Anal. Chem. **2005,** *77,* 3516−3524

Direct Detection of *S*-Nitrosothiols Using Planar Amperometric Nitric Oxide Sensor Modified with Polymeric Films Containing Catalytic Copper Species

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The direct amperometric detection of *S*-nitrosothiol species (RSNOs) is realized by modifying a previously reported amperometric nitric oxide gas sensor with thin hydrophilic polyurethane films containing catalytic Cu(II)/(I) sites. Catalytic Cu(II)/(I)-mediated decomposition of *S*-nitrosothiols generates $NO_{(g)}$ in the thin polymeric film at the distal tip of the NO sensor. Three different species are examined to create the catalytic layer: (1) a lipophilic Cu(II)−ligand complex; (2) Cu(II)−phosphate salt; and (3) small (3-μm) metallic Cu^0 particles. All three catalytic layers yield reversible amperometric response in proportion to the concentration of *S*-nitrosothiols (e.g., nitrosocysteine, nitrosoglutathione, *S*-nitroso-*N*-acetylcysteine, *S*-nitrosoalbumin) present in the aqueous test solution. Sensitivity toward the different RSNO species is dependent on the respective catalytic rates of decomposition of the RSNO species by reactive Cu(I), accessibility of the species into the polyurethane layer containing the catalyst, the level of reducing agents (ascorbate) used in solution to help generate reactive Cu(I) species, and the concentration of metal ion complexing agents present in the test solution (e.g., EDTA). Under optimized conditions, all RSNO species can be detected at ≤1 μM levels, with sensor lifetimes of at least 10 days for the sensors based on Cu(II)−phosphate and Cu^0 particles. It is further shown that the new RSNO sensors can be used to assess the "NO-generating" ability of fresh blood samples by effectively detecting the total level of reactive RSNO species present in such samples.

S-Nitrosothiols (RSNOs) are a group of bioactive molecules generated in vivo through the nitrosylation of thiols by oxidative intermediates of endogenous nitric oxide (NO) (e.g., N_2O_3 and NO^+) that form under physiological conditions.[1] Nitric oxide has been identified as the endothelium-derived relaxing factor (EDRF),[2] and several species are known to enhance NO production by the endothelial cells (e.g., bradykinin, ATP, etc.).[3,4] Further, nitric oxide's transient nature has led to the recognition that NO is possibly stored and transferred throughout the body in the form of RSNOs, species that exhibit physiological lifetimes comparable to that of EDRF.[5,6] Indeed, nitrosylation of sulfhydryl groups in amino acids, peptides, and proteins has been shown to provide NO-like biological activities including vascular smooth muscle relaxation, neurotransmission, and inhibition of platelet aggregation.[7−9] Such species, including low molecular weight RSNOs (e.g., *S*-nitrosocysteine (CysNO) and *S*-nitrosoglutathione (GSNO)) as well as *S*-nitrosoproteins, have all been found in mammalian systems, especially in blood plasma.[9−12] In addition, it has been suggested that *S*-nitrosoalbumin (AlbSNO) is the predominant reservoir for NO in mammalian blood.[10] RSNOs are also reportedly involved in the trans-S-nitrosylation of proteins by transferring the NO^+ moiety,[13,14] a process that may regulate the activity of enzymes and receptors by a reversible post-translational modification.[5,15,16]

Most of the biological effects of RSNO are mediated by the homolytic cleavage of the S−N bond leading to the release of

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(1) Wink, D. A.; Nims, R. W.; Darbyshire, J. F.; Christodoulou, D.; Hanbauer, I.; Cox, G. W.; Laval, F.; Laval, J.; Cook, J. A.; Krishna, M. C.; DeGraff, W. G.; Mitchell, J. B. *Chem. Res. Toxicol.* **1994**, *7*, 519−525.

(2) Ignarro, L. J.; Buga, G. M.; Wood, K. S.; Byrns, R. E.; Chaudhuri, G. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 9265−9269.

(3) Palmer, R. M. J. A., D. S.; Moncada, S. *Nature* **1988**, *333*, 664−666.

(4) Sprague, R. S.; Ellsworth, M. L.; Stephenson, A. H.; Lonigro, A. J. *Am. J. Physiol.* **1996**, *271*, H2717−H2722.

(5) Stamler, J. S.; Simon, D. I.; Osborne, J. A.; Mullins, M. E.; Jaraki, O.; Michel, T.; Singel, D. J.; Loscalzo, J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 444−448.

(6) Myers, P. R.; Minor, R. L.; Guerra, R.; Bates, J. N.; Harrison, D. G. *Nature* **1990**, *345*, 161−163.

(7) Keaney, J. F. J.; Simon, D. I.; Stamler, J. S.; Jaraki, O.; Scharfstein, J.; Vita, J. A.; Loscalzo, J. *J Clin. Invest.* **1993**, *91*, 1582−1589.

(8) Mathews, W.; Kerr, S. *J. Pharmacol. Exp. Ther.* **1993**, *267*, 1529−1537.

(9) Do, K. Q.; Benz, B.; Grima, G.; Gutteckamsler, U.; Kluge, I.; Salt, T. E. *Neurochem. Int.* **1996**, *29*, 213−224.

(10) Stamler, J. S.; Jaraki, O.; Osborne, J. A.; Simon, D. I.; Keaney, J.; Vita, J.; Singel, D. J.; Valeri, C. R.; Loscalzo, J. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7674−7677.

(11) Scharfstein, J. S.; Keaney, J. F. J.; Slivka, A.; Welch, G. N.; Vita, J. A.; Stamler, J. S.; Loscalzo, J. *J. Clin. Invest.* **1994**, *94*, 1432−1439.

(12) Gaston, B.; Reilly, J.; Drazen, J. M.; Fackler, J.; Ramdev, P.; Arnelle, D.; Mullins, M. E.; Sugarbaker, D. J.; Chee, C.; Singel, D. J.; Loscalzo, J.; Stamler, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 10957−10961.

(13) Simon, D. I.; Mullins, M. E.; Jia, L.; Gaston, B.; Singel, D. J.; Stamler, J. S. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4736−4741.

(14) Stamler, J. S.; Singel, D. J.; Loscalzo, J. *Science* **1992**, *258*, 1898−1902.

(15) Stamler, J. S.; Jia, L.; Eu, J. P.; McMahon, T. J.; Demchenko, I. T.; Bonaventura, J.; Gernert, K.; Piantadosi, C. A. *Science* **1997**, *276*, 2034−2037.

(16) Xu, L.; Eu, J. P.; Meissner, G.; Stamler, J. S. *Science* **1998**, *279*, 234−237.

10.1021/ac048192u CCC: $30.25
Published on Web 04/19/2005

NO. The decomposition of RSNOs is dependent on various factors, such as intensity of light, solution pH, metal ion concentration, and the presence of various reductants (e.g., ascorbate and thiols).[17−22] In aqueous solution, exposure to visible light or certain metal ions leads to the rapid decomposition of RSNOs. Williams et al. reported that RSNOs can undergo a Cu(II)-catalyzed reaction involving reduction of Cu(II) to Cu(I) by thiolate impurities or added reducing agents and subsequent reduction of the RSNO to RS^- and NO by the Cu(I) species.[17,23−25] Thiolate formed from this reaction can reduce the Cu(II) species also formed, regenerating Cu(I), yielding a catalytic cycle. Even a trace level of Cu(II) ions can greatly facilitate this reaction, including copper ions bound to peptides or proteins.[26] The reduction of Cu(II) to Cu(I) is a crucial first step for such decomposition, and the subsequent formation of a Cu(I) intermediate complex (five- or six-membered ring structures) with the RSNO species is also postulated to be involved in the decomposition mechanism.[23] The reaction of RSNOs via this pathway can be completely suppressed by adding a multidentate Cu(II) chelator such as EDTA to the solution.[17,23]

To detect RSNOs in biological systems, a variety of methods have been suggested. Most techniques depend on redox or substitution reactions of RSNOs to produce detectable products including other forms of nitrogen oxides (NO, NO_2^-, NO_3^-). These species can be monitored via either spectrophotometric or fluorometric assays, as well as chemiluminescence or electrochemical methods.[19,27,28] The rate and extent of these reactions are dependent on reaction conditions (e.g., pH), as well as the specific nature of the R group of the RSNO species.[19,25,29] In addition, the presence of interfering compounds often found in biological fluids can influence the accuracy in assaying the given RSNO species.[19,28,30] Therefore, separation steps, such as electrophoresis and HPLC, are frequently combined to avoid possible influence/interference from nitrite and other species (disulfides, thiols, or antioxidants) or proteins during analytical detection.[27] While current analytical methods provide good sensitivity, the intrinsic lability of RSNOs makes sample pretreatment steps difficult to implement.[27,31,32] Hence, the development of simple and reliable RSNO detection methods is still of great interest.

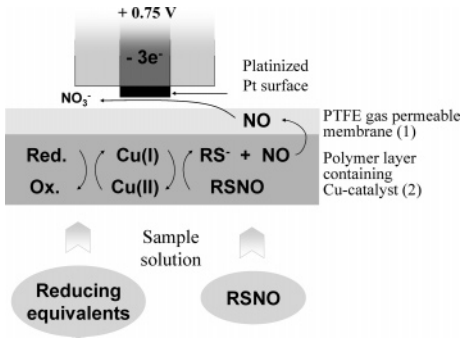

Figure 1. Amperometric detection scheme based on catalytic copper species within the outer polymeric layer of proposed RSNO sensor.

Recent research in this laboratory has demonstrated the possibility of preparing more thromboresistive coatings by incorporating Cu(II) sites within polymeric films.[33,34] When in contact with fresh blood, endogenous RSNO species in the blood can be catalytically converted to NO via the Cu(II)/Cu(I) redox chemistry described above, creating a locally elevated level of NO at the polymer/blood interface. The improved biocompatibility of the films is due to the potent inhibitory effect of NO on platelet activation and adhesion.[35] However, to use such polymers to enhance the blood compatibility of biomedical devices in clinical practice, it will be necessary to prove whether blood levels of endogenous reactive RSNO species do not vary greatly from subject to subject.

Herein we propose the concept of a direct and real-time RSNO sensor by combining the catalytic NO-generating capability of polymer films containing copper species with a highly sensitive amperometric NO sensor described previously.[36,37] While electrochemical RSNO detection using commercially available amperometric NO probes has been suggested,[19] RSNO measurements in this earlier work were accomplished by triggering solution-phase RSNO decomposition by adding Cu(II) ions and thiols as reagents to the bulk sample solution.[19] In the present work, various catalytic polymeric films containing copper species are used to modify the surface of the amperometric NO detector, thereby creating true sensors that respond reversibly to the levels of RSNO species in test samples. As illustrated schematically in Figure 1, the Cu(II)-mediated decomposition of RSNOs is achieved within a thin hydrophilic polymer layer (polyurethane (PU)) at the distal tip of the improved 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 planar platinized platinum anode, where oxidation of NO takes place. Three different catalytic layers are examined to prepare RSNO sensors including a lipophilic Cu(II)−ligand complex, a relatively insoluble copper−phosphate salt, and small (3-μm)

(17) Askew, S. C.; Barnett, D. J.; McAninly, J.; Williams, D. L. H. *J. Chem. Soc., Perkin Trans. 2* **1995**, 741−745.
(18) Oae, S.; Fukushima, D.; Kim, Y. H. *J. Chem. Soc., Chem. Commun.* **1977**, 407b−408.
(19) Pfeiffer, S.; Schrammel, A.; Schmidt, K.; Mayer, B. *Anal. Biochem.* **1998**, *258*, 68−73.
(20) Singh, S. P.; Wishnok, J. S.; Keshive, M.; Deen, W. M.; Tannenbaum, S. R. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 14434−14439.
(21) Scorza, G.; Pietraforte, D.; Minetti, M. *Free Radical Biol. Med.* **1996**, *22*, 633−642.
(22) Shishido, S. M.; Oliveira, M. G. d. *Photochem. Photobiol.* **2000**, *71*, 273−280.
(23) Williams, D. L. H. *Acc. Chem. Res.* **1999**, *32*, 869−876.
(24) Holmes, A. J.; Williams, D. L. H. *J. Chem. Soc., Chem. Commun.* **1998**, 1711−1712.
(25) Dicks, A. P.; Swift, H. R.; Williams, D. L. H.; Butler, A. R.; Al−Sa'doni, H. H.; Cox, B. G. *J. Chem. Soc., Perkin Trans. 2* **1996**, 481−487.
(26) Dicks, A. P.; Williams, D. L. H. *Chem. Biol.* **1996**, *3*, 655−659.
(27) Stamler, J. S.; Feelisch, M. In *Methods in Nitric Oxide Research*; Feelisch, M., Stamler, J. S., Eds.; John Wiley: Chichester, U.K., 1996; pp 521−539.
(28) Cook, J. A.; Kim, S. Y.; Teague, D.; Krishna, M. C.; Pacelli, R.; Mitchell, J. B.; Vodovotz, Y.; Nims, R. W.; Christodoulou, D.; Miles, A. M. *Anal. Biochem.* **1996**, *238*, 150−158.
(29) Wood, P. D.; Mutus, B.; Redmond, R. W. *Photochem. Photobiol.* **1996**, *64*, 518−524.
(30) Tsikas, D. *Nitric Oxide* **2003**, *9*, 53−55.
(31) Alpert, C.; Ramdev, N.; George, D.; Loscalzo, J. *Anal. Biochem.* **1997**, *245*, 1−7.
(32) Tashimo, O.; Ishibashi, T.; Yoshida, J.; Tsuchida, H.; Nishio, M. *Nitric Oxide* **2003**, *9*, 148−152.
(33) Oh, B. K.; Meyerhoff, M. E. *J. Am. Chem. Soc.* **2003**, *125*, 9552−9553.
(34) Oh, B. K.; Meyerhoff, M. E. *Biomaterials* **2004**, *25*, 283−293.
(35) Radomski, M.; Palmer, R.; Moncada, S. *Br. J. Pharmacol.* **1987**, *92*, 639−646.
(36) Lee, Y.; Oh, B. K.; Meyerhoff, M. E. *Anal. Chem.* **2004**, *76*, 536−544.
(37) Lee, Y.; Yang, J.; Rudich, S. M.; Schreiner, R. J.; Meyerhoff, M. E. *Anal. Chem.* **2004**, *76*, 545−551.

metallic Cu^0 particles, each incorporated into a thin PU layer. It will be shown that sensors prepared with all three types of films respond to various RSNO species at submicromolar levels and that those prepared with copper phosphate and metallic copper particles maintain amperometric response for at least 10 days. Further, it will be demonstrated that the resulting devices can be used to detect the presence and relative levels of NO-generating RSNO species within fresh blood samples.

EXPERIMENTAL SECTION

Materials. Microporous poly(tetrafluoroethylene) (PTFE) membranes (Tetratex, pore size 0.07 μm, thickness ~18 μm) and PTFE membrane filters (SM11803, pore size 1.2 μm, 16.6 cm^2 area) were obtained from Donalson Co., Inc. (Minneapolis, MN) and Sartorius (Goettingen, Germany), respectively. Silicone rubber (SR) (RTV3140) was from Dow Corning Corp. (Midland, MI), hydrophilic polyurethanes (HPU) (Tecophilic SP60D-20 or SP90A-100; 20 and 100 wt % water-uptake capability, respectively) were from Thermedics Polymer Products (Wilmington, MA), and *o*-nitrophenyloctyl ether and poly(vinyl chloride) (PVC) were from Fluka (Buchs, Switzerland). All chemicals were of analytical grade or better and used as received from the suppliers. Various phosphate buffers including phosphate-buffered saline (PBS) were prepared as needed in the laboratory. Cu(II)−dibenzo[*e*,*k*]-2,3,8,9-tetraphenyl-1,4,7,10-tetraazacyclododeca-1,3,7,9-tetraene (Cu(II)−DTTCT) was synthesized as described in the literature.[38] Cu(II)−phosphate and Cu^0 particles (dendritic, 3-μm average particle size) were purchased from Riedel-de Haën (Seelze, Germany) and Sigma-Aldrich (St. Louis, MO), respectively. All solutions were prepared with 18 MΩ cm^{-1} deionized distilled water by using a Milli-Q filter system (Millipore Corp., Billerica, MA).

Preparation of *S*-Nitrosothiols. Crystalline *S*-nitroso-*N*-acetylpenicillamine (SNAP) was synthesized according to a reported procedure[39] using a 5 mM solution of *N*-acetylpenicillamine containing 10 μM EDTA. Solutions (5 mM each) of other *S*-nitrosothiols (CysNO, GSNO, *S*-nitroso-*N*-acetylcysteine (SNAC)) were also prepared as previously described.[40] 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).

S-Nitrosylation of the Cys-34 in bovine serum albumin (BSA) was achieved by modifying the method of Stamler.[5] BSA was first reduced using dithiothreitol before S-nitrosylation. After incubation with equimolar dithiothreitol at 37 °C for 1 h, the BSA solution in 0.1 M Tris-HCl buffer (pH 8.0) was filtered three times with 10 μM EDTA solution to remove remaining dithiothreitol using a centrifugal filter device (molecular weight cutoff 30 000, Amicon Ultra, Millipore Corp.). The reduced BSA was then reacted with the acidified $NaNO_2$ solution. After standing for 30 min, the solution was neutralized with 1 M NaOH and filtered three times with PBS (pH 7.4) solution containing 10 μM EDTA. The total BSA concentration, sulfhydryl level, and *S*-nitroso-BSA concentration were determined by the BCP dye-binding method,[41] Ellman method,[42] and chemiluminescence measurements of NO liberated by Cu(II)-catalyzed decomposition, respectively. In this study, only the actual AlbSNO concentrations are reported based on the above analysis. It was found that more than 80% of the initially reduced BSA species contained free thiol groups, and more than 60% of the total BSA was converted to the desired *S*-nitroso-BSA.

Fabrication of Amperometric NO/RSNO Sensors. 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.[36] To create control NO sensors for blood measurements that respond to NO similarly to the RSNO sensors, a thin HPU (SP90A-100) film (~4-μm thickness) was attached over the PTFE membrane using an O-ring to hold the dual layer of membranes in place.

To fabricate the amperometric RSNO sensors, the additional catalytic polymer layer was prepared separately (see below) and physically held over the PTFE membrane of the NO sensor (see Figure 1a in ref 36 for basic sensor configuration) with an O-ring. Depending on the specific type of Cu catalyst used, i.e., Cu(II)−DTTCT, Cu(II)−phosphate, and metallic Cu^0 particles, the sensors will be referred to as type I, type II and type III, respectively. All sensors were 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. Calibration curves and current recordings were obtained as described in our previous work.[36]

Immobilization of Copper Catalysts in PU Polymer Matrix. A catalytic polymer layer containing the Cu(II)−DTTCT complex was obtained by wetting a PTFE membrane filter (pore size, 1.2 μm) with THF solution containing Cu(II)−DTTCT and HPU (SP60D-20). The Cu(II)−DTTCT (4 mg, 5.7 μmol) was dissolved in 2 mL of warm THF and then mixed with 40 mg of HPU. The clear cocktail solution (0.5 mL) was poured and evenly distributed over the entire surface of a PTFE membrane filter on a glass slide and allowed to dry slowly. A piece of this membrane was cut and mounted on the NO sensor to create the type I RSNO sensor.

To immobilize inorganic copper ion sources, 20 mg of Cu(II)−phosphate (for type II sensors) or Cu^0 particles (type III sensors) was mixed with 2 mL of THF solution containing HPU (SP90A-100) (80 mg). The solution was then sonicated to obtain a fine dispersion. An aliquot of this cocktail slurry (0.5 mL) was spread in a glass casting plate (area, ~25 cm^2) and dried using a mechanical shaker to prevent particle aggregation. A small section of this film was then attached to the NO sensor using an appropriate O-ring. A second pure HPU film (~4-μm thickness) was also affixed to the sensor as an outermost layer to block direct contact of copper sources with the bulk sample solution.

Detection of RSNOs with Polymer Film Modified Amperometric NO Sensors. All preliminary RSNO measurements

(38) Chandra, S.; Sharma, H. K. *Ind. J. Chem.* **1998**, *37A*, 1074−1078.
(39) Field, L.; Dilts, R. V.; Ravichandran, R.; Lenhert, P. G.; Carnahan, G. E. *J. Chem. Soc., Chem. Commun.* **1978**, 249−250.
(40) Stamler, J. S.; Loscalzo, J. *Anal. Chem.* **1992**, *64*, 779−785.
(41) Pinnell, A.; Northam, B. *Clin. Chem.* **1978**, *24*, 80−86.
(42) Riddles, P. W.; Blakeley, R. L.; Zerner, B. *Methods Enzymol.* **1983**, *91*, 49−60.

were performed in PBS buffer (pH 7.4) containing 10 μM EDTA and 10 μM ascorbate (added as reducing agent) and in a 100-mL amber reaction vessel at room temperature. Each RSNO solution was prepared fresh and used within 2 h. Current signals were monitored using a microchemical sensor analyzer (Diamond General, Ann Arbor, MI) in stirred solutions with polarization of the platinized platinum electrode of the sensor at +0.75V (vs Ag/AgCl). In some experiments, background NO levels in the bulk test solutions were monitored simultaneously using a plain unmodified NO sensor placed into the same sample solution as with the modified sensor. The measured current levels of RSNO sensors at specific times were converted to equivalent NO concentrations based on prior NO calibration data of the RSNO sensor.

For stability studies, the sensors' amperometric responses toward both NO (calibration) and SNAP were recorded periodically and compared to differentiate whether decreased response occurs due to deterioration of NO response or from loss of catalytic activity in the HPU polymeric layer.

Estimation of Copper Ion Leaching from Catalytic Polymeric Films. To correlate the change in sensor response with the dissolution of copper-containing catalytic sites, the copper content within the polymeric films and soaking solutions were measured after given time periods by using standard atomic absorption spectroscopy or ICPMS methods. The polymeric matrixes were dissolved with concentrated sulfuric acid and filtered before analysis.

Detection of RSNOs in Blood. For direct detection of endogenous RSNO species in blood, fresh sheep blood was obtained from Extracorporeal Membrane Oxygenation (ECMO) laboratory at the University of Michigan Medical School. Heparin was administered in the venous line of adult sheep to elevate the activated clotting time (measured in seconds) from an average range of 140−160 s (pre-heparin) to over 250 s (post-heparin), to decrease the propensity of blood clotting after obtaining the samples. Blood (~100 mL) was always drawn from an arterial line placed in the left carotid artery and then immediately isolated from ambient light by wrapping the syringe with aluminum foil. The amperometric measurements were made as quickly thereafter as possible (always < 2 h). The temperature of the sampled blood was maintained at 37 °C both before and during RSNO detection.

Two electrodes, a "control" NO sensor (no copper catalytic layer but with a thin HPU film over the PTFE membrane of the NO sensor) and a type II RSNO sensor were used in most of these blood experiments. Each sensor was first calibrated with respect to their inherent response to NO in PBS buffer. Then, the amperometric signals of both sensors were stabilized at 37 °C with the sensors placed into the same N_2-saturated 75-mL PBS (pH 7.4) solution. Finally, 25 mL of the fresh whole blood sample was added to the PBS solution to yield a 25% (v/v) dilution under a N_2 atmosphere, and the amperometric responses of each sensor to the sample were monitored.

RESULTS AND DISCUSSION

Catalytic Polymer Matrix. The proposed RSNO sensor design requires the use of immobilized copper species within a thin polymeric film in direct contact with the surface of the PTFE membrane of the NO sensor. Initial efforts focused on the use of the Cu(II)−DTTCT complex (type I sensor), which was shown previously to function catalytically in generating NO from RSNO species when incorporated into plasticized PVC films.[33] Earlier studies suggested that the ligated copper ions in this particular complex are able to carry out the same catalytic redox chemistry as free Cu(II) ions.[33] At the same time, any copper species that can provide a reservoir of low concentrations of free Cu(II) or Cu(I) ions within the polymeric film at the surface of the NO sensor could also be employed to convert RSNO species to NO. Toward this end, a relatively insoluble Cu(II) salt, i.e., cupric phosphate (K_{sp} = ~10^{-37})[43] (type II sensor) and metallic Cu^0 particles (type III sensor) were also examined as candidate materials within the outer polymer film of the RSNO sensor. In the case of Cu^0 particles, continuous slow corrosion of the particles in the presence of a phosphate buffered saline (typical sample test phase) could generate the necessary copper ions required to yield amperometric RSNO response.[44] In general, these different species were tested by adding them to an appropriate polymer coating at 10−20 wt % levels.

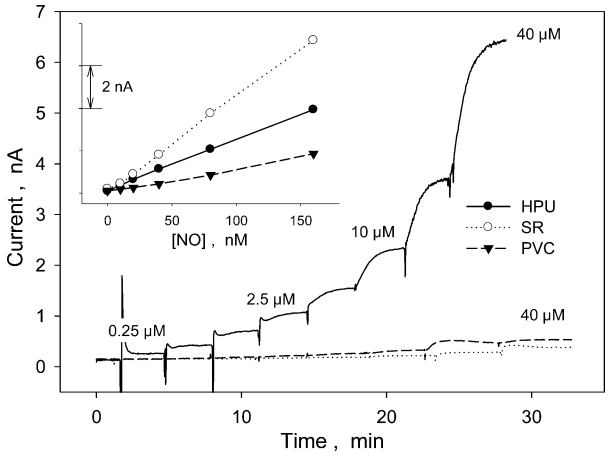

Figure 2. Influence of polymer film matrix containing Cu(II)−DTTCT in type I sensors on direct amperometric response toward SNAP and NO (inset). Sample solutions contain 10 μM EDTA and 10 μM ascorbate in pH 7.4 PBS solution.

The selection of the appropriate polymer matrix that contains the various copper species was found to be critical to achieve optimal RSNO sensitivity. Indeed, the permeability of this polymer matrix to the RSNO species as well as any reducing agents used to maintain high levels of Cu(I) species in the layer is a key element that dictates the degree of sensor response to RSNO species. Any NO generated in this layer due to the catalytic reaction must be able to diffuse quickly up to the underlying gas-permeable PTFE membrane of the NO sensor. In preliminary experiments, several possible polymer films were examined to confine the copper catalysts. Figure 2 illustrates how the specific nature of the film layer can dramatically affect the observed amperometric RSNO response. In this example, type I sensors were prepared using either PVC, silicone rubber, or HPU as the polymer matrix containing 10 wt % Cu(II)−DTTCT and all employed a thin PTFE membrane treated with each catalyst-doped polymer matrix as the outer catalytic film. As shown, a thin silicone

(43) Sillen, L. G.; Martell, A. E. *Stability Constants of Metal-ion Complexes*; The Chemical Society: London, 1964.
(44) Drogowska, M.; Brossard, L.; Menard, H. *J. Electrochem. Soc.* **1992**, *139*, 2787−2793.

rubber outer coating actually yields an RSNO sensor with the most sensitive response to solution-phase NO (see inset), but very little amperometric response toward SNAP, a relatively stable low molecular weight RSNO test species. A plasticized PVC coating containing the Cu(II)−DTTCT species yields a sensor with the lowest sensitivity to NO and also very poor response to SNAP. In contrast, a sensor made with an outer film of HPU provides modest direct amperometric NO response but a substantially enhanced sensitivity toward SNAP compared to either of the other two matrixes. Hydrophilic polyurethanes with poly(ethylene oxide) soft segments are well known to swell in water and possess good permeability toward small solute molecules.[45,46] Indeed, the polymer (HPU, Tecophilic SP60D-20) employed in this experiment has a high water uptake of 20 wt %, compared to ∼0.1 and <0.6% for SR and plasticized PVC, respectively.[47,48] It is believed that the partially swollen HPU allows the effective partitioning of the SNAP and ascorbate into the layer, thereby generating more NO, and closer to the PTFE membrane of the amperometric gas sensor.

Measurement Conditions. The following rate equation applies for most copper ion mediated RSNO decomposition reactions:[17]

$$\text{rate} = k[\text{Cu}^{2+}][\text{RSNO}] \qquad (1)$$

However, at low RSNO concentrations, zero-order kinetics is often observed due to the absence of thiolate (RS^-) reducing species within the given RSNO preparation (as impurity). Since reducing equivalents are required initially to generate Cu(I) species, the reaction rate is limited by the lack of reducing equivalents.[23,25] Moreover, the oxidation of Cu(I) to Cu(II) by ambient oxygen can compete with Cu(I)-mediated RSNO decomposition.[25] Thus, for the proposed RSNO sensor, a low level of exogenous reducing agent should be added to the sample phase to maximize the formation of reactant Cu(I) species within the catalytic polymer layer. While various thiol compounds can serve in this capacity, they can also activate NO release from RSNO via transnitrosylation reactions.[23] To avoid this complication, ascorbate was chosen as the primary reducing agent for the studies described herein. The level of ascorbate added to the test solution must be relatively low, since higher concentrations of ascorbate can promote NO release from RSNO species,[24,49] presumably by a direct redox reaction. Therefore, to maintain enhanced levels of Cu(I) species within the catalytic HPU layer of sensors, most experiments employed fresh 10 μM ascorbate (final concentration) in the sample phase. It was shown in separate experiments using chemiluminescence NO detection that this level of ascorbate does not generate NO from RSNO species.

The proposed RSNO sensor can also detect any solution-phase NO present. Therefore, the sample phase must not contain trace levels of Cu(II) ions 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 copper ions.[17,23] It was found that, in the presence of 10 μM EDTA, the amperometric NO sensors used in this work did not detect any NO in the solution phase while a corresponding RSNO sensor placed in the same solution responded to the added RSNO species (see Figure 1s in Supporting Information). However, at much higher levels of EDTA in the sample solution, a reduced response to the RSNO species is observed owing to greater chelation of copper ions within the catalytic layer (see below).

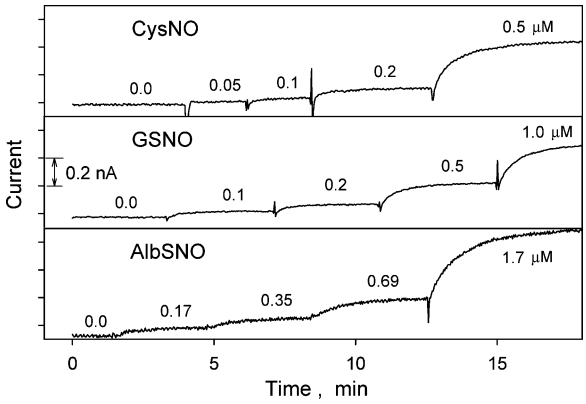

Figure 3. Typical real-time current recordings for type III sensor in response to low levels of three endogenous RSNO species. Sample solutions contain 10 μM EDTA and 10 μM ascorbate as a reducing agent, along with varying RSNO concentrations at room temperature.

Amperometric Responses to RSNO Species. To investigate the relationship between the recorded current and RSNO sample concentration, all sensors' responses at +0.75 V (vs Ag/AgCl) were recorded as increasing concentrations of the various RSNO species were added to PBS solution containing 10 μM ascorbate and 10 μM EDTA. Figure 3 illustrates the typical amperometric responses of a type III sensor at the low concentration range of CysNO, GSNO, and AlbSNO species, while panels a−c of Figure 4 illustrate representative complete calibration curve data for type I, type II, and type III sensors toward these same three species, as well as for SNAC, SNAP, and nitrite. As shown in Figure 3, typical response times required to achieve 95% of the steady-state current following changes in RSNO levels are less than 5 min. As expected, response and recovery (see below) times are dependent on the physical dimensions (especially thicknesses) of the various membranes/films and electrolyte layers that comprise the final sensor configuration.[50] All sensors exhibited fully reversible and reproducible amperometric responses (see Figure 5 for example of type II and III sensors' reversible response toward SNAP) when changing from low to high and then back down to lower concentrations of a given RSNO species.

As illustrated in Figure 4a−c, type III sensors were found to be much more sensitive than type I sensors in terms of the current response versus RSNO concentration change, even though type I sensors are more sensitive ((∼12 pA/nM) than type III (∼6 pA/nM) in their direct response toward NO (see two different *y*-axis

(45) Lamba, N. M. K.; Woodhouse, K. A.; Cooper, S. L.; Lelah, M. D. *Polyurethanes in biomedical applications*; CRC Press: Boca Raton, 1998.
(46) Lyman, D. J.; Loo, B. H. *J. Biomed. Mater. Res.* **1967**, *1*, 17−26.
(47) DeLassus, P. T.; Whiteman, N. F. In *Polymer Handbook,* 4 ed.; Brandrup, J., Immergut, E. H., Grulke, E. A., Eds.; Wiley-Interscience: Hoboken, NJ, 1999; Vol. 1.
(48) Chan, A. D. C.; Li, X.; Harrison, D. J. *Anal. Chem.* **1992**, *64*, 2512−2517.
(49) Holmes, A. J.; Williams, D. L. H. *J. Chem. Soc., Perkin Trans. 2* **2000**, 1639−1644.
(50) Hitchman, M. L. In *Measurement of Dissolved Oxygen*; Wiley: New York, 1978; pp 103−111.

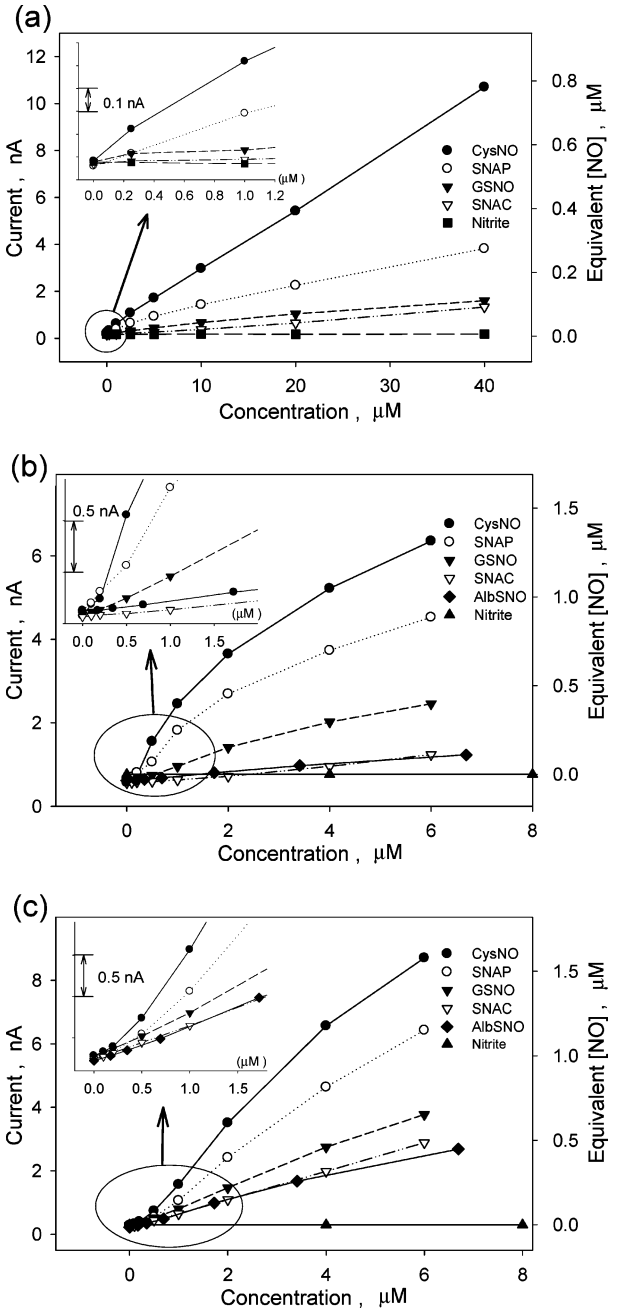

Figure 4. Typical calibration curves for three types of RSNO sensors toward various RSNO species and nitrite. (a) Cu(II)−DTTCT in HPU catalytic layer (type I); (b) Cu(II)−phosphate in HPU catalytic layer (type II); and (c) metallic Cu^0 particles in HPU catalytic layer (type III). PBS (pH 7.4) solutions containing 10 μM EDTA and 10 μM ascorbate were spiked with increasing levels of RSNOs to obtain the calibration plots. Insets in each panel show expanded view of low-concentration calibration data for all three sensors.

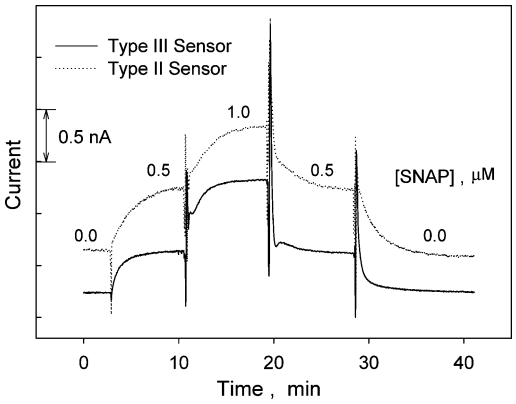

Figure 5. Dynamic recordings illustrating real-time reversibility of type II and type III RSNO sensors in amperometric response to varying concentrations of SNAP.

scales in figure). This observation suggests that the difference in sensor-to-sensor sensitivity toward a given RSNO species originates from the different NO-generating capability of the catalytic layer. Initially it was assumed that the actual species responsible for RSNO decomposition was the Cu(II)−DTTCT for type I and low levels of free copper ions within the HPU matrix for type II or III sensors. The lipophilic Cu(II)−DTTCT in the type I sensors was shown previously to exhibit catalytic function within a hydrophobic polymer matrix.[33,34] However, the Cu(II)−DTTCT may be more sequestered in the hydrophobic hard segment of the HPU matrix than free copper ions, and this may limit its catalytic function in the type I sensor configuration. On the other hand, in the presence of oxygen and in pH 7.4 PBS, Cu^0 metal corrodes slowly in a complex manner,[51] to yield passive layer of Cu_2O and Cu(II) compounds (CuO, $Cu(OH)_2$, or $Cu_3(PO_4)_2$),[44] which can generate low levels of Cu(I)/Cu(II) due to their solubility or further dissolution.[52] Thus, during the measurements, a small but continuous flux of copper ions from immobilized inorganic catalyst (either $Cu_3(PO_4)_2$ or Cu°) within the polymer film is expected to form a dynamic catalytic layer within/or at the surface of the HPU film/solution interface of the type II and III sensors. Indeed, the corrosion of metallic Cu^0 to Cu(II) is known to occur via Cu(I) formation, so both Cu(I) and Cu(II) can coexist within the polymer film of type III sensor.[44,52] By separate ICP-mass spectrometry analysis, the saturation level of the 10 mM phosphate buffer solution in contact with the inorganic copper phosphate sites used in the type II sensor was estimated to be below 2 μM at 37 °C (below 1 μM at room temperature). Hence, copper ions from both copper phosphate (type II sensor) and metallic copper (type III sensor) are likely to exist at certain levels in the HPU matrix and decompose the water-soluble RSNOs more effectively than the lipophilic Cu(II)−DTTCT species used in the type I sensor.

As shown in Figure 4a−c, all RSNO sensors yield concentration-dependent amperometric responses toward the RSNO species tested, but the relative response toward the various RSNO test species is quite different. The order of amperometric sensitivity is CysNO > SNAP > GSNO ≥ SNAC ∼ AlbSNO. This response pattern is essentially the same regardless of the nature of the copper catalytic layer employed. There are two possible factors that contribute to this response pattern. First, the diffusivity of the RSNOs within the HPU film should be dependent on the molecular weight of a given RSNO,[46] and this can influence the overall sensor sensitivity, especially the relatively small amperometric response observed for AlbSNO, a macromolecule. Second, the inherent reactivities arising from the structural characteristics of the RSNOs also appear to be critical. N-Acetylation in SNAP, SNAC, and GSNO has been considered as a structural feature

(51) Taylor, A. H. *J. Electrochem. Soc.* **1971**, *118*, 854−859.
(52) Ives, D. J. G.; Rawson, A. E. *J. Electrochem. Soc.* **1962**, *109*, 458−462.

that hampers the RSNO decomposition by blocking the formation of a cyclic intermediate complex (five- or six-membered ring structures) between the given RSNO and Cu(I)/Cu(II).[17,23] Interestingly, the observed amperometric sensitivity sequence for the RSNO sensors is quite comparable to the reported trend for the measured second-order RSNO decomposition rate constants (see eq 1) (without additional thiols) reported previously.[17] Such measured rate constants exhibit a large disparity between CysNO ($\sim$24 500 $\pm$ 500 M^{-1} s^{-1}) and SNAC/GSNO ($\sim$0 M^{-1} s^{-1}).[17] However, in this study, since exogenous reducing agents are employed, the rate for decomposition of SNAP, SNAC, and GSNO is likely to be enhanced (compared to their reported rate constants with Cu(II) in the absence of exogenous reducing equivalents), owing to faster recycling of Cu(II) to Cu(I) species in the catalytic layer by ascorbate added to the bulk solution.

For all RSNO sensor designs tested, there is essentially no response toward inorganic nitrite in the range of 0.1−100 μM. Although a previous report[34] suggests that nitrite can be catalytically reduced to NO by Cu(II)−DTTCT or free copper ions, such a reaction only occurs at a much higher concentration range (e.g., >1 mM) of nitrite and reducing agents, i.e., ascorbate.

Factors Influencing RSNO Sensor Sensitivity. Several experimental variables can influence the sensor sensitivity due to their effect on the Cu(II)-mediated RSNO decomposition chemistry. The most important factors appear to be the concentration of reducing agent (ascorbate) and metal chelator (EDTA) in the sample phase, as well as the specific nature of the buffer salts in the sample, oxygen levels, and catalyst leaching from the HPU polymeric layer. The latter determines the lifetimes of the RSNO sensors (see below).

To verify the influence of ascorbate or EDTA levels, the concentration of both species in the sample phase were varied. As shown in Figure 6, the level of both species can have a significant effect on the amperometric response of the RSNO sensors. Increasing EDTA levels in the sample phase decreases the amperometric response toward a given concentration (2 μM) of SNAP, as shown for types II and III sensors (Figure 6a). Since free copper ions within the catalytic layer are likely responsible for the NO generation process for these two types of sensors, this effect is anticipated. Type I sensors also exhibit a similar dependence on EDTA levels (data not shown); however, at this point it is not yet clear whether this effect is due to scavenging of low levels of free copper ions or direct ligation of EDTA to the Cu(II)−DTTCT complex within the polymeric layer, and concomitant inhibition of the catalytic reaction that can be carried out by the complex.

In contrast, increasing levels of ascorbate in the sample solution enhance the reactive Cu(I) species in the catalytic layer, thereby yielding faster NO generation rates and greater amperometric sensitivity toward RSNOs. This is illustrated in Figure 6b for type II and type III sensor responses to SNAP. A much greater effect is seen for the type II sensors based on cupric phosphate as the source of catalytic ions. Indeed, as mentioned above, corrosion of Cu^0 in type III sensors already yields a significant quantity of Cu(I) species, and hence even in the absence of reducing equivalents, there is a very large amperometric response toward 2 μM SNAP.

Given ions and oxygen levels present in the test solution also influence the amperometric response of the RSNO sensors. In

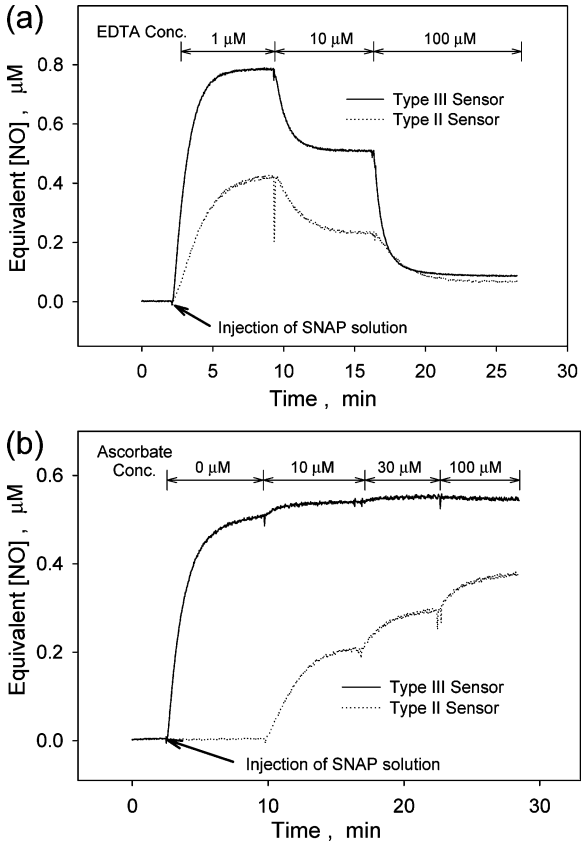

Figure 6. Influence of sample phase EDTA concentration (a) and ascorbate level (b) on the amperometric signal of type II and III sensors in response to constant SNAP at 2.0 μM. For (a), ascorbate was present at a constant level of 10 μM in PBS; for (b), EDTA was present at a constant level of 10 μM in PBS.

particular, the presence of chloride ions in the PBS buffer is very beneficial with respect to the responses observed with type III sensors (see Figure 2s in Supporting Information). The addition of chloride in PBS dramatically enhances the amperometric response toward SNAP compared to phosphate buffers that do not contain chloride ions. This can be explained by the fact that the dissolution kinetics of Cu^0 change substantially in saline media since preformed surface Cu_2O dissolves more readily under such conditions.[44,53] Even though phosphate ions are known to inhibit corrosion of Cu^0 by forming a passive layer containing $Cu_3(PO_4)_2$,[44] the concentration of chloride ions present in the PBS used in this study (100 mM) is in a region where chloride can sufficiently induce the breakdown of this passive layer and promote the continuous dissolution of Cu^0 by forming $CuCl_2^-$ as the predominant cuprous species.[51,54,55] Such species can be further oxidized to cupric complexes under aerated conditions.[56] In addition, chloride ions are known to form a more stable complex with cuprous ions ($\log \beta^\circ_2 = 5.68$ for $CuCl_2^-$) than cupric ions in aqueous media.[43,57]

The sensitivity of the type III sensor to SNAP in the presence of oxygen is somewhat less than in the absence of oxygen (see

(53) Ives, D. J. G.; Rawson, A. E. *J. Electrochem. Soc.* **1962**, *109*, 462−466.
(54) Puigdomenech, I.; Taxen, C. *Thermodynamic Data for Copper: Implication for the Corrosion of Copper under Repository Conditions*; Swedish Nuclear Fuel and Waste Management Co.: Stockholm, 2000.
(55) Lee, H. P.; Nobe, K. *J. Electrochem. Soc.* **1986**, *133*, 2035−2043.
(56) Hine, F.; Yamakawa, K. *Electrochim. Acta* **1970**, *15*, 769−781.

Figure 2s in Supporting Information). Two factors are likely responsible for this effect. First, the steady-state level of Cu(I) species in the catalytic layer will be oxygen dependent, since oxygen reacts quickly with free Cu(I) to generate Cu(II) species. Second, oxygen also reacts directly with NO, and hence, higher levels of RSNO generate greater surface concentrations of NO that can be partially scavenged by reaction with oxygen. All three types of sensors display a similar influence of oxygen on the measured amperometric RSNO response. Fortunately, for in vitro measurements, the level of oxygen can be readily controlled to essentially constant levels.

RSNO Sensor Lifetimes. To ascertain the practical lifetime of the RSNO sensors, their sensitivity (pA/nM) changes in response to SNAP were monitored with time. A relatively fast loss (~30% reduction after 1 d) in sensitivity toward SNAP is observed for type I sensors, but little change in response is found for type II and type III sensors (~20 and ~0% reduction after 10 d, respectively) (see Figure 3s in Supporting Information). The change in response of the type I sensors correlates with loss of copper species from the polymer film (as measured by atomic absorption; see Figure 4s). This loss of sensitivity and loss of copper species is accelerated by soaking the sensor in solutions containing EDTA and or thiols (see again Figure 4s). The influence of free thiols suggests that the presence of reducing agents can affect the binding constant for copper ions, with Cu(I) being much larger in size, and therefore bound less strongly to the DTTCT ligand. Hence, loss of copper ions from the ligand complex may well be responsible for the limited lifetime of type I sensors.

In contrast, type II and III sensors give relatively stable amperometric responses to RSNO species over an extended period of time, even when constantly soaked in PBS buffer containing 10 μM ascorbate and 10 μM EDTA (see Figure 3s b,c in Supporting Information). This is presumably due to the presence of a larger reservoir of total copper (in excess or continuously replenished with the newly generated ions from the dissolution of copper phosphate or the corrosion of metallic copper). However, even in these cases, the continuous leaching of copper ions is expected; for example, type II and III catalytic layers were found to saturate the 10 mM phosphate buffers with ~2 μM copper level at 37 °C. Therefore, the rate of loss of copper ions will be enhanced when the sensor is in frequent contact with a large volume of fresh solution. For the type III sensor, the copper content within the catalytic layer is most abundant among three types of sensors and likely supplies enough Cu(I) and Cu(II) through corrosion on the high surface area of metallic particles. This explains why type III sensors exhibit the highest sensitivity toward RSNO over a long period of time (at least 15 days; data not shown).

Direct Detection of RSNOs in Blood. To demonstrate the practical capability of the proposed RSNO sensors to detect endogenous RSNOs, both a control NO sensor and a type II RSNO sensor were employed to measure NO and RSNO levels, respectively, in diluted fresh sheep blood samples. As illustrated in Figure 7a, only the RSNO sensor exhibits a significant elevation in amperometric NO response upon the injection of sheep blood into PBS (pH 7.4) at 37 °C. The NO sensor within the same sample detects only a low-nanomolar level of NO, which matches the

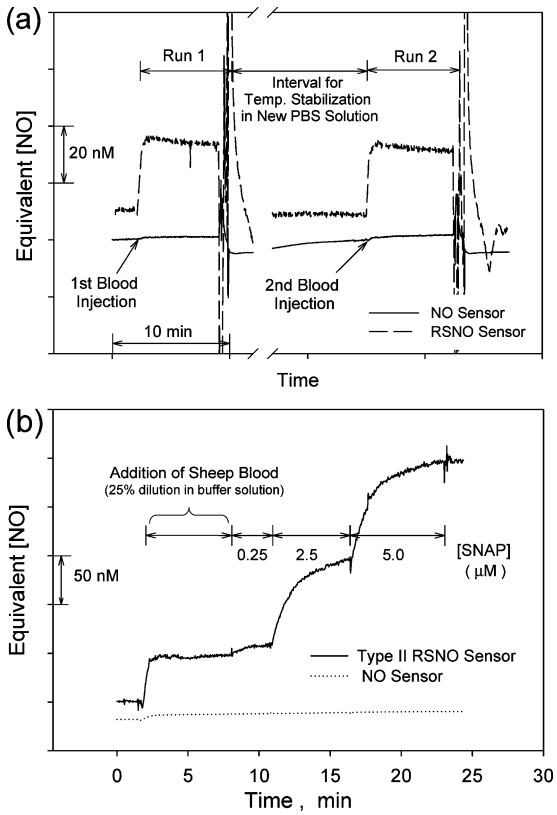

Figure 7. Direct detection of endogenous RSNOs in fresh sheep blood. (a) Two sensors (NO sensor and RSNO sensor utilizing copper phosphate as a catalyst) were initially stabilized at 37 °C in 75 mL of PBS under a N_2 environment. Upon injection of 25 mL of fresh sheep blood, only RSNO sensor displays significant amperometric current change. In (b), SNAP was added to a separate diluted blood solution at increasing concentrations to demonstrate the amperometric response to this nonendogenous RSNO in the presence of blood. EDTA and ascorbate were not added to the buffer.

reported NO concentrations in plasma from human blood.[10] The RSNO sensor, however, yields response equivalent to a change of ~25 nM in effective NO levels at the surface of the sensor (note: the RSNO sensor is precalibrated for its direct response to NO). No reducing equivalents were added to the blood samples in these experiments since fresh blood is known to possess a pool of potential reducing agents, such as protein-SH (452 μM), GSH (2.8 μM), cysteine (9.7 μM), and ascorbate (1−10 μM).[58,59] Similar amperometric responses were observed for multiple experiments with fresh sheep blood and when type III sensors were employed in such measurements (see Figure 5s in Supporting Information).

Figure 7b shows the amperometric response to added SNAP for a type II RSNO sensor and a control NO sensor placed in PBS buffer with a sample of diluted sheep blood added as well. Clearly, even in the absence of added reducing equivalents, very large response to the added SNAP is observed, above and beyond the initial response to the endogenous RSNOs already present in the sample.

CONCLUSIONS

It has been demonstrated here that direct, real-time measurements of RSNO species can be achieved by modifying a planar

(57) Wang, M.; Zhang, Y.; Muhammed, M. *Hydrometallurgy* **1997**, *45*, 53−72.

(58) Jones, D. P.; Carlson, J. L.; Mody, V. C.; Cai, J.; Lynn, M. J.; Sternberg, P. *Free Radical Biol. Med.* **2000**, *28*, 625−635.

(59) May, J. M. *Free Radical Biol. Med.* **2000**, *28*, 1421−1429.

amperometric NO gas sensor with an outer polymeric coating that contains catalytic copper species. Nitrosothiols that diffuse into the polymeric film can undergo catalytic decomposition to generate NO via complexed (e.g., DTTCT ligated) copper ions or local levels of Cu(II) or Cu(I) ions produced by cupric phosphate or metallic copper particles dispersed within the coating. All three types of modified sensors yield amperometric currents proportional to the concentrations of various RSNOs, including low molecular weight RSNOs as well as AlbSNO, and exhibit fully reversible electrochemical response. Sensitivity varies for the different *S*-nitrosothiol species, with the largest response observed for CysNO. The NO concentration changes observed for the RSNO sensor when in contact with fresh blood are a measure of the total RSNO species present that are reactive with the copper catalyst at the surface of the sensor. Indeed, all endogenous RSNO species in the blood sample can contribute to this amperometric response, including *S*-nitrosoproteins (primarily AlbSNO) and low molecular weight RSNO species (GSNO and CysNO), but with the different sensitivities as described above.

Several applications are envisioned for the RSNO sensor concept described here. As demonstrated already above, such sensors can be used to assess the relative levels of total reactive RSNO species in physiological fluids, and this measurement technique could be employed to predict the effectiveness of new copper-based polymer coatings as a means to reduce thrombosis on the surface of biomedical implants for patients that possess adequate levels of circulating RSNO species in their blood. Further, if the response and recovery times can be enhanced via use of thinner polymeric films, it should be possible to use the RSNO sensors as sensitive flow-through detectors in HPLC or other separation methods that will be able to quantitate the individual RSNO species in physiological samples. Finally, the sensor configuration described here can also provide a simple tool to assess the stability and purity of given nitrosothiol preparations, given their propensity to decompose in the presence of light as well as trace levels of copper ions in solution phase.

ACKNOWLEDGMENT

This research was supported by the National Institutes of Health under Grant EB-00783.

SUPPORTING INFORMATION AVAILABLE

(Figure 1s) Simultaneous detection of both SNAP and NO with type II sensor and NO sensor in the same sample solution; (Figure 2s) The effects of oxygen level and buffer composition on amperometric response of type III RSNO sensor toward SNAP; (Figure 3s) Long-term monitoring of RSNO sensors' amperometric sensitivity to SNAP; (Figure 4s) Changes in the copper content in HPU films with time as a result of leaching from the film of type I sensor; (Figure 5s) Response of type III RSNO sensor when in contact with diluted fresh blood. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review December 7, 2004. Accepted March 24, 2005.

AC048192U