Chiroptical Sensing of Homocysteine

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Abstract. The cyclization reaction between *ortho*-phthalaldehyde and L-homocysteine coincides with the generation of a pronounced positive CD signal at approximately 335 nm. Under identical conditions, other amino acids including cysteine produce very weak CD responses. This unusual substrate specificity allows accurate chiroptical analysis of the enantiomeric composition of homocysteine samples in the presence of cysteine without the need for time-consuming chromatographic separation. This significantly simplifies and speeds up *ee* determination at reduced solvent waste production.

Homocysteine (Hcy) and cysteine (Cys) are important biothiols that play essential physiological roles in redox homeostasis, intracellular defense against oxidative stress and as protein building blocks.[1] In addition, they are important disease biomarkers. Altered levels of cysteine have been associated with dementia, Parkinson's, Alzheimer's and Huntington's diseases.[2] Elevated levels of homocysteine in plasma, known as hyperhomocysteinemia, is considered a risk factor for Alzheimer's disease,[3] cardiovascular,[4] osteoporosis,[5] aneurysm[6] and renal disorders.[7] The traditional view of predominant homochirality in nature has been partially revised by emerging reports of co-existing enantiomers of amino acids and biothiols in the mammalian central nervous system and endocrine organs.[8] Both enantiomers of cysteine and homocysteine have been reported to be of physiological and clinical relevance.[9] Therefore, there is great interest in methods that allow enantioselective detection and quantification of these

biomarkers in aqueous media. Our group recently reported a chromophoric probe that allows enantioselective analysis of cysteine in the presence of structurally similar biothiols and other amino acids.[10] Less progress has been reported with regard to homocysteine. The determination of homocysteine enantiomers in the presence of cysteine and methionine has been reported but only partial resolution due to elution of D/L-Hcy over an approximately 10 minutes long time window was achieved. Moreover, this required the use of two columns connected in series resulting in long analysis time (40-50 minutes) per sample.[11] Herein, we describe a novel sensing assay that enables fast enantioselective quantification of homocysteine using circular dichroism (CD) spectroscopy, while eliminating elaborate sample preparation and time-consuming chromatographic separation (Figure 1). This chiroptical method simply requires mixing of the aqueous sample with equimolar amounts of inexpensive *ortho*-phthalaldehyde at room temperature and subsequent analysis of the generated CD maximum with the help of a calibration curve. One can now quickly determine the enantiomeric excess (*ee*) of aqueous homocysteine samples even in the presence of cysteine which nicely complements our previously reported work on substrate-specific chiroptical Cys sensing.

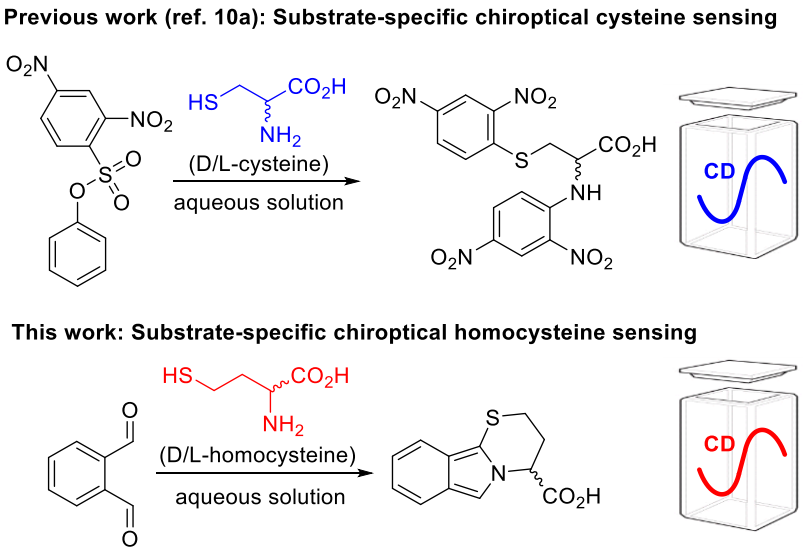

Figure 1. Comparison of Cys and Hcy specific chiroptical sensing approaches.

Hcy and *ortho*-phthalaldehyde, **1**, undergo an irreversible condensation/cyclization sequence to the tricyclic thioisoindole **2** in diluted aqueous solution (Figure 2).[12] This reaction gives a 6-membered heterocycle presumably via 6-*endo*-trig ring closure, which is favored according to Baldwin's rules,[13] and subsequent intramolecular imine condensation. The final step, the formation of the aromatic isoindole moiety, renders this chemistry irreversible. Because Cys has a shorter side chain the formation of a tricyclic scaffold requires a disfavored 5-*endo*-trig ring closure and the corresponding product **3** can be expected to have considerable angle strain. We reasoned that this could impede its formation or result in reduced stability, thus providing a unique opportunity for optical chirality sensing of Hcy in the presence of Cys which has not been accomplished to date.

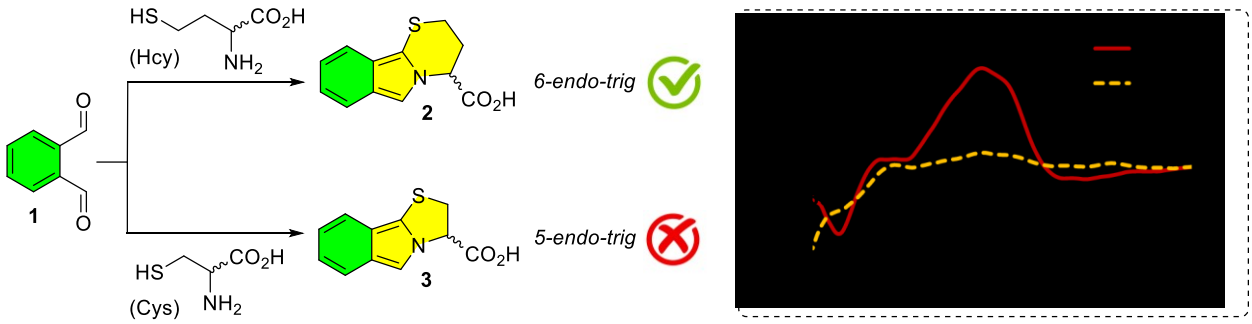

Figure 2. Optical chirality sensing of Hcy and Cys using probe **1**. CD measurements were taken at 0.28 mM in acetonitrile.

At the beginning of this study, we decided to examine if the reaction of L-Hcy and dialdehyde **1** can result in the formation of a quantifiable CD signal that would preferably occur above 300 nm to avoid interference from chiral impurities that might be present. We were pleased to find that

a strong CD signal with a maximum at approximately 335 nm appeared within a few minutes while the mixture with Cys remained almost CD-silent in the same region. UV analysis of the reaction product under similar conditions showed a distinctive maximum at approximately 350 nm which is in agreement with literature reports on thioisoindoles[14] and correlates well with the observed CD signal. Interestingly, the use of 2,3-naphthalenedicarboxaldehyde, **4**, having an extended chromophore did not improve the sensing results (see ESI). Probe **1** was therefore selected as the superior Hcy specific sensor for further optimization studies.

The speed and selectivity of the sensing assay were studied using CD spectroscopy and we chose to include alanine, serine, tyrosine, methionine, cystine and glutathione, GSH, for comparison (Figure 3A). The reaction between equimolar amounts of probe **1** and Hcy at 5.0 mM concentration was complete in less than 15 minutes at room temperature and the CD intensity proved stable for at least 2 hours (Figure 3B). Seven organic and inorganic bases as well as TRIZMA, potassium phosphate, borate and carbonate buffers (pH 8.0-9.0) were screened to find optimal reaction conditions. The highest CD intensities above 300 nm were obtained in the presence of Na_2CO_3 and K_2CO_3 but the amount of base had no effect on the CD intensity. We noticed that the colorless probe solution changed to yellow upon addition of Hcy. By contrast, the solution turned green in the presence of Cys, enabling naked eye detection (see SI). Most importantly, the CD response of probe **1** at 334 nm upon reaction with Hcy is significantly stronger when compared with structurally similar biothiols and amino acids (Figure 3C). Further analysis revealed that the reaction between **1** and homocysteine is complete within a few minutes and much faster than with cysteine. We monitored the reaction with the enantiomers of cysteine and found that the intensity of the CD signal originating from the formation of the isoindole **3** increases very slowly. After 2 hours, we obtained a slightly stronger CD response that is still significantly weaker

than the one obtained with Hcy after 15 minutes (see ESI). This indicated the possibility to develop a chiroptical assay suitable for selective Hcy sensing in the presence of other compounds such as Cys.

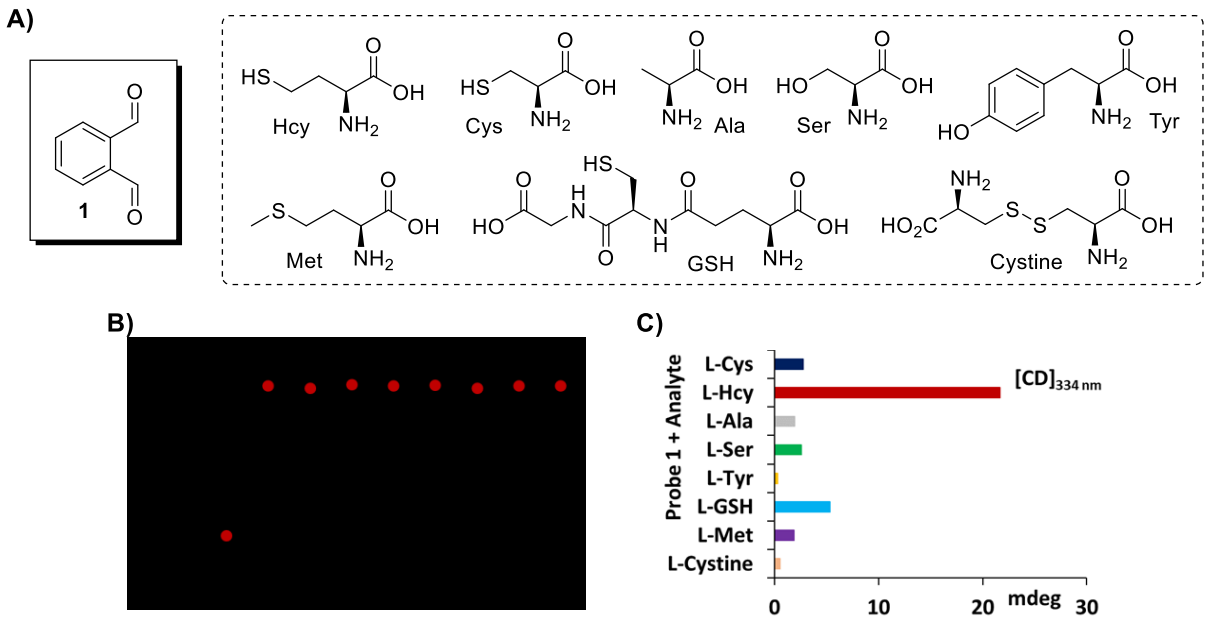

Figure 3. (A) Structures of **1**, representative amino acids and GSH. (B) Sensing time and CD signal stability. The CD intensity at 334 nm of probe **1** in the presence of Hcy was measured at different time intervals. (C) Selectivity. The CD responses of probe **1** obtained in the presence of enantiopure (>95% *ee*) Hcy, Cys, Ala, Ser, Tyr, Met, cystine and GSH at a concentration of 5.0 mM in acetonitrile: water (4:1). CD measurements were taken at 0.28 mM in acetonitrile.

Mass spectrometric analysis of a reaction mixture containing *ortho*-phthalaldehyde, **1**, and homocysteine confirmed formation of the thioisoindole **2** as discussed above. While we obtained a strong signal of the anticipated reaction product with Hcy a weaker, albeit clearly visible, signal was observed when Cys was used under the same conditions (ESI). Unfortunately, NMR analysis

of the thioisoindole formation was inconclusive. While both isoindoles **2** and **3** are apparently produced, at least initially, our ESI-MS and CD sensing results suggest that the less constrained tricyclic structure **2** is preferentially formed and also stable to degradation.

The apparent selectivity of our CD assay for Hcy encouraged us to attempt the determination of the enantiomeric composition of nonracemic samples in the presence of equimolar amounts of Cys which is probably the most challenging interferent. First, we used the L-enantiomer and racemic Hcy mixtures to examine the change in the CD signal intensity generated via the thioisoindole formation with **1** as a function of the sample *ee* (Figure 4). The plotting of the CD response at 334 nm versus Hcy *ee* showed a linear relationship.

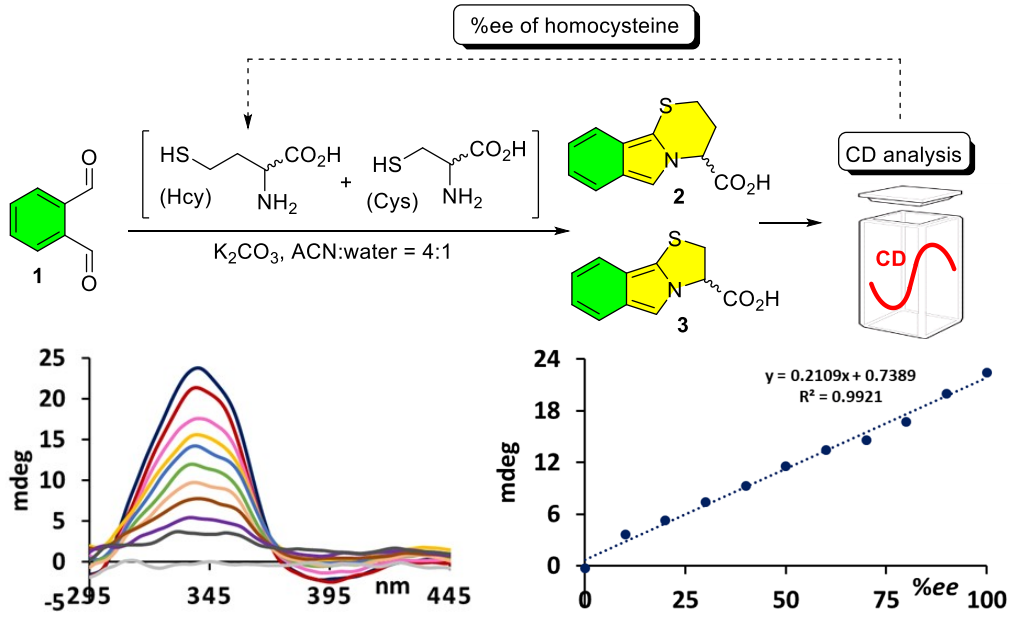

Figure 4. Sensing of the %*ee* of L-homocysteine in the presence of cysteine. The analyte concentrations were 5.0 mM and the probe **1** was used at 11.0 mM. The CD measurements were taken at 0.50 mM, see ESI for details.

With this calibration curve in hand we then prepared ten random Hcy samples at 5.0 mM covering 5.0-95.0% *ee* in the presence of equimolar amounts of L-Cys for CD analysis with our general sensing protocol. Gratifyingly, the determination of the enantiomeric composition was achieved with good accuracy and samples containing Hcy across the whole *ee* range were analyzed with a relatively small error margin averaging 3% (Table 1). Moreover, the presence of L-Cys does not interfere with the measurements. For example, the chiroptical sensing of Hcy samples with 85.0, 45.0 and 5.0 %*ee* using dialdehyde **1** gave 87.0, 42.6 and 5.2 %*ee*, respectively (entries 2, 6 and 10). To the best of our knowledge this is the first example of chiroptical *ee* sensing of homocysteine samples. We noticed, however, that chiroptical *ee* sensing of homocysteine at micromolar concentrations is not practical as the thioisoindole formation is slow and incomplete even after 2 hours.

Table 1. Chiroptical sensing of nonracemic Hcy samples on the presence of L-Cys.

Entry	**Hcy samples (%*ee*)**	**Sensing results (%*ee*)**
1	95.0	98.8
2	85.0	87.0
3	75.0	79.0
4	65.0	71.5
5	55.0	52.2
6	45.0	42.6
7	35.0	38.3
8	25.0	23.1
9	15.0	18.0
10	5.0	5.2

The CD responses at 334 nm were used to determine the %*ee* of homocysteine in the presence of cysteine. The analyte concentrations were 5.0 mM and the probe **1** was used at 11.0 mM. See ESI for details.

In conclusion, we have demonstrated quantitative optical sensing of the enantiomeric composition of homocysteine samples for the first time. The chiroptical assay is based on the cyclization reaction with a commercially available aromatic dialdehyde which yields a pronounced CD signal at approximately 335 nm just by mixing equimolar amounts in aqueous solution. The presence of other amino acids does not interfere with the analysis and we have shown that accurate %*ee* determination of homocysteine samples in the presence of cysteine is possible. Compared to chromatographic methods, this sensing method significantly simplifies and speeds up homocysteine *ee* determination at reduced cost and solvent waste production. To date, few chirality sensors that allow enantiomeric analysis of important biomolecules in the presence of structurally analogous compounds have been developed and we hope that this work will inspire other research groups to introduce new substrate-specific optical assays with potential in disease diagnosis and other medicinal applications.

Conflicts of interest

There are no conflicts to declare.

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