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Detection of biological thiols based on a colorimetric method*

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Abstract: Biological thiols (biothiols), an important kind of functional biomolecules, such as cysteine (Cys) and glutathione (GSH), play vital roles in maintaining the stability of the intracellular environment. In past decades, studies have demonstrated that metabolic disorder of biothiols is related to many serious disease processes and will lead to extreme damage in human and numerous animals. We carried out a series of experiments to detect biothiols in biosamples, including bovine plasma and cell lysates of seven different cell lines based on a simple colorimetric method. In a typical test, the color of the test solution could gradually change from blue to colorless after the addition of biothiols. Based on the color change displayed, experimental results reveal that the percentage of biothiols in the embryonic fibroblast cell line is significantly higher than those in the other six cell lines, which provides the basis for the following biothiols-related study.

Key words: Biothiols, Colorimetric detection, Bovine plasma, Cell lines
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1 Introduction

Biological thiols (biothiols), an important kind of functional biomolecules, such as cysteine (Cys) and glutathione (GSH), are pivotal in many biological processes. In recent decades, many studies have demonstrated that metabolic disorder of biothiols will lead to extreme damage in human and numerous other animals (Townsend *et al*., 2003; Reddie and Carroll, 2008; Weerapana *et al*., 2010). Specifically, a low level of biothiols in vivo may cause slowed growth, liver damage, weight loss, and some other adverse influences (Wang *et al*., 2005; Duan *et al*., 2008; Yang *et al*., 2012), while a high level of biothiols is closely related to Parkinson's, Alzheimer's, and cardiovascular diseases (Heafield *et al*., 1990; Rusin *et al*., 2004; Lin *et al*., 2008; Yang *et al*., 2011). Thus, a lot of effort has been put into quantifying the low-molecule-weight biothiols in serum (Ni *et al*., 2015; Ghasemi *et al*., 2015). However, there has been very little work on the detection in cellular lysates including high-molecule-weight biothiols, although biothiol determination of total proteins in cell lysates is of great diagnostic significance.

Several methods based on chromatography, electrophoresis, and fluorescent technology for biothiol detection have been reported, especially nanomaterial-based methods much facilitated by the development of the underlying nanotechnology (Lee *et al*., 2008; Hong *et al*., 2009; Yi *et al*., 2009; Shiu *et al*., 2010; Liu *et al*., 2014; Zhang *et al*., 2014; Zhu *et al*., 2014). Each method has its own advantage; however, pretreatments of the materials or expensive equipments are often necessary to obtain a good performance. It is worth noting that employing metal nanomaterials in analytical strategies attracts more and more interest (Ran *et al*., 2013) and makes great

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progress in accurate and convenient biothiol detection. Recently, a simple colorimetric method to assay biothiols in biosamples has been reported based on 3,3',5,5'-tetramethylbenzidine (TMB)-triggered silver (Ag) nanoparticles formation (Ni *et al*., 2015). In a typical test solution, the color of the solution changes from colorless to blue by mixing together TMB, a common chromogenic material, and silver ion (Ag^+) (Yang *et al*., 2008). When biothiols are present in the test system, biothiols would cause the reduction of oxidized TMB and at the same time could bind to Ag^+, both of which suppress the TMB oxidized process, and thus the test solution remains colorless. It makes the detection procedure convenient. Clearly, the simpler the detection process, the wider the potential usage of the method.

We have carried out a series of experiments based on the above reported principles to achieve an optical method by optimizing the experimental conditions (Ni *et al*., 2015). Then the optical method was used to detect biothiols in bovine plasma and total proteins in different kinds of cell lines.

2 Materials and methods

2.1 Chemicals and materials

TMB and silver nitrate were purchased from Sigma-Aldrich (Shanghai, China). Twenty amino acids (including alanine (A), arginine (R), asparagine (N), aspartic acid (D), cysteine (C), glutamine (Q), glutamic acid (E), glycine (G), histidine (H), isoleucine (I), leucine (L), lysine (K), methionine (M), phenylalanine (F), proline (P), serine (S), threonine (T), tryptophan (W), tyrosine (Y), and valine (V)), GSH and *N*-ethylmaleimide (NEM) were from the Shanghai Sangon Biological Engineering & Technology and Service Company (Shanghai, China). Fetal bovine serum (FBS) and Dulbecco's modified Eagle medium (DMEM) were bought from Sunshine Biotechnology Co., Ltd. (Nanjing, China). Ethanol, sodium acetate, and acetic acid were from the Nanjing Chemical Reagents Industry, China. All chemicals were used as supplied without further purification. TMB (20 mmol/L) was dissolved in ethanol. In addition, 1 mmol/L $AgNO_3$ and 1 mol/L NaAc reaction buffer (pH 4.0) were prepared by double-distilled water, which was purified with a Milli-Q purification system (Branstead, USA) to a specific resistance of 18.2 MΩ·cm and stored at 4 °C.

2.2 Preparation of biosamples

Bovine blood samples were collected from a jugular catheter. Heparin was used as an anticoagulant and the bovine plasma was prepared by centrifugation at 3000*g* at 4 °C for 10 min and stored at −20 °C.

The proteins were extracted from different cell lines by cell lysates. Cell lysates containing all proteins were obtained by the following procedure. All cell lines, including the hepatoma (Hepa), breast adenocarcinoma (MCF), breast cancer (4T1), the macrophage (RAW), the melanoma (F10), and the embryonic fibroblast cell line (3T3-H) were cultured in DMEM. Each cell line was first rinsed with phosphate buffered saline (PBS) three times. After incubation with 2 ml trypsin at 37 °C for 1.5 min, DMEM (FBS included) was added quickly and all solutions were collected by centrifugation at 1000 r/min for 5 min. Then, a cell pellet was suspended with 1 ml ice-cold PBS and centrifuged at 1000 r/min for 5 min. After that, 100 μl of cold lysis buffer was added to the cell pellet and placed on ice for 20 min and vortexed every 5 min. Finally, by centrifuging at 12000 r/min for 5 min, supernatant was collected and ready for use.

2.3 Detection of biothiols

The detection of biothiols was carried out at room temperature using TMB as an indicator in a NaAc buffer solution. In a typical run, biothiols (Cys or GSH) at different concentrations were first incubated with $AgNO_3$ for a few seconds. Then TMB was added to the above mixture. The UV-Vis spectral measurements were carried out by monitoring the absorbance change after 1 h reaction at room temperature. Cys or GSH was replaced by bovine plasma or cell lysates in real sample detection.

3 Results

3.1 Mechanism of the sensing system

The proposed method for biothiol detection is comprised of Ag^+ and TMB. TMB can deoxidize silver ions, causing Ag nanoparticle formation, accompanied with the color changes from colorless to

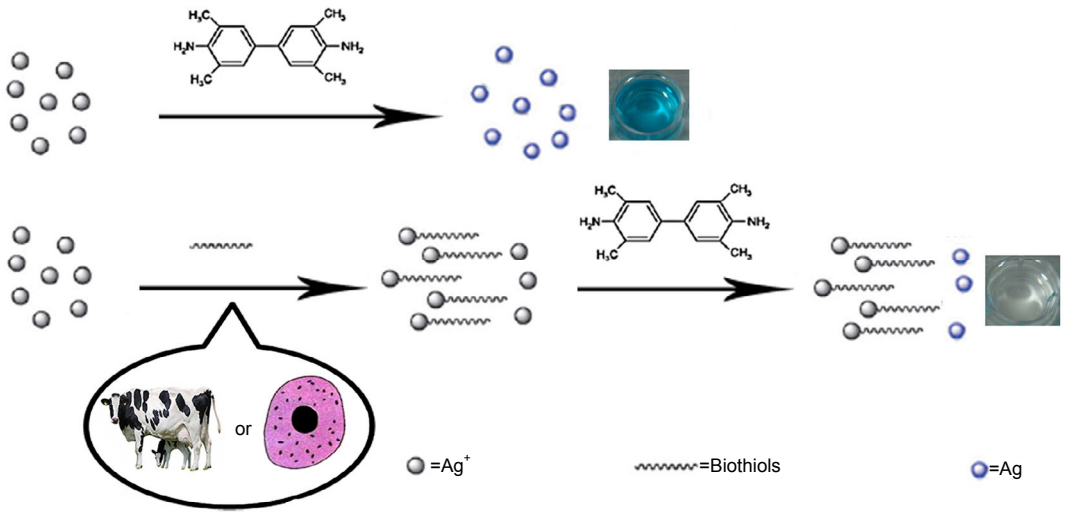

Fig. 1 Colorimetric assay of the detection of biothiols

blue (Yang *et al*., 2008). While biothiols can bind with Ag^+ first, this will further affect the oxidization of TMB and retain the colorlessness (Fig. 1). As the degree of color is closely negatively related with the quantity of biothiols in solution, a simple and sensitive assay for biothiol detection can be achieved.

3.2 Feasibility and selectivity of biothiol detection

The feasibility of this optical method is first identified by taking Cys as an example and experimental results from UV-Vis spectra and photographs are shown in Fig. 2. Without biothiols in solution, TMB is oxidized to TMB^+ by Ag^+ ion, which results in a significant peak at 656 nm in UV-Vis spectrum (curve A in Fig. 2). With the addition of biothiols, no peak at 656 nm can be detected (curve C in Fig. 2). An obvious difference can be observed in the presence and absence of biothiols. Furthermore, the effect of biothiols in this assay is further identified by NEM, a widely used thiol blocking agent (curve D in Fig. 2). There is almost no difference with curve A, which indicates that NEM-treated biothiols have nearly no influence on the oxide reaction between Ag^+ ions and TMB in this biothiols-containing system due to the blocking effect of NEM. The presence of NEM can impede the interaction between biothiols and Ag^+ ions, which will dispel the effect of biothiols on Ag^+ ions. As a result, the color of the solution changes from colorless to blue and the typical peak at 656 nm appears.

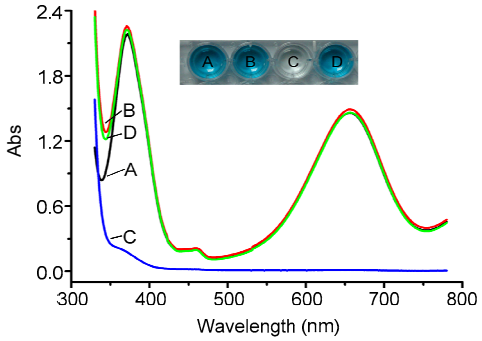

Fig. 2 UV-Vis spectra of different test solutions
Curve A: Ag^+ and TMB; Curve B: Ag^+, TMB and NEM (thiol blocking agent); Curve C: Ag^+, TMB and Cys; Curve D: Ag^+, TMB, NEM and Cys. Inserted photographs of the test solutions contain: (A) Ag^+ and TMB; (B) Ag^+, TMB and NEM; (C) Ag^+, TMB and Cys; (D) Ag^+, TMB, NEM and Cys

The selectivity of the method we proposed can be further confirmed. By testing 20 kinds of natural amino acids, it can be seen that only Cys can affect the oxide reaction between TMB and Ag^+ ions effectively. Thus, only the sample containing Cys remains colorless, while the other amino acid solutions all turn blue (Fig. 3a). The same results can be obtained by UV-Vis spectra and the histogram of UV-Vis spectral responses of different amino acids is shown in Fig. 3b.

3.3 Colorimetric detection of biothiols

After demonstrating the feasibility and selectivity of the method, we go on to detect the concentration

of biothiols. Two kinds of typical biothiols, Cys and GSH, are used as examples. Cys, which plays important roles in cell metabolism, is first detected. Experimental results reveal that this method can detect Cys with good sensitivity. Each Cys in the test system can greatly affect the oxide reaction between TMB and Ag^+ ions. As shown in Fig. 4, the intensity of the characteristic peak at 656 nm decreases when the concentration of Cys increases. The absorbance peak almost disappears when the Cys concentration is more than 100 μmol/L. By taking the concentration of Cys as the *x*-axis and the value of absorbance peak as the *y*-axis, the relationship between the absorbance at 656 nm deduct that at 753 nm ($Abs_{656–753\ nm}$) and Cys concentrations (C_{Cys}, in μmol/L) is obtained (Fig. 5a). A linear relationship between $Abs_{656–753\ nm}$ and C_{Cys} within the range of 0.5–50.0 μmol/L is also gained (Fig. 5a, inset). The linear equation is $Abs_{656–753\ nm}=-0.01544C_{Cys}+0.91206$ (n=3; R^2=0.99983) with a detection limit at 0.11 μmol/L. This method is not only suitable for detecting Cys but also biothiols in small peptides, such as GSH. As is shown in Fig. 5b, GSH can be detected in the linear range from 0.25 to 50.00 μmol/L. The linear regression equation is $Abs_{656–753\ nm}=-0.01924C_{GSH}+0.96297$ (n=3, R^2=0.99818) and the detection limit (3σ) is 0.09 μmol/L, where C_{GSH} is the concentration of GSH (μmol/L).

3.4 Biothiols in real samples

It has been widely accepted that the quantity of biothiols in vivo is highly related to certain diseases, so determination of biothiols in biosamples is of great diagnostic significance. To make sure of more explicit applications of our method in practical samples, we tested the biothiols of total proteins in bovine plasma and different cell lines. Bovine blood samples were collected from a jugular catheter and the bovine plasma was prepared by centrifugation. According to the sensor, the quantity of biothiols in bovine plasma

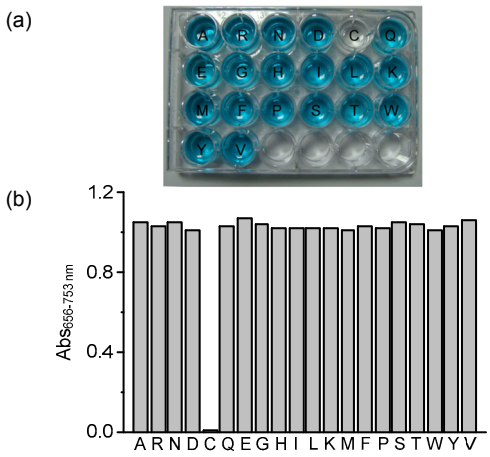

Fig. 3 Photographs (a) and histogram of UV-Vis spectra (b) for different amino acids

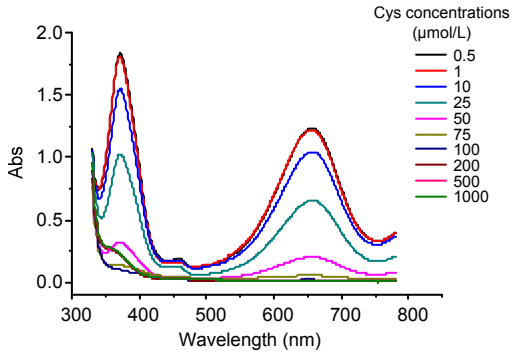

Fig. 4 UV-Vis spectra of mixed solution in the presence of different concentrations of Cys

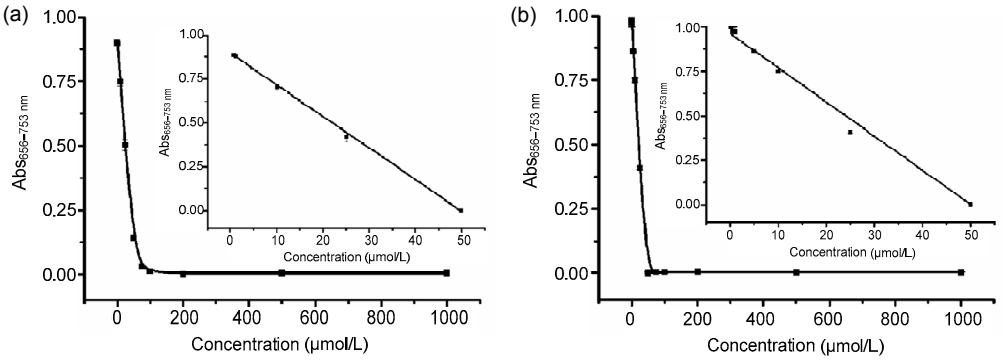

Fig. 5 Calibration curves of Cys (a) and GSH (b) with different concentrations

The inset indicates that the values of $Abs_{656–753\ nm}$ versus the Cys (a) or GSH (b) concentrations are linear over the range of 0.5–50.0 μmol/L. The data are presented as mean±standard deviation (SD) from three independent tests

Table 1 Determination of biothiols in bovine plasma

No.	Determined biothiol (μmol/L)	Added Cys (μmol/L)	Found Cys (μmol/L)	Recovery (%)	RSD (%)
1	486	1250	1743	100.56	0.794
2	486	2500	2988	100.08	2.101

RSD: relative standard deviation. The data are determined after three parallel experiments

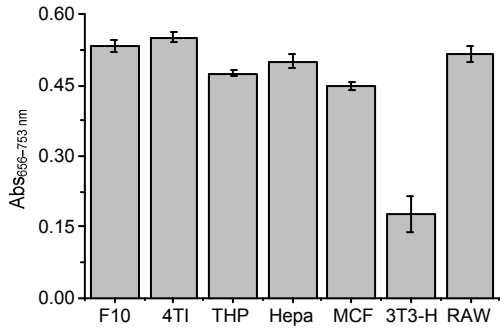

Fig. 6 Histogram of UV-Vis spectral responses to 7 kinds of cell lysates
The data are presented as mean±SD from three independent tests

was 486 μmol/L (Table 1). We added different quantities of Cys into bovine plasma and tested it again. After three parallel experiments (n=3), it can be seen that good recoveries can be obtained with relative standard deviation less than 2.5%, which means that this method can detect biothiols in complex biosamples effectively. We then successively evaluated the biothiol quantity of total proteins in different cell lines (Fig. 6). After cell disruption and adjusting the quantity of total protein equal to 1 mg/ml, the results revealed the quantities of biothiols in different cell lines. It can be seen that the contents of biothiols in 3T3-H cells are at least double that in the other six kinds of cell lines.

4 Conclusions

Following on from the previously reported work, not only the quantity of biothiols in bovine plasma is identified, but also biothiols including high-molecule-weight biothiols in different cell lysates are detected. By comparing biothiols of total proteins in different cell lines, it was found that the percentage of biothiols in 3T3-H cells is significantly higher than those in other cell lines, which offers great value in the related embryonic fibroblast cell study.

Contributors

Jin-feng MIAO and Yuan-yuan XU conceived the project and designed the experiments. Yuan-yuan XU and Yang-yang SUN performed the experiments. Yu-juan ZHANG and Chen-he LU prepared figures and wrote the manuscript.

Compliance with ethics guidelines

Yuan-yuan XU, Yang-yang SUN, Yu-juan ZHANG, Chen-he LU, and Jin-feng MIAO declare that they have no conflict of interest.

All institutional and national guidelines for the care and use of laboratory animals were followed. The animal care and use protocol employed is approved by the Institutional Animal Care and Use Committee of Nanjing Agricultural University and implemented in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals (China, 1988) and the Standards for the Administration of Experimental Practices (Jiangsu, China, 2008).

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中文概要

题　目：生物巯化物的可视化检测

目　的：通过简单可靠的可视化检测方法评估牛血清及各细胞系中生物巯化物的含量。

创新点：基于银纳米颗粒形成的比色变化过程对牛血清及细胞中生物巯化物进行了检测。

方　法：将 6 组不同的细胞系培养后进行裂解，其裂解产物分别与 3,3',5,5'-四甲基联苯胺（TMB）和硝酸银（$AgNO_3$）的混合液室温孵育后，用紫外可见分光光度计测量细胞中生物巯化物的含量。

结　论：通过不同细胞系中生物巯化物含量的比对，证实胚胎成纤维细胞中生物巯化物的含量明显高于其他细胞。

关键词：生物巯化物；可视检测；牛血清；细胞系