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S-Nitrosothiol Analysis via Photolysis and Amperometric Nitric Oxide Detection in a Microfluidic Device

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Abstract

A 530 nm light emitting diode was coupled to a microfluidic sensor to facilitate photolysis of nitrosothiols (i.e., *S*-nitrosoglutathione, *S*-nitrosocysteine, and *S*-nitrosoalbumin) and amperometric detection of the resulting nitric oxide (NO). This configuration allowed for maximum sensitivity and versatility while limiting potential interference from nitrate decomposition caused by ultraviolet light. Compared to similar measurements of total *S*-nitrosothiol content in bulk solution, use of the microfluidic platform permitted significantly enhanced analytical performance in both phosphate buffered saline and plasma (6–20× improvement in sensitivity depending on nitrosothiol type). Additionally, the ability to reduce sample volumes from milliliters to microliters provides increased clinical utility. To demonstrate its potential for biological analysis, this device was used to measure basal nitrosothiol levels in the vasculature of a healthy porcine model.

Keywords

S-nitrosothiols; nitric oxide; visible photolysis; amperometric sensor; microfluidic

Introduction

Nitric oxide (NO), a seemingly ubiquitous endogenous free radical, is intricately involved in a number of physiological processes including wound healing,^{1–4} angiogenesis,^{5–8} and the immune response.^{9–12} As such, the detection and quantification of NO and its metabolites is the subject of intense investigation, especially in biological environments.^{13–21} *S*-nitrosothiols (RSNOs), present in both low molecular weight (e.g., *S*-nitrosoglutathione and *S*-nitrosocysteine) and macromolecular (e.g., *S*-nitrosoalbumin) forms *in vivo*,^{22–29} are a class of metabolites believed to be the primary endogenous reservoirs and transporters of NO resulting from the nitrosation of thiols within blood plasma (which contains ~600 μM free thiols), cells, and tissues.³⁰ Furthermore, RSNO concentrations have been identified as important in a number of disease states, including sepsis, asthma, and tuberculosis.^{25,31–33} While some RSNOs have even been employed for clinical applications (e.g., *S*-nitroso-*N*-acetyl-penicillamine as a vasodilator),^{27,34} further understanding of the formation,

decomposition, and circulating concentrations of these species in both healthy and disease states is vital as future therapeutic uses emerge.

To date, nearly all RSNO measurement strategies are based on chemiluminescence, fluorescence, or electrochemistry.^{13,18,35–37} These methods rely on the indirect quantification of RSNOs via homolytic cleavage of the S—N bond with subsequent NO release and detection. Reductive catalytic bond cleavage can be achieved via copper ions or other reducing agents (e.g., ascorbate), but requires the addition of external reagents.^{38–46} Photolysis is an alternative means of achieving S—N bond cleavage that is easily coupled to any detection platform. In such experiments, the frequency of light has been proven to be an important parameter. For example, the use of visible over ultraviolet light allows for efficient photolysis while avoiding the possibility of overestimation due to the generation of NO from nitrate.^{47–50} Riccio et al. previously reported on the use of visible photolysis and amperometric detection for the quantification of RSNOs in both phosphate buffered saline (PBS) and blood plasma.⁵¹ To achieve adequate limits of detection, this detection scheme required deoxygenation of samples to avoid the reaction of NO with oxygen. The requirement of large sample volumes (40 mL), further limited the clinical utility of the setup.

In contrast, microfluidic devices permit the use of significantly reduced sample volumes, improving amenability to clinical analysis.^{52,53} The smaller sample volume also allows for more complete sample irradiation, thus increasing the RSNO to NO conversion efficiency of photolysis. Improvements in limits of detection and RSNO conversion to NO using a microfluidic platform have the potential for more accurate measurement of biologically relevant RSNO concentrations in physiological media (e.g., blood serum or plasma).

Herein, we report the use of a modified microfluidic platform for enhanced visible photolysis and amperometric detection of total RSNO concentration. The improved analytical performance of this device is demonstrated in both PBS and blood plasma. The device was also used to monitor the basal endogenous RSNO levels in healthy swine.

Materials and Methods

(Heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxysilane (17FTMS) was purchased from Gelest (Morrisville, PA). Methyltrimethoxysilane (MTMOS), (3-aminopropyl)triethoxysilane (APTES), reduced L-glutathione (GSH), bovine serum albumin (BSA), L-cysteine-hydrochloride (Cys), diethylene triamine pentaacetic acid (DTPA), water soluble cholesterol, and ferrous stabilized human hemoglobin A₀ were purchased from Sigma (St. Louis, MO). Nitric oxide gas (99.5% pure) was purchased from Praxair (Danbury, CT). AmiconTM Ultra-4 centrifugal filters (30 kDA cutoff) were obtained for EMD Millipore (Billerica, MA). Nitrogen and argon gases were purchased from National Welders Supply (Raleigh, NC). A Millipore Milli-Q UV Gradient A10 System (Billerica, MA) was used to purify distilled water to a final resistivity of 18.2 MΩ·cm and a total organic content of <6 ppb. A high-power mounted 530 nm light-emitting diode (LED; model M530L2) and driver were purchased from Thorlabs Inc. (Newton, NJ). Other solvents and chemicals were analytical-reagent grade and used as received.

Microfluidic device fabrication

Devices were fabricated as previously described.¹⁵ Briefly, 150 nm thick planar platinum (Pt) electrodes with a 10 nm titanium seed layer were patterned onto a glass substrate via photolithography and evaporative metal deposition. The resulting Pt electrodes were 100 μm wide. To impart selectivity to NO, a xerogel membrane was applied to each working electrode. Following the deposition of a 1002F-50 photoresist and exposure through a chrome mask, the electrode pattern was developed in SU-8 developer (Microchem Corp.; Newton, MA) and an adhesion layer of APTES (1% v/v in ethanol) was deposited via three passes with a spray coater. The substrate was then rinsed with water and dried in ambient conditions overnight. A fluoroalkoxysilane membrane solution was prepared via the acid catalyzed hydrolysis and condensation of 17FTMS and MTMOS as reported previously.^{15,21} Briefly, 600 μL absolute ethanol, 120 μL MTMOS, 30 μL 17FTMS, 160 μL distilled water, and 10 μL 0.5 M HCl were added sequentially to a 1.5 mL microcentrifuge tube with vigorous mixing between the addition of each component. This solution was then vortexed for 1 h. The sol solution (30 μL) was spread-cast across the working electrodes for 1 min using a pipette tip to ensure even coating (~ 150 nm thickness). The xerogel-coated substrate was then dried overnight under ambient conditions to facilitate adequate curing. The 1002F-50 photoresist was removed by soaking the substrate for 1 h in distilled water.

Reference electrodes were fabricated on separate glass microscope slides by sputtering a 10 nm Ti adhesion layer followed by a ~ 1 μm Ag layer in the magnetron sputtering system. To form channel walls, two parallel strips of 6.3 mm wide double-sided Kapton[®] polyimide tape (90 μm thick) were applied ~ 1 mm apart and perpendicular to the Pt electrodes on the working electrode substrate. The reference electrode slide was then clamped to the working electrode substrate bonded by heating at 100 $^{\circ}\text{C}$ for 5 min. After the ends of the channel were sealed, 8 mm diameter inlet/outlet reservoirs were affixed to the device. Prior to using the device, the Ag electrode was chemically oxidized by reaction in 50 mM ferric chloride for 10 s to create a pseudo-reference/counter electrode. Following this process, the device channel was rinsed with distilled water.

Preparation of S-nitrosothiols

Low molecular weight RSNOs were prepared using a previously reported procedure.^{51,54} Nitrosation of thiols was achieved by mixing 5 mM GSH or Cys dissolved in 120 mM sulfuric acid with an equal volume of 5 mM sodium nitrite in 20 μM EDTA. S-nitrosoalbumin (AlbSNO) was prepared by mixing a solution of BSA (200 mg mL^{-1} in water) with 1.5 mM sodium nitrite in 0.5 M hydrochloric acid in the dark for 30 min.²⁴ Final concentrations of the RSNO solutions (diluted 1:10 in PBS) were determined via their UV absorption maxima at 335 nm ($\epsilon = 503, 586, \text{ and } 3869 \text{ M}^{-1} \text{ cm}^{-1}$ for S-nitrosocysteine (CysNO), S-nitrosogluthathione (GSNO), and AlbSNO, respectively).⁵⁵ Nitrosothiol solutions were shielded from light and stored at 4 $^{\circ}\text{C}$ prior to use to prevent premature degradation.

Electrochemical analysis of S-nitrosothiols

The working and reference/counter electrodes of the microfluidic device were connected to a CH Instruments 1030A 8-channel potentiostat (Austin, TX). Prior to sample analysis, the

device was polarized at +800 mV vs. the Ag/AgCl pseudo-reference/counter electrode for at least 1 h in PBS with 500 μM DTPA (required to chelate free copper ion and avoid undesirable RSNO degradation). The LED was placed 100 mm above the device and focused using an adjustable biconvex lens to tune the focus and resulting spot size of the light upon the channel. An LED driver was used to vary light intensity. To calibrate the device, a saturated NO standard solution (prepared by purging deaerated PBS with NO gas for ~10 min to yield a 1.9 mM solution of NO) was diluted with PBS and introduced into the inlet reservoir. Nitrosothiol samples were introduced into the device in an identical manner. The light intensity of the LED at the device channel was measured at 530 nm using a Newport Model 840-C hand-held optical power meter (Irvine, CA). Plasma was prepared by collecting fresh blood into 3 mL EDTA-coated Vacutainer[®] tubes (Becton, Dickinson and Company; Franklin Lakes, NJ) and centrifugation of this sample at 4 °C (1300 $\times g$ for 10 min). Following introduction of plasma into the device, aliquots of each RSNO were added to facilitate calibration. To determine the ability to detect RSNOs added to whole blood, a similar calibration was performed where RSNOs were added to blood prior to centrifugation, with subsequent analysis of the resulting plasma.

Determination of the effect of cholesterol and hemoglobin on S-nitrosothiol measurement

Ferrous oxyhemoglobin was reconstituted in PBS and diluted to 1, 5, and 10 μM for immediate use. A calibration was performed in each hemoglobin sample using stock GSNO (up to 20 μM final concentration) to determine sensitivity changes relative to PBS alone. To determine the feasibility of Hb removal via centrifugal filtration, 5 μM Hb and 35 μM GSNO were added to a PBS sample (2 mL). A small volume of this sample (300 μL) was immediately analyzed using the microfluidic device. The remaining sample was placed into a centrifugal filter, centrifuged at 4 °C (4500 $\times g$ for 15 min), and then immediately analyzed. Cholesterol samples were prepared by adding water-soluble cholesterol (1.5, 2.5, and 3.0 mg mL⁻¹) to plasma samples. An aliquot of GSNO stock solution (35 μM final concentration) was added to each cholesterol solution and analyzed immediately using the microfluidic device.

Determination of basal S-nitrosothiol levels in blood

Arterial blood was collected from Yucatan[™] miniature swine (n=4 animals) into 3 mL EDTA-coated Vacutainer[®] tubes. The blood was immediately centrifuged at 4 °C (1300 $\times g$ for 10 min) to isolate blood plasma. This sample was subsequently run through the microfluidic device. Following sample introduction, the LED was turned on to facilitate RSNO decomposition to NO. Samples were also analyzed following centrifugal filtration using a 30 kDa cutoff membrane to remove AlbsNO and free hemoglobin. Following the collection of plasma, ~2 mL was introduced into the filter apparatus and centrifuged at 4 °C (4500 $\times g$ for 15 min).

Results and Discussion

The microfluidic NO sensor utilized herein has been previously characterized with respect to blood NO analysis.¹⁵ In other works, Riccio et al. established the effectiveness of green (500–550 nm) light for photolysis of RSNOs with subsequent amperometric detection of

NO. As such, initial analyses focused on optimizing a light source for incorporation of visible photolysis and amperometric detection of RSNOs within the microfluidic device platform. A 530 nm high-power LED was chosen as the ideal source due to its small size and amenability to tuning position, focus, and light intensity. The LED was positioned directly over the microfluidic channel at a distance of 100 mm to ensure complete irradiation of the sample prior to reaching the working electrode while also avoiding sample heating (Figure 1). The total transit time from the device inlet to the NO selective electrode was ~5 s.

Optimization of LED configuration

The low molecular weight nitrosothiol GSNO in oxygenated PBS was used to evaluate the effect of light intensity and irradiation area/focus. When evaluating intensity, the LED light was focused to a 10 mm diameter spot centered on the inlet channel. As indicated in Table 1, greater light intensity yielded a linear increase in sensitivity while improving the limit of detection (attributed to greater RSNO to NO conversion with the more intense source). Representative current traces as a function of light intensity are given in Figure 2. To evaluate the effect of irradiating a larger portion of the channel (the channel length from inlet to working electrode is ~20 mm), the focus of the light was altered to create a larger area of irradiation, centered along the length of the channel. Spot sizes of 5, 10, and 20 mm in diameter produced slightly varied output powers of 34, 40, and 27 mW, respectively. While a significant difference in sensitivity was observed between the 10 and 20 mm spot diameters (22.6 ± 1.6 and 12.3 ± 1.2 pA μM^{-1}), all sensitivities became equal when normalized to output power (~ 0.5 pA μM^{-1} mW $^{-1}$), indicating this difference resulted from the change in apparent light output instead of variation in irradiation area. The optimized configuration was used (focused 10 mm diameter light at 100% intensity) for subsequent experiments.

Detection of common low molecular weight and macromolecular nitrosothiols

In addition to GSNO, the utility of this device was evaluated for sensitivity to CysNO and AlbSNO in oxygenated PBS (to represent a physiologically relevant system). As indicated in Table 2, the device exhibited identical sensitivities for both low molecular weight RSNOs (i.e., GSNO and CysNO; 22.6 ± 1.6 and 25.5 ± 1.3 pA μM^{-1} , respectively), an expected result due to comparable molar absorptivities at the irradiation wavelength ($\epsilon = 17.2$ and 14.9 M $^{-1}$ cm $^{-1}$ at 530 nm, respectively). The maximum current signal was obtained after only 100 s, with linear response from 1–40 μM , well beyond expected in vivo concentrations (Figure S-1 and S-2, Supporting Information). Based on the oxidation current measured from dilutions of a saturated NO solution, utilizing visible photolysis within the microfluidic device configuration allows for a ~40% conversion of RSNO to free NO. Compared to 7–11% conversion in a 25 mL deoxygenated solution reported by Riccio et al. (using chemiluminescence detection),⁵¹ this result represents a significant improvement compared to utilizing this method in bulk solution. The ability of the device to detect NO from nitrosated serum albumin (AlbSNO) was also evaluated, as nitrosated proteins make up the majority of RSNO species in vivo.²³ Not surprisingly, an ~80% decrease in sensitivity was observed compared to low molecular weight RSNOs. The greater stability of AlbSNO relative to low molecular weight RSNOs is well known and attributed to the protection of

the nitrosocysteine group within a hydrophobic pocket of the protein.^{22,23,29,56} Of note, albumin contains only a single free cysteine residue for nitrosothiol formation despite its greater size,²³ making its theoretical NO storage/release equivalent to GSNO and CysNO.

After normalizing for differences in irradiation power and electrode surface area, a 6–8× improvement was observed for the low molecule weight RSNOs after 1 min irradiation, while the sensitivity for AlbsNO was improved by ~20×. In addition, the LOD for each was improved by at least 55%. Importantly, the results reported herein were obtained in oxygenated PBS. Further performance improvements would be expected in deoxygenated solutions. Indeed, a 44% increase in current was noted for a sample of GSNO upon dilution in deoxygenated versus oxygenated PBS (data not shown).

Detection in plasma

To fully evaluate the utility of this device for the determination of RSNOs in biologically relevant systems, it was important to establish whether measurement in physiological milieu (i.e., whole blood and plasma) was possible. Despite the significantly reduced sample cross-section (~90 μm) and irradiated volume (~450 nL), detection of exogenous RSNOs added to whole blood was not possible, likely due to light scattering and/or absorbance by red blood cells. Based on complete blood cell counts obtained from these samples, 450 nL of blood contains an average of 2×10^6 red blood cells. Nevertheless, measurements of RSNOs in oxygenated plasma were possible using the microfluidic device whether plasma was spiked with RSNOs or the RSNOs were first added to whole blood with subsequent centrifugation to collect plasma. Compared to measurements carried out in PBS, the sensitivities and limits of detection in plasma were greatly reduced, with an ~80% decrease in sensitivity for all RSNO species evaluated as shown in Table 2. Identical responses were achieved regardless of how the RSNO was added to the samples (Figure S-2, Supporting Information). Samples that were frozen (–20 °C) and reanalyzed after 24 h exhibited a ~50% decrease in sensitivity (data not shown). Based on these sensitivities, limits of detection of 400, 240, and 2660 nM were achieved for GSNO, CysNO, and AlbsNO, respectively. This diminished response is somewhat expected due to the opacity of plasma relative to PBS, as well as NO scavenging and light scattering due to the presence of proteins.^{57,58} Indeed, the transmittance of 530 nm light in blood plasma is decreased by ~26% relative to PBS.

Effect of cholesterol and hemoglobin concentration

Evaluation of NO scavenging and plasma turbidity is important for validating the potential clinical utility of the measurement technique. Small amounts of red blood cell hemolysis are common during blood centrifugation to plasma.⁵⁹ Free oxygenated hemoglobin (1, 5, and 10 μM) was thus added to PBS to determine its effect on the GSNO measurement. As shown in Table 3 (calibration data using 1–20 μM GSNO), the lowest free hemoglobin concentration (1 μM) did not significantly impact the measurement sensitivity. In contrast, the 5 and 10 μM free hemoglobin resulted in decreased sensitivity by ~35 and 71%, respectively. This result was somewhat anticipated as oxyhemoglobin rapidly scavenges NO.⁶⁰ Accurate measurement of GSNO using this approach might be challenging without knowing the extent of hemolysis in a given sample. Fortunately, a simple centrifugal filter of appropriate molecular weight cutoff could be utilized to remove free hemoglobin following blood

centrifugation and prior to analysis using the microfluidic device. Indeed, full signal restoration (a 33% improvement) was achieved following filtering of the sample containing 5 μM hemoglobin.

Changes in sample turbidity may also influence accurate RSNO measurement by changing the efficiency of RSNO photolysis. For example, large lipid concentrations may lead to underestimation of RSNO levels due to increased turbidity. We next evaluated the effects of blood cholesterol levels on the RSNO measurement. Of note, cholesterol concentrations $>3.0 \text{ mg mL}^{-1}$ are considered to be high and unhealthy in human patients.⁶¹ As shown in Table 3, the addition of 3.0 mg mL^{-1} cholesterol only slightly decreased the device sensitivity to RSNO relative to blanks (no additional lipid), while lower concentrations of cholesterol had little consequence. Nevertheless, a standard addition measurement could be employed to correct for sample turbidity variability between samples.

Measurement of endogenous nitrosothiol content

Despite the decreased sensitivity, this technique was used to determine total endogenous RSNO levels in fresh plasma separated from blood obtained from healthy swine, with an average of $1.5 \pm 1.0 \text{ }\mu\text{M}$ determined ($n=4$ animals), which falls within RSNO concentrations determined by numerous other methodologies (up to $9 \text{ }\mu\text{M}$).^{37,51} Samples were also analyzed following centrifugal filtration using a 30 kDa cutoff membrane to remove both AlbSNO and free hemoglobin. The subsequently measured RSNO concentrations were identical (Figure S-4, Supporting Information), indicating that 1) the RSNO concentration measured is due wholly to low molecular weight species (i.e., GSNO and CysNO) and 2) free hemoglobin did not plague these measurements (from healthy animals).

Conclusions

The work presented herein represents a significant enhancement in the measurement of RSNO in physiological fluids using photolytic cleavage with amperometric detection of NO. By utilizing a microfluidic device to reduce the sample cross-section and volume, irradiation and resulting RSNO decomposition to NO were increased, thus improving the sensitivity and limit of detection for both low molecular weight and protein RSNOs. Additionally, the required analysis time was minimal (i.e., <2 min for sample irradiation and detection). While detection of RSNOs directly in blood was not realized, analysis of blood plasma was feasible with measured RSNO levels in healthy swine falling within previously reported ranges.³⁷ Furthermore, the measured response was confirmed to be solely from low molecular weight RSNOs. This result signifies that low molecular weight RSNOs may be present in blood at significantly greater concentrations than protein RSNOs. Future studies should include the analysis of RSNO levels during disease states as well (e.g., sepsis) where both NO and RSNO concentrations are expected to rise.^{33,62–64}

Small LED sources can easily be coupled to future device designs, potentially allowing for simultaneous measurement of NO and RSNO in one sample. Future device designs should allow for on-chip separation of red blood cells from plasma to facilitate facile dual analysis of NO and RSNO, reduce sample handling, and prevent problems resulting from hemolysis. Light sources with greater intensity may also permit the measurement of RSNO directly in

whole blood. Within whole blood, S-nitrosohemoglobin is an especially relevant analyte of interest, as NO stored/transported in this form may be of importance to NO generation under hypoxic conditions.^{65–67}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Luo JD, Chen AF. *Acta Pharmacol Sin.* 2005; 26:259–264. [PubMed: 15715920]
2. Isenberg JS, Ridnour LA, Espey MG, Wink DA, Roberts DD. *Microsurg.* 2005; 25:442–451.
3. Soneja A, Drews M, Malinski T. *Pharmacological Reports.* 2005; 57:108–119. [PubMed: 16415491]
4. Nichols SP, Storm WL, Koh A, Schoenfisch MH. *Adv Drug Deliv Rev.* 2012
5. Donnini S, Ziche M. *Antioxid Redox Sign.* 2002; 4:817–823.
6. Morbidelli L, Donnini S, Ziche M. *Current Pharmaceutical Design.* 2003; 9:521–530. [PubMed: 12570800]
7. Al-Ani B, Hewett PW, Ahmed S, Cudmore M, Fujisawa T, Ahmad S, Ahmed A. *Plos One.* 2006; 1:e25. [PubMed: 17183652]
8. Kuwabara M, Kakinuma Y, Ando M, Katare RG, Yamasaki F, Doi Y, Sato T. *The Journal of Physiological Sciences.* 2006; 56:95–101. [PubMed: 16779917]
9. Snyder SH, Bredt DS. *Scientific American.* 1992; 266:74–77.
10. Lowenstein CJ, Alley EW, Raval P, Snowman AM, Snyder SH, Russell SW, Murphy WJ. *Proc Natl Acad Sci USA.* 1993; 90:9730–9734. [PubMed: 7692452]
11. Fang FC. *J Clin Invest.* 1997; 99:2818–2825. [PubMed: 9185502]
12. Thomas DD, Ridnour LA, Isenberg JS, Flores-Santana W, Switzer CH, Donzelli S, Hussain P, Vecoli C, Paolocci N, Ambs S, Colton CA, Harris CC, Roberts DD, Wink DA. *Free Radical Bio Med.* 2008; 45:18–31. [PubMed: 18439435]
13. Hetrick EM, Schoenfisch MH. *Annu Rev Anal Chem.* 2009; 2:409–433.
14. Hunter RA, Storm WL, Coneski PN, Schoenfisch MH. *Anal Chem.* 2013; 85:1957–1963. [PubMed: 23286383]
15. Hunter RA, Privett BJ, Henley WH, Breed ER, Liang Z, Mittal R, Yoseph BP, McDunn JE, Burd EM, Coopersmith CM, Ramsey JM, Schoenfisch MH. *Anal Chem.* 2013; 85:6066–6072. [PubMed: 23692300]
16. Coneski PN, Schoenfisch MH. *Chem Soc Rev.* 2012; 41:3753–3758. [PubMed: 22362308]
17. Davies IR, Zhang XJ. *Methods in Enzymology.* 2008; 436:63–95. [PubMed: 18237628]
18. Privett BJ, Shin JH, Schoenfisch MH. *Chem Soc Rev.* 2010; 39:1925–1935. [PubMed: 20502795]
19. Allen, BW.; Liu, J.; Piantadosi, CA.; Lester, P.; Enrique, C. *Methods in Enzymology.* Academic Press; 2005. p. 68–77.
20. Shin JH, Weinman SW, Schoenfisch MH. *Anal Chem.* 2005; 77:3494–3501. [PubMed: 15924380]
21. Shin JH, Privett BJ, Kita JM, Wightman RM, Schoenfisch MH. *Anal Chem.* 2008; 80:6850–6859. [PubMed: 18714964]
22. Williams DLH. *Accounts Chem Res.* 1999; 32:869–876.
23. Stamler JS, Jaraki O, Osborne J, Simon DI, Keaney J, Vita J, Singel D, Valeri CR, Loscalzo J. *PNAS.* 1992; 89:7674–7677. [PubMed: 1502182]

24. Stamler JS, Simon DI, Osborne JA, Mullins ME, Jaraki O, Michel T, Singel DJ, Loscalzo J. PNAS. 1992; 89:444–448. [PubMed: 1346070]
25. Foster MW, McMahon TJ, Stamler JS. Trends in Molecular Medicine. 2003; 9:160–168. [PubMed: 12727142]
26. Hogg N. Annu Rev Pharmacol. 2002; 42:585–600.
27. Hogg N. Free Radical Bio Med. 2000; 28:1478–1486. [PubMed: 10927172]
28. Giustarini D, Milzani A, Colombo R, Dalle-Donne I, Rossi R. Clin Chim Acta. 2003; 330:85–98. [PubMed: 12636927]
29. Giustarini D, Milzani A, Dalle-Donne I, Rossi R. J Chromatogr B. 2007; 851:124–139.
30. Stamler JS, Slivka A. Nutr Rev. 1996; 54:1–30. [PubMed: 8919695]
31. Doctor A, Platt R, Sheram ML, Eiseid A, McMahon T, Maxey T, Doherty J, Axelrod M, Kline J, Gurka M. PNAS. 2005; 102:5709–5714. [PubMed: 15824313]
32. Crawford JH, Chacko BK, Pruitt HM, Pikhova B, Hogg N, Patel RP. Blood. 2004; 104:1375–1382. [PubMed: 15150083]
33. Ottesen LH, Harry D, Frost M, Davies S, Khan K, Halliwell B, Moore K. Free Radical Bio Med. 2001; 31:790–798. [PubMed: 11557317]
34. Richardson G, Benjamin N. Clinical Science. 2002; 102:99–105. [PubMed: 11749666]
35. Nagano T, Yoshimura T. Chem Rev. 2002; 102:1235–1270. [PubMed: 11942795]
36. Bates JN. Neuroprotocols. 1992; 1:141–149.
37. Griveau S, Bedioui F. Analyst. 2013; 138:5173–5181. [PubMed: 23888299]
38. Cha W, Anderson MR, Zhang F, Meyerhoff ME. Biosens Bioelectron. 2009; 24:2441–2446. [PubMed: 19168347]
39. Cha W, Lee Y, Oh BK, Meyerhoff ME. Anal Chem. 2005; 77:3516–3524. [PubMed: 15924383]
40. Cha W, Meyerhoff ME. Langmuir. 2006; 22:10830–10836. [PubMed: 17129067]
41. Cha WS, Anderson MR, Zhang FH, Meyerhoff ME. Biosens Bioelectron. 2009; 24:2441–2446. [PubMed: 19168347]
42. Höfler L, Meyerhoff ME. Anal Chem. 2011; 83:619–624. [PubMed: 21230000]
43. Hwang S, Cha W, Meyerhoff ME. Electroanal. 2008; 20:270–279.
44. Kim LTT, Girard A, Griscom L, Razan F, Griveau S, Bedioui F. Electrochem Commun. 2011; 13:681–684.
45. Pfeiffer S, Schrammel A, Schmidt K, Mayer B. Anal Biochem. 1998; 258:68–73. [PubMed: 9527850]
46. Rafikova O, Rafikov R, Nudler E. PNAS. 2002; 99:5913–5918. [PubMed: 11983891]
47. Diers AR, Keszler A, Hogg N. BBA General subjects. 2014; 1840:892–900. [PubMed: 23988402]
48. Alpert C, Ramdev N, George D, Loscalzo J. Anal Biochem. 1997; 245:1–7. [PubMed: 9025962]
49. Dejam A, Kleinbongard P, Rassaf T, Hamada S, Gharini P, Rodriguez J, Feelisch M, Kelm M. Free Radical Bio Med. 2003; 35:1551–1559. [PubMed: 14680678]
50. Sexton DJ, Muruganandam A, McKenney DJ, Mutus B. Photochem Photobiol. 1994; 59:463–467. [PubMed: 8022889]
51. Riccio DA, Nutz ST, Schoenfisch MH. Anal Chem. 2012; 84:851–856. [PubMed: 22201553]
52. Tudos AJ, Besselink GAJ, Schasfoort RBM. Lab on a Chip. 2001; 1:83–95. [PubMed: 15100865]
53. Whitesides GM. Nature. 2006; 442:368–373. [PubMed: 16871203]
54. Musameh M, Moezzi N, Schauman LM, Meyerhoff ME. Electroanal. 2006; 18:2043–2048.
55. Feelisch, M.; Stamler, J. Methods in nitric oxide research. Wiley-Blackwell; 1996.
56. Mohny BK, Walker GC. JACS. 1997; 119:9311–9312.
57. Harding JL, Reynolds MM. Anal Chem. 2014; 86:2025–2032. [PubMed: 24444148]
58. Braughler JM, Mittal CK, Murad F. J Biol Chem. 1979; 254:12450–12454. [PubMed: 40996]
59. Sowemimo-Coker SO. Transfusion Medicine Reviews. 2002; 16:46–60. [PubMed: 11788929]
60. Doherty DH, Doyle MP, Curry SR, Vali RJ, Fattor TJ, Olson JS, Lemon DD. Nature biotechnology. 1998; 16:672–676.
61. Keaney JF, Curfman GD, Jarcho JA. N Engl J Med. 2014; 370:275–278. [PubMed: 24283199]

62. Carcillo JA. Crit Care Med. 1999; 27:1063–1065. [PubMed: 10397205]
63. Dhillon SS, Mahadevan K, Bandi V, Zheng Z, Smith CW, Rumbaut RE. CHEST. 2005; 128:1706–1712. [PubMed: 16162778]
64. Kirkebøen KA, Strand ØA. Acta Anaesth Scand. 1999; 43:275–288. [PubMed: 10081533]
65. Stamler JS, Jia L, Eu JP, McMahon TJ, Demchenko IT, Bonaventura J, Gernert K, Piantadosi CA. Science. 1997; 276:2034–2037. [PubMed: 9197264]
66. Gow AJ, Stamler JS. Nature. 1998; 391:169–173. [PubMed: 9428761]
67. Pawloski JR, Hess DT, Stamler JS. Nature. 2001; 409:622–626. [PubMed: 11214321]

Figure 1. Schematic of microfluidic device setup with LED and lens configuration.

Figure 2. Effect of light intensity on sensitivity. Representative amperometric responses for CysNO (75 μM) detection at 100 (blue dashed-dotted, 40 mW), 75 (purple dotted, 25 mW), 50 (red dashed, 6 mW), and 25% (black solid, 2 mW) light intensity at 10-mm irradiation diameter.

Table 1

Effect of light intensity and irradiation area on *S*-nitrosoglutathione sensitivity in oxygenated phosphate buffered saline. Data represent the mean $\pm$ standard deviation (n = 3)

Spot diameter (mm)	Apparent power (mW)	Sensitivity (pA μM^{-1})	LOD (nM)
5	34	17.6 ± 0.9	80
	40	22.6 ± 1.6	60
	25	11.7 ± 2.4	120
10	6	5.0 ± 1.7	280
	2	2.9 ± 0.1	480
	0	0.6 ± 0.0	2500
20	27	12.3 ± 1.2	110

Sensitivity and limit of detection as a function of S-nitrosothiol species in oxygenated phosphate buffered saline and plasma. Data represent the mean $\pm$ standard deviation (n = 3).

Table 2

RSNO type	PBS			Blood plasma		
	Sensitivity (pA μM^{-1})	LOD (nM)	Sensitivity (pA μM^{-1})	Sensitivity decrease from PBS (%)	LOD (nM)	
GSNO	22.6 $\pm$ 1.6	60	4.2 $\pm$ 1.8	82	400	
CysNO	25.5 $\pm$ 1.3	60	6.9 $\pm$ 3.2	73	240	
AlbSNO	5.0 $\pm$ 1.4	280	0.6 $\pm$ 0.2	87	2660	

Table 3

Effect of free hemoglobin (in PBS) and cholesterol (in plasma) concentrations on percent light transmittance through sample and sensitivity of GSNO measurement.

Hemoglobin		
Concentration (μM)	%T	Sensitivity ($\text{pA } \mu\text{M}^{-1}$)
0	0	22.6
1	-21	22.6
5	-30	14.6
10	-46	6.6

Cholesterol		
Concentration added (mg mL^{-1})	%T	Sensitivity ($\text{pA } \mu\text{M}^{-1}$)
0	0	4.5
1.5	-4	4.9
2.5	-21	4.1
3.0	-42	3.7