Full Paper

Glutathione Peroxidase-Based Amperometric Biosensor for the Detection of *S*-Nitrosothiols

*Mustafa Musameh, Neda Moezzi, Lisa M. Schauman, Mark E. Meyerhoff**

Department of Chemistry, University of Michigan, Ann Arbor, MI 48109-1055, USA
*e-mail: mmeyerho@umich.edu

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Abstract
A new biosensor is described for the detection of *S*-nitrosothiols (RSNOs) based on their decomposition by immobilized glutathione peroxidase (GPx), an enzyme containing selenocysteine residue that catalytically produces nitric oxide (NO) from RSNOs. The enzyme is entrapped at the distal tip of a planar amperometric NO sensor. The new biosensor shows good sensitivity, linearity, reversibility, and response times towards various RSNO species in PBS buffer, pH 7.4 . In most cases, the response time is less than 5 min, and the response is linear up to 6 μM of the tested RSNO species. The lowest detection limit is obtained for *S*-nitrosocysteine (CysNO), at approx. 0.2 μM. The biosensor's sensitivity is not affected by the addition of EDTA as a chelating agent; an advantage over other potential catalytic enzymes that contain copper ion centers, such as CuZn-superoxide dismutase and xanthine oxidase. However, lifetime of the new sensor is limited, with sensitivity decrease of 50% after two days of use. Nonetheless, the new amperometric GPx based RSNO sensor could prove useful for detecting relative RSNO levels in biological samples, including whole blood.

Keywords: Glutathione peroxidase, *S*-Nitrosothiols, nitric oxide, dialysis membrane, biosensor

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1. Introduction

S-Nitrosothiols (RSNOs) are potential nitric oxide (NO)-storage, transfer, and delivery vehicles that exist in blood and within living cells. They are generated in vivo via the nitrosylation of thiols by oxidative intermediates of endogenous NO (e.g., N_2O_3, and NO^+) that form under physiological conditions [1]. RSNOs have several functions that are related to NO directly. For example, they are considered the predominant bronchodilator in human airways [2]. Further, vasorelaxation of blood vessels can be controlled by RSNOs [3]. Indeed, *S*-nitrosohemoglobin can regulate blood flow via the release of NO [4, 5]. *S*-Nitroso-*N*-acetyl-penicillamine (SNAP) is a low molecular weight RSNO which has been used as a therapeutic drug. It has been found that this RSNO not only has the ability to inhibit platelet aggregation [6] but also has protective effects against intestinal damage induced by endotoxins [7]. As a direct vasodilator as well as an ACE inhibitor, *S*-nitrosocaptopril has been used to regulate blood pressure [8]. *S*-Nitrosoglutathione (GSNO), the most abundant *S*-nitrosothiol in the living body, has also been found to have the ability to be a potent inhibitor of platelet activity.

To understand their vital role in the body, it is important to devise reliable and fast methods for detecting RSNO species. In general, RSNOs can undergo homolytic cleavage of the S–N bond leading to the release of NO, with several factors affecting this reaction including the intensity of light (for photoreaction), solution pH, metal ion catalyst concentration, and the presence of various reductants (e.g., ascorbate and thiols) [9 – 14]. Metal ions, such as copper(II), are known to have a very powerful effect on decomposing RSNOs in the presence of a reducing agent (to convert Cu(II) to Cu(I)) [15 – 17]. Recently, such copper-based catalytic chemistry has been utilized in conjunction with an amperometric NO sensor to develop a sensitive, reversible sensor that responds to various RSNOs at sub-μM levels [18]. The copper catalysts were immobilized in polymeric films at the distal end of the NO probe. More recently, a novel synthetic organoselenium polymer was also immobilized on an amperometric NO sensor to create a device with good sensitivity for NO detection [19].

Certain enzymes have also been reported to have the ability to decompose RSNOs to release NO both in vitro and in vivo. Hence, such enzymes are also candidates for use in developing RSNO sensors. Zinc superoxide dismutase (CuZn-SOD) and xanthine oxidase are two enzymes already shown to carry out such a catalytic reaction [20 – 22]. A third enzyme that has been reported to decompose RSNOs is glutathione peroxidase (GPx). This selenoenzyme has several features that make it a superior catalyst for potentially developing an immobilized enzyme-based RSNO sensor. Indeed, GPx has a higher decomposition efficiency than the two other enzymes and has inherent resistance to inhibition by metal ion chelating agents such as EDTA. Glutathione peroxidase (PDB 1GP1, EC 1.11.1.9) is

a peroxidase found in the erythrocytes of mammals that helps prevent lipid peroxidation of the cell membrane. The function of GPx, therefore, is to reduce lipid hydroperoxides (LOOH) to their corresponding alcohols (LOH) in accordance with the following reaction:

$$2GSH + LOOH \rightarrow GSSG + LOH + H_2O \quad (1)$$

where GSH represents reduced monomeric glutathione, and GSSG represents oxidized glutathione. Glutathione peroxidase is a tetrameric glycoprotein possessing four selenocysteine amino acid residues.. The bovine erythrocyte enzyme has a molecular weight of 84 kDa. The three dimensional crystal structure for GPx has been solved [23] and shows that the enzyme contains four spherical subunits, each with a selenocysteine residue in a depression on the surface. Recent studies by Freedman indicate that GPx potentiates the inhibition of platelet function by RSNOs [24]. It was further suggested that GPx catalyzes the metabolism of endogenous GSNO to liberate NO in the presence of H_2O_2. Hou et al. found that diselenides could also catalyze the decomposition of RSNO to produce NO [25]. The initial activation is assumed to involve the interchange reaction of the diselenide and the thiol (Equations 2 and 3, below). The selenol generated from these exchange reactions reacts further with RSNO to release NO (in accordance with the net overall Equation 4) as shown in the scheme below.

$$R'Se{-}SeR' + RSH \rightleftharpoons RS{-}SeR' + R'SeH \quad (2)$$

$$RS{-}SeR' + RSH \rightleftharpoons RS{-}SR + R'SeH \quad (3)$$

$$2\,RS{-}NO \rightarrow R'Se{-}SeR'RSHRS{-}SR + 2\,NO \quad (4)$$

Herein, we report on the development of the first enzyme-based biosensor using GPx for the detection of RSNOs. The active GPx was physically entrapped between a dialysis membrane and the gas permeable membrane of a planar amperometric NO sensor. The sensor exhibits a reasonable response time (<5 min at 1 μM level of RSNO), with excellent sensitivity and reversibility toward a variety of physiological and nonphysiological RSNO species.

2. Experimental

2.1. Apparatus

Amperometric current signals were monitored using a microchemical sensor analyzer (Bioanalytical Systems (BAS) CV-37 voltammograph) in stirred solutions with polarization potential to the platinized platinum working electrode of the sensor at $+0.75$ V (vs. Ag/AgCl).

2.2. Chemicals/Materials

Microporous poly(tetrafluoroethylene) (PTFE) gas permeable membranes used to construct the NO sensors were obtained from Tetratex (Minneapolis, MN) with a pore size of 0.07 μm, and thickness 18 μm. Dialysis membranes (MW cut-off = 15 000) were purchased from Spectrum laboratories, Inc. (Rancho Dominguez, CA). Glutathione peroxidase, from bovine erythrocytes (lyophilized powder, 300 – 700 units/mg protein), crystalline *S*-nitroso-*N*-acetylpenicillamine (SNAP), glutathione (reduced form), cysteine, *N*-acetylcysteine, EDTA, and sodium nitrite were purchased from Sigma-Aldrich (St. Louis, MO). All solutions were prepared with 18 MΩ cm^{-1} deionized distilled water by using a Milli-Q filter system (Millipore Corp., Billerica, MA).

2.2.1. Preparation of S-Nitrosothiols (RSNOs)

Solutions (5 mM each) of *S*-nitrosothiols (CysNO, GSNO, and *S*-nitroso-*N*-acetylcysteine (SNAC)) were prepared as previously described [28]. Briefly, equal volumes of fresh 10 mM monothiol in 120 mM H_2SO_4 and 10 mM $NaNO_2$ (with 20 μM EDTA) were mixed at room temperature. Unless noted otherwise, these solutions were directly injected into PBS (pH 7.4) to obtain the desired concentration of RSNOs. The concentrations and stabilities of the synthesized RSNOs were determined by using a chemiluminescence NO analyzer (NOA; Seivers 280, Boulder, CO).

2.3. Electrode Preparation and Modification

The amperometric NO gas sensors used in this work were composed of a platinized Pt working electrode (Pt disk with 250-μm o.d.) sealed in glass wall tubing (with 2-mm o.d.) and a Ag/AgCl wire (127-μm o.d.) as reference/counter electrode. These two electrodes were incorporated behind a PTFE gas-permeable membrane (18-μm thickness, 0.07-μm pore size, Tetratex). A detailed preparation procedure for such sensors was reported previously [29].

To fabricate the amperometric RSNO sensors, a specified amount of GPx (typically 400 μg) was dissolved in 10 μL of PBS buffer, and then cast over the PTFE membrane of the NO sensor and allowed to dry for two h at room temperature. Then, a small piece of dialysis membrane was wet with PBS buffer and fixed on top of the enzyme layer with a plastic holder. The biosensor was washed with deionized water and stored at 4 °C in PBS until further use. Figure 1 shows a schematic diagram of the enzyme based biosensor assembled for these studies.

2.4. Response of Sensor to RSNOs

The sensor was polarized at $+0.75$ V versus Ag/AgCl for at least 12 h before use, and all subsequent amperometric measurements were carried out using the same applied

potential. After polarization, the complete biosensor was asserted by inserting the working electrode and reference electrode into the housing that holds the enzyme modified membrane.

All RSNO calibration measurements were carried out in PBS buffer (pH 7.4) containing 10 μM EDTA and 30 μM glutathione (added as a reducing agent) in a 100-mL amber reaction vessel at room temperature. Each RSNO solution was prepared fresh and used within 2 h.

3. Results and Discussion

As illustrated schematically in Figure 1, the GPx-mediated decomposition of RSNOs is achieved by the entrapped enzyme layer between the dialysis membrane and the NO gas permeable membrane at the distal tip of a planar amperometric NO sensor, leading to the production of NO in this confined region. The NO generated can diffuse through the gas-permeable membrane of the NO sensor to a platinized platinum anode, where electrooxidation of NO takes place. The choice of the GPx enzyme came after initially testing several other enzymes, each of which yielded sensors with much less RSNO sensitivity compared to that found with GPx (data not shown). For example, equal amounts of entrapped CuZn-SOD yielded amperometric responses 20-times less than that for GPx based sensors. Similar results were also observed when CuZn-SOD was immobilized in a similar fashion. Furthermore, it was found that response of the CuZn-SOD based sensors is completely lost when EDTA was added to the test solution mixture at levels higher than 10 μM. Such deactivation is not observed with GPx. In additional preliminary studies, another enzyme containing a selenium moiety, i.e., formate dehydrogenase, was also examined. However, response of sensors prepared with this enzyme was negligible compared to that observed for GPx.

In additional preliminary studies, the optimum amount of GPx enzyme entrapped was found to be 400 μg, equivalent to 40 units. The use of smaller amounts yielded sensors with lower RSNO sensitivity. Two immobilization methods were also tested. The first employed the use of glutaraldehyde as a crosslinking agent, and the second employed entrapment behind a dialysis membrane (as shown in Fig. 1). Unfortunately, the crosslinking strategy did not yield useful biosensors, since the activity of the enzyme was found to be greatly reduced. For this reason, the more classical dialysis membrane entrapment was the preferred choice to avoid enzyme deactivation. Two MW cut-off sizes of membranes were tested; 15 kDa and 50 kDa. It was found that the RSNO response times were less than 5 min when the 15 kDa cut-off membrane was used. Surprisingly, the larger pore size membrane yielded sensor response times that were longer than that obtained for sensors prepared with the smaller pores. This may be due to a greater thickness for this membrane. For subsequent work, all sensors were fabricated with the 15 kDa cut-off dialysis membrane.

Different reducing agents were examined for the optimum response of the GPx-based biosensor, including cysteine, glutathione, and ascorbate. Both cysteine and glutathione at 5 μM levels in the sample solution yielded equal responses with different RSNOs. However, it was surprising to note that use of ascorbate as the reducing agent resulted in enzyme deactivation rather than activation. The exact reason for this is not yet clear.

The use of EDTA at 10 μM level in the test solution was found to be essential to prevent catalytic reactions in the bulk solution from any free metal ions in the solution phase. Since the proposed RSNO sensor can detect NO present in solution as well, it is necessary to ensure that trace levels of unbound Cu(II) ions do not exist in the presence of the analyte RSNO species. It is well known that such bulk phase decomposition reactions can be completely suppressed by adding EDTA to the sample phase to chelate any free

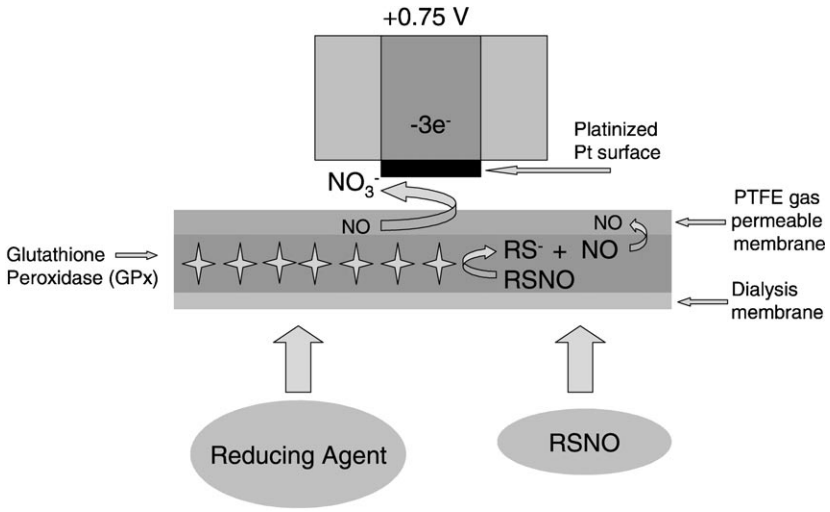

Fig. 1. Schematic design of the amperometric GPx biosensor for the indirect detection of RSNO via the detection of NO.

copper ions [9, 15]. It was found that in the presence of 10 μM EDTA, amperometric NO sensors without GPx immobilized yielded essentially no response when increasing levels of RSNO were added to the phosphate buffer test solution.

Once preliminary studies to optimize the configuration and test solution conditions were completed, the new biosensor's response towards a number of RSNOs was examined in detail in PBS, pH 7.4 buffer. Current was recorded as increasing levels of RSNO were added to a PBS solution containing 30 μM glutathione (as reducing agent) and 10 μM EDTA. Figure 2 illustrates the typical amperometric responses of the GPx based biosensor over the concentration range of 1 – 6 μM for SNAP, GSNO, CysNO, and SNAC. Next to each dynamic response recording is the corresponding calibration curve.

As shown in Figure 2, typical response times required to achieve 95% of the steady-state current following changes in RSNO levels are less than 5 min for all the RSNOs tested, but varied somewhat from one RSNO to another. A summary of the analytical data obtained with the optimized sensor is shown in Table 1.

Reversibility of the biosensor was evaluated by changing from low to high and then back down to low concentrations of GSNO species, as shown in Figure 3. The biosensor exhibited fully reversible and reproducible amperometric responses to GSNO as well as other RSNO species. The noise shown on the figure is due to the switching process in moving the sensor from one solution to another.

Table 1. Summary of the analytical performance.

RSNO	Linear range (μM)	R^2	Sensitivity (nA/μM)	*LOD* (μM)	Response time (min)
SNAP	0 – 6	0.9933	0.13	0.4	3
GSNO	0 – 6	0.9987	0.11	0.4	3
CysNO	0 – 6	0.9978	0.178	0.2	2
SNAC	0 – 6	0.9992	0.0446	0.4	4

The stability of the new RSNO sensor was examined by testing the amperometric response to 1 μM GSNO in PBS over a period of 4 days. It was found that the amperometric response decreased to 50% of its original value after 2 days and to about 30% of its initial response after 4 days when stored at room temperature in PBS. Denaturization of the enzyme, yielding conformations that prevent diselenide formation may be responsible for this instability.

Based on the scheme described in Equations 1 and 2, above, the need for a reducing agent should be essential for the activity of selenium containing species to function as a catalyst. Therefore, the effect of increasing glutathione concentration on the response of the GPx based biosensor towards the addition of 5.0 μM GSNO was evaluated. Surprisingly, as shown in Figure 4, the biosensor showed a fast and large response in the absence of any reducing agent. When 10 μM glutathione was added, a very slow increase in response was observed, much smaller than the initial response. It was also observed when GSNO was added to a solution containing glutathione, the response was very

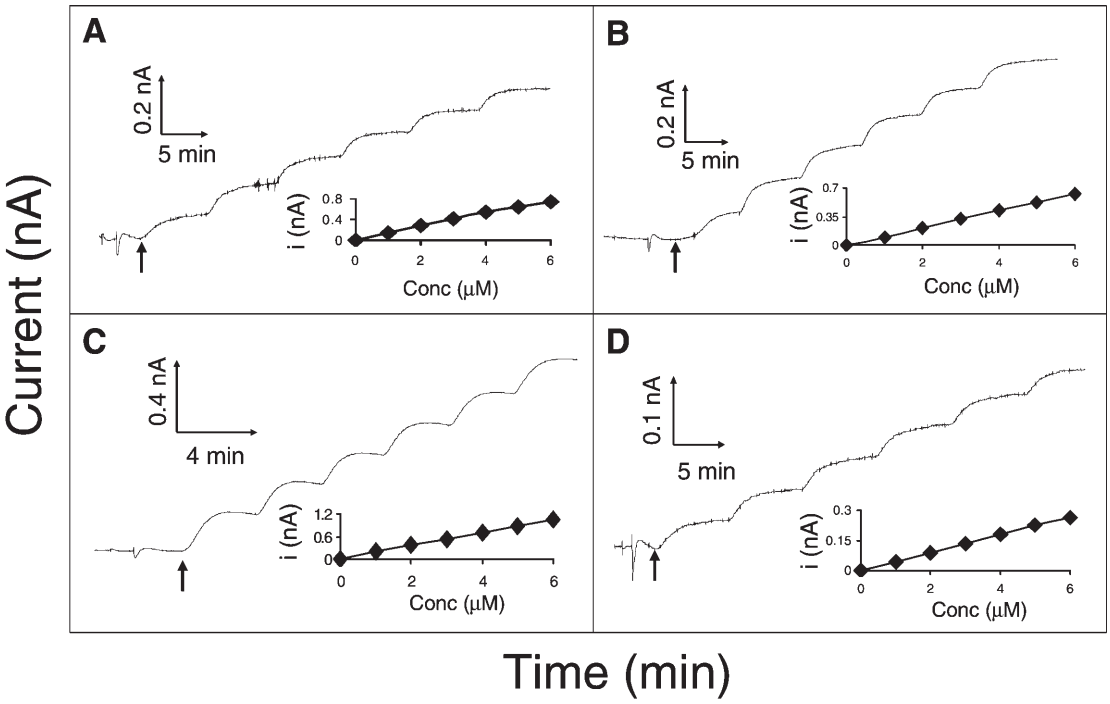

Fig. 2. Amperometric current-time recording using the GPx biosensor for the detection of different RSNOs. Stepwise additions of 1 μM of A) SNAP, B) GSNO, C) CysNO, and D) SNAC. Sample solution: 0.05 PBS buffer, pH 7.4, containing 10 μM EDTA and 30 μM glutathione.

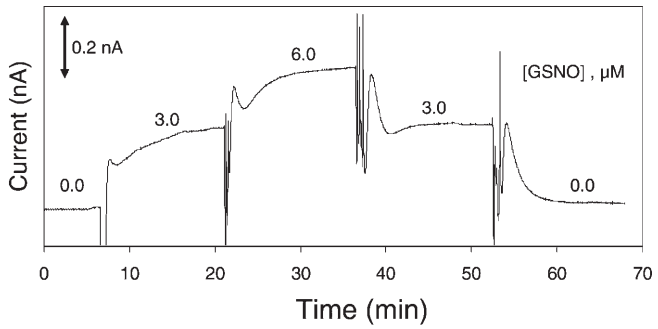

Fig. 3. Dynamic amperometric recordings illustrating real-time reversibility of the GPx biosensor in response to varying concentrations of GSNO. Other conditions as in Figure 2.

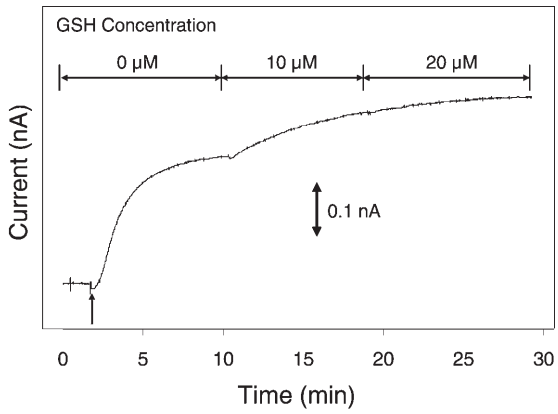

Fig. 4. Influence of sample phase glutathione concentration on the GPx biosensor amperometric signal in response to constant GSNO addition of 5.0 µM. EDTA was present at a constant level of 10 µM.

slow, while further additions of GSNO showed a faster response. The exact reason behind the functionality of the GPx biosensor in absence of glutathione is not yet clearly understood. Of course, RSH is produced after RSNO decomposition, and hence the analytical reaction itself locally produces some RSH reducing agent. Second, in any solution of RSNO, it is well known that there is already an equilibrium level of RSH contaminant [15]. Perhaps only very low levels of RSH are all that are required to effectively reduce the selenium sites in the GPx enzyme. This would explain why adding higher levels of GSH have little effect on the sensor's response toward RSNO species.

The effect of EDTA concentration on the sensor's RSNO response was also examined (see Fig. 5). As expected, addition of EDTA to a solution containing 5.0 µM GSNO did not affect the observed amperometric response. This was not the case when CuZn-SOD was used as the catalytic enzyme to prepare the RSNO sensors. Indeed, with immobilized CuZn-SOD, the amperometric response to 5 µM GSNO was totally suppressed upon the addition of 10 µM EDTA to the solution (data not shown).

It should be noted that variation in the levels of oxygen in the test sample may also influence the amperometric

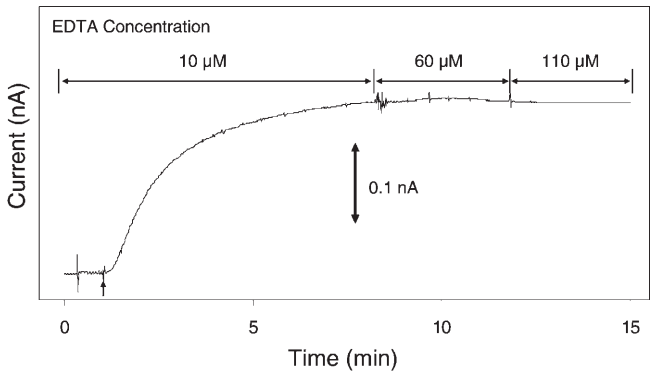

Fig. 5. Influence of sample phase EDTA concentration on the GPx biosensor amperometric signal in response to constant GSNO addition of 5.0 µM. No glutathione was for this experiment.

response of the RSNO sensor based on GPx. Indeed, recent studies regarding the effect of oxygen on an RSNO sensor that utilizes immobilized organo-selenium species, instead of GPx, as the catalytic layer has shown that the primary effect of oxygen is to lower surface levels of NO via a direct oxidation reaction with NO, yielding decreased sensitivity to the RSNO target species [19]. While generation of RSe–SeR species (from RSeH) should also be enhanced by increasing oxygen levels, this effect is outweighed by the scavenging of NO by oxygen, with a faster rate of this reaction as oxygen levels increase. In fact, direct sensor response to NO standards is reduced significantly in solutions saturated with 50% oxygen, compared to ambient conditions. This influence of oxygen is not critical in the intended application of the sensor for detecting relative RSNO levels in blood, where it is envisioned that samples would be diluted in buffers that possess ambient oxygen levels, and thus large differences in oxygen would not be expected in the final sample solution. Similarly, the effect of pH on the response of the organo-selenium based RSNO sensor follows the expected pattern of decreased response as pH is reduced [19], owing to the protonation of the strong organoselenide reducing agent. It is anticipated that the GPx-based sensor will exhibit the same pH influence. However, for all practical measurements, the pH of the sample will also be buffered at near physiological values (at or near pH 7.4), and thus the RSNO response behavior reported herein will be most relevant.

4. Conclusions

A new biosensor based on the use of glutathione peroxidase enzyme for the detection of RSNOs was described. It has been shown here that the direct, real-time measurements of RSNO species can be achieved by the incorporation of a GPx film at the surface of a planar amperometric NO gas sensor via an external dialysis membrane that entraps a concentrated solution of soluble enzyme. Decomposition of different RSNOs occurs upon the diffusion of these species

into the enzyme layer and when they interact with the selenium containing subunits within the enzyme. This biosensor shows amperometric currents proportional to the concentrations of various RSNOs, which included a number of low molecular weight physiologically relevant RSNOs, and exhibits fully reversible electrochemical response. Sensitivity varies for the different *S*-nitrosothiol species, with the largest response observed for CysNO.

The use of immobilized enzymes and proteins to design coatings for medical devices that would take advantage of the endogenous RSNOs present in the blood to create a locally elevated NO level at the device/blood interface would represent a novel approach to enhance the biocompatibility of medical devices. Indeed, production of NO locally would inhibit thrombosis on surfaces. However, to assess whether such an approach can be effective, it is necessary to determine the total levels of RSNO species that are be present in blood. A biosensor of the type described herein could potentially be useful for such purposes. Other applications that can utilize such a sensor could be as a sensitive flow-through detector in HPLC or other separation methods that will be able to quantify individual RSNO species in physiological samples. However, this will require the improvement of the sensor's response time and stability, which can be achieved by examining other immobilization methods and/or employing thinner external membranes.

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6. References

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