Published in final edited form as:

ACS Sens. 2019 July 26; 4(7): 1766–1773. doi:10.1021/acssensors.9b00170.

A selective and sensocompatible electrochemical nitric oxide sensor with a bilaminar design

Micah D. Brown^a, Mark H. Schoenfish^{a,*}

^aDepartment of Chemistry, University of North Carolina at Chapel Hill, CB 3290, Chapel Hill, North Carolina 27599, United States

Abstract

Macrophages mediate mammalian inflammation in part by the release of the gasotransmitter, nitric oxide (NO). Electrochemical methods represent the best means of direct, continuous measurement of NO, but monitoring continuous release from immunostimulated macrophages remains analytically challenging. Long release durations necessitate consistent sensor performance (i.e., sensitivity and selectivity for NO) in proteinaceous media. Herein, we describe the fabrication of an electrochemical sensor modified by an electropolymerized 5-amino-1-naphthol (poly(5A1N)) film in conjunction with a fluorinated xerogel topcoat. The unique combination of these membranes ensures selective detection of NO that is maintained over extended periods of use (>24 h) in biological media without performance deterioration. The hydrophobic xerogel topcoat protects the underlying NO-selective poly(5A1N) film from hydration-induced desorption. The bilaminar sensor is then readily adapted for measurement of the temporal and spatial NO-release profiles from immunostimulated macrophages.

Graphical Abstract

*Corresponding Author: schoenfish@unc.edu.

Supporting Information

The Supporting Information is available free of charge.

Three-step oxidation of NO; experimental setup; electroinactivity of stimulants; amperometric response of incubator door perturbation; illustration of distance-dependence; repeated trials of long-term macrophage NO release; microelectrode calibration.

The corresponding author declares the following competing financial interest: Mark Schoenfish is a founder of and maintains financial interest in Clinical Sensors, Inc. Clinical Sensors is an early-stage medical device company developing sensors for clinical use.

Keywords

nitric oxide; permselectivity; sensocompatibility; electrochemical detection; 5-amino-1-naphthol; xerogel; macrophage

From its discovery as the endothelium-derived smooth muscle relaxation factor, nitric oxide (NO) has been implicated in an ever-growing collection of biological processes.^{1, 2} With its ability to freely permeate cell membranes, NO acts as an intercellular gasotransmitter, mediating such processes as wound healing,³ vasodilation, angiogenesis,⁴ platelet aggregation,⁵ long-term memory potentiation,^{6, 7} and inflammation.⁸ In order for one molecule to exert such varied, sometimes opposing effects, the biological activity of NO is necessarily concentration-dependent. Low concentrations of NO (pM-nM) are biosynthesized from L-arginine substrate and either the endothelial or neuronal isoform of the nitric oxide synthase enzyme (eNOS and nNOS, respectively), initiating enzymatic cascades through cyclic GMP.⁹ High NO concentrations (nM-μM) are produced from inflammatory cells via the inducible isoform (iNOS; acting on L-arginine substrate) and create localized environments of nitrosative/oxidative stress to kill pathogens.¹⁰ As a potential biomarker of inflammation state, measurements of NO from relevant biological fluids (e.g., blood, serum, wound fluid) demonstrate prognostic utility in cases of sepsis and chronic wounds.^{11, 12} Probing the concentration dynamics of this seemingly ubiquitous molecule is essential to understanding these and other yet undiscovered functions of NO in mammalian physiology.

The high reactivity and diffusibility of NO present challenges to in situ detection and quantification.¹³ Nitric oxide's lifetime in biological milieu is limited to a few seconds due to scavenging by thiols, oxygen, free metal ions, and heme-containing proteins.¹⁴⁻¹⁶ Indirect detection methods can measure NO's more stable oxidative byproducts, nitrite and nitrate (i.e., NO_x species, collectively). Although the effects of scavenging are largely circumvented, indirect methods fail to capture NO fluctuations in real time and generally require extensive sample preparation (e.g., the Griess assay).¹⁷ Chemiluminescence and electron paramagnetic resonance spectroscopy offer direct and highly sensitive detection, but these techniques require complex instrumentation and are not well suited for real-time analysis in complex media.^{18, 19} Currently, electrochemical sensors offer the highest spatial and temporal resolution for in situ, real-time monitoring of NO in biological media.²⁰⁻²⁴

In the last thirty years, most biological applications of electrochemical NO sensors have involved measurement from either liver tissue (in the form of an excised organ or homogenate) or cultured cells.²⁵⁻²⁷ For the latter, macrophages and human umbilical vein endothelial cells (HUVECs) are generally preferred given their well-known NO-release response to certain stimuli, including L-arginine,²⁸ acetylcholine,²⁹ bradykinin,³⁰ and calcium ionophore.³¹ These stimuli activate the constitutively expressed (i.e., continually present in the cytoplasm) NOS isoforms, eNOS and nNOS, resulting in NO release within seconds. Detection of NO released from cultured cells is most often used as a demonstration of a sensor's suitability for measurement in biological systems (i.e., sensocompatibility).

Though measurement of cellular NO release derived from eNOS or nNOS may demonstrate initial sensocompatibility, these experiments are generally conducted over the course of minutes, often without any indication that suitable sensor performance has been maintained. A more analytically challenging biological application is the measurement of extended NO release (hours-days)^{32, 33}, specifically from iNOS. As part of the innate immune response, macrophages upregulate iNOS production in response to endotoxins and/or inflammatory cytokines, requiring 6–8 hours for complete enzyme expression.³⁴ Continuous electrochemical measurement of macrophage NO release on this time scale has been reported in far fewer reports (contrasted with constitutive NOS enzymes). Notably, Pekarova et al. demonstrated continuous NO measurement from lipopolysaccharide (LPS)-stimulated macrophages using a poly(porphyrin)- and Nafion-coated carbon fiber microelectrode (7 μm dia.) positioned above confluent cells.³⁵ The sensor's current response was converted to a rate of NO release ($\text{nmol NO h}^{-1} 10^6 \text{ cells}^{-1}$) using Griess analysis of the supernatant at 20 h and assuming a 1:1 conversion of NO to nitrite. Although this study marked the longest continuous monitoring of macrophage-derived NO release at the time, the sensor selectivity against nitrite was low (1:609, NO_2^- :NO signal response; -2.8 selectivity coefficient), a troublesome characteristic particularly in an environment with continuously rising nitrite concentrations ($20 \mu\text{M NO}_2^-$ at 20 h). In a different study, Cha et al. integrated an electrochemical NO sensor within a microfluidic device to measure NO release for 16 h from upstream cultured macrophages (stimulated with LPS).³⁶ Using a TeflonAF membrane, high selectivity was reported against both L-ascorbate and nitrite. However, the perfusate media did not contain serum, the primary source of proteins in standard culture media. Moreover, long response times (at most, 80 s) necessitated deoxygenation of the perfusate to prevent undesirable NO scavenging. While removing both oxygen and protein nutrients facilitated NO detection, such a configuration less accurately models the in vivo behavior of macrophages.

In order to meet the selectivity and sensocompatibility challenges of macrophage-derived, long-term NO measurement in proteinaceous media, we report the fabrication and characterization of a bilaminar sensor comprised of a platinum disk electrode modified by an electropolymerized 5-amino-1-naphthol (5A1N) film and a highly fluorinated xerogel (XG) topcoat. Previous studies have highlighted the benefits of poly(5A1N) for NO selectivity, particularly over nitrite.^{37–39} Likewise, XG membranes have been shown to possess NO permselective characteristics based on size-exclusion and hydrophobicity.^{40, 41} The bilaminar sensor design was evaluated for selectivity for NO over a wide panel of biologically relevant interferents and for sensocompatibility (i.e., retention of NO sensitivity, selectivity, and LOD with extended use in proteinaceous media). Sensor utility was demonstrated by continuously monitoring NO release from pro-inflammatory macrophages stimulated with LPS and interferon-gamma ($\text{IFN-}\gamma$) over a 24 h period. Scanning electrochemical microscope approach curves were also collected to probe the NO diffusion profile emanating from confluent pro-inflammatory macrophages.

Experimental

Materials, reagents, and apparatus

Trimethoxymethylsilane (MTMOS), 5-amino-1-naphthol (5A1N), hydrochloric acid (HCl), sodium nitrite (NaNO_2), sodium nitrite standard (0.1 M), L-ascorbic acid (AA), acetaminophen (AP), dopamine hydrochloride (DA), lipopolysaccharide (LPS), fetal bovine serum (FBS), lyophilized bovine oxyhemoglobin (Hb), *N*-(1-naphthyl)ethylenediamine dihydrochloride (NED), and sulfanilamide were purchased from Sigma Aldrich (St. Louis, MO). (Heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxysilane (17FTMS) was purchased from Gelest (Morrisville, PA). Ethanol (200 proof), ammonium hydroxide (NH_4OH), sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium chloride (NaCl), sulfuric acid (H_2SO_4), phosphoric acid (H_3PO_4), and hydrogen peroxide (H_2O_2 ; 30 wt%) were obtained from Fisher Scientific (Hampton, NH). Dulbecco's modification of Eagle's medium (DMEM; 4.5 g/mL glucose; no phenol or L-glutamine) was purchased from Corning (Corning, NY). Interferon-gamma ($\text{IFN-}\gamma$) was obtained from BioLegend (San Diego, CA). Murine RAW 264.7 macrophages were obtained from the American Type Culture Collection (Manassas, VA). Nitric oxide (99.5%; NO), carbon monoxide (99.3%; CO), nitrogen (N_2), and argon (Ar) gases were purchased from National Welders Supply (Raleigh, NC). Platinum (75 μm) and tungsten (0.20 mm) wires were obtained from A-M Systems (Sequim, WA). Other solvents and chemicals were analytical-reagent grade and used as received without further purification.

A Millipore Milli-Q UV Gradient A10 System (Bedford, MA) was used to purify distilled water to a resistivity of 18.2 M Ω -cm and a total organic content of 6 ppb. Saturated NO (1.9 mM) and CO (0.9 mM) were made by purging ~20 mL of phosphate buffered saline (PBS; 0.01 M, pH 7.4) first with Ar for 25 min and then with NO or CO gas for 25 min over ice. Saturated gas solutions were stored at 4 °C and used the day of preparation. Electrochemical experiments were carried out using either an 8-channel CH Instruments 1030C Electrochemical Analyzer or a CH Instruments 920D Scanning Electrochemical Microscope (SECM; Austin, TX). In total, eight inlaid 2 mm dia. polycrystalline platinum (Pt) disk working electrodes sealed in Kel-F (6 mm total diameter; CH Instruments), a silver-silver chloride (Ag|AgCl) reference electrode (3.0 M KCl; CH Instruments), and a coiled Pt wire counter electrode were employed for measurements on the 8-channel potentiostat. Platinum microelectrodes (75 μm dia.) were fabricated by hand for SECM measurements (detailed in Section 2.5.). All reported potentials hereafter are versus the Ag|AgCl reference electrode, and amperograms are presented in the polarographic convention. Contact angle measurements were collected using a CAM 200 optical contact angle meter (KSV Instruments; Bridgeport, CT), and an image of the xerogel was collected on a Hitachi S-4700 Scanning Electron Microscope (SEM; Tarrytown, NY).

Preparation of Pt/poly(5A1N)/XG sensors

Platinum working electrodes were mechanically polished with deagglomerated alumina slurries down to 0.05 μm in particle size (Buehler; Lake Bluff, IL) and ultrasonicated in ethanol to remove residual alumina. Electrodes were then electrochemically polished in 1 N H_2SO_4 by cycling between -0.4 and +1.8 V for 40 cycles (500 mV s^{-1}). Electrodes were

rinsed with water and transferred to a monomer solution of 5A1N. To improve solubility, 10 mM 5A1N was dissolved in PBS solution titrated to pH 1 with HCl. Poly(5A1N) films were electropolymerized via cyclic voltammetry (CV), sweeping the potential from 0 to +1.0 V (20 cycles; positive direction initial sweep) at a scan rate of 10 mV s⁻¹.³⁸ Film-modified electrodes were rinsed copiously with distilled water to remove unbound oligomer and allowed to dry for >1 h on the benchtop before spray-coating.

A fluoroalkoxysilane sol solution was prepared via acid-catalyzed hydrolysis and co-condensation of MTMOS and 17FTMS precursors (30% 17FTMS, v/v balance MTMOS). Ordered additions of 3.600 mL ethanol, 630 μ L MTMOS, 270 μ L 17FTMS, 960 μ L H₂O, and 60 μ L 0.5 M HCl constituted the sol solution, which was stirred vigorously for 1 h. The sol was spray-coated onto Pt disc electrodes (both bare and poly(5A1N)-modified) with an airbrush gun (Iwata HP-BC1 Plus; Yokohama, Japan) pressurized at 42 psi with a nozzle-target separation of 50 cm and 5 s of continuous dispersal. The sol-coated electrodes were then dried on the benchtop for >48 h to allow condensation of the xerogel matrix before further testing.

Steady-state amperometric measurements

Bare and modified electrodes were polarized at +0.8 V in deoxygenated PBS for 15 min to achieve a constant background current prior to measurements. Sensitivity for NO was determined with consecutive injections (475 nM each) of saturated NO solution into deoxygenated PBS. Calibrations were also done in FBS-supplemented DMEM to determine sensitivity retention relative to PBS trials. Limits of detection (LOD) were calculated with respect to 3 standard deviations of the noise ($S/N=3$). The current responses to injections of NaNO₂ (1 mM), AA (1 mM), AP (50 μ M), DA (100 μ M), CO (0.9 μ M), NH₄OH (1 mM), and H₂O₂ (1 mM) were measured and compared to the NO sensitivity. Interferent concentrations well exceeded anticipated biological concentrations.²⁰ Selectivity coefficients were calculated using Eq. 1, where $\log k_{NO,j}$ is the selectivity coefficient for NO over interferent j , S_j is the sensor sensitivity towards interferent j , and S_{NO} is the NO sensitivity of the sensor.⁴²

$$\log k_{NO,j} = \log \left(\frac{S_j}{S_{NO}} \right) \quad (\text{Eq. 1})$$

The long-term sensocompatibility of Pt/poly(5A1N)/XG, Pt/poly(5A1N), Pt/XG, and bare Pt electrodes were evaluated in PBS with 12 h of continuous operation (+0.8 V). Sensitivity towards NO, selectivity against nitrite, and the LOD were re-measured at the 12 h time-point and compared to initial measurements to assess performance maintenance. All sensor designs were also tested for sensitivity retention in FBS-supplemented (10% v/v) DMEM (without deoxygenation). The Pt/poly(5A1N)/XG sensors were evaluated for long-term stability in DMEM with 24 h of continuous operation (+0.8 V). Again, analytical metrics were re-measured at the 24 h time-point and compared to initial measurements to assess performance maintenance.

Continuous NO measurements from RAW 264.7 macrophages

The RAW 264.7 macrophages were seeded into a 12-well plate at a density of 1.0×10^6 cells/well (i.e., 2.5×10^5 cells cm^{-2}) and given 4 h to adhere. Each well contained 2.0 mL of phenol-free, FBS-supplemented DMEM. A custom 3D-printed well plate cover was designed to support a Pt/poly(5A1N)/XG working electrode and reference/counter electrodes in individual wells while the culture plate remained in an incubator at 37 °C and 5% CO₂ in air (Fig. S2). Sensors were positioned using rubber O-rings and calipers such that the electrode surface was 0 to 200 μm from the well bottom. Electrode lead attachments fed into the 8-channel potentiostat outside the incubator with the door closed. Electrodes were given ~8 h to polarize at +0.8 V in solution before the incubator door was opened and pro-inflammatory stimulants were injected into the cell (20 and 10 ng mL^{-1} LPS and IFN- γ , respectively). Continuous NO measurements were collected for 24 h post-stimulation. A least squares exponential regression was fit to the first 8 h pre-stimulation and used to background subtract the residual (i.e., non-analyte-specific) current from the entire trace. The current measured at $t = 2$ h post-stimulation was also background subtracted from all traces as a reference point where no NO evolution from the cells was anticipated. Data collection was repeated for unstimulated macrophage cells with parameters and data processing kept the same.

After electrochemical experiments were completed, supernatant solutions were collected and stored at -20 °C for 3 d before Griess analysis. Endpoint Griess analysis was carried out on samples to quantitate nitrite totals from both stimulated and unstimulated cell runs as a measure of total NO production. Briefly, sulphanilic acid was reacted with nitrite in the sample to form a diazonium salt before reaction with NED to yield the characteristic pink azo dye detectable via absorbance spectroscopy at $\lambda = 540$ nm. Nitrite calibration standards were prepared in FBS-supplemented DMEM (the same matrix as the samples).

SECM measurements of NO from RAW 264.7 macrophages

Given the size constraints of the SECM probe positioner, Pt microelectrodes were fabricated by hand. Briefly, glass capillary tubes (0.68 mm internal dia.) were pulled and beaded at the tip before introducing a ~1.0 cm length of 75 μm dia. Pt wire. Passage over a propane blowtorch melted the capillary walls to encapsulate the Pt wire, which was then soldered to a tungsten lead. The distal end was sanded down to expose the Pt and sharpen the tip to a diameter of ~400 μm . The poly(5A1N) and XG modification of the Pt microelectrode proceeded exactly as with the disk macroelectrodes (see Section 2.2.). Sensors were calibrated in DMEM immediately before use.

The RAW 264.7 macrophages were seeded into tissue culture dishes at a surface density of 2.5×10^5 cells cm^{-2} and given 4 h to adhere before stimulation with 20 and 10 ng mL^{-1} LPS and IFN- γ , respectively. In order to probe pro-inflammatory cells at their peak NO flux, 8 h of post-stimulation incubation were allotted for iNOS upregulation. Unstimulated control cells were incubated for the same period. Approach curves to the cells in the z-direction were collected on the SECM with an approach rate of 1.0 $\mu\text{m s}^{-1}$. The background current measured at 600 μm was subtracted from all traces. Oxyhemoglobin (Hb; 160 μM) scavenging of NO from stimulated macrophage cells was used as a negative control.

Statistical analysis

The NO sensitivity, sensitivity retention, selectivity coefficients, and LOD of the bare and modified electrodes are presented (either numerically or with error bars) as the mean $\pm$ standard error of the mean. Comparisons between data sets were performed using two-tailed *t*-tests where shown. Statistical significance is defined as $p < 0.05$.

Results and Discussion

Sensor fabrication

The electropolymerization of poly(5A1N) was carried out using a deposition procedure previously optimized for NO permselectivity against nitrite and AA.³⁸ As the potential is swept positively during electrodeposition, 5A1N monomers are oxidized through their amine groups to radical cations that dimerize, oligomerize, and ultimately precipitate out of solution onto the electroactive surface. The electrode surface is passivated against further monomer oxidation as the film grows with successive cycles. As a result, the process is self-terminating and results in thin (<100 nm), reproducible (<5% relative error in total charge passed) films (Fig. 1).⁴³ After deposition of poly(5A1N), the 17FTMS/MTMOS sol was spray-coated onto the electrode and allowed to dry/condense for at least 48 h. The poly(5A1N) film was predicted to improve xerogel compactness/adherence because amine functional groups have been reported to catalyze the condensation of alkoxy silane precursors in sol-gel systems.⁴⁴ The formation of a hydrophobic xerogel coating was confirmed by SEM and contact angle measurements, the latter increasing from 62° to >90° after membrane deposition (Fig. 1).

Sensor sensitivity and selectivity towards NO

As shown in Fig. 2A, the analytical performance characterization of bilaminar Pt/poly(5A1N)/XG sensors included determining the NO sensitivity ($12.4 \pm 8.0 \text{ pA nM}^{-1}$), detection limit ($\sim 1 \text{ nM}$), and dynamic range (0.01–10 μM). The NO selectivity of the sensors was also evaluated against a range of biological interferents of different size and charge state (Fig. 2B). Selectivity against a majority of interferents was markedly improved compared to the bare Pt electrode. In particular, selectivity improvements against larger interferents AA, AP, and DA (with net charges of -1 , 0 , and $+1$, respectively at physiological pH 7.4) demonstrated size-exclusion as a key selection mechanism. Among the smaller interferent species studied, cationic NH_4^+ and anionic NO_2^- were better rejected than uncharged CO and H_2O_2 . The differential rejection of charged, water-solvated interferents over uncharged species demonstrates the membranes' combined hydrophobic character. Previous work has reported that hydrophobic membranes are better suited for selective NO diffusion versus membranes also containing hydrophilic domains, such as Nafion.⁴⁵ Of note, selectivity over CO was not improved for poly(5A1N)/XG-modified sensors compared to bare Pt electrodes (Fig. 2B). The physicochemical properties of NO and CO, including size, lack of charge, and lipophilicity, are quite similar. These shared traits facilitate their roles as intercellular gasotransmitters and hemoprotein binders. As such, they regulate many of the same processes.^{46, 47} Lastly, the selectivity of the bilaminar sensor against nitrite was exceptional (selectivity coefficient of nearly -5) with $\sim 80,000$ -fold greater sensitivity towards NO over nitrite.

Sensor performance with extended use in proteinaceous media

The long-term sensocompatibility (i.e., ability to maintain analytical performance) of a sensor is arguably more important than its optimal, initial performance, particularly with respect to biological utility. Bare Pt, Pt/poly(5A1N), Pt/XG, and bilaminar Pt/poly(5A1N)/XG sensors were thus characterized before and after extended use in buffer and biologically representative solutions. Sensors were polarized for 12 h continuously at +0.8 V vs. Ag|AgCl in PBS. Endpoint measurements of analytical metrics (NO sensitivity, LOD, and selectivity over nitrite) were calculated and compared to initial measurements (Table 1). Of note, Pt/poly(5A1N) sensors initially had greater NO sensitivity than bare Pt. Electropolymerized films have demonstrated the ability to exert electrocatalytic effects and enhance electron transfer, thus increasing sensitivity.^{38, 48} The Pt/poly(5A1N) sensors showed good selectivity against nitrite initially (-3.8 ± 0.2 ; comparable to previous findings³⁸) but could not maintain selectivity after extended use (-2.3 ± 0.2). Concomitantly, the NO sensitivity of these sensors increased. Previous work by Shim et al. characterized the operation stability of poly(5A1N)-modified microelectrodes with similar findings.³⁷ The authors attributed the selectivity reduction to hydration-induced membrane desorption, a gradual process delayed by the intrinsic hydrophobicity of compact poly(5A1N) films. The initial selectivity of Pt/XG sensors versus nitrite (-2.3 ± 0.3) was unfavorable compared to Pt/poly(5A1N); however, these sensors notably maintained all performance metrics over 12 h of operation (Table 1). We concluded that the XG membrane was more stable and capable of withstanding hydration and polarization effects during continuous operation relative to poly(5A1N) films alone.

The combination of poly(5A1N) and XG membranes yielded markedly greater selectivity over nitrite (-4.9 ± 0.5) than either film individually with statistical significance ($p < 0.01$). The NO selectivity improvement suggests that XG deposition via spray-coating does not damage the poly(5A1N) film. After 12 h of continuous polarization in PBS, no change in LOD or nitrite selectivity were observed for Pt/poly(5A1N)/XG sensors, with negligible deviation in sensor sensitivity, indicative of membrane durability. The bilaminar design thus benefits from both the high selectivity of the poly(5A1N) film and the performance stability imparted by the XG topcoat. In this manner, the XG membrane effectively shields the underlying poly(5A1N) film from hydration-induced desorption reported by Shim et al., thus helping retain the selectivity characteristics of the sensor.³⁷

In addition to wear with continuous use, a critical challenge with in situ biological monitoring of NO is protein-related transducer biofouling. The effects of protein adsorption generally reduce sensor sensitivity and increase response time.^{49, 50} To study this potential effect, the NO sensitivity retention of bare and modified sensors was evaluated in FBS-supplemented DMEM solutions relative to PBS (Fig. 3). Without transducer modification, bare Pt electrodes maintained <20% of their original NO sensitivity in the proteinaceous media. Such sensitivity reduction is the result of both biofouling and matrix effects. The presence of oxygen, proteins, free metals, and thiol-containing residues in culture media are all effective scavengers of NO and reduce the apparent sensitivity. In addition, NO diffusion is diminished as a result of the greater viscosity of proteinaceous media.⁵¹ In contrast, Pt/poly(5A1N) and Pt/XG sensors had improved sensitivity retention compared to bare Pt, but

remained <70% relative to PBS trials. The bilaminar Pt/poly(5A1N)/XG sensors demonstrated ~80% sensitivity retention in DMEM. Clearly, sensors should be calibrated in relevant matrix before biological measurement. Of note, sensitivity retention returned to >95% when Pt/poly(5A1N)/XG sensors were re-calibrated in PBS, indicating that matrix effects play a greater role in sensitivity reduction relative to irreversible protein adsorption. This behavior is consistent with a previous report by Hunter et al.¹⁴ The Pt/poly(5A1N)/XG sensors were also evaluated for long-term (24 h) performance in FBS-supplemented DMEM under continuous operation (Table 1). The initial nitrite selectivity was slightly lower than in the PBS trials due to sensitivity reduction from matrix effects. However, the LOD, sensitivity, and selectivity each remained consistent over continuous operation in DMEM. To our knowledge, this demonstration of sensocompatibility for an electrochemical NO sensor has heretofore not been reported in the literature and strongly indicates the bilaminar sensor's capacity for use in biological samples.

Long-term NO measurement from RAW 264.7 macrophages

With the favorable sensocompatibility observed in proteinaceous media, the Pt/poly(5A1N)/XG sensor design was employed to measure long-term NO release from immunostimulated macrophages. Sensors were first polarized for 2 h and calibrated in FBS-supplemented DMEM before use to account for matrix effects and protein-related sensitivity reductions. The sensors were then positioned above confluent macrophages (Fig. S2). In all trials, the background current decreased in the beginning due to adaptations of the electrode to the applied potential from double layer charging, formation of Pt oxides, membrane polarization, and redox reactions of impurities.³⁵ The sum of these processes unrelated to analyte detection constitutes the residual current, the trial-to-trial reproducibility of which is generally poor among electrochemical techniques.⁵² In order to account for the residual current, least squares exponential regressions were fit to the first 8 h of polarization and subtracted from the remainder of the trace, a method comparable to other fit-based subtractions used to isolate the desired Faradaic current.^{52–54}

At the 0-h time-point, 20 and 10 ng mL⁻¹ lipopolysaccharide (LPS) and interferon-gamma (IFN- γ), respectively, were used to stimulate the macrophages (Fig. 4A). Neither stimulant is electroactive (Fig. S3). Lipopolysaccharide is recognized by macrophages as a structural element from the cell walls of *E. coli*. Interferon- γ is an inflammatory cytokine that, when combined with LPS, elicits a robust inflammatory response via iNOS upregulation.^{10, 55–57} Although macrophage proliferation is reduced by the presence of LPS and IFN- γ , long-term cell viability is not affected.⁵⁸ As shown in Fig. 4, the signal initially falls and returns to baseline during the first 2 h post-injection, an unavoidable, but consistent fluctuation attributed to the opening and closing of the incubator door (Fig. S4). Of greater significance, the NO release increased gradually after stimulation, plateauing to a steady flux approximately ~8 h post-injection. These results are in agreement with the reported rate of iNOS expression.^{34, 59} Approximately 14 h post-stimulation, the NO concentration began to decrease, attributable to consumption of L-arginine and/or metabolism of immunostimulatory molecules. This reduction in NO production is consistent with previous reports of immunostimulated murine macrophages.^{35, 60} Cha et al. did not observe this reduction in NO concentration over the 16 h duration of their experiment, possibly due to

perfusate replenishment of L-arginine compared to the use of static media herein. Griess analysis indicated that $56 \pm 3 \mu\text{M}$ of nitrite had accumulated in the supernatant, confirming proper immunostimulation of the macrophages. The behavior of unstimulated macrophages was also monitored continuously over the same duration (Fig. 4B). The lack of NO release indicates that an inflammatory response was not elicited in the unstimulated cells, as corroborated by endpoint Griess assay measurements ($<2.5 \mu\text{M}$ LOD). The comparison between stimulated and unstimulated experiments verifies that NO was detected in the former and that the background subtraction methods employed did not artificially create nor enhance the NO-release profiles reported.

SECM-assisted NO measurement from 264.7 macrophages

Nitric oxide diffuses radially from cells, with the equilibrium between NO supply and solution scavengers resulting in a dissipating NO concentration gradient surrounding the cell (Fig. S5).⁶¹ The NO concentration measured is therefore dependent on this gradient and subject to change in the synapse-like arrangement separating cell(s) and sensor (Fig. S6). Careful consideration of this distance-dependence must be taken into account for any sensing platform seeking to measure NO concentrations directly, particularly in efforts to examine the utility of NO as a biomarker.

For these experiments, the 2 mm dia. Pt electrode design was miniaturized to $75 \mu\text{m}$ and modified in exactly the same manner as macroelectrodes, thereby retaining analytical performance ($0.00703 \text{ pA nM}^{-1}$ NO sensitivity in DMEM, $\log k_{\text{NO}, \text{NO}_2^-} = -4.0$; Fig. S7). The microelectrode is approximately 3 orders of magnitude smaller in area than the macroelectrode, which correlates with the reduction in NO sensitivity (7.6 vs. $0.00703 \text{ pA nM}^{-1}$ in DMEM for the macro- and microelectrode, respectively). Identically stimulated macrophages were profiled 8 h post-stimulation at their peak NO release (Fig. 4A). As the SECM probe was moved from its location in bulk solution towards the confluent sheet of macrophages, the NO concentration sampled increased, beginning from baseline at $\sim 400 \mu\text{m}$ and reaching a maximum ($0.65 \pm 0.11 \mu\text{M}$) at the cell surface (Fig. 5A). Control trials of unstimulated and oxyhemoglobin-scavenged cells were profiled for verification of accurate NO measurement (Figs. 5B and 5C, respectively).

Our study represents the first application of SECM for continuously profiling the NO concentration gradient emanating from immunostimulated macrophages. Isik et al. previously reported variation in the peak NO response from bradykinin-stimulated HUVEC cells with SECM-guided electrode placement $5\text{--}30 \mu\text{m}$ above the cell surface (NO was not observed at distances greater than $30 \mu\text{m}$).⁶² Comparatively, we observe dissipation of the NO concentration gradient at $>400 \mu\text{m}$ above stimulated macrophages. The difference between spatial NO release profiles may be attributed to cell type and NOS expression. Macrophages are responsible for generating biocidal concentrations of NO during inflammation,⁶³ whereas HUVECs release lower, sustained NO fluxes to regulate vascular tone.⁶⁴ Once fully expressed, iNOS produces NO in 1000-fold larger quantities than the constitutive isoforms.⁵⁹

Conclusions

A bilaminar NO sensor was fabricated and evaluated for demanding use in biological media. The combination of a poly(5A1N) electropolymerized film and a xerogel topcoat significantly improved sensor selectivity over common biological interferents. The xerogel membrane also stabilized the sensor's analytical performance characteristics, enabling continuous operation over 24 h in proteinaceous media without reduction in the sensitivity or selectivity. These attributes facilitated the use of bilaminar sensors for monitoring long-term NO release from immunostimulated macrophages both temporally and spatially, the latter with SECM-assisted positioning. The proximal macrophage NO concentration at peak release was approximately 100× smaller than the concentration of accumulated nitrite (as measured by Griess), demonstrating the importance of imparting *and* maintaining selectivity towards NO. The accuracy of the bilaminar electrochemical NO sensor was assured by its sustained selectivity coefficient of −4.2 over nitrite. The distance-dependence of NO measurement from cells motivates the use of planar electrodes in future experiments, where direct culturing of cells on integrated electrode surfaces is anticipated to reduce sampling variability.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Funding Source

This research was supported by the National Institutes of Health (AI112064 and DK108318).

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Fig. 1. Schematic depiction of the Pt/poly(5-amino-1-naphthol)/xerogel (Pt/poly(5A1N)/XG) bilaminar sensor: (blue) XG deposition from condensed alkoxy silane precursors with SEM confirmation; (purple) electropolymerized film of poly(5A1N) and corresponding deposition trace under cyclic voltammetry.

Fig. 2.

(A) Example staircase amperogram collected on a Pt/poly(5-amino-1-naphthol)/xerogel electrode (2.0 mm dia.) in deoxygenated 10 mM PBS (pH 7.4) with 4 inj. of 0.25 μM , 3 inj. of 0.50 μM , and 3 inj. of 1.0 μM nitric oxide (NO) under constant potential amperometry (applied potential: +0.8 V). Inset: corresponding calibration curve from plateau currents. (B) Selectivity coefficients of bare (black) and modified (white) Pt electrodes against common biological interferents nitrite (NO_2^-), L-ascorbic acid (AA), acetaminophen (AP), dopamine (DA), carbon monoxide (CO), hydrogen peroxide (H_2O_2), and ammonia (NH_3). For all selectivity measurements, $n = 8$. ns = $p > 0.05$, * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Fig. 3. Retention of NO sensitivity of bare and modified Pt electrodes (2 mm dia.) in 10% FBS-supplemented DMEM relative to calibrations carried out in deoxygenated 10 mM PBS (n 8). $ns = p \geq 0.05$, $* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$.

Fig. 4.

The nitric oxide (NO)-release profiles of (A) stimulated and (B) unstimulated RAW 264.7 macrophages measured using Pt/poly(5-amino-1-naphthol)/xerogel sensors (2.0 mm dia.) inside an incubator. The current collected from the first 8 h of each trace was fit to a least-squares exponential regression, which was then background subtracted from the entire trace before conversion to NO concentration. The current measured at $t = 2$ h was also subtracted from the traces to address the effect of stimulant injection. Both stimulated (A) and unstimulated (B) received identical background current adjustments.

Fig. 5.

(A) The nitric oxide (NO) diffusion profiles of a confluent sheet of macrophages 8 h after stimulation with 20 ng mL^{-1} lipopolysaccharide and 10 ng mL^{-1} interferon- γ as measured by a Pt/poly(5-amino-1-naphthol)/xerogel microelectrode approaching at a rate of $1 \text{ } \mu\text{m s}^{-1}$ using a scanning electrochemical microscope (4 biological repetitions shown). The background current collected at $600 \text{ } \mu\text{m}$ was subtracted from the entire trace. Repeated with (B) unstimulated cells and (C) stimulated cells in the presence of $160 \text{ } \mu\text{M}$ hemoglobin, a potent NO scavenger. The background current collected at $400 \text{ } \mu\text{m}$ was subtracted from both traces.

Table 1.

Analytical performance metrics of bare and modified Pt electrodes with extended use (n = 3).

Electrode ^a	Medium	<i>t</i> ^b (h)	<i>S</i> _{NO} ^c (pA nM ⁻¹)	LOD ^d (nM)	log <i>k</i> _{NO,NO2} ^e
Pt	PBS	0	18.3 ± 1.6	5 ± 2	-1.2 ± 0.0
		12	14.2 ± 4.8	13 ± 8	-1.3 ± 0.1
Pt/poly(5A1N)	PBS	0	37.0 ± 6.4	1 ± 1	-3.8 ± 0.2
		12	81.5 ± 70.6	3 ± 3	-2.3 ± 0.2
Pt/XG	PBS	0	3.2 ± 1.3	4 ± 1	-2.3 ± 0.3
		12	2.7 ± 0.8	5 ± 5	-2.3 ± 0.1
Pt/poly(5A1N)/XG	PBS	0	12.4 ± 8.0	1 ± 1	-4.9 ± 0.5
		12	10.1 ± 4.9	1 ± 1	-4.8 ± 0.6
Pt/poly(5A1N)/XG	DMEM	0	7.6 ± 4.9	1.3 ± 0.4	-4.1 ± 0.4
		24	10.1 ± 4.0	0.8 ± 0.4	-4.2 ± 0.4

^aPt working electrodes (2 mm dia.).^bOperation time under an applied potential of +0.8 V.^cSensitivity towards NO.^d*S*/*N* = 3.^eCalculated from Eq. 1.