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Flow injection measurements of S-nitrosothiols species in biological samples using amperometric nitric oxide sensor and soluble organoselenium catalyst reagent

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Abstract

A novel flow injection analysis (FIA) system suitable for measurement of S-nitrosothiols (RSNOs) in blood plasma is described. In the proposed (FIA) system, samples and standards containing RSNO species are injected into a buffer carrier stream that is mixed with the reagent stream containing 3,3'-dipropionidiselene (SeDPA) and glutathione (GSH). SeDPA has been shown previously to catalytically decompose RSNOs in the presence of a reducing agent, such as GSH, to produce nitric oxide (NO). The liberated NO is then detected downstream by an amperometric NO sensor. This sensor is prepared using an electropolymerized *m*-phenylenediamine (*m*-PD)/resorcinol and Nafion composite films at the surface of a platinum electrode. Using optimized flow rates and reagent concentrations, detection of various RSNOs at levels in the range of 0.25–20 μ M is possible. For plasma samples, detection of background sensor interference levels within the samples must first be carried out using an identical FIA arrangement, but without the added SeDPA and GSH reagents. Subtraction of this background sensor current response allows good analytical recovery of RSNOs spiked into animal plasma samples, with recoveries in the range of 90.4–101.0%.

Keywords

Flow injection analysis; Plasma; S-nitrosothiols (RSNOs); Nitric oxide sensors

Introduction

Nitric oxide (NO) is recognized as a critically important biological messenger in the central and peripheral nervous systems and in the vascular endothelium. NO is also involved in host defense mechanisms and plays a critical role in inflammatory and degenerative diseases. Certain metabolites of NO, such as peroxynitrite (ONOO^-), and nitrous anhydride (N_2O_3), can be toxic or mutagenic when present at sufficiently high concentrations, increasing the importance of NO measurements in clinical chemistry. Furthermore, it has been recently found that nitrosylation of the sulfhydryl groups in amino acids, peptides, and proteins provide NO-like biological activities including vascular smooth muscle relaxation, neurotransmission, and inhibition of platelet aggregation. Indeed, *S*-nitrosothiols (RSNOs) are bioactive molecules generated in vivo, and their generation has been suggested to include the nitrosylation of thiols by endogenous NO or oxidative intermediates of NO (e.g., N_2O_3 and NO^+) [1–3]. It is believed that RSNOs are a carrier or reservoir of transient NO under physiological conditions as their decomposition, via thermal, photolytic, and catalytic pathways, can lead to localized NO generation [1, 2, 4]. Such species, including low-molecular-weight RSNOs (e.g., *S*-nitrosocysteine and *S*-nitrosogluthathione, GSNO) as well as *S*-nitrosoproteins, have all been found in mammalian systems, especially in blood plasma.

Various methodologies have been applied for the detection of RSNOs in human and animal biological fluids, such as chemiluminescence, high-performance liquid chromatography (HPLC), spectroscopy, fluorimetry, and gas chromatography–mass spectrometry. The RSNO values reported by different research groups using different methods cover several orders of magnitude. Stamler et al. [2] first reported the presence of 7 μM RSNOs in plasma of healthy humans. Over the last few years, the reported measured levels of RSNOs both in plasma and whole blood have ranged from low nanomolar to values greater than the first reported 7 μM [5]. Sophie et al. [6] applied electron paramagnetic resonance spectrometry to measure RSNOs in extracellular fluids from healthy human volunteers and rheumatoid arthritis patients. They reported median RSNO concentrations of 0, 109, and 309 nM in normal plasma, rheumatoid arthritis plasma, and synovial fluid, respectively. Funai et al. [7] performed spectrophotometric detection of RSNOs in rat blood and reported values around 3.4 μM . Napoli et al. [8] reported RSNOs levels of 1.78 μM for rat blood using a chemiluminescence-based assay. Wlodek et al. [9] determined RSNOs concentrations using a fluorometric method. In this method, 2,3-diaminonaphthalene is nitrosylated to 2,3-naphthotrizole as a fluorescent product which enables the determination of RSNOs via a degradation product. They found RSNO values in plasma of healthy subjects of approximately 8.8 μM . Therefore, measurement of RSNOs in physiological samples is quite challenging, with the range of normal values of RSNOs in blood varying widely depending on the methodologies employed [5, 10]. This is likely because of the inherent labile nature of RSNOs [11], with spontaneous decomposition influenced by trace metal ions, light, and heat. Hence, simpler methods to detect RSNOs, which do not require extensive sample preparation or utilize complex instrumentation, are sorely needed.

Previous research suggests that RSNOs can effectively be decomposed to generate NO by organoselenium catalysts in the presence of a reducing agent, in accordance with the reactions in Scheme 1 [12]. This chemistry has previously been utilized to develop electrochemical RSNO sensors that employ immobilized organoselenium species at the surface of amperometric NO sensors [13]. However, the response time and recovery time of such a complete sensor with the immobilized catalytic agent limit the throughput of the device, making it difficult to implement the sensor-based approach for high-throughput measurements of RSNOs in a large number of samples. In the present work, we utilize a soluble diselenide species, 3,3' dipropionicediselenide (SeDPA), as a catalytic reagent within a flow injection arrangement (Fig. 1) equipped with an amperometric NO detector to create

a system that can measure RSNOs in plasma. This is accomplished using a differential method in which the sample is injected and detected in the flow injection analysis (FIA) system in the absence of SeDPA and GSH reagents, allowing the detection of species that interfere with the electrochemical detector, as well as any trace NO in plasma. Subsequently, the same sample is injected in the presence of SeDPA and GSH reagents, which yields a signal proportional to the amount of interfering species, trace NO and RSNOs in the plasma. The difference in these two measurements (current recorded by amperometric NO sensor) yields a net current value that is directly proportional to the RSNO level in the blood plasma using a standard calibration curve, obtained with RSNO standards prepared in buffer. RSNO levels determined using our FIA method are in the range of those previously reported.

Experimental section

FIA system and method

The diagram of the flow injection system developed is shown in Fig. 1. To obtain a background signal (from any electrochemical interferences present) for the biological sample, only phosphate-buffered saline (PBS) buffer was used as the RS reagent. When SeDPA and GSH are in the buffer of the reagent stream (RS), NO is generated in the mixing coil proportional to the level of RSNO within the injected sample, and the electrochemical detector, at the distal tip of the tubing, responds to this NO and background interferences in the sample. A typical recording of output has the form of a current peak, the height, which is related to the concentration of the analyte [14]. In our system, a Minipuls 3 peristaltic pump (Gilson, Middleton, WI, USA) was used to pump the carrier stream (PBS buffer) and the RS to the NO detector. The PEEK tubing used for sample carrier stream, reagent carrier stream, and mixing coil, and the PTFE mixing tee were purchased from IDEX Health & Science LLC (Oak Harbor Manufacturing Facility, Oak Harbor, WA, USA). All current measurements from the NO sensor were made with a BioStat potentiostat (ESA Biosciences, Inc., Chelmsford, MA, USA). Samples were loaded into a 250- μ L sample loop and immediately injected into the carrier stream through the six-port rotary valve (Rhenodyne 9725i). Samples and reagents flowed to waste after passing by the NO sensor. Calibration curves were obtained by injecting freshly prepared GSNO standard solutions into the carrier stream of the FIA system. It should be noted that in order to avoid thermal, photo, and metal ion catalyzed decomposition of RSNOs, all samples were kept cold and all components of the FIA system were protected from light with aluminum foil (including the tubing, reagent bottles, etc.). Furthermore, ethylenediaminetetraacetic acid (EDTA) was added to buffers and to blood samples to inhibit metal ion decomposition reactions.

Reagents and materials—All chemicals were of analytical grade or better and were used as received. Sodium chloride, acetone, and ethanol were obtained from Fisher Scientific (Pittsburgh, PA, USA). Potassium chloride, glutathione, sodium nitrite, ammonium chloride, L-ascorbic acid, sodium hydroxide, concentrated sulfuric acid, hydrochloric acid, iron(III) chloride, EDTA, 1,3-phenylenediamine, resorcinol, and Nafion perfluorinated ion exchange resin (5 wt.% solution in lower aliphatic alcohols/H₂O mix containing 15–20% water) were obtained from Sigma-Aldrich (St. Louis, MO, USA) and used as received. RSNOs and 3,3'-dipropionidiselenide (SeDPA) were prepared as previously described in the literature [12, 15, 16]. Standard NO solution was freshly prepared as reported elsewhere [17, 18]. Briefly, 4 M H₂SO₄ was added dropwise to a deoxygenated NaNO₂ solution (~12 M). The generated NO gas was bubbled through a 20% NaOH solution to remove any interfering nitrogen dioxide species and then collected in deoxygenated PBS buffer. This standard solution was kept under an NO atmosphere at all times, stored in an airtight container sealed with a rubber septum. The concentration of NO

in the saturated solution was determined to be 1.72 mM at 20 °C by a chemiluminescence assay using a Sievers Nitric Oxide Analyzer 280 (Boulder, CO, USA).

Platinum wire coated with Teflon (bare, 127 μm in diameter; coated, 203.2 μm in diameter) and silver wire coated with Teflon (bare, 76.2 μm in diameter; coated, 139.7 μm in diameter) were purchased from A-M Systems, Inc. (Sequim, WA, USA). Buffers, including PBS (0.01 M, pH 7.4), were prepared fresh daily from appropriate reagents. All aqueous solutions were prepared with 18.2 M Ω deionized distilled water using a Milli-Q filter (Millipore Corp., Billerica, MA, USA).

Fabrication of the nitric oxide sensor

Figure 2 shows the configuration of the amperometric NO sensor that was used as a detector in the FIA arrangement to measure RSNO species. This design is a variant of the previously developed needle-type glucose sensor that detected hydrogen peroxide generated from an immobilized glucose oxidase reaction [19, 20]. The NO sensors are coated with Nafion as well as an electropolymerized film of an *m*-PD/resorcinol mixture. Nafion is an excellent material to block anionic molecules such as nitrite and ascorbate from reaching the underlying electrode, while poly(*m*-PD/resorcinol) can limit the access of any larger redox molecules to the surface of the electrode while offering free passage to NO. Previously, an *m*-PD/resorcinol film was used in developing glucose sensors [19, 20] and is used here with Nafion to further improve the selectivity of the NO sensor.

To prepare the electrochemical NO sensor, a clean tip of the working platinum electrode was created by removing the Teflon coating (0.5 cm in length) from the surface of the platinum wire (8 cm in length). The exposed tip (0.5 cm in length) was cleaned by soaking in acetone for 10 min, then in hydrochloric acid for 10 min, and finally rinsed with Milli-Q water. Electrodes were then modified with Nafion by dip-coating in a 5 wt.% solution of Nafion and cured in air for 10 min. This process was repeated twice to deposit three layers of Nafion film on the tip of working electrode. The electropolymerization solution containing *m*-phenylenediamine and resorcinol monomers (each at a concentration of 1.5 mM) in deoxygenated PBS buffer (0.01 M, pH 7.4) was freshly prepared and used immediately. The Nafion-coated Pt working electrodes were submerged in the electropolymerization solution and the potential of the working electrode was scanned from +0.00 to +0.83 V (vs. Ag/Ag⁺) at a 2-mV/s scan rate for 18–21 h. During the process of electropolymerization, the solution was protected from light and continuously purged with pure nitrogen. After electropolymerization, the platinum working electrodes were rinsed with Milli-Q water and coated with another three layers of Nafion via the same dip-coating process used prior to the electropolymerization step.

The reference electrode for the NO sensor was prepared and then wrapped around the Teflon surface of the platinum working electrode. To prepare the reference electrode, a thin Teflon-coated silver wire (15 cm in length) was first stripped off the Teflon at the distal end (5 cm in length) with a razor and then soaked in a solution consisting of 1 M HCl and 3 M FeCl₃ for 10 min. The exposed Ag wire was oxidized and, concomitantly, AgCl_(s) formed at the surface. The wire was then rinsed carefully with Milli-Q water twice. This Ag/AgCl wire was then tightly wrapped around the Teflon surface of the working electrode, covering about 5 mm of its length. The platinum working electrode above the portion wrapped with Ag wire and the extended Ag wire were placed into a 5-cm-long (1,500 μm i.d.) polyester heat-shrinkable tubing (Advanced Polymers Incorporated, Salem, NH, USA). A heat gun (model 750, MHT Products Inc.) operating at 600 °C was used to shrink this tubing. At the top of the electrode, 1-cm length of the Teflon coating was removed from the Pt and Ag wires to expose the wire for connection to the BioStat potentiostat. Sensors were soaked in deionized water for 3 h to release ethanol and other alcohols in which the Nafion is dissolved. Finally,

sensors were polarized at a potential of +0.75 V (vs. Ag/AgCl) overnight to obtain a steady baseline current for measurement. The same potential was applied for measurements of the NO in the FIA system.

Result and discussion

Selectivity evaluation of NO sensors

The ideal NO sensor for the FIA system should be sensitive enough to detect low concentrations of NO and insensitive to possible interferences in biological samples, such as nitrite, ammonium, and ascorbic acid. At the same time, the sensor must have reasonably fast response and recovery times in order to be used in a dynamic flow injection arrangement. Platinum and platinized platinum [21] working electrodes were compared to determine which would be best for incorporation into the FIA system. These sensors were coated with various combinations of the Nafion and *m*-PD/resorcinol films, as listed in Table 1. Selectivity coefficients of the various sensors were determined and are listed in Table 1. The amperometric selectivity coefficients ($\log K_{\text{NO},j}$) were calculated using the following equation [22]:

$$\log K_{\text{NO},j} = \log \left(\frac{\Delta I_j / c_j}{\Delta I_{\text{NO}} / c_{\text{NO}}} \right) \quad (1)$$

In this equation, ΔI_j and ΔI_{NO} were measured current changes in response to interfering species and NO, their concentrations being c_j and c_{NO} , respectively. The interfering species were added at 100 μM each and the NO concentration detected was 2 μM for this preliminary assessment of sensor selectivity. When comparing sensors with the same film composition, sensors with platinized platinum as the working electrode had poorer selectivity and higher sensitivity vs. those prepared with plain platinum as the working electrode. This is due to the fact that platinized platinum has a rougher and larger surface area, resulting in higher sensitivity. However, this rougher surface area had a negative effect on the electropolymerization process, likely leading to irregular coatings of the films and hence loss in selectivity. With respect to applying three layers of Nafion before or after electropolymerization, sensors coated with Nafion before electropolymerization possess better selectivity, but almost the same level of sensitivity compared to those coated with Nafion after electropolymerization. Thus, it is possible that the alcohols in which the Nafion was dissolved are capable of removing some of the poly(*m*-PD/resorcinol) layer. Interestingly, sensors coated with three layers of Nafion before and after electropolymerization yielded the best selectivity. As a result, platinum alone coated with Nafion before and after electropolymerization of the poly(*m*-PD/resorcinol) film provided the best combination for the selective detection of NO. The selectivity of such sensors over interfering species is adequate for the measurement of RSNOs in biological samples, provided that some correction for non-ideal selectivity is made using the differential measurement method employed.

Optimum applied potential for FIA system

Sensors have different performance levels at different applied potentials in terms of sensitivity and selectivity toward NO. The optimum potential was first determined prior to FIA experiments. As shown in Fig. 3a, there is a large peak at 0.737 ± 0.015 V in the linear sweep voltammogram of NO, which indicates that this is the optimal potential for NO oxidation and sensors will offer the highest sensitivity for NO measurement at this potential. Similarly, as shown in Fig. 3b, there is a large peak at 0.761 ± 0.006 V in the linear sweep

voltammogram of nitrite, which means nitrite will severely influence NO determination if potentials more positive than 0.76 V are applied. In addition, selectivity coefficients were obtained using the method mentioned above at different potentials and listed in Table 2. Selectivity to NO over interfering species obtained at 0.75 V proved to be optimal.

Optimum flow rate and concentration of catalyst and reducing reagent in FIA system

The flow rate along with the concentrations of catalyst and reducing agent can influence the RSNO sensitivity and detection limit of the FIA system. In this work, we used the NO sensor to detect NO liberated from the RSNOs that undergo a reduction reaction catalyzed by the organoselenium species (SeDPA) in the presence of a reducing agent (GSH). The key factors considered during method development were the reaction rate of RSNO decomposition by the selenium catalyst and reducing agent, the sensor's response time toward NO, and the lifetime of NO in solution. The optimum flow rate should not only be slow enough to allow complete decomposition of the RSNOs by the catalyst and to detect all of the NO generated but also fast enough to detect NO within its lifetime. In order not to cause pressure problems and not to produce noisy amperometric signals, we examined several different flow rates. As shown in Fig. 4, increasing the total flow rate from 1.14 to 2 mL/min, the RSNO sensitivity of the system increased significantly. This effect is likely due to reduced dispersion (and hence dilution) of the RSNO sample plug within the FIA system when the flow rate is greater [14]. As a result, lower amounts of GSNO can be detected at the higher flow rates. However, when further increasing the flow rate to 2.5 mL/min, the sensitivity of the system decreased when injecting high concentrations ($>10\text{ }\mu\text{M}$) of GSNO. At this high flow rate and high concentration of GSNO, it is likely that there is not adequate time to completely reduce the GSNO before the sample plug reaches the NO sensor. The GSNO converted compared to total GSNO injected was calculated as 95.1% (for $10\text{ }\mu\text{M}$ GSNO), 75.2% (for $30\text{ }\mu\text{M}$ GSNO), and 71.2% (for $40\text{ }\mu\text{M}$ GSNO). The optimum total flow rate (measured post-mixing tee) for RSNO measurements was determined to be 2 mL/min and was used for all measurements. Specific variations in flow rates studied were somewhat limited by the nature of the Minipuls peristaltic pump used within the FIA system. The current signals were recorded for GSNO standards when varying the concentrations of SeDPA and GSH used in the reagent stream while operating at the 2-mL/min total flow rate (Fig. 5). By increasing the concentrations of SeDPA and GSH, the sensitivity toward RSNOs also increased, as well as when the concentration ratio of SeDPA and GSH remained the same (10:1 in our experiment). As shown in Fig. 5b, this trend is most obvious for injections of standards containing high concentrations of GSNO ($>10\text{ }\mu\text{M}$). For low concentrations of GSNO, the sensitivity does not change significantly when the concentrations of SeDPA and GSH are increased. Again, this relates to the need for more catalyst activity and reducing agent (GSH, source of electrons for RSNO reduction) when trying to decompose higher concentrations of RSNOs. It should be noted that increasing SeDPA without increasing GSH would not be especially helpful because in order to create a greater concentration of active selenol species, a greater concentration of GSH would be needed. As previously reported, total RSNO levels in physiological samples are low, likely in the range of $0.2\text{--}2\text{ }\mu\text{M}$ [13, 23]. In addition, when using the proposed differential method to determine RSNO levels in plasma, it is crucial to use the catalyst and reducing agent concentrations that have a minimal effect on the baseline current as such species can in fact yield some anodic response at the NO sensor (see Electronic supplementary material (ESM) Fig. S1). We further tried to vary one of the concentrations of SeDPA and GSH while keeping the other reagent constant, but we did not find any better reagent ratio of SeDPA and GSH (see ESM Fig. S2). Consequently, $200\text{ }\mu\text{M}$ SeDPA and $20\text{ }\mu\text{M}$ GSH were determined to be the optimum reagent stream concentrations and were used in all subsequent measurements.

The influence of oxygen on the detection of RSNOs in FIA system

It is known that RSNOs can easily be decomposed by light, temperature changes, and the presence of trace metal ions. Therefore, care must be taken to prevent these factors from affecting RSNO measurement. In detail, samples were protected from light throughout the processes of preparation and analysis by using foil-covered containers, and the components of the FIA system were placed in foil-covered boxes to prevent photodecomposition during sample analysis. Furthermore, all samples were stored on ice in the dark and EDTA was added to all standards and buffers to protect samples from trace metal ion catalysis of RSNOs. In addition, oxygen can react with reduced selenium species to create superoxide [24], which can scavenge NO by the formation of peroxynitrite. In addition, oxygen can react with liberated NO to form nitrite and nitrate, which could reduce the observed amperometric responses. Hence, in the proposed FIA system, we examined the effect of oxygen in the carrier and reagent buffer streams on the response observed for injected GSNO. As illustrated in Fig. 6, signals for injected GSNO standards significantly decreased when the carrier and reagent streams were not deoxygenated. Sensitivity decreased by 37% when deoxygenated samples were injected into the buffer stream that had not been deoxygenated. However, signals remained almost the same for samples with and without normal oxygen levels when they were injected into a deoxygenated buffer stream (Fig. 7). These results suggest that ambient levels of oxygen in the FIA solutions can scavenge some of the NO generated from the RSNO species, either by direct reaction with NO or via the formation of superoxide that then reacts with NO. However, when the oxygen level in the sample plug is reduced simply by its dilution in deoxygenated carrier and reagent streams, the scavenging reactions are greatly reduced. Consequently, continuously purging the carrier and reagent flowing streams with nitrogen is required to achieve optimized RSNO sensitivity in the FIA system.

Response to GSNO using flow injection analysis system

As is shown in Fig. 8, the NO amperometric detector yielded a fast response to GSNO from 0.25 to 20 μM and also returned to the baseline very quickly. As also shown, the reproducibility of the signals is also quite good, with RSD values for $n=4$ injections typically 3.1%. To ensure that signals obtained were indeed from GSNO, the response to a 1- μM GSNO standard sample in the presence and absence of SeDPA and GSH within the FIA reagent stream was recorded. Signals obtained in the presence of SeDPA and GSH were approximately ten times larger than those observed in the absence of SeDPA and GSH (see ESM Fig. S3), which confirms that the signal increase was caused by GSNO and not by excess nitrite (used in standard GSNO preparation).

Detection of RSNOs in plasma

Six fresh blood samples (from pigs) were obtained from the University of Michigan Hospital and centrifuged (2,000 rpm, 4 $^{\circ}\text{C}$) for 20 min. The supernatant (plasma) was carefully aspirated at room temperature and pooled into a tube for assay with the flow injection system. The plasma was stored on ice in a refrigerator and assayed within 2 h after the blood was drawn. The levels of RSNO in the plasma samples as determined from prior calibration of the FIA system with GSNO were in the range of 0–1.36 μM (Table 3). It should be noted that one sample may have lost RSNOs through excessive exposure to light or heat prior to analysis, and no RSNOs were detected. The endogenous levels of RSNO in these samples were calculated by first running the samples in the same FIA system with the same NO sensor, but without SeDPA or GSH in the reagent stream. The height of the peaks obtained without the catalyst were subtracted from the corresponding measurements taken with optimized SeDPA and GSH concentrations in the reagent stream. The accuracy of this differential measurement method was evaluated by performing a recovery experiment with a plasma sample that was spiked with varying amounts of GSNO. Figure 9 and Table 4 show

the results of that study. Based on these recovery data, it is evident that the method can accurately quantitate the levels of GSNO added to the plasma sample. Note that in Fig. 9, when the spiked samples are injected into the FIA system, the peak height increases in proportion to the amount of GSNO added to the sample. It should also be noted that this method likely detects all low-molecular-weight RSNOs in the plasma phase (including *S*-nitrosocysteine that may form by a transnitrosation reaction between the added GSNO with cysteine within the blood sample). Furthermore, the FIA system also detects a fraction of the *S*-nitrosylated proteins (*S*-nitrosoalbumin) that may be present, owing to excess GSH in the reagent stream which can also be an NO⁺ acceptor for any transnitrosation reactions of *S*-nitrosoproteins in the plasma sample [25].

Conclusion

In summary, we have described a relatively simple FIA system that is capable of detecting RSNOs in the range of 0.25–20 μM. Blood plasma can be injected directly into the system that employs an organoselenium catalyst and reducing agent in order to decompose the RSNOs releasing NO, which can be easily detected with an amperometric NO sensor. This method possesses good performance over a wide range of RSNO concentrations. The differential approach employed offers a new method to measure species in biological samples when the detecting sensor does not have ideal selectivity over potential interferences in such samples. The main advantage of this new FIA system is the potential to have a high-throughput assay system when compared to measuring RSNOs in plasma or whole blood using the previously described RSNO sensor [13] that employs an immobilized RSe species at the surface of the electrochemical NO sensor. In addition, the soluble catalytic reagents and electrochemical NO sensor arrangement used here for FIA measurements of RSNOs could also be employed as a selective post-column detection method when separating various RSNOs via HPLC methods [26]. Efforts to demonstrate this application using the same chemistry and detection strategy are planned for the future.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Schematic diagram of *S*-nitrosothiols (*RSNOs*) assay flow injection analysis system.

Components of the FIA system: *CS* carrier stream (PBS buffer), *RS* reagent stream (200 μ M SeDPA and 20 μ M GSH dissolved in PBS buffer), *P* Minipuls 3 Peristaltic Pump, *V* six-port rotary injection valve, *SL* sample loop, *MT* mixing tee, *MC* mixing coil, *D* flow through detector (amperometric NO sensor), *R* recorder (BioStat), *W* waste

Fig. 2. Schematic diagram of poly(*m*-phenylenediamine/resorcinol) and Nafion composite film-modified amperometric NO sensor

Fig. 3. Linear sweep voltammograms of nitric oxide and nitrite. **a** Nitric oxide (from *bottom to top*): 10, 25, and 35 μM. **b** Nitrite (from *bottom to top*): 50 and 75 mM

Fig. 4. Effect of the total flow rate of carrying buffer and buffer containing reagents on the response to different concentrations of GSNO: *a* 2 mL/min; *b* 1.4 mL/min; *c* 2.5 mL/min; *d* 1.15 mL/min

Fig. 5.
a, b Effect of concentration of selenium catalyst (SeDPA) and reducing agent (GSH) on the response to different concentrations of GSNO. *a* 250 μ M SeDPA, 25 μ M GSH; *b* 200 μ M SeDPA, 20 μ M GSH; *c* 150 μ M SeDPA, 15 μ M GSH; *d* 100 μ M SeDPA, 10 μ M GSH

Fig. 6. Effect of oxygen level in carrier and reagent stream on the response to different concentrations of RSNOs. **a** FIA response to standard GSNO (from 0.05 to 20 μM , deoxygenated) when carrier and reagent streams were deoxygenated and not deoxygenated. **b** Resulting calibration curves toward GSNO (from 0.05 to 20 μM , deoxygenated) when carrier and reagent streams were deoxygenated and not deoxygenated

Fig. 7. Effect of oxygen level in sample solutions on the response to different concentrations of RSNOs: **a** FIA response signals to standard GSNO samples (from 0.05 to 20 μM) when sample solutions were deoxygenated and not deoxygenated. **b** Calibration curves obtained toward GSNO (from 0.05 to 20 μM) when sample solutions were and were not deoxygenated while flowing streams were always deoxygenated

Fig. 8. Response to different GSNO standard solutions (a) and calibration curve using flow injection analysis system (b). Total flow rate, 2 mL/min; concentration of SeDPA, 200 μM ; GSH, 20 μM . Data are represented as means $\pm$ SD ($n=4$)

Fig. 9.
a Typical response to plasma in the presence and absence of selenium catalyst (200 μM SeDPA) and reducing agent (20 μM GSH) and response to plasma when spiked with a given amount of GSNO. **b** GSNO calibration curve in the presence of 200 μM SeDPA and 20 μM GSH; total flow rate, 2.0 mL/min

Scheme 1.

Scheme of RSNO decomposition reactions by organoselenium catalyst using a reducing agent. Each species represents: diselenide (RSe-Se-R), thiol (R'-SH), selenosulfide (RSe-SR'), selenolate (RSe^- , a conjugate base of selenol), and *S*-nitrosothiol (R''SNO)

Table 1
Selectivity and sensitivity of various NO sensors prepared with different working electrodes and combination of electropolymerized films and Nafion ($n=5$ for each sensor type)

Sensor	Selectivity ($\log K_{\text{NO, Nitrite}}$)	Selectivity ($\log K_{\text{NO, Ammonium}}$)	Selectivity ($\log K_{\text{NO, Ascorbic Acid}}$)	Sensitivity (pA/nM)
Platinum, poly(<i>m</i> -PD/resorcinol)	-1.91 ± 0.15	-4.60 ± 0.20	-3.60 ± 0.27	41.40 ± 2.25
Platinized platinum, poly(<i>m</i> -PD/-resorcinol)	-1.75 ± 0.09	-3.60 ± 0.21	-3.60 ± 0.20	51.20 ± 2.27
Platinum, poly(<i>m</i> -PD/resorcinol) first, 3 layers of Nafion second	-2.05 ± 0.11	-4.14 ± 0.32	-2.96 ± 0.17	28.80 ± 1.27
Platinized platinum, poly(<i>m</i> -PD/resorcinol) first, 3 layers of Nafion second	-1.69 ± 0.10	-3.10 ± 0.19	-3.23 ± 0.15	43.90 ± 3.19
Platinum, 3 layers of Nafion first, poly(<i>m</i> -PD/resorcinol) second	-2.62 ± 0.12	-4.58 ± 0.20	-2.64 ± 0.12	25.70 ± 2.26
Platinum, 3 layers of Nafion first, poly(<i>m</i> -PD/resorcinol) second, 3 layers of nafion third	-2.80 ± 0.16	-4.89 ± 0.27	-4.06 ± 0.37	17.10 ± 1.20
Platinized platinum, 3 layers of Nafion first, poly(<i>m</i> -PD/resorcinol) second, 3 layers of Nafion third	-1.98 ± 0.14	-2.58 ± 0.10	-4.05 ± 0.31	23.70 ± 2.01

Table 2
Selectivity of various NO sensors prepared with working electrodes coated composite films (three layers of Nafion first, poly(*m*-PD/resorcinol) second, three layers of Nafion third) at different potentials ($n=5$ for each sensor type)

Applied potential (V)	Selectivity ($\log K_{\text{NO}, \text{Nitrite}}$)	Selectivity ($\log K_{\text{NO}, \text{Ammonium}}$)	Selectivity ($\log K_{\text{NO}, \text{Ascorbic Acid}}$)
0.7	-2.45 ± 0.15	-4.09 ± 0.20	-3.25 ± 0.19
0.75	-2.80 ± 0.16	-4.89 ± 0.27	-4.06 ± 0.37
0.8	-2.59 ± 0.21	-3.09 ± 0.15	-2.77 ± 0.09
0.85	-2.38 ± 0.12	-3.78 ± 0.18	-2.72 ± 0.14

Table 3
Summary of RSNO concentration in fresh animal plasma using the FIA system

Sample	RSNOs (nM)
1	498±56
2	597±65
3	865±83
4	674±40
5	1360±96
6	0±35

Average of peak measurements $\pm$ range ($n=2$)

Table 4
Summary of RSNO recovery experiments in plasma

Amount of GSNO		%Recovery
Added (μM) ^a	Found (μM) ^b	
1.0	1.0	100.0
1.66	1.05	90.4
12.5	12.6	101.0
25.0	23.1	92.4

^a Aliquots 5 mM GSNO standard were added to 0.6 mL fresh plasma to obtain known amount of GSNO concentration

^b Average of measurements ($n=2$)