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A reversible competition colorimetric assay for the detection of biothiols based on ruthenium-containing complex

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ABSTRACT

A novel reversible colorimetric sensor, which based on a competitive ligation of Hg^{2+} by thiols, cysteine (Cys) or glutathione (GSH), and thiocyanate (SCN) on the N3 dye (bis(4,4′-dicarboxy-2,2′-bipyridine) dithiocyanato ruthenium (II)), was developed for the detection of biothiols. First, Hg^{2+} ions coordinate to the sulfur atom of the dyes' SCN groups, and this interaction induces a change in color from red to yellow, owing to the formation of a complex of Hg^{2+}–N3. Then, in the presence of biothiols, the red color of N3 is recovered concomitantly with the dissociation of the Hg^{2+}–N3 complex, due to the extraction of Hg^{2+} by biothiols. Thus the corresponding color variation in the process of the dissociation of the Hg^{2+}–N3 complex can be employed for the quantitative detection of thiols using UV–vis spectroscopy. In particular, the transformation can be readily viewed with the naked eye. A good linear relationship between the change in absorbance (ΔAbs) of Hg^{2+}–N3 at 461 nm and the thiol concentration was obtained in the range of 0.5–25 μM, and the detection limits are then calculated to be 57 and 52 nM for Cys and GSH, respectively. The proposed colorimetric assay displays a high selectivity for Cys over various other amino acids and GSSG (oxidized glutathione).

1. Introduction

Biothiols, such as Cys (cysteine), Hcy (homocysteine) and GSH (glutathione), play important biological roles in the human body [1,2]. The levels of Cys are linked to many health issues [3]. For example, excess Cys has been associated with neurotoxicity [4], while a deficiency of Cys is involved in many syndromes such as edema, retarded growth, hair depigmentation, liver damage, skin lesions and cancer [5]. GSH, the most predominant nonprotein thiol within the human cellular system, serves as an antioxidant and protects cells from oxidative stress [6]. Therefore, the development of rapid, simple and accurate techniques for biothiols detection is highly acquired. To date, a wide variety of analytical methods have been developed for biothiol detection, including HPLC [7,8], voltammetry [9,10] and spectrofluorometry [1,11]. However, several drawbacks, such as the lower selectivity against other amino acids, the requirement of cumbersome laboratory procedures, and the use of sophisticated instruments, limit their further applications.

Colorimetric methods have become an attractive technique since the detection results can be easily read out with the naked eye [12]. Moreover, they have advantages of simplicity, rapidity, low cost and no requirement of any sophisticated instrumentation [13,14]. Recently, Mirkin and co-workers [15] developed a brilliant colorimetric competition assay for the detection of cysteine using oligonucleotide-functionalized AuNPs probes. The probes contained strategically placed thymidine–thymidine (T–T) mismatches which could selectively coordinated with Hg^{2+}. Upon the very selective coordination of Hg^{2+} with Cys, the extraction of Hg^{2+} from T–Hg^{2+}–T complexes and the dissociation of aggregated AuNPs occurred, and accompanied by color changes. Thus the concentration of Cys could be determined by monitoring changes in UV–vis spectra of AuNPs. This strategy has been widely adopted to the development of various sensors, such as “molecule beacon” fluorescent assays [16–18], polymer probes [19,20], sensors based on nanoparticles [21,22], and DNAzyme-dependent techniques [23–26]. However, to our knowledge, no efforts have been put to utilize this strategy for devising single dye molecule probes for the colorimetric detection of Cys.

Ru-containing dyes, such as N3 (bis(4,4′-dicarboxy-2,2′-bipyridine) dithiocyanato ruthenium (II)) and N719 (bis(2,2′-bipyridyl-4,

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4′-dicarboxylato) ruthenium(II) bis(tetrabutylammo-nium) bis(thiocyanate)), have been widely used as sensitizers for dye-sensitized solar cells, due to their strong absorptions in the wavelength range of visible light and their favorable photovoltaic properties [27–29]. Such excellent properties thus make them effective candidates for colorimetric detections sensors. Coronado et al. discovered that N719 were effective colorimetric sensors for Hg^{2+}, due to the selective coordination between Hg^{2+} with thiocyanate groups on the N719 dyes, which resulted in the corresponding changes of displayed color from dark red-purple to yellow [30].

Inspired by these distinguished findings, we selected a Ru-containing dye (N3) as a chromophore serving to achieve the Hg^{2+} recognition, and developed a facile assay for the detection of thiols based on the N3–Hg^{2+} complex. The N3–Hg^{2+} complex was the first time to be used as a colorimetric competition probe for the detection of Cys. Moreover, the detection process based on the N3–Hg^{2+} complex is reversible because of the reversible binding ability of N3 with Hg^{2+}. Moreover, the N3 dye in our assay is commercially available.

2. Experimental

2.1. Materials

N3 dye was purchased from Suzhou Sheng Chemical Co., Ltd (Suzhou, China). $HgCl_2$, GSH and all the amino acids were obtained from Sigma-Aldrich (St. Louis, MO). All other chemical reagents were analytical grade, and used directly without further purification. Deionized water (18 MΩ cm^{-1}) from a water purification system (Simplicity Plus, Millipore Corporation, Billerica, MA) was used throughout.

2.2. UV–vis measurements

UV–vis absorbance spectra were collected using a Shimadzu UV2450 spectrophotometer at room temperature. The quantification experiments were carried out on a quartz cuvette with a total volume of 2 mL.

2.3. HPLC conditions

Chromatographic analysis was performed using a reversed-phase (RP) HPLC system (Shimadzu 6AD, Columbia, MO) equipped with a column (Jupiter-10 μm-C18-300 Å, dimension of 250 × 4.6 mm i.d.) from Phenomenex (Torrance, CA). Injection volumes were fixed at 10 μL. The mobile phases were 0.1% trifluoroacetic acid in water (v/v, mobile phase A) and 0.1% trifluoroacetic acid in methanol (v/v, mobile phase B). The flow rate was 1 mL min^{-1}, and the gradient conditions started at 30% B, ramped to 100% B in 20 min, and were held at 100% B for 7 min. For the diode array detector, the data were collected at 308 nm.

2.4. Preparation of N3–Hg^{2+} complex probe solution

All the solutions were prepared in CH_3OH/H_2O buffer (50:50, v/v, 10 mM HEPES, pH = 7.4). The N3–Hg^{2+} complex probe solution was obtained by mixing 50 μM of N3 with 25 μM of Hg^{2+} in the buffer solution, and stocked in the refrigerator.

2.5. Colorimetric detection of Cys/GSH

A various concentrations of Cys/GSH were added to the probe solution and mixed for 30 s. Then the changes in the UV–vis absorption spectra were recorded. For quantification, we measured the absorption variations (ΔAbs) at 461 nm between signals of the blank (probe solution) and samples (probe solution in the presence of biothiols or other amino acids).

2.6. Detection of Cys in serum

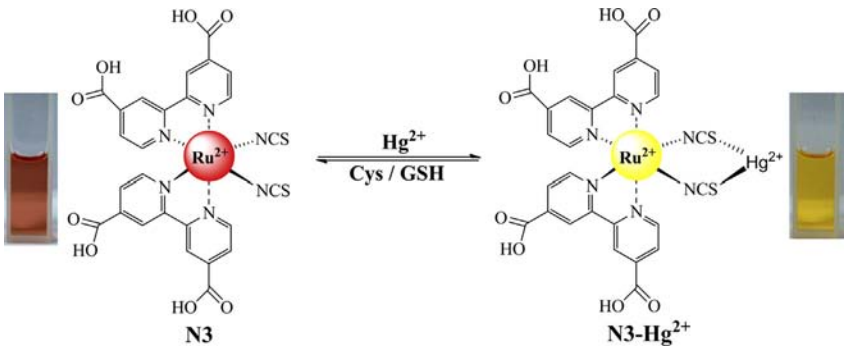
For the analysis of Cys in human serum system, the serum sample was treated by mixing with equivalent volume of ethanol to precipitate large molecules, which were subsequently removed via centrifugation at 13,000 rpm for 10 min. The supernatant was collected and passed through a 0.22-μm PTFE filter. Then 20 μL of the pretreated serum was added into 2 mL probe solution. Subsequently, various concentrations of Cys were transferred into the above mixtures. And then UV–vis spectra were recorded at room temperature.

3. Results and discussion

3.1. Preparation and properties of the N3–Hg^{2+} complex

N3 dye shows unique optical properties, such as the strong absorption in the visible region and a high extinction coefficient at ~500 nm ($\varepsilon = 1.3 \times 10^4$ M^{-1} cm^{-1} at 518 nm in ethanol) [31]. As shown in Scheme 1, due to the selective coordination between Hg^{2+} with sulfur atom of the dyes' SCN, N3 can readily be combined with Hg^{2+} leading to the formation of N3–Hg^{2+} complex. Consequently, the red color of the probe solution turns to yellow (photographic images in Scheme 1). With the further addition of thiol-containing compounds, the Hg^{2+} can be extracted from N3–Hg^{2+} complex due to the stronger interaction between the thiol groups with Hg^{2+}, and the corresponding yellow-to-red color change takes place.

The binding model and the reaction stoichiometry between N3 and Hg^{2+} were investigated by using Job's method. The total



Scheme 1. Schematic illustration of the reversible colorimetric detection of biothiols based on N3 dye. Photographic images show the corresponding colors of the complexes. (For interpretation of the references to color in this scheme legend, the reader is referred to the web version of this article.)

concentration of the N3 and Hg^{2+} was kept at 20 μM. Under different molar ratios of N3 to Hg^{2+}, the absorbance values were recorded at a wavelength of 508 nm which was the absorbance peak of N3 in the visible region (data not shown). The maximum ΔAbs (absorption variations at 508 nm of N3 in the presence and absence of Hg^{2+}) was achieved at 0.6 mol fraction of N3, which indicates that N3 can form stable complex with Hg^{2+} at a 3:2 stoichiometry. The result is in agreement with Coronado's findings that N3 and Hg^{2+} formed a precipitate with a Ru:Hg ratio of 3:2 at low Hg^{2+} concentration [30]. Accordingly, we speculated that each Hg^{2+} is tetrahedrally coordinated to three sulfur atoms from adjacent ruthenium complexes and one Cl^- anion. This binding mode was also found in the crystalline structure of $[Ru(N_2C_{12}O_4H_8)_2(NCS)_2HgCl_2]\cdot 5H_2O$ [30].

Prior to quantitative analysis of thiols (taking Cys as a model), the UV–vis spectrum properties of N3 and N3–Hg^{2+} was studied. N3 showed a broad visible band with the maximum wavelength of 508 nm ($\varepsilon = 1.33\times10^4\ M^{-1}\ cm^{-1}$) (see Fig. 1), which can be attributed to metal-to-ligand charge transfer transitions (MLCT) of ruthenium complex N3 [32]. As expected, with the addition of 25 μM Hg^{2+}, the visible absorption band shifted from 508 to 461 nm. This approximately 47 nm blue shift corresponds to a color change from red to yellow (see the inset in Fig. 1). With the further addition of 25 μM Cys or GSH, the red color of N3 recovered, the optical photos also clearly showed the yellow-to-red changes. Due to the strong bonds between thiol group with Hg^{2+}, Cys/GSH can extract Hg^{2+} from the N3–Hg^{2+} complex. Therefore, N3–Hg^{2+} complex was dissociated, resulting in free N3, and the restoration of the displayed red color.

This reversible binding process was further investigated and confirmed by HPLC measurements. To identify the formation of the N3–Hg^{2+} complex, HPLC with a UV–vis detector was used to detect the complex by comparing their Rt (retention time). The detection wavelength was set at 308 nm which is the typical absorbance peak of the bipyridine [33]. N3 dye (100 μM) alone showed a main peak with Rt of 15.7 min (Fig. 2, curve A). After mixing N3 (100 μM) with Hg^{2+} (66.7 μM), the peak at 15.7 min was completely disappeared, and a new broadened peak in the range about 21–25 min appeared. This is attributed to the conversion of N3 to N3–Hg^{2+} complex. As mentioned before, the N3 molecule was reacted with Hg^{2+} at a 3:2 stoichiometry, while each N3 molecule contains two –SCN groups. Thus Hg^{2+} and N3 can form

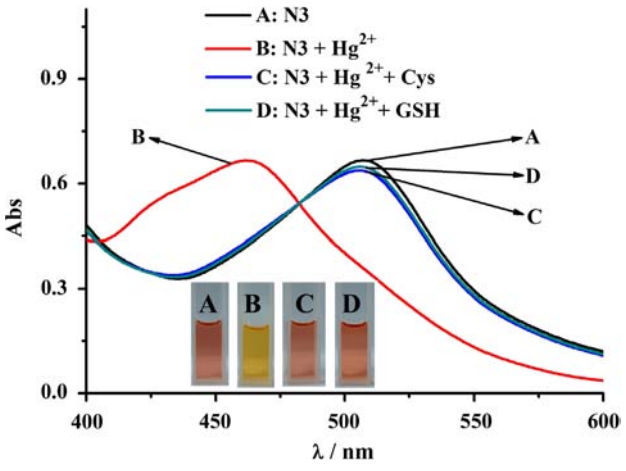


Fig. 1. Absorption spectra of 50 μM N3 (curve A) in CH_3OH/H_2O (50/50, v/v, 10 mM HEPES, pH = 7.4); after adding 25 μM Hg^{2+} (curve B); and further mixing with 25 μM Cys (curve C) or 25 μM GSH (curve D). The inset shows the corresponding photographic images. (For interpretation of the references to color in this figure, the reader is referred to the web version of this article.)

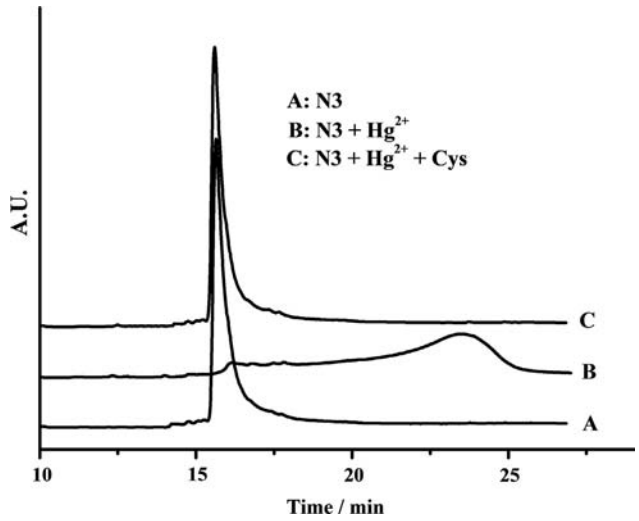


Fig. 2. HPLC chromatograms obtained by injecting a solution of CH_3OH/H_2O (50/50, v/v, 10 mM HEPES, pH = 7.4) containing (A) 100 μM N3; (B) 100 μM N3 and 66.7 μM Hg^{2+}; (C) 100 μM N3, 50 μM Hg^{2+} and 100 μM Cys.

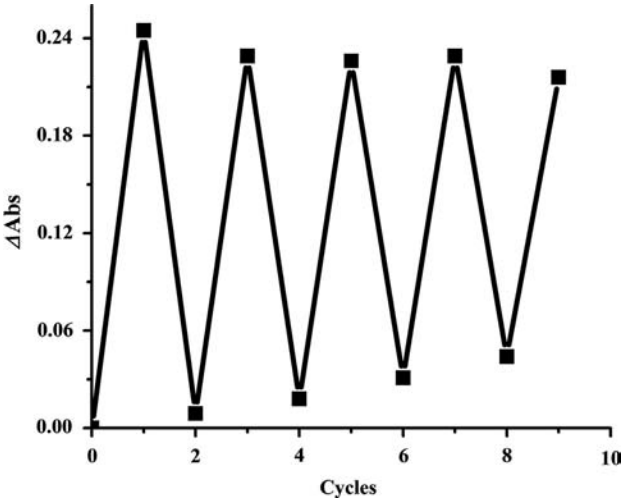


Fig. 3. Reversible switching of ΔAbs of N3–Hg^{2+} complex (N3: 50 μM; Hg^{2+}: 25 μM) in CH_3OH/H_2O (50/50, v/v, 10 mM HEPES, pH = 7.4) upon the alternate addition of Cys/Hg^{2+} with various concentrations (0:0, 26.5:0, 26.5:25, 53:25, 53:50, 79.5:50, 79.5:75, 106:75, 106:100 and 132.5:100 μM, respectively).

inter-crossed structures instead of a single-species. As a result, a much broadened peak was shown in the HPLC spectrum. After the N3–Hg^{2+} complex solution was treated with enough Cys, the typical HPLC spectrum of N3 was entirely restored (Fig. 2C), indicating that the N3 molecule can coordinate with Hg^{2+} reversibly, and implying that the assay can be used for the reversible detection of Cys.

Sequentially, we investigated the reversible and repetitive transformations between N3–Hg^{2+} and N3 by alternately adding Hg^{2+} and Cys into the N3 solution and monitoring the absorbance changes of the solution. Interestingly, the alternate addition of a constant amount of Hg^{2+} and Cys to the N3 solution gives rise to a switchable change of ΔAbs at 461 nm. Such a reversible interconversion of N3–Hg^{2+}/N3 can be repeated more than 10 times by the modulation of Cys/Hg^{2+} (as shown in Fig. 3), indicating that N3–Hg^{2+} complex can be developed as a reversible colorimetric probe for Cys. The reversible interconversion between N3–Hg^{2+} and N3 was illustrated in Scheme 1.

3.2. Detection of Cys and GSH

As mentioned above, N3 reacts with Hg^{2+} at 3:2 stoichiometry, but to avoid the interference of the free Hg^{2+}, which can hinder the detection of Cys at lower concentrations, the ratio of Hg^{2+} to N3 was set at 1:2. Upon adding biothiols (taking Cys as a mode) to the N3–Hg^{2+} complex, the color change of the probe solution (from yellow to red) could be viewed by naked-eye inspection (Fig. 4A). To have a more quantitative measure of this change, the buffer solution of N3–Hg^{2+} was titrated with Cys, and the UV–vis absorption spectra were recorded.

The dependence of the UV–vis spectra of N3–Hg^{2+} on Cys concentrations is shown in Fig. 4B. In the absence of Cys the visible peak absorbance at a wavelength 461 nm was observed, with the addition of Cys, the absorbance at 461 nm decreased systematically, while the absorbance at 508 nm increased. When the Cys concentration reached 30 μM, no further variation in the spectrum could be observed and the absorption spectrum almost completely recovered to that of the free N3. The ΔAbs of the N3–Hg^{2+} complex at 461 nm was applied to monitor the Cys concentrations. With the increase of Cys concentration, ΔAbs increased linearly with the concentration of Cys in the range of 0.5–25 μM, the linear equation can be expressed by $\Delta Abs = (0.01351 \pm 0.00111) + (0.00925 \pm 0.000165) \times [Cys/\mu M]$ ($R^2 = 0.999$). The detection limit (3σ) of the method for Cys was estimated to be 57 ± 3 nM ($n = 3$). All the relative standard deviations (RSDs) at different concentrations were less than 5.7%, indicating that the optical response of this assay for Cys is highly reproducible. In addition, this assay was also used to detect GSH, a good linear relationship in the range of 0.5–25 μM was also obtained ($R^2 = 0.996$), with a detection limit of 52 ± 2 nM.

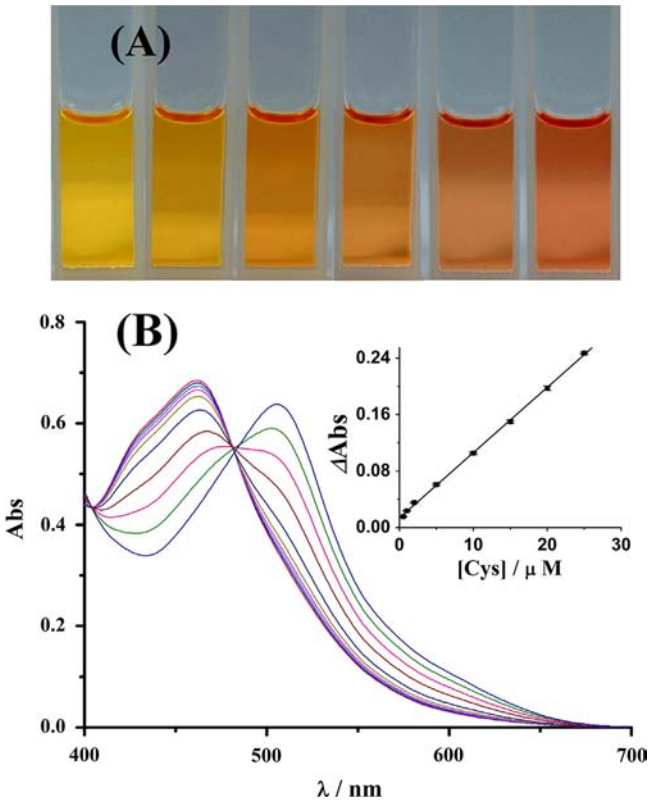


Fig. 4. (A) Visual color comparison of N3–Hg^{2+} complex upon addition of Cys at various concentrations (from left to right: 0, 5, 10, 15, 20 and 25 μM). (B) Absorption spectra of N3–Hg^{2+} in the presence of various concentrations of Cys (0.5, 1, 2, 5, 10, 15, 20 and 25 μM). Inset: calibration curve of ΔAbs vs. Cys concentrations. Error bars represent the standard deviation of three measurements. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.3. Selectivity

To evaluate the selectivity of this assay for biothiols, we investigated its UV–vis absorbance response to other amino acids as well as a common disulfide GSSG under identical conditions. In Fig. 5, ΔAbs in the assay did not change distinctly in the presence of the other amino acids. It is likely that these amino acids do not have any significant interaction with Hg^{2+}, resulting in no color changes. However, in presence of biothiols (Cys or GSH), the displayed color dramatically changed from yellow to red (see the inset of Fig. 5). Notably, methionine (Met), which contains sulfur in the form of the thioester group, does not affect the displayed color. While, histidine (His) has slight interference. This may be due to its imidazole ring which can bond with Hg^{2+} [34]. GSSG also has little disturbance. But the interference caused by either GSSG or His is negligible compared with the biothiols. Hence, the proposed assay has high selectivity for Cys/GSH among other amino acids.

3.4. Detection of Cys in human serum matrix

To investigate the performance of the proposed method in complicated matrix, a set of more meaningful Cys determination were conducted in the buffer solution with spiked human serum, using a similar reported procedure [18]. We used a standard addition method to test Cys in the 200-fold diluted human serum. The result indicated the present method responses effectively to Cys in human serum matrix. The assay exhibits a good linear correlation between ΔAbs (at 461 nm) and Cys concentration over the range of 1–20 μM ($R^2 = 0.999$). The method can detect Cys with a much lower concentration than that in human blood plasma (about 240–360 μM) [35,36], suggesting that the proposed method has great potential for diagnostic purposes.

4. Conclusions

A facile and reversible colorimetric competition assay for the accurate determination of biothiols based on the N3–Hg^{2+} complex was developed. In the absence of Cys/GSH, N3 dye and Hg^{2+} can form a stable complex (N3–Hg^{2+}) due to the coordinate of Hg^{2+} to the sulfur atom of the –SCN groups on N3 dye. Accordingly, the red color of the dye will turn to be yellow, which is the color of the N3–Hg^{2+} complex. We found that by adding biothiols (Cys or

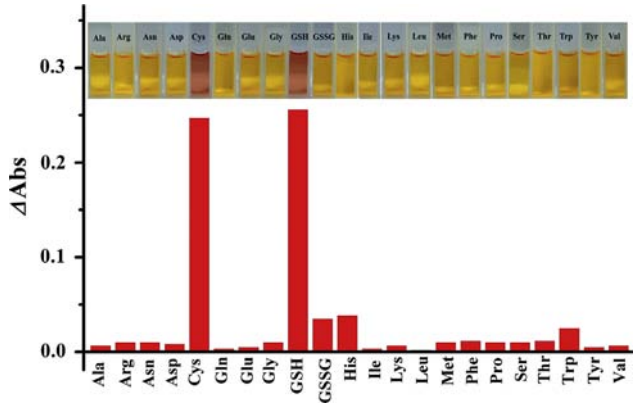


Fig. 5. ΔAbs of the N3–Hg^{2+} complex at 461 nm upon the addition of various amino acids and GSSG (each at 25 μM), [N3] = 25 μM, [Hg^{2+}] = 25 μM. Photographic images show corresponding colors of probe solutions. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

GSH) to N3–Hg^{2+} complex, Hg^{2+} can be efficiently extracted from the complex due to strong binding between Hg^{2+} and thiol group of the target analyte (Cys/GSH). The colorimetric competition assay thus developed shows high sensitivity (LOD: 50 nM, based on the 3σ method) and excellent selectivity with respect to other amino acids and GSSG. Importantly, color changes are readily viewed with naked eye. And the detection process is reversible, due to the reversible interconversion between N3–Hg^{2+} and N3. In summary, this work provides an alternative colorimetric sensor suitable for the facile, low cost detection of Cys/GSH. And the new proposed competition reaction between Hg^{2+} with SCN group and Cys can inspire to the development of more novel competitive sensors based on other suitable ligands.

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