated equations could be easily achieved as follow:

$$\Delta A_{1,\,450\ nm} = 2.9248\times[C] + 0.3239\times[H] - 0.0042$$
$$\Delta A_{1,\,540\ nm} = 1.9170\times[C] + 3.1975\times[H] + 4.0147\times[G] + 0.0033$$
$$\Delta A_{2,\,530\ nm} = 3.7869\times[C] + 6.9357\times[H] + 9.6161\times[G] + 0.0176$$

To verify the feasibility of simultaneously quantitative determination of Cys, Hcy and GSH, we prepared six mixtures of three biothiols with various ratios. Once the absorption spectra of **1** and **2** in the presence of biothiols were recorded, after simple calculation, the concentrations of each single biothiol in the mixture could be achieved. As shown in Fig. 5, the biothiols' concentrations calculated from three uncorrelated equations showed good agreements to the experimental concentrations. Other wavelength combination also gave reasonable results (see the ESI,† Fig. S4). The results showed that it is a simple and effective method for the quantitative detection of Cys, Hcy and GSH in a mixture.

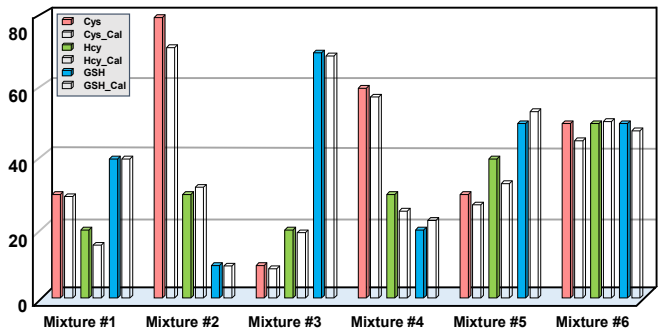

Fig. 5 The experimental and calculated concentrations of Cys/Hcy/GSH in the mixture.

In summary, we have developed a simple approach to quantitatively discriminate Cys, Hcy, and GSH in the mixture. The discrimination capability is attributed to two chlorinated BODIPY sensors **1** and **2**, which show different sensing behaviours to the three biothiols with the different reaction rates. A simple "three equations with three unknows" model was performed. To the best of our knowledge, this is the first optical method of simultaneous sensing of Cys, Hcy, and GSH in a mixture, which avoids complicated manipulation and analysis. We are now working on the design of superior sensors with higher reaction reactivity and better solubility. A stop-flow injection-based prototype machine, which controls reaction time accurately, is also developing in our lab (see the ESI,† Fig. S5).

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Notes and references

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