Chinese Journal of Chemistry, **2004**, *22*, 445—449 **Article**

Spectrofluorimetric Determination of Reduced Glutathione Using Organic Nanoparticle Probes

WANG, Le-Yu(汪乐余) WANG, Lun*(王伦) ZHU, Chang-Qing(朱昌青)
DONG, Ling(董玲) XIA, Ting-Ting(夏婷婷) CHEN, Hong-Qi(陈红旗)
BIAN, Gui-Rong(卞桂荣)

College of Chemistry and Materials Science, Anhui Normal University, Wuhu, Anhui 241000, China

Nanometer-sized fluorescent particles (poly-4-vinylaniline nanoparticles) have been prepared under ultrasonic radiation. The fluorescence of poly-4-vinylaniline nanoparticles (PVN) is *ca*. 50 folds higher than that of 4-vinylaniline. In comparison with single organic fluorophores, these nanoparticle probes are brighter, more stable against photobleaching, and do not suffer from blinking of light emission. A new fluorimetric method for the determination of reduced glutathione (GSH) has been developed with poly-4-vinylaniline nanoparticles as a probe. The maximal fluorescence for the reagent occurs at excitation and emission wavelengths of 306 and 380 nm, respectively. Under optimal conditions, PVN reacts with GSH and *o*-phthalaldehyde (OPA) to give a highly fluorescent derivative in Na_2CO_3-HCl buffer (pH＝10.55). The fluorescence excitation and emission wavelengths of the fluorescent derivative are located at 340 and 428 nm, respectively. The relative fluorescence intensity (RF) is linear in the range of the GSH concentration from 3.0×10^{-7} to 1.2×10^{-5} mol/L. The method has been applied to the determination of GSH in synthetic samples with satisfactory results.

Keywords fluorescent probe, glutathione, organic nanoparticle

Introduction

Reduced glutathione (GSH) is a very important tripeptide.[1] GSH widely exists in living tissues. In animal organization, the concentration of free glutathione is in the range 0.5—10.0 mmol/L. Usually over 99% of glutathione is present in the reduced form in all organisms.[2] Intermediates of GSH biosynthesis such as cysteine, γ-glutamyl-cysteine (γ-Glu-Cys) or cysteinyl-glycine (Cys-Gly) also occur in the cell but at much lower concentrations.[3]

GSH plays an important biological role in the whole living process. For example, GSH is capable of scavenging oxygen-derived free radicals, which are thought to contribute to the development of many common diseases including arthritis, stroke, heart attack and cancer.[4-6] Mirroring the increasing interest in the role of GSH, several analytical methods have been reported for its quantification, including electrochemistry,[7] spectrophotometry,[8,9] enzymatic method,[10,11] HPLC[12-14] and spectrofluorimetry.[15,16] In recent years, spectrofluorimetric method is widely used in the field of biological science for its sensitivity, simplicity and low cost. In the spectrofluorimetric determination of GSH, some fluorescent reagents have been proposed such as bimanes,[17,18] *o*-phthaldialdehyde (OPA),[19,20] *N*-substituted maleimides,[21,22] and so on. All these reagents have advantages of high sensitivity and selectivity, which is favorable for the determination of trace GSH in biological sample.

Fluorescent nanoparticles have been widely used in the fields of biological science and analytical chemistry for their good optical properties.[23-26] In this paper, we prepare a highly fluorescent nanoparticle, poly-4-vinylaniline, under ultrasonic radiation. PVN reacts with GSH in the presence of OPA at pH＝10.55 to give a highly fluorescent derivative when the mixture is incubated for 20 min in 33 ℃ water bath. The fluorescence of the derivative is stable and its relative fluorescence intensity (RF) is linear in the range of the GSH concentration from 3.0×10^{-7} to 1.2×10^{-5} mol/L. The detection limit (DL) is 1.1×10^{-8} mol/L. The present method has been applied to the determination of GSH in synthetic samples with satisfactory results.

Experimental

Apparatus

Fluorescence spectra and relative fluorescence intensities were measured on a Hitachi F-4500 fluorescence spectrophotometer (Tokyo, Japan) equipped with a Xenon lamp and a 1 cm×1 cm quartz cell. The spectral bandpass for both excitation and emission was set at 5

* E-mail: lunwang56@hotmail.com
Received August 8, 2003; revised and accepted January 5, 2004.
Project supported by the Education Young Teacher Foundation of Anhui Province (No. 2004jq121), the National Natural Science Foundation of China (No. 20375001) and the Natural Science Foundation of Anhui Province (No. 03044904).

nm. A pHS-3C pH-meter (Shanghai Dazhong Analytical Instruments Factory, China) was used for accurate adjustments of pH. Nanoparticles were prepared with a VCX-500 ultrasonic processor (Sonic, U.S.A.) and transmission electron microscopy (TEM) images of nanoparticles were acquired on a Hitachi H-600 transmission electron microscope (Tokyo, Japan).

Reagent

All chemicals used were of analytical-reagent grade or the highest available purity. All aqueous solutions of the reagents were made up in distilled water. GSH and cysteine were prepared by directly dissolving commercially available reagents in water at a concentration of 1.0×10^{-3} mol/L. Working solutions were prepared by diluting the stock solutions with water. Solutions were stored at 0—4 ℃. 4-Vinylaniline and $K_2S_2O_8$ (KSP) were purchased from Acros (USA) and used without further purification. Poly-4-vinylaniline nanoparticles were synthesized according to the procedures as follows: 0.2 mL of 4-vinylaniline (7.268 mol/L) was dissolved in 200 mL of acetone. Under vigorous stirring, 200 mL of water were added. Then the poly-4-vinylaniline nanoparticles were prepared under ultrasonic radiation in the presence of $K_2S_2O_8$. The PVN stock solution of 1.0×10^{-3} mol/L was prepared. A stock standard solution of o-phthaldialdehyde (OPA) (Sigma) was prepared by dissolving it in 95% ethanol to a final concentration of 1.0×10^{-3} mol/L. A Na_2CO_3-HCl buffer solution (pH =10.55) at a concentration of 0.2 mol/L was used. Distilled and deionized water was used throughout.

Procedure

Into a 10.0 mL standard flask 1.0 mL of PVN, 0.3 mL of OPA and 1.0 mL of buffer solution (pH=10.55) were transferred. A known volume of GSH standard solution was added. The solution was dilute with water to the volume and mixed. The relative fluorescence intensity at *ca*. 428 nm with excitation at 340 nm was measured.

Results and discussion

Spectral characteristics of fluorescence

Figure 1 shows the fluorescence spectra of 4-vinylaniline (1) and poly-4-vinylaniline (2) in the absence of buffer. In our research, it was found that the excitation and emission maxima of PVN are similar to those of 4-vinylaniline, but the fluorescence intensity is significantly enhanced. Fluorescence spectra obtained from the reaction mixture of PVN with GSH and OPA are also illustrated in Figure 1 (4—6). The fluorescence maximum for the derivative was at excitation and emission wavelengths of 340 and 428 nm, respectively. The fluorescence intensity of derivative was enhanced greatly by increasing the GSH concentration, which was convenient for the measurement of GSH.

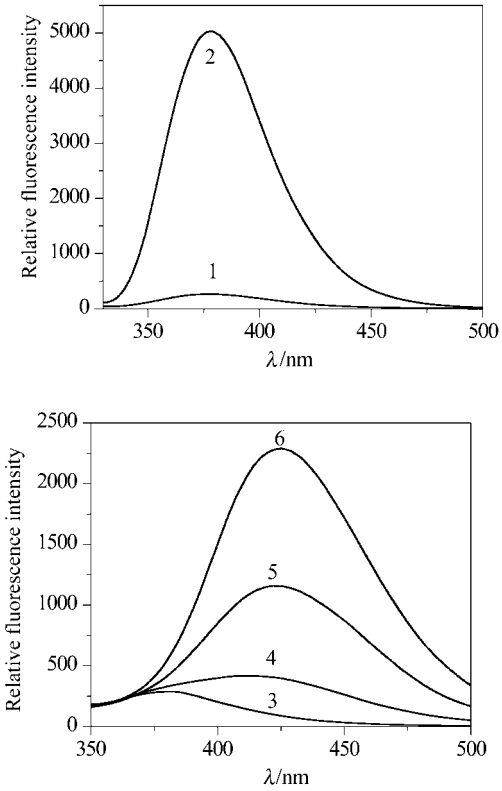

Figure 1 Fluorescence spectra of 4-vinylaniline (1, $\lambda_{em}/\lambda_{ex}$= 380/306 nm), PVN (2, $\lambda_{em}/\lambda_{ex}$=380/306 nm) and derivative (3—6, $\lambda_{em}/\lambda_{ex}$=428/340 nm). 4-Vinylaniline: 1.0×10^{-4} mol/L; PVN: 1.0×10^{-4} mol/L; pH=10.55; GSH ($\times10^{-6}$ mol/L): 3, 0.0; 4, 0.6; 5, 2.5; 6, 5.5

TEM image of PVN

TEM image of PVN is shown in Figure 2. It can be seen that the diameter of PVN is about 35 nm, indicating that the polymerization is successful under ultrasonic radiation.

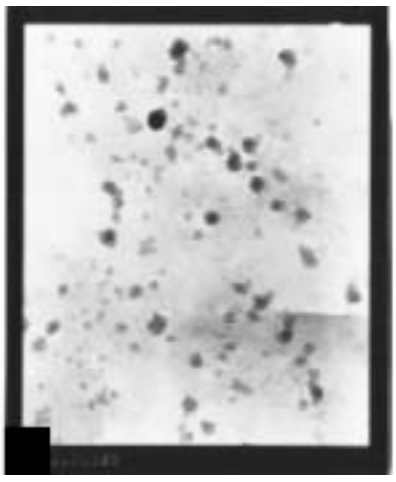

Figure 2 TEM image of PVN.

Optimization of general procedure

The apparent effect of pH on the enhancement of de-

rivative was studied in the pH range of 8.08—11.36 and the results indicated that the optimum pH value was around 10.55 (Figure 3). The influence of PVN concentration on the fluorescence of PVN in the absence of buffer was investigated. It was found that the maximum and constant fluorescence were reached when PVN was in the concentration (here, represented by the concentration of 4-vinylaniline existing in single molecules) range of 5.5×10^{-5}—15.5×10^{-5} mol/L (Figure 4). In subsequent studies, 1.0×10^{-4} mol/L was chosen.

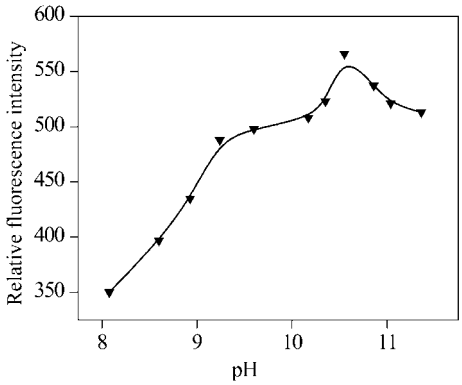

Figure 3 Effect of pH on the fluorescence of derivative. PVN: 1.0×10^{-4} mol/L; GSH: 1.0×10^{-6} mol/L; OPA: 3.0×10^{-5} mol/L.

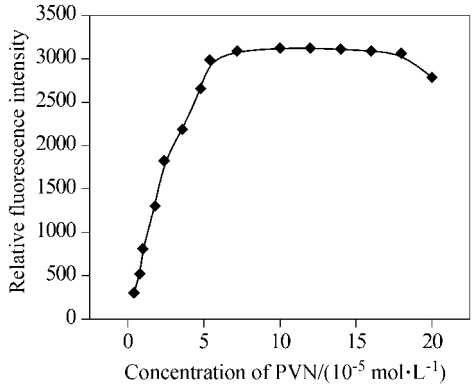

Figure 4 Effect of concentration of PVN on the fluorescence of PVN in the absence of buffer solution.

The effect of OPA concentration was also investigated with constant concentration of PVN and GSH at pH=10.55. The results are shown in Figure 5. The experimental results indicated that the maximum and constant fluorescence intensity of derivative occurred when OPA concentration was in the range of 2.0×10^{-5}—4.0×10^{-5} mol/L. In this work, an OPA concentration of 3.0×10^{-5} mol/L was recommended. The influence of incubation time on fluorescence intensity was also investigated. The results showed that the maximal fluorescence intensity was reached when the solutions were mixed and incubated for 20 min in 33 ℃ water bath. The fluorescence remained constant for at least 3 h at room temperature. Therefore, a 20 min incubation time was adopted in this work. The adding sequence of PVN, OPA, GSH and buffer solution had no obvious effect on the fluorescence system. However, considering the consistent manipulation, in all experiments, PVN and OPA were first mixed, then buffer solution was added, and finally the GSH was added.

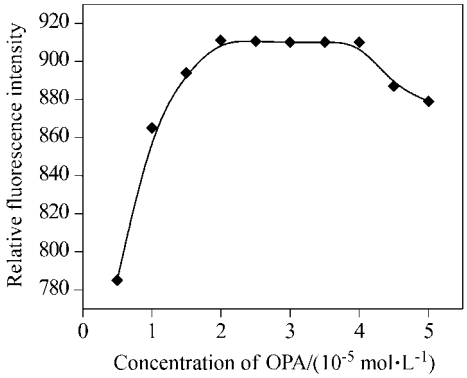

Figure 5 Effect of concentration of OPA on the fluorescence of derivative. PVN: 1.0×10^{-4} mol/L; GSH: 2.0×10^{-6} mol/L; pH 10.55.

Calibration graph, sensitivity and precision

Under the optimal conditions stated above, calibration graph, detection limit, and precision were obtained. The regression equation of calibration curve was as follows: $F=192.3+384.2c$ (mol/L). Linear relationship was obtained from 3.0×10^{-7} to 1.2×10^{-5} mol/L. A correlation coefficient of 0.9989 ($n=12$) was also acquired. The limit of detection (3σ) for GSH is 1.1×10^{-8} mol/L (here σ represents the standard deviation of 9 blank measurements). The precision was established by repeated determinations ($n=6$) using 2.0×10^{-6} mol/L GSH. The relative standard derivations (RSD) did not exceed 2.5%.

Interferences

When the amount of GSH was 2.0×10^{-6} mol/L, the influence of foreign substances on the determination was studied (Table 1). Of the tested substances, aminoacetic acid, glucose, proteins, and nucleic acids did not interfere with the determination of GSH. In addition, several common metal ions such as Na^+, K^+, Ca^{2+}, Zn^{2+}, Fe^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+} in the whole blood also had no obvious interference. Cysteine interferes with the detection when its concentration is higher than 1.0×10^{-5} mol/L. However, Cysteine content is very low in whole blood. Thiol-containing compounds in whole blood include GSH, Cys, *etc*. As the GSH content is higher than 90% of the total thiol-containing compounds in blood, thiol compounds in whole blood can be regarded as GSH.[27] The experimental results indicate that the method has a high selectivity. So the method is practical and it should be able to be used to determine GSH in whole blood.

Sample analysis

Under optimal conditions according to the standard procedures, three synthetic samples, in which a series of foreign substances had been added based on the tolerance level of foreign substances displayed in Table 1, were determined according to the given linear relationship. Recovery experiments were performed and the results are listed in Table 2. The obtained GSH concentrations in synthetic samples showed good agreement with those of addition. Good recoveries were obtained from 96.3% to 102.3% with a satisfactory analytical precision (RSD$\leqslant$2.5%), which confirmed the feasibility and reliability of the present method.

Table 1 Tests for the interference of coexisting substances [a]

Coexisting substance	Coexisting conc./ ($\mu g \cdot mL^{-1}$)	Change of RF/%	Coexisting substance	Coexisting conc./ ($\mu g \cdot mL^{-1}$)	Change of *RF*/%
Glucose	12.5	−2.8	Ca(II), Cl^-	10.0	−2.4
Fish sperm DNA	15.0	−4.3	Zn(II), Cl^-	10.0	+3.4
Calf thymus DNA	15.0	−3.2	Mg(II), Cl^-	10.0	−2.7
Gamma globulin	10.0	+2.6	Fe(II), SO_4^{2-}	10.0	−3.9
Human serum albumin	10.0	−3.3	Al(III), Cl^-	10.0	−4.4
Bovine serum albumin	10.0	−2.4	K(I), Cl^-	25.0	−1.8
Aminoacetic acid	12.5	+3.2	Na(I), Cl^-	25.0	−1.6
Cysteine	1.0×10^{-5} mol/L	−5.1	Fe(III), Cl^-	10.0	−3.1

[a] GSH: 2.0×10^{-6} mol/L (0.615 μg/mL); OPA: 3.0×10^{-5} mol/L; PVN: 1.0×10^{-4} mol/L; pH=10.55.

Table 2 Results of determination for synthetic samples [a]

GSH in samples/ ($\mu mol \cdot L^{-1}$)	Coexisting substances and concentration/ ($\mu g \cdot mL^{-1}$)	GSH found [b]/ ($\mu mol \cdot L^{-1}$)	Recovery/ %	RSD/ %
2.0	Cysytein (4.0×10^{-6} mol/L); HSA (5.0); Glucose (5.0); Ca (II) (5.0); Zn (II) (5.0)	1.95	97.5	2.3
3.0	Aminoacetic acid (5.0); HSA (6.0); Ca (II) (6.0); Mg (II) (6.0); Gamma globulin (5.0)	3.07	102.3	1.9
4.0	Cysytein (8.0×10^{-6} mol/L); Ca (II) (5.0); Zn (II) (5.0); Gamma globulin (5.0)	3.85	96.3	2.5

[a] OPA: 3.0×10^{-5} mol/L; PVN: 1.0×10^{-4} mol/L; pH=10.55. [b] Average of six determinations.

Conclusion

In this paper, poly-4-vinylaniline nanoparticles have been prepared under ultrasonic radiation. Its fluorescence is *ca*. 50-folds higher than that of 4-vinylaniline. Ultrasonic synthesis is simpler and more rapid than general method. The diameter of nanoparticles is larger even than that of nanoparticles prepared by other methods. Unlike single dye molecules, each nanoparticle contains about 100 molecules of an embedded dye that is protected from the outside environment. As such, these fluorescent nanoparticles are highly resistant to photobleach and emit bright and steady fluorescence.

Compared with other fluorescence-labeling reagents for thiol, PVN reacts with GSH very rapidly. PVN is more readily prepared from inexpensive and commercially available precursors using ultrasonic synthetic method. The proposed procedure is simple, inexpensive, and rapid. Further studies in this field will open up the way to the application of organic nanoparticles in analytical chemistry and analytical biochemistry. The use of fluorescent nanoparticles in biochemistry and analytical biochemistry can solve some of the problems associated with single fluorophore such as rapid photobleaching and blinking. The good optical properties of these nanoparticles should make them particularly useful for further development of immunosensor and bioanalytical applications.[28,29]

References

1 Dolphin, D.; Poulsen, R.; Avramovic, O. P. *Glutathione: Chemical, Biochemical and Medical Aspects*, Vol. III, Wiley & Sons, J., New York, **1989**, p. 367.

2 Meister, A.; Anderson, M. E. *Ann. Rev. Biochem.* **1983**, *52*, 711.

3 Winters, R. A.; Inkowski, J.; Ercal, N.; Matthens, R. H.; Spitz, D. R. *Anal. Biochem.* **1995**, *277*, 14.

4 Ames, B. N. *Science* **1983**, *211*, 1256.
5 Cerutti, P. A. *Science* **1985**, *227*, 375.
6 Cross, C. E.; Halliwell, B.; Borish, E. T.; Pryor, W. A.; Ames, B. N.; Saul, R. L.; Cord, J. M. Mc; Haroman, D. *Ann. Intern. Med.* **1987**, *107*, 526.
7 Johll, M. E.; Willimas, D. G.; Johnson, D. C. *Electroanalysis* **1997**, *9*, 1397.
8 Swaitditat, A.; Tsen, C. C. *Anal. Biochem.* **1072**, *45*, 349.
9 Catala, I. M.; Lahuerta, I. L.; Martinez, C. J. *Analyst* **1998**, *123*, 1685.
10 Tietze, F. *Anal. Biochem.* **1969**, *27*, 502.
11 Griffith, O. W. *Anal. Biochem.* **1980**, *106*, 207.
12 Noctor, G.; Foyer, C. H. *Anal. Biochem.* **1998**, *264*, 98.
13 Ivanov, A. R.; Nazimov, I. V.; Baratova, L. A. *J. Chromatogr.* **2000**, *870*, 433.
14 Lenton, K. J.; Therriault, H.; Wagner, J. R. *Anal. Biochem.* **1999**, *274*, 125.
15 Keiji, T.; Shukuro, I. *Chem. Pharm. Bull.* **1996**, *44*, 226.
16 Gala, S. M.; Korany, M. A. *Bull. Fac. Pharm.* **1997**, *35*, 73.
17 Kosower, N. S.; Kosower, S. M.; Mewton, C. L.; Ranny, H. M. *Proc. Natl. Acad. Sci. U. S. A.* **1979**, *76*, 3382.
18 Ferandez-Checa, J. C.; Kaplowiz, N. *Anal. Biochem.* **1990**, *190*, 212.
19 Leroy, P.; Nicolas, A.; Wellmann, M.; Michelet, F.; Oster, T.; Siost, G. *Chromatographia* **1993**, *36*, 130.
20 Hissin, P. J.; Hilf, R. *Anal. Biochem.* **1976**, *74*, 214.
21 Nakashima, K.; Niishida, K.; Nakatsuji, S.; Aikyama, S. *Chem. Pharm. Bull.* **1986**, *34*, 1678.
22 Takahashi, H.; Nara, Y.; Yoshida, T.; Tuzimura, K. *Agric. Biol. Chem.* **1978**, *42*, 769.
23 Chan, W. C. W.; Nie, S. *Science* **1998**, *281*, 2016.
24 Bruchez, M. Jr; Moronne, M.; Gin, P.; Weiss, S.; Alivisatos, A. P. *Science* **1998**, *281*, 2013.
25 Jason, R. T.; Michelle, M. F.; Nie, S. *Anal. Chem.* **2000,** *72*, 1979.
26 (a) Wang, L. Y.; Guo, C.; Li, M. G.; Xu, F. G.; Zhu, C. Q.; Wang, L. *Chin. J. Anal. Chem.* **2003**, *31*, 83 (in Chinese).
(b) Wang, L. Y.; Zhou, Y. Y.; Zhu, C. Q.; Zhang, M. C.; Tao, H. S.; Wang, L. *Chem. J. Chin. Univ.* **2003**, *24*, 612 (in Chinese)
(c) Wang, L. Y.; Wang, L.; Dong, L.; Hu, Y. L.; Xia, T. T.; Chen, H. Q.; Li, L.; Zhu, C. Q. *Talanta* **2004**, *62*, 237.
27 Jocelyn, P. C. *Biochemistry of SH Group*, Academic Press, London, **1973**, p. 10.
28 Pathak, S.; Choi, S. K.; Arnheim, N.; Thompson, M. E. *J. Am. Chem. Soc.* **2001,** *123*, 4103.
29 Willard, D. M.; Carillo, L. L.; Jung, J.; Van Orden, A. *Nano. Lett.* **2001**, *1*, 469.

(E0308072 ZHAO, X. J.)