Amperometric Detection of Oxidized and Reduced Glutathione at Anodically Pretreated Diamond Electrodes

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(Received October 1, 2002; CL-020845)

Anodically oxidized diamond electrodes have enabled direct oxidation of not only thiols but also disulfides such as oxidized glutathione. Coupled with LC separation after sample pretreatment, simultaneous determination of oxidized and reduced glutathione in rat whole blood was achieved at 1.50 V vs Ag/AgCl. The present method was comparable with enzymatic assay in real sample analysis.

Glutathione (GSH) with a thiol moiety, which is widely distributed in living cells, has an important feature of antioxidants. It forms glutathione disulfide (GSSG) in the presence of oxidants. Therefore, the GSSG/GSH ratio serves as a sensitive indicator of oxidative stress and is a key marker for the redox status of cells.[1] The detection methods dealt with these compounds should exhibit high sensitivity due to the low available concentrations of GSSG in biological samples.

Although analytical method adapting electrochemical detection is simple, rapid, sensitive, and versatile, the electrochemical determination of disulfides is very difficult due to their high oxidation potentials. The oxidative reaction of disulfides at Au and Pt electrodes occurs in the potential region of surface oxide,[2] and may complicate analytical applications. Carbon electrodes such as glassy carbon (GC) generally show the signal for disulfides and methionine at high overpotentials ($\geq$1.4 V vs SCE) in comparison to those for thiols ($\approx$1 V vs SCE),[3] resulting in low signal to background currents; electrode fouling is a major problem with these electrodes. Moreover, chemically modified electrodes have been used to oxidize thiols, but they do not respond to disulfides.[4]

In the present work, we evaluate the potential of anodically oxidized (AO) diamond electrodes for the GSH and the GSSG oxidations, and demonstrate the application of this electrode for the LC-amperometric detection of both GSH and GSSG in rat whole blood.

Conductive diamond electrodes were deposited on Si (100) wafers using a high-pressure microwave plasma-assisted chemical vapor deposition system. The details of the preparation have been described previously.[5] Anodic pretreatment of the diamond electrode was carried out by constant current density of $+8\,\mathrm{mA\,cm^{-2}}$ in Britton-Robinson buffer (pH 2) for 20 min. The XPS results obtained before and after anodization for the as-deposited diamond revealed that the surface O/C ratio increased from 0.02 to 0.15. Female rats whole blood was freshly drawn using Na_2EDTA as an anticoagulant. For deproteinization, 200 µL whole blood was mixed with 600 µL ice-cold 5% metaphosphoric acid, and the mixture was centrifuged for 10 min at 3000 G. A 50 µL aliquot of supernatant, after centrifuging, was diluted to 1 mL with the LC mobile phase. Sample was injected into the LC system without any further treatment.

Figure 1 shows the cyclic voltammograms for GSSG at AO diamond, GC, and Pt electrodes in acidic medium (pH 2). At the Pt electrodes the oxidation of GSSG is mediated by surface oxide and a small oxidation current is observed in the same potential as the peak of surface oxide formation. Increasing potential results in overlapping the peak current with oxygen evolution. In case of the GC electrodes, surface fouling due to the building of adsorbed reaction products is observed during GSSG oxidation. The oxidation of GC surface itself also causes an increase in the background current because of the relatively high potential. In contrast, the response of the GSSG at the AO diamond exhibits two well-defined peaks with diffusion controlled limiting currents, which can be explained based on the ion-dipole interactions at the electrode surface. The as-grown hydrogen-terminated diamond is not so active for GSSG. As mentioned in our previous reports,[6] we believe that the oxygen functional groups such as carbonyl or hydroxyl groups formed on the facets of the AO diamond microcrystals form a negative dipolar field, which electrostatically attracts the positively charged GSSG molecule in acidic pH. It is interesting to note that the voltammetric waves shift positive with increasing pH, which is in contrast to the behavior at other electrodes. This behavior

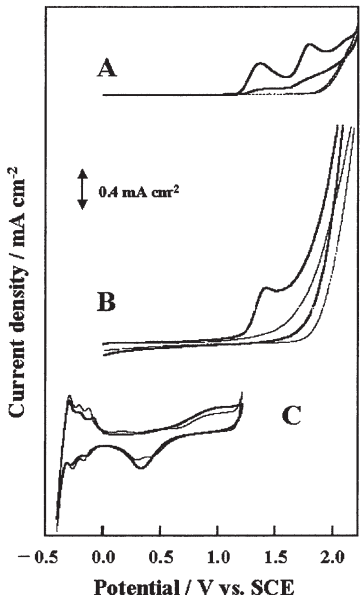

Figure 1. Cyclic voltammograms for 1 mM GSSG in pH 2 Britton-Robinson buffer at (A) AO diamond, (B) glassy carbon, and (C) platinum electrodes. The potential sweep rate was $0.1\,\mathrm{V\,s^{-1}}$. Thin lines represent background current.

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Table 1. Rat whole blood concentrations of GSH and GSSG determined by LC-BDD method and enzymatic method[a]

	LC-BDD method	Enzymatic method	LC-BDD/enzymatic
GSH (μM)	722.1 ± 28.9	839.7 ± 61.5	0.86 ± 0.06
GSSG (μM)	24.2 ± 2.4	27.1 ± 8.1	0.97 ± 0.27

[a]Results are expressed as mean ± S.D. (n = 10 for each Method.)

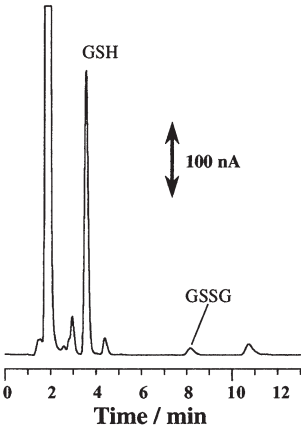

Figure 2. Chromatogram of rat whole blood (after protein precipitation, 1:80 dilution) showing peaks for GSH and GSSG. Separation column; Inertsil ODS–3 (4.6 mm i.d × 75 mm, dp = 3 μm), Mobile phase; MeCN/0.1% TFA = 2/98, Temp.; 25 deg., Flow rate; 0.7 mL min^{-1}, Injection volume; 20 μL. AO diamond was subjected to 1.50 V vs Ag/AgCl.

provides a clear evidence for our hypothesis that the attractive electrostatic interaction between the negative dipolar field of diamond surface and the positively charged GSSG in acidic solution improves the kinetics for the oxygen transfer reaction.

Exhaustive electrolysis of GSH and GSSG at AO diamond was performed to understand the reaction mechanism. The oxidation products, GSO_3H for GSH, and GSO_3H, GSOSG, and GSOOSG for GSSG, were identified by tandem mass spectrometry. Similar oxygen transfer reactions were observed for GSH at a mixed-valence ruthenium oxide polymer as mediator[7] and a Bi-doped PbO_2 electrodes.[8] These oxygen transfer reactions are believed to occur mainly by mediation of electrogenerated OH radicals (from water discharge), which is believed to occur primarily at the defects on the diamond electrodes without significant co-evolution of O_2. This is of great significance for the use of AO diamond electrodes as amperometric sensors.

The analytical performance of the AO diamond was examined by LC coupled with electrochemical detection. The present method (LC-BDD method) was found to produce linear calibration curves to up 250 μM with detection limits of 1.4 nM for GSH and 1.9 nM for GSSG (S/N = 3). These minimum detectable amounts are the lowest values reported so far.[9] The within-day reproducibility and the day-to-day stability of the response during 3 days were less than 3% (R.S.D.), indicating a highly stable amperometric response.

Figure 2 shows the typical chromatogram for rat whole blood after deproteinization, which was subjected to 80 times dilution during pretreatments. The peaks of GSH and GSSG have no interference with other sulfur containing compounds, such as methionine at $t_R \approx 4.4$ min or cysteine and cystine, which are merged with Na_2EDTA signal at 1.8 min. Although the GSH recovery was relatively less (92–94%) compared with the case of GSSG (99–105%), the recovery levels were fairly satisfactory.

LC-BDD method was compared with the enzymatic method reported by Richie et al.,[10] in order to check the validation. Table 1 shows the comparison of results obtained from two methods (n = 10 for the pooled samples). While the value for GSSG is in good agreement (LC-BDD/Enzymatic = 0.97) between two methods, the value for GSH is somewhat lower for enzymatic method (LC-BDD/Enzymatic = 0.86). One of the reasons for this difference could be the relatively low recovery of GSH, while other possibility being the difficulty of precise pipetting for whole blood.

In conclusion, the comparison of LC-BDD method and the enzymatic method for GSH and GSSG evidenced that the application of LC coupled with diamond electrode has enabled a simple, sensitive, and selective method for determination of biogenic sulfur compounds in biological samples.

References

1 Y. Morel and R. Barouki, *Biochem. J.*, **342**, 481 (1999).
2 a) P. Vandeberg and D. C. Johnson, *Anal. Chem.*, **65**, 2713 (1993). b) J. C. Hoekstra and D. C. Johnson, *Anal. Chem.*, **70**, 83 (1998).
3 T. R. Ralph, M. L. Hichman, J. P. Millington, and F. C. Walsh, *J. Electroanal. Chem.*, **375**, 1 (1994).
4 a) B. Nalini and S. S. Narayanan, *Electroanalysis*, **10**, 779 (1998). b) T. Inoue and J. R. Kirchhoff, *Anal. Chem.*, **72**, 5755 (2000). c) J.-M. Zen, A. Senthil Kumar, and J.-C. Chen, *Anal. Chem.*, **73**, 1169 (2001).
5 T. Yano, D. A. Tryk, K. Hashimoto, and A. Fujishima, *J. Electrochem. Soc.*, **145**, 1870 (1998).
6 a) H. Notsu, I. Yagi, T. Tatsuma, D. A. Tryk, and A. Fujishima, *J. Electroanal. Chem.*, **492**, 31 (2000). b) H. Notsu, T. Fukazawa, T. Tatsuma, D. A. Tryk, and A. Fujishima, *Electrochem. Solid-State Lett.*, **4**, H1 (2001). c) C. Terashima, T. N. Rao, B. V. Sarada, D. A. Tryk, and A. Fujishima, *Anal. Chem.*, **74**, 895 (2002).
7 J. B. Laughlin, K. Miecznikowski, P. J. Kulesza, and J. A. Cox, *Electrocehm. Solid-State Lett.*, **2**, 574 (1999).
8 N. D. Popovic, J. A. Cox, and D. C. Johnson, *J. Electroanal. Chem.*, **455**, 153 (1998).
9 P. C. White, N. S. Lawrence, J. Davis, and R. G. Compton, *Electroanalysis*, **14**, 89 (2002).
10 J. P. Richie, L. Skowrouski, P. Abraham, and Y. Leutzinger, *Clin. Chem.*, **42**, 64 (1996).