ELECTROCHEMICAL MEASUREMENT OF NITRIC OXIDE
FROM BIOLOGICAL SYSTEMS

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ABSTRACT

REBECCA ANNE HUNTER: Electrochemical Detection of Nitric Oxide
from Biological Systems
(Under the direction of Mark H. Schoenfisch)

Nitric oxide (NO) is known to be involved in a number of physiological processes, including the immune response. As such, its role in severe infection and sepsis has been investigated, but previous measurement techniques have relied on complicated instrumentation or the quantification of NO byproducts (e.g., nitrate and nitrite). Herein, the fabrication of a microfluidic amperometric sensor for the direct detection of NO in whole blood is described. These sensors were used to evaluate the potential of NO and nitrosothiols (a stable transporter) as prognostic and/or diagnostic biomarkers for infection and sepsis.

The microfluidic devices facilitated the selective electrochemical measurement of NO in small volumes of blood at the point-of-care, with adequate sensitivity and limits of detection achieved in buffer, wound fluid, and whole blood. A green (530 nm) light-emitting diode was coupled to the device to enable photolysis of *S*-nitrosothiol species with subsequent NO detection. While inefficient photolysis prevented the measurement of nitrosothiols in whole blood, detection in serum was achieved.

A porcine model of sepsis permitted monitoring of temporal changes in NO and nitrosothiols throughout disease progression. While increases in NO were observed concurrently with other indicators (e.g., increased blood lactate and base deficit), the

accumulation of nitrosothiols was observed hours prior to the onset of other symptoms, despite a dramatic drop in the circulating white blood cells that produce NO.

A murine model of sepsis was utilized to understand the effects of bacterial virulence and immune suppression on NO during an infection. A non-lethal pneumonia with *Pseudomonas aeruginosa* resulted in elevated NO levels at 72 h that returned to baseline concentrations after 1 wk. A more virulent bacterium, *Klebsiella pneumoniae*, resulted in much greater increases in NO, reflecting its pathogenicity. Conversely, in a murine model of post-burn immune suppression and infection, blood NO concentrations remained unchanged relative to uninfected animals despite increased infection severity.

Nitric oxide-selective microelectrodes were also used to study NO release at the single cell level, from both immune cells and neurons. Upregulation of carbon monoxide production by the macrophages was demonstrated to inhibit their ability to release NO following immune stimulation. Additionally, the concentration and kinetics of NO release from neurons were determined.

This work is dedicated to my parents,
who have always encouraged me to work hard and follow my dreams.

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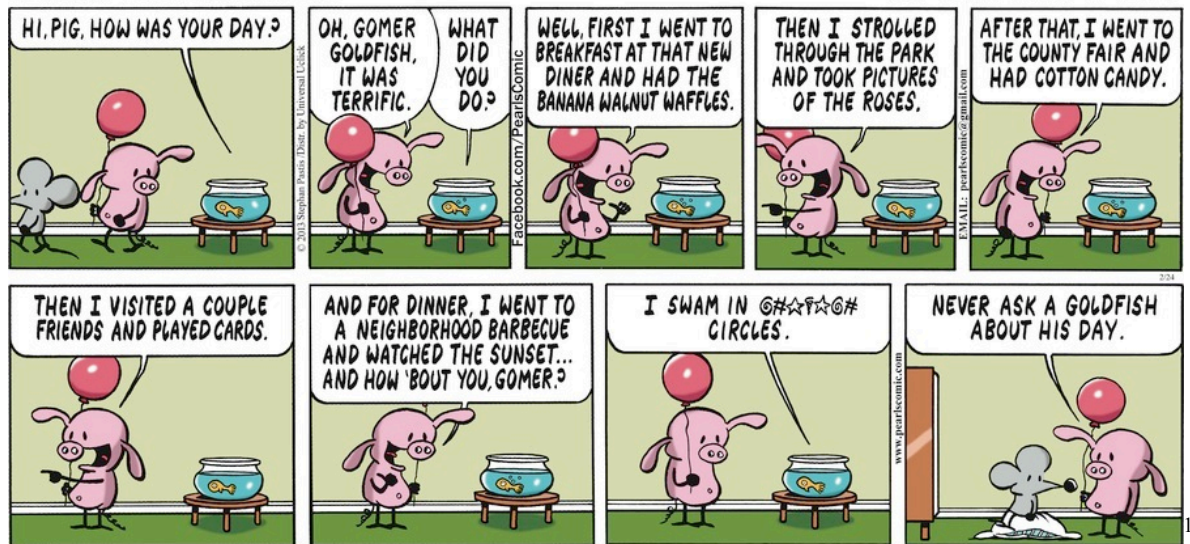
Due in part to the interdisciplinary nature of my research, I've worked with numerous collaborators during my graduate school career. The study of nitric oxide release from snail neurons would not have been possible without our collaborators from the lab of Professor Vincent Rehder at Georgia State University, especially Liana Artinian who always provided us with a place to stay during our visits and even drove a tank full of snails up to our lab for further experiments. The microfluidic devices used to measure nitric oxide in blood were designed with the help of W. Hampton Henley in the lab of Professor J. Michael Ramsey at UNC. All of the porcine sepsis studies in swine were made possible by the lab of Dr. James Manning in the Department of Emergency Medicine at UNC, especially Shane McCurdy and Ben Ellington who endured the long and sometimes frustrating days and nights with me and two pigs. Work in the murine model of sepsis began with the lab of Dr. Craig Coopersmith at Emory University, thanks to the help of Dr. Jonathan McDunn. Studies in this model were able to continue at UNC in collaboration with the lab of Drs. Bruce Cairns and Robert Maile of the North Carolina Jaycee Burn Center and Department of Microbiology and Immunology. I owe special thanks to a fellow graduate student, Julie Malik, for her direct involvement with all of these experiments in the burn model of sepsis. Michelle Palacio, of Professor Virginia Miller's lab at UNC, also assisted with some of these experiments. I am also particularly thankful to the medical doctors we collaborated with, Bruce Cairns and James Manning, for their valuable insight on clinical utility of nitric oxide measurements.

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LIST OF ABBREVIATIONS AND SYMBOLS

~	approximately
°	degree(s)
°C	degree(s) Celsius
%	percentage(s)
±	statistical margin of error
$	dollar(s)
× g	times the force of gravity
$[NO]_m$	maximum NO concentration
7-NI	7-nitroindazole
17FTMS	(heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxysilane
µA	microampere(s)
µg	microgram(s)
µL	microliter(s)
µm	micrometer(s)
µmol	micromole(s)
µM	micromolar
Ag	silver
Ag/AgCl	silver/silver chloride
Ag^+	silver ion
AlbSNO	nitrosoalbumin
Ar	argon gas

ATCC	American Type Culture Collection
Au	gold
CARS	compensatory anti-inflammatory response syndrome
CFU	colony forming unit(s)
CO	carbon monoxide
CO_2	carbon dioxide
CRP	C-reactive protein
Cu	copper
CV	cyclic voltammetry
CVC	central venous catheter(s)
cGMP	cyclic guanosine monophosphate
Cys	L-cysteine
CysNO	nitrosocysteine
d	day(s)
DI	deionized
DNA	deoxyribonucleic acid
DPV	differential pulsed voltammetry
DTPA	diethylene triamine pentaacetic acid
e^-	electron
EDTA	ethylenediaminetetraacetic acid
e.g.	for example
eNOS	endothelial nitric oxide synthase
EPR	electron paramagnetic resonance spectroscopy

et al.	and others
etc.	and so forth
EtOH	ethanol
FBS	fetal bovine serum
FDA	Food and Drug Administration
Fe	iron
GSH	L-gluathione
GSNO	nitrosoglutathione
g	gram(s)
h	hour(s)
H_2O	water
HCl	hydrochloric acid
H_2S	hydrogen sulfide
i.e.	that is
I.V.	intravenous
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
IVC	intravascular catheter
KCl	potassium chloride
KOH	potassium hydroxide
kg	kilogram(s)
L -NAME	*N*-monomethyl-L-arginine

LOD	limit of detection
LPS	lipopolysaccharide
M	molar
MeOH	methanol
mg	milligram(s)
min	minute(s)
mL	milliliter(s)
mm	millimeter(s)
mM	millimolar
mmol	millimole(s)
mV	millivolt(s)
mol	mole(s)
MTMOS	methyltrimethoxysilane
$M\Omega$	megaohm
N_2	nitrogen gas
nA	nanoampere(s)
NaCl	sodium chloride
NADPH	nicotinamide adenine dinucleotide phosphate
NH_4OH	ammonium hydroxide
nM	nanomolar
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NO_2	nitrogen dioxide

NO_2^-	nitrite
NO_3^-	nitrate
NO_X	nitrogen oxdie species
NOS	nitric oxide synthase
o-PD	*o*-phenylenediamine
O_2	oxygen gas
$OONO^-$	peroxynitrite
P	partial pressure
pA	picoamperes
PBS	phosphate buffered saline, pH 7.4
pH	–log of proton concentration
PDMS	polydimethylsiloxane
pM	picomolar
ppb	parts per billion
ppm	parts per million
PROLI/NO	*N*-diazeniumdiolated L-proline
Pt	platinum
PtB	platinum black
PTFE	polytetrafluoroethylene
PVP	poly(vinyl pyrollidone)
rpm	revolutions per minute
RSNO	*S*-nitrosothiol
RT	room temperature

s	second(s)
S1813	S1813 photoresist
SECM	scanning electrochemical microscopy
SIRS	systemic inflammatory response syndrome
Sn	tin
t	time
$[NO]_{tot}$	total concentration of NO
$t_{1/2}$	NO release half life
Ti	titanium
TNF	tumor necrosis factor
TSB	tryptic soy broth
U.S.	United States
UV	ultraviolet
v:v	volume to volume ratio
V	volt(s)
wt	weight
×	times
Zn	zinc
ZnGB	zinc deuteroporphyrin 2,4-bis glycol

CHAPTER 1. PHYSIOLOGICAL SIGNIFICANCE OF NITRIC OXIDE AND ITS MEASUREMENT WITHIN BIOLOGICAL SYSTEMS

1.1 The ubiquitous roles of nitric oxide

Investigation into the physiological roles of nitric oxide (NO) has expanded immensely since the realization that the endothelial derived relaxation factor was, in fact, likely to be NO.[1] Since that time, the pivotal role of this gaseous free radical species has been recognized in numerous biological processes including the immune response to infection,[2-4] vasodilation,[5] would healing,[6,7] and cancer biology.[8] Nitric oxide is known to impart physiological activity through binding to the heme center of soluble guanylyl cyclase (sGC), an enzyme involved in the formation of the second messenger cyclic guanosine monophosphate (cGMP). A multitude of physiological processes are linked to cGMP, including vascular smooth muscle relaxation, platelet activation, and protein kinase signaling cascades.[9] As such, the physiological roles of NO are far-reaching.

Despite the breadth of knowledge that has been gained since its discovery, much remains unknown about NO, especially with regard to absolute concentrations, its role in specific disease states, and how its dysfunction can be corrected for. As such, new detection methodologies are sought after to further the understanding of this small but immensely important free radical species.

1.1.1 *Nitric oxide in the immune system*

Within the immune system, NO is produced by specific cells (i.e., monocytes/macrophages, neutrophils, eosinophils) by the inducible isoform of nitric oxide synthase (iNOS), an enzyme whose expression is regulated by complex cascades of cytokines from different immune cells.[4,10] All isoforms of NOS (i.e., inducible, endothelial, neuronal) produce NO from L-arginine, which is oxidized to produce *N*-hydroxy-L-arginine. Further oxidation of this intermediate yields both L-citrulline and NO. Stimulation of iNOS by cytokines or components of the bacterial cell wall allows for continuous release of NO for up to 5 d, as long as the stimuli remains present and the intracellular L-arginine supply is not depleted.[4] When released by these cells, NO acts as a signaling molecule and may have antimicrobial/anti-tumor (pro-inflammatory) activity or immunosuppressive (anti-inflammatory) effects, depending on the concentration released. For example, the presence of bacteria within the body will signal an upregulation in iNOS production and subsequent release of NO from immune cells will cause killing of nearby microbial organisms.[3,4,11-13] The bactericidal properties of NO are attributed to both nitrosative and oxidative stress.[14] For example, the reaction of NO with oxygen produces toxic byproducts such as dinitrogen trioxide, which initiates nitrosation of protein thiols and DNA deamination. Nitric oxide may also react with superoxide (a radical product of normal cell respiration) to yield peroxynitrite. Buildup of peroxynitrite results in lipid peroxidation and membrane damage.[15-17] While eukaryotic cells have evolved mechanisms for preventing buildup of such toxic byproducts (e.g., production of superoxide dismutase to scavenge superoxide and limit peroxynitrite accumulation), excessive inflammation and NO release may still damage host tissue. For this reason, NO is believed to play an important role in disease states that are caused by severe

infection and/or injury.[18,19] While NO may be elevated to dangerous levels in the presence of high concentrations of bacteria, low NO concentration may indicate immune suppression and/or an insufficient response to infection.

Nitric oxide may also serve an opposing role in the immune system as an anti-inflammatory molecule or immune suppressant, providing a mechanism to defend the host from itself. For example, NO suppresses T helper cell proliferation and cytokine production, thus limiting the chronic immune response.[20] Neutrophils, which are involved in the body's non-specific response to infection, while stimulated by low NO concentrations, may experience inhibited adhesion to endothelial cells when exposed to high concentrations of NO, thus hindering their ability to traverse the vascular wall and migrate to local sites of infection.[21] Further, NO has been observed to inhibit mast cell degranulation.[22] While these negative feedback mechanisms help to prevent potentially dangerous chronic inflammatory states, immune dysfunction is still prevalent in severe diseases such as sepsis.

1.1.2 Nitric oxide in the nervous and cardiovascular systems

Unlike the immune system, NO in the nervous and cardiovascular systems is generally not produced by an inducible NOS isoform. Instead, it is generated by NOS isoforms that are triggered by increases in intracellular calcium: endothelial (eNOS) and neuronal (nNOS) nitric oxide synthase for the cardiovascular and nervous systems, respectively.[23-25] Small, transient increases in intracellular calcium concentrations will thus cause short-lived production of NO (nM amounts within 10 min),[26] in contrast to the sustained production resulting from iNOS stimulation.

The physiological importance of NO was first realized within the cardiovascular system, where it is now known to regulate vascular tone and prevent platelet and leukocyte

adhesion.[5,23,27,28] Due to its function as a blood pressure regulator, reduced bioavailability of NO may be of importance in a number of cardiovascular diseases.[29] Naseem reported that reduced NO release from the endothelium may lead to more rapid progression of diseases such as atherosclerosis, where adhesion of platelets and leukocytes contribute to buildup and blockage along the vascular wall.[29] Additionally, hindered NO production could lead to decreased angiogenesis. Conversely, overproduction of NO would cause dangerous systemic vasodilation.

Within the central nervous system, NO is involved with general neurotransmission, thermal regulation, hormone release, and sleep cycles.[30-33] Nitric oxide is also known to play a key role in long-term potentiation (memory formation).[34] Studies by Rehder et al. have indicated the importance of NO in neuronal development, as it regulates the extension of neuronal growth cone filopodia.[35,36] Nitric oxide produced by nNOS within the peripheral nervous system is also involved with smooth muscle relaxation.[25] Similar to the cardiovascular and immune systems, dysfunctional NO production within the nervous system can contribute to disease. Inflammation and the resulting NO production contributes to numerous neurodegenerative diseases, including Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, and multiple sclerosis.[37]

1.2 Nitrosothiols as physiological transporters of nitric oxide

Nitric oxide is a free radical gaseous species with a half-life ranging from milliseconds to seconds depending upon its concentration and the medium in which it exists.[38] As such, its lifetime in the body, most notably in blood, is believed to be as short as 2 msec due to the presence of scavengers (i.e., proteins and oxygen).[39] Conversely, *S*-nitrosothiol species, originally known as thionitrites, are more stable biological transporters

of NO and transducers of NO bioactivity. Nitrosothiols have been shown to exhibit effects similar to NO in vivo, including smooth muscle relaxation[40] and reduced platelet adhesion.[41] Unlike free NO radicals, nitrosothiols do not react directly with metalloproteins (e.g., hemoglobin) or other radical species (e.g., superoxide), thus increasing their lifetime and preventing the buildup of toxic species such as peroxynitrite.

S-nitrosothiols are synthesized on the bench top via a reaction between thiols and species such as nitrous acid, dinitrogen trioxide, dinitrogen tetraoxide, or nitrosyl chloride.[42] In vivo, nitrosothiols may be formed via multiple reactions involving NO and/or its byproducts with the thiols of proteins (e.g., albumin and hemoglobin), amino acids (e.g., L-cysteine), and other small molecules (e.g., glutathione).[43] Blood plasma alone contains ~600 μM thiol "groups", including ~500 μM albumin and 20–45 μM low molecular weight thiols (i.e., L-cysteine and glutathione).[44] Despite it's greater size and abundance, albumin contains only one free cysteine residue at which nitrosothiols form. Though once considered a major scavenger of NO, Stamler et al. demonstrated the ability of hemoglobin to accommodate nitrosation of its β-93-cysteine group, thus conserving the biological reactivity of NO by forming S-nitrosohemoglobin.[45] Nitric oxide stored in this manner may later be released by multiple mechanisms, most notably under hypoxic conditions.[46-48]

As shown in Figure 1.1, a common route of nitrosothiol formation requires a mixture of NO, oxygen (O_2), and a thiol.[49] One of many nitrosothiol formation pathways relies on the oxidation of NO by O_2, where a peroxynitrite radical is generated and then further reacts with NO to produce nitrogen dioxide ($\cdot NO_2$). At normal physiological concentrations of NO (i.e., <100 nM), this reaction is unlikely as it may take hours to proceed.[50] In the event that this reaction does occur, $\cdot NO_2$ reacts directly with a thiol to form a thiyl radical, which can

subsequently combine with NO to form nitrosothiol. Alternatively, $\cdot NO_2$ may react with another molecule of NO to form dinitrogen trioxide (N_2O_3), which directly nitrosates a thiol species. In the event that NO autooxidation does not take place, NO reacts directly with a thiol, forming an aminoxyl radical that can be oxidized to form nitrosothiol. Evidence suggests that copper and iron ions can also generate *S*-nitrosothiols. The proposed mechanisms involve either a one-electron oxidation of a thiol (with subsequent reaction of the thiyl radical with NO) or the formation of a complex between NO and the metal.[51,52] This mechanism is complicated by the fact that these same metals also initiate nitrosothiol decomposition. Within cellular compartments, transnitrosation is important in the formation and function of *S*-nitrosothiols.[53] In this reaction, a thiolate anion attacks a nitroso nitrogen, allowing for transfer of the *S*-nitroso functional group as shown below:

$$RS^- + R'SNO \rightleftharpoons RSNO + R'S^-$$
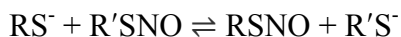
Of note, transnitrosation rates are not equal between all thiols, and may vary between 0.1 and 500 $M^{-1}s^{-1}$.[50]

While *S*-nitrosothiols species are considered stable relative to the lifetime of free NO in vivo, numerous mechanisms exist by which nitrosothiols decompose to release NO. As shown in Figure 1.2, thermal and photolytic cleavage are two decomposition mechanisms that result in hemolytic cleavage of the S–N bond, generating a thiyl radical and gaseous NO.[54] While trace metals such as copper have been implicated in the formation of nitrosothiols in vivo, they are also closely linked with their catalytic decomposition. Copper(II) may be reduced by trace thiolate, with the resulting copper(I) reacting with nitrosothiols to liberate NO upon returning to its oxidized state.[54] As mentioned previously,
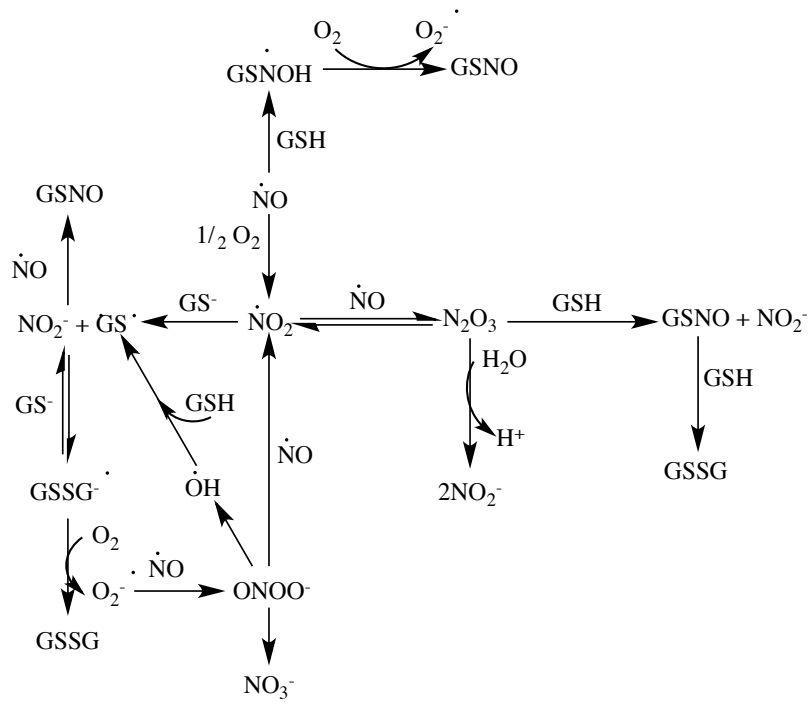
Figure 1.1 Routes of nitrosothiol formation in vivo requiring nitric oxide, oxygen, and glutathione.
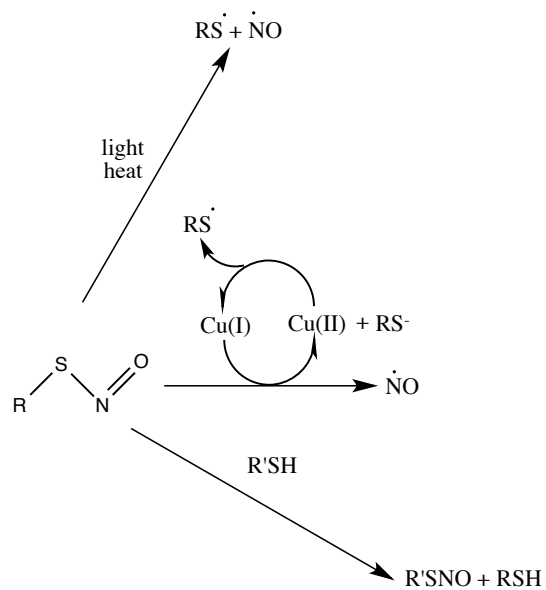
Figure 1.2   Routes of decomposition of nitrosothiols.

transnitrosation between a nitrosothiol and free thiols is an alternative and biologically relevant route of decomposition.

As the formation and decomposition of nitrosothiols in vivo are so closely linked with NO-dependent pathways, a major goal has been to study their concentrations within cells and tissues. Within mouse macrophage cells (RAW 264.7), *S*-nitrosothiol levels are reported to be ~5 pmol mg^{-1} protein.[55] Reported basal concentrations of nitrosothiols in blood plasma range widely, from 1 nM in rats[56] to µM levels in humans, depending on the measurement technique utilized.[57] Certain disease states lead to even greater levels of nitrosothiol formation and accumulation. In one example, nitrosothiol levels were observed to increase from 300 nM in healthy subjects to 4 µM in pneumonia patients.[58]

While the roles of both NO and nitrosothiols in the body are apparent, much remains unknown regarding specific pathways and mechanisms of action. As such, techniques to accurately monitor small concentration changes and pinpoint specific protein targets are critical for future studies.

1.3   Detection of nitric oxide and nitrosothiols

1.3.1   *Spectroscopic detection of nitric oxide*

To date, NO can be measured in a number of ways, each with distinct advantages. Spectroscopic methods (i.e., absorbance, fluorescence, chemiluminescence, and electron paramagnetic resonance) rely on the indirect detection of NO via byproducts of reactions with other chemicals or adducts formed between NO and other compounds.[59] First described in 1864,[60] the Griess assay, formally know as the diazotization assay, allows for the quantification of total NO concentrations via nitrite analysis. Nitrite is a stable byproduct of

the reaction of NO with oxygen in aqueous media, and upon reaction with sulfanilamide and

N-(1-napthyl)ethylenediamine an azo dye is formed. Absorbance spectroscopy is then used to

relate the concentration of dye to the concentration of nitrite (and thus NO that was in

solution). Although inexpensive and easily obtained commercially, the Griess assay only

provides a limit of detection of ~0.5 µM in buffer solution and is mainly useful for

determination of NO totals.[59,61,62] The lack of real-time information and poor limit of

detection of this assay preclude its use for analysis of biological solutions.

In contrast, chemiluminescence detection, a more costly method due to

instrumentation requirements, measures NO directly via reaction with ozone.[63] This reaction

produces an excited state nitrogen dioxide, which emits a photon upon relaxation to the

ground state. Nitric oxide-release kinetics may be determined directly from materials in

solution, as the NO is immediately transported from solution to the detector by an inert

carrier gas. As an alternative, nitrate and nitrite in solution can be measured following their

reduction to NO. These spectroscopic techniques generally require complex instrumentation

and thus are not amenable to in vivo applications. Additionally, their use with biological

media (e.g., cell culture solutions and blood/serum) is limited due the need to purge solutions

of oxygen.

1.3.2   Real-time amperometric detection of nitric oxide

Due to NO's redox activity, electrochemistry is also commonly used to quantify NO

in a variety of formats (e.g., direct oxidation via amperometry) and even at the single cell

level due to the ability to miniaturize sensors.[62] Currently, electrochemical methods for

detecting NO remain the most pliable for biological applications given their adaptability and

capacity to measure real-time release. Compared to other techniques, the electrochemical

measurement of NO provides some distinct advantages, including superior spatial and temporal resolution, the potential for low limits of detection, and the ability to tune selectivity and sensitivity by changing the potential applied or modifying the electrode with a catalyst and/or permselective membrane.[59,64] Electrode platforms of varied shapes and sizes can be fabricated for the amperometric detection of NO, providing excellent spatial resolution when required.

Despite the simplicity of this measurement technique, a number of important considerations must be made to carry out accurate physiological measurements. The sensitivity, limit of detection (LOD), and linear response range of the sensor are especially important given the varied, but generally small concentrations of NO (i.e., picomolar to micromolar) that exist physiologically. For an amperometric measurement, selectivity is equally important given the number of potential electroactive interferents that may be present in vivo (e.g., nitrite, nitrate, hydrogen sulfide, ascorbic acid, dopamine). Due to its reactive nature, not all the NO released from a system may reach the electrode surface to be detected. As such, the size and shape of the electrode must be carefully considered for each application. For example, measurement of NO from a single cell adhered to a surface would best be achieved by placing a small electrode (similar in size to the cell) directly above the cell, thus limiting the distance traveled and the amount of NO that is allowed to diffuse and/or react without being detected. Despite such operational challenges, a number of sensor systems have been developed and demonstrated to accurately measure physiological levels of NO.

Regardless of sensor design, each transducer must include a surface at which to oxidize or reduce NO and a means of excluding potential interferents (i.e., species that are

oxidized/reduced at a similar potential). The most common electrodes are platinum,[2] carbon fiber,[65] glassy carbon,[66] and gold.[67] Platinum is perhaps most widely utilized as it can be coated with small platinum black particles to increase microscopic surface area, thus significantly improving sensitivity and detection limits.[68-70]

Amperometric measurements in biological systems are made even more challenging by a number of interferents that may be present in whole blood, plasma/serum, or cell/bacteria culture medium. Such species are electroactive at working potentials similar to or lower than NO (i.e., +0.7–1.0 V for oxidation). Common biological interferents and their concentrations are given in Table 1.1. The membranes utilized for enhancing selectivity toward NO vary significantly depending on other sensor characteristics. Most selective membranes rely on exclusion of interferents based on size and/or charge. For example, Nafion® is a widely used hydrophobic, cation exchange fluoropolymer that is applied via a dip-coating process and excludes interferents based on charge.[71] While applied in a manner similar to Nafion®, xerogel-based permselective membranes allow for greater control of hydrophobicity and porosity. Shin et al. first described the use of xerogels derived from fluorinated alkylalkoxysilane precursors to maximize selectivity of electrodes toward NO.[72,73] Additionally, xerogels may be applied to any sensor geometry (i.e., disk type, conical, planar) via dip coating, casting, or spray coating application of the sol.[74] To exclude interferents based on size, Ferreira et al. electropolymerized o-phenylenediamine (o-PD) onto carbon film electrodes that had previously been dip coated with Nafion®.[75] This treatment allowed for a 30:1 selectivity for NO over interferents tested (e.g., nitrite and ascorbic acid), but was not particularly effective against small cationic species (e.g., dopamine and serotonin). In another study, Kato et al. utilized cross-linked Langmuir-Blodgett (LB)

Table 1.1 Common biological interferents and their basal concentrations in vivo.

Interferent	In vivo concentration	Tissue/media	Reference
Hydrogen sulfide	< 100 nM	blood	76
Ammonium (pH 7.4)	30 µM	blood	77
Nitrite	176 nM	blood	78
Uric acid	254 µM	serum	79
Ascorbic acid	43 µM	serum	80
Acetaminophen	130 µM	plasma	81
Ammonia (pH 11)	35 pM	blood	77
Peroxynitrite (ONOOH/ONOO⁻)	30 pM	endothelium	82
Carbon monoxide	0.5–1.5 µM	mouse kidney	83

films of oligo(dimethyl-siloxane) copolymer as permselective coatings, allowing for a 25:1 selectivity for NO over nitrite while maintaining sensitivities to NO that were similar to bare electrodes.[84] An alternative means of enhancing selectivity without coatings is the use of nanopore electrodes. Shim et al. developed a nanopore-platinized platinum electrode for NO detection by electrochemically etching a platinum nanodisk and subsequently depositing layers of porous platinum black onto the disk.[85] The inside of the pore was then silanized to increase hydrophobicity and further enhance sensitivity and selectivity.

While all electrochemical NO sensors utilize a specific working electrode material and selective membrane, varied sensor styles can also be considered depending on the application. The Shibuki-style sensor, based on the original oxygen electrode developed by Clark et al.,[86] is comprised of a micropipette which contains both the working (platinum) and reference (silver) electrodes. This micropipette is then sealed with a gas-permeable membrane and filled with electrolyte solution.[87] As the construction of the Shibuki sensor limits its amenability to miniaturization, new sensors were designed to avoid the requirement of an internal filling solution. Solid permselective electrodes allow for direct modification of the electroactive area with a hydrophobic membrane, typically via a simple dip coating or casting process. This advancement accommodates the use of electrodes of varied shapes and size. Designed in a manner similar to the solid permselective electrodes, solid catalytic sensors were created to improve the efficiency of the oxidation or reduction of NO at the electrode surface and/or allow for a reduction in the working potential. Such electrodes usually include a mediator capable of catalyzing the electrochemical process directly on the electrode surface or within a permselective membrane.[88]

Each of the aforementioned sensor designs is capable of detecting NO via its oxidation or reduction. While electrooxidation is primarily used, some reports of NO measurement via electroreduction exist.[89-91] This reaction proceeds via the following two electron process at potentials ranging from -0.5 to -1.4 V vs. Ag/AgCl:[88]

$$2NO + 2e^- \rightarrow N_2O_2^-$$
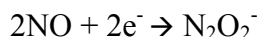
Of note, oxygen is a major interferent in this process as it is also reduced at -0.6 V vs. Ag/AgCl via the process given below:

$$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$$
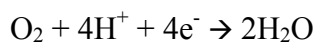
While most other interfering species are avoided when utilizing electroreduction, reduced sensitivity is a caveat. Furthermore, electroreductive NO sensors do not operate efficiently at physiological pH and are characterized by poor detection limits (i.e., μM range).[92] Improved sensitivity is possible by coating electrodes with complexes that catalyze the reduction of NO (e.g., chromium complexes,[93] hemoglobin[94]). Despite these few reports of NO detection via electroreduction, oxidation of NO remains the most common means of detecting NO electrochemically.

Nitric oxide is readily oxidized at potentials ranging from +700–900 mV vs. Ag/AgCl reference via the three-electron process below:[95,96]

$$NO \rightarrow NO^+ + e^-$$

$$NO^+ + OH^- \rightarrow HNO_2$$

$$HNO_2 + H_2O \rightarrow NO_3^- + 2e^- + 3H^+$$
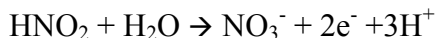
Conversion of the nitrosonium cation (NO^+) to nitrite is irreversible.[64] Due to the aforementioned presence of interferents (Table 1.1) and the relatively high working potential, the use of a selective membrane is required for sensors that utilize this process to detect NO.

Catalytic oxidation of NO allows for enhancement of both selectivity and sensitivity by reducing the potential at which NO is oxidized, thus limiting the effect of interfering species. Recently reported electrode modifications include metalloporphyrins, metallophthalocyanines, self-assembled monolayers (SAMs), and protein catalysts. For example, Hrbáč et al. utilized the electropolymerization of nickel porphyin onto carbon fiber microelectrodes to achieve a 6-fold increase in sensitivity while also enhancing selectivity.[97] Unfortunately, metalloporphyrins degrade rapidly, so metallophthalocyanines have been adopted as a more stable alternative electrocatalytic layer.[98] Self-assembled monolayers form highly stable chemical bonds with glassy carbon electrodes, making them useful as electrocatalysts for compounds with relatively high oxidation potentials (e.g., NO). Sivanesan et al. utilized SAMs of 1,8,15,22-tetraaminophthalocyanatocobalt(II) to decrease NO's oxidation potential by 310 mV while simultaneously increasing the oxidation current produced.[99]

Electrochemical detection continues to be one of the most promising methods for NO measurement, especially from biological systems. Further enhancements in sensitivity and selectivity, as well as new sensor configurations (e.g., microfluidic devices) will allow for greater understanding of the many essential roles of NO in vivo.

1.3.3 Detection of nitrosothiols

To date, the techniques most commonly utilized for nitrosothiol analysis exploit indirect detection schemes.[100] Indirect detection of *S*-nitrosothiols is achieved via decomposition and detection of the resulting products (i.e., NO, NO^+).[62,100] Despite their relative stability, nitrosothiol species will decompose via multiple pathways (i.e., heat, light,

and chemical reduction) as shown in Figure 1.2. The figures of merit for the most commonly utilized methods for nitrosothiol detection are outlined in Table 1.2.

The Saville assay is a colorimetric assay modeled after the Griess assay.[101] Mercuric ions (Hg^{2+}) are used to cleave the S–NO bond. The NO^+ generated from this cleavage then reacts with O_2 to form nitrite (NO_2^-). The reaction then proceeds in the same manner as the Griess assay, where nitrite is reacted with acidic sulfanilamide and *N*-(1-naphthyl)ethylenediamine to form an azo dye which is detected spectrophotometrically.[60] Despite the simplicity of this assay, in vivo nitrite concentrations are usually much greater than that of nitrosothiols, making accurate quantification of nitrosothiols challenging. Indeed, the limit of detection for the Saville assay is ~500 nM in buffer solution. Analogous to the Saville assay, nitrite generated from nitrosothiols can be reacted with 2,3-diaminonaphthalene to form the fluorescent 2,3-naphthotriazole ($\lambda_{excitation}$ = 365 nm, $\lambda_{emission}$ = 405 nm) for in situ detection.[102] As a more selective alternative, 4,5-diaminofluorescein can be used to react directly with NO released from nitrosothiols.[103] However, while the UV photolysis utilized to decompose nitrosothiols is efficient, it also causes NO generation from nitrate, leading to artificially elevated NO totals.[78] Chemiluminescence NO detection has also been combined with reductive or photolytic cleavage of nitrosothiols.[104] Detection limits have been reported in the nM range with various reducing agents employed depending on the application (e.g., Cu^+/cysteine, iodine/triiodide).[105,106] Although less common due to cost, high performance liquid chromatography (HPLC) has been used to separate and detect different classes of nitrosothiols. Specifically, Marzinzig et al. coupled reverse phase HPLC with electrochemical or fluorescence detection to separate and quantify mixtures of low molecular weight nitrosothiols.[107] While reductive cleavage of the S–NO bond would

Table 1.2 Summary of predominant analytical methods for detecting *S*-nitrosothiols in biological samples.

Method	Detected species	LOD	RSNOs analyzed	Sample
Saville assay	Azo dye	500 nM	LMW[1]	Plasma
Chemiluminescence	NO_2^*	pM	LMW + AlbSNO	Blood Plasma Serum
Fluorimetry	Fluorescent adduct	nM	LMW + AlbSNO	Blood
Electrochemistry	NO	10 nM	LMW + AlbSNO	Plasma Serum Diluted blood

[1]LMW: low molecular weight nitrosothiols

theoretically allow for a means of direct nitrosothiol detection via electrochemistry, this method would suffer from O_2 interference and not accommodate selective detection of different types of nitrosothiols due to the similar reduction potentials required. As such, indirect electrochemical detection of nitrosothiols is achieved in a manner similar to other nitrosothiol assays, where NO detection (via electrochemical oxidation) follows nitrosothiol decomposition.

Compared to other techniques, detection of nitrosothiols via electrochemistry provides superior spatial and temporal resolution without the requirement of complex or costly instrumentation. *S*-nitrosothiol decomposition to NO may be achieved in a manner identical to other techniques, including chemical reduction and photolysis. Copper catalyzed reduction is one such method and whereby the addition of copper chloride salt or the addition of copper(II) in the presence of an additional reducing agent (e.g., glutathione, L-cysteine, ascorbate) breaks down the nitrosothiols to NO. Despite the facile nature of this method, NO generation (and thus nitrosothiol amount detected) varies significantly based on the amount of Cu^+ or the ratio of Cu^{2+} to reducing agent.[108-110] Furthermore, the addition of catalyst solutions to small biological samples is inconvenient and has the potential to dilute already small quantities of nitrosothiols to undetectable levels. To deal with these shortcomings, Meyerhoff et al. developed immobilized catalysts for the decomposition of NO and incorporated them into electrochemical NO sensors. A number of catalysts (e.g., copper, organoselenium, organotelluride) were immobilized into sensor membranes that coated the tip of the working electrode to facilitate nitrosothiol decomposition with immediate NO detection.[111-114] Organoselenium-based sensors generated superoxide upon reaction of oxygen with the catalyst, and thus their utility was limited by NO scavenging.[115]

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Nevertheless, these devices were utilized to estimate levels of both low and high molecular weight nitrosothiols in biological samples. Meyerhoff et al. also developed nitrosothiols sensors that entrapped glutathione peroxidase behind a NO-selective PTFE membrane. Exploiting a similar idea, Bedioui et al. designed a ring-disk ultramicroelectrode where the center disk oxidized electrodeposited Cu(0) to Cu(I).[116] The Cu(I) released facilitated nitrosothiol decomposition and the outer ring was used to detect the resulting NO. The same group also demonstrated the utility of this method using both dual disk-disk and band-band electrode configurations.[116]

Alternatively, nitrosothiol decomposition may be triggered by light, specifically in the wavelength ranges of 290–270 nm and 505–625 nm.[117] Decomposition via photolysis occurs by direct homolytic cleavage of the S–NO bond without involvement of side reactions.[117] Coupling this decomposition pathway with electrochemical detection of NO is particularly appealing as the light intensity (and thus NO generation) is highly tunable. Light sources can easily be adapted to a variety of electrochemical configurations, including small microfluidic devices. Additionally, this method does not require the addition of chemicals to samples, which is especially important if the sample volume is small and/or when measurements must be made rapidly. Riccio et al. described the combination of visible photolysis and amperometric detection of *S*-nitrosothiols.[118] Visible light (532 nm) was chosen to avoid electrochemical interference, as ultraviolet (UV) light generates NO from nitrite in solution, and would thus overestimate nitrosothiol concentrations.[78] Despite slightly decreased sensitivity relative to copper catalysts, this work demonstrated how tuning the properties of a light source (e.g., varying light intensity) could change the rate and amount of NO generation from both low and high molecular weight nitrosothiols in phosphate buffered saline and

blood plasma. Despite these advantages, the measurement required deoxygenation of the sample to overcome NO scavenging by oxygen. Additionally, relatively large sample volumes (~20 mL) were required for analysis. While these shortcomings limit the current clinical utility of this measurement technique, a light source could easily be coupled with numerous sensor configurations. For example, incorporation into a microfluidic device would allow for improved sample irradiation with enhanced sensitivities and detection limits. Additionally, a microfluidic device is more amenable to the small sample volumes required for future clinical analysis applications.

1.4   Nitric oxide and nitrosothiols in sepsis

The general understanding of sepsis within the medical community has varied greatly throughout the years. During the sixteenth century, what we now understand to be sepsis was referred to as "hectic fever."[119] Even then, Machiavelli understood that this mysterious malady was easy to treat but difficult to detect at its inception, while later becoming simple to recognize but challenging to cure.[119] More recently, sepsis was simply considered to be a systemic host response to infection.[120] A more detailed, concrete definition of sepsis was not outlined until 1992, when the American College of Chest Physicians and the Society of Critical Care Medicine convened and came to a consensus on the differences between the spectrum of syndromes related to the body's response to infection.[121] As outlined in Table 1.3, this spectrum includes systemic inflammatory response syndrome, sepsis, severe sepsis, and septic shock, each presenting with different symptoms.[122] To date, these definitions have not been modified.

Table 1.3   Definition of systemic inflammatory response syndrome, sepsis, severe sepsis, and septic shock in humans.

Syndrome	Definition
Systemic inflammatory response syndrome (SIRS)	2 or more of the following: - Body temperature >38°C or <36°C - Heart rate >90 beats per minute - Respiration >20 breaths per minute or $PaCO_2$ <32 mmHg - White blood cell count >12,000 mm^{-3} or <4,000 mm^{-3}
Sepsis	SIRS caused by suspected or confirmed infection
Severe sepsis	Sepsis with acute organ dysfunction, hypotension, or hypoperfusion
Septic shock	Sepsis with hypotension/hypoperfusion despite adequate fluid resuscitation

The current framework for classifying patients with sepsis is extremely important given its severity, the challenge of diagnosis, and associated treatment strategies. A recent epidemiological study examined trends in sepsis from 1979 through 2000, revealing that >750,000 cases of sepsis occur annually in the United States, accounting for 1.3% of all hospitalizations.[123] While mortality has decreased over the years and currently ranges from 18–30%, sepsis remains the leading cause of death in critically-ill patients. Recent statistics indicate sepsis-related incidences are increasing by 8.7% annually. In the United States alone, healthcare costs for treating sepsis exceed $16 billion.[124] Clearly the need for improved understanding, diagnosis, and treatment of the disease and its underlying causes is highly warranted.

1.4.1   Sepsis disease progression

The immune response to pathogens broadly is a normal physiological response and is usually tightly regulated. During a typical infection by a limited number of bacteria, the innate immune response is initiated upon detection of the invading organisms and the polarization of local macrophages to the M1 (pro-inflammatory) phenotype.[125] Detection of foreign pathogens can occur through a number of mechanisms, although the best understood are pathogen-recognition receptors (e.g., Toll-like receptors) that identify bacterial cell wall components (e.g., lipopolysaccharide). The resulting "activated" macrophages (along with other immune cells) phagocytose the invading organisms and produce pro-inflammatory cytokines (e.g., tumor necrosis factor, IL-6, and IL-8) in order to recruit other circulating white blood cells (e.g., neutrophils, lymphocytes, monocytes) to assist. Following uptake of the bacteria by the macrophage, it becomes entrapped within the phagolysosome, where the

combination of reduced pH, proteases, and other antimicrobial agents (including NO) cause rapid killing and clearance of the invading species.

Sepsis, being a systemic inflammatory response, occurs when this normal response to infection becomes so severe or dysfunctional that it causes harm to the host. Such action may be caused when the bacterial load present is too large for the body to handle, when the infection is due to a particularly virulent strain, or when the immune system is not functioning properly. This dysfunction will cause a number of physiological changes, both at the cellular and systemic levels, leading to severe and dangerous symptoms. During the later stages of sepsis, the body enters an immunosuppressive phase, termed the compensatory anti-inflammatory response syndrome (CARS).[126] This phase of the disease is particularly dangerous, as it increases patient susceptibility to secondary infections and death. Figure 1.3 illustrates this changing immune response over time during the progression of sepsis.

During the first phase of sepsis, a dramatic increase in production of pro-inflammatory cytokines is observed. This phenomenon, know as the "cytokine storm," allows the innate immune system (i.e., macrophages and neutrophils) to communicate with the body's adaptive immune system.[127,128] This overstimulation also causes a significant upregulation of the expression of iNOS[129-131] and certain cell surface markers (e.g., CD80 and CD86), which permit juxtacrine signaling between phagocytes and T cells.[132,133]

Cell death is another major hallmark of sepsis, including both necrosis and apoptosis. Necrosis, which usually occurs following an ischemic injury and results in slow death due to lack of nutrient supply, causes leakage of caustic enzymes which may also damage surrounding tissue.[134,135] Conversely, apoptosis is a programmed cell death that can occur via binding of proteins to specific receptors or a mitochondrial-mediated pathway. This process

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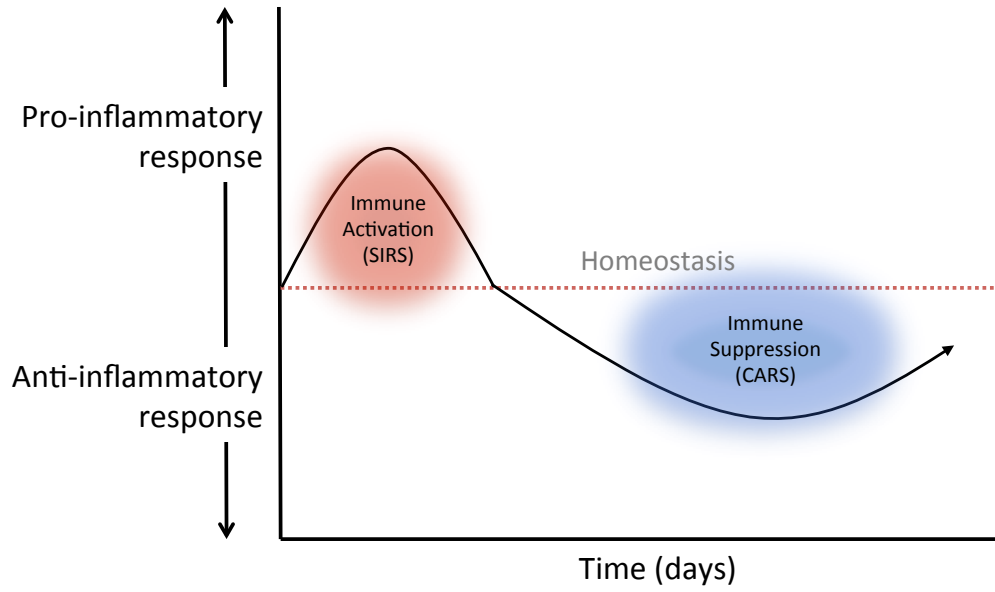
Figure 1.3 Changes in immune response during the progression of sepsis in human patients. Adapted from Hotchkiss et al.[136]

is a normal part of cellular proliferation, but occurs to an extreme and detrimental extent during sepsis. Of note, apoptosis is more pronounced in lymphocytes and gastrointestinal epithelial cells during sepsis.[137,138] Lymphocyte death is linked to the CARS that is observed in later stages of sepsis.[139,140] Under normal circumstances, the intestine has an unusually high cell turnover rate, making it particularly sensitive to changes in apoptosis.[141] In contrast to this increased cell death, neutrophils often undergo delayed apoptosis during sepsis, leading to further damage to tissue by the release of toxic antimicrobial products.[142]

1.4.2 Clinical manifestations of sepsis

These phenotypic and chemical changes at the cellular level manifest a number of systemic physiological changes that may be monitored in the hospital setting. As provided in Table 1.3, physicians consider a set of symptoms that define sepsis within a spectrum of disorders. In addition to these basic physiological metrics, a number of potential biomarkers for sepsis have been proposed for diagnostic and/or prognostic use. Due to the shear number of physiological processes involved in sepsis and its systemic nature, the quantity of biomarkers that have been evaluated to date is immense, with at least 178 independent biomarkers since 2010 alone.[143] Most biomarkers have been tested for their prognostic ability, but of greater importance may be the ability to improve the diagnosis of sepsis, as early and appropriate treatment is known to improve patient outcomes.[144-147] The most commonly studied categories of biomarkers include cytokines/chemokines and acute phase proteins. A number of cells (most notably immune cells) release cytokines and chemokines in order to mediate the pathophysiology of sepsis. For example, pro-inflammatory cytokines such as tumor necrosis factor[148,149] and various interleukins[150-169] are released at high levels in response to infection. Similarly, anti-inflammatory cytokines and chemokines may be

examined to monitor the immunosuppressive phase of sepsis.[170] While clinical and experimental studies have revealed that these biomarkers are elevated in sepsis and may predict survival,[171] they are neither sensitive nor specific enough to be clinically useful. Acute phase proteins such as C-reactive protein may aid in predicting a patient's response to therapy, but serve no diagnostic value.[172-174] Other categories of molecules that have been studied for their diagnostic and/or prognostic value include cell surface markers, coagulation biomarkers, vasodilation biomarkers, organ dysfunction biomarkers, vascular endothelial damage biomarkers, and receptor biomarkers.[143] However, no one biomarker to date has proven both sensitive and specific enough to become routinely implemented in sepsis monitoring. Future approaches must include the evaluation of new potential biomarkers as well as multi-biomarker panels for more accurate diagnosis.[170]

1.4.3   Nitric oxide and nitrosothiols as prognostic/diagnostic biomarkers

Due to the well-established roles of NO in the immune system and inflammation, as well as its role in blood pressure maintenance, many have hypothesized that endogenous NO levels may become erratic during the development and progression of sepsis. For example, multiple studies have indicated increases in iNOS during progression of sepsis.[129,131,175,176] Regulation of iNOS has been examined to determine whether its up- or down-regulation may be beneficial for the treatment of sepsis, but the results have been mixed. For example, some findings have indicated that inhibition of iNOS provides a protective effect and thus improves outcomes.[177-179] In contrast, others have reported that NO scavenging or iNOS deficiencies actually worsen outcomes, likely due to the role of NO in mediation of infection.[180,181] Such conflicting results indicate a delicate balance and the need for methods that more directly determine circulating NO and nitrosothiol levels in vivo. To date, a

number of studies have examined changes in metabolites of NO (i.e., nitrate, nitrite, and nitrosothiols) more directly during sepsis in humans and other animal species, most frequently utilizing chemiluminescence and colorimetric detection methods. The results of select studies have been summarized in Table 1.4. While the reported concentrations vary widely, an increase in endogenous NO relative to controls was observed in almost all cases. One notable exception was reported by Jacob et al., who observed a decrease in plasma nitrate/nitrite levels in patients with trauma and infection relative to control (no trauma or infection) patients.[182] Despite this trend, an increase was observed relative to patients with trauma but without infection. The ability to measure NO and nitrosothiols directly in whole blood and/or plasma would provide a clearer understanding of the mechanisms involved in this immune dysfunction.

1.4.4   Animal models of sepsis

Outcomes for sepsis may only be further improved through advancements in understanding mechanisms of disease pathophysiology and the development of new therapeutics. Well-controlled and reproducible studies to achieve such an outcome are only possible through the use of animal models.[183] Preclinical (i.e., animal) studies are especially necessary for testing of potential therapeutic agents prior to human clinical trials. The main concern in planning such studies is that the model accurately replicates the complex physiological state of human sepsis, in which the inflammatory response develops over days and leads to multi-organ failure when not treated promptly.

While mimicking human sepsis remains a priority,[184,185] the use of nonhuman primates in sepsis research is rare, due in part to ethical objections.[186] Large animal species—including dogs, sheep, and pigs—are often used and share some similar disease pathways

Table 1.4   Summary of reported changes in NO and its metabolites during sepsis.

Species	Analyte	Medium	Detection Method	Control	Septic	Reference
Human	NO_X[a]	Plasma	Capillary electrophoresis	20 ± 3 μM	144 ± 39 μM	130
Human	NO_X	Plasma	Reduction/ chemiluminescence		133 ± 48 μM	187
Human	NO_X	Plasma	Griess assay	29 ± 4 μM	72 ± 9 μM	19
Human	NO_X	Plasma	Griess assay		90–150 μM	188
Human	NO_X	Serum	Griess assay	43 ± 24 μM	118–145 μM	189
Human	RSNO	Blood	Reduction/ chemiluminescence		1.2–6.25 μM	190
Mouse	RSNO	Plasma	Reduction/ chemiluminescence	5×10^{-6} SNO[b] per heme	4×10^{-5} SNO per heme	191
Rat	RSNO	Plasma	Reduction/ chemiluminescence	108 ± 23 nM	1335 ± 423 nM	192
Pig	NO_X	Plasma	Reduction/ chemiluminescence	No difference, but did observe upregulated iNOS during sepsis		175

[a] NO_X refers to byproducts of NO oxidation, nitrate and nitrite.
[b] SNO refers to a nitrosothiol moiety.

with humans. These species also allow for clinical monitoring (e.g., heart rate, cardiac output, blood gasses) that is similar to an intensive care unit setting. Despite these advantages, the use of large animals are often cost prohibitive. As such, their use in studying sepsis is less prevalent than rodent models (i.e., mice and rats). Unlike other models for sepsis, rodent models afford the ability to study disease mechanisms in greater detail, especially with the use of genetically engineered knockouts. Due to low total blood volumes (~2 mL in mice) however, serial sampling in a mouse is not possible without the induction of hypovolemic shock.[185] Of note, each of these species has a varied timeframe for sepsis development and mortality, as well as differing sensitivities to bacteria and endotoxin.[185] Another important consideration in terms of relevance of this model is animal age. While most human patients who develop sepsis are >65 years of age,[193] animals used for modeling sepsis and other diseases are generally juvenile, leading to significantly decreased mortality.[194,195]

Once the appropriate species has been chosen, one must consider the means of inducing sepsis. Three main categories of sepsis models exist: administration of a toxin (e.g., lipopolysaccharide), administration of a viable pathogen (e.g., bacteria), and surgical alteration of a protective barrier (e.g., bowel perforation). Each method has its own distinct advantages and caveats, as outlined in Table 1.5. Direct administration of live bacteria is another means of inducing sepsis in animal models that can be useful for studying specific mechanisms of host response to infection by particular bacteria. The two main routes of infection include direct administration into the blood or peritoneum, and intra-tracheal administration for lung infection. Direct inoculation of the blood will have a rapid effect on the cardiovascular system, while a lung or peritoneal infection will first induce immune cell

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Table 1.5 Advantages and caveats of various animal models of sepsis. Adapted from Buras et al.[196] and Zanotti-Cavazzoni et al.[185]

Model	Advantages	Caveats	Variable Parameters
Endotoxemia	- well-controlled - easy to perform - changes in cardiovascular state and cytokine levels	- single toxin dissimilar to human sepsis - variable response depending on species - variable response depending on dose	- type of toxin - route of administration - dose - animal species
Bacterial infection	- allows measurement of host response to pathogen - response tunable with bacteria load	- single pathogen does not always mimic human sepsis - requires prior bacteria quantification - genetic background affects host response	- bacterial strain - compartment of infection - timing of infusion - antibiotics - animal species
CLP and CASP	- polymicrobial sepsis - most closely mimics human disease states (hemodynamic, metabolic, and immune)	- polymicrobial sepsis leads to variability between animals - uncontrolled bacterial load (CLP model) - abscess formation in CLP model - age variability	- size of puncture (CLP) or stent (CASP) - number of perforations - antibiotics - animal species

differentiation and migration.[197] Other important considerations for this model include bacterial strain and load.[198] This method is also desirable because the strain causing the infection is known, allowing for the assessment of specific targeted treatments.

The host-barrier disruption model of sepsis usually involves perforation of the bowel. Cecal ligation and puncture (CLP), considered the "gold standard" in sepsis research, is meant to mimic ruptured appendicitis in humans.[199-201] The bowel perforation created using this model allows for fecal leakage into the abdomen, creating a polymicrobial infection and necrotic tissue.[202,203] Disease severity is usually adjusted by changing the puncture size or number of punctures.[199] This model is especially clinically relevant as it mimics the hemodynamic, metabolic, and immune responses most often observed in humans during sepsis.[199,204] However, disease severity and progression is more difficult to control, as the load of fecal material and bacteria is variable. Similar to CLP, the colon ascendens stent peritonitis (CASP) model also creates bowel leakage into the abdomen, but seepage is prolonged due to stent placement. As a result, increases in systemic cytokine and bacteria levels are generally higher in the CASP model.[196] Disease severity can be modified by altering the size of the stent placed. This model is typically used to study the acute phase of septic peritonitis.

Choosing an appropriate model and means of inducing sepsis relies greatly on the desired result of a given experiment. While no model is perfect, careful selection of parameters can provide data that is relevant and translatable to human subjects.

1.5   Summary of dissertation research

The focus of my dissertation research was to develop improved methods for detecting NO and nitrosothiols from a variety of complex biological systems. To accomplish this, I made use of electrochemical detection of NO and nitrosothiols using an assortment of platforms suited for each individual measurement environment. The specific aims of my research included the:

1) understanding of commonly utilized methods for detection NO, including their advantages and shortcomings, specifically in complex biological media;

2) design and evaluation of a microfluidic electrochemical NO sensor specifically suited for detection of NO in small volumes of blood;

3) adaption and evaluation of the microfluidic sensor to accommodate on-chip photolytic cleavage and amperometric detection of *S*-nitrosothiols in physiological fluids;

4) utilization of the microfluidic sensor to evaluate NO as a diagnostic and/or prognostic biomarker for sepsis using controlled animal models;

5) use of a microsensor for understanding the dynamics of NO release from single cell systems including neurons and macrophage cells.

The goal of this introductory chapter was to provide an overview of the current understanding of the physiological roles of NO and *S*-nitrosothiols, including their role in sepsis, and how electrochemistry can be utilized for both NO and nitrosothiol detection in complex biological systems. In Chapter 2, a more thorough evaluation of the most common NO detection schemes (i.e., Griess assay, chemiluminescence, and electrochemistry) in multiple types of biological media will be provided. Chapters 3 and 4 will focus on the

development of a microfluidic sensor to facilitate NO measurement in small volumes of physiological fluid and its adaption to allow for photolysis and amperometric detection of *S*-nitrosothiols. The study of NO and nitrosothiols as potential diagnostic/prognostic biomarkers for sepsis in two animal models (i.e., mice and pigs) will be detailed in Chapter 5. In Chapter 6, the use of a microelectrode to study NO release from single cell systems (i.e., macrophages and neurons) will be discussed. Finally, a summary of my work along with a description of future studies will be provided in Chapter 7.

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CHAPTER 2: CHALLENGES OF NITRIC OXIDE MEASUREMENT FROM BIOLOGICAL SYSTEMS[2]

2.1   Introduction

Nitric oxide (NO), an endogenous free radical produced by a collection of enzymes known as NO synthases (NOS), is a physiological mediator of the cardiovascular, immune and nervous systems.[1] For example, NO produced in the vasculature by endothelial NOS serves as a vasodilator and blood pressure regulator.[2,3] The immune system produces NO at high concentrations via inducible NOS to serve as a signaling molecule[4,5] and potent antimicrobial agent.[6] In the brain, NO produced by neuronal NOS functions as a neurotransmitter and is involved in memory formation.[7] The physiological significance of NO has led to increased research on NO and NO-releasing scaffolds as potential therapeutics.[8-12] Given that the location and concentration of NO governs its biological effect, the release characteristics of NO-based therapies often influence their success. Consequently, developing and assessing methods for the accurate quantitative measurement of NO is critical.

While understanding NO's behavior in vivo is obviously important, this task is far from trivial. Nitric oxide is highly reactive with a lifetime on the order of seconds in most biological media.[13] Additionally, the diffusion of NO is rapid, with diffusion coefficients

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approaching 3300 μm^2 s^{-1} in physiological buffer.[14,15] Complicating matters further, physiological concentrations of NO span six orders of magnitude (pM to μM),[16] requiring sensitive measurement techniques over wide dynamic ranges.[17] To date, three analytical techniques account for the majority of NO measurements in the literature.[12,18]

First described in 1864,[19] the Griess assay, formally know as the diazotization assay, allows for the quantification of total NO concentrations via nitrite analysis. Nitrite is a stable byproduct of the reaction of NO with oxygen in aqueous media, and upon reaction with sulfanilamide and *N*-(1-napthyl)ethylenediamine an azo dye is formed. Absorbance spectroscopy is then used to relate the concentration of dye to the concentration of nitrite (and thus NO that was released into solution). Although inexpensive and easily obtained commercially, the Griess assay only provides a limit of detection around 0.5 μM and is mainly useful for determination of NO totals.[12,20,21]

In contrast, chemiluminescence detection, a more costly method due to instrumentation requirements, measures NO directly via reaction with ozone.[22] This reaction produces and excited state nitrogen dioxide, which emits a photon upon relaxation to the ground state. Nitric oxide-release kinetics may be determined directly from materials in solution, as the NO is immediately purged from solution to the detector. As an alternative, nitrate and nitrite in solution can be measured following their reduction to NO.

Due to NO's redox activity, electrochemistry is also commonly used to quantify NO in a variety of formats (e.g., direct oxidation via amperometry) and even the single cell level due to sensor miniaturization.[21] This detection scheme is especially advantageous for in vivo and in situ application due to the spatial and temporal resolution provided by the measurement technique.

While each of these techniques has inherent advantages for measuring NO, it is important to consider the environment in which the NO is measured. Since NO readily reacts with thiols, other free radical species (e.g., superoxide, thiyl radicals, and lipid peroxyls), and metal-containing proteins (e.g., hemoglobin) in situ, the available (i.e., free) NO will vary depending on the sample medium.[23-25] While a number of reviews have focused on describing the available methodologies for biological measurement of NO, [21,22,26-33] a systematic study of the effects of biological media on the validity of such methods is lacking. Herein, we evaluate the accuracy of NO analysis with respect to measuring NO in physiological buffers and fluids, cell culture media, and bacterial broth using the Griess assay, a chemiluminescence analyzer, and an amperometric sensor.

2.2 Materials and Methods

(Heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxysilane (17FTMS) was purchased from Gelest (Morrisville, PA). Methyltrimethoxysilane (MTMOS) was purchased from Fluka (Buchs, Switzerland). Griess assay reagents were purchased from Promega (Madison, WI). Tryptic soy broth (TSB) and brain heart infusion (BHI) broth were purchased from BD Biosciences (San Jose, CA). Dulbecco's Modified Eagle Medium (DMEM), McCoy's Medium 5A Modified, fetal bovine serum (FBS), dipotassium ethylenediaminetetraacetic acid (K_2EDTA), nicotinamide adenine dinucleotide phosphate (NADPH), and nitrate reductase (from *Aspergillus niger)* were purchased from Sigma (St. Louis, MO). Opti-MEM I (a reduced serum medium) was purchased from Life Technologies (Grand Isle, NY). Leibovitz medium (L-15; a carbon dioxide-free cell culture medium) was purchased from Lonza (Basel, Switzerland). Porcine blood was obtained from the Francis Owen Blood

Research Laboratory (University of North Carolina; Chapel Hill, NC). Blood serum was obtained by collecting porcine blood without the addition of anticoagulant. After allowing the blood to clot, it was centrifuged at 2500 rpm for 15 min and the supernatant (i.e., serum) was removed. To obtain blood plasma, porcine blood was drawn into a tube with K_2EDTA (~1.8 mg mL^{-1}), mixed immediately, and then centrifuged at 2500 rpm for 15 min. After centrifugation, three layers were present (from top to bottom: plasma, leukocytes, and erythrocytes); the top layer was removed. A Millipore Milli-Q UV Gradient A10 System (Bedford, MA) was used to purify distilled water to a final resistivity of 18.2 MΩ·cm and a total organic content of ≤6 ppb. Nitrogen and argon gases were purchased from AirGas National Welders (Raleigh, NC). Nitric oxide gas was purchased from Praxair (Danbury, CT). Other solvents and chemicals were analytical-reagent grade and used as received.

2.2.1 *Preparation of physiological media*

Artificial saliva solution was prepared as described by Arvidson et al.[34] via the addition of the following components to 500 mL of Milli-Q water: 279 mg monopotassium phosphate, 284 mg sodium phosphate dibasic, 1.24 g potassium bicarbonate, 482 mg sodium chloride, 254 mg magnesium chloride, 184 mg calcium chloride, and 394 mg citric acid. The pH of the solution was subsequently adjusted to 6.7. A 500 mL solution of artificial normal human urine was prepared according to Kark et al.[35] by adding the following to Milli-Q water: 2.40 g sodium phosphate, 2.25 g potassium chloride, 3.75 mg sodium chloride, and 9.1 g urea. After the pH of this solution was adjusted to 5.9, 25 mg bovine serum albumin and 1.0 g creatinine were added. For a 500 mL solution of physiosol in Milli-Q water the following were added: 2.63 g sodium chloride, 185 mg potassium chloride, 150 mg magnesium chloride, 1.11 g sodium gluconate, and 2.51 g sodium acetate.[36] The pH of the

solution was then adjusted to 6.0. A saturated NO solution (1.9 mM NO) was made by purging ~20 mL of PBS with argon for 30 min to remove oxygen, followed by NO gas for 20 min.

2.2.2 Synthesis of PROLI/NO

N-diazeniumdiolated L-proline (PROLI/NO) was prepared following a previously published protocol.[37] Briefly, L-proline (2.05 g) was dissolved in a solution of methanol (25 mL) and sodium methoxide (2.00 g). The solution was then placed in a stainless steel reaction vessel and flushed with Ar six times (three in succession, three for 10 min each), then charged with NO at a pressure of 10 atm for 3 d with constant stirring. Six additional Ar purges were performed after 3 d. The solution was then precipitated by the addition of diethyl ether (150 mL) at −20 °C for 4 h. The white precipitate was isolated by vacuum filtration and dried in vacuo to yield PROLI/NO, which was stored at −20 °C until use. Ultraviolet spectra of a 14.9 $\mu g \cdot mL^{-1}$ solution of the product (in 1.0 M sodium hydroxide) were acquired on a Thermo Scientific evolution array UV–visible spectrophotometer (Figure 2.1). The molecular weight of pure PROLI/NO was taken to be 251 $g \cdot mol^{-1}$.

2.2.3 Griess assay

To quantify NO via the Griess assay,[38] 50 μL of a 2 mg mL^{-1} solution of PROLI/NO in 100 mM sodium hydroxide (NaOH) was added to 15 mL of desired media and incubated at room temperature for at least 24 h. Aliquots (50 μL) of this sample were added to a sulfanilamide solution (50 μL) and incubated in the dark at room temperature for 5 min. Naphthylethylenediamine (50 μL) was added to the mixture to form a colorimetric product with concomitant absorbance measured in each well at 540 nm using a LabSystems

MultiSkan RC microplate reader (Helsinki, Finland). Sodium nitrite standards were used to normalize the assay reactivity and associated absorbance.

For analysis of blood constituents (i.e., plasma and serum), NADPH (25 µL) and nitrate reductase (2 µL) were added to the samples and allowed to incubate for at least 30 min prior to the addition of the Griess reagents.

2.2.4   Chemiluminescence detection

Real-time NO release was monitored using a Sievers 280 Chemiluminescent NO Analyzer (Boulder, CO). The instrument was calibrated with a 25.6 ppm gas standard (balance N_2) and an atmospheric sample that had been passed through a NO zero filter. Samples were prepared by adding 10 µL of a 2 mg mL^{-1} solution of PROLI/NO in 100 mM NaOH to 30 mL of desired media that had been degassed in a sample vessel for at least 20 min. Nitric oxide produced in the vessel was carried to the NO analyzer by a stream of nitrogen gas bubbled into the solution (80 mL min^{-1}) across the headspace of the flask (120 mL min^{-1}), equivalent to 200 mL min^{-1} flow to the instrument.

2.2.5   Electrochemical detection

Inlaid 2 mm diameter polycrystalline platinum (Pt) disk electrodes sealed in Kel-F (CH Instruments; Austin, TX) were mechanically polished with successively finer grades of deagglomerated alumina slurries down to 0.05 µm particles (Buehler; Lake Bluff, IL). Residual alumina was removed using an ultrasonic cleaner (in water) and the electrodes were dried with nitrogen. A fluorinated NO selective xerogel membrane was applied to the electrode as previously described to minimize response to common interferents.[39,40] Briefly, a silane solution was prepared by mixing MTMOS (60 µL) in ethanol (300 µL). To this solution, 17FTMS (15 µL) was added, resulting in a 20% v/v fluoroalkoxysilane (balance

MTMOS) mixture. The silane solution was subsequently mixed with water (80 μL) and 0.5 M HCl (5 μL) for 1 h. The resulting sol (1.5 μL) was cast onto Pt working electrodes and allowed to cure for 24 h under ambient conditions. To evaluate the analytical performance of the NO sensors, amperometric measurements were performed using a CH Instruments 730B bipotentiostat (Austin, TX). The electrode assembly (3-electrode configuration) consisted of the xerogel-modified Pt working electrode, a Pt-coiled counter electrode, and a Ag/AgCl reference electrode (3.0 M KCl; CH Instruments). Electrooxidation currents were recorded at an applied potential of +700 mV (vs. Ag/AgCl). To measure NO release in the various media using PROLI/NO as the NO source, a 2 mg mL^{-1} solution of the NO donor was added to a constantly stirring bulk solution (30 mL) for a final concentration of 0.1 mg mL^{-1}. Of note, the larger volume of media was necessary to accommodate the working, reference, and counter electrodes in the flask.

2.3   Results and Discussion

Diverse biological media were chosen for these experiments to properly represent environments in which NO measurements are relevant (e.g., in vivo, cell/tissue culture, bacteria culture). Solutions included simple physiological buffers (PBS and physiosol), simulated biological fluids (saliva, urine, and wound fluid), cell culture media (DMEM, McCoy's, L-15, and Opti-MEM), bacterial broth (TSB and BHI), whole blood, plasma, and serum. The salt, amino acid, protein, and vitamin content of these media vary significantly (Tables 2.1–2.4) and thus likely impact the validity of NO measurement data.

2.3.1   Nitric oxide determination via Griess

The Griess assay allows for indirect measurement of NO via nitrite, NO's reaction product in oxygenated media (eqs 1–3):

$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

$$NO + NO_2 \rightarrow N_2O_3 \quad (2)$$

$$N_2O_3 + H_2O \rightarrow 2NO_2^- + 2H^+ \quad (3)$$
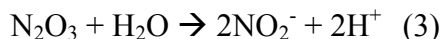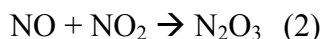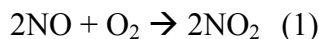
The resulting nitrite is reacted with the sulfanilamide and *N*-(1-naphyl)ethylenediamine producing an azo dye with an absorbance maximum at 540 nm. To accurately quantify NO, calibration curves are constructed with a standard nitrite solution in the sample medium.[38]

In theory, 1 molecule of PROLI/NO decomposes to release 2 molecules of NO, for a theoretical total NO release of 7.9 μmol·mg^{-1}.[37] Following synthesis, PROLI/NO was characterized by UV/vis spectroscopy (Figure 2.1). An observed λ_{max} at 252 nm confirmed *N*-diazeniumdiolate formation from the L-proline precursor, consistent with prior reports.[37] A 14.9 μg·mL^{-1} solution was prepared in 1.0 M sodium hydroxide and the molar absorptivity coefficient (ε) reported previously by Saavedra et al.[37] at 252 nm (8.4 mM^{-1}·cm^{-1}) was used to determine a PROLI/NO concentration of 55.7 μM. If total purity were assumed, the concentration of this solution would be 59.5 μM, implying a relative purity of 93.5%. This value is also supported by the chemiluminescent NO release totals in buffers lacking scavenging components (i.e., PBS).
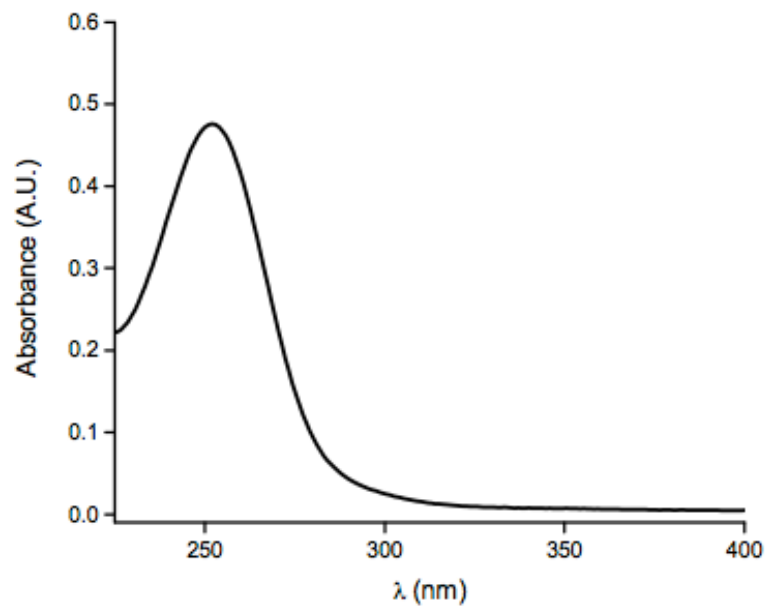
Figure 2.1   UV-visible spectra of 14.9 µg mL^{-1} PROLI/NO in 1.0 M sodium hydroxide.

Table 2.1   Salt concentrations (g L^{-1}) and pH of common biological media.

	Media									
	PBS	Physiosol	Saliva	Urine	DMEM [41,42]	McCoy's [42-44]	L-15 [45]	Opti-MEM [46]*	TSB [47]	BHI [47]
pH	7.4	6.0	6.7	5.9	7.4	7.3	7.4	7.3	7.5	7.4
$CaCl_2$			0.37		0.20	0.14	0.14#	0.10		
$MgCl_2$		0.30#	0.51				0.094			
$Fe(NO_3)_3$					0.0001					
$MgSO_4$					0.98#	0.98#	0.098#			
KCl	0.20	0.37		4.5	0.40	0.40	0.40			
KH_2PO_4	0.54		0.56				0.060		2.5	
$NaHCO_3$			2.5		3.7	2.2		X		
NaCl	8.1	5.3	0.96	7.5	6.4	6.5	8.0		5.0	5.0
Na_2HPO_4	0.87#		0.57#	4.8#	0.11#	0.50#	0.19#			2.5#
Citric acid			0.79							
Sodium gluconate		2.2								
Sodium acetate		5.0								

*The complete Opti-MEM formulation is proprietary, so not all components and their concentrations are available (those known to be present are denoted with an X). #Denotes anhydrous form of salt.

Table 2.2   Amino acid concentrations (g L^{-1}) of common biological media.

	PBS	Physiosol	Saliva	Urine	DMEM [41,42]	McCoy's [42-44]	L-15 [45]	Opti-MEM [46]*	TSB [47]	BHI [47]
L-Alanine						0.013	0.45		0.82	0.39
L-Arginine •HCl					0.084	0.042	0.50		0.63	0.19
L-Asparagine •H$_2$O						0.045	0.25		0.11	0.024
Aspartic acid									0.58	0.23
L-Cysteine •HCl					0.063	0.024	0.12		0.051	0.03
L-Glutamine					0.58			X	0.0085	0.0060
Glutamic acid						0.022			2.8	0.38
Glycine					0.030	0.0075	0.20		0.77	0.22
L-Histidine •HCl •H$_2$O					0.042	0.021	0.25		0.35	0.072
Hydroxy-L-proline						0.020				
L-Isoleucine					0.11	0.39	0.25		1.0	0.14
L-Leucine					0.11	0.39	0.13		1.4	0.26
L-Lysine •HCl					0.15	0.037	0.075		1.2	0.25
L-Methionine					0.030	0.015	0.15		0.38	0.060
L-Phenylalanine					0.066	0.017	0.25		0.97	0.14
L-Proline						0.017			1.4	0.20
L-Serine					0.042	0.026	0.20		0.42	0.018
L-Threonine					0.095	0.018	0.60		0.34	0.042
L-Tryptophan					0.016	0.0031	0.020		0.15	0.018
L-Tyrosine •2Na •2H$_2$O					0.10	0.026	0.37		0.24	0.072
L-Valine					0.094	0.018	0.20		1.1	0.19

*The complete Opti-MEM formulation is proprietary, so not all components and their concentrations are available (those known to be present are denoted with an X). [#]Amino acid content derived from tryptone and peptone. [‡]Amino acid content derived from brain heart infusion.

Table 2.3   Vitamin concentrations (mg L^{-1}) of common biological media.

	Media									
	PBS	Physiosol	Saliva	Urine	DMEM [41,42]	McCoy's [42-44]	L-15 [45]	Opti-MEM [46]*	TSB [47]	BHI [47]
Ascorbic acid						0.60				
D-Biotin						0.20				
Choline chloride					4.0	5.0	1.0			
Riboflavin mononucleotide							0.10			
Folic acid					4.0	10	1.0			
Inositol					7.2	36	2.0			
Nicotinamide					4.0	0.50	1.0			
p-Aminobenzoic acid						1.0				
Niacin (B$_3$)						0.50				
D-Pantothenic acid (B$_5$)					4.0	0.20	1.0			
Pyridoxine •HCl (B$_6$)					4.0	0.50	1.0			
Pyridoxal •HCl (B$_6$)						0.50				
Riboflavin (B$_2$)					0.40	0.20				
Thiamine •HCl					4.0	0.20				
Thiamine monophosphate							1.0			
Cobalamin (B$_{12}$)						2.0				

*The complete Opti-MEM formulation is proprietary, so not all components and their concentrations are available.

Table 2.4 Concentrations (g L^{-1}) of other additives in common biological media.

	PBS	Physiosol	Saliva	Urine	DMEM [41,42]	McCoy's [42-44]	L-15 [45]	Opti-MEM [46]*	TSB [47]	BHI [47]
D-Glucose					4.5	3.0		X	2.5	
D+Galactose							0.90			
Creatinine				2.0						
Dextrose										3.0
Urea				18.2						
HEPES								X		
Hypoxanthine								X		
Thymidine								X		
Phenol red •Na					0.016	0.011	0.010	X		
Pyruvic acid •Na					0.11		0.55	X		
Tyrptone									17	
Peptone						0.60			3.0	
Glutathione						0.00050				
Insulin								X		
Transferrin								X		

*The complete Opti-MEM formulation is proprietary, so not all components and their concentrations are available (those known to be present are denoted with an X). [#]BHI also contains infusion from brain and heart (6.0 g L^{-1}), peptic digest of animal tissue (6.0 g L^{-1}), and pancreatic digest of gelatin (14.5 g L^{-1}).

Estimated NO release totals derived from the total nitrite present in each type of media are given in Figure 2.2 along with totals derived from chemiluminescence detection. The total NO measured in PBS via the Griess assay was only 5.67 ± 0.27 µmol mg^{-1}, ~30% lower than the theoretical amount and that detected via the chemiluminescence method (7.24 ± 0.04 µmol mg^{-1}). Of note, care was taken to add fresh PROLI/NO solution to the media quickly. In addition, multiple samples were analyzed to obtain standard deviations. Similar depressed NO levels were observed in all of the media types tested by Griess. We attribute the deviation between Griess and theoretical to lost (e.g., escaped) NO gas (from solution) that subsequently was not accounted for in the total nitrite levels. Indeed, when the headspace in a 20 mL scintillation vial was reduced by adding 20 mL of PBS versus 15 mL, the total nitrite detected was increased by $44\% \pm 12\%$. Conversely, when the total buffer volume was decreased to 10 mL, the total nitrite recovered was reduced by $32\% \pm 21\%$ (data not shown). The most significant decreases in measured NO levels were observed for bacterial broth (i.e., TSB and BHI), certain cell culture media (i.e., McCoy's and L-15), and blood constituents (i.e., plasma and serum), likely the result of NO's reaction with proteinaceous components in the media. Both cell culture and bacterial growth media consist of complex mixtures of amino acids, proteins, sugars, and vitamins of various concentrations (Tables 2.1–4). Proteins and other additives present in some media have been shown to interfere with the Griess assay previously,[48-50] acting as either positive or negative interferents. For example, positive interferents such as NOS and hemoglobin also absorb light around 540 nm. A number of other interferents including cysteine, tyrosine, ascorbate, and NADPH react with nitrite to negatively skew NO totals. Although interfering proteins could be removed by chemical precipitation or ultrafiltration,[26] such solution conditioning is tedious and would be at the

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expense of biological relevance. As anticipated, NO release in whole blood was not quantifiable via Griess due to both the opacity of blood and the presence of interfering proteins.[49] Additionally, NO and nitrite are rapidly oxidized to nitrate in whole blood.[51,52] Nevertheless, nitrite levels were measureable from plasma and serum samples extracted from the blood with the caveat that nitrate reductase and NADPH were used to revert any nitrate that formed back to nitrite. In both plasma and serum, the totals of NO detected were >15% lower (4.75 ± 1.1 μmol mg^{-1} and 4.18 ± 0.85 μmol mg^{-1}, respectively) than that in PBS, indicating the challenge of using the Griess assay to determine NO levels in blood constituents. Indeed, NADPH has been indicated as an interferent for Griess. Furthermore, nitrite recovery is highly variable and dependent on enzyme activity.[26,31,53]

The accurate detection of NO via the Griess assay is also concentration-dependent in certain types of media. While no significant differences were observed for non-proteinaceous media, detection in the more complex media (i.e., cell culture media and bacterial broth) varied significantly depending on the amount of PROLI/NO added to solution (Figure 2.3). For example, while the addition of a low PROLI/NO concentration (0.67 μg·mL^{-1}) in DMEM yielded nitrite totals that were only $48\% \pm 9\%$ of those achieved at the highest concentration of PROLI/NO (67 μg·mL^{-1}), an intermediate concentration (67 μg·mL^{-1}) yielded nitrite amounts that nearly matched the highest concentration ($97\% \pm 1\%$). The same trend held true for the bacterial broth TSB. This can be attributed to the effect of scavenging, as the concentration of such scavengers in a given medium is constant, so adding more NO allows this effect to be overcome to some extent.

Given these results, the use of Griess for quantifying NO in most biological media leads to questionable results. Nevertheless, the potential for high-throughput analysis via

microtiter plates and readers makes Griess useful for initial screening of NO-release materials in less complex media (e.g., PBS) so long as such data are supplemented with more rigorous analysis in relevant milieu prior to drawing conclusions regarding clinical utility. In addition, some kinetic data are obtainable via Griess by taking aliquots from a sample solution at set periods or moving bulk (i.e., larger) substrates (e.g., polymer-coated slides) in and out of a soak solution and subsequently sampling those solutions. For example, AHAP films soaked in select media yielded totals similar to those obtained under the same conditions by chemiluminescent detection (Table 2.5) after 1 week. Although the low levels of NO release were undetectable with chemiluminescence after 2 weeks, the accumulation of nitrite was still measurable by the Griess assay and indicated a continued release of NO from the substrates.
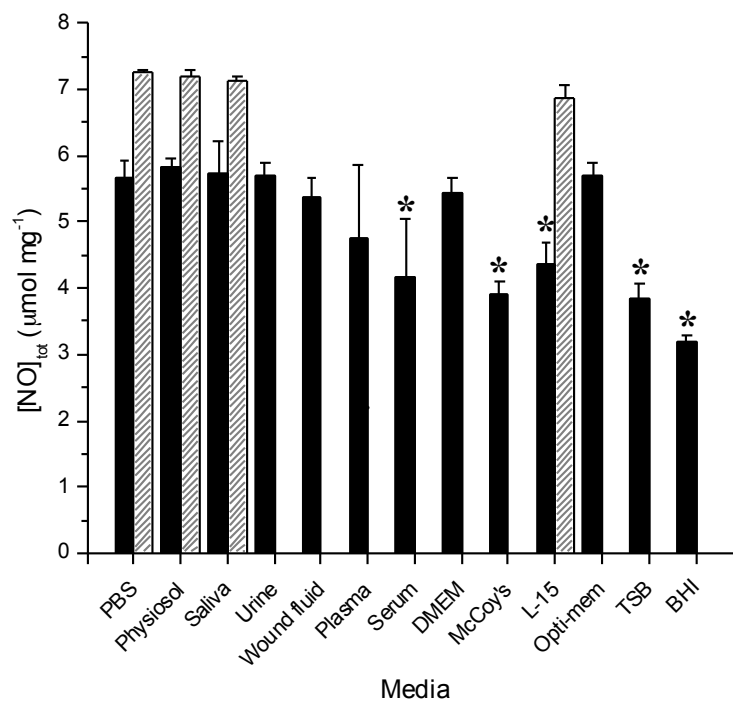
Figure 2.2 Total NO released from PROLI/NO in several types of biological media determined via Griess assay (solid) and chemiluminescence (striped). Theoretical NO release from PROLI/NO is 7.9 μmol mg^{-1}. *Denotes a significant difference (p <0.05) relative to PBS.
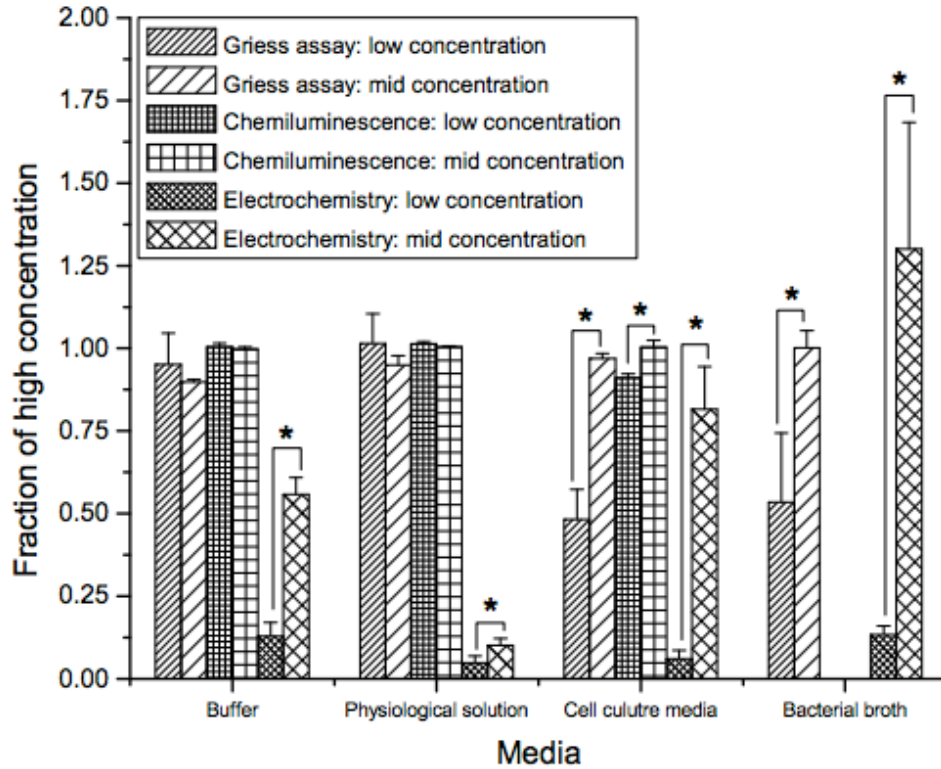
Figure 2.3 Concentration dependence trends of PROLI/NO in different types of media for each technique. For the Griess assay and electrochemistry the final concentrations of PROLI/NO were 0.67, 6.7, and 67 μmol mg^{-1}. For chemiluminescence analysis, the concentrations used were 0.167, 1.67, and 16.7 μmol mg^{-1}. Chemiluminescence analysis could not be performed in bacterial broth due to frothing. Results were normalized to the highest concentration used for each. *Denotes a significant difference ($p<0.05$).

Table 2.5   Total NO release from AHAP xerogel films in various media at 1 and 2 weeks as determined using nitrite concentrations via the Griess assay. Total NO as determined using chemiluminescence was 1.29 µmol cm^{-2} in PBS at 1 week.

Media	Week 1	Week 2
PBS	1.12 ± 0.00	2.18 ± 0.00
Artificial saliva	1.49 ± 0.00	2.06 ± 0.01
TSB	0.69 ± 0.01	1.63 ± 0.02

2.3.2   Chemiluminescence nitric oxide analyzer

To facilitate real-time measurement with improved (lower) limits of detection relative to the Griess assay, chemiluminescence has become an important tool for solution-based NO analysis.[18] Comparable to Griess, the measurement principles for chemiluminescence are straightforward. Nitric oxide is reacted with ozone in an instrument separate from the solution/sample vessel to form an excited-state nitrogen dioxide species that emits a photon upon relaxation back to the ground state. This light (600–875 nm) is detected by a photomultiplier tube. The reaction is both specific for and sensitive to NO with a detection limit approaching 0.5 ppb (0.66 pM in 100 mL) in PBS.[12]

Analogous to Griess experiments, samples of PROLI/NO (0.67 μg mL^{-1}) were introduced into a solution, but analyzed using chemiluminescence detection to quantify NO release. The media compatible with this method was limited to solutions that did not foam as a result of purging with nitrogen gas. Foaming due to nitrogen bubbling was most prominent for culture media, bacterial broth solutions, and blood (i.e., whole, plasma, serum) due to their high protein content.[54] Unfortunately, purging the sample is necessary to both deoxygenate the media (to reduce autooxidation) and carry NO to the instrument, making this technique incompatible with samples containing high concentrations of proteins. Nitric oxide release totals for the compatible media are shown in Figure 2.2, allowing for direct comparison of total NO detected with the Griess assay as a function of media type. Of note, the 7.24 ± 0.04 μmol NO mg^{-1} measured in PBS via chemiluminescence was near the predicted NO payload for PROLI/NO and further confirmed the integrity of our NO donor as synthesized. Surprisingly, no significant differences in NO totals were observed for low protein content media using chemiluminescence, indicating the high accuracy of this

technique for measuring total NO. While L-15 cell culture media contains biomolecular components that may scavenge NO as described above, such reactivity was limited as nitrogen rapidly carried the NO from the sample vessel to the instrument upon formation.

Another advantage of chemiluminescence detection is the ability to extract kinetic information about the NO release. For example, the maximum NO flux, time to the maximum release (t_{max}) and NO release half-lives ($t_{1/2}$) may be determined for a particular NO source (e.g., macromolecular scaffold) in addition to NO totals. The kinetic NO release profiles from PROLI/NO in PBS, physiosol, artificial saliva, and L-15 are provided in Table 2.6. Little variation in the NO release kinetics for PROLI/NO in these media was noted using chemiluminescence, with two exceptions. In artificial saliva, the maximum NO release was significantly lower than that in PBS ($73,360 \pm 1,100$ vs. $86,200 \pm 3,700$ pmol s^{-1} mg^{-1}, respectively). This behavior may be attributed to a lower pH compared to PBS (6.7 vs. 7.4, respectively). The pH effects on NO release from *N*-diazeniumdiolate NO donors are well known.[9] Likewise, the half-life of PROLI/NO was reduced by ~8 s in L-15 cell culture media. These variances are not surprising given the complex and varying nature of biological media (Tables 2.1–2.4).

Despite the advantage of providing real-time NO-release kinetic information, the instrumental setup for chemiluminescence remains problematic for performing measurements in biological samples (e.g., undesirable frothing). In addition, the reaction vessel should be free of oxygen to minimize side reactions that would result in decreased NO detection.[12,22] Unfortunately, most NO-producing cells require an oxygenated environment (for survival), thus precluding chemiluminescence for measuring long-term, real-time cellular NO release.

Table 2.6   Kinetic parameters of NO release from PROLI/NO in PBS, physiosol, L-15, and artificial saliva. *Denotes a significant difference ($p < 0.05$) relative to PBS.

	t_{max} (s)	max NO release (pmol s^{-1} mg^{-1})	$t_{1/2}$ (s)
PBS	42 ± 1.9	$86{,}200 \pm 3{,}700$	65 ± 3.0
Physiosol	40 ± 1.7	$81{,}200 \pm 36$	64 ± 1.1
L-15	40 ± 1.2	$96{,}600 \pm 3{,}100$	$57 \pm 0.7*$
Saliva	47 ± 5.2	$73{,}400 \pm 1{,}100*$	74 ± 3.5

2.3.3 *Amperometric nitric oxide sensor*

In contrast to other NO measurement approaches, electrochemical sensors allow for the measurement of NO in almost any biological setting, including at the single cell level.[55-57] The advantages of electrochemical detection of NO include superior spatial and temporal resolution, excellent limits of detection and dynamic ranges, and the potential for miniaturization. In addition, both the selectivity and sensitivity of the sensor are tunable by changing the applied potential to the working electrode and modifying the working electrode with a catalyst and/or permselective membrane.[12,58] Due to these inherent advantages, amperometric sensors have been used extensively to examine both endogenous[11,56,57,59-62] and exogenous[63-65] NO production. In particular, constant potential amperometry is most often utilized for NO detection as it allows for sub second temporal resolution,[56] and produces a current proportional to NO concentration upon the oxidation of NO via a three-electron process (eqs 4–6):

$$NO \rightarrow NO^+ + e^- \quad (4)$$

$$NO^+ + OH^- \rightarrow HNO_2 \quad (5)$$

$$HNO_2 + H_2O \rightarrow NO_3^- + 2e^- + 3H^+ \quad (6)$$
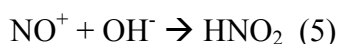
A NO-selective fluorosilane-based xerogel-coated platinum working electrode[39] was used to measure the electrooxidation of NO as current at an applied potential of +700 mV vs. Ag/AgCl reference electrode in a well-stirred solution. Total NO release was determined by integrating the current vs. time response of each sample, since the charge at the electrode surface is proportional to the moles of analyte oxidized. The integrated totals obtained yielded NO concentrations significantly lower than the theoretical amount of NO released from PROLI/NO regardless of media. These results were not surprising since the electrode is

only able to oxidize a portion of the NO liberated in its vicinity due to its small size. Consequently, the total NO release calculated is not representative of the actual total amount of NO produced by PROLI/NO in bulk solution. This effect may be further compounded in biological media where several sinks for NO exist (e.g., proteins). While electrochemical sensors are clearly less suitable for characterization of potential NO donors, useful information may be deduced upon comparison of relative NO totals in different media and certainly when quantifying localized NO release (e.g., from a surface or single cell). As shown in Figure 2.4, the greatest total NO detected for oxygenated media was in PBS at $16 \pm 1.4 \times 10^{-4}$ µmol mg^{-1}. Unsurprisingly, the NO release from PROLI/NO in oxygenated physiosol was not significantly different than PBS due to their similarity; only their salt concentration and pH differ slightly. Nitric oxide release totals in oxygenated L-15 and Opti-MEM cell culture media were also similar to those in PBS despite significant protein content. However, L-15 and Opti-MEM did not contain added fetal bovine serum (FBS) as do DMEM and McCoy's, the latter resulting in NO scavenging via the presence of sulfhydryl-containing proteins (e.g. albumin, fibrinogen, macroglobulins, glycoproteins).[66,67] For example, the amount of NO measured in simulated wound fluid (10% v/v FBS in water) was significantly lower ($60.4 \pm 7.4 \times 10^{-5}$ µmol mg^{-1}). In whole blood, undoubtedly the most complex of all the media tested, the measured NO total was the lowest ($17.3 \pm 4.8 \times 10^{-7}$ µmol mg^{-1}), due to the expected reaction of NO with numerous blood proteins including oxyhemoglobin.[68] Despite the low total NO measured in whole blood, it was still quantifiable using the amperometric sensor. Nitric oxide totals in blood constituents (i.e., plasma and serum) were also determined with the amperometric sensor. Due to the reduced hemoglobin

concentration in these samples, the NO totals were greater than those in blood (4.02 ± 1.3 × 10^{-4} μmol mg^{-1} and 1.63 ± 0.67 × 10^{-4} μmol mg^{-1} for plasma and serum, respectively).

While testing of deoxygenated media was limited to solutions that did not foam, NO levels were larger after the removal of oxygen (Figure 2.4). These results were expected since oxygen reacts readily with NO.[69,70] In blood and other media containing high protein content or cells, fouling due to protein/cell adsorption may also impact the analytical accuracy of the measurement for electrochemical sensors.[71-74] Of note, such fouling for sensors used in the short experiments described herein only accounted for a 3–5% decrease in sensitivity (data not shown), and therefore did not contribute significantly to the differences observed in NO totals.

Analogous to the Griess assay and chemiluminescent detection, the accurate electrochemical detection of NO was also dependent on the concentration of the NO donor. However, a concentration-dependent effect was observed in all media (Figure 2.3). We attribute this effect to the finite surface area of the working electrode, whereby greater concentrations of the NO donor readily alter the local NO concentrations.

As the amount of NO donor near the electrode surface influences the concomitant NO measured, the electrode distance from an NO source should have a similar effect on NO measurement. To examine this methodically, a NO-selective electrode was placed 25, 50, and 100 μm above a NO-releasing surface (xerogel polymer cast on glass) under ambient conditions in PBS. The amperometric signal (current) obtained when the working electrode was placed 50 μm above the surface at 95 min was ∼45% of that recorded at 25 μm (Figure 2.5). Similarly, the signal was reduced by ∼73% at 100 μm above the surface (relative to the 25 μm working electrode placement). While such NO source/electrode distance dependence

will clearly impact the analytical accuracy of a measurement, it may be useful for measuring the diffusion of NO from surfaces or cells as a function of biological medium.

Despite the overall low NO totals that would be quantified electrochemically, the use of amperometric sensors remains beneficial due to unequalled spatial and temporal resolutions. As such, electrochemistry has proven to be quite useful for the characterization of cellular NO release[11,56,57,59-62] and may prove useful for measurements from small NO-releasing surfaces. However, electrochemical NO analysis is generally the least robust of the methods described herein, requiring frequent calibration and performance testing to maintain data integrity.
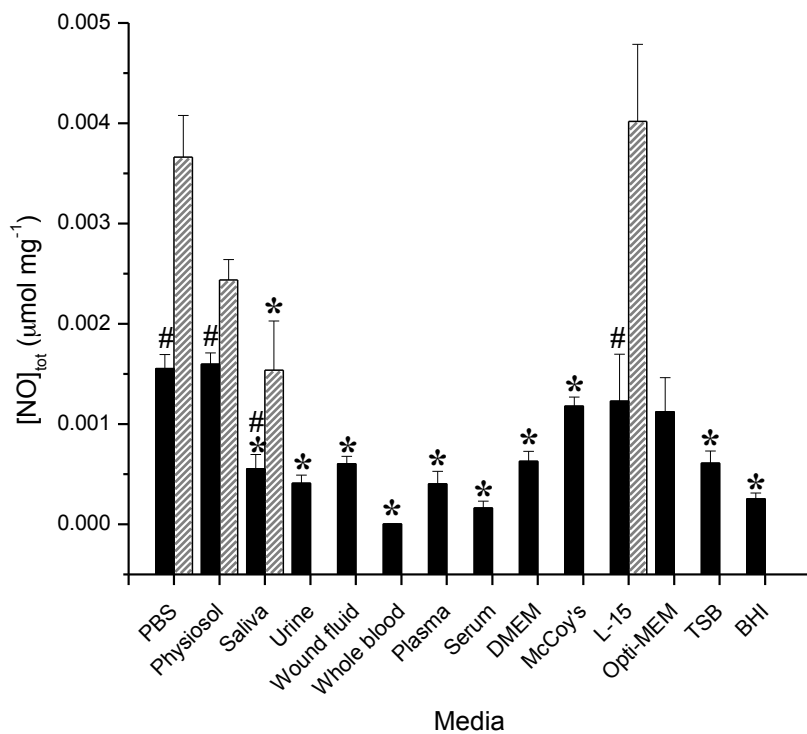
Figure 2.4 Nitric oxide totals measured using PROLI/NO as the NO source in several types of biological media as determined via amperometry in oxygenated (solid) and deoxygenated (striped) solutions. Theoretical NO release from PROLI/NO is 7.9 μmol mg^{-1}. *Significant difference ($p < 0.05$) relative to PBS. #Significant difference ($p < 0.05$) relative to deoxygenated solution.
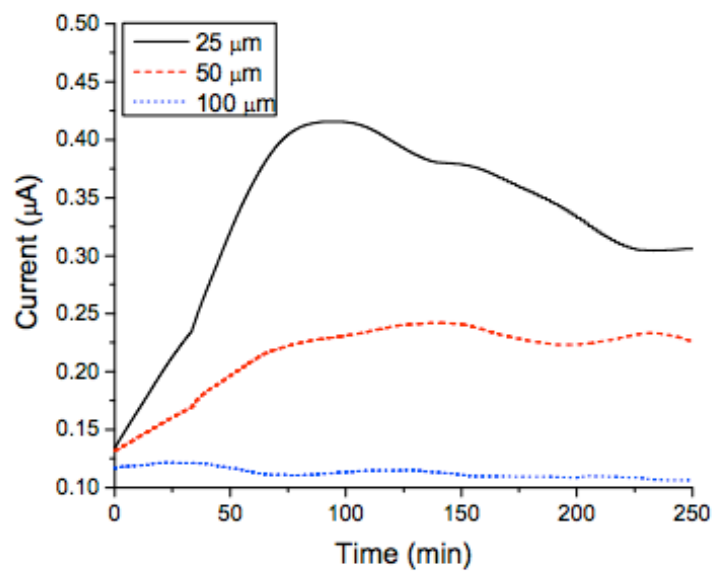
Figure 2.5   Distance-dependent NO release from xerogel membranes in PBS detected via constant potential amperometry.

2.4 Conclusions

The analytical measurement of NO is complex,[12] requiring careful scrutiny of both the analysis method and its performance as a function of solution milieu. While prior work has described the utility of the Griess assay, chemiluminescence analyzers, and electrochemical sensors for measuring NO in solution, performance discrepancies as a function of sample type have been disregarded. The data reported here clearly demonstrate the significant variations between analysis technique and sample composition. As such, the caveats of the analytical method employed must be carefully considered with respect to sample, desired data (e.g., NO totals, flux, kinetics and bioavailability), and result integrity.

2.5   References

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CHAPTER 3: MICROFLUIDIC DEVICE FOR AMPEROMETRIC NITRIC OXIDE DETECTION IN BIOLOGICAL MEDIA[3]

3.1   Introduction

Nitric oxide (NO), a diatomic free radical endogenously produced by a class of enzymes known as nitric oxide synthases (NOS),[1-9] plays a role in a number of physiological processes including wound healing,[10-12] angiogenesis,[13-16] and the immune response.[6,17,18] As might be expected, the detection and quantification of NO in vivo and from NO donor scaffolds has been the subject of intense research.[19-25] Measuring NO in biological systems is challenging due to NO's reactivity (i.e., short half-life), wide concentration range (pM to μM),[1-3,9] and sample complexity.[19] Despite such challenges, direct and indirect methods for measuring NO are routinely employed to determine its concentration in biological samples.[26] Often, NO is most easily quantified by measuring its oxidative byproducts (e.g., nitrite and nitrate). In this respect, absorbance or fluorescence may be used to quantify NO upon its reaction with an assay reagent after oxidation to nitrite or nitrate (i.e., NO_X).[3,27-29] Depending on the sample, pre-existing NO_X species could be measured simultaneously and thus must be considered when determining the signal from solely NO. For example, the Griess assay is widely used to quantify NO indirectly as nitrite in solution. Nitrite and nitrate levels fluctuate

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in physiological milieu,[1-3,9] making real-time NO concentration determination complex.[3] Strategies for measuring NO more directly include chemiluminescence,[30,31] electron paramagnetic resonance (EPR) spectroscopy,[30,32,33] and the use of electrochemical sensors.[19,26] Although measurement of NO by chemiluminescence or EPR allows for sensitive and direct analysis, the required instrumentation for these methods is expensive, requires extensive user training, and is difficult to adapt for analysis of challenging biological matrices such as whole blood.[26,34] In contrast, electrochemistry allows for real-time measurement of NO in physiological media using a sensor platform that is tunable (i.e., sensor style, geometry, material, and size) based on the application at a generally low cost.[19,20,22,26,32,35-48] For biomedical use, electrochemical sensors are amenable to miniaturization and thus can facilitate both in vivo and ex vivo analysis.[19,20,49,50]

A notable obstacle for measuring NO accurately in biological milieu via amperometry is the presence of interfering species such as nitrite, acetaminophen, ascorbic acid, uric acid, hydrogen sulfide and ammonium/ammonia that may also be redox active at the working electrode potential required for NO analysis.[19,51,52] Almost all effective NO sensor designs include a membrane-modified working electrode to eliminate or reduce the diffusion of interferents and concomitant erroneous sensor response. For example, working electrodes have been modified with Teflon,® Nafion,® and silicon rubber membranes to restrict the diffusion of nitrite and larger molecules such as dopamine and ascorbic acid to the electrode surface, relative to NO.[43,44,50,53] To simplify NO sensor fabrication, we have employed xerogel sensor membranes that enable straightforward modification of a number of electrode geometries via dip coating or casting of a sol solution.[45,46] The ensuing xerogel-modified

sensors are characterized by superior analytical performance (i.e., sensitivity and selectivity for NO).

The style of electrode platform (i.e., needle-type, planar, microfluidic) is dependent on the intended measuring environment. For example, needle-type sensors provide adequate NO sensitivity for single cell analysis.[42,47] The sensor design requirements for blood analysis are more complex. Malinski et al. reported the fabrication of a Teflon®-coated NO microsensor (5 μm tip diameter) to measure NO intravascularly in human subjects before and after the administration of bradykinin.[47] However, the clinical utility of such in vivo devices proved poor primarily due to biofouling (i.e., protein adsorption, platelet adhesion, and clot formation) that leads to erratic or unreliable sensor response.[54] While ex vivo measurements are possible, the most common sensor designs require large sample volumes (>1 mL) and convection that increases background noise and negatively impacts analytical performance.

In contrast to stand-alone sensors, the use of microfluidics allows for reduced sample volume and handling (e.g., elimination of mechanical convection), thus addressing the shortcomings of prior devices and analytical methodology required for clinical analysis.[55,56] With respect to NO, Spence et al. combined microfluidics with planar carbon ink electrodes to measure NO from stimulated endothelial cells.[57] The device was fabricated using polydimethylsiloxane (PDMS) channel walls. Recognizing that NO and other gases may diffuse through PDMS, Cha et al. reported the fabrication of a catalytic gold/indium tin oxide microfluidic NO sensor using polyethylene tetraphthalate/polyurethane channels to minimize loss of NO.[58] Despite suitable analytical performance in phosphate buffered saline (PBS; 10 pA nM^{-1} NO), the design of this device was complex, requiring hand assembly.

Herein, we report the use of sol-gel chemistry and standard photolithographic techniques amenable to rapid, reproducible, and inexpensive fabrication of microfluidic NO sensors. The analytical performance of the device is demonstrated in simulated wound fluid and whole blood, indicating the ability to measure NO in complex media. Toward more clinical applications, the device is used to monitor NO concentrations in a murine model of sepsis confirming that NO levels increase during a systemic inflammatory response to infection.[2,3]

3.2   Materials and Methods

(Heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxysilane (17FTMS) was purchased from Gelest (Tullytown, PA). Methyltrimethoxysilane (MTMOS), (3-aminopropyl)triethoxysilane (APTES), ascorbic acid, acetaminophen, sodium sulfide and sodium nitrite were purchased from Sigma (St. Louis, MO). Ammonium hydroxide solution (14.8 M) was purchased from Fisher Scientific (Hampton, NH). Nitric oxide gas (99.5%) was purchased from Praxair (Danbury, CT). Nitrogen and argon gases were purchased from National Welders Supply (Raleigh, NC). Other solvents and chemicals were analytical-reagent grade and used as received. A Millipore Milli-Q UV Gradient A10 System (Bedford, MA) was used to purify distilled water to a final resistivity of 18.2 MΩ·cm and a total organic content of <6 ppb. Simulated wound fluid was produced by diluting fetal bovine serum (FBS) obtained from Sigma in distilled water (1:10, v:v). Whole porcine blood was drawn into 1:10 (v:v) 40 mM ethylenediaminetetraacetic acid (EDTA) from healthy pigs at the Francis Owen Blood Lab (University of North Carolina, Chapel Hill, NC).

3.2.1 Preparation of working electrodes

Planar platinum (Pt) electrodes were patterned onto on a 0.9 mm thick SCHOTT B270 glass substrate (Telic Company; Valencia, CA) via photolithography and evaporative metal deposition. Glass substrates (100 × 100 mm) were cleaned with distilled water, isopropanol, nitrogen gas, and then dried at 95 °C for 5 min. After cooling to room temperature, Microposit S1813 photoresist (Microchem Corp.; Newton, MA) was deposited via spincoating at 3000 rpm for 45 s. The substrate was then soft-baked at 115 °C for 2 min. An electrode pattern was exposed through a polyester film/emulsion photomask (Infinite Graphics Incorporated; Minneapolis, MN) for 10 s using a Karl SÜSS MA6/BA6 mask aligner (hard contact, 100 μm gap) equipped with a 350 W UV lamp (SÜSS Microtec; Garching, Germany). The pattern was developed in an AZ400 alkaline developer (1:3 dilution in water) for 1 min, rinsed with distilled water, dried with nitrogen gas, and then baked on a hotplate at 115 °C for 2 min. The exposed glass surface was oxygen plasma cleaned at 100 W for 1 min. To fabricate working electrodes, 10 nm Ti and 150 nm Pt were deposited in a Kurt J. Lesker PVD 75 magnetron sputtering system (Clairton, PA). The substrate was soaked in acetone to liftoff the remaining photoresist and excess metal resulting in 100 μm wide patterned Pt electrodes on glass.

3.2.2 Membrane synthesis and deposition

Working electrode-modified glass substrates were rinsed with distilled water, dried with nitrogen, and heated to 95 °C for 5 min. Substrates were then oxygen plasma cleaned at 100 W for 1 min. The membrane deposition regions were masked by evenly applying 1002F-50 photoresist (prepared as previously described[59]) by spin-coating at 500 rpm for 10 s, 3000 rpm for 40 s, and heating at 95 °C for 1 h on a hot plate. The electrode pattern was exposed

through a chrome mask for 80 s using the mask aligner. Following exposure, the substrate was baked on a hot plate at 95 °C for 10 min. The pattern was developed in SU-8 developer (Microchem Corp.; Newton, MA) for 6 min, rinsed with isopropanol, dried with nitrogen, and baked on a hotplate at 115 °C for an additional 10 min. An adhesion layer of APTES was deposited via three 5 mL injections at 130 °C using a LabKote vapor deposition system (Yield Engineering Systems; Lawrence, CA). The fluoroalkoxysilane membrane solution was prepared via the acid catalyzed hydrolysis and condensation of 17FTMS and MTMOS as reported previously.[45] 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 micro centrifuge tube with vigorous mixing between the addition of each component. This solution was then vortexed for 1 h. Working with 16 electrode batches, 30 µL of the sol solution was spread-cast across the working electrodes using a pipette tip for 1 min to ensure even coating. 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. Membrane thickness was characterized using a P15 Profilometer (KLA-Tencor Corp.; San Jose, CA).

3.2.3 Microfluidic device fabrication

Reference electrodes were fabricated on separate glass microscope slides. The slides were oxygen plasma cleaned (100 W, 5 min) and masked with tape so that only the middle third of each slide remained exposed. Reference electrodes were deposited in this region by first 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 double-sided Kapton® polyimide tape (90 µm thick, KaptonTape.com) were applied ~3 mm apart and

perpendicular to the Pt electrodes on the working electrode substrate. The reference electrode slide was then bonded to the working electrode substrate (reference electrode facing down) by aligning and clamping the components together with spring clamps and heating at 100 °C for 5 min. After the ends of the channel were sealed, 8 mm diameter inlet/outlet reservoirs were affixed to the device using a high-strength, chemical-resistant epoxy (Loctite® Professional Heavy Duty 5 min; Westlake, OH). Electrical wires were soldered directly to the solder-on pads of each electrode facilitating an electrical connection to the potentiostat. Prior to using the device to measure NO, 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.

3.2.4   Microfluidic device characterization

To evaluate the performance of the microfluidic device, the working and reference/counter electrodes were connected to a CH Instruments 1030A 8-channel potentiostat (Austin, TX). Gravity solution flow (~100 µL min^{-1}) was employed to move sample through the device by attaching a 40 mm piece of Tygon® tubing to the inlet reservoir. This location served as the introduction site. Prior to sample analysis, the device was polarized at 700 mV vs. the AgCl pseudo-reference/counter electrode for up to 1 h in PBS. To calibrate the device, a saturated NO standard solution (prepared by purging deaerated PBS with NO gas for ~10 min resulting in a 1.9 mM solution of NO) was diluted with PBS and introduced into the inlet reservoir. To assess the selectivity of the sensor for NO, separate solutions of nitrite, acetaminophen, ascorbic acid, uric acid, hydrogen sulfide, ammonia/ammonium, and peroxynitrite (protonated and deprotonated) were injected into the device. The sensitivity of the microfluidic sensor to NO was also tested in both simulated

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wound fluid (10% v/v fetal bovine serum in water) and anti-coagulated porcine whole blood. In these experiments, select volumes of the saturated NO solution were added to 2 mL aliquots of blood, mixed briefly, and added to the sample reservoir. For wound fluid testing, increasing volumes of saturated NO were added to 30 mL of wound fluid, mixed, and added to the sample reservoir.

3.2.5 Animals

Murine sepsis experiments were performed using C57Bl6/J mice that had free access to food and water, and were maintained on a 12 h light/dark schedule. Animal studies were performed in accordance with National Institutes of Health Guidelines and approved by the Emory University Institutional Animal Care and Use Committee (IACUC). To initiate pneumonia-induced sepsis, mice were anesthetized using isoflurane and then received a mid-line cervical incision. A total of 40 µL of *Pseudomonas aeruginosa* (ATCC 27853) suspended in normal saline was then introduced by direct intratracheal installation using a 28-gauge needle, corresponding to 2–4 x 10^7 colony-forming units.[60,61] To enhance delivery of the bacteria into the lungs, mice were held vertically for 10 s. All mice received a subcutaneous injection of saline (1 mL) post-operatively to compensate for insensible fluid losses. For NO measurement, 250 µL of blood was obtained via cardiac puncture (while the animals were under anesthesia) at the time of sacrifice. The blood was immediately transferred to an EDTA tube, mixed, and injected onto the sample port of the microfluidic device. Blood samples from control mice in the absence of bacteria exposure were used as the 0 h time point for NO analysis and were run throughout the experiment. Additionally, all sensors were calibrated before, during, and after animal experiments.

3.2.6   Statistical analysis

Murine sepsis data were analyzed using OriginPro 7.0 (OriginLab; Northampton, MA) and presented as mean ± standard error of the mean. Comparisons between groups were performed using the Wilcoxon rank-sum test with $P < 0.05$ considered to be statistically significant.

3.3   Results and Discussion

Working electrode compositions for NO analysis have spanned many materials including carbon ink,[57] gold/indium tin oxide,[62] and platinum.[46,63] Platinum (Pt) working electrodes were utilized for this study due to availability, compatibility with our microfabrication equipment, and inherent robustness for sensor applications. Working electrodes were deposited by metal evaporation using standard photolithographic techniques. Clean glass was first modified with a thin (10 nm) layer of Ti to improve the adhesion of Pt at the desired thickness (150 nm), with metal thickness monitored using a quartz crystal microbalance.

Prior to xerogel modification, an ethanol-resistant photoresist (1002F-50) mask was applied over the entire substrate to enable selective deposition of the membrane solution over only the working electrodes after UV exposure and processing. To ensure selectivity for NO over interfering species, a 20% (v/v) 17FTMS-MTMOS fluorinated alkoxysilane xerogel membrane was deposited onto the microfabricated working electrodes. Selectivity for NO using xerogel sensor membranes was modified slightly from what we reported previously for Pt-coated tungsten conical wire electrodes.[45] Of note, spread-casting of the sol was necessary to enable reproducible coating of the planar Pt electrodes; dip-coating of this substrate (in sol) did not allow for sufficient control over the ensuing xerogel thickness. The spread-

casting process consistently produced xerogel membranes that were 129 ± 59 nm thick, robust (i.e., scratch resistant) and capable of withstanding subsequent solution immersion (for use as sensors) without delamination or cracking of the films.

Before microfluidic device fabrication, the xerogel-coated Pt electrodes were characterized with respect to NO sensitivity and selectivity over common interferents in a stirred solution of PBS. An unforeseen benefit of the cast NO-selective membrane was reduced background signal and noise while making measurements. While the sensitivity of the membrane-coated Pt electrodes to NO was reduced by ~10% relative to bare electrodes (2.2 to 2.0 pA nM^{-1} NO, respectively), the decreased noise allowed for an improved limit of detection (260 vs. 6 nM NO for bare vs. xerogel-coated, respectively). Analogous to our previous wire-based electrodes,[45] the sensitivity of the xerogel-modified electrodes to NO was ~4 orders of magnitude greater than any of the interferents tested (nitrite, ascorbic acid, acetaminophen, uric acid, hydrogen sulfide, ammonia/ammonium).

A microfluidic device was fabricated by placing a glass substrate patterned with a Ag reference electrode on top of a ~3 mm wide microfluidic channel formed by applying two strips of double-sided Kapton$^{®}$ polyimide tape (~90 µm thick) across the working electrode substrate. A cutaway illustration of the device fabricated in the manner is shown in Figure 3.1, with a cross-section given in Figure 3.2. A deep, wide channel was chosen for this design to allow for adequate flow of more viscous biological fluids like blood or plasma. The addition and removal of sample were accomplished by fixing glass (8 mm diameter) reservoirs over the inlet and outlet vias with epoxy.

The fully assembled device was characterized using constant potential amperometry at a working electrode potential of +700 mV vs. Ag/AgCl pseudo-reference/counter

electrode. To achieve a steady baseline current, the device was polarized in PBS for ~1 h prior to testing. Nitric oxide calibration curves were constructed by adding 1.6 μL aliquots of saturated NO to a PBS solution and transferring ~1 mL of this solution to the sample inlet reservoir of the device. Measurements were recorded upon stabilization of the oxidation current. The NO solution in the reservoir was then replaced with another NO solution of a different concentration. As shown in Figure 3.3, the response to NO for bare and membrane-coated electrodes in the microfluidic device geometry was 2.0 and 1.4 pA nM^{-1} NO, respectively. As would be expected, the xerogel-coated electrode was characterized by a lower sensitivity than the bare electrode due to slowed NO diffusion across the membrane to the electrode surface. In addition, sensor response to NO for both the bare and coated electrodes was lower for the microfluidic devices relative to the entire glass substrate (described previously) because the channel exposes only a portion of the actual working electrode area patterned onto the glass.

Despite lower sensitivity when encased within the microfluidic device, the limit of detection for both the bare and coated microfluidic electrodes were 880 and 840 pM NO, roughly 1 log lower than the same electrodes prior to device fabrication. The improved limit of detection is attributed to lower noise due to the elimination of bulk convection and oscillating magnetic field from the stir plate. Furthermore, the use of gravity flow avoids pulsatile noise common to peristaltic pumps. For the device configuration described, volumes as low as 250 μL were adequate for successful NO analysis. By integrating a pulsatile-free flow control device (e.g., a venturi or syringe pump) and/or reducing channel width, even smaller volumes are conceivable. The response of the xerogel-coated electrode to nitrite, ascorbic acid, acetaminophen, uric acid, hydrogen sulfide, ammonium, ammonia, and
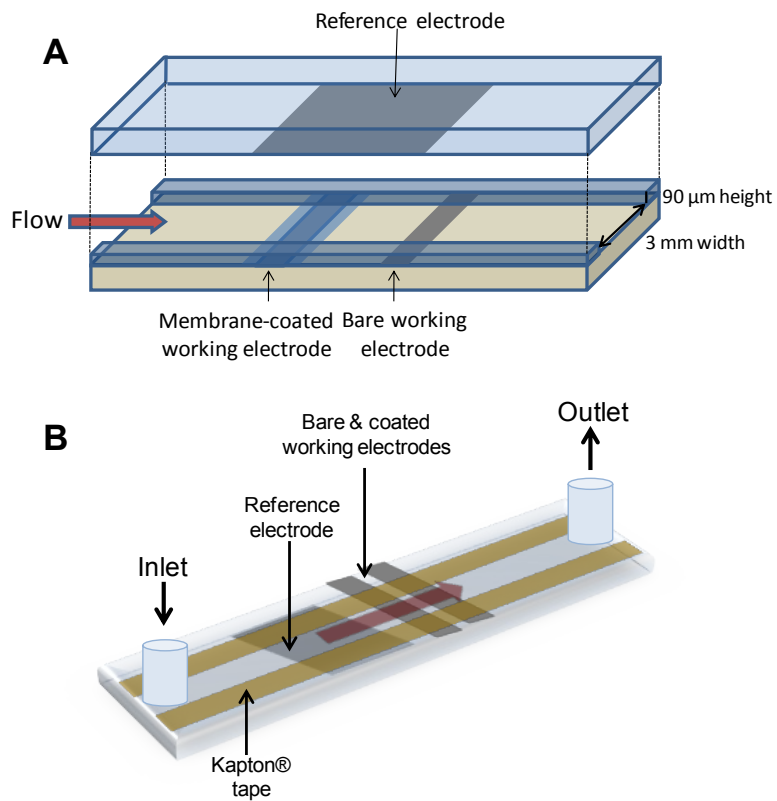
Figure 3.1 A) Cutaway illustration of electrode locations and channel construction; and, B) fully assembled device with inlet and outlet reservoirs.
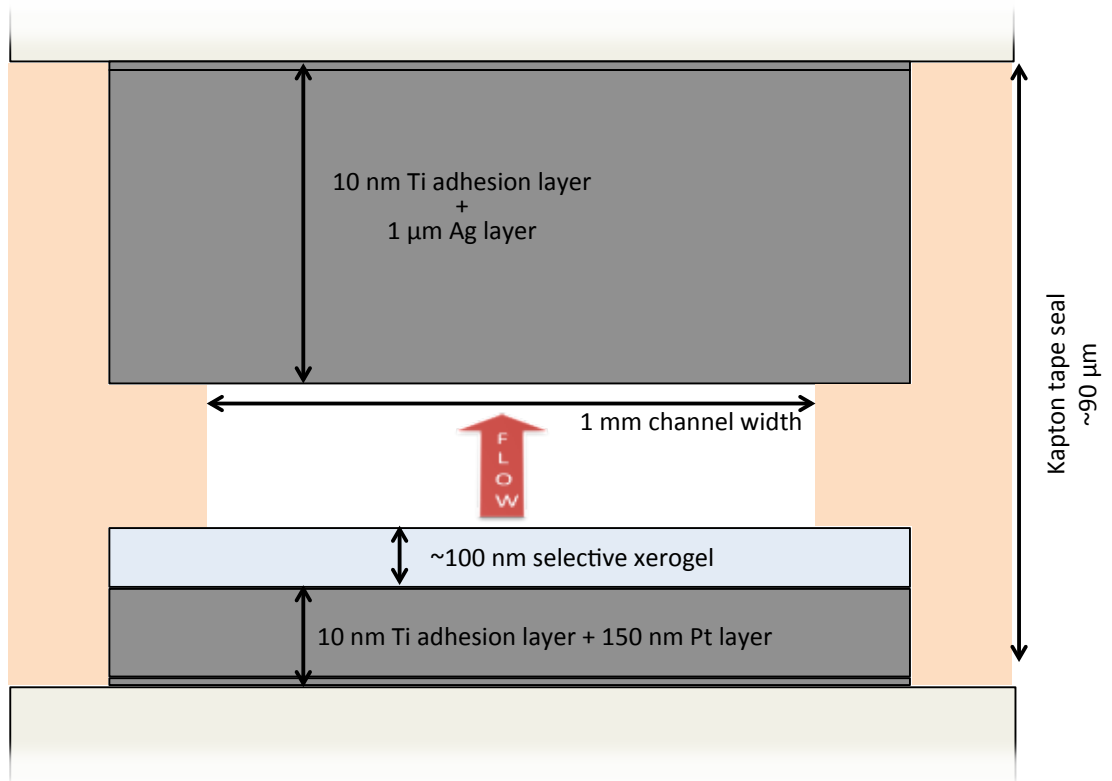
Figure 3.2 Illustration of device cross-section (not shown to scale).
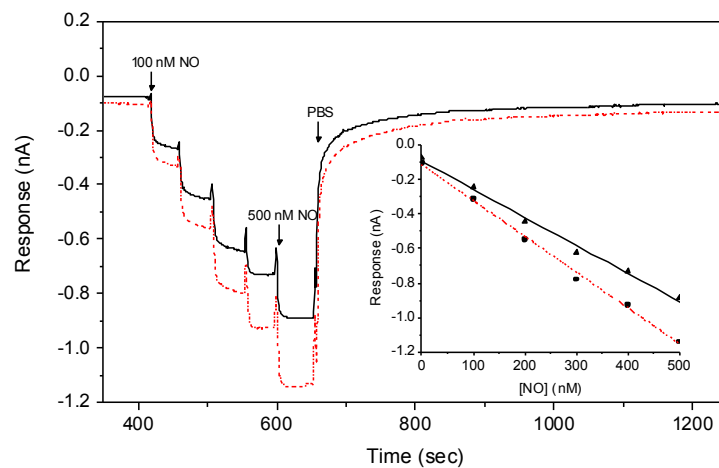
Figure 3.3   Typical sensor response of bare (dotted red line) and xerogel-coated (solid black line) electrodes in microfluidic geometry to NO in PBS flowing at 15 µL/min. Inset shows NO calibration curves for bare (●) and xerogel-coated (▲) sensors.

Interferent	Selectivity (coated)	Selectivity (bare)
Hydrogen sulfide	< -6.0	-0.5
Ammonium	-5.8	-5.6
Nitrite	-5.3	-4.2
Uric acid	-5.0	-2.8
Ascorbic acid	-4.2	-2.4
Acetaminophen	-4.0	-2.7
Ammonia	-3.8	-3.8
Peroxynitrite ($ONOO^-$)	-4.0	-2.7
Peroxynitrite ($ONOOH$)	-1.5	-0.5

Table 3.1   Selectivity coefficients of common interferents for both bare and xerogel-coated platinum working electrodes

peroxynitrite (both protonated and deprotonated) was exceptionally low. The difference in selectivity between bare and xerogel-coated platinum electrodes is given in Table 3.1. Of note, an improvement in selectivity over most interferents was observed with the addition of the xerogel membrane. One exception to the selectivity improvement was ammonia, which is not a concern as it wouldn't be present in high concentrations at physiological pH (7.4).

3.3.1 *Microfluidic nitric oxide sensor response in physiological fluids*

The analytical performance (i.e., sensitivity and selectivity) of the microfluidic NO sensor was characterized in wound fluid and whole blood to determine clinical analysis potential. Despite the presence of proteins, the microfluidic NO sensor proved capable of measuring NO in simulated wound fluid (10% FBS in water, v/v, based on the composition of interstitial fluid), a clinically relevant sample for which NO analysis may prove useful due to NO's role in wound healing.[10-12,64] Boykin recently suggested that NO could be used as a prognostic biomarker for assessing wound healing if easily measured in wound extracts.[64] The microfluidic sensor responded to NO additions in the simulated wound fluid sample with a LOD of 18 nM, roughly 20 times higher than in PBS. The increased NO detection limit is attributed to NO scavenging, as would be expected for proteinaceous solutions. Indeed, the LOD was increased further in whole blood.

Measurement of NO in whole blood is believed to be difficult due to NO scavengers such as hemoglobin and oxygen.[65-67] To calibrate NO sensitivity accurately, aliquots of a saturated NO solution were added to 1 mL samples of porcine blood prior to transfer into the microfluidic device. As shown in Figure 3.4, the addition of saturated NO solution was readily measurable, producing currents proportional to the amount of NO spiked into the blood up to 100 μM. Of note, care was taken to minimize the period between NO injection

into the blood and the analysis to avoid degradation of the NO signal due to reaction with scavenging species present in the blood. As shown in Figure 3.4, the signal did decay over time (within ~100 s), indicating the prevalence of, and problems associated with, NO scavenging/loss.

Although the sensitivity to NO in blood was decreased relative to PBS (0.0035 vs. 1.4 pA nM^{-1} NO), the reduced noise characteristic of the microfluidic device configuration still enabled a satisfactory LOD (472 nM NO). A LOD of ~500 nM is likely sufficient for studying a number of NO-mediated disease states where NO concentrations have been reported in the μM range.[2,3,36,68-71] Of note, slightly higher sensitivities (0.0125 vs. 0.0035 pA nM^{-1} NO) were observed for higher NO concentration ranges (i.e., 20–100 μM NO), likely attributed to the greater concentration of NO relative to potential scavengers in blood.

3.3.2   Nitric oxide levels in sepsis

Sepsis is a systemic inflammatory response caused by severe infection and characterized by a multifaceted immunologic response consisting of an initial hyperinflammatory phase and subsequent immunosuppression.[72-75] Due to NO's role in the immune response to pathogens,[6,17,18] previous studies have monitored plasma concentrations of nitrate and nitrite—both stable end products of NO oxidation in vivo—using the Griess assay to assess their role as biomarkers of sepsis. As expected, large concentration changes were observed for severely septic patients.[2,3,76] While indirect NO monitoring via the Griess assay may provide some insight into changes in NO dynamics throughout the progression of disease, blood sample processing and poor LOD preclude bedside monitoring and/or sepsis risk assessment (at pre-severe disease levels). In contrast, the microfluidic NO sensor described herein may represent a strategy for point-of-care monitoring of NO directly in

whole blood. To evaluate the ability to assess sepsis progression, we infected mice with a single but lethal (96% mortality within 48 h) dose of *P. aeruginosa* (2–4×10^7 colony-forming units). A group of unmanipulated control animals (n=8) was used as the 0 h-time point to represent healthy or baseline blood NO concentrations. Blood was sampled from infected mice at 2, 6, and 24 h (n = 8, 10, and 13 animals per group, respectively) throughout the course of sepsis progression. As expected, blood NO concentration changes over 2, 6, and up to 24 h after introduction of bacteria were statistically significant, reaching 82 ± 12 µM at 24 h (Figure 3.5). This 8-fold increase agrees with those reported previously by indirect detection of NO_X species.[2,3,76] For example, Strand et al. reported a 7-fold increase in average NO_X concentration in human patients with sepsis (144 ± 39 µM) relative to healthy patients (20 ± 3 µM).[2] The observed large increase in blood NO in this study is not surprising given the lethal bacterial dose administered. Future experiments will employ a lower, less lethal dose of *P. aeruginosa* and smaller measurement intervals, if necessary, to study the onset of sepsis. Additionally, the effects of other bacterial strains and antibiotic treatment on blood NO levels will be assessed to more fully gauge the suitability of NO as a sepsis biomarker and prognosis indicator upon treatment.
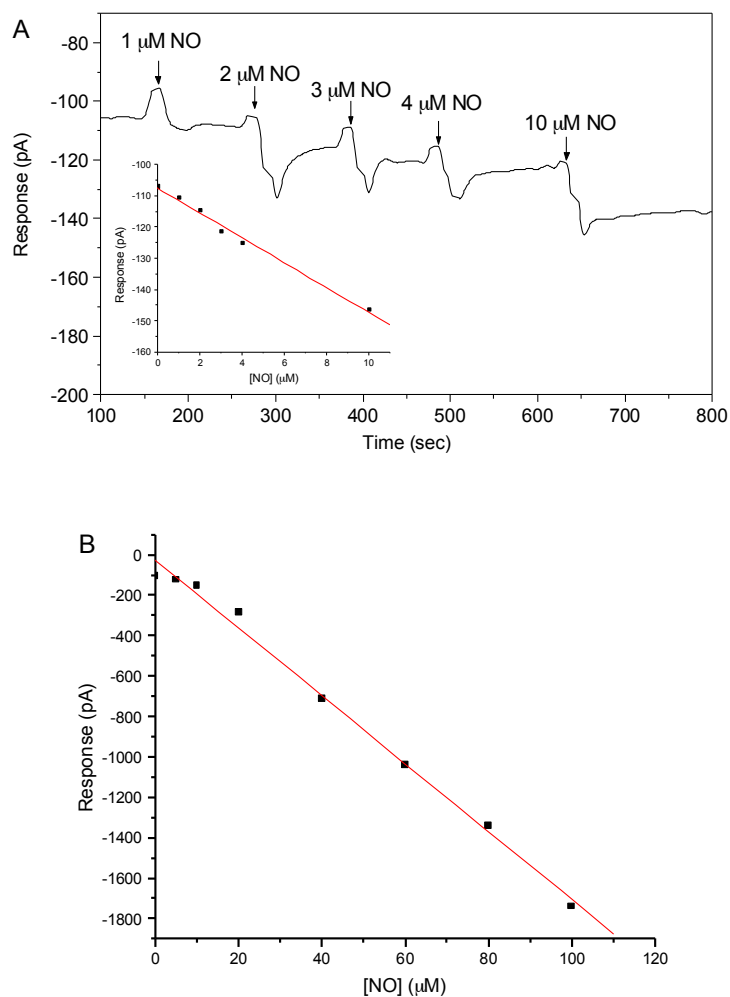
Figure 3.4 Typical microfluidic sensor response to 1 µM increases in NO concentration (A) and full dynamic range (B) in porcine whole blood.
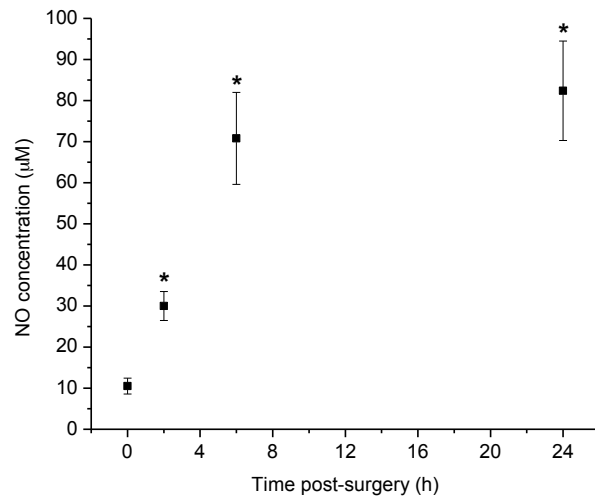
Figure 3.5 Temporal changes in blood NO concentrations during the progression of sepsis in a pneumonia murine model of sepsis. Each data point represents the average ± standard error of the mean for a group of mice of n ≥ 8. *denotes a significant difference in blood NO relative to the 0 h unmanipulated group ($p < 0.05$).

3.4   Conclusions

Herein we describe the use of standard photolithographic microfabrication techniques to construct a microfluidic NO sensor. The use of a NO-selective working electrode and microfluidic geometry enable highly sensitive detection of NO in both PBS and more challenging biological matrices including simulated wound fluid and whole blood. The analytical performance of the microfluidic sensor was dependent on sample milieu, with excellent sensitivity and selectivity for NO in PBS. As expected, the response of the device to NO in biological fluids was attenuated due to scavenging of NO by proteins. Nevertheless, the 472 nM LOD in blood is the lowest reported to date using direct electrochemical detection. The microfluidic device configuration enables rapid analysis of NO at low concentrations and in small (~250 µL) sample volumes that may prove useful for studying NO's action as a potential disease biomarker and/or therapeutic. As an example, whole blood NO levels changed dramatically in a pneumonia mouse model of infection indicating NO's potential as a biomarker for sepsis.

3.5   References

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CHAPTER 4: IMPROVED DETECTION OF *S*-NITROSOTHIOLS VIA VISIBLE PHOTOLYSIS AND AMPEROMETRIC DETECTION IN A MICROFLUIDIC DEVICE

4.1 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 due to the nitrosation of thiols within blood plasma (which contains ~600 μM free thiols), cells, and tissues.[30] Futhermore, 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 or other reducing agents (e.g., ascorbate), but requires the addition of external reagents or the immobilization of a catalyst.[38-45] Photolysis is an alternative means of achieving S—N bond cleavage that is easily coupled to a 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 of RSNOs due to the generation of NO from nitrate.[46-49] 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.[50] 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.[51,52] 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 *S*-nitrosothiols. 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.

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4.2   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), and diethylene triamine pentaacetic acid (DTPA) were purchased from Sigma (St. Louis, MO). Nitric oxide gas (99.5% pure) was purchased from Praxair (Danbury, CT). Nitrogen and argon gases were purchased from National Welders Supply (Raleigh, NC). A Millipore Milli-Q UV Gradient A10 System (Bedford, 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.

4.2.1   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 mask, 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 (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxysilane (17FTMS) and

methyltrimethoxysilane (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.   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 °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.

4.2.2   Preparation of S-nitrosothiols

Low molecular weight RSNOs were prepared using a previously reported procedure.[50,53] 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 were determined via their UV absorption maxima at 335 nm (ε = 503, 586, and 3869 $M^{-1} cm^{-1}$ for *S*-nitrosocysteine (CysNO), *S*-nitrosoglutathione (GSNO), and AlbSNO, respectively).[54] Nitrosothiol solutions were shielded from light and stored at 4 °C prior to use to prevent premature degradation.

4.2.3 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 ×*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.

4.2.4 *Determination of basal S-nitrosothiols levels in blood*

Arterial blood was collected from Yucatan™ miniature swine (n=4) into 3 mL EDTA-coated Vacutainer® tubes. The blood was immediately centrifuged at 4 °C (1300 ×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.

4.3   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. The total transit time from the device inlet to the NO selective electrode was ~5 s.

4.3.1 *Optimization of LED configuration*

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

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light was focused to a 10 mm diameter spot centered on the inlet channel. As indicated in Table 4.1, 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 4.1. 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 $\pm$ 1.6 and 12.3 $\pm$ 1.2 pA μM^{-1}), all sensitivities became equal when normalized to output power (~0.5 pA $\mu M^{-1}\, mW^{-1}$), 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.

4.3.2   *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 $\pm$ 1.6 and 25.5 $\pm$ 1.3 pA μM^{-1}, respectively), an expected result due to comparable molar absorptivities (ε = 17.2 and 14.9 $M^{-1}\, cm^{-1}$, respectively). The maximum current signal was obtained after only 100 s, with linear responses up to 150 μM, well beyond expected in vivo concentrations. 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),[50] 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.[22,23,29,55] 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
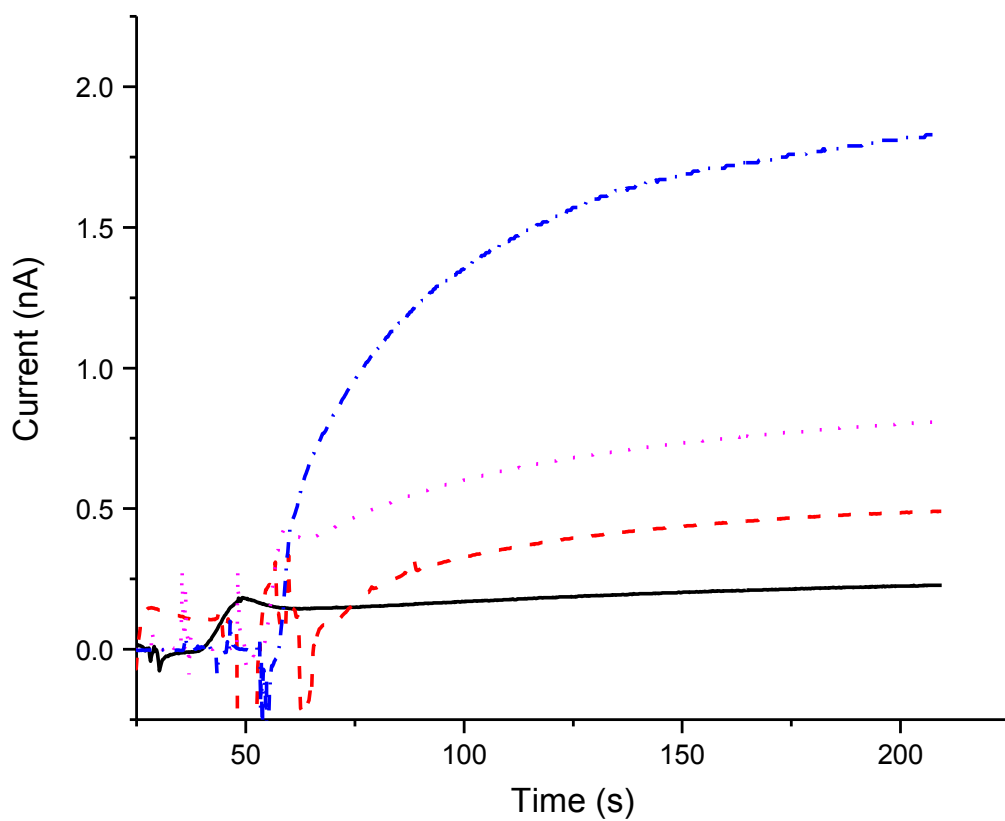
Figure 4.1 Representative amperometric responses for CysNO detection at 100 (blue dashed-dotted, 40 mW), 75 (purple dotted, 25 mW), 50 (red dashed, 6 mW), and 25% (black solid, 2 mW) light intensity and 10 mm irradiation diameter.

Table 4.1   Effect of light intensity and irradiation area on *S*-nitrosoglutathione sensitivity in oxygenated phosphate buffered saline. Data represent the mean ± standard deviation (n≥3).

Spot diameter (mm)	Apparent power (mW)	Sensitivity (pA µM^{-1})	Limit of detection (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

solutions. Indeed, a 44% increase in current was noted for a sample of GSNO upon dilution in deoxygenated versus oxygenated PBS (data not shown).

4.3.3 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 4-2. Identical responses were achieved regardless of how the RSNO was added to the samples (Figure 4-2). Samples that were frozen (−20 °C) and reanalyzed after 24 h exhibited a ~50% decrease in sensitivity (data not shown). Based on these sensitivities, theoretical 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.[56,57] Indeed, the transmittance of 530 nm light in blood plasma is limited to 26%.

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Table 4.2 Sensitivity and limit of detection of a range of *S*-nitrosothiol species in oxygenated phosphate buffered saline and plasma. Data represent the mean ± standard deviation (n≥3).

RSNO type	PBS		Blood plasma		
	Sensitivity (pA µM^{-1})	LOD (nM)	Sensitivity (pA µM^{-1})	Sensitivity decrease from PBS (%)	LOD (nM)
GSNO	22.6 ± 1.6	60	4.2 ± 1.8	82	400
CysNO	25.5 ± 1.3	60	6.9 ± 3.2	73	240
AlbSNO	5.0 ± 1.4	280	0.6 ± 0.2	87	2660
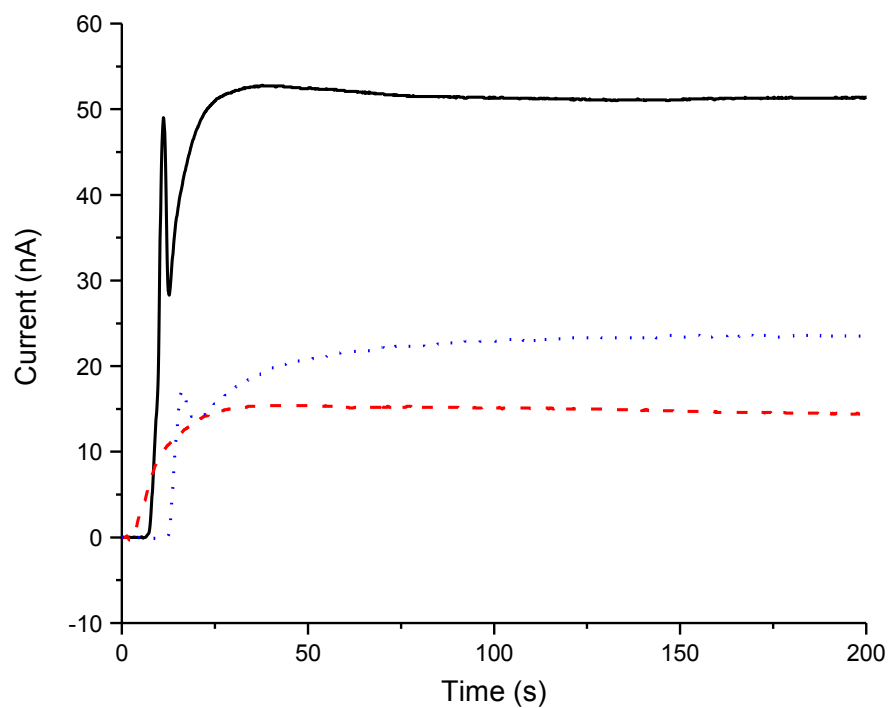
Figure 4.2   Representative amperometric responses for CysNO added directly to phosphate buffered saline (solid black), plasma (dashed red), and whole blood (dotted blue). Following addition to whole blood, the sample was centrifuged to separate plasma and immediately analyzed.

Despite the decreased sensitivity, this technique was used to determine endogenous RSNO levels in fresh plasma separated from blood obtained from healthy swine, with an average of 1.5 ± 1.0 μM determined (n=4). This concentration likely represents only low molecular weight RSNOs due to the elevated limit of detection for AlbSNO in plasma (2.7 μM), yet falls within plasma RSNO concentrations determined by numerous other methodologies (up to 9 μM).[37]

4.4   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 nitrosothiols. Additionally, the required analysis time was minimal (i.e., <2 min for sample irradiation and detection) was required. While detection of RSNOs directly in blood was not realized, analysis in the plasma separated from whole blood was possible with RSNO levels in healthy swine falling within previously reported ranges.[37] 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.[33,58-60]

Small LED sources can easily be coupled to future device designs, potentially allowing for simultaneous measurement of NO and RSNO in one sample. Light sources with greater intensity may permit the measurement of RSNO directly in whole blood, but care must be taken to avoid sample heating. 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.[61-63]

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CHAPTER 5: TEMPORAL MONITORING OF NITRIC OXIDE AND NITROSOTHIOLS IN A PORCINE MODEL OF SEPSIS

5.1   Introduction

Sepsis, a systemic inflammatory response syndrome (SIRS), occurs when the body's normal response to infection becomes so severe or dysfunctional that it causes harm to the host. This dysfunction may initiate a number of physiological changes, both at the cellular and systemic levels, leading to severe symptoms including a dramatic increase in the production of pro-inflammatory cytokines. This "cytokine storm" has the potential to cause multiple organ dysfunction and death.[1,2] A recent epidemiological study revealed that >750,000 cases of sepsis occur annually in the United States and account for 1.3% of all hospitalizations.[3,4] While mortality has decreased and currently ranges from 18–30%, sepsis remains the leading cause of death in critically-ill patients. Recent statistics indicate that sepsis-related illness is increasing by 8.7% annually. In the United States alone, the cost for treating sepsis exceeds $16 billion per year.[3,4] Clearly the need for improved understanding, diagnosis, and treatment of this disease and its underlying causes is highly warranted.

Changes at the cellular level manifest a number of systemic physiological manifestations that may be monitored in the hospital setting. In addition to basic physiological metrics (e.g., lactate, base excess, heart rate), a number of potential biomarkers for sepsis have been proposed for diagnostic use. Indeed, the number of biomarkers evaluated to date is immense, with >178 independent biomarkers considered since 2010,[5,6]

due in part to the complex physiology associated with sepsis. Unfortunately, no single biomarker has proven both sensitive and specific enough to be routinely implemented for sepsis monitoring. Potential future approaches must include the evaluation of and new biomarkers and multi-biomarker panels for more accurate diagnosis.

Much research has pointed to endogenous NO levels as a sepsis biomarker due to NO's roles in the immune system, inflammation, and blood pressure maintenance. For example, many studies have indicated increased iNOS expression during the progression of sepsis.[7-9] Additionally, a number of studies monitored changes in NO metabolites (i.e., nitrate, nitrite, and nitrosothiols) during sepsis using chemiluminescence or colorimetric detection methods.[7,10-17] While reported "NO" concentrations vary and range up to ~150 μM, an increase in endogenous NO relative to controls has been observed in most cases. [7,15-18] Similar to NO, nitrosothiols have been shown to induce smooth muscle relaxation[19] and reduced platelet adhesion.[20] Unlike free NO radicals, nitrosothiols do not react directly with metalloproteins (e.g., hemoglobin) or other radical species (e.g., superoxide), thus increasing their lifetime and preventing the buildup of toxic species such as peroxynitrite. The ability to directly measure NO and nitrosothiols in whole blood and/or plasma at the bedside may provide a clearer understanding of the mechanisms involved in this immune dysfunction given NO's significant chemical reactivity.

Herein, we describe the use of a microfluidic amperometric sensor to monitor temporal changes in NO throughout the progression of sepsis in a porcine model of cecal ligation and puncture (CLP). The changes in NO are compared to more traditionally monitored indicators in intensive care unit patients (e.g., lactate, mean arterial pressure) to determine the usefulness of NO as a prognostic and/or diagnostic biomarker for sepsis. In a

separate study, this device was fitted with a light-emitting diode (LED) to facilitate nitrosothiol and subsequent NO detection. Nitrosothiols levels during sepsis are directly compared to temporal changes in NO.

5.2   Materials and Methods

5.2.1   *Microfluidic device fabrication and characterization*

Microfluidic NO sensors were fabricated as described previously.[21] Briefly, 150 nm thick planar platinum (Pt) electrodes with a 10 nm titanium (Ti) seed layer were patterned onto a glass substrate via photolithography and evaporative metal deposition. The resulting Pt electrodes were 100 μm wide. To provide selectivity to NO, a xerogel membrane was applied to each working electrode. Following the deposition of 1002F-50 photoresist mask, an adhesion layer of (3-aminopropyl)triethoxysilane (1% v/v in ethanol) was deposited via three passes with a spray coater. The substrate was then rinsed with water and allowed to dry in ambient conditions overnight. The fluoroalkoxysilane membrane solution was prepared via the acid catalyzed hydrolysis and condensation of (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxysilane (17FTMS) and methyltrimethoxysilane (MTMOS).[22,23] Briefly, 600 μL absolute ethanol, 120 μL MTMOS, 30 μL 17FTMS, 160 μL distilled water, and 10 μL 0.5 M hydrochloric acid 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 using a pipette tip for 1 min to ensure even coating. The xerogel-coated substrate was then dried overnight under ambient conditions to facilitate curing of the sensor membrane. The 1002F-50 photoresist was removed by soaking the substrate for 1 h in distilled water.

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Reference electrodes were fabricated on separate glass microscope slides by sputtering a 10 nm Ti adhesion layer followed by a ~1 μm Ag layer. To form channel walls, two parallel strips of 6.3 mm wide double-sided Kapton® polyimide tape (90 μm thick, KaptonTape.com) were applied ~1 mm apart and perpendicular to the Pt electrodes on the working electrode substrate. The reference electrode slide was then bonded to the working electrode substrate by clamping the components and heating at 100 °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. The device channel was rinsed with distilled water following this process.

The working and reference/counter electrodes of each 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. For nitrosothiol measurement, a 530 nm LED (Thorlabs Inc.; Newton, NJ) at 100% intensity was placed 100 mm above the device and focused on the microfluidic channel (just before the working electrode) using an adjustable biconvex lens. To calibrate the devices, 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. For nitrosothiols, aliquots of nitrosothiols were added following introduction of serum into the device.

5.2.2 *Porcine model for sepsis*

All experiments were conducted in compliance with The University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. For temporal

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monitoring of NO, twelve male swine were obtained from the North Carolina State University breeding colony in Raleigh, NC. Swine were subjected to overnight fasting prior to each experiment. Anesthesia was induced intramuscularly using a mixture of ketamine and buprenorphine (500 and 0.3 mg) and animals were administered isoflurane and intubated for mechanical ventilation. A femoral venous catheter was used for fluid maintenance throughout the duration of the study and femoral artery catheter allowed for hourly blood draws and constant monitoring of heart rate and mean arterial pressure. A midline laparotomy was performed following catheter placement. The cecum was ligated with suture and an incision was made to allow for removal of intestinal contents. The cecum contents were weighed and ~1 g fecal material per kg body weight (in 500 mL saline) was placed into the abdominal cavity. The cecum was then replaced in the abdominal cavity and the laparotomy incision was closed. Control animals (n=5) underwent a sham surgery consisting of a laparotomy with only cecal manipulation (i.e., no perforation). The time of cecal perforation was considered to be zero for the experiment. During the entire study duration, animals were maintained on isotonic fluids at a maintenance rate and buprenorphine was administered as necessary.

For a follow-up study comparing temporal changes in NO and nitrosothiols, the Yucatan minipig was utilized. The CLP surgery and all other procedures were performed in the same manner as previously, but pain medication (i.e., buprenorphine) was administered at a constant low dose of 1.5 μg kg^{-1} h^{-1}.

Physiological monitoring

Temperature, heart rate (HR), mean arterial pressure (MAP), central venous pressure (CVP), and electrocardiogram (ECG) were monitored throughout the duration of the

experiment (up to 26 h or until termination). Arterial blood (~3 mL) was drawn into ethylenediamine triacetic acid (EDTA)-coated Vacutainer® tubes hourly for standard monitoring of blood gasses and complete blood counts. For blood gas analysis, a Radiometer ABL800 Flex (Copenhagen, Denmark) was utilized. A Horiba scil Vet ABC hematology analyzer (Kyoto, Japan) was used to facilitate complete blood counts. Fresh volumes of blood (~500 μL) were used (immediately after collection) for the microfluidic NO analysis. The remaining blood was immediately centrifuged at 4 °C (1300 ×g for 10 min) to isolate blood serum. This sample was subsequently run through the microfluidic device. Following sample introduction, the LED was turned on to facilitate RSNO decomposition to NO.

5.2.3 *Statistical analysis*

Porcine sepsis data are presented as the mean ± standard error of the mean. Comparisons between groups and from basal levels were performed using the Wilcoxon rank-sum test with $p < 0.05$ considered to be statistically significant.

5.3   Results and Discussion

The swine model was selected for this study because of their physiological similarity to humans compared to other models (e.g., rodents).[24] In particular, their large size allows for routine physiological monitoring and serial blood draws. Cecal ligation and puncture was used to replicate the complex physiological state of human sepsis, as it mimics ruptured appendicitis and polymicrobial sepsis. Cecal ligation and puncture is often referred to as the "gold standard" in modeling sepsis because it mirrors the hemodynamic, metabolic, and immune responses observed in humans during sepsis.[25-27]

5.3.1 *Physiological changes in a porcine model of sepsis and their effect on circulating nitric oxide concentrations*

In the mixed-breed study, temporal changes in NO were monitored in order to compare its rate and magnitude of change to other physiological metrics. Of note, survival varied between control and septic groups, as shown in Figure 5.1. Similar to humans, sepsis in this swine model resulted in significant changes in hemodynamic and blood chemistry profiles. Within the control group, HR (Figure 5.2) and MAP (Figure 5.3) were maintained at ~110 beats per minute (BPM) and ~100 mmHg, respectively. While the development of sepsis led to few significant changes in HR, disease progression resulted in decreased MAP at ~13 h post-surgery. Systemic hypotension and rapid HR are known outcomes of sepsis,[28] and others have suggested NO's potential role in this process due to its role as a vasodilator.[29]

Using the microfluidic NO sensor, we monitored blood NO concentrations hourly. Similar to previous reports using alternative detection schemes and NO metabolites (e.g., nitrate and nitrite), a significant increase in NO was observed over the course of the experiment and progression of sepsis. In general, the changes in blood NO were noted when other physiological metrics also began to change in favor of enhanced NO circulation. In contrast, NO levels in control animals decreased slightly over the 24 h experiment (Figure 5.4). When averaged across all CLP animals (Figure 5.4A), significant increases in NO relative to controls were noted at 6 h. Amongst the CLP swine that survived the entire study duration (Figure 5.4B), a significant increase in NO relative to controls was first observed 8 h ($40 \pm 19\%$ increase from baseline levels). Significant increases were also observed at 11, 14, 15, and 20 h post-surgery.
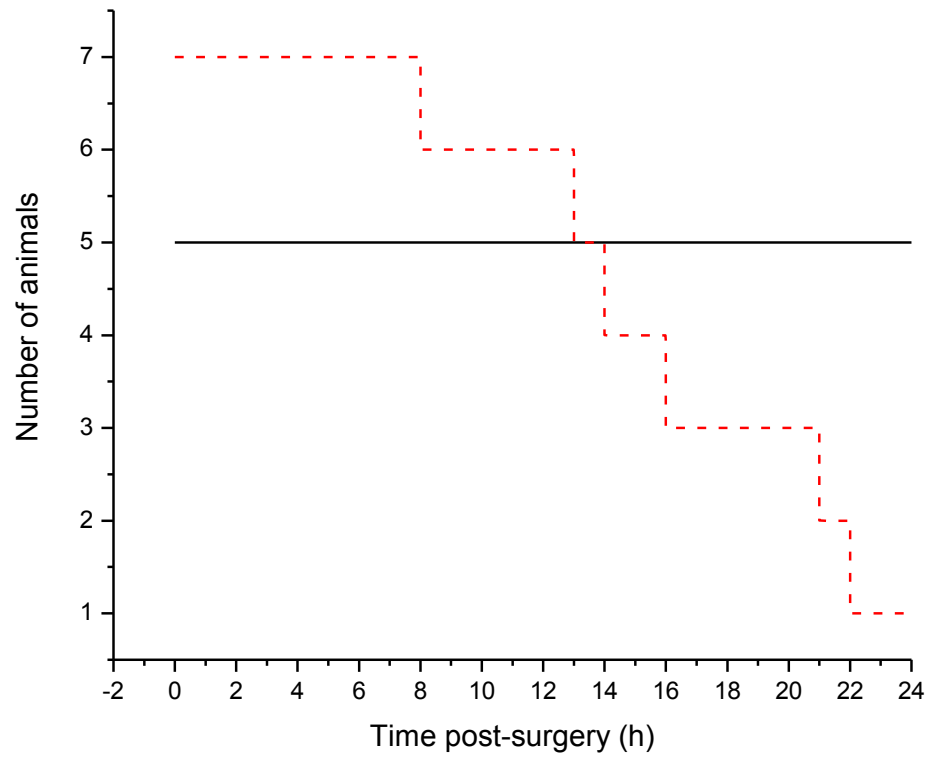
Figure 5.1   Survival for control (solid black) versus cecal ligation and puncture (dashed red) swine over 24 h post-surgery.
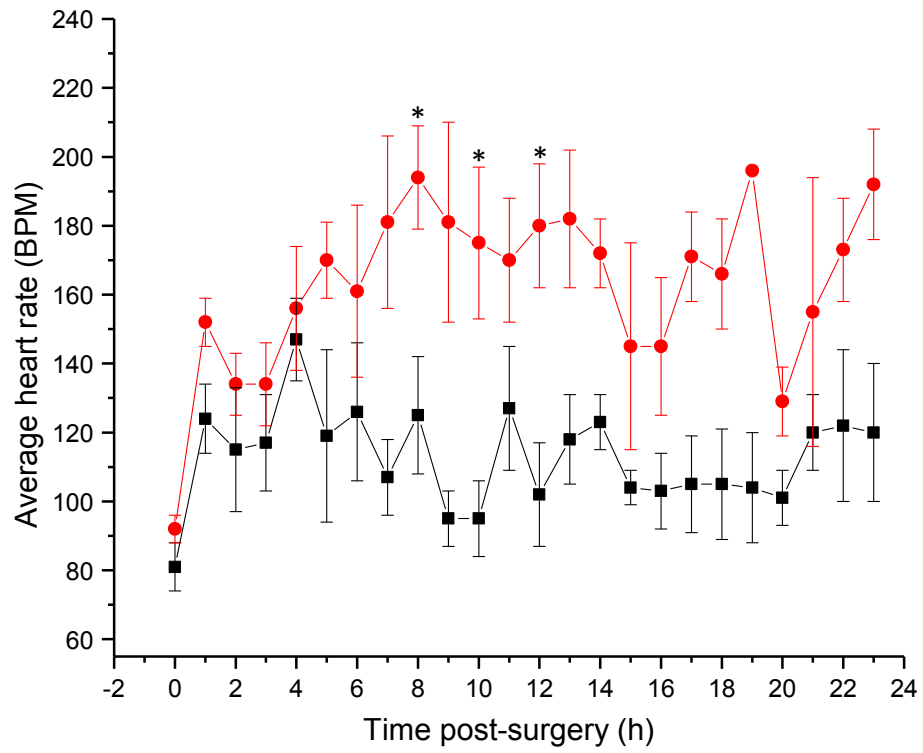
Figure 5.2 Changes in heart rate of septic (●) versus control (■) swine. Data are presented as the mean ± standard error of the mean. * denotes a significant difference relative to the control group at the given time point ($p < 0.05$).
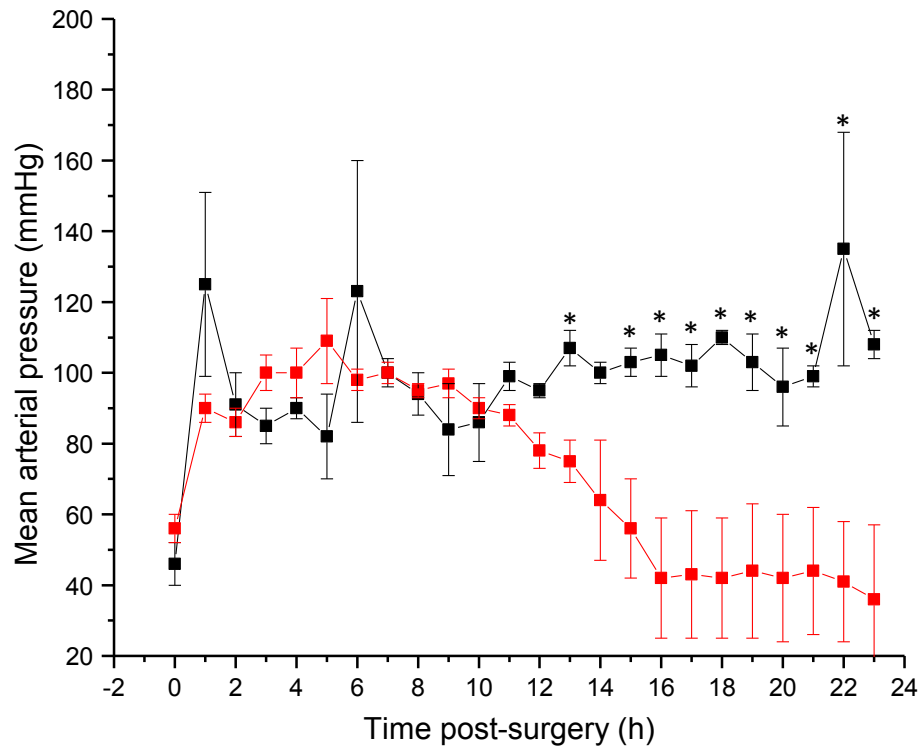
Figure 5.3   Changes in mean arterial pressure of septic (●) versus control (■) swine. Data are presented as the mean ± standard error of the mean. * denotes a significant difference relative to the control group at the given time point (p <0.05).

As shown in Figure 5.4C, elevation of blood NO was even greater during severe sepsis, which was classified by early mortality (≤15 h survival). Within this group, a significant increase in NO relative to controls was apparent as early as 3 h (88 ± 33% increase from baseline levels). In addition to occurring earlier, this increase in blood NO was larger than the initial changes in survival CLP swine. At 14 (final time point), we measured a 6-fold increase in NO relative to baseline levels. This trend is similar to our observations in a lethal model of murine sepsis, where NO increased ~800% within 24 h, indicating that NO is a predictor of mortality.[21] To more accurately compare all animals within both the control and septic groups, the final percent change in NO for each group was averaged, based on the last sample collected for each animal (Figure 5.4D). While the variation amongst CLP swine was large (415 ± 44% increase from baseline), NO increases were consistently greater than those observed for control animals (-23 ± 7% change from baseline).

While full blood chemistry profiles (i.e., blood gasses and complete blood counts) were collected hourly for each animal following CLP surgery, lactate, average base excess (ABE), white blood cell (WBC) counts and hemoglobin concentrations were monitored most closely as these parameters are currently considered most relevant in the hospital setting. Nitric oxide production during sepsis is most often attributed to the robust immune response and stimulation of immune cells.[14,16,30,31] Not surprisingly, the number of circulating WBCs increased by 133 ± 38% in control swine (Figure 5.5), peaking at 8 h following surgery. Such WBC behavior was expected as leukocytosis is a normal response to surgical trauma.[32] Despite the WBC surge, no increase in NO levels was noted for controls. In contrast, septic animals experienced an immediate and dramatic reduction in WBC counts, with counts
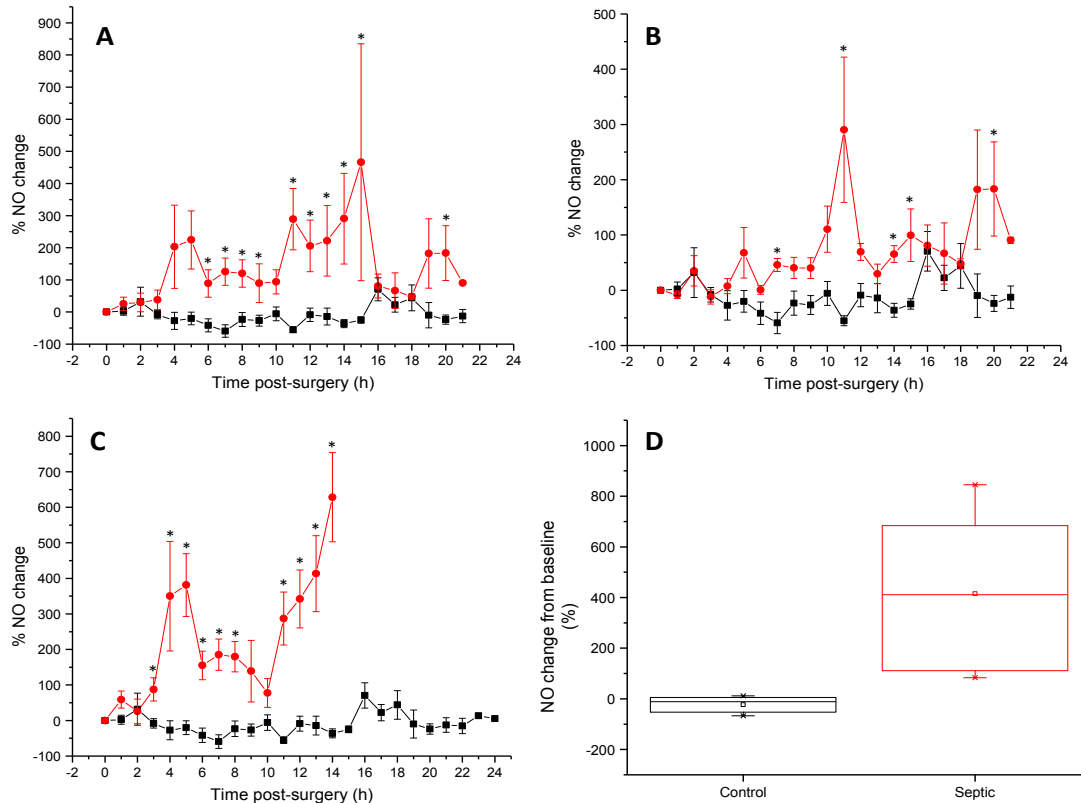
Figure 5.4 Percent change from baseline NO concentrations in septic (●) versus control (■) swine. (A) Data from all animals; (B) Data from only animals that survived the experiment duration; (C) Averages of the septic animals that survived <15 h; and (D) The final end-of-life change for all animals. Data are presented as the mean ± standard error of the mean. * denotes a significant difference relative to the control group at the given time point (p <0.05).

decreasing from (13.5 ± 0.4) to (5.4 ± 1.1) × 10^3 cells mm^{-3} at the time of death for all septic animals. Leukopenia during sepsis has been well documented, and is not necessarily the result of apoptosis. Rather, studies in both rodents and swine have indicated the recruitment of WBC into the liver or infection loci during abdominal sepsis.[33-35] While not circulating, these leukocytes still produce NO, and do so at concentrations up to 10-fold higher than cells obtained from sham animals.[33] While unlikely that the NO produced from localized WBCs was able to circulate systemically and be detected by our device, buildup of other metabolites (nitrite, nitrate, nitrosothiols) is likely and the altered blood chemistry in a severely compromised physiological state may facilitate the regeneration of NO.[36-39]

Similar to initial changes in NO levels, total hemoglobin concentrations also peaked at 5 h, with an increase of 63 ± 12% from baseline level in septic animals (Figure 5.6). Red blood cell counts increased by ~40% (data not shown), indicating hemolysis and a subsequent increase in free hemoglobin. Elevated concentrations of free hemoglobin (and thus heme), caused by the presence of blood borne pathogens,[40] has been linked with an increased risk of mortality for patients with sepsis.[41] Larsen et al. reported that mice lacking heme oxygenase-1 had greater concentrations of circulating heme making them more susceptible to death following CLP-induced polymicrobial sepsis,[42] attributed to the ability of heme to induce programmed cell death in the presence of other pro-inflammatory mediators, leading to multiple organ dysfunction.[42,43] In addition, it is possible that increased circulating hemoglobin concentrations, in conjunction with the metabolic acidosis of severe sepsis, contributed to enhanced circulation of NO with concomitant systemic vasodilation.

Figure 5.5 Percent change from baseline white blood cell counts in septic (●) versus control (■) swine. Data are presented as the mean ± standard error of the mean. * denotes a significant difference relative to the control group at the given time point ($p < 0.05$).

Figure 5.6 Percent change from baseline hemoglobin concentrations in septic (●) versus control (■) swine. Data are presented as the mean ± standard error of the mean. * denotes a significant difference relative to the control group at the given time point ($p < 0.05$).

Lactate, a product of anaerobic cellular metabolism following glycolysis, becomes elevated during tissue hypoxia.[44] Prior work has indicated that consistently increased lactate levels are predictive of poor patient outcomes.[45,46] Base excess (or base deficit), calculated based on the measured carbon dioxide partial pressure (PCO_2), blood pH, and bicarbonate concentration, has also been reported as a predictor of morbidity and mortality in patients with sepsis.[46] While the lactate and ABE levels remained consistent within the control group throughout the study, large changes were observed for both in the septic animals. Lactate was increased up to $175 \pm 127\%$ at 4 h post-CLP, reflective of the severity of this model (Figure 5.6). Similarly, changes in ABE were significant, with deviations from basal levels of up to $-153 \pm 69\%$ at 6 h post-surgery (Figure 5.7). Collectively, these fluctuations indicate ongoing tissue hypoxia and slight acidification of the blood, which may directly impact the circulating concentration of NO. Indeed, the lactate increase at 4 h correlated with NO increases between 3–7 h. Lactic acidosis has previously been reported to stimulate inducible NO synthase[47] and increases concentrations of the pro-inflammatory cytokine interleukin-6.[48] Additionally, the slightly hypoxic conditions that contribute to increased lactate levels are optimal for the generation of NO from nitrite via reaction with hemoglobin.[36-38]
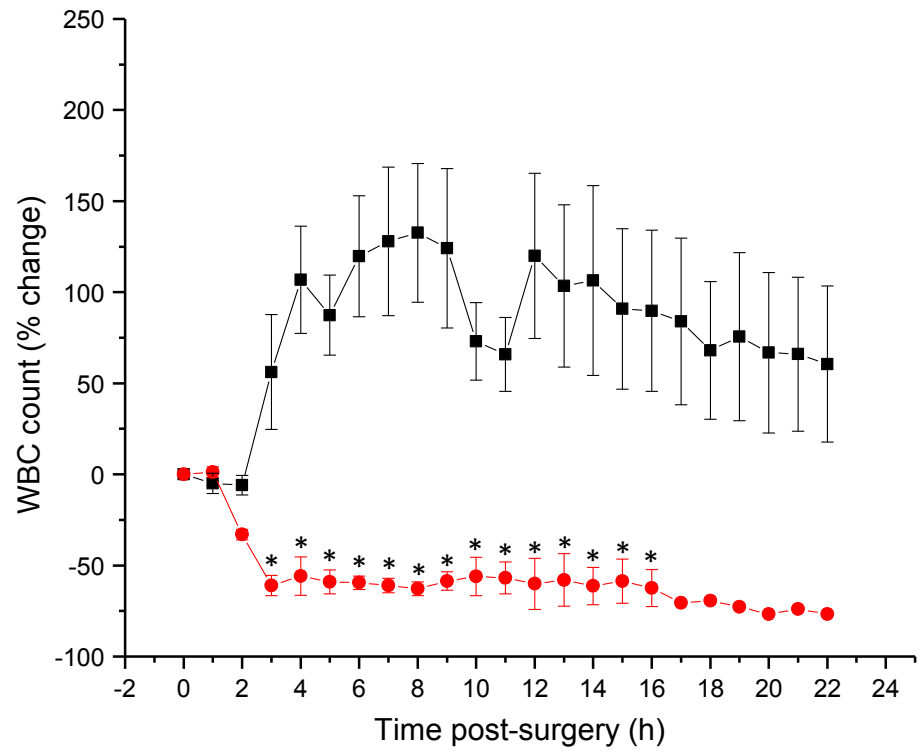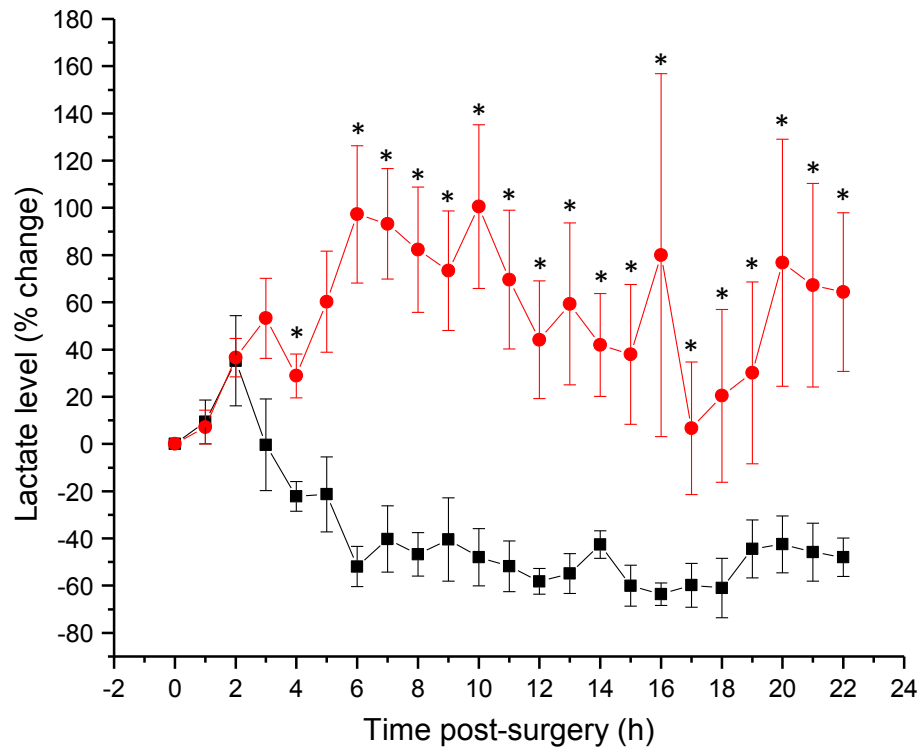
Figure 5.7 Percent change from baseline lactate concentrations in septic (●) versus control (■) swine. Data are presented as the mean ± standard error of the mean. * denotes a significant difference relative to the control group at the given time point ($p < 0.05$).
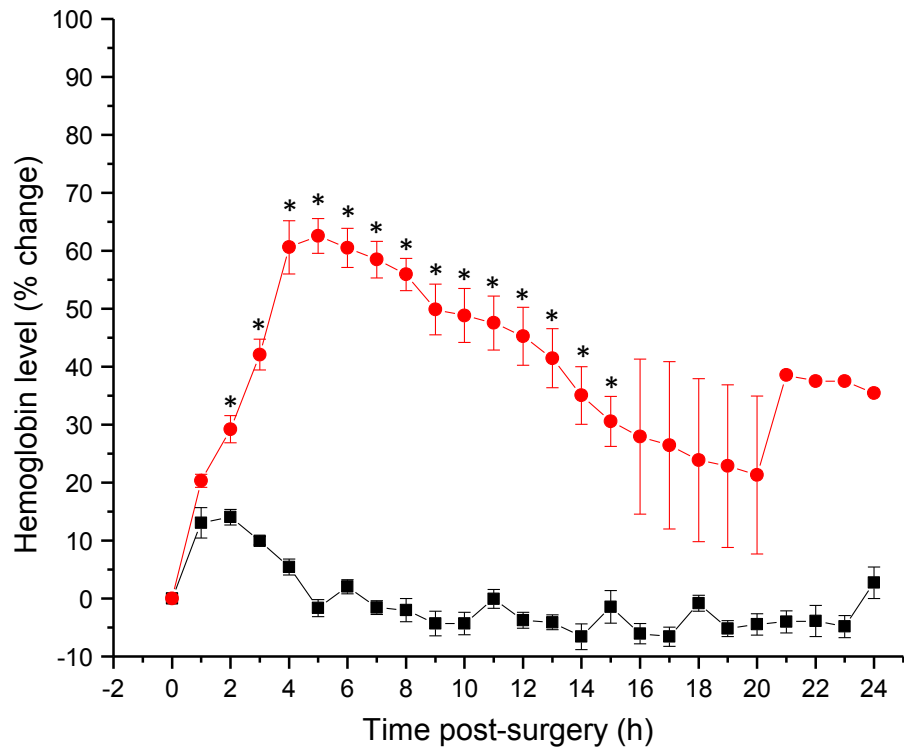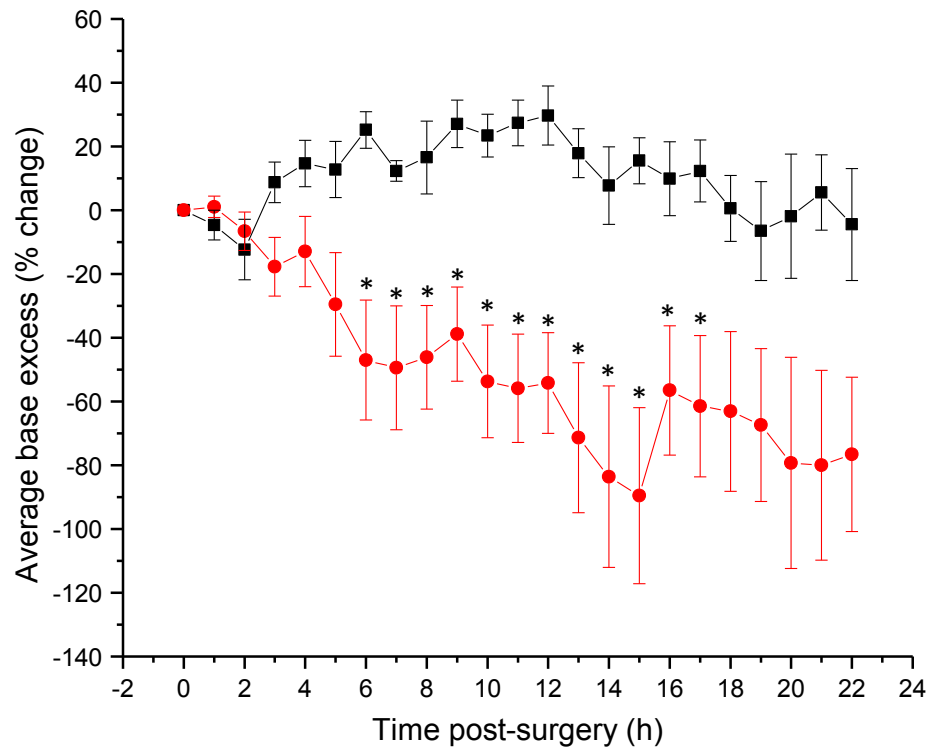
Figure 5.8 Percent change from baseline base excess in septic (●) versus control (■) swine. Data are presented as the mean ± standard error of the mean. * denotes a significant difference relative to the control group at the given time point ($p < 0.05$).

Table 5.1   Levels of nitric oxide, lactate, base excess, white blood cells, and hemoglobin at 0, 4, and 15 h post-surgery. Data are presented as the mean ± standard error of the mean. * denotes a significant difference relative to the control group at the given time point and ‡ denotes a significant difference from basal levels ($p < 0.05$).

Analyte	Group	Time post-surgery (h)		
		0	4	15
Nitric oxide (nM)	Control	210 ± 40	150 ± 40	160 ± 10
	Septic	160 ± 20	350 ± 50	920 ± 250*‡
Lactate (mM)	Control	1.9 ± 0.1	1.6 ± 0.2	0.7 ± 0.1‡
	Septic	1.7 ± 0.1	2.7 ± 0.2*	4.6 ± 1.2*
Average base excess (mM)	Control	5.2 ± 0.1	6.0 ± 0.1	6.0 ± 0.2
	Septic	7.1 ± 0.2	4.3 ± 0.4‡	-0.8 ± 1.8*‡
White blood cells (10^3 mm^{-3})	Control	13.2 ± 1.0	27.0 ± 1.9‡	19.9 ± 1.8‡
	Septic	13.5 ± 0.4	5.6 ± 0.5*‡	5.4 ± 1.1*‡
Hemoglobin (g dL^{-1})	Control	10.7 ± 0.2	11.2 ± 0.3	10.1 ± 0.3
	Septic	10.0 ± 0.1	16.0 ± 0.4*‡	12.5 ± 1.1*

While nitrite was once considered a useless oxidation product of NO metabolism, new evidence suggests the possibility of nitrite recycling to NO under hypoxic and/or acidic conditions.[39] Although the potential pathways/mechanisms are many and complex, the most likely scenarios involve the reaction of nitrite with deoxy-hemoglobin to regenerate NO directly or provide dinitrogen trioxide that subsequently reacts with thiol species to produce nitrosothiols. Nitric oxide can also form a complex with oxy-hemoglobin that may be oxidized to regenerate free NO in the presence of nitrogen dioxide radicals (formed via the reaction of nitrite with oxy-hemoglobin). Stamler et al. also demonstrated the ability of hemoglobin to accommodate nitrosation of its β-93-cysteine group, thus conserving the biological reactivity of NO by forming *S*-nitrosohemoglobin.[49] Nitric oxide stored in this manner may be liberated via multiple mechanisms, most notably under hypoxic conditions.[50-52] The increased lactate and base deficit observed in our study indicate the development of tissue hypoxia and an increase in blood acidity, conditions that are ideal for the recycling of nitrite to NO. As shown in Figure 5.9, the fraction of oxy-hemoglobin decreased in the most severe cases of sepsis, and NO concentrations appeared to increase at a similar rate. These results suggest that dramatic increases in NO during the progression of sepsis are more likely due to nitrite accumulation and blood acidification and deoxygenation that favored the recycling of nitrite to NO, rather than directly from stimulated immune cells. The most dramatic NO elevations during severe sepsis correlate with significantly decreased MAP and thus mortality.
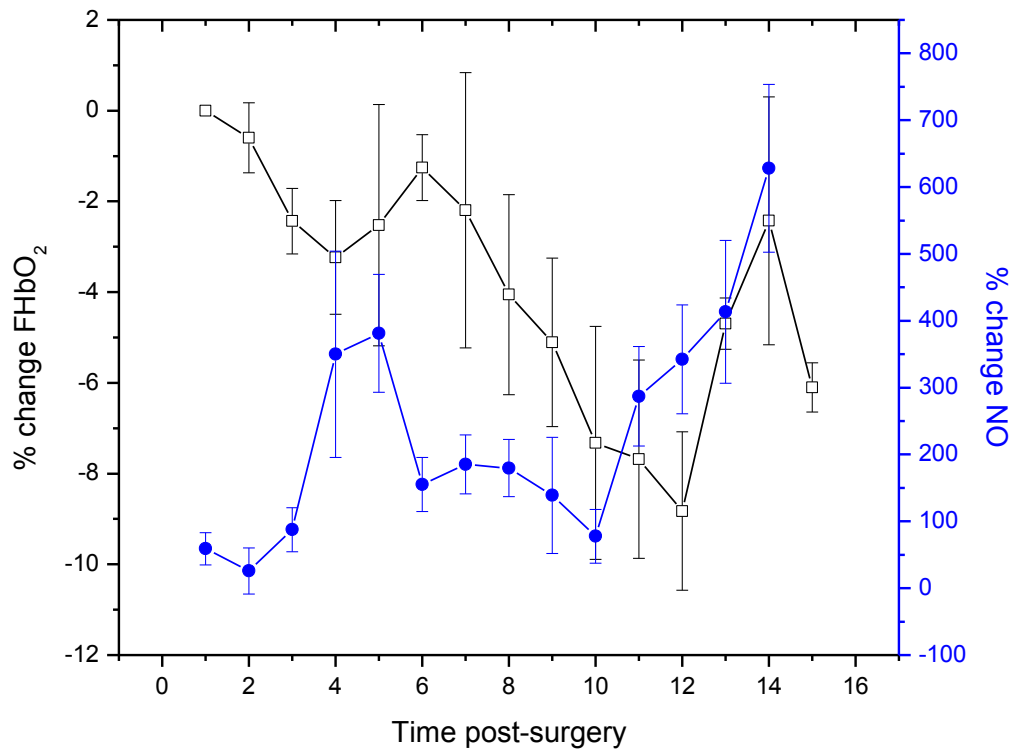
Figure 5.9 Correlation between changes in nitric oxide and fraction of oxyhemoglobin in blood for severely septic swine.

5.3.2   Nitrosothiols versus nitric oxide in a porcine model of sepsis

The results from the mixed-breed swine model of sepsis indicated a possible buildup of byproducts of NO, which may include nitrite, nitrate, and/or nitrosothiols. As nitrosothiols have long been considered stable, physiological transporters of NO,[19,53-56] we sought to monitor temporal changes in NO and nitrosothiols simultaneously. For this study, Yucatan miniature swine were utilized. This breed of swine is commonly used for laboratory studies due to decrease variability between animals.[57] A previous study utilizing this breed for monitoring of peritoneal sepsis demonstrated the slow onset of disease over multiple days.[35] Overall, the severity of sepsis within a 24 h period was reduced in this breed of swine compared to the mixed-breed swine utilized in the first study. All animals in the Yucatan study survived the experiment (n=6 CLP, n=4 sham control). Figure 5.10 depicts the stark difference in NO changes between the two separate models of sepsis. While control groups were reproducible between studies, sepsis severity and NO levels varied greatly. Lactate concentrations did not increase significantly relative to controls until 20 h following CLP, with increases from baseline of only 35 ± 34% (Figure 5.11). In comparison, lactate levels were doubled at 6 h in the mixed-breed model. Increases in circulating NO concentrations (61 ± 14% from basal levels) also occurred at 20 h (Figure 5.12). As our mixed-breed study alluded to, measurable changes in circulating NO may be delayed due to the late shift in blood chemistry that occurred.

Despite the delayed increase in NO, nitrosothiol levels began to increase as early at 8 h following CLP (Figure 5.13) in the Yucatan study. At this time, serum nitrosothiol levels were 61 ± 21% greater than basal concentrations. An initial burst in NO produced by immune
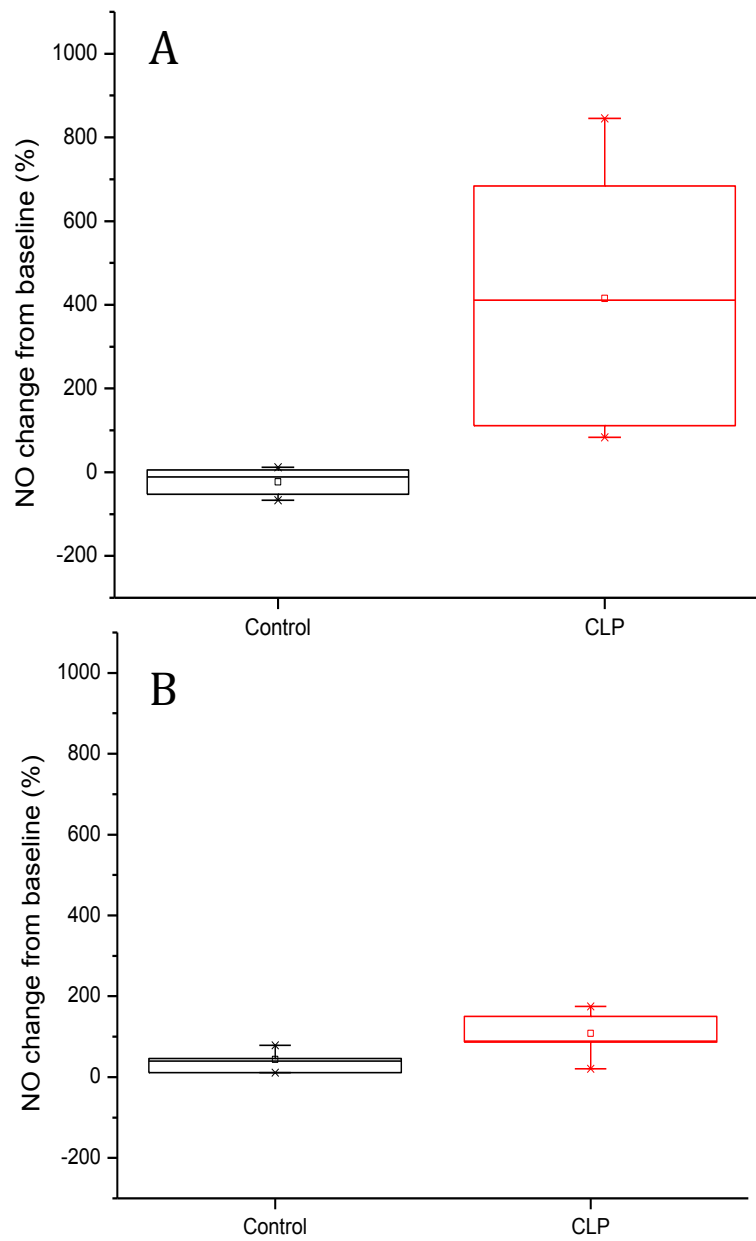
Figure 5.10 Box-and-whisker plots depicting percent change in NO for control and septic animals in mixed-breed (A) and Yucatan (B) mini pigs.
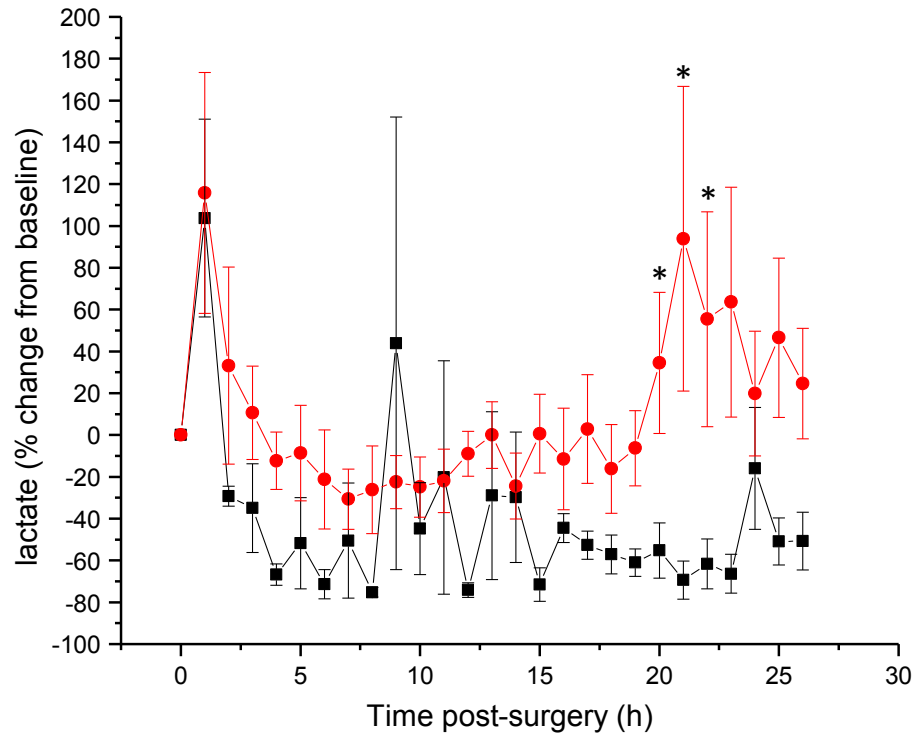
Figure 5.11 Percent change from baseline lactate concentrations in septic (●) versus control (■) Yucatan swine. Data are presented as the mean ± standard error of the mean. * denotes a significant difference relative to the control group at the given time point ($p < 0.05$).
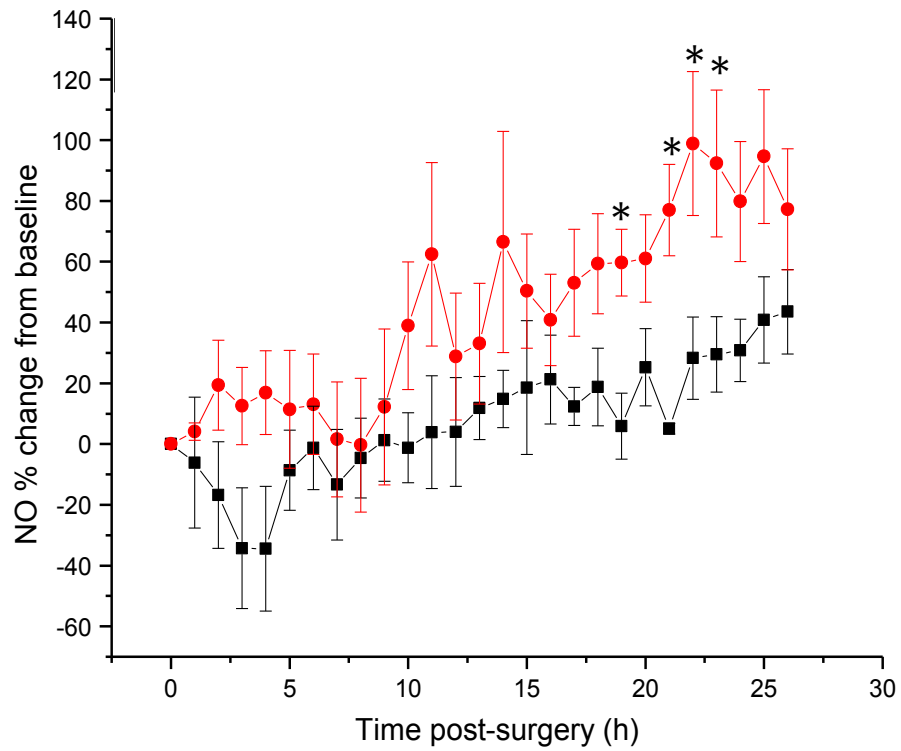
Figure 5.12   Percent change from baseline NO concentrations in septic (●) versus control (■) Yucatan swine. Data are presented as the mean ± standard error of the mean. * denotes a significant difference relative to the control group at the given time point ($p < 0.05$).
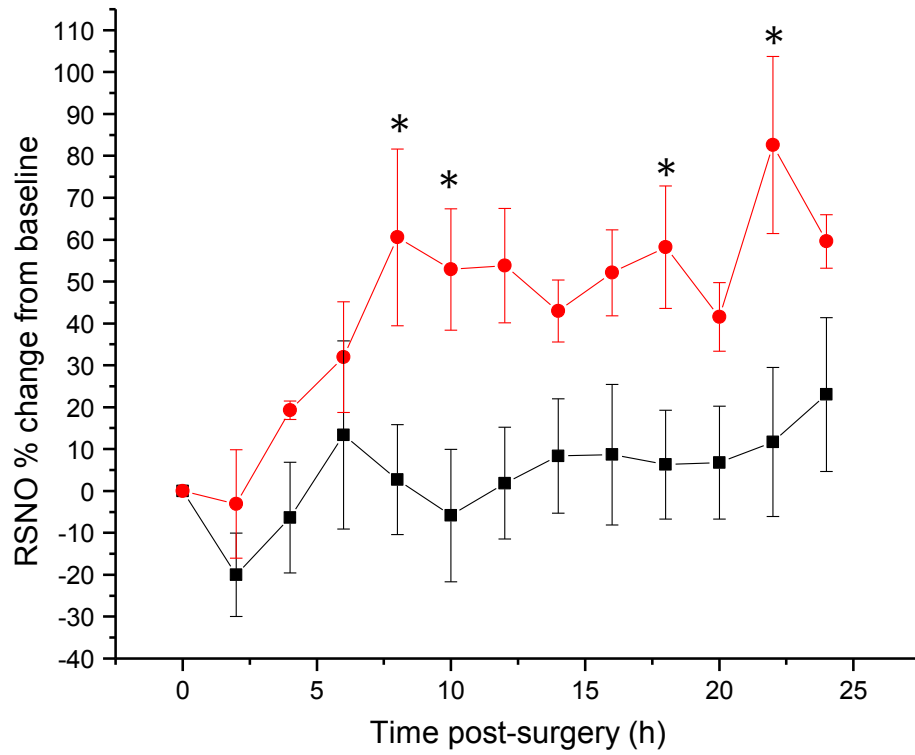
Figure 5.13 Percent change from baseline nitrosothiol concentrations in septic (●) versus control (■) Yucatan swine. Data are presented as the mean ± standard error of the mean. * denotes a significant difference relative to the control group at the given time point ($p < 0.05$).

cells may cause accumulation of nitrosothiols via a number of mechanisms. The most common routes of nitrosothiol formation requires a mixture of NO, oxygen (O_2), and thiols.[58] One of many nitrosothiol formation pathways relies on the oxidation of NO by O_2, where a peroxynitrite radical is generated that further reacts with NO to produce a nitrogen dioxide radical ($\cdot NO_2$). Nitrogen dioxide will react directly with a thiol to form a thiyl radical, that subsequently combines with NO to form nitrosothiol. Alternatively, $\cdot NO_2$ may react with another molecule of NO to form dinitrogen trioxide (N_2O_3) that directly nitrosates a thiol species. In the event that NO autooxidation does not take place, NO reacts directly with a thiol, forming an aminoxyl radical that can be oxidized to form a nitrosothiol. Following this initial increase in nitrosothiols concentration, their level remained relatively stable, indicating an early increase in NO generation.

5.4   Conclusions

These two CLP studies using the mixed-breed and Yucatan swine indicate that NO and nitrosothiols play a key role in the onset, development, and outcome of sepsis. In less severe cases of sepsis (Yucatan swine model), nitrosothiols appear to accumulate in the blood 12 h prior to the onset of other symptoms. While increased nitrosothiol levels do not appear to be linked directly to decreased blood pressure that can ultimately result in multiple organ dysfunction and death, the accumulation of these and other byproducts of NO production (e.g., nitrite) may prime the bloodstream for late-stage regeneration of NO as the blood chemistry becomes optimal (i.e., more acidic and hypoxic) for this process. Late production of large quantities of free NO was especially evident during severe sepsis, resulting in sizeable arterial pressure decreases and often death (n=4 animals). While NO is important for

the killing and clearance of bacterial pathogens during infection, its seemingly unregulated production in late-stage sepsis has the potential to cause significant physiological dysfuntion and death.

5.5   References

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CHAPTER 6: NITRIC OXIDE MONITORING IN A MURINE MODEL OF SEPSIS—EFFECTS OF BURN INJURY AND BACTERIAL VIRULENCE

6.1   Introduction

Infection and resulting sepsis are especially common amongst patients in the intensive care unit due to their immunocompromised state. Pneumonia, often a ventilator-associated infection, is the most common nosocomial (i.e., hospital acquired) infection, with an estimated annual incidence of 160,000 cases in the United States alone.[1,2] While the immune response to pathogens is a normal physiological response to infection and is usually a tightly regulated cascade, sepsis results when the body's response to infection becomes dysfunctional to the point of causing harm to tissues and organ systems. To date, sepsis remains the leading cause of mortality amongst critically-ill patients. In addition, the incidence of sepsis is increasing annually by 8.7%.[3,4] As such, the significant healthcare burden of sepsis motivates the need for improved understanding, diagnosis, and treatment of sepsis and its underlying causes.

While many types of bacteria have been implicated in nosocomial infection, *Pseudomona aeruginosa* and *Klebsiella pneumonia* are among the most prominent, particularly in ventilator-associated pneumonia.[1,5,6] Infection severity may vary significantly depending on the virulence (i.e., degree of pathogenicity) of a particular bacterial strain.[7] *K. pneumoniae* is known to be particularly virulent, sometimes resulting in immune paralysis.[8] In addition to other common virulence factors, this species of bacteria develops a thick,

acidic polysaccharide capsule, protecting it from phagocytosis and other immune defense mechanisms.[9]

Physical trauma such as burn injury also causes severe immune dysfunction, increasing the risk of infection, sepsis, multiple organ dysfunction, and death.[10] The immune response following trauma is characterized by an initial pro-inflammatory period and a subsequent anti-inflammatory phase.[10,11] The early stage is similar to the systemic inflammatory response syndrome (SIRS) that occurs during severe infection and sepsis,[12] with the release of pro-inflammatory mediators (e.g., tumor necrosis factor, interferon gamma, interleukin-6, interleukin-1β).[13] In the struggle to maintain homeostasis, a compensatory anti-inflammatory response syndrome (CARS) ensues following SIRS.[11,14,15] While limiting damage due to chronic inflammation, the CARS phase also increases a patient's susceptibility to infection.[6,14,16] Burn patients are especially prone to ventilator-associated and wound infections,[16,17] and the leading cause of death following burn injury is related to infection and sepsis.[16,18]

The ability to monitor the immune response during and following trauma and infection is key for understanding the physiological changes that occur and evaluating treatment options. The measurement of endogenous nitric oxide (NO), a free radical species that is intricately involved with the innate immune response,[19-34] may reflect the immune status of an organism. Indeed, the upregulation of inducible nitric oxide synthase (iNOS) during SIRS has been reported, in addition to the accumulation of NO byproducts in blood and tissue.[6,20,25,35-39] Conversely, Jacob et al. reported decreased serum concentrations of nitrate and nitrite in trauma patients, indicating suppressed NO production resulting from

CARS.[40] Cairns et al. reported that burn injury induced a late decrease in expression of Toll-like receptor (TLR),[41] a macrophage receptor linked with the induction of iNOS.[42-44]

Until recently, direct detection of NO in whole blood was not possible, but instead required the measurement of its byproducts (i.e., nitrate and nitrate) or the use of complex instrumentation (i.e., electron paramagnetic resonance spectroscopy).[45] As described in Chapter 3, a microfluidic amperometric sensor was developed to enable the measurement of NO in small volumes of blood.[36] To date, the effects of bacterial virulence and trauma-induced CARS on NO levels during infection have not been elucidated, and thus this device was used to study blood NO changes in a murine model of sepsis. Small animal (e.g., rodent) models of sepsis are particularly attractive as they permit the study of more fundamental mechanisms of sepsis due the availability of genetic variants and routes of infection (e.g., CLP, pneumonia).[46-49] Direct administration of live bacteria into the trachea or nasal passage produces severe pneumonia that represents ventilator-associated infection.[47,49,50] The effect of virulence is studied by comparing two separate strains of bacteria (i.e., *P. aeruginosa* and *K. pneumoniae*) with known pathogenicity variation. Lastly, we used a murine model of burn injury to examine the effect of trauma on NO levels during a late-stage infection.

6.2   Materials and Methods

6.2.1   *Microfluidic device fabrication and characterization*

Devices were fabricated as described previously.[36] Briefly, 150 nm thick planar platinum (Pt) electrodes with a 10 nm titanium (Ti) seed layer were patterned onto a glass substrate via photolithography and evaporative metal deposition. The resulting Pt electrodes were 100 μm wide. To provide selectivity to NO, a xerogel membrane was applied to each

working electrode. Following the deposition of 1002F-50 photoresist mask, an adhesion layer of (3-aminopropyl)triethoxysilane (1% v/v in ethanol) was deposited via three passes with a spray coater. The substrate was then rinsed with water and allowed to dry under ambient conditions overnight. The fluoroalkoxysilane membrane solution was prepared via the acid catalyzed hydrolysis and condensation of (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxysilane (17FTMS) and methyltrimethoxysilane (MTMOS) as reported previously.[51,52] Briefly, 600 µL absolute ethanol, 120 µL MTMOS, 30 µL 17FTMS, 160 µL distilled water, and 10 µL 0.5 M hydrochloric acid 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 using a pipette tip for 1 min to ensure even coating. 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 the deposition of a ~1 µm Ag layer. 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 bonded to the working electrode substrate by clamping the components together and heating at 100 °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.

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 to 1 h in PBS. Prior to use, 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 used to calibrate the device.

6.2.2 Murine model of infection with Klebsiella pneumonia and Pseudomonas aeruginosa

To evaluate the effect of bacterial virulence, two strains were used to induce pneumonia: 1) intratracheal administration of 50 μL *P. aeruginosa* (PAK strain, 1×10^6 colony forming units mL^{-1}) or 2) intranasal administration of 20 μL *K. pneumoniae* (2×10^5 colony forming units mL^{-1}). Uninfected groups were administered identical volumes of phosphate buffered saline (PBS) containing protease peptone. At select times following infection, ~300 μL blood was drawn into ethylenediaminetetraacetic acid (EDTA)-coated microcentrifuge tubes via submandibular puncture. This blood was immediately (within 10 s) injected into the microfluidic device and analyzed amperometrically to determine NO concentrations.

6.2.3 Murine model for sepsis and burn injury

All protocols were performed in accordance with the National Institutes of Health guidelines and approved by the University of North Carolina at Chapel Hill Institutional Animal Care and Use Committee. Nine week-old female C57BL/6 mice weighing ~18 g underwent a 20% total body surface area (TBSA) burn injury as previously described.[53] Briefly, mice were anesthetized with gaseous isofluorane, their dorsal flanks shaved, and they received a subcutaneous injection of morphine sulphate prior to receiving a full-

thickness burn with 4 applications of a copper rod heated in boiling water. Following burn injury, mice were resuscitated via an intraperitoneal injection of lactated Ringer's solution. Throughout the experiment duration, mice were monitored and received morphine in their drinking water (0.02 mg mL^{-1}; 4 mg kg^{-1} body weight per day) ad libitum. Sham (0% TBSA) mice also underwent these treatments as described, except the application of the copper rod. Rapamycin-treated mice received an intraperitoneal injection of rapamycin (4 mg kg^{-1}) 5 d prior to burn injury, with continued daily treatment following injury.

At 14 d following burn injury, pneumonia was induced via the intratracheal administration of 50 μL *Pseudomonas aeruginosa* (PAK strain, 1×10^6 colony forming units mL^{-1}) following sedation via Avertin. Uninfected groups were administered 50 μL PBS with protease peptone in the same manner. At 48 h following infection, ~300 μL blood was drawn into EDTA-coated microcentrifuge tubes via submandibular puncture. This blood was immediately injected into the microfluidic device and analyzed amperometrically to determine NO concentrations.

6.2.4   *Statistical analysis*

An unpaired, two-sided Student's t-test was used to determine statistical significant between groups, with $p < 0.05$ considered to be significant.

6.3   **Results and Discussion**

6.3.1   *Nitric oxide levels during systemic infection and the effect of virulence*

Following infection with *P. aeruginosa*, blood NO levels were monitored at 1, 3, and 7 d. At the dose of bacteria administered (1×10^6 colony forming units mL^{-1}), mice were expected to clear the infection and fully recover without treatment. Conversely, the virulence

associated with *K. pneumoniae* in mice often leads to mortality within 72 h following infection[54] and thus blood was drawn to monitor NO concentrations at 12, 24, and 72 h following infection. As shown in Figures 6.1—6.3, the blood NO levels remained relatively unchanged for uninfected mice in both experiments. In contrast, both infections resulted in greater circulating NO concentrations, although there were differences in the magnitude of change. Indeed, an infection of *P. aeruginosa* resulted in significant increases in NO at 72 h following infection, with a 90 ± 50% change relative to uninfected mice (Figure 6.4). By 7 d after the initial infection, NO concentrations returned to basal levels (Figure 6.5). For the more virulent *K. pneumoniae*, the most substantial increases in NO also occurred at 72 h following infection, with a much greater magnitude than that observed for *P. aeruginosa* at the same time point. Relative to uninfected mice, NO concentrations were increased by 600 ± 110% at 72 h. These mice do not generally recover from *K. pneumoniae* infection,[54] and the large increase in NO is likely attributable to the infection severity. The increase in NO observed in this model is similar to fatal infections that we previously reported on in both murine and porcine models of sepsis.[36] Given these results, it is clear that circulating NO concentrations reflect the severity of an infection, with infections by more pathogenic bacteria resulting in the generation of more NO.
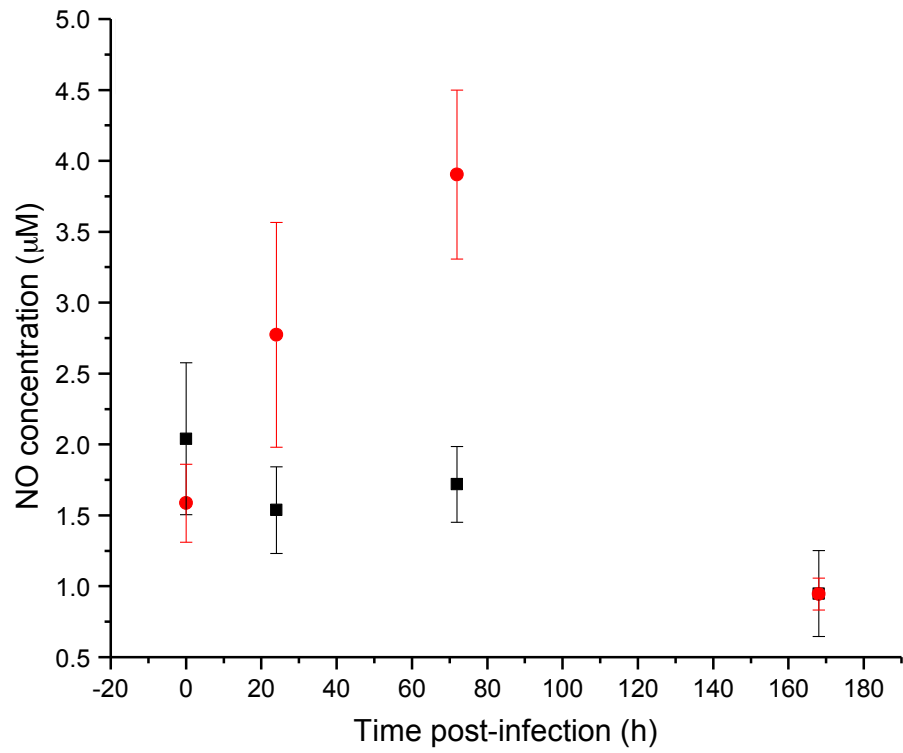
Figure 6.1   Change in blood NO concentrations for uninfected (■) and *Pseudomonas aeruginosa* infected mice (●). Data are given as mean ± standard error of the mean. For each time point, n = 4 for uninfected and n = 6 for infected.
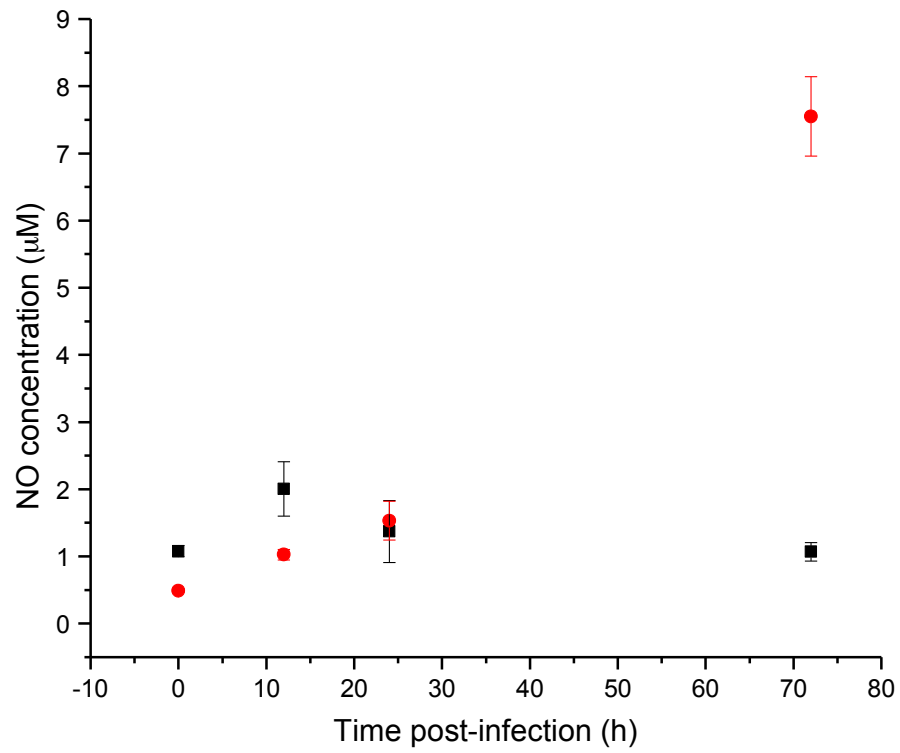
Figure 6.2 Change in blood NO concentrations for uninfected (■) and *Klebsiella pneumoniae* infected mice (●). Data are given as mean ± standard error of the mean. For each time point, n = 2–3.
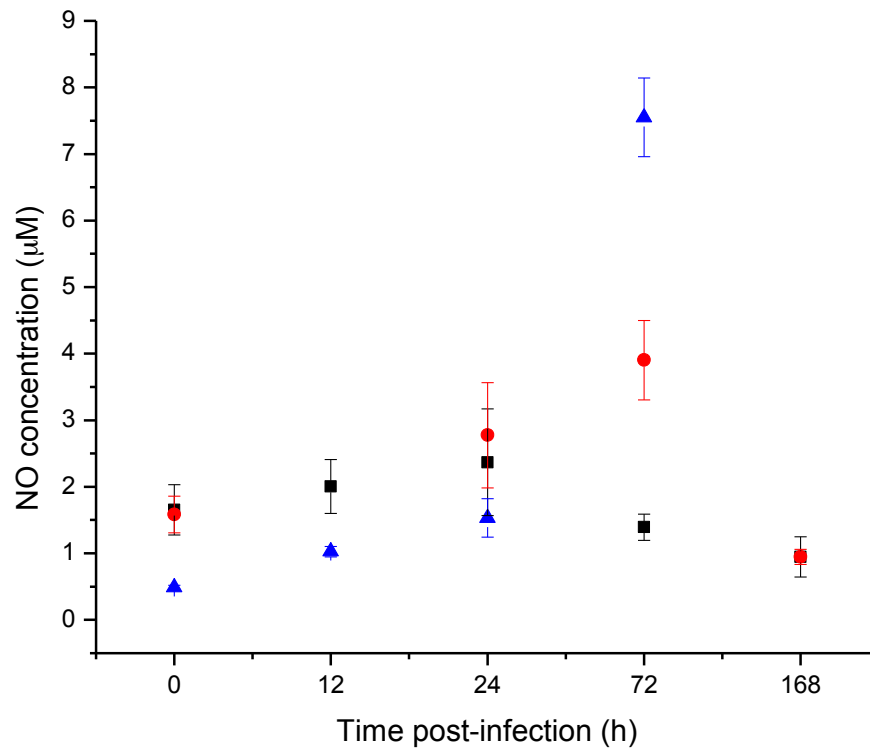
Figure 6.3   Change in blood NO concentrations over time during *Pseudomonas aeruginosa* (●) and *Klebsiella pneumoniae* (▲) infections in a murine model. Shown relative to uninfected mice (■). Data are given as mean ± standard error of the mean.
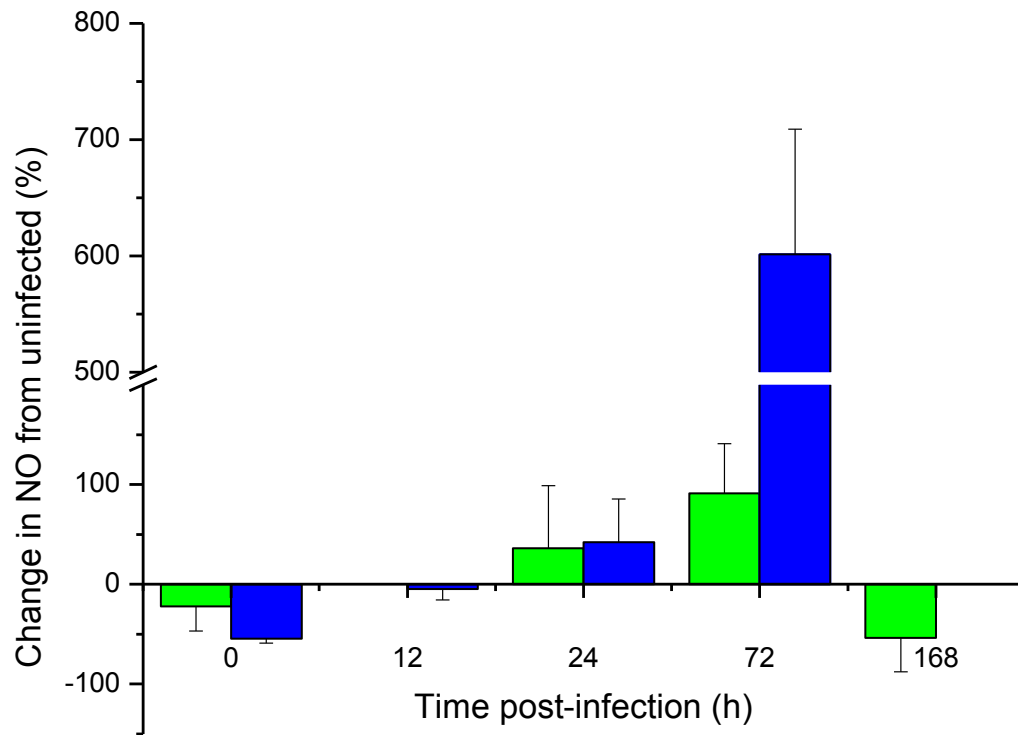
Figure 6.4   Percent change in murine blood NO concentrations relative to uninfected animals during infections with *Pseudomonas aeruginosa* (green) and *Klebsiella pneumoniae* (blue).
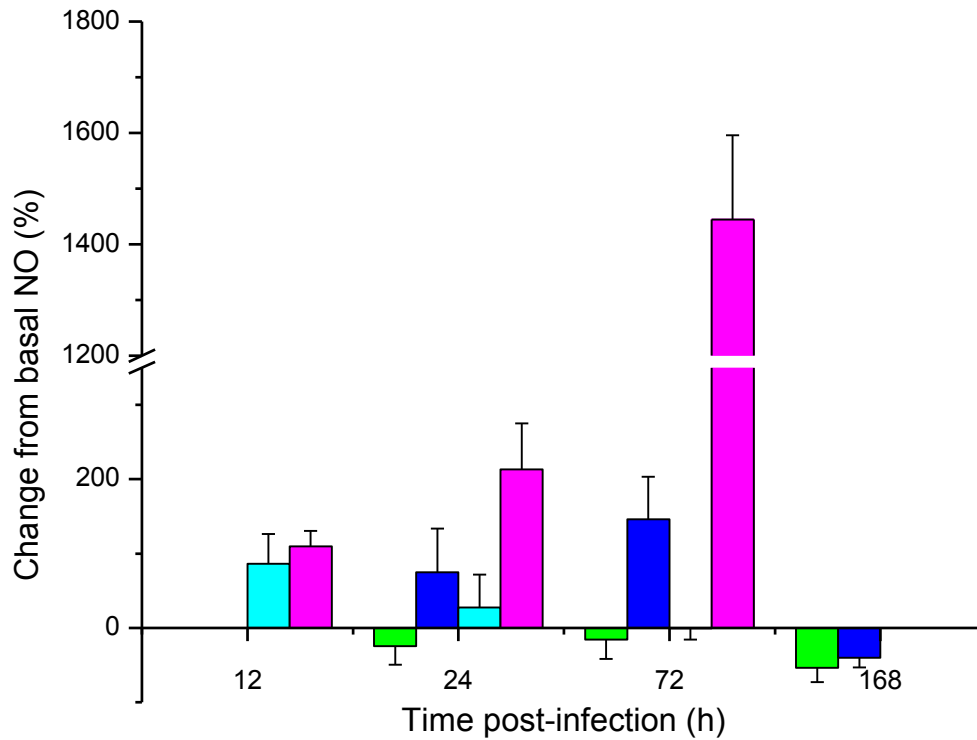
Figure 6.5 Percent change in murine blood NO concentrations relative to basal levels during infections with *Pseudomonas aeruginosa* (blue) and *Klebsiella pneumoniae* (magenta). Shown relative to uninfected mice for *P. aeruginosa* (green) and *K. pneumoniae* (cyan).

6.3.2   Nitric oxide during the compensatory anti-inflammatory response syndrome following burn injury

While these studies have made the link between NO and SIRS evident, the effect of CARS on systemic NO concentrations remains unknown. As burn injury elicits late-stage CARS,[14,55,56] a murine burn model was used to evaluate changes in NO production during periods of immune suppression. A previous report by Cairns et al. demonstrated that while burn injury induced upregulated expression of Toll-like receptor (TLR) by macrophages early (3 d) following trauma, decreased TLR expression occurred at later time points (14 d).[41] Other studies have linked TLR with the induction of iNOS and concomitant release of NO by innate immune cells (e.g., macrophages).[42-44] Indeed, TLR is directly involved in microbe recognition by innate immune cells (TLR2 for Gram-positive peptidoglycan and TLR4 for Gram-negative lipopolysaccharide) and thus mediates subsequent inflammatory signals, including NO.[57] As shown in Figure 6.6, blood NO levels were significantly increased during a *P. aeruginosa* infection without previous trauma. At 48 h following infection, NO concentrations were 810 ± 180 and 370 ± 40 nM for infected and uninfected mice, respectively. This deviation is not surprising, as NO is known to be produced in response to bacteria,[58] with increased endogenous NO levels having been observed during systemic infection.[36] However, similarly increased NO levels were not observed during *P. aeruginosa* infection occurring 14 d following burn injury. Nitric oxide concentrations were 410 ± 110 and 290 ± 80 nM for uninfected and infected mice, respectively. The differences between the infected and uninfected groups with burn injury were insignificant and indicate a late-stage effect of trauma on immune function (i.e., immune suppression) and NO production

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Figure 6.6 Nitric oxide levels 48 h following *P. aeruginosa* infection in a murine model of sepsis, both with and without prior burn injury. Data are given as mean ± standard error of the mean. For each treatment, n = 3–4 and 4–6 for uninfected and infected groups, respectively. Statistical significance is indicated by * based on $p < 0.05$.
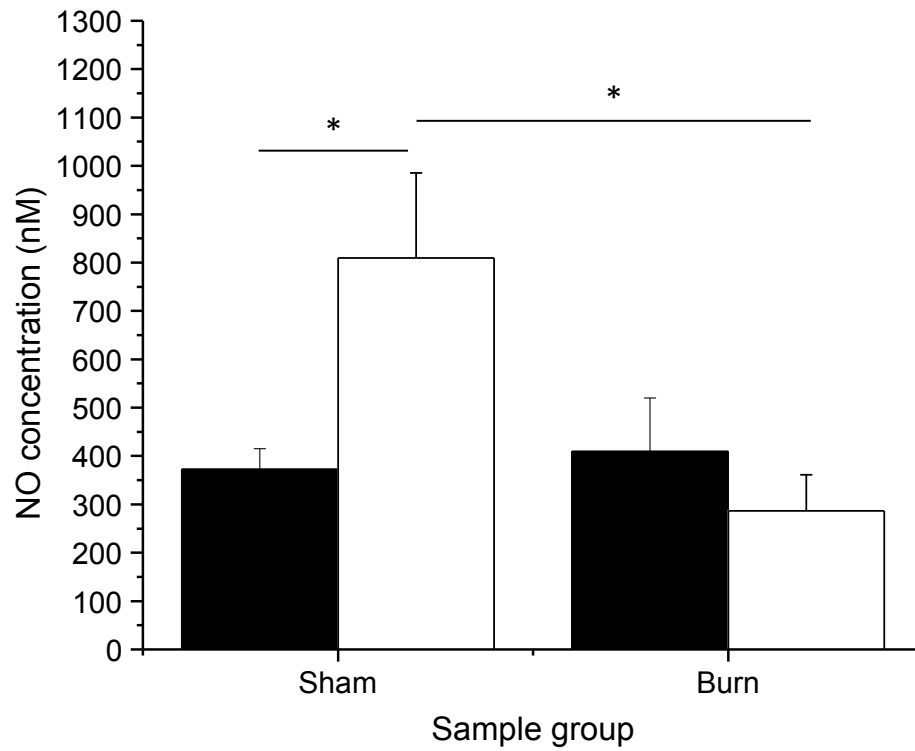
during an infection. Of note, the NO concentrations observed in burn mice (both infected and uninfected) were equivalent to those in the uninfected sham mice. The dysfunctional immune response following burn injury has been well documented,[11] with CARS frequently observed late after burn injury. Multiple studies have suggested that early hyperactivity of macrophage cells contributes to the development of immune suppression.[59-61] The observed decrease in NO production is not surprising given the numerous immune defense systems linked to TLR, including the induction of iNOS.[57] Reduction in NO production by innate immune cells, along with other aspects of immune suppression (e.g., shifts in T cell phenotype,[61] altered cytokine profiles[62]), contribute to the increased infection susceptibility of burn patients.

Similar to burn injury, treatment with rapamycin also led to decreased NO levels despite infection. Rapamycin is well known for its immunosuppressive action by its blockage of the mammalian target of rapamycin (mTOR), which is believed to regulate the innate immune response.[63] In particular, inhibition of mTOR has been proven to decrease NO production by lipopolysaccharide-stimulated macrophage cells, possibly due to decreased secretion of interferon-β.[64] As shown in Figure 6.7, rapamycin treatment negated the expected NO upregulation relative to uninfected animals, both with and without prior burn injury. A study by Malik et al. has also demonstrated the ability of rapamycin treatment to inhibit the clearance of a *P. aeruginosa* lung infection, likely due in part to decreased oxidative burst from pulmonary neutrophils.[65]

Nitric oxide concentrations during infection were similar between the rapamycin-treated and burn groups. Some studies have indicated that all isoforms of NOS are upregulated during severe infection and sepsis,[66,67] but our studies suggest the iNOS is the
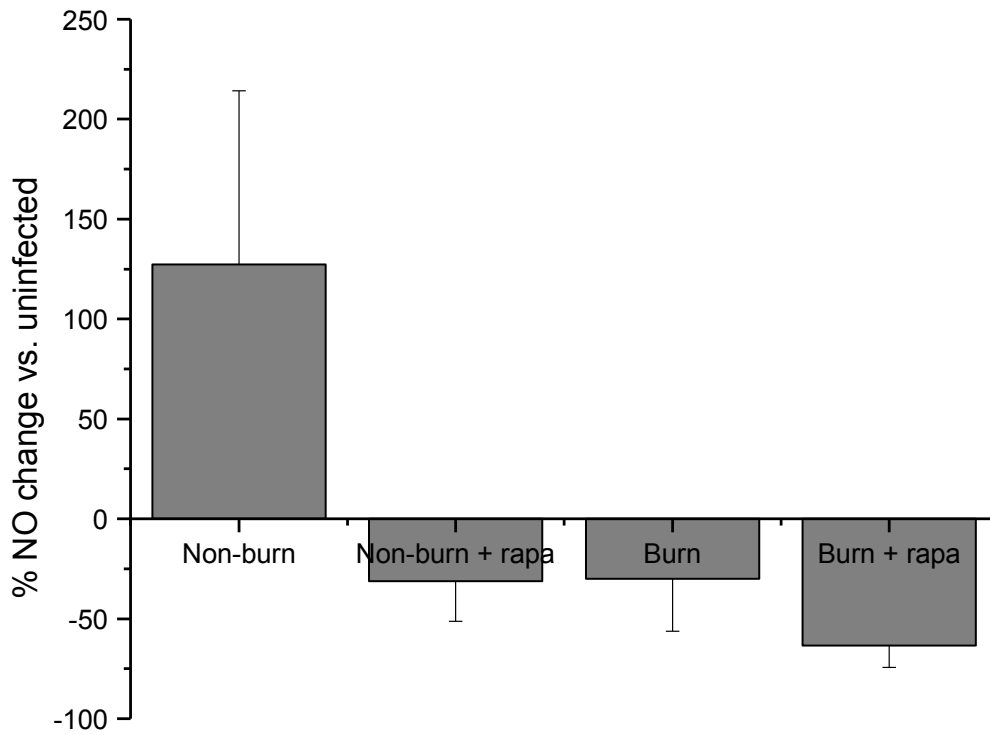
Figure 6.7   Percent change in NO relative to uninfected mice during *Pseudomonas aeruginosa* with and without 14 d prior burn injury and after rapamycin treatment. Each treatment group has a separate uninfected control group. For each treatment, n = 3–4 and 3–6 for uninfected and infected groups, respectively.

major contributor to NO production. While the physiological response to burn trauma and the development of immune suppression are complex, modulation of NO release may be key in improving patient outcomes. Especially during CARS, supplementing endogenous NO production (via NO-release vehicles or modulation of physiological pathways to stimulate NO release) may prevent the spread of serious systemic infections. Diminishing the initial inflammatory burst that results in later immune suppression is likely of equal importance.

6.4   Conclusions

While increased levels of endogenous NO may indicate development of a severe infection and SIRS in otherwise healthy animals, the immune dysfunction during CARS caused by prior trauma (e.g., burn injury) will also significantly alter its production. As such, monitoring in vivo NO production may provide insight into emerging infection as well as immune dysfunction. As such, understanding normal circulating NO concentrations is vital to comprehending the immune response to infection. The clinical utility of blood NO measurements should be evaluated further by monitoring concentration changes at additional time points (i.e., more frequently) throughout the course of infection/sepsis and throughout the SIRS/CARS phase following trauma. Additionally, the effect of pathogenicity must be evaluated with the use of identical strains of varied virulence.

6.5   References

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CHAPTER 7: SUMMARY AND FUTURE DIRECTIONS

7.1   Summary of work

In the preceding chapters, the development of sensors for NO and nitrosothiol measurements was described, along with select biologically relevant applications. Most notably, they were used to measure the production of NO and nitrosothiols during the infection and inflammation associated with sepsis. Before such measurements were possible, it was necessary to understand the various techniques available for the measurement of NO and their caveats in complex biological environments. In Chapter 2, the three main analytical techniques for the measurement of NO (i.e., Griess assay, chemiluminescence, and electrochemistry) were evaluated for accuracy in variety of complex media, including cell/bacterial culture broths and blood (whole, serum, and plasma). This work was especially important given the relatively low physiological concentrations of NO. The Griess assay was quite useful for estimating total NO concentrations based on the buildup of its byproducts (i.e., nitrate and nitrite) in solution, but accuracy depended on the sample milieu. In addition, sample volume (and thus headspace within the sample vial) was important, as a large headspace volumes permitted the loss of NO and resulted in underestimations. A dependence on sample concentration was also observed. The chemiluminescence analyzer was especially useful for obtaining real-time kinetic information and totals. However, this technique required the use of very specialized equipment and was limited to non-proteinaceous media, as purging with

nitrogen was required to carry NO to the detector. As such, scavenging effects were minimized due to the lack of oxygen and the limited time allowed for NO to interact with media components. Electrochemical detection (i.e., constant potential amperometry) allowed for the greatest versatility, as it could be utilized in all types of media (including whole blood), whether they were deoxygenated or not. Due to the small electrode size (3 mm diameter) relative to the large volume of media (~30 mL), overall totals calculated were low, as detection is limited to the NO oxidized at the surface of the electrode. Similar to the Griess assay, a dependence on concentration and headspace volume was observed for electrochemistry as well. When measuring from a NO-releasing surface (e.g., films or cells) a distance-dependence also existed. Despite what seem like numerous disadvantages, electrochemical techniques provide unparalleled versatility and as such are ideal for measuring NO from biological samples, including cells and whole blood.

The advantages of electrochemical NO detection were exploited to develop a microfluidic amperometric sensor for the measurement of small volumes of biological fluids (e.g., blood). The fabrication and utilization of this device was described in Chapter 3. Standard photolithographic techniques were used to pattern working, counter, and reference electrodes onto glass slides. The microfluidic channels were formed using Kapton® tape to seal the slides together. The device was evaluated in phosphate buffered saline, wound fluid (10% v/v fetal bovine serum in water), and whole blood. While sensitivity was decreased in whole blood relative to phosphate buffered saline, limits of detection down to 400 nM were still achievable. Additionally, the device was selective against a number of important physiological interferents (i.e., nitrite, ascorbic acid,

acetaminophen, uric acid, hydrogen sulfide, ammonium, ammonia, and both protonated and deprotonated peroxynitrite). Blood samples as small as ~250 μL was measurable, which is especially necessary for collecting samples from small mammals (e.g., rodents). To demonstrate the clinical utility of the device, it was used to measure temporal changes in blood NO levels in a murine model of sepsis. For the lethal dose of bacteria administered, seven-fold increases in NO were observed over a period of 24 h.

In Chapter 4, the utility of the microfluidic sensor was expanded to incorporate the detection of *S*-nitrosothiols (i.e., *S*-nitrosoglutathione, *S*-nitrosocysteine, and *S*-nitrosoalbumin) via visible photolysis and subsequent detection of NO. The device itself was identical to that detailed in Chapter 3, but with the addition of an external 530 nm LED. As nitrosothiols absorb light in this region of the spectrum, photolysis can be facilitated without interference from nitrite. The light source was tuned to achieve efficient nitrosothiol photolysis and detection. As expected, increased light intensity yielded improved sensitivity. Irradiation area was also tuned, but light intensity varied as the spot size was changed, and no change in sensitivity was noted due to irradiation area alone. Once the optimal LED arrangement was achieved, a significant improvement in sensitivity and limit of detection compared to previously reported results in bulk solution (~40 mL) was noted due to the small sample volume and cross-section of the device. Indeed, a 6–20× improvement in sensitivity (depending on nitrosothiol type) was noted in phosphate buffered saline when utilizing the microfluidic configuration. Additionally, minimal analysis time (i.e., <2 min for sample irradiation and detection) was required. While detection of RSNOs directly in blood was not realized, analysis in the serum

separated from whole blood was achieved. Using this device, basal nitrosothiol levels in healthy swine were quantified at 1.5 ± 1.0 μM, falling within previously reported ranges.

The application of the microfluidic amperometric NO and nitrosothiol sensors for numerous animal models was described in Chapter 5. Both murine and porcine models for sepsis were utilized to determine the link between NO and this disease state and evaluate its potential as a prognostic and/or diagnostic biomarker. In order to monitor disease progression longitudinally, a porcine cecal ligation and puncture model for sepsis was chosen. Utilizing large animals allowed for the hourly collection of blood and the use of traditional intensive care unit monitoring (i.e., heart rate, blood pressure, body temperature, blood gasses, and blood cell counts). Hemodynamic trends were as expected, with steadily increased heart rate and decreased mean arterial pressure observed. In the preliminary porcine study, NO levels increased in conjunction with other potential indicators (e.g., lactate), but the percent change from basal levels was significantly more dramatic for NO. A large rise in NO concentration was observed at ~4–5 h post-surgery. Significant increases (up to 800%) were also observed at much later time points, especially in animals where the model was lethal prior to 24 h. Surprising, circulating white blood cells counts were significantly diminished within a few hours following the surgery in the septic animals. A follow-up study allowed for some modifications, including continuous (rather than bolus) administration of lower levels of buprenorphine and the detection of nitrosothiols in addition to NO. The swine were also changed from a mixed breed to a more standardized breed, in the hopes of reducing variability between animals. While this breed appeared to be much more robust and tolerant of the procedure, the additional measurement of nitrosothiols proved promising,

as significant changes were noticed in septic versus control animals at ~8 h post-surgery. Overall, changes in lactate and NO were less dramatic relative to the first group of swine, with significant increases not occurring until 19 and 20 h for NO and lactate, respectively. This study highlighted the link between circulating NO concentrations and other physiological changes (e.g., blood lactate), but also indicated that metabolites of NO (i.e., nitrosothiols) may accumulate early in sepsis.

Unlike the porcine model, a murine model of sepsis is particularly useful for studying mechanistic details of the role of NO in sepsis and general inflammation. Thus far, work in this model has focused solely on the measurement of NO. The murine model involved the intratracheal administration of pure strains of bacteria at known concentrations. Initial experiments monitored NO changes over a multi-day period to determine its role as infection developed and resolved. This was carried out for two separate strains of bacteria, *Pseudomona aeruginosa* and *Klebsiella pneumoniae*, to determine if strain virulence had an effect on NO levels. Indeed, mice easily clear the dose of *P. aeruginosa* administered in <1 wk, while mice infected with *K. pneumoniae* usually do not recover. As such, mice infected with *P. aeruginosa* had increased concentrations of NO at 72 h following infection (91 ± 50% increase relative to controls), but returned to basal levels by 1 wk, indicating their ability to recover from this infection. Conversely, during an infection with *K. pneumoniae*, NO levels were increased much more dramatically at 72 h (600 ± 110% increase relative to controls). This infection is fatal, and these increases in NO are similar to those observed during a high dose *P. aeruginosa* infection (lethal within 48 h). Finally, as patients with prior trauma are especially prone to nosocomial infection,[1,2] we sought to evaluate the effect of burn

injury on NO levels in this murine model of infection. As expected, an infection that occurred 14 d following a burn injury yielded no detectable increase in NO relative to uninfected animals, demonstrating the significant immunosuppressive effect of CARS following burn injury. While this may limit to ability to "track" the progression of an infection in burn patients, it does offer insight into the immune suppression that is occurring which must also be controlled.

At a more fundamental level, electrochemical sensors can also be used to measure NO from various cell systems. Chapter 6 detailed the use of microelectrodes to probe NO and CO release from cultured macrophage cells as well as snail buccal ganglia. Increases in NO and CO generation from RAW 264.7 macrophage cells was noted following stimulation with lipopolysaccharide/interferon-gamma and hemin/NADPH, respectively. Promotion of increased CO generation prior to iNOS stimulation resulted in reduced NO production, with higher hemin concentrations resulting in lower amounts of NO release. The cross talk between NO and CO was also obvious in snail buccal ganglia, as inhibited CO generation led to a significant increase in the production of NO.

7.2   Future studies

Initial results from animal models of infection and sepsis have indicated the pivotal role of NO in inflammation. Monitoring of endogenous NO production may eventually prove to be of diagnostic and/or prognostic value to patients in the intensive care unit, but more systematic studies in animal models must first be carried out. In particular, mouse models will allow for the study of fundamental roles of NO during infection and following treatment. To build on the work completed thus far, future studies

must assess whether infection with different types of bacteria has an effect on NO production or its duration and rate of release. For example, more virulent strains may induce more rapid NO release or higher concentrations, as these strains are more challenging to clear.[3] For example, Matsumoto et al. discovered that a virulence regulatory factor CvfB (common in some strains of *Staphylococcus aureus* and *Streptococcus pneumonia*) binds RNA and plays a role in infection severity.[4] Additionally, Gram-positive bacteria must be evaluated as they induce an immune response via different receptors on innate immune cells.[5] In particular, infections by *Staphylococcus aureus* are especially common in hospital patients.[1,6] While we have observed that NO levels in mice decrease once the infection has been eradicated, there is also an interest in understanding whether antibiotic administration will produce a similar effect. In particular, it is necessary to understand if the use of bactericidal versus bacteriostatic antibiotics will produce differential effects. While bacteriostatic antibiotics keep bacteria in the stationary phase of growth, bactericidal agents kill >99.9% of the inoculum. However, the lytic action of bactericidal agents may cause an intensified immune response,[7] thus altering NO release.

The effect of physical trauma such as burn injury, with or without infection, is also of immense interest as these patients are particuarly prone to nosocomial infection. Even without a subsequent infection, the inflammation caused by injury may result in changing in NO levels over the course of many weeks following trauma.[8] Indeed, while the SIRS/CARS phenomenon following trauma has been elucidated at discrete time points, it still poorly understood overall.[9] The physiological response to infection following injury will also vary significantly depending on when exposure to bacteria

occurs (i.e., early or late after trauma).[10,11] As such, NO levels should be monitored in infections that begin at varying time points following a burn or other traumatic injury.

To date, it is unclear whether NO produced during inflammation is due solely to iNOS or also originates from endothelial NOS and/or neuronal NOS. While our studies involving rapamycin treatment revealed the ability of this drug to inhibit NO production via iNOS during infection, NO release was not completely diminished. In the future, the study of NO concentrations in iNOS knockout mice with and without infection could be directly compared to rapamycin treatment. Additionally, nNOS and eNOS knockout mice (and/or drugs to inhibit these enzymes) should be utilized to understand the role of each enzyme in inflammation due to infection and trauma.

While ventilator-associated infection (pneumonia) is common in hospital patients, other types of infection also persist.[12,13] Utilizing alternative models of sepsis in rodents, especially cecal ligation and puncture, will provide additional information on the role of NO in multiple routes of infection, as septic peritonitis and polymicrobial infections are common.[14] Additionally, a two-hit model of sepsis may provide relevant information, as sepsis often occurs after bacteria exposure that follows another type of injury and/or inflammation.[15]

In addition to building upon previous animal studies, future work will also require refinement of the measurement device. Most importantly, the device must be miniaturized in order to accommodate smaller sample volumes. The current minimum volume (~250 μL) prevents serial measurements in a single mouse, as drawing a large volume such as this induces hypovolemic shock.[16] Ideally, a single drop of blood (<50 μL) could be used for future measurements. Further tuning of selectivity and sensitivity

will also be important. Enhanced sensitivity would allow for detection of much smaller changes in NO concentrations and is most easily achieved by tuning electrode surface area. For example, platinization of the working electrode (with lead acetate and platinum black) has been utilized on disk-type microelectrodes, but not planar electrodes within a microfluidic device. Thickness of this platinization layer will be important, as a layer that is too thick may impede flow through the device. This modification may necessitate recessing the working electrode so it does not significantly protrude into the channel. Further tuning of the composition and application of the selective xerogel membrane may allow for a simultaneous improvement in selectivity and sensitivity. In particular, spray-coating procedures should be evaluated, as they will allow for more even and reproducible coatings.

While NO and nitrosothiol detection are currently carried out in separate devices, it is possible to eventually incorporate both into a single platform. The current challenge in achieving this lies in the inability to detect nitrosothiols in whole blood, resulting from significant light absorbance/scattering by red blood cells. The two main options that exist for addressing this problem are 1) the use of a much more intense light source and 2) the removal of red blood cells from the measurement entirely. While a more intense light source may help overcome the poor transmittance of blood, there is also a risk of sample heating, which will initiate non-specific nitrosothiol degradation. It is also disadvantageous to remove red blood cells from both the NO and nitrosothiol measurement, as they play an important role in NO regeneration from nitrite. A useful alternative would be the creation of a device that allows for detection of NO from whole blood with subsequent exclusion of red blood cells to allow for photolysis and detection

of nitrosothiols. This would be possible with the incorporation of a filter or a narrowing channel. Numerous examples that utilize both currently exist in the literature.[17-20] Having a single device to make both measurements simultaneously will significantly decrease analysis time and increase the feasibility of future clinical implementation.

Finally, there are a number of studies outside the scope of animal models that can help further elucidate the physiological roles of NO. Related to the murine sepsis studies that have been carried out, it is also of interest to study NO in inflammation at the cellular level. During in vivo studies, immune cells are routinely harvested, sorted, and analyzed to determine release of expression of various markers, cytokines, and other mediators. These cells could be cultured and monitored electrochemically to determine their NO release profiles or their ability to be stimulated by lipopolysaccharide or peptidoglycan. The study of CO release would also be possible using the dual microelectrodes pioneered by the Lee lab.[21,22]

As our lab also designs nanomaterials for clinical applications, there is interest in characterizing their immune stimulation characteristics in addition to cytotoxicity. While many of the materials generated in the lab have proven non-cytotoxic, they may still generate a host immune response. While this response is normal, a prolonged or overzealous response—possibly elicited by certain types of materials or functional groups—will create a risk of chronic inflammation. Both types of NO sensors developed in our lab (i.e., microfluidic devices and disk-type microelectrodes) coupled with more traditional assays (e.g., cytotoxicity and cytokine profiling) could be used to assess this response in cultured macrophage cells. While scanning electrochemical microscopy is useful for spatial resolution of NO release from individual cells, these cells can also be

cultured within the channel of our microfluidic device. This setup would facilitate incubation with different types of nanomaterials to assess their biocompatibility. The measurement of NO in addition to pro-inflammatory cytokines (e.g., tumor necrosis factor and interferon gamma) will provide insight into macrophage phenotype, with increased concentrations indicating a shift toward the M1 (pro-inflammatory) phenotype. Assessment of cytotoxicity is possible using a lactate dehydrogenase assay.[23] As transition to the M1 phenotype requires a significant increase in metabolic activity, the MTT/MTS assay can be used to monitor mitochondrial activity.[24] In particular, changes in macrophage phenotype as a function of exposure time, material type or functionalization, and concentration can be determined. For nanoparticles, assessment as a function of size is also important.

7.3   Conclusions

Electrochemical devices are well-suited for tuning to specific biological systems and as such, will help to elucidate the complex physiological roles of NO and nitrosothiols, potentially providing new diagnostic tools. Induced NO release from immune and other cells types is especially of interest, as various triggers and complex pathways are involved. Despite knowing little about the exact mechanism of action, many hypothesize that NO plays a vital role in disease states related to severe infection and inflammation, such as sepsis. Our research provides the first example of direct monitoring of endogenous NO levels during the progression of sepsis in two separate animal models. The ability to measure NO directly, rather than its metabolites and/or byproducts, provides unique real-time information about the circulation of NO, whether

via its direct production from cells or recycling in the blood and tissues. As previous indirect assays have indicated, NO levels do increase quite significantly in sepsis, and it seems that other physiological changes also play an important role in its generation, scavenging, recycling, and circulation. Future devices will expand on the versatility of such measurements and further enhance the understanding of NO's role in biology, in areas including wound healing, organ transplant, cystic fibrosis, and neurological diseases.

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**APPENDIX: CELLULAR NITRIC OXIDE AND CARBON MONOXIDE—
UNDERSTANDING RELEASE FROM PHAGOCYTES AND NEURONS**

A.1   Introduction

Since the realization that the endothelial derived relaxation factor was likely to be nitric oxide (NO),[1] the pivotal role of this gaseous free radical species has been recognized in a number of biological processes including the immune response to infection,[2-4] vasodilation,[5] would healing,[6,7] and cancer biology.[8] Other biological gasses have also been recognized as significant, including oxygen (O_2), hydrogen sulfide (H_2S), and carbon monoxide (CO).[9] Much interest in how these gasses interact with one another while exerting their individual physiological roles now exists, particularly with respect to certain diseases. Both NO and CO are known to impart physiological activity through binding to the heme center of soluble guanylyl cyclase (sGC), an enzyme involved in the formation of the second messenger cyclic guanosine monophosphate (cGMP). This shared target means NO and CO serve similar physiological roles (i.e., vasodilation,[10,11] inflammation,[12,13] and neurotransmission[14,15]).

Within the innate immune system, NO is produced by specific cells (i.e., monocytes/macrophages, neutrophils, eosinophils) via the inducible isoform of nitric oxide synthase (iNOS), an enzyme whose expression is regulated by complex cascades of cytokines from immune cells.[4,16] All isoforms of NOS (i.e., inducible, endothelial, and neuronal) produce NO from L-arginine, which is oxidized to produce *N*-hydroxy-L-arginine. Further oxidation of this intermediate yields both L-citrulline and NO. Stimulation of iNOS by cytokines or components of the bacterial cell wall (e.g., lipopolysaccharide or peptidoglycan) initiates NO release for up to 5 d as long as the

stimuli remains present and the intracellular L-arginine supply is not depleted.[4] When released by these cells, NO acts as a signaling molecule and may also have pro-inflammatory activity or immunosuppressive (i.e., anti-inflammatory) effects, depending on the concentration. For example, the presence of bacteria within the body will upregulate iNOS production by immune cells, with the NO release killing nearby microbial organisms.[3,4,17-19] The bactericidal properties of NO are attributed to both nitrosative and oxidative stress, caused by toxic byproducts such as dinitrogen trioxide, which initiates nitrosation of protein thiols and DNA deamination.[20] Nitric oxide may also react with superoxide (a radical product of normal cell respiration) to yield peroxynitrite, the buildup of which results in lipid peroxidation and membrane damage.[21-23]

Nitric oxide also serves as an anti-inflammatory molecule or immune suppressant, providing a mechanism to defend the host from itself. For example, NO has been shown to suppress T-helper cell proliferation and cytokine production, thus limiting the chronic immune response.[24] While neutrophils are stimulated by low NO concentrations, they may experience inhibited adhesion to endothelial cells in the presence of elevated NO levels, thus hindering their ability to traverse the vascular wall and migrate to local sites of infection.[25] Further, NO has been observed to inhibit mast cell degranulation.[26]

Carbon monoxide, in addition to ferrous iron and biliverdin, is produced by cells by the degradation of free heme via heme oxygenase (HO) in the presence of O_2 and nicotinamide adenine dinucleotide phosphate (NADPH).[9] Similar to NOS, three isoforms of HO exist, including an inducible form (HO-1) linked to the immune response.[27] Unlike NO, CO serves a protective role in the immune system as both an anti-inflammatory and

anti-oxidative molecule.[28] Up-regulation of HO-1 occurs during oxidative stress,[29] suggesting coordinating functions of NO and CO. Additionally, HO-1 is associated with the production of superoxide dismutase, which will limit the cytotoxic effects of NO by decreasing peroxynitrite formation.[30] Increased CO generation can also down regulate NO production as CO can bind to and/or degrade the heme active site of NOS.[31]

In contrast to the immune system, NO in the nervous system is not produced by an inducible NOS isoform. Rather, it is generated by neuronal nitric oxide synthase (nNOS), which is triggered by increases in intracellular calcium.[32-34] Small, transient increases in intracellular calcium concentrations will thus cause short-lived production of NO (nM amounts for <10 min).[35] Within the central nervous system, NO is involved in general neurotransmission, thermal regulation, hormone release, and sleep cycles.[36-39] Nitric oxide is also plays a role in long-term potentiation (i.e., memory formation).[40] Rehder et al. reported the importance of NO in neuronal development, as it regulates the extension of neuronal growth cone filopodia.[41,42] Nitric oxide produced by nNOS within the peripheral nervous system is involved in smooth muscle relaxation.[34] Similar to the cardiovascular and immune systems, dysfunctional NO production within the nervous system can contribute to disease. Inflammation and resulting NO production contributes to numerous neurodegenerative diseases, including Alzheimer's, Parkinson's, amyotrophic lateral sclerosis, and multiple sclerosis.[43]

Heme oxygenase-2 is concentrated in the brain and produces CO as a neuronal messenger with functions comparable to NO.[38] For example, CO is involved with long term potentiation in the brain.[44] Verma et al. demonstrated the ability of CO to inhibit the release of certain endocrine factors, including oxytocin and arginine vasopressin.[14]

Additionally, CO may be linked to circadian rhythm regulation.[45] While NO is implicated in vasodilation, CO can function as a vasoconstrictor or vasodilator, depending on the presence of reactive oxygen species.[46] Carbon monoxide has recently been implicated in neurological disease, including stroke.[47] Cross-talk between NO and CO can occur in the same manner as in the immune system and remains misunderstood to date.

The most common detection strategies for NO and CO analysis from cells and tissues involves the measurement of their byproducts. The accumulation of nitrate and nitrite from NO released into solution (i.e., culture medium or blood plasma) is easily measured using the Griess assay.[48] Carbon monoxide production is quantified via detection of biliverdin, a byproduct of CO generation by heme oxygenase.[49] Immunohistochemical staining has been used to quantify the upregulation of NOS or HO within tissues.[50] While only recently gaining in popularity, electrochemical methods offer the unique advantage of allowing for the detection of NO and CO directly from its source in real time. The first microelectrode for the simultaneous measurement of NO and CO was reported by Lee et al. in 2007.[51,52] These sensors were used to measure NO and CO from brain and kidney tissue. A final goal of my thesis was to measure NO release from snail buccal ganglia and quantify both NO and CO simultaneously from cultured macrophage cells, further expanding the knowledge of the in vivo relationship between NO and CO generation.

A.2   Materials and Methods

A.2.1   Sensor fabrication and characterization

A dual amperometric sensor for the simultaneous measurement of NO and CO was prepared as described previously.[51] Briefly, two platinum (Pt) wires (25 and 75 μm) were inserted into the openings of a pulled dual barrel capillary. The tapered region of the capillary was then thermally fused. The end of the electrode was polished using 3.0, 1.0, 0.05 μm diamond paper and then electrochemically cleaned in 0.5 M sulfuring acid using cyclic voltammetry (+1.2 to −0.3 V vs. Ag/AgCl). The 75 μm (NO/CO) working electrode was platinized in a 1% (v/v) chloroplatinic acid aqueous solution using chronocoulometry at −0.1 V vs. Ag/AgCl (2 C cm^{-2}). The surface was further modified with the addition of a tin layer, deposited electrochemically at −0.6 V vs. Ag/AgCl in a stirred aqueous solution containing 0.01 M tin chloride and 3 M sulfuric acid for 1 h. The 25 μm (NO) working electrode was platinized in a 3% (v/v) chloroplatinic acid aqueous solution using cyclic voltammetry (+0.6 to −0.35 V vs. Ag/AgCl) at a scan rate of 20 mV s^{-1}. Single microelectrodes for the measurement of NO alone were fabricated in a similar manner utilizing a 50 μm Pt wire. To impart selectivity against common interferents, the microsensors were dip-coated sequentially into a (3-aminopropyl)triethoxysilane solution (1% v/v in ethanol) and a fluorosilane-based sol, prepared as described previously.[53,54] Briefly, 600 μL absolute ethanol, 120 μL methyltrimethoxysilane, 30 μL (heptadecafluoro-1,1,2,2-tetrahydrodecyl)trimethoxysilane, 160 μL distilled water, and 10 μL 0.5 M hydrochloric acid were added sequentially to a 1.5 mL microcentrifuge tube with vigorous mixing between the addition of each component. The sol was then vortexed for 1 h prior to use.

Sensors were calibrated before and after each measurement using saturated NO (1.9 mM) and CO (0.9 mM) solutions, prepared by the deoxygenation of phosphate buffered saline (pH 7.4) using argon gas for 30 min, with subsequent purging using either NO or CO for another 30 min. All the electrochemical experiments were carried out using a scanning electrochemical microscope (SECM, model 900C, CH Instruments; Austin, TX). For measurements from cells (cultured macrophages or snail buccal ganglia) the electrode was positioned ~50 μm above the surface of the cells. Prior to measurement, the electrode was polarized for >1 h (+0.8 and +0.6 V vs. Ag/AgCl for NO and CO electrodes, respectively).

A.2.2   Macrophage cell culture

RAW 264.7 murine macrophages (American Type Culture Collection, TIB-71) was used for the macrophage experiments. Cells were cultured in 60 × 15 mm CellBIND® dishes (Corning Life Sciences; Cornina, NY) at 37 °C under 5% carbon dioxide atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 1.0 g L^{-1} D-glucose and L-glutamine, and supplemented with 10% (v/v) fetal bovine serum, 100 units mL^{-1} penicillin, and 100 μg·mL^{-1} streptomycin. All stimulants were added following the growth of a confluent monolayer of cells (~24 h). The addition of interferon gamma (IFN-γ, 20 units mL^{-1}) and lipopolysaccharide (LPS, 50 ng mL^{-1}) was required to induce NO release. For combined NO and CO generation, hemin (20–100 μM) and NADPH (80 μM) were added 2 h before IFN-γ and LPS. Prior to electrochemical measurement, the RAW 264.7 cells were mechanically removed from half the culture dish. For lateral scanning experiments, the sensor was traversed from the blank side of the dish to the side containing cells. For long-term measurements, the

electrode was positioned directly above cells to facilitate continuous measurement from a single group of cells.

A.2.3 *Preparation of ganglia and neurons*

The buccal ganglion from *Helisoma trivolvis* was dissected as described by Cohan et al.[55] The snail was deshelled and then anesthetized in a 25% v/v solution of Listerine® in saline. After 20 min in the anesthetic solution, the snail was pinned onto a dissecting dish. An incision was made on the dorsal side of the snail, from the mid-back region to the top between the antennae. Following this incision, the penis was pinned to the side and the esophagus cut to expose the buccal mass. The buccal ganglion was then detached from the attaching nerves and muscles, and pinned in a separate dish containing saline.

A.3 Results and Discussion

A.3.1 *Nitric oxide and carbon monoxide from macrophages*

As NO and CO both serve important roles in the immune response, cultured macrophage cells were used to study the dynamics of their release following stimulation with LPS and IFN-γ or hemin. To establish the time course of NO and CO release, the dual electrochemical microelectrode was positioned ~50 μm above a confluent monolayer of cells for 18 h. As shown in Figure A.1, NO production peaked ~13 h following stimulation with LPS and IFN-γ, increasing 170 nM from baseline concentrations. A lateral scan of blank and LPS/IFN-γ stimulated macrophage cells
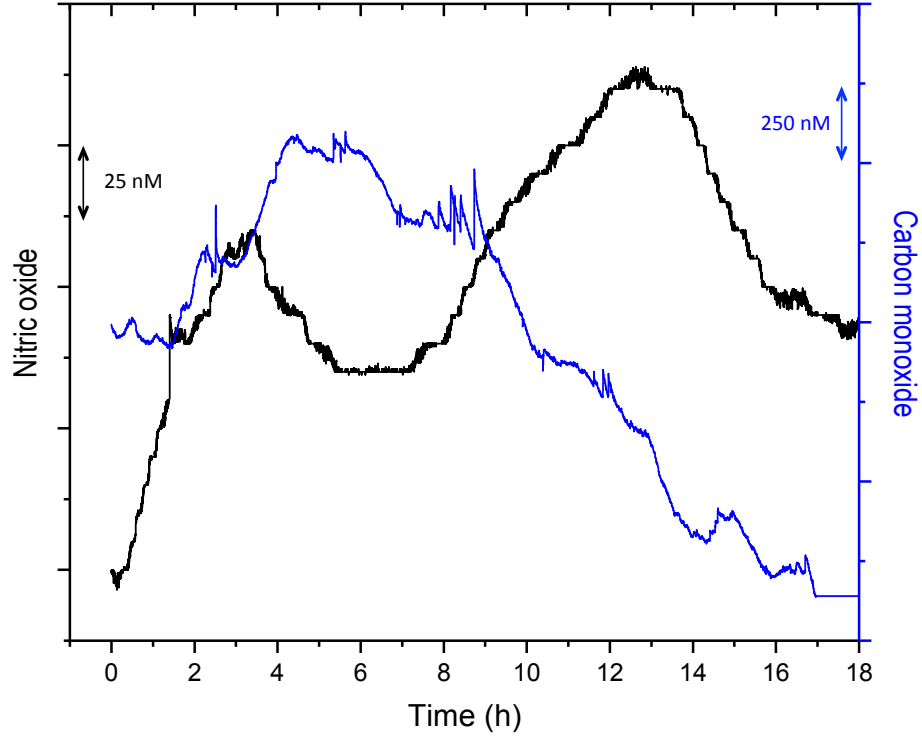
Figure A.1 Release profiles of NO (black) and CO (blue) following stimulation with LPS/ IFN-γ and hemin/NADPH, respectively. Basal concentrations of NO and CO prior to stimulation were below the limit of detection.

revealed similarly elevated NO concentrations following a 15 h incubation period (Figure A.2). Carbon monoxide levels did not increase after stimulation with LPS and IFN-γ, as a source of heme is required and was not present in the media. In the presence of hemin (20 μM) and NADPH (80 μM), CO generation was promoted, peaking much earlier than NO, with an increase of 580 nM relative to baseline levels observed at ~5 h (Figure A.1).

Carbon monoxide generation from macrophage cells was also examined as a function of hemin concentration. Cells were incubated overnight (~18 h), with 20, 50, and 100 μM hemin (and 80 μM NADPH) in DMEM with LPS/IFN-γ. Following the incubation period, the media was replaced with fresh PBS and a constant-distance lateral scan was performed across the cell dish. While the increase in CO generation between 20 and 50 μM hemin was minimal (Figure A.3), 100 μM hemin caused CO levels to increase by ~1 μM. In all cases, NO generation was suppressed to non-detectable levels. Others have observed similar decreases in NO generation upon upregulation of HO-1, although via indirect measurement of these analytes (i.e., nitrite and biliverdin for NO and CO, respectively).[56]

These results demonstrate the cross-talk phenomena between NO and CO. Common immune stimulants, such as LPS and IFN-γ, seemed to shift the macrophages toward a pro-inflammatory (M1) phenotype, resulting in the generation of large quantities of NO over many hours. Amatore et al. have demonstrated that these cells also release other reactive oxygen and nitrogen species.[57-60] While in this pro-inflammatory state and without a source of heme, CO generation was extremely limited due to down-regulation of HO-1. Conversely, the presence of hemin resulted in enhanced CO production with concomitant suppression of NO generation. In addition to its involvement in the
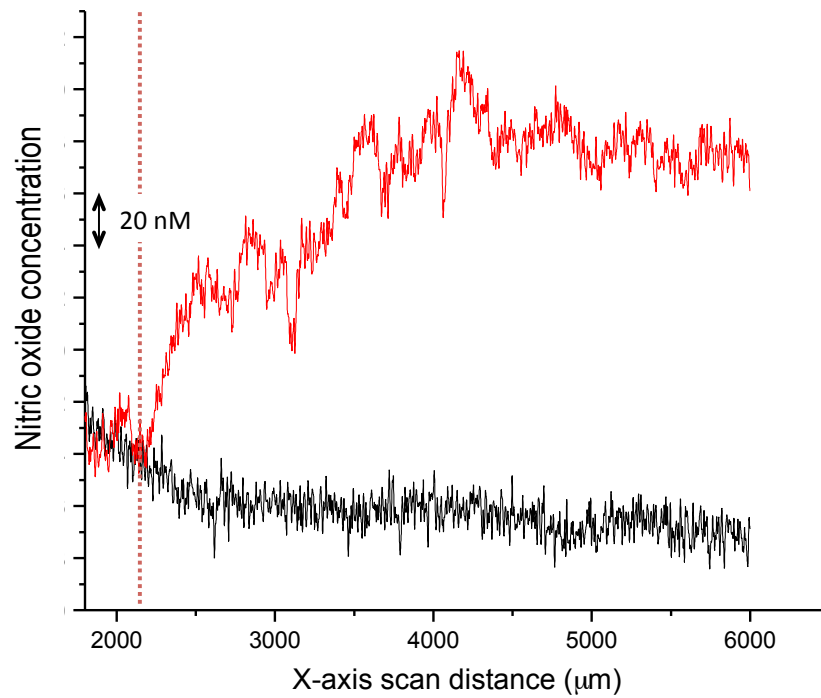
Figure A.2 Nitric oxide release from unstimulated (black) macrophage cells and those stimulated with LPS and IFN-γ (red). Nitric oxide concentrations from unstimulated cells were undetectable and thus considered to be zero. Dashed red line indicates barrier between blank dish and cultured cells.
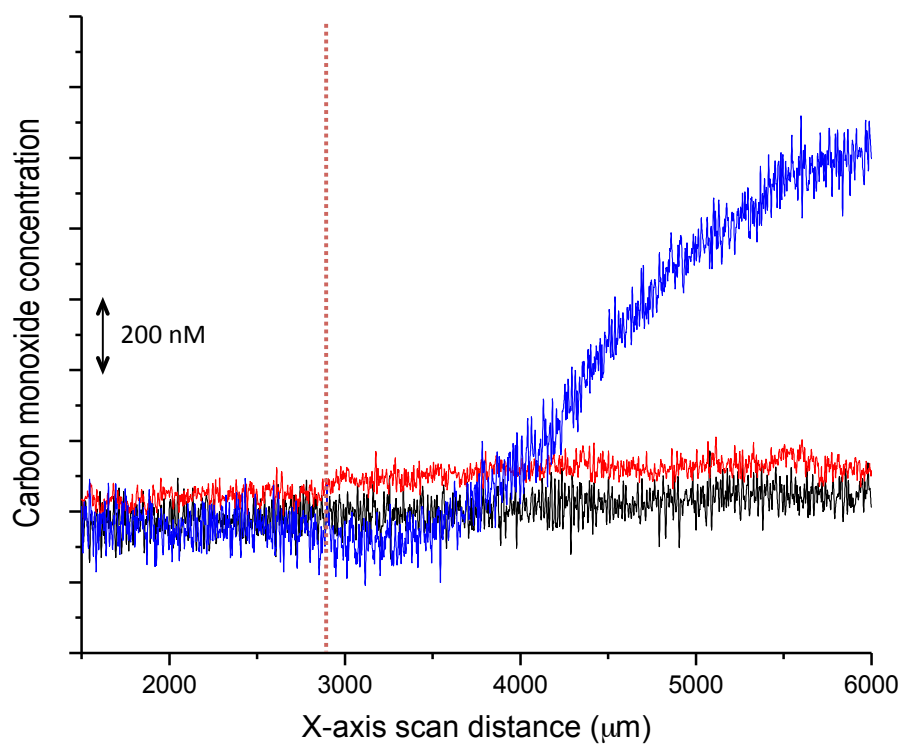
Figure A.3 Carbon monoxide release from macrophage cells incubated with 20 (black), 50 (red) and 100 μM (blue) hemin and LPS/IFN-γ for ~18 h. Dashed red line indicates barrier between blank dish and cultured cells. Release of CO was not measured from the blank side of the dish.

production of superoxide dismutase,[30] HO plays a role in the degradation of free heme (often from lysed red blood cells).[61] During a severe bacterial infection (sepsis) where hemolysis and extreme upregulation of NO are common,[62] the down-regulation of heme oxygenase may prove detrimental. Indeed, increased concentrations of free heme in the blood have been associated with poor patient outcomes.[63] Future experiments should include the study of NO release as a function of duration of CO upregulation (i.e., preincubation with hemin/NADPH at varied intervals prior to iNOS stimulation to determine if extended exposure to CO will inhibit immune stimulation of NO to a greater extent. In addition, HO and NOS inhibitors (e.g., zinc deuteroporphyrin 2,4-bis glycol and 1400-W, respectively) should be utilized.

A.3.2   Nitric oxide release from buccal ganglia

Helisoma trivolvis snail ganglia and neurons were utilized in a subsequent study due to their size (up to 100 μm) and the ease at which they can be cultured.[55] Little is known about rate and concentration of NO released from these cell bodies and how NO rates/levels may affect neighboring neurons. Microelectrodes offer the spatial and temporal resolution required to examine NO release from such systems. As nNOS is dependent on transient increases in intracellular calcium, potassium chloride (KCl, 17 mM) was added to the surgically extracted ganglia at varying intervals to cause membrane depolarization. As shown in Figure A.4, the subsequent calcium influx resulted in rapid and short-lived NO release. The addition of sodium chloride (17 mM) resulted in no detectable signal change, indicating that this effect is not the result of a change in ionic strength. The use of a selective nNOS inhibitor (7-nitroindazole (7-NI),
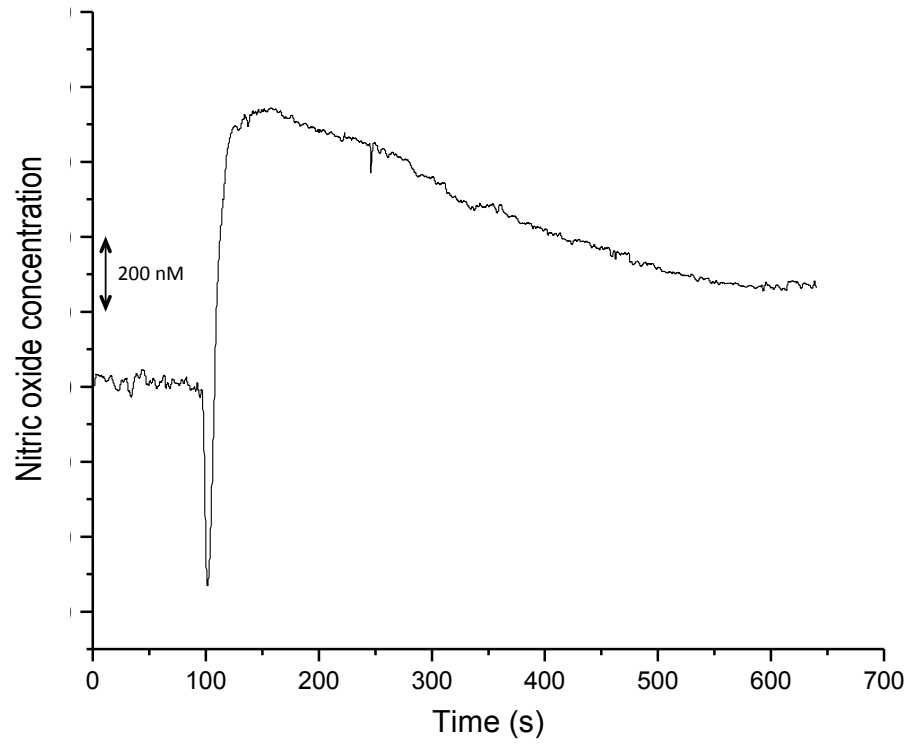
Figure A.4   Nitric oxide release from a *H. trivolvis* buccal ganglia following stimulation with 17 mM KCl. Nitric oxide concentrations prior to stimulation were undetectable. Signal returns to baseline after ~10 min.

400 μM, 30 min incubation), resulted in a significant reduction (of ~40%) in NO generation following stimulation with KCl (Table A.1).

To date, dual NO/CO measurements from ganglia have been challenging due to irreproducible placement of the dual electrode system. Buccal ganglia are bundles of nerve cells with specific locations, so care must be taken to place the electrodes in an identical manner across experiments. While this problem may be minimized by using SECM to scan the entire ganglia, the transient release of NO and CO following stimulation would make timing of the measurement challenging. We predict that the cross-talk between NO and CO in this system would be similar to macrophage cells, with inhibition of one resulting in the upregulated production of the other. Future experiments should study the effects of specific inhibitors such as 7-nitroindazole (a nNOS inhibitor) and zinc deuteroporphyrin 2,4-bis glycol (ZnBG, a HO inhibitor).

A.4   Summary

The spatial and temporal resolution enabled by using microelectrodes allows for the measurement of NO and CO from cell systems. The results presented herein represent the first simultaneous direct measurement of NO and CO from cultured macrophage cells, and suggest that upregulation of CO generation may prevent pro-inflammatory macrophage polarization. In addition, a NO sensor was used to determine NO-release kinetics and concentration from extracted snail buccal ganglia, indicating the transient release of NO from nNOS relative to iNOS.

Table A.1   Nitric oxide concentrations released from *H. trivolvis* buccal ganglia following stimulation with 17 mM KCl with and without 7-nitroindazole inhibition. Data are presented as average ± standard error of the mean.

Stimulation	NO concentration (nM)	*n*
KCl	435 ± 77	8
NaCl	no detectable signal	8
KCl after 7-NI incubation	272 ± 91	4

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