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A label-free fluorimetric detection of biothiols based on the oxidase-like activity of Ag^{+} ions

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ABSTRACT

In this work, a label-free and sensitive fluorimetric method has been developed for the detections of biothiols including cysteine (Cys), homocysteine (Hcy), and glutathione (GSH), based on the specific biothiol-induced inhibition of the oxidase-like activity of silver ions (Ag^{+}). It is well established that *o*-phenylenediamine (OPD) can be oxidized by Ag^{+} ions to generate fluorescent 2,3-diaminophenazine (OPDox). The introduction of biothiols would inhibit the oxidation of OPD by Ag^{+} due to the strong coordination between biothiols and Ag^{+}. The changes of fluorescence intensities obtained in the Ag^{+}-OPD system exhibited good linear correlations in the ranges of 0.50–30.0 μM for Cys, 1.0–45.0 μM for Hcy and 0.50–40.0 μM for GSH. The detection limits (S/N = 3) of Cys, Hcy and GSH were 110 nM, 200 nM and 150 nM, respectively. Subsequently, the developed fluorimetric method was successfully applied for the detection of biothiols in human serum.

1. Introduction

Biothiols such as cysteine (Cys), homocysteine (Hcy), and glutathione (GSH) are important components of many proteins and play crucial roles in human physiological functions and pathological conductions [1]. Abnormal levels of biothiols are connected with various chronic diseases, such as arthritis, cancer, HIV/AIDS, and so on [2,3]. For example, deficiency of Cys is usually associated with slowed growth, hair depigmentation, edema, liver damage, lethargy, muscle, and fat loss, and skin lesions etc. [4]. Abnormal Hcy levels are an indicator of chronic renal failure and are associated with cardiovascular disease [5,6]. Also, GSH, as an important antioxidant in vivo, plays an important role in physiological processes such as oxidative stress and cell growth [7]. Therefore, it is of great interest to develop efficient methods for the detection of biothiols in biological samples.

During the past decade, a number of effective analytical methods have been developed for the detection of biothiols, typically including high performance liquid chromatography (HPLC) [8], capillary electrophoresis [9], electrochemical method [10], and mass spectrometry (MS) [11,12]. Although these strategies showed promising results for biothiols detection, there are still some hindrances such as the time-consuming process, the utilization of sophisticated and expensive instrumentation, and the requirement of technical expertise. Recently, fluorescence-based bioanalytical methods have become extremely popular due to their remarkable advantages, such as high sensitivity, simplicity, economy, and real-time detection [13–27]. Particularly, a variety of fluorescent determination methods have been developed for biothiols by using organic dyes [20,21], nanomaterials [22–26] and DNA-based biosensors [27]. However, most of these fluorescent probes used may encounter with the time-consuming preparation procedure and high cost. Therefore, it is still highly desirable to establish more convenient and sensitive assays for biothiols.

Aiming to address the aforementioned drawbacks, we have concentrated on the utilization of conventional yet promising organic fluorophore without the time-consuming synthesis process and complicated post-treatment process. Previous reports have shown that *o*-phenylenediamine (OPD) could be oxidized by Ag^{+} ions to yield 2,3-diaminophenazine (usually called OPDox) exhibiting an orange-yellow fluorescence when irradiated by ultraviolet light [28,29]. It is also reported that biothiols possess the strong affinity towards metal cations especially Ag^{+} and Hg^{2+} [26,27,30,31]. Inspired by these facts, a novel and simple fluorescent assay for biothiols with Ag^{+}-regulated and biothiols-inhibited oxidation of OPD has been innovatively developed for the first time. The proposed method is simple and fast for biothiols detection without the complex synthesis procedure and the use of expensive instruments. The developed method was successfully applied to the direct analysis of biothiols in biological fluid.

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2. Experimental

2.1. Materials and Reagents

Cysteine (Cys), homocysteine (Hcy), glutathione (GSH), $AgNO_3$, *o*-phenylenediamine (OPD), $Na_2HPO_4 \cdot 12H_2O$, $NaH_2PO_4 \cdot 2H_2O$, and 11 other amino acids were purchased from Aladdin Company (Shanghai, China). 10 mM phosphate buffer solutions (PBS) were prepared by mixing stock standard solutions of Na_2HPO_4 and NaH_2PO_4. All reagents were of analytical reagent grade, and used as received. Doubly deionized water was used throughout.

2.2. Apparatus

All fluorescence measurements were carried out on a fluorescence spectrophotometer (FluoroMax-4, SPEX, USA) operated at an excitation wavelength at 417 nm. UV–vis spectra were recorded on a UV–vis spectrophotometer (Shimadzu, UV-3600, Japan).

2.3. Procedure for the Detection of Biothiols

Cys, Hcy and GSH with different concentrations were freshly prepared before use. First, 5.0 μL of 6.0 mM $AgNO_3$ were incubated with different concentrations of biothiol solution for a certain time, followed by the addition of 30.0 μL of 6.0 mM OPD for a certain time incubation and then diluted to 500.0 μL with 10 mM PBS at pH 6.0. The fluorescence intensities were recorded in the wavelength range from 450 nm to 700 nm. To investigate the selectivity of this assay, influence of the other 11 amino acids to the Ag^+-OPD system was investigated. All the fluorescence detection was under the same conditions throughout the experiment: the slit widths of both excitation and emission were 5 nm, respectively.

2.4. Determination of Practical Samples

The human serum samples were obtained from the local hospital. For determination of total thiols in serum samples, the disulfide bonds were reduced to the protein-bound thiols by addition of triphenylphosphine (PPh_3) as catalyst [32,33]. Briefly, 500.0 μL of collected serum were vigorously mixed with 40.0 μL of 0.2 M HCl and 20.0 μL of 0.4 M PPh_3 (in water-acetonitrile 20:80 V/V and 2.0 M HCl). After incubation for 15 min, the hydrolysed serum was mixed with 500.0 μL of acetonitrile to precipitate proteins, followed by centrifugation at 4000 rpm for 20 min. The supernatant containing biothiols in serum was used for further analysis, and the known amount of thiols was estimated using a standard addition method. Since the thiols content of serum is beyond the dynamic range of the proposed method, the serum samples were appropriately diluted with 10 mM PBS before measurement.

3. Results and Discussion

3.1. Design and Establishment of Biothiols Sensing System

It is well recognized that the aqueous solution of pure OPD was colorless and exhibited negligible fluorescence as shown in Fig. S1. In contrast, the introduction of Ag^+ ions essentially triggered the oxidative reaction of OPD, during which the original colorless and nonfluorescent solution has gradually turned to pale yellow in color and showed intense orange-yellow fluorescent under ultraviolet light. Neither Ag^+ ions nor OPD solution have obvious absorption peak in the visible range, while the Ag^+-OPD mixed solution has an obvious absorption peak at 417 nm, which belongs to the characteristic absorption spectrum of OPDox, the main oxidation product of OPD [34]. Meanwhile, the corresponding fluorescence excitation peak and emission peak of the Ag^+-OPD mixed solution were observed at about 417 nm and 560 nm (Fig. S2), respectively, which is similar to that of OPDox in previous work as well [28,29]. Importantly, we found that both the fluorescence and absorption intensities could decrease obviously in the presence of biothiols (Fig. 1B and Fig. S3). This indicated that the oxidative reaction of OPD could be partially inhibited in the presence of biothiols because of the preferential complexation affinity of biothiols to Ag^+. Thus, the above phenomena illustrate that biothiols could restrain the Ag^+-OPD oxidative reaction. Therefore, a convenient fluorimetric assay could be designed and established for sensing biothiols sensitively and selectively (Fig. 1A).

3.2. Optimization of the Sensing System

The reaction conditions were optimized to establish the optimum analytical conditions. We used the changes of the fluorescence intensities, that is F_0-F, as a criterion to optimize the detection conditions, where F_0 and F are the fluorescence intensities at 560 nm in the absence and presence of biothiols, respectively.

3.2.1. Effect of Reaction Time

The effect of reaction time on the detection of biothiols was studied, and the experimental results were shown in Fig. 2. The results indicated that the fluorescence intensity of Ag^+-OPD system increased gradually and reached the maximum when the reaction time reached 10 min, which means that the oxidation reaction was almost completed within 10 min. However, the fluorescence intensity of Ag^+-OPD system rapidly decreased in the first 1 min and then changed slightly in the following 15 min upon the addition of biothiols. Obviously, the F_0-F reached the maximum in 10 min. In the following experiments, reaction time of 10 min was chosen.

3.2.2. Effect of pH Values

The pH value is a crucial factor for almost every sensing system. According to the previous reports, Ag^+ can exhibit excellent oxidation ability towards OPD in acidic media [29]. Thus, we investigated the effects of pH in the range of 4.0–7.0 on the detection of biothiols. As shown in Fig. 3, the Ag^+-OPD system showed a stronger fluorescence intensity in the weak acidic media (pH 4.0–7.0), whereas the fluorescence intensity of Ag^+-OPD system decreased obviously in the presence of biothiols. The fluorescence intensity showed the largest difference when the pH value of the media was around 7.0. Therefore, pH 7.0 was the optimal condition for the sensing system.

3.3. Sensitivity of the Sensing System

Under the optimization conditions, we evaluated the capability of this analytical system for quantitative detection of biothiols. As shown in Fig. 4, it was clearly seen that with the increase of biothiols concentrations, the fluorescence intensities of Ag^+-OPD system decreased gradually while the F_0-F increased systematically. The F_0-F exhibited a good linear relationship with biothiols in the concentration ranges of 0.50–30.0 μM, 1.0–45.0 μM, and 0.50–40.0 μM for Cys, Hcy, and GSH, respectively. The regression equations were F_0-$F = 18.99 + 11.22C$ ($R^2 = 0.9967$) for Cys, F_0-$F = 17.32 + 6.491C$ ($R^2 = 0.9979$) for Hcy, and F_0-$F = 14.58 + 8.456C$ ($R^2 = 0.9943$) for GSH, respectively. The detection limit (S/N = 3) can reach as low as 110 nM, 200 nM and 150 nM for Cys, Hcy and GSH, respectively. Moreover, the repeatability of the proposed method was evaluated by five repeated measurements of 5.0 μM Cys, Hcy and GSH and the relative standard deviation (RSD) was 3.56%, 2.67% and 3.12%, respectively, demonstrating the reliability of the proposed method.

In addition, we compared the characteristics of the proposed sensor with other fluorescent biothiols sensors reported elsewhere. As shown in Table 1, the Ag^+-OPD system could provide better sensitivity in comparison with DNA-CuNCs [36], $CuInS_2$ QDs [23], CdTe QDs [22], and diethanol amine modified graphene quantum dots (GQD-DEA) [38].