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Redox-based selective fluorometric detection of homocysteine†

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Plasma homocysteine (Hcy) is an important risk factor for various diseases. A novel redox-sensitive fluorescent probe is developed for the selective detection of Hcy. A linear calibration curve has been obtained in buffer and plasma for the quantitative determination of Hcy in such media.

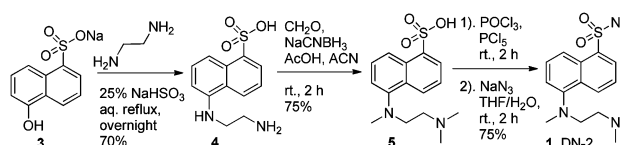
Biological thiols such as cysteine (Cys), homocysteine (Hcy) and glutathione (GSH) play versatile roles in various processes, including protein structure and functions, antioxidant activity, and redox signaling. Hcy is a key intermediate generated during the biosynthesis of Cys.¹ Plasma Hcy exists in three major forms including free reduced (~1%), free oxidized (30%) and protein-bound oxidized (70%) forms.² The sum of all forms, total homocysteine (tHcy) has been identified as an independent risk factor for cardiovascular disease (CVD),³ diabetes,⁴ renal disease,⁵ and neurodegenerative diseases such as Alzheimer's disease (AD).^{11,12} Impaired methionine and Hcy metabolism leads to hyperhomocysteinemia (HHcy), which is demonstrated by an elevated level of plasma tHcy.⁶ According to the American Heart Association (AHA) advisory statement, normal tHcy concentrations range from 5–15 μM; moderate, intermediate, and severe hyperhomocysteinemia refer to concentrations between 16 and 30, between 31 and 100, and >100 μM, respectively, and are essentially pathognomonic.⁷ As a result, accurate determinations of Hcy in biological systems such as blood plasma and urine are of great importance in diagnostic applications.

Due to high degree of similarity in both structure and chemical properties between Hcy and other thiols such as Cys, the selective detection of Hcy has never been a trivial issue. Current detection methods for Hcy involve the derivatization at the sulfhydryl group using electrophilic fluorogenic reagents.⁸ However, due to the lack of selectivity among different biological thiols, these methods require tedious separating techniques

such as GC and HPLC. This has led to an increased cost and reduced efficiency and thus limited the applicability of Hcy as an important biomarker. Therefore, selective recognition of Hcy over other biological thiols is of great interest. Fluorescent probes based on cyclization mechanisms using both the amino and sulfhydryl groups have been reported mostly selective for Cys.⁹ The Strongin group found a colorimetric method for selective Hcy detection based on a radical mechanism.¹⁰ They also reported a fluorescent probe showing different reaction profiles for Hcy and Cys and thus their simultaneous detection.^{9b} The Yoon lab recently reported a pyrene-based fluorescent probe functionalized with an aldehyde moiety selective for Hcy.¹¹ Herein we report a redox-sensitive fluorometric detection of Hcy using a dansyl azide analogue **1** (DN-2).

During our search for redox-sensitive fluorescent probes for H₂S,¹² we discovered a dansyl-based fluorescent probe (DN-2) with selectivity towards Hcy over other biological thiols such as Cys and GSH in aqueous media. It is well known that thiols show reducing ability and help to maintain the redox states in biological systems. Redox reactions have been employed in thiol detections. For example, Ellman's reagent (5,5'-dithiobis-2-nitrobenzoic acid or DTNB)¹³ is widely used in the quantitation of sulfhydryl groups through the reductive cleavage of a disulfide bond, which releases the chromogenic indicator. However, only a few studies have been reported on the difference in reducing ability among thiols.¹⁴

Specifically, we have synthesized dansyl derivative **1** (DN-2) as a fluorescent probe. The synthesis of DN-2 is accomplished in 3 steps from commercially available starting materials (Scheme 1). The substitution reaction between 5-naphthol-1-sulfonate **3** and



Scheme 1 Synthesis of the fluorescent probe DN-2.

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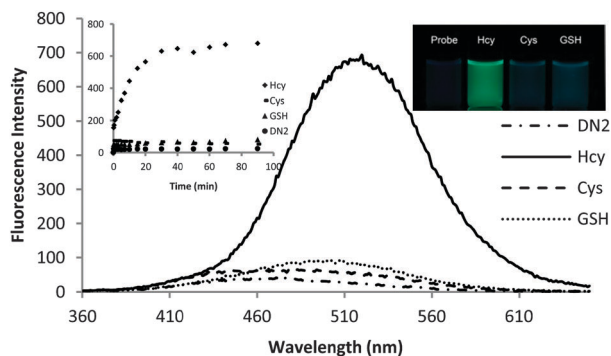
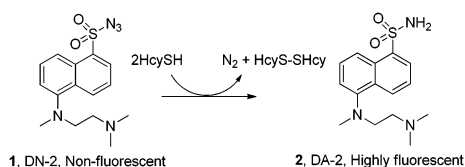


Fig. 1 Fluorescence spectrum of DN-2 in the absence and presence of different amino thiols; inset: time dependent fluorescence emission (517 nm) of DN-2 in the presence of different Hcy and Cys, and comparison of fluorescence intensity change with the addition of different amino thiols. (DN-2 120 μM, amino thiols 100 μM in 100 mM sodium phosphate buffer at pH 7.4 with 10% ethanol, fluorescence spectrum and pictures were recorded 1 h after the addition of thiols).

ethylene diamine was followed by reductive amination, which installed three methyl groups on the amino groups.¹⁵ Then the sulfonate was converted to sulfonyl chloride, which reacted quantitatively with sodium azide to form the sulfonyl azide, DN-2 (**1**). This short and convenient synthetic route has provided the probe in a 37% isolated yield.

Fluorescence spectroscopy was used to monitor the reaction between DN-2 and biological thiols, Cys, Hcy, and GSH. As is shown in Fig. 1, a significant fluorescence increase (~25-fold) at around 517 nm was observed for Hcy. Such fluorescent changes are also readily visible to the naked eyes (Fig. 1, inset). However, with Cys and GSH, only 2 and 3-fold fluorescence intensity change was observed, respectively. Spectroscopic (¹H NMR, ¹³C NMR, and MS) identification of the product confirmed that the sulfonyl azide was reduced by Hcy, forming the corresponding sulfonamide and homocysteine (Scheme 2). The fluorescence increase was due to the formation of highly fluorescent DA-2 (Φ_{FL} (DN-2) = 0.007 and Φ_{FL} (DA-2) = 0.58 in acetonitrile, Φ_{FL} (DN-2) = 0.02 and Φ_{FL} (DA-2) = 0.29 in water, Fig. S1, ESI†).

Next we compared the reaction time profile of DN-2 to thiols. Specifically, DN-2 was dissolved in the solution to a final concentration of 120 μM, while thiol concentrations were 100 μM. The probe, DN-2 was only weakly fluorescent in solvent systems, including acetonitrile (ACN), 100 mM sodium phosphate buffer at pH 7.4, and water. Immediately after the addition of thiols, an initial small increase in fluorescence was observed for all species (Fig. 1, left inset). However, the fluorescent intensity quickly reached a plateau for Cys and GSH. For Hcy, the fluorescence intensity reached a plateau at about 1 h point at



Scheme 2 Sensing reaction of DN-2 with Hcy.

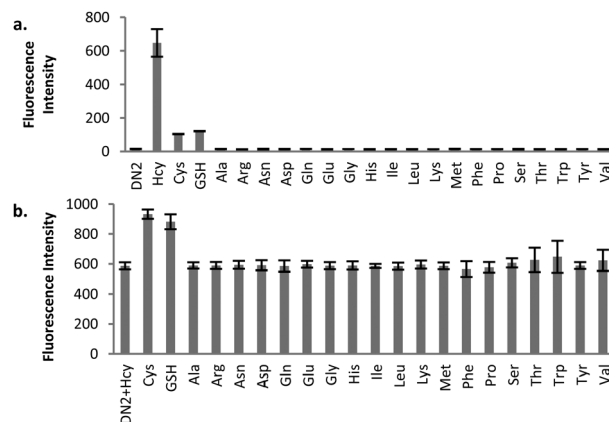


Fig. 2 (a) Fluorescence response of DN-2 to amino acids; (b) fluorescence response of DN-2 to Hcy in the presence of amino acids. (DN-2 120 μM, amino acids 100 μM in 100 mM sodium phosphate buffer at pH 7.4 with 10% ethanol, fluorescence intensities were recorded 1 h after the addition of amino acids. Data represents the average of three independent experiments.)

a much higher level. The selectivity for Hcy over Cys and GSH was found to be 8–12-fold. However, since GSH concentration in plasma is as low as ~2 μM,¹⁶ one would not expect interference problem with GSH.

To further test the feasibility of DN-2 as a fluorescent probe in a complex biological system, the selectivity of DN-2 was tested among various amino acids in phosphate buffer with 10% ethanol (Fig. 2a). It was found that DN-2 did not respond to most amino acids. A selectivity of over 45-fold was found among these amino acids at 100 μM. The reaction between DN-2 and Hcy was carried out in the presence of various amino acids (100 μM) (Fig. 2b) in order to test how the presence of other amino acids affects the fluorescence response of DN-2 to Hcy. It was found that the fluorescence response of DN-2 to Hcy was elevated by ~40% in the presence of 100 μM Cys. GSH showed similar effect. In contrast, none of the other amino acids affected the detection of Hcy. In addition, other possible reducing species in biological samples, such as Fe(II), Cu(I), and ascorbate, showed no reactivity with DN-2 (Fig. S2, ESI†).

A fluorescent probe is especially useful for quantitation if a linear calibration curve could be obtained. Therefore, experiments were performed to obtain a calibration curve using a series of concentrations for Hcy. As expected, a linear calibration curve with $R^2 > 0.99$ was obtained for Hcy (Fig. 3). This linearity is very important for quantitative analysis of Hcy.

Since the presence of Cys was found to increase the fluorescence response of DN-2 to Hcy by ~40%, we were interested whether endogenous Cys fluctuations (~200–250 μM) would affect Hcy detection. In order to test the effect of Cys fluctuation, calibration curves of Hcy at 0–50 μM was generated in the presence of 200 and 250 μM of Cys (Fig. S3, ESI†). It was found that these two curves are very close, though not identical. Therefore, the accuracy of Hcy quantitation may be affected by Cys fluctuation. However, the selectivity of DN-2 to Hcy still allows a good estimation of Hcy level with large (~50 μM) fluctuation of Cys concentrations.

As arylsulfonyl azide was found to be a very sensitive fluorescent probe for sulfide,^{12,17} DN-2 also reacts with sulfide.

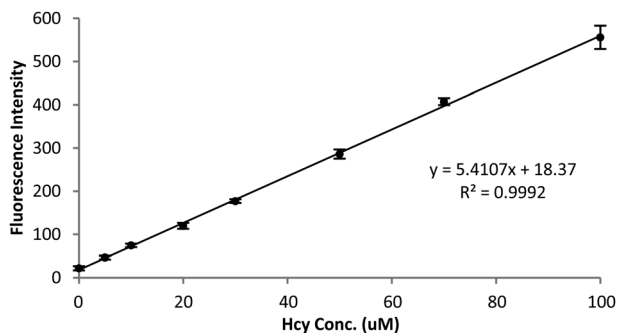


Fig. 3 Calibration curve for Hcy, (DN-2 120 μ M, Hcy 100 μ M in 100 mM sodium phosphate buffer at pH 7.4, with 10% ethanol; fluorescence intensities at 517 nm were recorded 60 min after the addition of Hcy. Data represents the average of three independent experiments).

In order to exclude the influence of sulfide in the selective detection of Hcy, a solution of ZnCl_2 could be added into the reaction buffer to precipitate sulfide out of the solution in the form of ZnS ($K_{\text{sp}}(\text{ZnS}) \sim 2 \times 10^{-25}$). It was found that the presence of high concentration of Zn^{2+} (at 300 μ M) shows very little interference with the fluorescence response of DN-2 to Hcy (Fig. S4, ESI †).

Due to the significance of Hcy in biological system, the application of this probe in serum was examined. In diluted (10%) deproteinized fetal bovine serum (FBS), with addition of Hcy, this probe selectively responded to Hcy over Cys (Fig. 4, inset). Furthermore, concentration-dependent fluorescence changes of DN-2 in diluted deproteinized FBS were observed. A linear calibration curve ($R^2 = 0.9997$) was obtained suggesting the potential utility of DN-2 in serum. The detection limit of Hcy in deproteinized FBS was 10 μ M based on a 3-fold signal-to-noise ratio.

Our experiments have found that DN-2 selectively oxidizes Hcy in buffer and diluted deproteinized FBS. Although Cys is reduced by DN-2 at very high concentrations (~ 100 mM), there is apparently a large difference at lower concentrations. Thus, we were interested in achieving an understanding of the difference in reducing ability between Cys and Hcy. Hcy has one extra methylene group compared to Cys. However, this does not make its sulfhydryl group a stronger nucleophile since

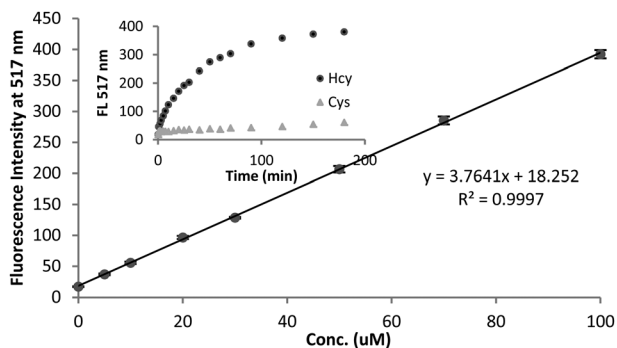


Fig. 4 Calibration curve for Hcy (DN-2, 120 μ M, Hcy, 0–100 μ M in diluted deproteinized FBS, fluorescence intensities at 517 nm were recorded 180 min after addition of Hcy. Data represents the average of three independent experiments); inset shows time-dependent fluorescence response of DN-2 (120 μ M) to Hcy (100 μ M) or Cys (100 μ M) in diluted deproteinized FBS.

most nucleophilic substitution-based fluorescent probes show similar reactivity with both thiols. We have also examined the $\text{p}K_{\text{a}}$ of the sulfhydryl groups on Cys and Hcy using pH titration.¹⁸ It was found that the $\text{p}K_{\text{a}}$ was 8.25 for Cys and 8.9 for Hcy (Fig. S5, ESI †), similar to most previously reported values.^{18,19} (In another paper, the $\text{p}K_{\text{a}}$ of $-\text{SH}$ group was reported to be as high as 10.²⁰) According to these $\text{p}K_{\text{a}}$ values, Cys should be deprotonated more easily than Hcy. Therefore, one would expect that Cys being a stronger reducing agent than Hcy if $\text{p}K_{\text{a}}$ was the determining factor. Fluorescent/colorimetric probes with disulfide moieties show comparative or slightly higher reactivity to Cys.²¹ This may be due to their difference in $\text{p}K_{\text{a}}$. However, this is not the case in the redox reaction between these thiols and DN-2, where a different mechanism is indicated. In fact, it has been reported that oxidation of Hcy by albumin is faster than that of Cys and the oxidation is accelerated by basic conditions or addition of a copper catalyst.^{14b,22}

The reduction mechanism of an azido compound by a dithiol has been proposed in a previous report.²³ Based on the experimental results, we propose that the initial attack by thiol leads to the formation of an intermediate **6** (Fig. 5), which is responsible for the initial 2–3-fold fluorescence increase. Proton transfer then occurs to neutralize the negatively charged nitrogen leading to intermediates **6b** and **6c**. In the case of Hcy, the amino group then attacks the sulfur to form a five-membered ring intermediate **7**, which then reacts with another molecule of thiol to form the disulfide product. The intermediate **8** then releases N_2 to provide the sulfonamide product **2**. In the case of Cys, a similar intramolecular attack would lead to a strained four membered ring **9**, which would be unstable and thus unfavorable. In addition, the reversibility of the initial step means that the intermediate (**6**) exists in equilibrium with the starting materials and thus the probe (**1**) is available to react with Hcy even in the presence of Cys. In fact, cyclic transition states/intermediates have been used to explain the mechanisms of other Cys- or Hcy-selective probes.^{9b,10a,b} It is entirely possible that there are other conformational factors, which affect the reactivity of intermediate **6**. In the last step, another thiol comes to attack the sulfur to form a disulfide. Although gaseous diatomic bond energy for general S–N bond is higher than S–S bond,²⁴ the formation of disulfide bond from the attack of sulfhydryl group on an electron-deficient S–N bond has been reported previously.²⁵ In this step,

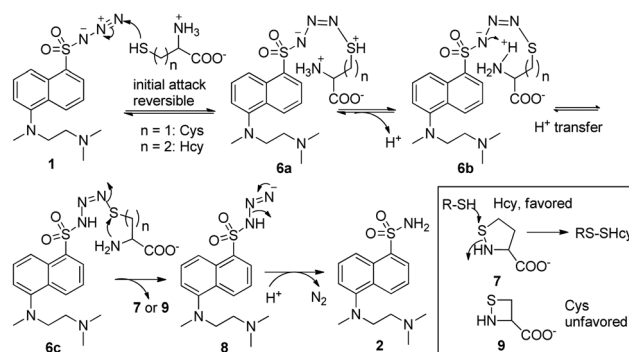


Fig. 5 Proposed mechanism.

it is conceivable that when excessive amount of Cys exists in the reaction media, a Cys-Hcy mixed disulfide could also form, leading to an increase of the "effective" concentration of Hcy. This explains why Cys alone does not trigger fluorescence increase but causes a ~40% increase in fluorescence response to Hcy. It should be noted that a previously published dansyl azide analogue, 1,5-DNS-Az also shows selectivity for Hcy (Fig. S6, ESI†).

In addition, the influence of pH on the selectivity has also been tested using 96-well plate and a microplate reader (Fig. S7, ESI†). Sodium phosphate buffer (100 mM) in the pH range of 5.3–11.5 was used in the experiments. A pH dependent fluorescence response was observed for DN-2 in the presence of thiols. It was found that DN-2 itself remains fluorescently stable at pH values lower than 10.5. The selectivity for Hcy increased with increasing pH from 5–7.4. In buffers at pH higher than 7.4, reactions with Cys and GSH were promoted. Therefore, the highest selectivity was observed at pH 7.4, which is the physiological pH. We have also examined the reaction time profile in phosphate buffer at 37 °C (Fig. S8, ESI†). The result has suggested that heat facilitated the reaction. The reaction time for 120 µM of DN-2 and 100 µM of Hcy decreased from 60 min at room temperature (about 22 °C) to 30 min at 37 °C.

Hcy is a very important biomarker for the diagnosis and prognosis of various diseases. However, due to the structural similarity of Hcy and Cys, direct detection of this biomarker still remains a major challenge. We have developed a novel redox sensitive fluorescent probe for quantitative Hcy analysis. The sensing reaction is selective for Hcy over other biological thiols such as Cys and GSH. In addition, a linear calibration curve could be obtained in both buffer and diluted deproteinized FBS with a detection limit at 10 µM. Endogenous Cys level fluctuation (200–250 µM) can also be tolerated to provide a good estimation of Hcy concentration. This probe will be very useful for the selective detection of Hcy in complex biological samples.

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