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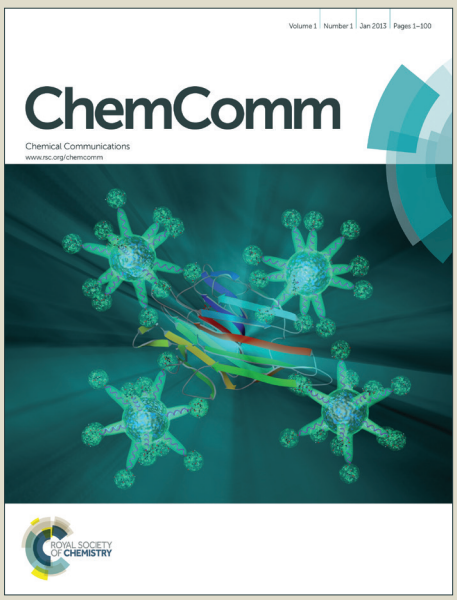

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COMMUNICATION

A novel graphene oxide based fluorescent nanosensing strategy with hybridization chain reaction signal amplification for highly sensitive biothiol detection

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A novel fluorescent nanosensor has been developed for detecting biothiols including cysteine and glutathione using graphene oxide based hairpin DNA-selective fluorescence quenching and thymine-Hg(II)-thymine coordination-controlled hybridization chain reaction, which provides a simple but the most sensitive platform for biothiol assays.

Biothiols play a crucial role in the physiological matrix for their participation in the process of reversible redox reactions and important cellular functions including detoxification and metabolism.[1] Deficiency of cysteine (Cys) is usually associated with slowed growth, edema, liver damage, and skin lesions etc.[2] Glutathione (GSH), the most abundant intracellular nonprotein thiol, participates in a number of cellular processes including maintenance the reducing environment with in cells, neutralization of the free radicals and peroxides, and regulation of the nitric oxide cycle.[3] Altered levels of these two important biothiols have been implicated in a number of pathological conditions, including Alzheimer's and Parkinson's diseases for cysteine,[4] diabetes and HIV disease for glutathione.[5] Thus, a facile, sensitive, and selective detection of biothiols in biological samples is highly required.

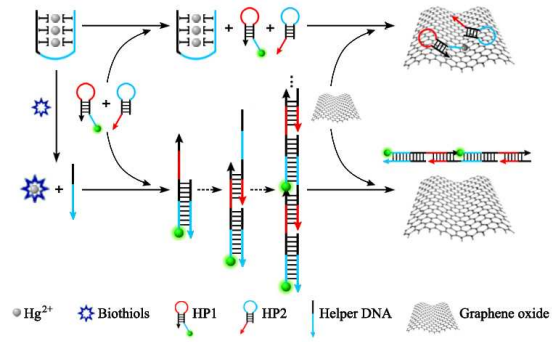
A number of methods to detect biothiols have been described, including capillary electrophoresis, high-performance liquid chromatography, and mass spectrometry. However, in most cases, these approaches require sophisticated instrumentation and technical expertise, as well as long analysis time, which have limited their practical applications. Due to the intrinsical high sensitivity, simplicity, and ease of operation, fluorescence-based strategies have received intensive attention. For instance, many organic small-molecule fluorescent probes have been developed for the biothiols, even imaging studies for intracellular thiols.[6] Quantum dot-based fluorescent sensors were also reported.[7] These strategies have good sensitivity and selectivity for biothiols, but most of them require time-consuming procedures for synthesis of small-molecule fluorescent probes and quantum dots. Therefore, facile routes with simplicity and high selectivity are still highly desirable in terms of the inherent limitation of the methods available.

Novel designs of DNA sensing platforms have recently been studied extensively in view of increasing applications of biosensors for disease biomarkers in the field and in point-of-care testing.[8] One of these sensing platforms consists of a hairpin-DNA molecular-beacon (MB) system which offers functionality and unique biorecognition propensity of nucleic acids and provides a variety of analytical signal transduction options.[9] The concept of hybridization chain reaction (HCR) has become a fascinating and effective amplification technique for enzyme-free amplified detection of DNA and proteins.[10] However, HCR has not been used for biothiols detection. Graphene oxide (GO), a single-atom-thick two-dimensional nanosheet possessing nice water-solubility and superior fluorescence quenching ability.[11] Its fluorescence quenching ability for fluorophores is also superior to many other materials.[12] Hence, graphene oxide has attracted significant research interest and hold great potential for many applications.[13] In addition, GO is a low-cost and environmentally friendly material.

Herein we report for the first time that a "turn-on", sensitive, and selective method for biothiol detection in aqueous solutions by using GO-based hairpin DNA-selective fluorescence quenching. In this sensing system, we design a helper probe with a few intramolecular thymine-thymine (T-T) mismatchs which can form a stem-loop hairpin structure due to T-Hg^{2+}-T coordination to respond to the biothiols.[14] In the presence of biothiols, thiol-Hg^{2+} interactions can release the helper probe in a single-stranded conformation, which then induces HCR of the hairpins and amplify the fluorescence signal. The produced dye-labeled double-stranded DNA (dsDNA) HCR product remains highly fluorescent on addition of GO nanosheets because of the weak adsorption of dsDNA on GO nanosheets. The novel method demonstrates superior ultrasensitivity with a detection limit for cysteine and glutathione have been obtained as 0.08 nM and 0.1 nM. To the best of our knowledge, our strategy is one of the most sensitive methods for biothiol assay.[15] In addition, the proposed nanosensing method exhibits a "turn-on" response to biothiols, which can increase the sensing reliability and overcome the disadvantages of previous "turn-off" techniques such as susceptibility to false positive response or inferior sensitivity.[16]

The developed strategy for biothiol detection is illustrated in Scheme 1. The sensing system consists of three probes, the helper DNA probe (DNA 1 in Table S1), FAM-labeled hairpin probe (HP1), and label-free hairpin probe (HP2). When incubated with Hg^{2+} in the absence of biothiols, the helper DNA probe forms a stem-loop hairpin due to the Hg^{2+}-mediated T-T base pairing. The hairpin structured helper probe disables the assembly of the HCR product such that the unreacted hairpin DNA probes, HP1 and

HP2, can be adsorbed on GO surface through their sticky ends and loops, delivering a very weak fluorescence signal due to the super quenching capability of GO to the fluorescence of FAM labels. After the addition of biothiols in the reaction mixture of the two hairpin DNA probes, the initiator probe and Hg^{2+}, the strong interaction between biothiols and Hg^{2+} enables the displacement of Hg^{2+} from the T-Hg^{2+}-T complex and destroys the hairpin-like structure. The helper DNA probe can then mediate the chain-like assembly of HP1 and HP2 through the HCR reaction, generating a long chain of helper DNA leading complex of HP1 and HP2. The produced dye-labeled double-stranded DNA (dsDNA) HCR product cannot be adsorbed on GO surface, thereby giving a strong fluorescence signal indicating the concentration of biothiols. Moreover, because several biothiol molecules can release a helper probe, which can trigger the formation of a long chain of HP1-HP2 assembly with a number of activated fluorophores, this strategy provides the possibility of substantial amplification of the fluorescence signal and enables a highly sensitive assay for biothiols.



Scheme. 1. Illustration of GO based platform couple with hybridization chain reactions for biothiol analysis.

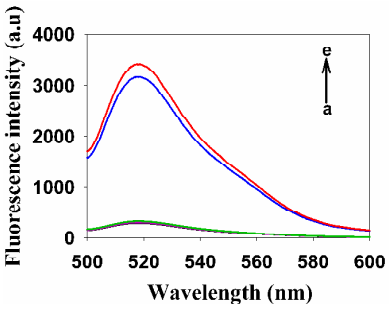
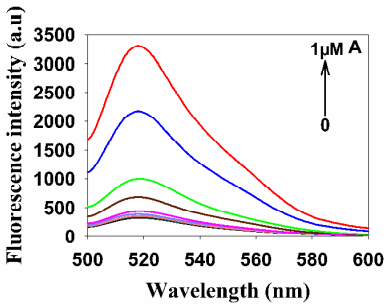


Fig. 1. The fluorescence emission spectra of different sensing systems: (a) Helper DNA1 + HP1 + HP2 + Hg^{2+} + GO; (b) Helper DNA2 + HP1 + HP2 + Hg^{2+} + GO; (c) Helper DNA2+ HP1+ HP2 +Hg^{2+} + cysteine+ GO; (d) Helper DNA1 + HP1+ HP2 + Hg^{2+} + cysteine + GO; (e) Helper DNA1 + HP1 + HP2 + Hg^{2+}. (GO 5 μg mL^{-1}, Helper DNA1 50 nM, Helper DNA2 50 nM, HP1 1 μM, HP2 1 μM, Hg^{2+} 500 nM, cysteine 2 μM).

To confirm the feasibility of this strategy, the fluorescence emission spectra of various phases of the assay are show in Fig. 1. The sample containing Hg^{2+}, HP1, HP2, and helper DNA1 was found to exhibit high fluorescence signal (curve e). However, up to 90% fluorescence emission was quenched upon the addition of 5 μg mL^{-1} GO (curve a). The fact was attributed to the strong adsorption of the hairpin DNA on the GO nanosheets surface and the super fluorescence quenching ability of GO nanosheets originated from the effective FRET between the fluorescent dye and GO nanosheets. In contrast, in the presence of 2 μM cysteine, the response signal recovered even after GO was added into the solution (curve d). On the other hand, if helper DNA1 was replaced by DNA2 that was not able to trigger the HCR, the response signal remained almost unchanged (curve c). Taken together, these results suggested that the HCR was triggered by biothiols with the aid of helper DNA1, which demonstrated our proposed strategy had satisfactory response performance for biothiol assay. Additionally, the formation of long dsDNA via the HCR in response to biothiols was also proved by electrophoresis analysis (Fig. S2 in ESI).



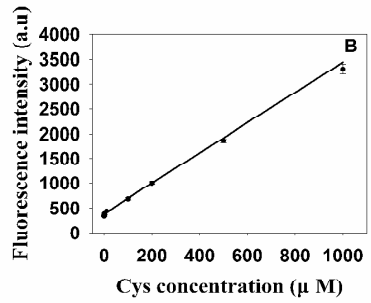


Fig. 2. (A) Fluorescence emission spectra of GO nanosheet platform in the presence of increasing amounts of cysteine, the arrow indicating the signal changes with increases in cysteine (0, 0.1, 0.5, 1, 10, 100, 200, 500 and 1000 nM). (B) Calibration curve of GO nanosensor in the presence of increasing amount of cysteine dependence of fluorescence intensity on the concentration of cysteine. The final concentrations of GO, Helper DNA1, HP1, HP2 and Hg^{2+} ions are 5 μg mL^{-1}, 50 nM, 1 μM, 1 μM and 500 nM, respectively.

In order to assess the applicability of this method, cysteine was used for testing. After Hg^{2+} was mixed with the helper DNA1, cysteine with different concentration (from 0 to 1000 nM) was added to the solution and incubated for 10 min. Due to the chelation between cysteine and Hg^{2+} ion, the more cysteine was added, the higher dissociation of Hg^{2+} from T-Hg^{2+}-T base pair occurred. As a result HCR will continue and generate a long chain of HP1-HP2-helper DNA-target complex. As shown in Fig. 2A, the fluorescence responses of the system gradually increased with the increase of cysteine concentration. A good linear relationship over the range from 0.1 to 1000 nM with a correlation coefficient of 0.998 was obtained (Fig. 2B, F and F_0 are fluorescence intensities in the presence and absence of cysteine, respectively). The detection limit of cysteine was 0.08 nM according to the 3σ rule, which was lower than that of the previous polymeride templated Ag clusters and quantum dots methods.[7a,16] Glutathione could also react with Hg^{2+} ion due to the

existence of thiol group. All procedures for glutathione detection were the same as those for cysteine assay. Similarly, detection of glutathione displays the same concentration dependence with a linear relationship in the range of 0.1-1000 nM and a detection limit of 0.1 nM according to the 3σ rule, which was much lower than those obtained from reported fluorescent probe and quantum dots assays (Fig. S6 in ESI).[17] The result indicated that the GO nanosheet platform could be applied to highly sensitive biothiol analysis in a wide concentration range.

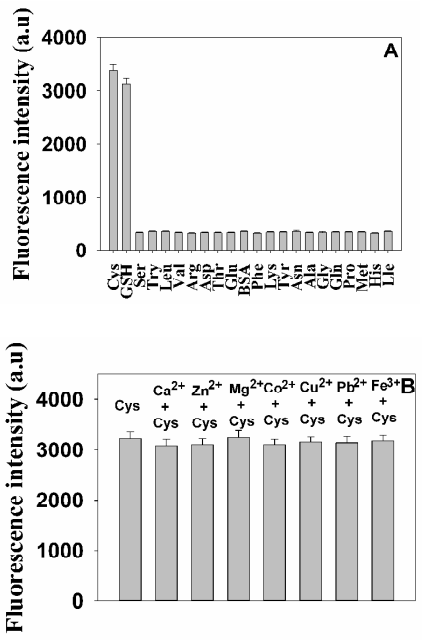


Fig. 3. Fluorescence intensity change(F-F_0) of GO nanosheet platform in the presence of various amino acids, BSA (A) and meta lions(B), where F and F_0 are fluorescence intensities of GO nanosheet platform in the presence and absence of different amino acid, BSA and metal ions. The error bar represents the standard deviation of three measurements. The final concentrations of GO, Helper DNA1, HP1, HP2, Hg^{2+} ions , cysteine, glutathione and the other amino acids are 5 μg mL^{-1}, 50 nM, 1 μM, 1 μM, 500 nM, 1 μM and 10 μM respectively. The concentration of BSA was 20mg/L. The concentrations of Ca^{2+}, Fe^{3+}, Mg^{2+}, Cu^{2+}, Zn^{2+}, Co^{2+}, and Pb^{2+} were 5 μM.

High selectivity is essential to the development of a good sensor. Considering the structural similarity of amino acids, the potentially effects of other amino acids on the system were investigated to evaluate the selectivity. The concentration of other amino acids was 10-fold higher than cysteine and glutathione. As shown in Fig. 3A, the changes of fluorescence intensities in the presence of cysteine or glutathione were strikingly larger than that of other amino acids. Besides, the possible interference of other substances that might exist in biological samples, such as BSA (20 mg/L) and metal ions were also examined. The concentration ratio of metal ions to cysteine used in the assay is much larger than it in real human serum sample, but they almost unaffected the response of this biosensor to cysteine (Fig. 3B). This excellent selectivity is attributed to the specific and stable interaction between biothiols and Hg^{2+} ions. These results indicate that the proposed assay has high selectivity for biothiols and it is practical for the determination of biothiols in biological samples.

In order to evaluate the applicability of the present sensing assay in biological samples, the determination of total biothiols content in human serum was performed, which generally contains proteins, hormones, glucose, and other biological substances. Obviously, the biological samples exhibited high fluorescence signal, indicating that human serum contained biothiols (Fig. S7 in ESI). In contrast, the pretreatment of human serum samples with the thiol-blocking reagent N-ethylmaleimide for 2 h resulted in no distinct increase of the fluorescence intensity (Fig. S7 in ESI). This observation shows that fluorescence changes in the assay system is entirely caused by biothiols present in the test sample. The treated human serum samples were firstly diluted by phosphate buffer (pH 7.4) to fall into the linear range of our method and to obtain quantitative recovery of the spiked thiols. The total biothiols content in human serum was determined by the standard addition method using cysteine as the standard.[18] The obtained biothiol levels were within the reported normal range.[19] The recovery results ranged from 99% to 105%, indicating that no significant interference with the determination of biothiols in human serum samples was encountered after an appropriate dilution of the samples (Table S2 in ESI). The good recoveries of known a mount cysteine in the serum sample definitely demonstrate the accuracy and reliability of the present method for biothiol determination in practical applications.

In summary, an HCR- and GO-based fluorescent sensor has been developed for the "turn-on" detection of biothiols in aqueous solutions. In the sensing system, the target, biothiols, induced the formation of long duplex chains through destroying the T-Hg^{2+}-T coordination chemistry and amplified fluorescence emission. The HCR guaranteed high sensitivity of the method. GO was employed as an excellent fluorescence quencher to lower the background signal and help strengthen the detection sensitivity further. GO also functioned as the signal controller by selectively adsorbing hairpin DNA and liberating long dsDNA products. The detection limit for cysteine and glutathione were experimentally obtained as 0.08 nM and 0.1 nM, respectively. Experiments for human serum samples demonstrated that the detection method was applicable for detection of biothiols in real samples.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Experimental details and additional Figures. See DOI: 10.1039/b000000x/

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