DEDICATIONS

This Work is Dedicated To:

My parents, my grandparents, and my husband whose encouragement and love is unending

ACKNOWLEDGEMENTS

During my time completing my PhD, numerous people have been influential in my success. First, Dr. Mark Meyerhoff, thank you for being a wonderful advisor. I appreciate all of the advice, support, encouragement and pep talks to help keep me going. I now know the true meaning of "taking what the defense gives me," and it is a valuable lesson I'll take with me. I deeply value your guidance and can not thank you enough for everything you have done for me over the last 6 years.

Thank you to Dr. Kristina Hakannson, Dr. Raoul Kopelman and Dr. Joerg Lahann for serving on my committee. I appreciate the comments and suggestions you have had and I thank you for giving me your time.

For their assistance with the many blood experiments I performed, thank you to Terry Major and Hitesh Handa in the Extracorporeal Life Support Research laboratory. Thank you for helping me to obtain the blood samples, for letting me use some of your lab space, and for your helpful comments.

During my time as a member of the Meyerhoff lab, I have been privileged to work with a wonderful group of people. Thank you to Sangyeul Hwang, Yiduo Wu, Wansik Cha, Youngjea Kang, Mike Shen, Biyun Wu, Lin Wang, Jun Yang, Qinyi Yan, Laura Zimmerman, Wenyi Cai, Bo Peng, Andrea Bell, Liz Brisbois, Si Yang, Alex Wolf, and Alex Ketchum for sharing in the graduate student experience with me. Thank you also to

the post docs who have passed along advice and offered many helpful suggestions, specifically Melissa Reynolds, Jason Bennett, Mariusz Pietrzak, Kebede Gemene, Lajos Hofler, Gary Jensen, Kun Liu, and Dipankar Koley. I appreciate all of the conversations, the suggestions, the dinners, and all of the times in between with our group. A special thank you to Penny for being a wonderful aislemate and for sharing with me your thoughts on e-readers and all of the various types of Chinese candy. Drea, thanks for all of the fro-yo runs and ridiculous stories. You always know how to make me laugh.

While working on my thesis over the years, I have been fortunate enough to mentor several undergraduates completing research in our lab. In particular, thank you to Alessandro Colletta and Ian Rust for all your work on Chapter 4 of this thesis. You saved me so much time and I had a great time getting to know you in the process.

Over the last 6 years I have come to realize that teaching is my passion, and I owe much of my teaching experience to Nancy Kerner and Stacey Buchanan. Nancy helped me gain confidence as a GSI during my first two years and gave me the opportunity to work with the Women in Science and Engineering camp every summer. The camp quickly became one of my favorite events of the summer and I am so thankful for having had that experience. My experience at Henry Ford Community College was largely made possible because of Stacey. Thank you for letting me know about the opportunity and for providing me feedback and assistance throughout that year.

A special thank you to Roy Wentz, the glassblower for the department, for spending hours with me fabricating the optimal piece of glassware for my various projects. Your hard work and quick turnaround are very much appreciated. Thank you

also to Patti Fitzpatrick for ordering the numerous chemicals and other supplies I've need over the years and for being patient and answering my many questions.

I am fortunate to have made some invaluable friendships during my time as a graduate student. Thank you to Laura Zimmerman, Brittany Worley, and Chris Avery for endless hours of entertainment. From playing games, to watching movies or "running" a half marathon, your friendships have enriched my life and have created memories I will never forget.

Finally, I thank my family for always believing in me and for making me feel like anything is possible. Over my 25 year career as a student, you have always been there for me. To my parents, brother, sister-in-law, grandparents, aunt, uncles, cousins, and in-laws, your encouragement has pulled me through the difficult times and has helped me more than you'll even know. I love all of you. Lastly, thank you to my husband, Brian, for being my best friend and for being with me throughout this process. Thank you for celebrating my successes and for comforting me when things were not going well. You have made graduate school a more enjoyable experience and I'm excited to see what happens next.

TABLE OF CONTENTS

DEDICATION	ii
ACKNOWLEDGEMENTS	iii
LIST OF FIGURES	ix
LIST OF TABLES	xiv
ABSTRACT	XV
CHAPTER 1 – INTRODUCTION	1
1.1 Biological Significance of S-Nitrosothiols and Nitric Oxide	
1.1.1 Formation of Nitric Oxide	
1.1.2 Reactions with Nitric Oxide	
1.2 Current NO and RSNO Detection Methods	
1.2.1 Fluorescence	4
1.2.2 Absorbance	
1.2.3 Mass Spectrometry	
1.2.4 Chemiluminescence	8
1.2.5 Electron Paramagnetic Resonance Spectroscopy	
1.2.6 Oxyhemoglobin Assay	
1.2.7 Electrochemical	
1.3 Nasal Nitric Oxide Detection	
1.3.1 Origin and Function of Nasal NO	15
1.3.2 Detection of Nasal NO	
1.3.3 Nasal NO in Disease	
1.4 Statement of Research	17
1.5 References	
CHAPTER 2 – CHEMILUMINESCENT DETECTION OF S-NITROSOTH	
USING A SENSITIVE AND SELECTIVE ORGANOSELENIUM CATALY	ST 26
2.1 Introduction	
2.2 Experimental	
2.2.1 Materials and Instruments	
2.2.2 Synthesis of RSNOs	
2.2.3 Synthesis of Selenocystamine	
2.2.4 Nitric Oxide Analyzer (NOA) Assays	
2.2.5 Testing Transnitrosation	
2.2.6 Plasma RSNO Testing	
2.2.7 Detection of HbSNO	
2.3 Results and Discussion	
2.3.1 Optimization of Selenocystamine/Glutathione Assay	
2.3.2 Characterization of Selenocystamine/Glutathione Assay	

	2.3.3	Transnitrosation Analysis	. 42
		Spiked Plasma Testing	
		S-Nitrosohemoglobin Detection	
2.4		usions	
2.5	Refere	ences	. 52
CHAPT	ER 3 –	CHEMILUMINESCENT AND OPTICAL DETECTION OF S-	
NITRO S	SOTHI	OLS IN EXHALED BREATH CONDENSATE	. 54
3.1	Introd	luction	. 54
		imental	
	3.2.1	Materials and Instruments	. 56
	3.2.2	Collection of Breath Condensate	. 57
		Nitric Oxide Analyzer Analysis	
	C	opper/Cysteine Assay System	. 59
	Se	elenocystamine/Glutathione Assay System	. 59
	3.2.4	Optical Detection using the Saville-Griess Assay	. 59
3.3		s and Discussion	
		Nitric Oxide Analyzer Analysis	
		Characterization of Saville-Griess Assay	
		Saville-Griess Assay Results	
		Explanation of previous studies	
		Potential Downfalls of EBC Collection	
		usions	
		ences	. 70
		QUANTIFYING EXHALED NASAL NITRIC OXIDE USING	
		OBIN AND NITRATE ION-SELECTIVE ELECTRODES	
		luction	
4.2	-	imental	
		Materials and Instruments	
		Oxyhemoglobin Preparation	
		Carbon Monoxide and Nitric Oxide Solutions	
		Nitric Oxide and Carbon Monoxide Calibrations with oxyHb	
		Synthesis of Vanadyl Salen Ionophore	
		Nitrate Ion-Selective Electrode Preparation	. 80
	4.2./	Asymmetrical Cellulose Triacetate Ion-Selective Membrane	0.0
	420	Preparation	. 80
	4.2.8	Determining Potentiometric Response of Nitrate Sensing Membrane	0.1
	120	Electrodes	
	4.2.9	Nitric Oxide Detection Through Gas Permeable Tubing	. 81
12		0 Nasal Nitric Oxide Analysiss and Discussion	
4.3		Characterization of oxyHb with Nitric Oxide and Carbon Monoxide.	
		Determining oxyHb Affinity for CO and NO	
		Synthesis of Vanadyl Salen Ionophore	
		Nitrate Ion-Selective Electrode Membrane Optimization	
		Potentiometric Response to oxyHb	
	7.5.5	1 OTOTICOTION TO INCOMPLIANCE TO ONVIIO	・ノコ

4.3.6 Nitric Oxide Calibration with Asymmetric Cellulose Tria	cetate
Membrane	98
4.3.7 Gas Phase Testing	
4.3.8 Nasal Nitric Oxide Testing	101
4.4 Conclusions	106
4.5 References	107
CHAPTER 5 – CONCLUSIONS	110
5.1 Summary of Results and Contributions	110
5.2 Future Work	
5.3 References	117

LIST OF FIGURES

Figure 1.1:	Structures of the three low molecular weight RSNOs used in this study.	2
Figure 1.2:	Synthesis of NO and citrulline from L-arginine.	3
Figure 1.3:	Reaction of DAF-2 with NO and oxygen to form the fluorescent triazole compound, DAF-2T.	5
Figure 1.4:	Schematic representation of the Griess Assay with acidified sulfanilamide reacting with nitrite to form an intermediate which reacts with NED.	7
Figure 1.5:	Amperometric RSNO sensor consisting of a platinized platinum electrode behind a gas permeable membrane and an outer dialysis membrane with a selenium catalyst covalently attached.	14
Figure 1.6:	Diagram showing NO concentrations detected in different regions of the airways using a chemiluminescence analyzer.	15
Figure 2.1:	a) Structure of selenocystamine. b) Catalytic cycle for the selenocystamine.	27
Figure 2.2:	Schematic of the biotin-switch method for protein RSNO detection.	28
Figure 2.3:	Model structure of S -nitrosohemoglobin showing the NO located on the cysteine β 93 residue.	30
Figure 2.4:	Schematic HbSNO detection with the NO ⁺ moving across the cell membrane to the free NAC to form SNAC.	31
Figure 2.5:	The cell used for the NOA assays with the assay reagents in the bottom being purged with $N_2(g)$.	34
Figure 2.6:	Samples containing 25 µM AlbSNO with either 25, 100, or 250 µM of one of the LMW thiols were loaded into a centrifuge tube containing a 30,000 MW cutoff filter.	35

Figure 2.7:	To detect HbSNO, fresh sheep or pig blood was obtained and spun down to remove the plasma.	37
Figure 2.8:	UV-Vis spectra of SeCA over time.	38
Figure 2.9:	S-Nitrosoglutathione calibration in 2 mL with 0.5 mM SeCA and 5 μ M GSH. Inset. The lowest concentration tested, 20 nM, is clearly observed.	39
Figure 2.10:	a) Typical calibration curves at 2 mL and 3 mL total volume for GSNO, CysNO, and SNAC. b) Reproducibility of the SeCA/GSH assay in 2 mL, for n = 3 measurements.	40
Figure 2.11:	Calibration curves for GSNO, CysNO, and SNAC using the Cu(II)/Cys assay system at both 2 mL and 3 mL total volume.	40
Figure 2.12:	Response to nitrite for the a) Cu(II)/Cys assasy and for the b) SeCA/GSH assay.	41
Figure 2.13:	Calibration curve for AlbSNO in 2 mL total volume using the SeCA/GSH assay (n = 3 for each calibration point).	42
Figure 2.14:	Injections of AlbSNO with and without GSH present.	42
Figure 2.15:	Precent transnitrosation for GSH, Cys, and NAC at the three concentrations of thiols tested and the two incubation times.	43
Figure 2.16:	Samples of 1 μM GSNO analyzed with and without AF present.	45
Figure 2.17:	Time trials of sheep plasma spiked with 10 μM GSNO at a) 23 °C, b) 37 °C, and c) 4 °C.	48
Figure 2.18:	Detection of HbSNO from pig blood using NAC for transnitrosation.	50
Figure 3.1:	Schematic of exhaled breath condensate collection device fabricated and used in this research.	58
Figure 3.2:	The chemistry of the Griess Assay showing the final azo product with a maximum absorbance at 540 nm.	60
Figure 3.3:	The chemistry of the Saville Assay.	61
Figure 3.4:	Analysis of the same EBC sample using the Cu/Cys assay showing a) initial results of two – 1 mL aliquots and b) analysis after 24 h.	62

Figure 3.5:	Analysis of 1 mL of EBC using 5 mM CuCl ₂ and 3 mM Cys in PBS (pH 7.4).	63
Figure 3.6:	Analysis of 1 mL of EBC using 5 mM SeCA with 0.5 mM GSH.	64
Figure 3.7:	Exhaled breath condensate from the same subject analyzed using a) 5 mM CuCl ₂ with 3 mM Cys and b) using 5 mM SeCA with 0.5 mM GSH.	64
Figure 3.8:	A typical calibration curve for a) the Griess Assay using nitrite as the standard and b) for the Saville Assay using GSNO as the standard.	65
Figure 4.1:	Schematic diagram demonstrating the collection and testing of exhaled nasal air.	83
Figure 4.2:	Spectra of the three oxyHb species showing the Soret band around 415 nm.	84
Figure 4.3:	Spectra of bovine oxyHb and metHb with the Soret band shifting from 413 nm to 406 nm, and the two smaller peaks between 500 and 600 nm diminishing.	84
Figure 4.4:	As NO is added to the oxyHb solution, a) the Soret band shifts and the secondary peaks decrease. b) The difference spectrum shows the peak at 401 nm increasing with each NO addition.	85
Figure 4.5:	Calibration curves toward NO for 5 μM and 10 μM bovine oxyHb.	85
Figure 4.6:	a) Oxyhemoglobin binding to CO to form carboxyHb. b) As CO is added, a difference plot can be generated, with a maximum at 420 nm.	86
Figure 4.7:	Calibration curve for CO using 10 µM bovine oxyHb.	86
Figure 4.8:	Spectrum of oxyHb with CO added followed by NO.	87
Figure 4.9:	Spectrum of oxyHb with NO added followed by CO.	87
Figure 4.10:	Spectrum of oxyHb with NO and CO added at the same time.	88
Figure 4.11:	Structure of the synthesized vanadyl salen ionophore.	88
Figure 4.12:	Mass spectrum of the synthesized vanadyl salen ionophore with the expected m/z value of 611.0.	89

Figure 4.13:	Spectra of the salen ligand and the vanadyl salen complex.	89
Figure 4.14:	a) Typical nitrate response for sensors containing 0.56 wt% and 0.94 wt% TDMAC, 0 wt% ionophore and 1:2 PVC:NPOE. b) Calibration curve with the dotted line indicating how LOD is calculated.	90
Figure 4.15:	Structures of TDMAC and the various plasticizers used to prepare nitrate responsive membrane electrodes.	91
Figure 4.16:	Nitrate response with membranes containing 2 wt% ionophore and 1:2 polymer:NPOE ratio, with the polymer being either PVC or polyurethane (PU).	94
Figure 4.17:	Oxyhemoglobin added to plasticized PVC sensors with 0.94 wt% TDMAC.	95
Figure 4.18:	Asymmetry potential caused by oxyHb interacting at the membrane/sample interface.	95
Figure 4.19:	Schematic diagram demonstrating the preparation of the new asymmetric cellulose triacetate nitrate ISE.	96
Figure 4.20:	Comparison of nitrate response for ASMs and PVC membranes.	97
Figure 4.21:	Response toward oxyHb added for both ASMs and PVC membranes.	97
Figure 4.22:	Calibration of nitrate produced from 50 μM oxyHb reacting with NO.	99
Figure 4.23:	Calibration of nitrate produced from 10 μM oxyHb reacting with NO.	99
Figure 4.24:	Potentiometric response to a nasal air sample bubbled into 25 μM oxyHb.	103
Figure 4.25:	(Left) Potentiometric response to a nasal air sample bubbled into a solution containing 50 μ M oxyHb. (Right) The control response for the air bubbled through buffer only.	103
Figure 4.26:	The potentiometric response for another nasal air sample bubbled through a solution containing 50 μM oxyHb.	103
Figure 4.27:	a) A sample of nitrogen gas bubbled through buffer, as well as b) through 50 μ M oxyHb.	104

Figure 4.28:	A control experiment with the addition of a sample containing only 50 μM oxyHb.	105
Figure 4.29:	a) A sample of compressed air bubbled through buffer and b) through 25 μM oxyHb.	105
Figure 5.1:	a) The structure of the cobalt-corrole ionophore. b) Preliminary data showing the sensor response to 7 anions, with the highest selectivity shown for nitrate.	113
Figure 5.2:	Schematic diagram showing the composition of the NO releasing film, along with the mechanism of NO release from $DBHD/N_2O_2$.	115
Figure 5.3	Nitric oxide flux from the NO releasing film over the course of 10 d.	115
Figure 5.4:	Nitric oxide released from the wrap that reacted with oxyHb to produce nitrate.	116

LIST OF TABLES

Table 2.1:	<i>K</i> values for three LMW thiols used in the transnitrosation studies.	44
Table 2.2:	<i>K</i> values for AlbSNO incubated with GSH at varying concentrations, as reported by Hogg et al.	44
Table 2.3:	Summary of spiked plasma results	46
Table 2.4:	Enzymes present in plasma that are known to breakdown GSNO.	47
Table 3.1:	Nitrite concentrations detected from 3 subjects using the Cu/Cys chemiluminescent assay.	62
Table 3.2:	A comparison of the nitrite concentrations determined using the chemiluminescent assay and the Griess Assay.	66
Table 4.1:	Summary of results from different buffer compositions used.	90
Table 4.2:	Different membrane compositions made for nitrate detection.	92
Table 4.3:	Selectivity over nitrite for both asymmetric membranes and plasticized PVC membranes.	98
Table 4.4:	Varying incubation time of oxyHb in silicone rubber tubing with and without NO.	101

ABSTRACT

Methods for the Detection of S-Nitrosothiols and Nitric Oxide in Blood and Breath

by

Natalie R. Crist

Chair: Mark E. Meyerhoff

Nitric oxide (NO) and S-nitrosothiols (RSNO) are important biological molecules

with multiple functions throughout the body, including acting as a vasodilator and

preventing platelet aggregation. The detection of these molecules in blood and breath has

previously been examined for disease diagnostics by various analytical techniques, each

with its own limitations, revealing a wide range of values reported and thus the need for

new detection methods. For RSNO detection, interference from nitrite remains one of the

largest obstacles, while for NO detection in breath, the complexity and cost of the

detection methods pose the most difficult challenge to overcome. In this thesis, work is

presented that addresses these issues with new methods developed and demonstrated.

XV

First, a new chemiluminescent assay system for RSNO detection is described using an organoselenium (RSe) catalyst. The system is capable of detecting RSNO concentrations as low as 20 nM, without any response to nitrite. The RSe-based method is used to detect S-nitrosohemoglobin in blood. Using the newly developed assay, exhaled breath condensate (EBC) is collected and analyzed for RSNOs. Despite previous literature reports, no detectable RSNOs are found in the EBC from 5 healthy volunteers. Finally, a simple and inexpensive method for determining the NO concentration in exhaled nasal air is presented. Utilizing the oxyhemoglobin reaction with NO to produce nitrate and methemoglobin, both potentiometric and optical detection of nasal NO is possible. Using asymmetric cellulose triacetate ion-selective membranes, calibration curves for the nitrate generated can be made. Collecting exhaled nasal air and bubbling the air through a solution of oxyHb allows for the NO to be trapped and quantitated with both detection methods. Overall, an average of 210 ppb NO is measured from the exhaled nasal air. With further improvements of the nitrate ion-selective membrane, this method of quantifying NO could be used in other applications, including monitoring the NO flux from new NO-releasing polymeric materials.