Electrochemical Detection of Glutathione on SAMs on Gold Using an Electroactive Quininoid-Type Molecule

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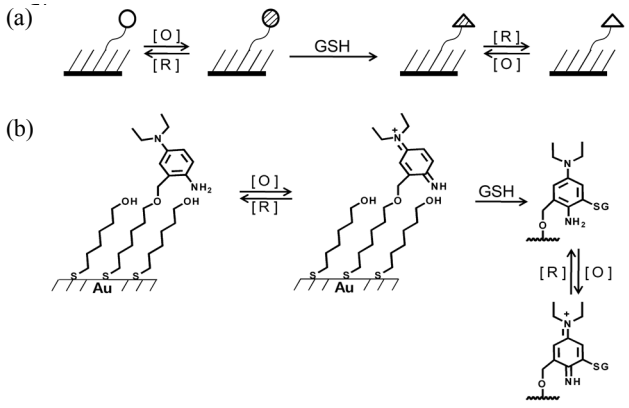
Received July 15, 2009, Accepted September 9, 2009

In this work, we describe a new sensor that specifically responds to biothiols, i.e., glutathione (GSH), in solution. An electrochemical transducing strategy was utilized and cyclic voltammetry (CV) was employed to monitor the presence of GSH in real time. Our approach harnessed self-assembled monolayers (SAMs) on gold consisting of an alkanethiolate which was terminated by electroactive quininoid moiety. Prior to thiol molecule treatment, the characterisitc reversible redox peaks of the electroactive quininoid group was observed, while the reduction peak was dramatically shifted upon a treatment of GSH. This sensor showed the capability to detect the GSH in solution in the range of 1 mM ~ 100 aM. We believe that this strategy will provide an important tool for accurate, sensitive, rapid, and low-cost determination of GSH.

Key Words: Sensor, Cyclic voltammetry, Electrochemical detection, Glutathione, Self-assembled monolayers

Introduction

A biosensor is an analytical tool that equips with two functions, capturing biological targets and transducing target binding events to measurable signals. Over the past decade there have been huge efforts to develop new biomolecular sensors, which are mostly driven by the demands for fundamental studies in biology and many applications in biotechnology. Such demands include enzyme activity assays,[1] determining the level of the analytes,[2] and detection of the biomolecules such as oligonucleotides, sugars, and proteins.[3-5] Particularly, the last need has drawn enormous attention because those biomolecules are in many cases the indicators to detect a number of diseases, and hence, the sensors of them can be actually used as medical diagnostic tools. Recent theme in development of biosensors is to raise sensitivity and accuracy for the reliable detection of low-abundant analytes, which requires a suitable signal transducing strategy. Various conventional methods have been reported for those purpose encompassing fluorescence,[6] surface-enhanced Raman spectrometry,[7] surface plasmon resonance,[8] electrical/electrochemical conversion,[9] *etc*. Among them, electrochemical signal transduction is advantageous over other signal transducing strategies in terms of simplicity, accuracy, sensitivity, and cost. Indeed, it can provide a simple, inexpensive, rapid, easy- to-miniaturize platform, and therefore, many of commercialized biochip-based biosensors have adopted electrochemical sensing platforms.



Scheme 1. (a) Strategy for designing a sensor for the electrochemical detection of GSH; (b) The structure of the monolayer used in this work and the electrochemical conversions of the molecules on the surface upon a treatment of GSH.

Here, we describe a new sensor that specifically responds to biothiols, i.e., glutathione (GSH), in solution. The GSH, found in the tissues and cells, are well recognized to be physiologically important. For example, GSH is proven to be critical for maintaining cellular homeostasis.[10] Furthermore, the level of certain form of glutathione in biological fluids serves as biomarkers for some diseases.[11] Routinely, Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid) or DTNB) is widely used for colorimetric measurment of the amount of thiols in solutions. Although it provides a simple and accurate protocol, sometimes this method is hampered by thiols which contains a deleterious chromophore and/or by a large background absorbance.

Our approach in this report utilizes an eletrochemical signal transducing stratey to detect GSH. We harness self-assembled monolayers (SAMs) on gold consisting of an alkanethiolate which is terminated by an electroactive moiety, *N*,*N*-diethyl-*p*-phenylenediamine (Scheme 1).[12]

This electroactive moiety on the monolayer shows the distinctive, reversible redox activity. When oxidized, the resulting product reveals the chemical reactivity towards GSH and forms a covalent tethering with GSH. The GSH-conjugated molecule then provides different electrochemical signals, which are used for detection of GSH. Scheme 1b illustrates the structure of the monolayer used in this work and the electrochemical conversions of molecules on the surface. The monolayer presenting *N*,*N*-diethyl-*p*-phenylenediamine is oxidized to quininoid type molecule, which efficiently reacts with a thiol of GSH by way of Michael addition. The reversible electrochemical behaviour of the resulting GSH-adduct produces electrochemical signals which are an evidence for the presence of GSH in solution.

Experimental

Reagents and instruments. Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise mentioned. Phosphate buffered saline (PBS) was from WelGENE Inc. (Seoul, Korea) and ethanol was obtained from Merck (Darmstadt, Germany). Acetonitrile was obtained from Burdick & Jackson (Morristown, NJ, USA). Cyclic voltammetry was performed using a WPG100 Potentiostat/Galvanostat (Wonatech, Seoul, Korea) with Pt Auxiliary electrode and Ag/AgCl reference electrode. The gold working electrode was prepared by vacuum deposition of titanium (100 Å) followed by gold (900 Å) onto silicon wafers. Mass analysis was performed with an Autoflex III MALDI-TOF mass spectrometer (Bruker Daltonics, Germany) with a Smartbeam^{TM} laser as an ionization source.

Preparations of DEPT-presenting monolayers. Gold-coated silicon wafers (~0.5 cm^2) were immersed in a solution of *N*,*N*-diethyl-*p*-phenylenediamine-thiol (DEPT) and a 6-mercapto-1-hexanol in ethanol in a ratio of 1:1 for 12 h (the total concentration of thiol was 1 mM). The monolayers were washed with copious amount of ethanol and dried under a stream of nitrogen.

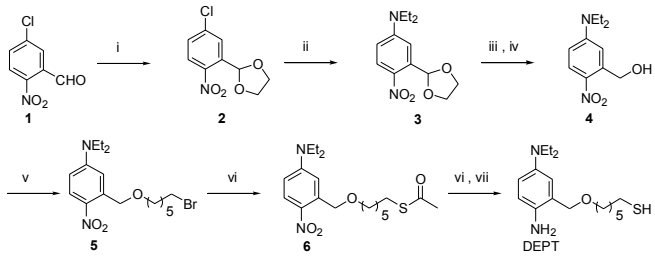
MALDI TOF-MS analysis. Spectra were acquired with an accelerating voltage of 19 kV, a 50 Hz repetition rate, and positive mode with an average of about 500 shots. To confirm the reaction progression, monolayers were analyzed with THAP (5 mg/mL in acetonitrile) as a matrix.

Cyclic voltammetry. The parameters for cyclic voltammetry were; initial potential of −300 mV, scan rate of 100 mVs^{-1}, sample interval of 1 mV, interval time of 2 s. All scans were performed from −300 mV to 300 mV in PBS at pH 7.4 which was purged with nitrogen for 10 min before experiment. Glutathione was prepared at various concentrations ranging from 1 mM to 100 aM in deoxygenated PBS (pH 7.4).

Results and Discussion

Synthesis of DEPT. The electroactive molecule *N*,*N*-diethyl-*p*-phenylenediamine-thiol (DEPT) used in this study was synthesized in 8 steps from commercially available reagents (Scheme 2). All intermediates and final product gave satisfactory ^{1}H NMR spectra. Details will be described in a subsequent full report. Briefly, 5-chloro-2-nitrobenzaldehyde **1** was protected by ethylene glycol to afford **2**, which was subsequently substituted with diethyl amine to give **3**. Removal of ethylene glycol protecting group followed by reduction with $NaBH_4$ afforded alcohol **4**. The resulting alcohol was alkylated with 1,6-dibromohexane (**5**) and thioacetate group was introduced to give **6**. Reduction of nitro group and hydrolysis resulted in the final product DEPT.

Mass analysis of the chemical conversion on the monolayer. We used matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) to establish that the chemical conversion on the monolayer proceeded to give the intended product. MALDI-TOF MS is well suited for the analysis of SAMs on gold and now used routinely to confirm the chemical/biochemical transformations on SAMs on gold.[13,14]



Scheme 2. Synthetic scheme for electroactive molecule *N*,*N*-diethyl-*p*-phenylenediamine-thiol (DEPT); i) ethylene glycol, *p*-toluenesulfonic acid (*p*-TsOH), toluene, 80 °C; ii) diethylamine, dimethyl sulfoxide (DMSO), 100 °C; iii) *p*-TsOH, acetone/water (1:1), 80 °C; iv) sodium borohydride ($NaBH_4$), tetrahydrofuran (THF)/ethanol; v) sodium hydride (NaH), THF, 1,6-dibromohexane; vi) potassium thioacetate, THF; vii) Pd/C, H_2, methanol, ethyl acetate; viii) lithium hydroxide (LiOH), THF, water.

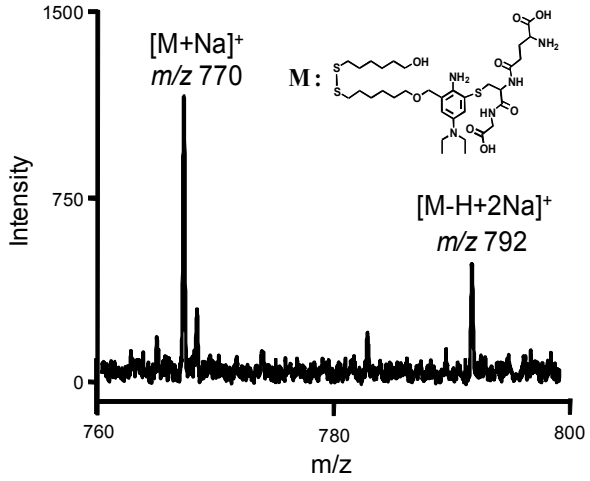


Figure 1. MS anaysis of the DEPT presenting monolayer after the GSH treatment.

We prepared a mixed monolayer presenting DEPT among a background of 6-mercapto-1-hexanol in a ratio of 1 : 1. The monolayer was subjected to an electrical potential of 400 mV and subsequently treated with GSH (10 mM, PBS). The mass analysis of the monolayer showed two major peaks at *m*/*z* 770 ([M+Na]$^+$), *m*/*z* 792 ([M-H+2Na]$^+$) corresponding to the mixed disulfides containing GSH-conjugated product (Fig. 1).

This result clearly verifies that the *N*,*N*-diethyl-*p*-phenylenediamine moiety was electrochemically oxidized to the quininoid group, which was then covalently anchored to the thiol group of GSH as proposed in Scheme 1b.

Real-time detection of GSH in solution. Next, the electrochemical behavior of the DEPT was analyzed. The cyclic voltammogram of the monolayer presenting DEPT showed the reversible redox peaks at potentials of 55 mV and 160 mV for reduction, and at potentials of 90 mV and 160 mV for oxidation, respectively (Figure 2a, dotted line). Upon a treatment of GSH (1 mM in PBS for 1hr), two peaks for reduction were merged into the one peak at 100 mV and gave rise to an additional peak at −190 mV, while two peaks for oxidation were combined into the one peak at 150 mV.[15] In response to GSH, the peak at 100 mV (reduction) gradually decreased as

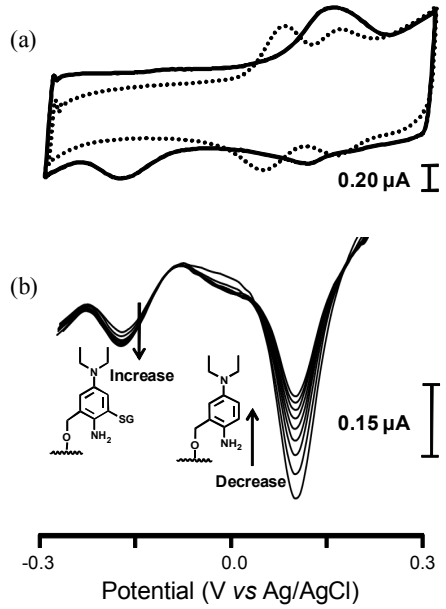


Figure 2. Cyclic voltammogram obtained from the DEPT presenting monolayer (a) before the GSH treatment (dotted line) and after the GSH treatment (solid line), (b) detailing the response to GSH in sloution (100 μM) in real-time (scan rate of 100 mVs^{-1}).

the new peak at −190 mV (reduction) gradually increased to finally result in the voltammogram which can be attributed to the redox couple of GSH-conjugated product as depicted in Scheme 1b (Figure 2a, solid line). Figure 2b displays the real-time result at 100 μM concentration of GSH, clearly showing that the reduction peak at 100 mV was replaced by the new peak at −190 mV.

Detection of GSH at various concentrations in solution. Finally, in order to explore the capability of our system as a sensor for GSH, we monitored the response of DEPT presenting monolayers to GSH. Figure 3 shows the reduction waves of cyclic voltamogram in reponse to GSH in various concentrations (second cycles, scan rate of 100 mVs^{-1}, intial scan from −300 mV). As expected, the reduction peak of the monolayers increased with the GSH concentrations. Importantly, GSH in 100 attomolar concentration was clearly observed, verifying the ultrahigh sensitivity of our strategy. This result implies that our appoach using electrochemical signal changes of DEPT on monolayers can provide a sensitive, rapid, and low-cost sensor for the real-time detection of GSH.

The measurements were performed using DEPT coated gold chips which were manually cut and using a custom-made cell. The current experimental method caused the slightly different electrode area and solution exposure time in each measurement, and therefore, gave rise to inaccuracy of the target detection. In fact, we found that the response of sensor didn't show the clear dependence on the low target concentration range. We are currently improving the experimental set-up for better performance of our sensor such as use of a regular chip size and multianalyses of the several target solutions at the same time, which, we believe, will allow quantification informations of the target and kinetic analysis of the surface reactions.

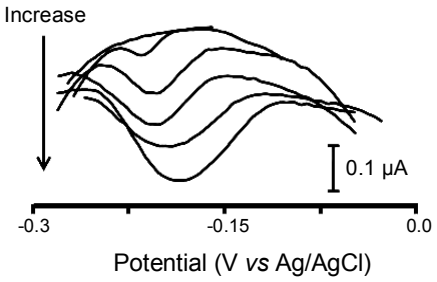


Figure 3. The reduction waves of cyclic voltammograms from the DEPT presenting monolayers in reponse to GSH in various concentrations (0 M, 100 aM, 1 pM, 10 μM, 100 μM, 1 mM).

Conclusions

In order to detect GSH, we proposed an electrochemical method based on the SAMs on gold which presented the analyte-responsive molecule, DEPT. The presence of GSH in solution induced a change in the electrochemical behavior of DEPT. The strategy described here showed the ultrahigh sensitivity to the detecion of GSH in solution at the concentration of 100 aM. We believe that this strategy will provide an important tool for sensitive, rapid, and low-cost determination of GSH. In addition, by taking advantage of the electrochemical signal transduction such as easy-to-miniaturize platform, our sensor will prove useful in biology and biotechnology.

Acknowledgments. This work was supported by the faculty research fund of Konkuk University in 2007.

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15. We have not yet fully understood this electrochemical behaviour of the DEPT-presenting monolayer. We assumed that it could be attributed to the instability of the quininoid moiety of the DEPT on gold surfaces.