Amperometric Nitrosothiol Sensor Using Immobilized Organoditelluride Species as Selective Catalytic Layer

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Abstract
A new amperometric sensor capable of responding to various biological S-nitrosothiol species (RSNOs) is described. The sensor is prepared using an organoditelluride-tethered poly(allylamine hydrochloride) (PAH) polymer crosslinked within a dialysis membrane support mounted at the distal surface of an amperometric NO probe. The surface immobilized organoditelluride layer serves as a selective catalyst to decompose various RSNO species to NO in the presence of a thiol reducing agent added to the sample. The proposed sensor responds directly and reversibly to various low molecular weight (LMW) RSNOs in the range of 0.1 μM to 10 μM with nearly equal sensitivity. The main advantage of this sensor over previously reported Cu(II/I) and organodiselenium-based RSNO sensors is its long operational life-time (at least one month). A discussion regarding solution phase transnitrosation reactions potentially allowing the measurement of higher molecular weight S-nitrosoproteins is provided, along with data showing preliminary results in this direction. Further, the direct detection of endogenous RSNO species in diluted fresh whole sheep blood is also demonstrated using this new sensor.

Keywords: Nitric oxide, S-Nitrosothiols, Organoditelluride, Catalytic denitrosation, Amperometric nitric oxide sensor

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Dedicated to Professor Ernő Pretsch on the Occasion of His Retirement from ETH Zürich

1. Introduction
S-Nitrosothiols (RSNOs) are an important group of molecules found in blood and consist of both low molecular weight (LMW) species such as S-nitrosoglutathione (GSNO) and S-nitroscysteine (CySNO), as well as high molecular weight (HMW) RSNO macromolecules such as S-nitrosoalbumin (AlbSNO) and S-nitrosohemoglobin (HbSNO) [1]. The typical biological pathway to form RSNOs is nitric oxide (NO)-mediated S-nitrosation of thiol groups in amino acids, peptides, and proteins. For example, one of the identified processes for RSNO formation involves reactions with intermediates of the NO oxidation pathway, such as nitrogen dioxide (NO₂) and dinitrogen trioxide (N₂O₃), as nitrosating agents of free thiols (RSHs) [2, 3]. Nitrosylated metal centers (copper or iron) [4, 5] as well as a radical adducts of RSH and NO (RS−N′•/COH) [6] are also recognized intermediates in the production of RSNOs in the presence of an electron acceptor. Additionally, another important conduit for RSNO formation is the reallocation of NO from species as a result of an exchange reaction with sulfhydryl groups, which is termed transnitrosation [7].

Endogenous RSNOs have been recognized as a molecular vehicle to transfer and store transient NO molecules produced enzymatically by nitric oxide synthase (NOS) within endothelial and other cells. Nitric oxide participates in many physiological processes ranging from cytoprotection to cytotoxicity. In fact, endogenous RSNOs exhibit many of the same physiological functions as NO, such as inhibition of platelet aggregation [8] and the relaxation of vascular smooth muscle cells [9]. RSNOs are also known to participate in host defense processes such as killing tumors [10] and intracellular pathogens [11], in addition to intracellular signaling [12], ion channel regulation [13, 14], apoptosis [15, 16], etc. Hence, the decrease or increase in the physiological production of NO, which appears to occur in certain diseases such as atherosclerosis, hypertension, and diabetes, has been associated with not only a dysfunctional endothelial cell layer [17–20], but also the improper storage and/or delivery of NO by means of RSNOs [21, 22]. Indeed, an inverse correlation between NO and RSNO concentrations has been recently reported in specific NO-related disorders. For instance, the suppressed NO levels due to the endothelial dysfunction caused by oxidative stress (typically by lack of adequate ascorbate) are characteristic of preeclampsia [23, 24], a pregnancy-specific syndrome known to be the major cause of both maternal and fetal morbidity and mortality, where the RSNO levels in preeclampsia plasma were found to be higher compared with normal pregnancy and nonpregnancy plasma [22]. Further, it has been reported that while NO concentrations are
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generally higher in the expired air of asthma patients (vs. controls), the average RSNO levels of the expired air in asthmatic children are lower than in normal children [21]. In addition, a recent report demonstrated that patients who had more risk factors associated with heart attacks and strokes have a lower level of RSNOs in their blood, presumably since these risk factors (high cholesterol, high blood pressure, smoking, etc.) contribute to endothelial dysfunction in these individuals [25]. Therefore, the monitoring of endogenous RSNO levels in blood is of potential interest for the purpose of disease diagnosis and treatment as well as risk assessment.

There are several known approaches to measure RSNO species. Fundamentally, NO and oxidative products (NO$_2^-$, and/or NO$_3^-$) resulting from the reductive reaction of RSNOs using reduct agents, for example cysteine/HS-CuCl$_2$, VCl$_3$/HCl, or ferricyanide, etc.) can be detected by chemiluminescence or electrochemical methods [26–29]. Sometimes, these NO products react with reagents to form a detectable product (Griess nitrite reagent or fluorescent chemicals) that can be quantified by spectrophotometric or fluorometric assays [30, 31]. Despite the good sensitivity of present approaches (especially chemiluminescence based assays), their ability to quantify RSNOs accurately are often doubted because all existing methods require a number of tedious pretreatment or separation steps to eliminate interfering substances (e.g. nitrite, antioxidants, and/or proteins, etc.) [30, 32–34]. Since RSNOs are known to be highly labile molecules that can decompose in the presence of light or trace metal ions [35, 36], such pretreatment steps can lead to loss of the analyte.

To deal with the accuracy problems as well as existing complicated procedures, recent research in this laboratory has proposed the direct measurement of RSNO levels in fresh whole blood samples via planar amperometric RSNO sensors [37, 38]. These sensors are prepared by immobilizing a nitric oxide generating polymer (NOGPs) at the surface of a highly selective amperometric NO sensor [39, 40]. The NOGP is a polymeric material appended with an NO generating catalyst, which can rapidly denitrosate RSNOs when in contact with blood to locally increase the NO concentration within the polymer layer. Hence, the amperometric NO sensor with the immobilized NOGP can detect the NO produced from the RSNO decomposition via this polymeric layer diffuses through a gas permeable membrane (teflon) of the NO sensor to a platinized platinum anode, where it is oxidized and the resulting current in proportion to the NO levels present. It will be shown that this new RSNO sensor configuration is able to directly detect various LMW RSNO species at $>0.1$ µM levels with nearly equal sensitivity, with an excellent operational lifetime (at least one month). In addition, speculation regarding transnitrosation between RSNO/RSHs is discussed with respect to the potential to use the sensor to detect higher molecular weight RSNOs. Finally, the measurement of global RSNO species in the fresh animal blood sample is demonstrated via this new device.

2. Experimental

2.1. Materials

Dialysis membranes were purchased from Spectrum Laboratory Inc. (Rancho Dominguez, CA). Bovine serum albumin (BSA, gamma-globulin and protease free, fatty acid content = 0.005%), glutaraldehyde (25 wt%), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl), N-hydroxysuccinimide (NHS), N-acetyl-penicillamine (NAP), glutathione (GSH) and cysteine (CySH) were products of Sigma (St. Louis, MO). All other chemicals including poly(allylamine hydrochloride) (PAH, $\text{Avg. M. W.}=15$ k) from Aldrich (Milwaukee, WI) and solvents from Fisher Scientific (Fair Lawn, NJ) were used without further purification unless otherwise noted. Distillation was employed for the purification of Et$_3$N, and THF prior to use. DI water was prepared by a Milli-Q filter system (18 MΩ cm$^{-1}$; Millipore Corp., Billerica, MA, USA).

2.2. Preparation of S-Nitrosothiols

S-Nitrosocysteine (CySNO), S-nitroso-N-acetyl penicillamine (SNAP), and S-nitrosoglutathione (GSNO) solutions.
were prepared according to the method described in the literature [44]. All solutions were freshly prepared in a timely manner as needed from stock solutions prepared daily. S-Nitroso-albumin (AlbSNO) was also prepared using BSA as previously reported [45]. The concentration of AlbSNO was determined by using a chemiluminescence NO analyzer (NOA) (Seivers 280, Boulder, CO) and excess copper ions to quantitatively liberate the NO [37].

2.3. Preparation of the Organoditelluride-Linked Hydrogel

The conjugation of 5'-ditelluro-2,2'-dithiophenecarboxylic acid (DTDTCA, refer to Figure 1 for the structure; prepared as described in previous report [43]) with poly(allylamine hydrochloride) (PAH) was carried out via an EDC coupling chemistry. The resulting polymer obtained by filtering was washed with DI water using a membrane filter (MWCO, 5 kD) via the centrifuge at several times to remove small molecules. The polymer (DTDTCA-tethered PAH) was determined to possess 7.5 wt% of Te, as measured by inductively coupled plasma-high resolution mass spectrometry (ICP-HRMS). Then, a small volume (40 μL) of a mixture (DTDTCA-tethered PAH (0.2 mL, 4 wt%), Et3N (0.1 mL, 1 wt%), and glutaraldehyde (0.1 mL, 1 wt%) in DI water) was placed onto a dried dialysis membrane (MWCO, 50 kD; 1 cm²) that was previously purified by extensively washing with 0.1 mM EDTA solution and DI water to eliminate metal ion impurities within the membrane. The resulting membrane was allowed to stand a few days at RT and washed with DI water prior to use.

2.4. Fabrication of Amperometric RSNO/NO Sensors

Amperometric NO sensors were fabricated according to the procedures described elsewhere [38]. In brief, a platinized Pt working electrode (Pt disk with 250 μm o.d.) sealed in glass wall tubing (2 mm o.d.) was surrounded by a coiled Ag/AgCl wire reference/counter electrode (127 μm o.d.), and these two electrodes were integrated behind a PTFE gas-permeable membrane (GPM, Tetratex; 18 μm thickness, 0.07 μm pore size) treated with 0.5 μL of 1% Teflon AF solution (Dupont Fluoroproducts, Wilmington, DE) [46] using a housing (1 mL of disposable tuberculin slip tip; Becton, Dickinson and Company, Franklin Lakes, NJ) along with an internal solution (0.4 – 0.5 mL of 100 mM phosphate buffer, pH 4.4, with 50 mM NaCl and 0.005 % methyl cellulose). A plain dialysis membrane was used as a control layer for one sensor (control sensor with no NO generation) by mounting it over the GPM of a NO sensor using an O-ring. To prepare the RSNO sensor, a piece of the organoditelluride-linked hydrogel impregnated dialysis membrane was mounted over the GPM of a second NO sensor. Finally, both sensors were polarized at +0.75 V vs. Ag/AgCl for at least 12 h prior

Fig. 1. A) Schematic of RSNO detection using the proposed amperometric sensor modified with a NO generating catalytic layer (see Sec. 2.4 for the detailed procedure). B) Synthetic scheme used for the preparation of NO generating catalytic layer (2) by crosslinking the polymer (1), organoditelluride-tethered poly(allylamine hydrochloride)) within a dialysis membrane, where the polymer (1) was prepared by coupling of the organoditelluride species (5'-ditelluro-2,2'-dithiophenecarboxylic acid) with a poly(allylamine hydrochloride) (see Sec. 2.3 for the details).
to use. All amperometric measurements were performed using the same applied potential.

2.5. Detection of S-Nitrosothiols

All measurements of RSNO solutions were carried out in PBS buffer (10 mM, pH 7.4) containing 0.1 mM EDTA (necessary to sequester metal ion impurities, especially copper ions known to be an excellent catalyst for RSNO decomposition) in a brown amber vial (100 mL) at RT. Prior to use, calibration curves for both the RSNO and NO sensors were obtained by measuring their intrinsic amperometric responses toward a NO standard solution. The background NO levels in bulk RSNO solutions were also monitored via the NO sensor, as needed, by simultaneously placing both the RSNO and NO sensors into the same solution and then converting to NO concentrations based on the prior calibration curve for each sensor using the standard NO solutions. The long-term stability of the RSNO sensor was determined by assessing the sensitivity of amperometric response toward GSNO (using GSH as reducing agent) and NO every few days.

2.6. Detection of RSNOs in Blood

The experimental methods for the detection of endogenous RSNOs in animal blood were analogous to those reported previously [37, 38]. In brief, heparinized fresh sheep blood (obtained from Extracorporeal Membrane Oxygenation (ECMO) laboratory at the University of Michigan Medical School) was kept at 35°C before and during the experiments. Both NO and RSNO sensors were pre-calibrated to determine their inherent amperometric NO response in stirred PBS buffer (pH 7.4) at 35°C. Then, the amperometric signal of each sensor was monitored by adding the fresh sheep blood (30 mL) into the PBS buffer, pH 7.4 (70 mL, saturated with N₂ containing GSH and EDTA (final concentrations of 50 mM and 0.1 mM, respectively, after the blood was added). For the estimation of RSNO concentrations in blood, the RSNO sensor’s inherent response to the exogenous GSNO was post-calibrated by injecting the GSNO standard solution in the same blood sample (i.e., standard addition method).

3. Results and Discussion

3.1. RSNO Sensor Performance

Due to the catalytic activity of organoditelluride species and corresponding polymeric materials that possess covalently linked RTe sites in denitrosating RSNOs [43], an amperometric NO sensor with an external dialysis membrane containing an organoditelluride-linked polymer is expected to detect NO and RSNO levels in test samples. An electrochemical NO sensor covered with a blank dialysis mem-

brane should only detect NO concentrations in the same samples. Thus, to evaluate the basic analytical performance of the proposed RSNO sensor, both an RSNO and NO sensor were prepared and tested for their direct response toward NO and various RSNO species.

For preliminary studies, both sensors were concurrently placed in a working buffer solution containing a suitable reducing agent (50 μM GSH) (along with 0.1 mM EDTA in phosphate buffered saline (PBS), pH 7.4) and were monitored for their amperometric responses toward added NO or GSNO solutions as shown in Figure 2. The expected current responses of both sensors toward added NO standard solution confirmed their suitable function for amperometric NO detection (see Fig. 2A). The intrinsic NO sensitivities obtained from this experiment can be used to prepare calibration curves for each sensor to convert the current values recorded for each device into NO equivalent levels.

Upon adding increasing concentrations of GSNO into a fresh working buffer solution (with added GSH), the RSNO sensor displays a significant current change in proportional to the GSNO levels present (see Fig. 2B), while the current of the “control” NO sensor in the same solution does not change appreciably. This confirms that the presence of the catalytic layer mounted over the gas permeable membrane

<table>
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<tr>
<th>Current (nA)</th>
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<td>Time (min)</td>
<td>0</td>
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<td>RSNO sensor</td>
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Fig. 2. The inherent amperometric responses of the a) NO and b) RSNO sensors monitored by adding small aliquots of a standard solution of A) 2 mM NO (0.5, 0.5, 1.0, 2.0, and 4.0 μL, respectively) or B) 2.5 mM GSNO (40, 40, 80, and 160 μL, respectively) into a stirred solution of PBS buffer, pH 7.4, containing 0.1 mM EDTA and 50 μM GSH at room temperature. Each aliquot was intermittently added after the steady-state of current signal was reached. (A) and (B) are separate experiments; the numbers in the graph indicate the accumulated concentrations of the given test species (NO or RSNO) in the working buffer solution.
is responsible for the catalytic conversion of GSNO to NO at the surface of the RSNO sensor.

Such catalytic function of the organoditelluride-linked hydrogel on the surface of the RSNO sensor was also demonstrated by performing a real-time reversibility test. As shown in Figure 3, the RSNO sensor dynamically responds to changes in GSNO concentration in real-time as the level of GSNO is increased and then decreased within the same test vessel.

When comparing the relatively short response times (2–4 min) for both sensors to reach the steady-state current levels with respect to NO concentrations (see Fig. 2A), a somewhat slower response (4–8 min) toward changes in GSNO concentrations was observed with the RSNO sensor, suggesting that the dialysis membrane (thickness, 20–40 μm; MWCO, 50 kD) filled with the crosslinked catalytic polymer acts as a diffusion barrier for mass transfer of GSNO/GSH species into the catalytic layer, and this leads to the slower dynamic response. Indeed, the slower response time (> 10 min) was observed when the dialysis membrane with a lower MWCO (15 kD or 8 kD) was employed. It is important to note, however, that the response time of the proposed RSNO sensor reported herein is comparable to a previously reported organodiselenide-based RSNO sensor [38] also prepared using an outer dialysis membrane to immobilize the polymeric catalyst (i.e., the catalytic layer was between the gas permeable membrane and dialysis membrane, see Fig. 1).

The main advantage of the RSNO sensor prepared with the immobilized organoditelluride species is a very long operational lifetime (at least one month), which was determined by frequently monitoring the sensitivity (nA/M) changes in response to both NO and GSNO. Remarkably, the sensitivity for the GSNO response is maintained with time while only a slight decrease in the NO response originating from the platinized platinum working electrode is observed over the same given period. Hence, this behavior yields an overall small increase in the relative sensitivity (S) for GSNO vs. NO response (S_{GSNO}/S_{NO}) as a function of time (see Fig. 4).

The excellent stability of the proposed RSNO sensor compared to any of the previously reported amperometric RSNO sensors modified with NO generating catalysts (e.g., copper [37] and/or organodiselenide [38]-based RSNO sensors; up to 2 weeks) likely stems from the greater stability of the active organoditelluride species (DTDTCA) employed within the catalytic layer of the new device. Considering the redox mechanism involved for NO generating catalysts (i.e., copper ions, RSeSR, or RTeTeR), the least stable forms of the catalysts (i.e., Cu(I), RSe-/selenolate, or RTe-/tellurolate) in their redox cycles very likely determines their catalytic lifetimes, which is reflected in the RSNO sensors’ stabilities. In fact, the organoditelluride species (DTDTCA) used in this study possesses a unique structure that can be further stabilized in the reduced form (RTe-, where R = thiophenecarboxylic acid) by the aromatic resonance that can occur (refer to Fig. 1B). This decreases the possibility of side reactions to form Te(Se) element and/or the adduct of a small molecule thiolate (RS-, the reducing agent), RSTe-/tellurolate- [47, 48] that can be washed out from the catalytic layer. Indeed, the amperometric RSNO sensors reported in previous studies did not possess such a structural benefit for the NO generating catalyst, resulting in reduced long-term stability [38]. Further, the fact that RSNO sensitivity can actually remain the same while the inherent amperometric NO response of the RSNO sensor decreases with time, suggests that the mass transfer into the catalytic layer of the RSNO analytes and reducing agent may actually increase as a function of time, as the layer becomes more fully hydrated and the crosslinked catalytic polymeric material reorganizes and/or the cellulose membrane support hydrolyzes.

**Fig. 3.** The dynamic amperometric response and reversibility of RSNO sensor by varying three different concentrations (0, 2, and 4 μM) of GSNO in the working buffer solution at RT. The RSNO sensor was initially placed in the working buffer solution (with GSH present) without GSNO, while recording the current signal, and then placed in fresh working buffer solution with the new solution containing 2 μM GSNO. After a new steady-state current signal was observed, the solution was again exchanged with a 4 μM GSNO solution in working buffer. This process was repeated sequentially with solutions containing the given concentrations of GSNO in working buffer.

**Fig. 4.** Stability of the RSNO sensor, plotted as relative sensitivity (S_{GSNO}/S_{NO}) of the RSNO sensor as a function of time, where S_{GSNO} is the sensitivity (nA/μM) of the RSNO sensor toward GSNO and S_{NO} is the sensitivity (nA/μM) of the RSNO sensor toward NO. The sensitivity (nA/μM) is the slope of data set obtained from recording the steady-state current values for various concentrations of samples (NO and GSNO) (see Fig. 2). The sensor was kept in the PBS buffer, pH 7.4, at room temperature and a polarizing potential of +0.75 V was maintained continuously for the entire test period (1 month).
3.2. Direct Amperometric Detection of S-Nitrosothiols

As depicted in Figures 5 and 6, the new organoditelluride-based RSNO sensor is capable of directly detecting various RSNO species from sub-μM to μM levels, which are the suggested ranges for RSNO species in blood [49], although their exact concentrations in blood plasma are still in considerable debate [50, 51]. The measurements of RSNO species by the sensor exhibit reasonable detection limits (ca. 0.1 μM) with respect to the LMW RSNOs including GSNO, CySNO, and SNAP. An attractive feature of the proposed RSNO sensor is that the responses toward GSNO and CySNO are nearly equal in the range of 0.1–4 μM as illustrated by their respective calibration curves when GSH (50 μM) is employed as a reducing agent (see Fig. 6A and B). Such similar sensitivities toward the various LMW endogenous RSNOs were reported previously for low concentrations (<1 μM) of RSNOs using the organodiselenide-based RSNO sensor [38]. In contrast, the Cu(II/I)-based RSNO sensor reported earlier exhibits a much greater amperometric response toward CySNO than GSNO as well as other RSNO species [37].

3.3. Role of Reducing Agents in Amperometric Response

An interesting phenomenon observed in the detection of macromolecular AlbSNO is that the current signals continuously increase, without yielding a final steady-state response (see Fig. 5C). Due to the large molecular weight of AlbSNO (69 kD) compared to the molecular cut-off (MWCO, 50 kD) of the dialysis membrane mounted on the RSNO sensor, it is unlikely that AlbSNO passes through this membrane and reacts directly with the organoditelluride catalyst. However, a transnitrosation reaction between AlbSNO and excess GSH reducing agent (50 μM GSH is already present in the working buffer solution) can provide a low molecular RSNO species (i.e., GSNO) from the higher molecular weight AlbSNO species [52], and this can be the origin of the response observed.

The calibration curves for LMW RSNOs measured by the RSNO sensor (see Fig. 6B) exhibit enhanced sensitivities in the presence of CySH compared to GSH, in addition to a reversed order of sensitivity in the case of SNAP. Such increases in the sensitivity toward the LMW RSNOs could also be associated with transnitrosation reactions because the amperometric responses for LMW RSNOs at various
concentrations in the presence of CySH reducing agent are much slower in reaching a steady-state amperometric signal (compared to when using GSH as the reducing agent), especially, the very long response time observed for SNAP additions (data not shown). In fact, it has been reported that CySH and its residues in many proteins are widely involved in biological transnitrosation reactions [53, 54]. Thus, the equilibrium constant for the transnitrosating reaction with cysteine as the reducing agent present in excess is likely more favorable than for GSH [52, 55, 56]. Another explanation is that increases in the sensitivity toward the LMW RSNOs could also be associated with the enhanced kinetics in generating catalytically active species, i.e., RTe− (tellurolate) in the presence of CySH compared to GSH. The more facile reaction between RTe-SCy and CySH may elevate the steady-state catalytic site (tellurolate) levels within the modified dialysis membrane than that between RTe-SG and GSH.

Figure 6C clearly shows the elongated response time observed when using CySH as a reducing agent in place of GSH. Despite its lower molecular weight (vs. GSH) and faster transnitrosation tendency, a much more sluggish amperometric response toward GSNO additions can be seen. Since this elongation in response time is also observed for other RSNO substrates when tested (data not shown) with the exact same RSNO sensor, it appears that when CySH is employed as a reducing agent an additional unidentified equilibrium reaction process may occur that slows down the establishment of a steady-state surface NO concentration. We speculate that the capability of CySH (unlike GSH) to form HNO and other reduced intermediates [57] from the direct reaction with RSNO could be at play.

In contrast, any significant S-nitrosation of GSH from the GSNO, CySNO, or SNAP is not obvious from the response patterns observed, which is also supported by the fact that no significant background NO level in the bulk solution is detected by the NO sensor when simultaneously monitored with the RSNO sensor in the same solution (see Fig. 2B). However, as shown Figure 5C, the S-nitrosation of GSH from AlbSNO as an analyte does seem to take place [52]. Therefore, there appear to be rather complicated relationships of transnitrosation between endogenous RSNOs, which are important in understanding the characteristics of RSHs/RSNOs in biological systems. Although further studies are necessary to fully understand the transnitrosation equilibria involved between all endogenous RSH/RSNO species, based on the observed experimental results presented here, the proposed RSNO sensor can directly detect various LMW RSNOs at low concentrations (0.1–4 μM) in the presence of GSH (50 μM), and there is also some sluggish yet considerable amperometric response observed toward protein RSNOs due to transnitrosation chemistry with the added reducing thiol agent (GSH).

Another interesting observation is the effect of GSH concentration on the observed sensitivity of the proposed
RSNO sensor. Based on our recent mechanistic work \[43\], the reducing agent, RSH, participates in the rate-determining reaction step of organoditelluride-mediated RSNO denitrosation reaction. Hence, the RSNO sensor’s sensitivity is expected to be influenced by the concentration of RSH in the working buffer. Indeed, as shown Figure 6C, the addition of GSH (up to 40 \(\mu\)M) into the working buffer solution greatly improves the sensitivity of the RSNO sensor without changing the response time. At higher concentrations of GSH, the effect levels off, and saturates, with no change in observed response to further increases in the concentration of GSH. Therefore, the amount of GSH recommended for use in the working buffer (50 \(\mu\)M) seems appropriate to detect various RSNO species with optimal sensitivity.

### 3.4. Direct Detection of Endogenous S-Nitrosothiols in Blood

Initially, both the RSNO and NO sensors, which were precalibrated for their inherent responses with respect to a NO standard solution at 35 \(^\circ\)C, were concurrently placed in a new working buffer solution (70 mL) at 35 \(^\circ\)C and their baseline amperometric signals were recorded. Upon adding fresh sheep whole blood (30 mL) into the working buffer solution, the RSNO sensor detects the RSNO/NO levels in the blood sample, while the control NO sensor only detects NO levels in the same blood sample (see Fig. 7). The change in current signal observed between the two sensors can be converted to the NO equivalent levels by means of the prior NO calibration curves, yielding a noticeable difference between two sensors in terms of surface NO concentrations detected, as represented by \(\Delta\) value (ca. 60 nM NO concentration) in Figure 7A.

Finally, the injection of a GSNO standard solution into the same blood sample illustrates clearly that the two sensors exhibit their expected functions, with the RSNO sensor having significant response, while little or no response is seen from the control NO sensor in the same blood sample after the RSNO additions (see Fig. 7B). From the initial response of the RSNO to the added blood, and the change in current detected when the sample is further spiked with several additions of GSNO, it is possible to utilize the method of multiple standard addition \[58, 59\] to determine the total GSNO equivalent concentration in the original blood sample.

Based on the NO and GSNO calibration curves, the current difference between the two sensors translates to ca. 2.6 \(\mu\)M GSNO equivalents in the sheep blood. Given the studies shown above (see Fig. 5C) some fraction of this value comes from the AlbSNO present in the blood, which yields amperometric response via the transnitrosation chemistry with the GSH reducing agent. If conditions can be found where this reaction proceeds with near 100% efficiency, then the sensor value reported would be a measure of the total RSNO species in the plasma phase of the blood. Efforts to find conditions where such high efficiency takes place are currently in progress.

In this work, a new type of RSNO sensor has been prepared by employing a crosslinked organoditelluride-tethered PAH polymer within a dialysis membrane support mounted as an outer layer of a planar amperometric NO sensor. Due to the catalytic activity of organoditelluride-linked hydrogel for the decomposition of RSNO species to NO in the presence of appropriate RSH reducing agent, various LMW RSNO/RSH species that can pass through the diffusion barrier and liberate NO to increase the local NO levels within this catalytic layer and this results in an increase in current proportional to the RSNO concentration present in solution. Compared to previously reported RSNO sensors based on immobilized Cu(II) \[37\], organodiselenide \[38\] and enzyme layers \[60\], this new organoditelluride modified sensor has a superior operational lifetime (more than a month), presumably due to the enhanced stability of the catalyst employed. In addition, the proposed RSNO sensor responds directly and reversibly to various LMW RSNOs such as CySNO, GSNO, and SNAP in the range of 0.1 \(\mu\)M to 10 \(\mu\)M with nearly equal sensitivity, which is comparable to a previously reported organodiselenide-based RSNO sensor \[38\], while Cu(II)-based \[37\] and enzyme-based \[60\] RSNO...
sensor exhibits quite varied sensitivities toward different LMW RSNOs. It has been further shown that a trans-nitrosation reaction between high molecular weight RSNO species and the necessary low molecular weight thiol reducing agent actually yields an increase in LMW RSNO in the test solution that also allows significant amperometric response to the HMW AlbSNO species to be observed. The sensor provides an output signal in diluted whole blood samples that is likely proportional to the level of total RSNO species in the plasma phase of the blood sample. With further optimization of response times and more studies to determine conditions where the catalytic layer can directly liberate NO from HMW endogenous RSNOs by changing the sensor configurations and/or via more efficient trans-nitrosation reactions, the proposed sensor could ultimately provide a useful new tool for rapidly determining total RSNO species concentrations in whole blood samples, without mandatory pretreatment steps that can dramatically alter the levels of RSNO detected by other methodologies.

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6. References