Testing of glutathione contents by calcein-Cu(II) fluorescence spectra

L Liu\textsuperscript{1}, W Wu, Y Zhang, W Chen, J Y Guo and S Z Li

School of Electronic and Electrical Engineering, Wuhan Textile University, No.1 Yangguang Street, Wuhan, China
*E-mail: liuli@wtu.edu.cn

Abstract. Glutathione (GSH) is a natural active tripeptide formed by glutamic acid, glycine, and cysteine, which plays a vital role in many physiological processes of the living body. The GSH contents of the sample were detected by using calcein-Cu(II) fluorescence system in our work. In a Na\textsubscript{2}HPO\textsubscript{4}-NaH\textsubscript{2}PO\textsubscript{4} buffer with a pH of 8.0, Cu(II) could coordinate with calcein. Calcein-Cu(II) complex was not fluorescent which led to a reduction of calcein fluorescence intensity. For the strong affinity of GSH with copper (II), the addition of GSH solution would make calcein free from calcein-Cu(II) complex and recover fluorescence. Excitation spectra and emission spectra of calcein and calcein-Cu(II) system were researched. And fluorescence spectra of calcein-Cu(II) system with different GSH contents were investigated. The influence of experimental parameters such as reaction time and the addition sequence of the solution on results was also studied. We got the following conclusions: first, Cu(II) can make the calcein fluorescence be quenched, but the wavelength at the peak of fluorescence is unchanged. Second, the addition of Glutathione can make the fluorescence of calcein - Cu (II) system recover. And the change of the fluorescence intensity has a linear relationship with the concentration of GSH in the range from 2×10\textsuperscript{-6} to 1.0×10\textsuperscript{-4} mol/l. Third, response time may be extended appropriately for complete reaction in the system. At last, the experimental results have shown that calcein - GSH - Cu(II) system has higher sensitivity and better stability than calcein - Cu(II)- GSH system. This test method is of great importance in practical application.

1. Introduction
Glutathione (GSH) is a kind of important tripeptide containing sulfhydryl group, amino group, and gamma amide bond, which is formed by condensation of glutamic acid, glycine, and cysteine. It is widely distributed in body fluid and tissue fluid of human and animals and is a kind of metabolic regulators in cells [1]. It not only removes peroxide and other free radicals in the body but also promotes the activity of the liver enzyme and maintains the integrity of the erythrocyte membrane. At the same time, it also has many physiological functions such as maintaining DNA's biosynthesis and cellular immunity. Therefore, rapid, efficient and accurate detection on GSH is vital to the application of prevention, investigation, and treatment of some diseases.

In recent years, many test methods have been used, such as spectrophotometry, chromatography, capillary electrophoresis, an electrochemical method, surface enhanced Raman scattering (SERS), fluorescent probe and so on [2]. Fluorescence analysis has the advantages of a simple test, high selectivity and short response time [3, 4], and more importantly, it can also be utilized in real-time monitoring and imaging of organisms and has been widely applied in biomedicine, analytical chemistry, chemical biology, and many other fields.

GSH could be tested usually when it was combined with a fluorescence system. Cao et al. determined glutathione in cucumber by using orthophthalalddehyde as a complexing agent, the determining limit was 0.1μg/ml [5]. Xu et al. synthesized water-soluble ZnS quantum dot by using mercaptopropanoic acid (MPA) as the capping ligand for the detection of GSH [6]. The fluorescence intensity of the ZnS QDs was significantly quenched in the presence of Ni\textsuperscript{2+} ions. In the presence of GSH, the fluorescence of ZnS QDs was recovered because Ni\textsuperscript{2+} preferably reacted with GSH. The degree of recovery of the fluorescence
intensity of ZnS QDs was proportional to the GSH concentration. Huang reported a method to realize precise and rapid GSH detection by using silver-nanoclusters as a fluorescent probe [7]. Yang designed and synthesized a fluorescent probe for the detection of biothiols and investigated its properties for labeling glutathione [8]. After reaction with glutathione in 2-[4-(2-hydroxyethyl)-piperazinyl]ethanesulfonic acid (HEPES) buffer, the color of the solution changed from light yellow to pink, which could be detected by naked eyes.

Contents of acetylcy steine, traces of sulfide and GSH were tested by fluorescence spectroscopy using calcein-Cu(II) system [9-10]. In a buffer with a suitable pH, copper would coordinate with calcein, which caused the quenching of fluorescence. Due to strong affinity of sulfide with copper (II), copper (II) would be seized from the calcein-Cu (II) complex and the released calcein leading to the recovery of fluorescence. Recovery degree of the fluorescence was linear with a concentration of sulfide ion.

Based on the principle of fluorescence analysis, a method of quantitative determination of GSH was established according to the variation of fluorescence intensity with different concentrations of GSH standard solution in calcein-Cu(II) system. The comparison of our results with Jing et al. [10] is provided.

2. Experimental
2.1. Reagents and instruments

The following reagents were used: Glutathione (Shanghai Aladdin Bio-Chem Technology Co., Ltd), calcein (Sinopharm Chemical Reagent Co., Ltd), CuSO₄·5H₂O (Sinopharm Chemical Reagent Co., Ltd), NaOH (Sinopharm Chemical Reagent Co., Ltd), Na₂HPO₄·12H₂O (Sinopharm Chemical Reagent Co., Ltd), NaH₄PO₃·2H₂O (Sinopharm Chemical Reagent Co., Ltd). All reagents used in the experiment were AR.

Spectropro® 500 fluorescence spectroscopy (Acton Company, USA), FA2004 analytical balance (Shanghai Precision Instrument Co., Ltd), SZ-96 automatic water distiller (Shanghai Yarong Biochemical Instrument Factory).

2.2. Method
2.2.1. Selection of concentration ratio of calcein to Cu(II) and optimum concentration. It was reported that the relative contents of copper(II) and calcein would influence the intensity of the background signal and the linear range of detection results. When the content ratio of copper(II) to calcein was less than 1:1, the fluorescence quenching of calcein-Cu(II) system increased with the addition of copper (II). When the content ratio of copper(II) to calcein was over than 1:1, the degree of fluorescence quenching was tending to stable. Compared with calcein, sulfide had a stronger affinity with copper(II), if there were excessive copper(II) in the solution, sulfide ion would first react with the excess of copper(II) instead of seizing the copper (II) ions in calcein-Cu(II) complex, which would result in the lower sensitivity, and destroyed the linear relationship of fluorescence intensity with calcein concentrations in low concentration. Therefore, the concentration ratio of copper (II) and calcein was 1:1 in their experiments. Because GSH molecules contain active sulfhydryl groups, which chemical properties are similar to sulfur ions, we consulted the reference [9] and chose the concentration ratio of copper(II) and calcein as 1:1 and the optimum concentration of calcein was 1×10⁻⁵ mol/l. The concentrations of GSH were 2×10⁻⁶, 4×10⁻⁶, 6×10⁻⁶, 8×10⁻⁶, and 1.0×10⁻⁵ mol/l, respectively.

2.2.2. Preparation of stock solution. Calcein standard solution (1.0×10⁻³mol/l): 0.0623g calcein solid was accurately weighed by analytical balance and 25ml NaOH solution of 0.1mol/l concentration was added into it. After the calcein solid was dissolved completely, the solution was transferred into a 100ml volumetric flask, and distilled water was added to the volume. The calcein standard solution should be stored at 4°C, which was diluted with distilled water to the desired concentration step by step when used.

Copper(II) ion standard solution(1.0×10⁻²mol/l): 0.0160g CuSO₄ was accurately weighed by analytical balance. After CuSO₄ was dissolved completely by little-distilled water, the solution was transferred into a 100ml volumetric flask, and distilled water was added to the final volume. The calcein standard solution was diluted with distilled water to the desired concentration step-by-step when used.
The GSH standard solution (1.0×10-3 mol/l): 0.0307g GSH was accurately weighed by analytical balance, which was dissolved completely by little-distilled water. The solution was transferred into a 100ml volumetric flask, and distilled water was added to the final volume. The GSH standard solution was diluted with distilled water to the desired concentration step by step when used. Because the reduced GSH could be easily oxidized, GSH standard solution should be prepared before detection.

Buffer solution (0.2 mol/l): 7.1600g NaH2PO4·12H2O was accurately weighed by analytical balance, which was dissolved completely by little-distilled water and cooled down to room temperature. The solution was transferred into a 100ml volumetric flask, and distilled water was added to scale line, and 0.2 mol/l NaH2PO4 solution was obtained. 3.1200g NaH2PO4·2H2O was accurately weighed by analytical balance, which was dissolved completely by little-distilled water. According to the above steps, 0.2 mol/l NaH2PO4 solution was got. The mixed 94.7 ml NaH2PO4 solution with a 5.3 ml NaH2PO4 solution, the 100ml buffer with a pH of 8 was prepared.

2.2.3. Detection. Calcein standard solution (1×10-3 mol/l), GSH standard solution (1.0×10-3 mol/l) and Copper(II) ion standard solution (1.0×10-3 mol/l) was diluted by distilled water to the standard solution with 1×10-4 mol/l concentration respectively. 2.5ml of 0.2 mol/l buffer solution and 2.5ml of 1×10-4 mol/l calcein standard solution were transferred into five 50ml volumetric flasks. Then 1×10-4 mol/l GSH standard solution of 1.0, 2.0, 3.0, 4.0, and 5.0 ml were added, respectively. 2.5ml 1×10-4 mol/l Copper(II) standard solution was added subsequently and distilled water was dropped to final volume. Five samples were prepared.

These samples were heated in water bath at 40℃ for 20 min, and their excitation spectra and emission spectra were obtained. The standard curve of concentration of GSH with the peak intensity of fluorescence was constructed.

3. Results and Discussion
3.1. Excitation spectra and emission spectra of calcein and calcein-Cu (II)
From figure 1, it can be seen when the concentration of calcein solution was 1×10-5 mol/l, and pH value of buffer solution equals to 8.0, strong fluorescence of calcein appeared. The wavelength at excitation peak and emission peak was 497 nm and 518 nm. After adding copper (II) to calcein solution, the quenching of fluorescence occurred, and the wavelength at excitation peak and emission peak had no apparent change. In a Na2HPO4-NaH2PO4 buffer with pH of 8.0, copper (II) could coordinate with calcein, Calcein-Cu(II) complex was not fluorescent which led to the reduction of calcein fluorescence intensity.

Figure 1. Excitation spectra and emission spectra of calcein and calcein-Cu(II) curves labeled 1, 2 corresponding to excitation and emission spectra of calcein and curves labeled 3, 4 corresponding to excitation and emission spectra of calcein-Cu(II).

3.2. Fluorescence spectra of calcein-GSH-Cu(II) system
For simplicity, according to the sequence of adding reagents, the solution obtained by the above steps were referred to as calcein-GSH-Cu (II) system. Figure 2 depicts its fluorescence spectra with different concentration of GSH. For a strong affinity of GSH with copper (II), the addition of GSH solution would make calcein free from the calcein-Cu(II) complex and recover the fluorescence intensity. With an
increase in GSH concentration, the fluorescence intensity enhanced and the wavelength at peak intensity remained unchanged.

3.3. Comparative analysis of experimental results of calcein-GSH- Cu(II) with calcein-Cu(II) -GSH

To study the influence of the sequence of adding GSH and copper, the sequence of reagents was changed. For the calcein-Cu(II)-GSH system, after 1×10⁻⁴ mol/l GSH standard solution of 1.0, 2.0, 3.0, 4.0, and 5.0 ml was added, and samples were heated in water bath at 40°C for 20 minutes, the fluorescence spectra were determined, as shown in figure 3. With an increase of GSH concentration, fluorescence intensity enhanced and the wavelength at peak intensity had no change, which was similar to calcein-GSH-Cu(II) system.

3.3.1. Comparison of sensitivity of calcein-GSH-Cu(II) with calcein-Cu(II) -GSH. The difference between the maximum and minimum of fluorescence peak intensity was written as ΔF, while ΔF₁ corresponding to calcein-GSH-Cu(II) system and ΔF₂ corresponding to calcein-Cu(II) -GSH, which is shown in figure 4 by a bar graph. ΔF₁ = 176.34, ΔF₂ = 99.33, ΔF₁ was much larger than ΔF₂, so calcein-GSH-Cu(II) system was more sensitive than calcein-Cu(II) -GSH system.

3.3.2. Comparison of stability of calcein-GSH-Cu (II) with calcein-Cu(II)-GSH. For calcein-GSH-Cu(II) system, there were five sets of data corresponding to different concentrations of GSH, and the time...
required for stability was 20, 20, 35, 40, and 60 min, respectively. The higher the concentration of GSH, the longer the time required for stability. For calcein-Cu(II)-GSH system, the time required for stability was 20, 20, 35, 100, and 115 min, respectively. As compared to the time necessary for stability in two fluorescence systems, calcein-Cu(II)-GSH system required less time to stabilize, and its stability was better than calcein-C(II)-GSH. calcein-GSH-Cu(II) system should be chosen in practical application.

3.3.3. Influence of reaction time on the results. The reaction time of the reagents had significant impact on the results. When the concentrations of calcein, copper (II) ion and GSH were all 5×10^{-6} mol/l and the pH was 8.0 in PBS buffer, the reaction time and fluorescence intensity values are shown in table 1 and figure 5.

In both calcein-GSH-Cu(II) and calcein-Cu-GSH(II) systems, the longer the reaction time, the better the stability. Under the same concentration of GSH, calcein-GSH-Cu(II) system would need less time for the reaction and stabilization.

### Table 1. Reaction time and fluorescence intensity of two systems

| Serial number | Reaction time /min | Fluorescence intensity /a.u. | Reaction time /min | Fluorescence intensity /a.u. |
|---------------|--------------------|-----------------------------|--------------------|-----------------------------|
| 1             | 20                 | 497.00                      | 20                 | 583.67                      |
| 2             | 25                 | 548.67                      | 35                 | 676.83                      |
| 3             | 35                 | 612.33                      | 55                 | 744.67                      |
| 4             | 55                 | 656.67                      | 70                 | 783.00                      |
| 5             | 80                 | 683.00                      | 100                | 809.67                      |
| 6             | 100                | 687.67                      | 130                | 812.33                      |

### Figure 5. Relation of fluorescence intensity with reaction time. (a) calcein-GSH-Cu(II) system, (b) calcein-Cu-GSH(II) system

3.3.4. Standard curve and linear equation. GSH concentration with relative fluorescence intensity was plotted, and standard curves of calcein-GSH-Cu(II) system and calcein-Cu-GSH(II) system were given by using the linear fitting method in figure 6. Concentration of GSH was used as X-axis, and fluorescence intensity was as Y-axis, the linear equation was \( F=22.35C+480.43 \) for calcein-GSH-Cu(II) system and \( F=11.183C+690.37 \) for calcein-Cu-GSH(II) system, the correlation coefficient was 0.991 and 0.972 respectively, the linear range of these two equations was from 2×10^{-6} mol/l to 1.0×10^{-5} mol/l. Compared with calcein-Cu-GSH(II) system, the standard curve for calcein-GSH-Cu(II) system had a
larger slope and more sensitivity. The standard glutathione solution with a content of $2.0 \times 10^{-5}$ mol·L$^{-1}$ was tested ten times and a relative standard deviation of 1.2%.

3.3.5. Effects of other ions on results. The impact of some common ions on the determination of glutathione was investigated. Interference experiments were performed on $2.0 \times 10^{-5}$ mol/l glutathione under the selected experimental conditions. The results showed that in the range of $\pm$ 5% of the interference level, 200 times of the amount of PO$_4^{3-}$, NO$_3^-$, Na$^+$, K$^+$ and ten times of the amount of Al$^{3+}$ had no interference, and ten times of the amount of Fe$^{2+}$ would interfere the results.

![Figure 6. Standard curves of (a) calcein-GSH-Cu(II) system, (b) calcein-Cu-GSH(II) system](image)

![Figure 7. A standard curve of GSH reported by Jing et al. [10]](image)

3.3.6. Comparison of our results with reference [10]. Jing B.B et al. had done the similar work on testing the GSH concentration. The wavelength at excitation peak and emission peak was 492nm and 519nm. In the sodium borate buffer solution with pH of 9.18, according to the dropping step of buffer solution $\rightarrow$ calcein $\rightarrow$ GSH $\rightarrow$ Cu$^{2+}$, the standard curve equation was $F=8.1964C+196.43$, the linear range is in $2.0 \times 10^{-6}$-$1.4 \times 10^{-5}$ mol/l, and the detecting limit is $1.0 \times 10^{-6}$mol L$^{-1}$. We adopted Na$_2$HPO$_4$·NaH$_2$PO$_4$ buffer with pH of 8.0, while wavelength at excitation peak and emission peak was 497 and 518nm in our experiment. The wavelength at fluorescence peak has no noticeable difference with Jing’s report, but excitation wavelength is red-shifted. From the linear equations and figures 6 and 7, because of having a larger slope, our standard curve has more sensitivity. Therefore, it can be considered that the pH value and the type of buffer solution would also influence the results.

4. Conclusions
The GSH content was determined by fluorescence spectra of calcein-Cu(II) system in our work. In a Na$_2$HPO$_4$·NaH$_2$PO$_4$ buffer with pH of 8.0, copper(II) can coordinate with calcein which led to the reduction of fluorescence intensity of calcein. The addition of GSH solution would make calcein free from the calcein-Cu(II) complex and recover the fluorescence intensity. The change of the fluorescence
intensity had a linear relationship with the concentration of GSH in the range of $2 \times 10^{-6}$ mol/l - $1 \times 10^{-5}$ mol/l, the linear equation was $F = 22.35C + 480.43$. We also researched the influence of reaction time and the sequence of adding reagents on the experimental results. It was shown that the more the concentration of GSH, the longer the time needing for stability. We could appropriately extend reaction time for more stability. Calcein - GSH- Cu(II) system has a higher sensitivity and better stability than the calcein - Cu(II)- GSH system. This test method is of great importance in practical applications.

Acknowledgment
This work was supported by the National Natural Science Fund of China (61108033).

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