ADVANCES IN POLYMERIC NANOSENSOR TECHNOLOGY
FOR BIOLOGICAL ANALYSIS

By
Mark S. Ferris
A thesis submitted to the Faculty and the Board of Trustees of the Colorado School of Mines in partial fulfillment of the requirements for the degree of Doctor of Philosophy (Chemical Engineering).

Golden, Colorado

Date ______________________

Signed: ______________________

Mark Ferris

Signed: ______________________

Dr. Kevin J. Cash
Thesis Advisor

Golden, Colorado

Date ______________________

Signed: ______________________

Dr. Anuj Chauhan
Professor and Head
Department of Chemical Engineering
ABSTRACT

Polymeric nanosensors are a next-generation sensing technology with the promise to improve the way that scientists, engineers, and healthcare professionals collect analyte data. They have a diameter on the order of 100 nanometers, a polyethylene glycol based lipid coating for biocompatibility, and they utilize luminescence techniques for signal transduction which allows for remote and non-invasive sensor read-outs. This makes them ideal for complex in vitro and in vivo applications in biological environments where the currently available sensor technology falls short. However, being an emerging technology, more research and development is needed to address several current limitations. This thesis presents advancements in polymeric nanosensor technology in three key areas of need: (1) attachment strategies and range control methods in enzyme-based detection mechanisms (2) tools for dynamic range control and extension for ionophore-based detection mechanisms, and (3) methods for background noise elimination. These areas of need are addressed through three reports of technological innovation. The first details a novel method for attaching glucose oxidase to polymeric nanosensors through a biotin/avidin approach, with broader implications for any type of enzyme-based (biomolecule-detecting) polymeric nanosensor. It also demonstrates three methods increasing the apparent enzyme activity associated with each nanoparticle and therefore shifting the response range toward lower glucose concentrations: by tuning the amount of biotin groups on the nanosensor surface, by adjusting the amount of biotinylated-glucose oxidase used during synthesis, and by adjusting the amount of avidin linkers used during synthesis. More biotin groups on the nanosensor surface and more biotinylated-glucose oxidase during synthesis both led to lower response ranges, while an optimal amount of avidin (0.22 mg) lead to the lowest response range. The second report details two designs for dual indicator use in ionophore-based (ion-detecting) polymeric nanosensors with supporting theoretical response models for each. This tool is shown control the sensor LogEC50 over 1.5 orders of magnitude and expand the total range span by 47%. The third report details a bulk optode membrane sensor that incorporates persistent luminescent microparticles into an ionophore-based mechanism for sodium detection. The signal from this ‘glow sensor’ can avoid background noise from biological autofluorescence by programming a delay in between sensor excitation and signal collection. The sensor is also shown to reversibly respond to sodium with a response range of 2.4 – 414 mM sodium and a LogEC50 of 52 mM sodium, with selectivity coefficients of -2.2 and -3.3 over the potentially interfering cations potassium and lithium,
respectively, and with a shelf-life of at least 14 days. These three developments solve key issues and help push polymeric-nanosensors toward application in real-world settings.
# TABLE OF CONTENTS

ABSTRACT ................................................................................................................................. iii  
LIST OF FIGURES ....................................................................................................................... ix  
LIST OF TABLES ........................................................................................................................ xv  
ACKNOWLEDGEMENTS ........................................................................................................ xviii  
CHAPTER 1 INTRODUCTION .................................................................................................... 1  
  1.1 General Introduction ....................................................................................................... 1  
  1.2 Literature Review ............................................................................................................ 2  
    1.2.1 Analytes of Interest ............................................................................................... 2  
    1.2.2 Overview of Bioanalytical Sensors ....................................................................... 3  
       1.2.2.1 Detection Strategies .................................................................................... 4  
       1.2.2.2 Sensor Classifications ............................................................................... 10  
  1.2.3 Theory ....................................................................................................................... 14  
    1.2.3.1 Luminescence Theory ............................................................................... 14  
    1.2.3.2 Theoretical Ionophore-Based Mechanism Response ................................ 18  
    1.2.3.3 Discussion on Theoretical Enzyme-Based Mechanism Response............ 21  
  1.3 Thesis Problem Statement ............................................................................................. 22  
  1.4 Thesis Organization ....................................................................................................... 23  
  1.5 References Cited ........................................................................................................... 23  
CHAPTER 2 ENZYME CONJUGATED NANOSENSOR WITH TUNABLE DETECTION LIMITS FOR SMALL BIO-MOLECULE DETERMINATION ......................... 31  
  2.1 Abstract ......................................................................................................................... 31  
  2.2 Introduction .................................................................................................................... 31  
  2.3 Results ........................................................................................................................... 35  
    2.3.1 Confirmation of ELiNS Conjugation ................................................................. 35
2.3.2 NSB-A-BGOx Sensors – Effect of BGOx Concentration ........................... 36
2.3.3 NSB-A-BGOx Sensors – Effect of Avidin Concentration ..................... 37
2.3.4 Dynamic Range Control through Surface Biotinylation ....................... 38

2.4 Discussion ................................................................................................................. 41
2.5 Conclusion .................................................................................................................. 43
2.6 Experimental Section .............................................................................................. 43
   2.6.1 Reagents and Materials ..................................................................................... 43
   2.6.2 Nanosensor Synthesis ....................................................................................... 44
   2.6.3 Enzyme-Linked Nanosensor Synthesis ............................................................ 44
   2.6.4 Nanosensor Characterization .......................................................................... 44

2.7 Notation ....................................................................................................................... 45

CHAPTER 3 A DUAL-INDICATOR STRATEGY FOR CONTROLLING THE DYNAMIC
RANGE IN IONOPHORE-BASED OPTICAL NANOSENSOR .................................. 49

3.1 Abstract ...................................................................................................................... 49
3.2 Introduction ................................................................................................................ 50
3.3 Theory ........................................................................................................................ 53
   3.3.1 Derivation .......................................................................................................... 53
   3.3.2 Response Prediction ........................................................................................... 55
   3.3.3 pK_a Separation Analysis .................................................................................. 56

3.4 Experimental .............................................................................................................. 57
   3.4.1 Reagents and Materials ..................................................................................... 57
   3.4.2 Optode cocktail and Nanosensor Formulation .................................................. 57
      3.4.2.1 Synthesis of Lithium Optode Cocktail ....................................................... 57
      3.4.2.2 Synthesis of Lithium Nanosensor ............................................................. 58
      3.4.2.3 Synthesis of Calcium Optode Cocktail ..................................................... 58
   3.4.3 Procedures .......................................................................................................... 58
Figure 1.1  (Top) Schematic of sensing mechanism for an ionophore-based bulk optode sensor made with a calcium ionophore with a 3:1 complex ratio. A charge-balancing additive is used to hold the pH indicators (Ind) in a protonated state in the absence of $\text{Ca}^{2+}$. As $\text{Ca}^{2+}$ increases in the sample, it is extracted into the sensor film where it binds with calcium ionophores (L). The +2 charge of the calcium ion causes the deprotonation of two pH indicator molecules, causing the color of the indicators to shift from blue to purple: (Bottom) Molecular structures of the calcium ionophore, charge-balancing additive, and pH indicator. Reproduced with permission from reference 19 (Annual Review of Analytical Chemistry, Vol 7, 2014; Vol. 7, pp 483-512. Copyright 2014, Annual Reviews). .......................................................... 5

Figure 1.2  Ion-Selective Electrode Schematic. Reproduced with permission from Reference 21 (Carrier-Based Ion-Selective Electrodes and Bulk Optodes. 1. General Characteristics. Chemical Reviews 97 (8), 3083-3132. Copyright (1997) American Chemical Society). .......................................................... 11

Figure 1.3  Jablonski Diagram. Absorption of a photon excites an electron to an excited singlet state. Relaxation to a lower energy excited state is known as internal conversion and conversion to an excited triplet state is known as intersystem crossing. The return to the ground state causes the release of a new photon. Reproduced with permission from reference 79 (Principles of Fluorescence Spectroscopy: Third Edition. Copyright (2006) Springer Science and Business Media, LLC). .......................................................... 15

Figure 1.4  Simplified Jablonski diagram showing quantum yield and fluorescence lifetime. Reproduced with permission from reference 79 (Principles of Fluorescence Spectroscopy: Third Edition. Copyright (2006) Springer Science and Business Media, LLC). .......................................................... 16

Figure 1.5  Three equilibrium expressions to determine ionophore-based sensor response: (1) Extraction of the target ion into the sensor phase, corresponding with a release of hydrogen, (2) target ion binding with the ionophore, and (3) acid/base equilibria of the pH indicator. Reproduced with permission from reference 19 (Annual Review of Analytical Chemistry, Vol 7, 2014; Vol. 7, pp 483-512. Copyright 2014, Annual Reviews). .......................................................... 18

Figure 1.6  Theoretical $\text{O}_2$ concentration profile at surface of an enzyme-linked nanosensor. (A) The lower limit of the sensors dynamic range corresponds to an analyte concentration that cause an $\text{O}_2$ gradient to form at the sensor surface based on the enzyme-catalyzed reaction. (B) The upper limit of the sensor’s dynamic range corresponds to an analyte concentration that depletes all $\text{O}_2$ at the nanosensor surface based on the enzyme-catalyzed reaction. ............................ 22

Figure 2.7  Sensor mechanism. Nanosensors loaded with an oxygen-responsive fluorescent dye have the enzyme glucose oxidase (GOx) conjugated to their lipid layer. When the local glucose concentration increases, a reaction between glucose and oxygen and glucose is catalyzed by GOx, reducing the local oxygen levels and increasing the fluorescence of the oxygen-responsive dye. Nanosensors also contained iron oxide ($\text{Fe}_3\text{O}_4$) nanoparticles for magnetic control. .......................................................... 32
Figure 2.8  Biotin/Avidin based conjugation strategies. (Left) NSB-A-BGOx particles consist of typical nanosensor formulations created with a substituted biotinylated lipid and mixed in solution with free avidin linker (A) and biotinylated glucose oxidase (BGOx). (Right) NSB-AGOx particles consist of typical nanosensor formulations created with a substituted biotinylated lipid and mixed in solution with avidinylated glucose oxidase (AGOx). ................................................................. 33

Figure 2.9  Effect of enzyme concentration (BGOx) on NSB-A-BGOx sensor response. Kinetic traces of sensor response with high (A), medium (B), and low (C) concentrations of BGOx. Analysis of initial slope across the test range of glucose concentrations shows fastest response to high BGOx, slower response to medium, BGOx, and a minimal response to low BGOx (D). ...................................................................................... 37

Figure 2.10  Combined, normalized dose/response curves for nine batches of NSB-A-BGOx sensors over seven unique avidin levels, generated by plotting the initial slope of the kinetic trace against glucose concentration for each sensor batch (A). Response midpoint (LogEC50) vs. avidin amount, showing a minimum LogEC50 (maximum responsiveness) in the range of 0.11-0.44 mg avidin (B). .............................................. 38

Figure 2.11  Effect of surface biotinylation on response of NSB-A-BGOx particles. Higher surface biotinylation (A) leads to a stronger response to glucose than a lower amount of surface biotinylation (B). .......................................................................................... 39

Figure 2.12  Response tuning with NSB-AGOx sensors based on surface biotinylation. Kinetic traces of sensor response to a range or glucose for 100% (A), 25% (B), 1% (C), and 0% (D) biotinylation....................................................................................................... 40

Figure 3.1  Graphical Abstract. The dynamic range of ionophore-based nanosensors is controllable through changes to the indicator composition.................................................. 49

Figure 3.2  Mechanism of IBOS response to increasing analyte (I') concentration with two pH indicators (Ind1 and Ind2) co-loaded into a sensor matrix with additive (R-), and ionophore (L). At low analyte concentration, both pH indicators are protonated and both give off a fluorescent signal. At a higher concentration, the analyte begins to diffuse into the sensor and binds to the ionophore that in turn deprotonates the pH indicator with the lower pK_a. At high analyte concentrations, both pH indicators begin to deprotonate until a point where there is no signal from either molecule at the selected wavelength................................................................................................. 52

Figure 3.3  Theoretical normalized protonation dose-response curves for lithium-sensitive IBOS. (A) Mixed nanosensor method: changing \( \theta \) does not impact how the pH indicators deprotonate. (B) Mixed optode method: By mixing the pH indicators together in one sensor, the thermodynamics of deprotonation change based on \( \theta \). The linear range and EC_50 shifts toward higher concentrations as Ind2 is replaced with Ind1. ....................................................................................................................... 55

Figure 3.4  Theoretical normalized dose-response curves. Solid red curves represent the mixed optode method while dashed black curves represent the mixed nanosensor method. \( \theta \) increases from 0 to 1 at an increment of 0.2 from right to left. While the
deprotonation of the individual pH indicators is very different, the detectable combined-fluorescent output is similar between the two methods at any value of $\theta$.

Figure 3.5  Mixed optode lithium-sensitive IBOS response. (A) Normalized fluorescence dose-response curves. $\theta$ increases from 0 to 1 as the color shifts from blue to pink. (B) The linear range and LogEC$_{50}$ shifts toward lower concentrations as ChII is replaced with ChVII.

Figure 3.6  Control of LogEC$_{50}$ with sensor composition. More deviation from theory observed with the mixed optode method than with the mixed nanosensor method.

Figure 4.1  Graphical Abstract. The brightness of the ‘glow’ emanating from the sensor decreases as the sodium concentration increases.

Figure 4.2  Glow Sensor mechanism. A charge balancing additive holds the Blueberry dye in a protonated state in the absence of sodium. Sodium from the test sample is extracted into the sensor core where it binds with the ionophore. Charged sodium ions force the deprotonation of the Blueberry dye to maintain electroneutrality in the organic phase. When deprotonated, the Blueberry dye absorbs photon emission from the persistent luminescence microparticles at a higher rate, minimizing the observed phosphorescence.

Figure 4.3  Signal analysis for Glow Sensor spots. The Blueberry dye in the sensor turns from clear to blue upon deprotonation, increasing the absorbance of the glow from the persistent luminescence microparticles, thereby decreasing the amount of measured luminescence in basic solution. Due to the presence of sodium ionophore, the addition of sodium causes the same deprotonation of the Blueberry dye. (A) Phosphorescent decay curves ($n=3$) from a single sensor spot averaged together at increasing sodium concentrations. Dotted lines show area of integration used to calculate sensor response. (B) Dose/response curve showing average response to sodium of the four individual sensor spots. This shows that the sensor phosphorescence decreases as a function of sodium concentration. (B, inset) images of the phosphorescent spots under acidic (bright) and basic (dim) conditions.

Figure 4.4  Full glow sensor characterization. (A) Sensor signal after addition of 100 mM sodium. Response time of the sensor is 9.6 minutes ($T_{95}$). (B) Reversibility of the sensor analyzed by exposing sensor to alternating solutions of 0 mM and 100 mM sodium. (C) The sensor is highly selective against the potentially interfering ions Li$^+$ and K$^+$. (D) The sensor response to sodium is stable over 14 days.

Figure 5.1  EnzNS under ‘steady-state’ operation. Sensors are sealed in a microdialysis tube, adhered to the bottom of a well plate, and HEPES/TRIS solution is added on top. (A) A 3x7 area scan is taken of the center of the well every minute for 6 minutes before the glucose solution is changed and the scan is repeated. (B) The 7 points of interest from the brightest (middle) row of the area scan are processed into a dose/response curve.

Figure 5.2  Dual-ionophore use for dynamic range tuning with (A) mixed optode method and (B) mixed optode method. A single ionophore tuning experiments suggests that the
dynamic range can be adjusted without dual-ionophore use, but at the loss of signal-strength. ................................................................. 85

Figure 5.3  (A) The silicon nanocrystal sensor is selective for sodium against potentially interfering cations (B) and is responsive for at least one week. ................................. 86

Figure A.1 Demonstration of enzyme/nanosensor separation scheme. ELiNS and EnzNS undergo three rounds of magnetic separation and purification to remove unbound GOx from the magnetic nanosensors. EnzNS formulations (without avidin) have no attachment between the magnetic nanosensors and enzyme, so all enzyme is removed and the nanosensors lose their responsiveness to glucose. ELiNS formulations (with avidin) contain linkages between enzyme and the magnetic nanosensors, and so they retain their responsiveness to glucose after separation. ........... 90

Figure A.2 Triplicate data for NSB-A-BGOx sensors with highest enzyme loading (1 mg). ................. 90

Figure A.3 Kinetic traces for three separate experiments that explored the effect of seven avidin levels on NSB-A-BGOx sensor response. The first experiment explored low levels of avidin (A, B, and C), the second explored intermediate levels of avidin (D, E, and F), and the third explored high levels of avidin (G, H, and I). Low enzyme activity is observed with low amounts of avidin (0.02 and 0.05 mg), and slightly diminished enzyme activity is observed with high avidin amounts (0.88 and 1.32 mg). .......... 91

Figure A.4 Response to 4mM in the low avidin experiment (A), the intermediate avidin experiment (B), and the high avidin experiment (C). Summary of response to 4 mM glucose across the three experiments, with error bars showing standard deviation between overlapping levels of avidin (0.11 mg and 0.44 mg) tested between the two experiments (D). ................................................................................................. 92

Figure A.5 Effect of surface biotinylation on response of NSB-A-BGOx particles. Replica of Figure 4 with a narrow y-axis range in panel B showing that the sensors are still slightly responsive to glucose at 6.3% biotinylation. ................................................................. 92

Figure A.6 Dose response curve generated by plotting initial slope of kinetic trace vs. glucose concentration for NSB-A-BGOx particles with different amounts of surface biotinylation. ................................................................. 92

Figure A.7 Dose/response curves for NSB-AGOx sensors. ................................................................. 93

Figure A.8 TEM image of Fe₃O₄ nanoparticles in nanosensors. The darker shaded circles show nanosensors with no Fe₃O₄ nanoparticles encapsulated (blue, top), a low amount of Fe₃O₄ nanoparticles encapsulated (red, lower left), and a high amount of Fe₃O₄ nanoparticles encapsulated (green, lower right). .................................................. 94

Figure B.1 Theoretical predictions of mixed optode (red solid lines) and mixed nanosensor (black dashed lines) dose-response curves when both pH indicators have the same maximum fluorescence. .................................................................................. 97

Figure B.2 Nanosensor response curves using theoretical predictions from the mixed optode theory at various pH indicator pK_a separations. Blue curves show the response of sensors made with indicator 1, pink curves show the response of sensors made with
indicator 2 and solid purple lines are the response of the mixed optode approach with $\theta = 0.5$. ................................................................. 98

Figure B.3  Nanosensor response curves using theoretical predictions at various pH indicator $pK_a$ separations. Blue curves show the response of sensors made with indicator 1, pink curves show the response of sensors made with indicator 2 and solid purple lines are the response of the mixed nanosensor approach with $\theta = 0.5$. ................................................................. 99

Figure B.4  Absorbance spectra of lithium-selective IBOS with (A) ChII or (B) ChVII....... 100

Figure B.5  Emission Spectra of lithium-selective IBOS with (A) ChII or (B) ChVII after 660 nm excitation................................................................. 100

Figure B.6  Mixed nanosensor lithium-sensitive IBOS response. (A) Normalized fluorescence dose-response curves. $\theta$ increases from 0 to 1 as the color shifts from blue to pink. (B) The linear range and LogEC$_{50}$ shifts toward lower concentrations as ChII is replaced with ChVII. A * represents a significant difference in LogEC$_{50}$ with p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001................................................................. 100

Figure B.7  Dynamic range extension of lithium-selective IBOS.............................................. 101

Figure B.8  Apparent overall equilibrium constant vs. $\theta$............................................................ 101

Figure B.9  Single-Chromoionophore control experiment. Changing the concentration of chromoionophore VII changes only the intensity of the nanosensor without affecting the LogEC$_{50}$................................................................................................................. 101

Figure B.10  Response of single-pH indicator nanosensors and dual pH indicator nanosensors made with the mixed optode method to lithium, sodium, and potassium. ................. 102

Figure B.11  Control of nanosensor response with mixed optode method in PBS solution.......... 102

Figure B.12  (A) Dose/response curve obtained from sample with unknown lithium content. Two capsules of lithium orotate were dissolved in 40 mL HEPES/TRIS and then diluted by a factor of ten, five times. (B) Dose/response curve obtained from dilutions of lithium at known concentration. The x-axis value of ‘test sample’ (-1) was shifted by the difference in LogEC$_{50}$ between the two curves, and then the lithium content of one pill was back-calculated. ....................................................................................... 102

Figure B.13  Mixed optode method for calcium-sensitive IBOS.................................................... 103

Figure B.14  Mixed nanosensor method for calcium-sensitive IBOS........................................... 103

Figure B.15  LogEC$_{50}$ tuning of calcium-sensitive nanosensors............................................... 103

Figure B.16  Dynamic range extension of calcium-sensitive nanosensors................................. 104

Figure C.1  (Left Column) Glow Sensor luminescence (fluorescence and phosphorescence when the shutter is open, phosphorescence only when the shutter is closed) during shutter program for four spots: A, B, C, and D. (Right Column) The average of the three
phosphorescent decay curves collected while the shutter is closed for spots A, B, C, and D.

Figure C.2 Spot A under basic conditions compared to background (no sensor) signal. This shows that the phosphorescence is predominantly quenched by full deprotonation of the blueberry dye, although some residual signal remains.

Figure C.3 Luminescent signal from the Glow Sensor compared to background noise over time after ending excitation. The trend is described by a two-phase exponential decay with a fast half-life of 1.6 seconds and a slow half-life of 14.08 seconds.

Figure C.4 (Left column) Luminescence during shutter program for Glow Sensor spots without Blueberry dye. (Right Column) Averaged phosphorescent decay curves for Glow Sensor spots without Blueberry dye.

Figure C.5 (Left) Dose/Response curve for Glow Sensor spots made without Blueberry Dye. Minimal response to Na⁺ is observed. (Right) Glow Sensor spots made without persistent luminescence microparticles show no phosphorescence under acid and base conditions (see Fig S2 red curve for comparison of background signal). Time zero in this panel is the time when the shutter is closed and the excitation source blocked from the sample.

Figure C.6 (Top) Absorbance of an optode spot made without phosphorescence microparticles under acidic and basic conditions, showing a change in absorption over a wide range. (Bottom) Phosphorescent spectra of Glow Sensor (Blue) and no-blueberry dye control spot made without blueberry dye (Red) under acidic (solid circles) and basic (hollow circles) conditions. This demonstrates that the glow sensor phosphorescence is greatly reduced in basic conditions, which corresponds with a rise in absorbance from the blueberry dye at the same range of wavelengths. Without blueberry dye, however, the Glow Sensor does not change its phosphorescence between acidic and basic conditions. The energy coupling between the blueberry dye and phosphorescent microparticles is likely either due to the inner filter effect or from resonance energy transfer, both of which would require a rise in absorbance in the blueberry dye to correspond to a decrease in phosphorescence from the phosphorescent microparticles.

Figure C.7 Luminescence during extended shutter program for Glow Sensor spots during response time experiment.

Figure C.8 Drift of Glow Sensor $\alpha_{50}$ over the course of the experiment. ** represents a significant difference in $\alpha_{50}$ with $p < 0.01$.

Figure C.9 (Left) Dose/Response curve and (Right) dynamic range for spots A, B, C, and D.

Figure C.10 Compilation of all Glow Sensor dose/response curves demonstrating excellent reproducibility between sensor batches.
LIST OF TABLES

Table 1.1  Commercially Available Sodium Ionophores
Table 1.2  Commercially Available Potassium Ionophores
Table 1.3  Commercially available Lithium Ionophores
Table 1.4  Commercially Available Calcium Ionophores
Table 1.5  Commercially Available Magnesium Ionophores
Table 1.6  Commercially Available Chromoionophores
LIST OF TERMS, SYMBOLS, AND ABBREVIATIONS

LiNS: Lithium detecting nanosensor
Ch: Chromoionophore
NaI: Sodium Ionophore
CaI: Calcium Ionophore
LiI: Lithium Ionophore
Ind1: Indicator 1
Ind2: Indicator 2
ISE: Ion selective electrode
Fe₃O₄NP: Iron oxide nanoparticle
ChII: Chromoionophore II
ChVII: Chromoionophore VII
Ind1: Indicator 1
Ind2: Indicator 2
ULR: upper linear range
LLR: lower linear range
L: analyte-binding ligand
R−: charge-balancing additive
I⁺: cation

Enzyme-coupled nanosensor (EnzNS): a general term for any nanosensor that uses an enzyme as its recognition element

Enzyme-associated nanosensor: an enzyme/nanosensor platform where the two are simply mixed together with no attempt for chemical binding

Enzyme-linked nanosensor (ELiNS): an enzyme/nanosensor platform where the two are bound to each other with some type of chemical bond

Mixed Optode Method: A method of creating a dual-chromoionophore or dual-ionophore nanosensor platform where both chromoionophores or both ionophore are loaded into a single nanosensor
Mixed Nanosensor Method: A method of creating a dual-chromoionophore or dual-ionophore nanosensor platform where two batches of single-chromoionophore or single-ionophore sensors are created and then mixed together in different ratios

EnzNS: Enzyme-coupled nanosensors (a solution of enzymes and polymeric nanosensors with no specific interaction between the two entities)

ELiNS: Enzyme-linked nanosensors (a structure that consists of enzymes conjugated to a polymeric nanosensor with a specific interaction holding them together)

GOx: Glucose oxidase

AGOx: Avidin/glucose oxidase conjugates

BGOx: Biotin/glucose oxidase conjugates

NSB-A-BGOx sensors: ELiNS consisting of polymeric nanosensors with biotin groups on the surface and an avidin linker connecting BGOx to the nanosensors

NSB-AGOx sensors: EliNS consisting of polymeric nanosensors with biotin groups on the surface to which AGOx is bound
ACKNOWLEDGEMENTS

First, I would like to thank my advisor, Professor Kevin J. Cash, for inviting me to his research group and for supporting me through the entire process. Specifically, I want to thank him for his constant enthusiasm for science and for teaching me, and trusting me, to think independently from day one. Additionally, I want to thank the rest of my committee, Professors Kim Williams, Ning Wu, and Keith Neeves for their valuable advice and continued guidance for my research plans, and for teaching me in depth on the subjects of analytical chemistry, soft matter, and bioengineering. I consider the knowledge I gained in each of these subjects to be essential to my Ph.D. education.

I want to thank Dr. Anne Galyean for her guidance through the Ph.D. process, her career advice, and for helping me learn to mountain bike without crashing, an essential component of any Colorado-based Ph.D. program. I also want to thank my officemates, Dr. Anne Galyean, Aakash Katageri, and Megan Jewell for help working through experimental problems, for the lively discussions, and for making the office and lab an enjoyable place to work. Additionally, I want to thank all of my labmates for their hard work and support, especially Greta Gohring, Makayla Elms, and Madeline Behr for their help with data collection in my publications.

In addition to the knowledge gained about chemical engineering, analytical chemistry, polymeric nanosensors, and the scientific process, I found the Ph.D. process to be incredibly valuable in expanding my worldview and building personal relationships over shared suffering and the pursuit of common goals. For that I want to thank all of my classmates in the CSM Chemical and Biological Engineering program and all of my CSM triathlon teammates. Finally, I want to thank all of my housemates, including Kate Sciamanna, Javier Vargas-Johnson, Scott Nicholson, Tommy Feurst, John Czerski, Alexis Dubois, Meriel Young, Dr. Tadesse Weldu, and Andrew Schied for ensuring there were never any dull moments in my life and for making Golden an affordable place to live.

Most importantly, I would like to thank my parents for the 28 years of encouragement, motivation, patience, and moral support necessary to complete a Ph.D.
CHAPTER 1

GENERAL INTRODUCTION

1.1 General Introduction

Bioanalysis

Key research, engineering, and healthcare decisions are based on analyte concentration data collected during human health and biological monitoring. The standard approach in modern health care is a medical professional collecting a sample (e.g. blood, urine, saliva) for offline analysis. This procedure is effective for providing a snapshot of analyte levels in a patient, but it comes with significant limitations. Fluctuations in analyte levels cannot be detected without repeated sample collection, an inconvenience for both the patient and provider. Furthermore, analysis is restricted to being performed at health institutes where the proper equipment is available. In addition to clinical use, analyte monitoring is important in cell cultures and 3D cellular meshworks which are often used for drug development. Improved analyte monitoring technology can help quantify the chance a new compound has for clinical success. The potential for improvement in new drug development, where the recent trend has been an increase in research and development spending and a decline in clinical success rate, is profound. Analyte monitoring also provides insight into questions of scientific interest such as the basis of neuronal signaling in bacterial cultures. These examples represent just a small number of the numerous scientific and medical fields where analyte monitoring plays a key role. The continued development of new methods to improve data collection will lead to more accurate diagnoses, better treatment plans, faster technology development, a more efficient allocation of resources, and scientific insight.

Bioanalytical Sensors

A device used to quantify analyte concentration is known as a sensor. A distinction should be made between sensing and detection in that detection requires merely determining whether an analyte is present or not (above a certain threshold), whereas sensing requires quantifying the amount of analyte present. Any sensor used for biological analysis is known as a bioanalytical sensor, whereas the term biosensor is reserved for sensors that contain a biological element (e.g. DNA fragments, enzymes). The field of bioanalytical sensing is complex and a wide variety of
sensors with overlapping classifications have been developed for various applications. However, all sensors must consist of an analyte recognition element and a signal transduction element.\textsuperscript{4-6} For a limited number of analytes, a single element can serve both purposes,\textsuperscript{7,8} but usually two separate elements are used and must be couple together. Classification of bioanalytical sensors is usually based on the sensing mechanism, the signal transduction mechanism, or by the physical shape, structure, and material make-up of the device. Due to the diversity of applications, no one sensor attribute dominates the entire field, but for many \textit{in vivo} sensing applications, a list of desirable traits includes biocompatibility, miniature size, reversibility/reuse, low invasiveness, the ability for spatial mapping, and ability for continuous detection.\textsuperscript{9}

\textbf{Polymeric Nanosensors}

Luminescent polymeric nanosensors are a relatively new sensor technology with significant advantages over more established sensor types that give them the potential to answer challenging biological questions through advancements in the field of bioanalytical monitoring. Polymeric nanosensors are a miniaturized form of bulk optode membrane sensors, made by dissolving the optode components (e.g. polymer, plasticizer, recognition groups) in organic solvent and emulsifying that solution with an amphiphilic lipid surfactant. This results in water-soluble spherical nanoparticles \textasciitilde{}180 nm in diameter. The nanosensor size makes them minimally invasive and biocompatible for \textit{in vivo} and \textit{in vitro} use and gives them the ability to create spatial maps of analyte concentrations. Being an emerging sensor technology, more research and development is needed to unlock the potential of nanosensors to solve challenging, real-world problems where other sensor classes fall short.

\textbf{1.2 Literature Review}

\textbf{1.2.1 Analytes of Interest}

In this thesis, we present sensor designs for detecting three different analytes (glucose, lithium, and sodium) with a focus on bioanalytical applications (i.e. cell culture analysis and \textit{in vivo} physiological monitoring). Therefore, we formulated the sensors for higher analyte levels near physiological concentrations and do not intend for them to be applied for trace detection. Glucose, lithium, and sodium have extensive analytical interest in numerous fields.
Sodium is a monovalent cation that is essential to maintaining normal physiological function. Hyponatremia, a condition of low blood sodium concentration, is the most common electrolyte disorder. Dysfunction of sodium channels in the brain has been implicated in epilepsy, long QT syndrome, heart failure, and other diseases. Continuous sodium sensors have been developed for monitoring plasma blood electrolyte levels and creating spatial maps of sodium activity during action potentials in isolated cardiomyocytes.

Lithium is a monovalent cation that is considered one of the five biologically most important alkali and alkaline earth metal cations. Ionic lithium acts as a mood stabilizer and is most commonly used for the treatment of manic-depressive disorder. After intake as a therapeutic drug, the actual concentration of lithium in the blood varies from person to person and therefore must be monitored regularly to ensure proper dosage.

Glucose plays an essential role in many cellular processes, including cellular respiration. The most prevalent example of glucose monitoring is for diabetes, a well-known chronic, metabolic disorder that results in abnormal glucose levels. Diabetes is a major cause of blindness, kidney failure, heart attacks, stroke, and lower limb amputation and in 2012 an estimated 1.5 million deaths were caused by diabetes while another 2.2 million deaths were caused by high blood glucose. Because of the inherent dangers, it is critical for diabetics to closely monitor their blood glucose. The World Health Organization estimates there to be 422 million people in the world and 8.5% of people over 18 years of age to have diabetes as of 2014. Because of this large demand, roughly 85% of the current biosensor market is for glucose sensors. While in this work we focused on these three analytes, the methods we developed are applicable to many small cations, anions, and molecular targets through simple, well-known formulation changes.

### 1.2.2 Overview of Bioanalytical Sensors

Sensor design is often thought of in terms of analyte recognition and signal transduction: Some aspect of the sensor must be able to determine the presence of the intended analyte and that recognition event must trigger a change in some property of the sensor that can be measured with available equipment. Greater quantities of the analyte should trigger larger measurable changes for the device to be considered a sensor instead of a detector. For a select group of analytes, one element can serve as both recognition element and signal transducer, a strategy referred to here as
**direct detection.** This is exemplified by pH indicator dyes – where the protonation/deprotonation event directly changes the dye color.\(^7\) However, to reach a greater number of analytes, separate elements are used for analyte recognition and signal transduction and must be coupled together, a strategy known here as *indirect detection.* An example is the modern immunoassay, where an antibody recognizes a target protein, and then a secondary signal is generated by other sensor components.\(^{17, 18}\) Modern polymeric nanosensors usually rely on one of two indirect strategies for analyte recognition (ionophore-based and enzymatic) and mostly use light emission for signal transduction. Ionophore-based recognition\(^{19, 20}\) is used to build ion sensors, while enzymatic approaches\(^9\) are used to reach small biomolecule targets. Both strategies were initially developed for electrochemical sensors\(^5\) and later used in bulk optode sensors,\(^{20, 21}\) which are earlier generation sensor classes that led to the development of polymeric nanosensors.

### 1.2.2.1 Detection Strategies

**Organic Fluorescent Molecular Dyes**

Organic fluorescent molecular dyes are a class of natural and synthetic molecules with fluorescent properties. While fluorescent dyes are commonly used for imaging purposes where their only duty is to render cells or specific structures fluorescent and therefore visible through microscopy, some dyes have intrinsic sensing capabilities, meaning they are able to serve both as analyte recognition element and signal transducer (e.g. serve by themselves as a direct sensor).\(^{22}\) However, only a limited number of analyte targets are accessible through fluorescent dyes. A large number of molecules exist that can directly detect oxygen\(^8\) and pH,\(^{23}\) while a relatively fewer options exist for ion targets, though recent synthesis efforts are aimed at expanding the available selection.\(^6, 24\) These dyes have been deployed for imaging/sensing applications such as biofilm pH analysis\(^{25}\) and have been used to gain insight into key questions of scientific interest such as understanding sodium and potassium dynamics in bacterial ion channels.\(^3\) However, use of these dyes without a larger sensor construct for applications such as cellular imaging makes them susceptible to issues such as unwanted binding to proteins, cellular toxicity, and intracellular sequestration.\(^{26}\) Use of free fluorescent dyes also limits their capacity for optimized response and further sensor functionalization.
**Ionophore Recognition**

Ionophore-based strategies\(^{21}\) are based on molecules which can reversibly bind to specific ions, known as ionophores. Ionophore use began in 1964 after a discovery by Moore and Pressman that some antibiotics induce ion transport in mitochondria.\(^{27}\) Simon and Stefanac soon investigated further and found that the phenomenon is due to the selective formation of these complexes and certain cations.\(^{28}\) Around the same time, some groups were synthesizing macrocyclic polyethers and macroheterobicyclic compounds and showed their utility for complexing alkali and alkaline-earth metals.\(^{29}\) In a few years, many natural and synthetic ionophores were realized\(^ {21}\) and soon they were being used in ion-selective electrodes, a type of electrochemical sensor, for cation sensing.\(^ {30}\)

![Figure 1.1](image-url)

**Figure 1.1** (Top) Schematic of sensing mechanism for an ionophore-based bulk optode sensor made with a calcium ionophore with a 3:1 complex ratio. A charge-balancing additive is used to hold the pH indicators (Ind) in a protonated state in the absence of Ca\(^ {2+}\). As Ca\(^ {2+}\) increases in the sample, it is extracted into the sensor film where it binds with calcium ionophores (L). The +2 charge of the calcium ion causes the deprotonation of two pH indicator molecules, causing the color of the indicators to shift from blue to purple: (Bottom) Molecular structures of the calcium ionophore, charge-balancing additive, and pH indicator. Reproduced with permission from reference 19 (*Annual Review of Analytical Chemistry, Vol 7, 2014; Vol. 7, pp 483-512. Copyright 2014, Annual Reviews*).
Since ionophores are typically optically silent, they are paired with a fluorescent pH indicator and a charge balancing additive for use in optical sensors such as bulk optodes and polymeric nanosensors. The fluorescent pH indicator is usually from a family of organic fluorescent indicators derived from the molecules Nile blue and fluorescein known as chromoionophores that exist in an acid/base equilibrium and change color as they shift their protonation state. The response of ionophore-based sensors is then based on a model of thermodynamic equilibrium (see Section 1.2.3.2) that takes into account the binding strength of the ionophore and the protonation state of the chromoionophore, coupled together by charge balance in the sensing phase. The mechanism can be thought of in three steps, illustrated in Figure 1.1: (1) analyte extraction from the sample into the sensing phase, (2) analyte binding with the ionophore, and (3) deprotonation of the pH indicator, causing a change in fluorescence. Realistically, these three steps are all equilibrium reactions happening simultaneously, but do highlight the three distinct equilibria.

Important considerations when choosing an ionophore during sensor design are the complexing coefficient and the equilibrium constant ($\beta$). The equilibrium constant is known to change depending on the dielectric constant of the local environment.\textsuperscript{31} Likewise, the pK\textsubscript{a} of the chromoionophore should be considered, as well as the charge of the chromoionophore, and it should be taken into account that the local dielectric constant can affect the observed pK\textsubscript{a}.\textsuperscript{7, 32-34} Present day, there are a wealth of ionophores available commercially for common cations such as Na\textsuperscript{+} (Table 1.1), K\textsuperscript{+} (Table 1.2), Li\textsuperscript{+} (Table 1.3), Ca\textsuperscript{2+} (Table 1.4), and Mg\textsuperscript{2+} (Table 1.5). A list of commercially available chromoionophores is shown in Table 1.6. Tables 1.1-1.6 also show equilibrium constants and complexing coefficients in different environments for select compounds.

<table>
<thead>
<tr>
<th>Sodium Ionophore\textsuperscript{35}</th>
<th>Alternate name</th>
<th>Log($\beta$) in BEHS\textsuperscript{31}</th>
<th>Log($\beta$) in NPOE\textsuperscript{31}</th>
<th>Complexing Coefficient (n)\textsuperscript{31}</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>ETH 227</td>
<td>7.71±0.05</td>
<td>9.41±0.11</td>
<td>2</td>
</tr>
<tr>
<td>II</td>
<td>ETH 157</td>
<td>8.76±0.05</td>
<td>10.91±0.04</td>
<td>2</td>
</tr>
<tr>
<td>III</td>
<td>ETH 2120</td>
<td>7.71±0.05</td>
<td>9.41±0.11</td>
<td>2</td>
</tr>
</tbody>
</table>
Table 1.1 Commercially Available Sodium Ionophores (continued)

<table>
<thead>
<tr>
<th>IV</th>
<th>DD-16-C-5</th>
</tr>
</thead>
<tbody>
<tr>
<td>V</td>
<td>ETH 4120</td>
</tr>
<tr>
<td>VI</td>
<td>6.55±0.06</td>
</tr>
<tr>
<td>VIII</td>
<td>X</td>
</tr>
</tbody>
</table>

| VI  | 7.69±0.05  | 10.27±0.05 | 1 |

Table 1.8 Commercially Available Potassium Ionophores

<table>
<thead>
<tr>
<th>Potassium Ionophore$^35$</th>
<th>Alternate name</th>
<th>Log(β) in DOS$^{31}$</th>
<th>Log(β) in NPOE$^{31}$</th>
<th>Complexing Coefficient (n)$^{31}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Valinomycin</td>
<td>10.1±0.07</td>
<td>11.63±0.08</td>
<td>1 (assumed)</td>
</tr>
<tr>
<td>II</td>
<td>BB15C5</td>
<td>7.84±0.02</td>
<td>10.04±0.01</td>
<td>1</td>
</tr>
<tr>
<td>III</td>
<td>BME 44</td>
<td>6.88±0.05</td>
<td>10.22±0.07</td>
<td>1</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 1.9 Commercially available Lithium Ionophores

<table>
<thead>
<tr>
<th>Lithium Ionophore$^35$</th>
<th>Alternate name</th>
<th>Log(Beta) in NPOE$^{31}$</th>
<th>Complexing Coefficient (n)$^{31}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>ETH 149</td>
<td>10.71±0.04</td>
<td>2</td>
</tr>
<tr>
<td>II</td>
<td>ETH 1644</td>
<td>8.24±0.04</td>
<td>2</td>
</tr>
<tr>
<td>III</td>
<td>ETH 1810</td>
<td>8.77±0.02</td>
<td>2</td>
</tr>
<tr>
<td>IV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td></td>
<td>5.33±0.03</td>
<td>1</td>
</tr>
<tr>
<td>VII</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td></td>
<td>10.40±0.07</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 1.10 Commercially Available Calcium Ionophores

<table>
<thead>
<tr>
<th>Calcium Ionophore$^35$</th>
<th>Alternate name</th>
<th>Log(β) in DOS$^{31}$</th>
<th>Log(β) in NPOE$^{31}$</th>
<th>Complexing Coefficient (n)$^{31}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>ETH 1001</td>
<td>19.70±0.07</td>
<td>24.54±0.09</td>
<td>2</td>
</tr>
<tr>
<td>II</td>
<td>ETH 129</td>
<td>25.5±0.1</td>
<td>29.2±0.2</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 1.4 Commercially Available Calcium Ionophores (Continued)

<table>
<thead>
<tr>
<th></th>
<th>Calcium Ionophore</th>
<th>Log(β) in DOS 31</th>
<th>Log(β) in NPOE 31</th>
<th>Complexing Coefficient (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>III</td>
<td>Calimycin, Antibiotic A 23187</td>
<td>8.67±0.07</td>
<td>12.39±0.18</td>
<td>2</td>
</tr>
<tr>
<td>IV</td>
<td>ETH 5234</td>
<td>8.67±0.08</td>
<td>27.39±0.04</td>
<td>3</td>
</tr>
</tbody>
</table>

Table 1.11 Commercially Available Magnesium Ionophores

<table>
<thead>
<tr>
<th>Magnesium Ionophore 35</th>
<th>Alternate name</th>
<th>Log(β) in DOS 31</th>
<th>Log(β) in NPOE 31</th>
<th>Complexing Coefficient (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>ETH 1117</td>
<td>9.72±0.18</td>
<td>13.84±0.16</td>
<td>3</td>
</tr>
<tr>
<td>II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>ETH 4030</td>
<td>7.25±0.11</td>
<td>12.15±0.11</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 1.12 Commercially Available Chromoionophores

<table>
<thead>
<tr>
<th>Chromoionophore 35</th>
<th>Alternative Name</th>
<th>pKa 32</th>
<th>pKa in PVC/DOS 33</th>
<th>pKₐ in PVC/NPOE 33</th>
<th>pKₐ in membrane (PVC/DOS) 7</th>
<th>Derivative Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>ETH 5294</td>
<td>11.1±0.1</td>
<td>11.41±0.03</td>
<td>14.82±0.03</td>
<td>12</td>
<td>Nile Blue</td>
</tr>
<tr>
<td>II</td>
<td>ETH 2439</td>
<td>9.16 ± 0.02</td>
<td>12.3±0.02</td>
<td>10.2</td>
<td>Nile Blue</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>ETH 5350</td>
<td>8±0.04</td>
<td>9.59±0.04</td>
<td>13.4</td>
<td>Nile Blue</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>ETH 2412</td>
<td>17±0.04</td>
<td>20.4±0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nile Blue</td>
</tr>
<tr>
<td>VI</td>
<td>ETH 7075</td>
<td>12.53 ± 0.01</td>
<td>15.43±0.05</td>
<td></td>
<td>Fluoroscein</td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>ETH 5418</td>
<td>8.56 ± 0.004</td>
<td>11.72±0.06</td>
<td>9</td>
<td>Nile Blue</td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>TBPE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IX</td>
<td>ETH 4003</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XI</td>
<td>ETH 7061</td>
<td>18.04±0.01</td>
<td>20±0.04</td>
<td></td>
<td>Fluoroscein</td>
<td></td>
</tr>
<tr>
<td>XV</td>
<td>ETH 4001</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>XVII</td>
<td>GJM-541</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Enzymatic Recognition

Enzymatic recognition was used by Clark and Lyons in 1962, in the first biosensor described in the literature. They coupled the enzyme glucose oxidase, which catalyzes the oxidation of glucose according to Equation 1.1, to an amperometric electrode that could measure $P_{O_2}$.

$$\beta - D - Glucose + O_2 \rightarrow D - glucono - 1,5 - lactone + H_2O_2$$

(1.1)

The concentration of glucose in the sample was shown to be proportional to the lowering of $P_{O_2}$ which was sensed by the electrode. In the following several years, this type of sensing mechanism was adopted to make sensors for other small biomolecule targets by combining an existing electrode with the appropriate enzyme. The wide variety of oxidase enzymes available (e.g. enzymes that catalyze the oxidation of some small biomolecule with the depletion of $O_2$) makes it easy to change the sensor target with by replacing the oxidase enzyme and retaining $O_2$ based signal transduction method. Equation 1.1 also causes a local drop in pH, meaning that pH reporters can be utilized for signal transduction. Goldfinch and Lowe demonstrated this concept in 1984 for penicillin, urea, and glucose using the appropriate enzymes. Enzymatic recognition can also be used with optical bulk optode membrane sensors, though ionophore-based mechanisms are far more common with this sensor class. While polymeric nanosensors are also more commonly designed with ionophore-based mechanisms, they have been shown to be compatible with enzyme-based mechanisms and their viability for use in *in vivo* mice studies has been demonstrated.

Other recognition strategies

While ionophores are key for ion detection and enzymes are key for small bio-molecule detection, other strategies are needed to branch outside of these two families of analytes. Antibodies are naturally occurring protein structures that are deployed by the immune system for recognition and elimination of harmful foreign substances. As a result, their excellent analyte target specificity and affinity can be utilized in sensor devices for detection of a large number of disease markers, food and environmental contaminants, biological warfare agents, and illicit drugs. Nucleic acid sequences can be formulated for the recognition of specific DNA fragments through highly specific base-pair sequence matching. Similarly, single-stranded oligonucleotide sequences
known as aptamers can be made to have high binding affinities for select analytes through an iterative selection process from large random sequence pools. Aptamers have been isolated to bind to a wide variety of analytes including ions, small molecules, proteins, and whole cells.\textsuperscript{4, 45} In addition to the above ‘natural’ recognition elements, analyte receptors can also be synthesized such as with molecularly imprinted polymers (MIPs). MIPs are templated polymer matrices that are design to achieve specific binding with a target analyte by creating size- and shape- specific pockets.\textsuperscript{46} These recognition strategies are potential avenues for expanding the range of analytes accessible with the polymeric nanosensor platform.

1.2.2.2 Sensor Classifications

Modern day polymeric nanosensors are the result of miniaturization of optical bulk optode membrane sensors, often referred to simply as ‘optodes,’ meaning they consist of the same sensor components and utilize the same response mechanisms but simply differ in packaging. Many of the response mechanisms used with bulk optode sensors originated from the reports on electrochemical sensors that came earlier and adjusted for optical signal transduction. Because of this progression, referring to the literature on bulk optodes and electrochemical sensors is often helpful for the design and development of polymeric nanosensors.

Electrochemical

An electrochemical sensor is generally defined as any sensor with an electrochemical signal transduction element, which can take the form of amperometry, potentiometry, and conductometry among other methods.\textsuperscript{5} Electrochemical sensor reports predate those of bulk optode membranes or polymeric nanosensors,\textsuperscript{36} making them more robust and more fully studied. Among the most relevant subclassifications of electrochemical sensors for polymeric nanosensors are ion-selective electrodes and enzyme-based electrochemical films. The essential feature of ion-selective electrodes (ISEs) and many other electrochemical sensors is the solvent polymeric membrane (i.e. plasticized polymeric matrix), which is a water immiscible, high viscosity liquid used to house hydrophobic sensing components. James Ross\textsuperscript{47} and Adam Shatkay\textsuperscript{48} introduced the use of plasticized polymer matrices into sensor concepts in 1967 in their respective calcium sensor reports. Since then, poly(vinyl) chloride (PVC) quickly became widely accepted as the standard polymer material,\textsuperscript{21, 49} often paired with bis(2-ethylhexyl) sebacate as a plasticizer, though other
membrane materials are sometimes used as well. For use in ISEs, this sensing membrane is placed between the sample and an internal electrolyte solution and responds to the activity of the target ion, as shown in Figure 1.2.

**Figure 1.2 Ion-Selective Electrode Schematic.** Reproduced with permission from Reference 21 (Carrier-Based Ion-Selective Electrodes and Bulk Optodes. 1. General Characteristics. Chemical Reviews 97 (8), 3083-3132. Copyright (1997) American Chemical Society).

Electrochemical sensors such as ISEs have extraordinarily large measurement ranges that can span from about 1 to $10^{-6}$M and are capable of continuous measurements, but they require relatively complex devices for signal transduction. Due to the ionophore-based response mechanism, they have cross-sensitivity to pH, though this can be overcome by co-monitoring pH with a separate electrode. They are also not easily miniaturized and cannot monitor two- and three-dimensional spaces without complex sensor arrays. While they have shown some capacity for *in vivo* sensing applications, they have low biocompatibility and require transdermal implantation which invokes an immune response from the host organism.

**Bulk Optodes Membranes**

Bulk optode membranes consists of a hydrophobic, solvent polymeric membrane, with millimeter- to centimeter-scale dimensions, that can interface with an aqueous test sample. They utilize many of the same analyte recognition strategies as electrochemical sensors but instead rely
on optical signal transduction. The first sensor that closely resembled modern optical bulk optodes was reported by Charlton et al. in 1982. The design consisted of simply a plasticized PVC matrix with the commonly used potassium ionophore (i.e. ion-binding molecule) valinomycin and used an anionic dye, erythrosine B, that was mixed with the test sample for an optical signal transduction. The sensor served as an irreversible, single-use test strip. The field grew more rapidly beginning in 1989, when Morf et al. added a lipophilic pH indicator (i.e. chromoionophore) to the sensing phase and monitored its absorbance to make reversible carbonate, ammonium, and calcium optodes. The concept quickly to include spread to include cation targets such as phenylethylammonium ions, lead, potassium, sodium, and zinc. Bulk optodes have also been designed using enzymatic recognition, as described in Section 1.2.2.1 Detection Strategies.

The optical signal transduction strategies utilized by bulk optode membranes have a number of advantages over electrochemical sensors. Optical transduction allows for remote data collection, meaning analyte monitoring can occur without bulky devices that interfere with the samples they are trying to measure. While still being too bulky for ideal in vivo use, bulk optodes are more amenable to miniaturization and alterations to make them more biocompatible, which has been the focus of many research efforts since ~1999.

PEBBLES

An important development for bioanalytical sensors came in 1999, when Clark et. al. miniaturized the bulk optode sensor platform to a nanoscale device which they termed PEBBLEs (Probes Encapsulated By Biologically Localized Embedding). PEBBLEs consist of spherical polymer spheres, with indicator dyes trapped within the matrix. The PEBBLE sensor construct combined the size advantage of fluorescent indicator dyes with the advantage of the protective polymer matrix that comes with bulk optode sensors. The miniature size of PEBBLEs made them viable for intracellular monitoring, since their total volume is negligible to that of a cell. PEBBLEs also provided the advantage of greatly improved response time (1 ms) since they greatly increase the surface area to volume ratio and therefore reduce the time for analyte diffusion. PEBBLEs were also shown to be compatible with a diverse set of detection mechanisms adopted from bulk optodes and ISE membranes. By simply incorporating hydrophobic, organic fluorescent indicators along with ratiometric dyes, PEBBLEs could be made to directly sense pH, oxygen, calcium, and magnesium. By using a hydrophobic, decyl methacrylate matrix, ionophore-based
components (i.e. ionophore, additive, and pH indicator) could be incorporated to reach more ion targets via an ionophore-based mechanism. Enzymes could also be incorporated and paired with oxygen-sensitive fluorophores for glucose detection.

While PEBBLEs were an important sensor advancement that showcased the ability for bulk optode miniaturization, they had several fatal flaws that limited their practical applications and would soon be improved upon with second-generation miniaturized sensor constructs. In addition to the preparation being time-consuming, the size of the particles were not well controlled. The particle synthesis often resulted in a bimodal distribution, and while the majority of the particles per batch would be as small as 20 nm, a majority of the sensor components would be incorporated into a smaller number of larger 200 nm particles. But most importantly, PEBBLEs were shown to leach as much as 50% of the loaded indicator dye over a 48 hour period, limiting their application to short-term use.

Polymeric Nanosensors

In 2007, many of the negative attributes of PEBBLE sensors were addressed with a report for polymeric nanosensors by Clark and co-workers. Instead of the crosslinked polymer matrices used by PEBBLEs, these sensors took the plasticized PVC matrix preferred by bulk optodes and enclosed microscopic spherical particles with an amphiphilic lipid layer through a simple sonication synthesis. These sensors were shown to be stable for about one week and were applied for in vivo applications such as visualizing sodium dynamics in cardiomyocytes and in mice. The polymeric nanosensor platform has been continually researched since this report and progress is still being made today. In addition to adopting response mechanisms from bulk optode and electrochemical sensors for use in polymeric nanosensors, many new polymeric nanosensor reports showcase new modifications to the ionophore-based and enzyme-based response mechanisms. For example, in order to widen the possible reporters to use with an ionophore-based mechanism, so-called ‘static’ reporters (i.e. luminescence elements that are not sensitive to pH or any other analyte) have been paired with pH-sensitive dyes that can changed the perceived luminescence of the static reporter through a number of mechanisms. Bakker and co-workers also pioneered a method for overcoming the pH dependency of ionophore-based mechanisms by operating polymeric nanosensors in an ‘exhaustive sensing mode’ where the sample analyte is completely consumed by the probe.
Other Types of Miniaturized Optical Sensors

In addition to polymeric nanosensors from Clark and co-workers and PEBBLEs, there are a variety of other miniaturized optical sensor platforms based on bulk-optode sensors. Bakker and co-workers created monodisperse, optode-based beads on the order of several micrometers in diameter with a sonicating particle caster for a many different ion targets including $\text{Na}^+$, $\text{K}^+$, $\text{Ag}^+$, $\text{Cl}^-$, and $\text{NO}_2^-$ among others. Tohda and Gratzl created color-changing optode-based micron-scale sensor beads through a spray-drying method. Adopting both enzymatic and ionophore-based recognition strategies, they incorporated an array of beads into their “silver sensor,” a bar-shaped device, roughly 250 μm x 2 mm that could simultaneously potassium, sodium, and glucose. Finally, they optimized the components with a goal of monitoring interstitial fluids with the silver sensor implanted under the skin. Ruckh et al. demonstrated a polymer-free optode-based nanosensor in an effort to further reduce the sensor size for intracellular experiments. Balconis and Clark created lipase-degradable optode-based nanosensors with a solvent displacement method by replacing the PVC/BEHS core with polycaprolactone and a citric acid ester plasticizer. These efforts represent just a few of the many miniaturized platforms based on bulk optode membranes. The relative advantages and disadvantages of each means that no one sensor construct dominates research interest.

1.2.3 Theory

This section covers theoretical aspects of luminescence theory, ionophore-based sensor response, and enzyme-based sensor response that are relevant to the completed work.

1.2.3.1 Luminescence Theory

Luminescence is the process by which a substance emits light. An electron in a luminescent molecule will jump to an excited state (higher energy orbital) upon the input of energy, such as by absorbing a photon. Relaxation of the electron to the ground state from an electronically excited state will then emit a new photon, producing light. Emission from an excited singlet state is known as fluorescence while emission from excited triplet states is known as phosphorescence. The transition from an excited singlet state to the ground state is spin allowed and rapid since the excited electron has the opposite spin of its corresponding electron in the ground state orbital. Phosphorescence is a slower process since a transition from an excited triplet state to the ground state is spin forbidden due to the presence of a change in spin.
state is spin forbidden. To reach an excited triplet state, the electron must additionally undergo a change in spin, known as intersystem crossing. Photon emission rates for fluorescence are on the order of $10^8 \text{s}^{-1}$ while photon emission rates for phosphorescence are on the order of $10^3$ to $10^0 \text{s}^{-1}$.

Figure 1.3 Jablonski Diagram. Absorption of a photon excites an electron to an excited singlet state. Relaxation to a lower energy excited state is known as internal conversion and conversion to an excited triplet state is known as intersystem crossing. The return to the ground state causes the release of a new photon. Reproduced with permission from reference 79 (Principles of Fluorescence Spectroscopy: Third Edition. Copyright (2006) Springer Science and Business Media, LLC).

Emitted photons are typically at a lower wavelength due to energy losses to heat and other non-radiative decay processes, known as internal conversion, that occur before fluorescence or phosphorescence. A Jablonski diagram, shown in Figure 1.3, is typically used to illustrate electron movement during luminescence. A red-shift (toward higher wavelength) of the emission spectra from the absorption spectra is due to internal conversion and is known as the Stokes’ shift.

A luminescent material can be characterized by its emission lifetime and quantum yield. Quantum yield, which indicates the brightness of the fluorophore, is defined as the ratio of the number of photons emitted to the number absorbed. The rate of photon absorption is equal to the rate of photon emission plus the rate of non-radiative decay to the ground state, so quantum yield can be expressed by Equation 1.2, where $Q$ is the quantum yield, $\Gamma'$ is the photon emission rate from the fluorophore, and $k_{nr}$ is the rate of non-radiative decay to the ground state.
Fluorescence lifetime can therefore be described by Equation 1.3.

\[ \tau = \frac{1}{\Gamma + k_{nr}} \]  

(1.3)

The simplified Jablonski diagram in Figure 1.4 depicts the processes that determine quantum yield and fluorescence lifetime. Fluorescent materials have emission lifetimes on the order of $10^{-9} - 10^{-7}$ seconds\(^8\) while phosphorescent materials have emission lifetimes on the order of microseconds to hours.\(^8\) The phenomena of phosphorescent materials displaying emission lifetimes that last an exceptionally long time (minutes to hours and even days) is known as long-lifetime phosphorescence or persistent luminescence.\(^8, 8^3\) These materials have recently attracted research attention for bioanalytical sensing applications due to their ability to avoid background noise in biological samples.\(^8^4\)

Figure 1.4 Simplified Jablonski diagram showing quantum yield and fluorescence lifetime. Reproduced with permission from reference 79 (Principles of Fluorescence Spectroscopy: Third Edition. Copyright (2006) Springer Science and Business Media, LLC).

To build a luminescent nanosensor, the recorded luminescence from the signal transduction element must change in response to fluctuations in analyte concentration. This change could be a decrease in intensity, increase in intensity, shifts in peak wavelength or spectral signature, or changes in other measured properties such as excited state lifetime or polarization. In addition to
many mechanisms by which the light-emission from a luminescent element can be quenched, there are other processes by which the apparent luminescence from a material can be altered by interaction with another molecule. In this thesis, we exploit the following quenching and non-quenching mechanisms for sensor design: collisional quenching, static quenching, ionization of the fluorophore, resonance energy transfer, and the inner filter effect. Collisional quenching occurs when a luminescent molecule in an excited state is deactivated upon collision with another molecule (the ‘quencher’). Decrease in fluorescence through collisional quenching is described by the Stern-Volmer equation, shown in Equation 1.4.

\[
\frac{F_0}{F} = 1 + K[Q] = 1 + k_q \tau_0 [Q]
\] (1.4)

In Equation 1.3, K is the Stern-Volmer quenching constant, \(\tau_0\) is the fluorescence lifetime in the absence of quenching, and \(k_q\) is the bimolecular quenching constant which reflects both the efficiency of quenching and accessibility of the fluorophore to the quencher. The Stern-Volmer equation indicates a linear response of fluorescence intensity to quencher concentration. One of the most common quenchers, which we utilize in Chapter 2, is \(O_2\).

Some luminescent materials exist in an acid/base equilibrium where the acidic version of the molecule has different spectral properties than its conjugate. These light-emitting materials serve as natural pH sensors since their fluorescence properties can be altered over a range of pH where the light-emitting material is not completely at one end of the acid/base equilibrium base. One form of the molecule may be fluorescent while the other is not so that the fluorescence intensity changes with increasing or decreasing pH near the molecule’s acid-dissociation constant. Alternatively, the two forms may both be fluorescent but with distinctly different peaks so that as pH increases the intensity of one peak increases while the intensity of the other peak decreases and the color of light emitted by the compound gradually changes.\(^{23}\)

When the emission spectrum of a light-emitting molecule (the donor) overlaps with the absorption spectrum of another molecule (the acceptor), the energy from an excited state electron can be transferred from the donor to the acceptor, causing quenching of luminescent emission from the donor in a process known as Resonance Energy Transfer (RET). With RET, the acceptor does not need to be a luminescent material, and it’s important to note that no actual emission from the
donor is occurring. RET is commonly confused with a similar, non-quenching process called the inner filter effect (IFE) where an emitted photon from a luminescent material is absorbed by a nearby molecule, causing the apparent quenching of luminescence from the luminescent material. We utilize this approach in Chapter 3 with typical chromoionophore based sensors where the chromoionophore changes spectral properties in response to protonation and deprotonation.

Figure 1.5 Three equilibrium expressions to determine ionophore-based sensor response: (1) Extraction of the target ion into the sensor phase, corresponding with a release of hydrogen, (2) target ion binding with the ionophore, and (3) acid/base equilibria of the pH indicator. Reproduced with permission from reference 19 (Annual Review of Analytical Chemistry, Vol 7, 2014; Vol. 7, pp 483-512. Copyright 2014, Annual Reviews).

1.2.3.2 Theoretical Ionophore-Based Mechanism Response

The theoretical response of ionophore-based optical sensors is based on a well understood model thermodynamic equilibrium expressions.\textsuperscript{19,85} The derivation of the response equation has a few variations based on the charge of the pH indicator and for anion vs. cation detection, but the following will focus on cation sensing with a neutral pH indicator. Response of ionophore-based optical sensors can be thought of in three steps, depicted in Figure 1.5: (1) extraction of the target ion into the organic sensor core (i.e. cation exchange), (2) binding of the target ion to the ionophore, and (3) deprotonation of the pH indicator. The pH indicators used in IBOS are typically from a series of commercially available organic fluorescent molecular probes known as
‘chromoionophores’ (see Table 1.6) whose fluorescence properties can be altered through acid/base deprotonation, as described above in Section 2.3.1.

These three expressions are written as depicted in Figure 1.5 (page 18) and written below in Equation 1.5:

\[
\begin{align*}
\text{Equation 1.5:} \\
zH^+(aq) + I^{z+}(org) & \overset{K_{\text{exch}}}{\longleftrightarrow} zH^+(org) + I^{z+}(aq) \\
nL(org) + I^{z+}(org) & \overset{\beta_{L_nI^{z+}}}{\longleftrightarrow} L_nI^{z+}(org) \\
zIndH^+(org) & \overset{K_a}{\leftrightarrow} zInd^0(org) + zH^+(org)
\end{align*}
\]

Here, \( z \) is the charge of the target cation, \( H^+ \) is a hydrogen cation (proton), \( I^{z+} \) is the target cation, \( L \) is the ionophore, \( n \) is the complexing coefficient of the ionophore, \( \text{Ind} \) is the pH indicator, \( aq \) and \( org \) refer to the aqueous and organic phases, respectively, and \( K_a, \beta_{L_nI^{z+}}, \) and \( K_{\text{exch}}^{H^+,I^{z+}} \) are equilibrium constants (acid dissociation constant of the pH indicator, ionophore binding constant, and naked ion exchange constant, respectively). From these equations, the equilibrium constants can be expressed as shown in Equation 1.6.

\[
\begin{align*}
K_{\text{exch}}^{H^+,I^{z+}} &= \frac{(a_H)^z[L^{z+}]}{(a_I)[H^+]^z} \\
\beta_{L_nI^{z+}} &= \frac{[L_nI^{z+}]}{[I^{z+}][L]^n} \\
(K_a)^z &= \frac{[Ind^0]^z[H^+]^z}{[Ind1H^+]^z}
\end{align*}
\]

where bracketed terms represent species in the organic phase and ionic species in the aqueous phase are represented by their activities. The overall exchange constant, \( K_{\text{Overall}} \), can then be expressed as the product of the naked cation exchange constant, the ionophore binding constant, and the acid dissociation constant of the pH indicator as shown in Equation 1.7.

\[
K_{\text{Overall}} = K_{\text{exch}}^{H^+,I^{z+}} \beta_{L_nI^{z+}}(K_a)^z
\]
Electroneutrality is assumed in the organic phase meaning the total additive concentration is
assumed to equal the concentration of positive charges as shown in Equation 1.8.

\[ R_T = [\text{Ind1}H^+] + [\text{Ind2}H^+] + z[L_nI^{z^+}] \]  

(1.8)

\( \text{Ind}_T \) and \( \text{L}_T \) are defined as the total amount of pH indicator and ionophore in the organic phase, as shown in Equation 1.9:

\[ \text{Ind}1_T = [\text{Ind1}H^+] + [\text{Ind1}^0] \]

\[ \text{L}_T = [L] + n[L_nI^{z^+}] \]  

(1.9)

And finally, protonation degree of the indicator (1 - \( \alpha \)) is defined in Equation 1.10.

\[ 1 - \alpha = \frac{[\text{Ind}H^+]}{\text{Ind}_T} \]  

(1.10)

The expressions in Equations 1.9 and 1.10 can then be rearranged as shown in Equation 1.11.

\[ [\text{Ind}H^+] = (1 - \alpha)\text{Ind}_T \]

\[ [L_nI^{z^+}] = \frac{R_T - [\text{Ind}H^+]}{z} \]  

(1.11)

\[ [L] = L_T - n[L_nI^{z^+}] = [L] = L_T - \frac{n}{z}(R_T - [\text{Ind}H^+]) \]

The expressions from Equation 1.11 are inserted into Equation 1.7 to remove all unknown terms, and rearranged to obtain a response function which correlates the target cation activity \( (a_i) \) to the degree of protonation of the pH indicator based on known species concentrations and thermodynamic constants, as shown in Equation 1.12.

\[ a_i = \frac{(a_H)^z}{zK_{\text{overall}}} \left[ \frac{R_T - (1 - \alpha)\text{Ind}_T}{L_T - \frac{n}{z}(R_T - (1 - \alpha)\text{Ind}_T)} \right]^n \left( \frac{\alpha}{1 - \alpha} \right)^z \]  

(1.12)
Finally, the degree of protonation can be related to the fluorescence of the sensor with Equation 1.13, where $F_{\text{prot}}$ and $F_{\text{deprot}}$ are the fluorescence intensities of the pH indicator in its protonated and deprotonated states, respectively.

$$F = (1 - \alpha)(F_{\text{prot}} - F_{\text{deprot}}) + F_{\text{deprot}} \quad (1.13)$$

### 1.2.3.3 Discussion on Theoretical Enzyme-Based Mechanism Response

As discussed in the previous section, the ionophore-based response mechanism is rooted in thermodynamic equations, while enzyme-based response is rooted in transport phenomena and reaction kinetics. As a result, the shape of the sensor device becomes an important factor in modelling the response, and models of planar electrochemical sensors won’t translate well to predicting nanosensor response, despite utilizing the same recognition strategy. Many mathematical models of enzyme-based, planar, electrochemical sensors have been reported based on diffusion equations that rely on simplifying assumptions.\(^{86-89}\) Recently, more detailed models have been built to aid optimization of enzyme-based electrochemical sensor design.\(^{90}\)

To the best of our knowledge, no enzyme-based response models have been built for polymeric nanosensors or for any other type of miniaturized, spherical sensor. Regardless, considering the basics of the interactions of transport phenomena and reaction kinetics can be instructive for sensor design. Away from the sensor surface, there will exist some bulk concentration of $O_2$ in the solution. When the target analyte is present, a reaction will occur within the enzyme layer to deplete both the analyte and $O_2$. Depletion of $O_2$ within the enzyme layer will then drive diffusion of oxygen from the bulk supply (away from the enzyme layer), forming a concentration gradient at the nanosensor surface. Some minimum analyte concentration, which corresponds to the lower limit of the sensor’s dynamic range, will be necessary to overcome $O_2$ diffusion from the bulk and cause this gradient. The upper limit of the sensor’s dynamic range should correspond to an analyte concentration that causes a fast enough reaction in the enzyme layer to deplete all $O_2$ at the nanosensors surface. Analyte concentrations above this upper limit will not cause a change in the sensor’s luminescent signal, since with no $O_2$ present at the sensor surface, the sensor signal will be at its maximum. $O_2$ concentration profiles corresponding to the lower and upper limits of the sensor dynamic range are depicted in Figure 1.6. Higher enzyme
concentrations in the enzyme layer should therefore speed the reaction rate and shift the lower and upper limits of the sensor’s dynamic range toward lower concentrations.

Figure 1.6 Theoretical $O_2$ concentration profile at surface of an enzyme-linked nanosensor. (A) The lower limit of the sensors dynamic range corresponds to an analyte concentration that cause an $O_2$ gradient to form at the sensor surface at the nanosensor surface based on the enzyme-catalyzed reaction. (B) The upper limit of the sensor’s dynamic range corresponds to an analyte concentration that depletes all $O_2$ at the nanosensor surface based on the enzyme-catalyzed reaction.

1.3 Thesis Problem Statement

Polymeric nanosensors have an advanced set of properties, including miniature size, biocompatibility, non-invasive and remote readout, continuous monitoring and ability to generate 3D images that make them poised to revolutionize biological and clinical monitoring by giving scientists and clinicians the ability to analyze cell cultures and patients in ways not possible with currently available technology. However, being an emergent technology, polymeric nanosensors have several weaknesses that limit their practical application. To further the development of polymeric nanosensors toward practical use, we have identified several key areas where more research and development is needed to overcome these limitations. These limitations include, but are not limited to:

1. Lack of enzyme attachment strategies and range control techniques in small biomolecule-selective enzyme-based nanosensors
2. Lack of range control techniques for cation-selective (ionophore-based) nanosensors
(3) Use of organic dyes leads to background noise interference from light scattering and biological autofluorescence

1.4 Thesis Organization

The main content of this thesis is divided into three chapters based on three scientific manuscripts submitted to and/or accepted in various scientific journals. Chapter 2 is a report on a biotin/avidin-based enzyme-attachment and range control method for small biomolecule-selective nanosensors published in AIChE Journal. Chapter 3 is a report on a tool for dynamic range control and extension for ionophore-based optical nanosensors published in Sensors and Actuators: B: Chemistry. Chapter 4 is a report on the use of persistent luminescent microparticles in sodium-selective bulk optode membrane sensors as a replacement for organic dyes and as a method for avoiding biological autofluorescence and light scattering in biological samples that is under review at RSC Advances. Chapter 5 contains a summary of the conclusions from each of the main chapters and a discussion on recommendations for future work. Supplemental material for Chapters 2, 3, and 4 are found in Appendix A, B, and C, respectively. Appendix D contains copyright permissions for Figures and publications reproduced in this Thesis.

1.5 References Cited


47. Ross, J. W., Calcium-Selective Electrode with Liquid Ion Exchanger. *Science* 1967, 156 (3780), 1378-&.


62. Brasuel, M.; Kopelman, R.; Miller, T. J.; Tjalkens, R.; Philbert, M. A., Fluorescent nanosensors for intracellular chemical analysis: Decyl methacrylate liquid polymer matrix


82. Li, Y.; Gecevicius, M.; Qiu, J. R., Long persistent phosphors-from fundamentals to applications. *Chemical Society Reviews* 2016, 45 (8), 2090-2136.


CHAPTER 2

ENZYME-CONJUGATED NANOSENSORS WITH TUNABLE DETECTION LIMITS FOR SMALL BIO-MOLECULE DETERMINATION

A paper accepted for publication in AIChE Journal\(^1\)

Mark Ferris\(^2\), Makayla Elms\(^3\), Kevin Cash\(^4\)

2.1 Abstract

Polymeric nanosensors are more appealing than electrochemical sensors for advanced biological imaging applications, but more development is required before polymeric nanosensors can achieve widespread application. The detection of small biomolecules without a suitable ionophore, such as the detection of glucose, depends on enzymatic recognition by mixing oxidase enzymes with nanosensors. However, conjugation schemes and response control methods are needed to make polymeric nanosensors suitable for real-world applications. In this work, we develop enzyme-conjugated nanosensors based on the biotin/avidin interaction with two unique structures. The response of the first structure is controllable through changes to the concentrations of enzyme and avidin as well as surface biotinylation level during synthesis, while the second structure requires a simpler synthesis and has a controllable response through changes to the surface biotinylation level. We also report findings about optimal avidin concentration and separation of unbound enzyme from the enzyme-nanosensor conjugates through a magnetic purification approach.

2.2 Introduction

Fluorescent, polymeric nanosensors are a next-generation sensing technology that can be used for complex applications where currently available sensors fall short. Their small size (<200nm), biocompatible outer layer, and optical readout make them ideal for use in biological environments and for generating continuous, 3-dimensional maps of analyte concentration with

---

\(^1\) Reprinted with permission of AIChE J. 2019:e16698. https://doi.org/10.1002/aic.16698
\(^2\) Primary author and editor, Department of Chemical and Biological Engineering, Colorado School of Mines
\(^3\) Co-author, undergraduate researcher, Department of Chemical and Biological Engineering, Colorado School of Mines
\(^4\) Co-author, Assistant Professor, Department of Chemical and Biological Engineering, Colorado School of Mines
Despite these features, polymeric nanosensors tend to have limited dynamic ranges, limited recognition elements, and other design limitations that prevent their practical, widespread use for advanced sensing applications. Electrochemical sensors, even with relatively less desirable attributes that includes high invasiveness, inability for remote monitoring, and lack of multi-dimensional resolution without complex sensor arrays, are a more robust technology due to research dating back decades, and are more commonly used than polymeric nanosensors. Therefore, to unlock the potential for polymeric nanosensors to meet the demand of more challenging sensing applications, more research and development is needed.

Figure 2.7 Sensor mechanism. Nanosensors loaded with an oxygen-responsive fluorescent dye have the enzyme glucose oxidase (GOx) conjugated to their lipid layer. When the local glucose concentration increases, a reaction between glucose and oxygen and glucose is catalyzed by GOx, reducing the local oxygen levels and increasing the fluorescence of the oxygen-responsive dye. Nanosensors also contained iron oxide (Fe₃O₄) nanoparticles for magnetic control.

The polymeric nanosensor platform is modular so that it is easy to change the selectivity of the sensor to a range of analyte targets. Initially, polymeric nanosensors were developed to detect ions such as sodium, potassium, lithium, and calcium, by including ion-binding molecules (ionophores) as recognition elements and pairing with charge balancing additives and pH-sensitive fluorophores for the optical readout. The response of ion-responsive polymeric nanosensors are based on combined thermodynamic equilibriums and the underlying theory has been described in detail. Dynamic range control and extension for ion-sensitive polymeric nanosensors is achievable through formulation approaches such as chromoionophore changes. However, for analytes without a readily available ionophore (or no charge), this approach
falls short. Similar to many other biosensor approaches, enzymatic recognition can be paired with our nanosensors to broaden the range of detectable analytes. As examples, glucose and histamine have been detected by using oxidase enzymes as a recognition group and oxygen-sensitive nanosensors as the reporting agent. With this type of sensor, termed enzyme-based nanosensors (EnzNS), the enzyme consumes the analyte and oxygen simultaneously, dropping the local oxygen levels and increasing the fluorescence of the nanosensors which are quenched by oxygen – shown conceptually in Figure 2.1. For EnzNS the response is dominated by enzyme kinetics and oxygen transport in the test system. A tunable response range can be obtained by controlling the enzyme loading in the nanosensor formulation, with higher enzyme amounts leading to lower detection limits, as has been demonstrated in enzyme-based electrochemical sensors.

**Figure 2.8** Biotin/Avidin based conjugation strategies. (Left) NSB-A-BGOx particles consist of typical nanosensor formulations created with a substituted biotinylated lipid and mixed in solution with free avidin linker (A) and biotinylated glucose oxidase (BGOx). (Right) NSB-AGOx particles consist of typical nanosensor formulations created with a substituted biotinylated lipid and mixed in solution with avidinylated glucose oxidase (AGOx).
However, there is a key limitation of our EnzNS preventing their application. As previously demonstrated, the nanosensors and enzymes are not conjugated, but simply mixed together instead. While this is a suitable approach in a lab setting, in realistic *in vivo* and *in vitro* settings it is possible for the enzymes to diffuse away from the nanosensors, causing a drop in response from the nanosensors that isn’t related to the analyte concentration. Eventually, the enzymes could diffuse far enough away from the nanosensors that they do not respond to the target analyte at all. While for short term experiments this likely doesn’t impact results, for long term experiments this is a severe limitation. Without a conjugation approach this system is limited in both quantitation and long-term application.

There are numerous attachment strategies that exist for coupling enzymes to planar surfaces for electrochemical sensors and to other types of miniaturized optical sensors, such as polymer dots, gold nanoparticles, magnetic nanoparticles, and silicon oxide nanodots. One simple and effective strategy for controllably attaching enzymes to planar surfaces for electrochemical sensors is based on the biotin/avidin or biotin/streptavidin interaction. Avidin and streptavidin, homologs of each other, are tetrameric binding proteins that form strong interactions with biotin. The linkage between biotin and either avidin or streptavidin is one of the strongest known non-covalent bonds ($k_a \sim 10^{15} \text{ M}^{-1}$). Snejdarkova et al. created the first biotin/streptavidin based enzyme-conjugated electrochemical sensor by attaching biotin-modified glucose oxidase enzymes to a biotin-modified phospholipid bilayer with a streptavidin linker. Since then, this basic strategy has been used to immobilize enzymes such as lactate oxidase, alcohol oxidase, glutamate oxidase, horseradish peroxidase, and hydrogenase, as well as many other recognition and reporting groups. Multiple reports have also demonstrated that increasing the number of enzyme layers increases the magnitude of the response current and leads to dynamic range extension of the sensor toward lower analyte concentrations.

In this paper, we create an optical glucose sensitive Enzyme-Linked NanoSensor, which we term ELiNS, and show that the response can be tuned by controlling the enzyme density on the nanosensor surface. We create the enzyme/nanosensor linkage by adopting the biotin/avidin attachment strategies for electrochemical sensors outlined above. We accomplish this with two different ELiNS designs (NSB-A-BGOx particles and NSB-AGOx particles), shown schematically in Figure 2.2. In both ELiNS designs, we replace some portion of the amphiphilic
lipid layer with a biotinylated version of the same lipid during nanosensor synthesis, so that there are biotin functional groups on the surface of the nanosensor. The key differences between the two architectures is how the enzyme is conjugated to the nanoparticles. For NSB-A-BGOx particles, we mix a biotinylated version of glucose oxidase and free avidin linker in solution to form a linkage from the biotinylated nanosensors (NSB) to the avidin (A) to the biotinylated glucose oxidase (BGOx). We show that the response of these ELiNS is tunable through changes to the concentrations of BGOx and avidin, as well as changes to the surface density of reactive biotin sites on the nanosensor surface. We also find an optimal concentration of avidin above and below which enzyme activity is inhibited. For the NSB-AGOx particles, we use an avidin/GOx conjugate (AGOx) to directly attach GOx to the nanosensor surface using only the two subunits (biotinylated nanosensor (NSB) and avidinylated enzyme (AGOx)). This approach also created an ELiNS with a response range tunable through control of the surface density of biotin, similar to what has been seen for electrochemical approaches.

2.3 Results

2.3.1 Confirmation of ELiNS Conjugation

During ELiNS development, the nanosensors were characterized by recording the increase in fluorescence intensity of the oxygen-sensitive dye in response to the addition of glucose in a well plate. The nanosensor solution is covered with a mineral oil layer to slow oxygen dissolution so that the fluorescence increase is indicative of oxygen depletion in a fixed volume, a method adopted from literature. The initial rate of fluorescence increase, maximum fluorescence of the sensor, and shape of the response curve can then all be analyzed to characterize the response to glucose of the sensor. Importantly, with this method of analysis, the response of ELiNS and EnzNS should look the same, since in the closed volume there is no possibility for enzyme to diffuse away from the nanosensors. Therefore, to demonstrate the efficacy of enzyme and nanosensor conjugation, a separation scheme was devised to separate unbound enzyme from nanosensors – forcing the tested sensors to respond as they would in an open system after time for the enzymes to diffuse away. This was accomplished by adding iron oxide (Fe₃O₄) nanoparticles to the optode cocktail formulation which is used to make nanosensors, giving us magnetic control over the sensors. Nanosensors were synthesized with a biotinylated lipid, then mixed with either BGOx to form a control enzyme/nanosensor mixture (EnzNS) or BGOx and the avidin linker to form the
ELiNS described in Figure 2.1 (left, NSB-A-BGOx particles). Both EnzNS and ELiNS underwent magnetic separation to isolate either plain nanosensors (in the control sample) or ELiNS from free enzyme. Figure A.1 shows the response to addition of 28 mM glucose for ELiNS and EnzNS in a mineral oil sealed well, after separation. The EnzNS no longer respond to glucose after separation, indicating that all of the unbound BGOx has been removed. Therefore, as expected, there is no reaction with glucose. However, when avidin is added to the mixture before separation, the nanosensors retain their response to glucose. This is a clear indication that BGOx has been attached to the nanosensors through the avidin linker, forming the desired ELiNS. All ELiNS formulations described in this paper were put through this separation scheme before analysis to obtain only the response of nanosensors with bound enzymes.

2.3.2 NSB-A-BGOx Sensors – Effect of BGOx Concentration

The effect of the BGOx concentration used during ELiNS is demonstrated in Figure 2.3. With the highest concentration of BGOx tested (1 mg / batch), there is no obvious response to 0 and 0.1 mM glucose, as evidenced by the shape of the response curve being roughly flat. 1 mM glucose causes a clear increase in signal from the nanosensor, while glucose concentrations between 10-100 mM all produce a similar response in terms of shape, maximum fluorescence, and initial slope. This sensor formulation is therefore most sensitive between 1 and 10 mM glucose. With an intermediate concentration of BGOx (0.05 mg / batch), the sensor shows no response to 0, 0.1, and 1 mM glucose. Unlike the higher BGOx formulation, this sensor shows a different response to each of 10, 20, 30, 50, and 100 mM glucose in terms of initial slope and maximum fluorescence. With the lowest concentration of BGOx (0.02 mg / batch), the sensor showed a diminished response to glucose across the range tested, evidenced by the response being straight lines instead of curves, even at high glucose concentrations, and by the significantly lower maximum fluorescence than the other two ELiNS samples. Comparing the initial slope of response curves for these three samples shows that over a ~2 order of magnitude range of enzyme concentration, higher enzyme loading leads to a steeper initial slope, regardless of glucose concentration. It also indicates that higher enzyme loading leads to a lower detection limit. Multi-batch data for highest enzyme loading (1 mg BGOx) sample are shown in Figure A.2.
Figure 2.9 Effect of enzyme concentration (BGOx) on NSB-A-BGOx sensor response. Kinetic traces of sensor response with high (A), medium (B), and low (C) concentrations of BGOx. Analysis of initial slope across the test range of glucose concentrations shows fastest response to high BGOx, slower response to medium, BGOx, and a minimal response to low BGOx (D).

2.3.3 NSB-A-BGOx Sensors – Effect of Avidin Concentration

Similar to understanding the impact of BGOx on sensor response, we analyzed the effect of avidin amount during synthesis on ELiNS response. For logistical reasons, this was analyzed over three separate experiments, each comparing ELiNS synthesized with different concentrations of avidin, the results of which are summarized in Figure 2.4. Supporting data for Figure 2.4 includes kinetic traces (Figure A.3), response to 4 mM glucose between the three experiments (Figures A.4A, A.4B, and A.4C), and combined response to 4 mM glucose (Figure A.4D). Figure A.4 shows that the sensors are most responsive to glucose in the range of 0.11 to 0.44 mg avidin / batch. With low avidin amounts of only 0.02 or 0.05 mg, the response to glucose at 4 mM is linear with a shallow slope. At higher avidin levels of 0.88 and 1.32 mg, the response to 4 mM glucose
is also weaker, as seen by the shallower response slopes and lower maximum fluorescence with 1.32 mg avidin. Figure 2.4A shows the combined, normalized dose/response curves for all batches of ELiNS tested during these experiments, generated by plotting the initial slope of each response curve against glucose concentration. Each data set was fit with a four-parameter logistic response curve in order to determine its dynamic range. In Figure 2.4B, the midpoint of the dynamic range of each response curve (LogEC$_{50}$) is plotted against the avidin concentration. This figure shows a minimum LogEC$_{50}$ corresponding to the same avidin range (0.11-0.44 mg) that led to the strongest response to glucose, further indicating this range as an optimal avidin linker concentration for sensor sensitivity.

![Figure 2.10](image)

Figure 2.10 Combined, normalized dose/response curves for nine batches of NSB-A-BGOx sensors over seven unique avidin levels, generated by plotting the initial slope of the kinetic trace against glucose concentration for each sensor batch (A). Response midpoint (LogEC$_{50}$) vs. avidin amount, showing a minimum LogEC50 (maximum responsiveness) in the range of 0.11-0.44 mg avidin (B).

### 2.3.4 Dynamic Range Control through Surface Biotinylation

The dynamic range of the ELiNS is controllable by adjusting the percent biotinylated lipid on the nanosensor surface for the two particle designs described in Figure 2.2. Figure 2.5 shows the effect of surface biotinylation control with NSB-A-BGOx sensors. To control the surface coverage we varied the formulation of surfactant in the nanosensor fabrication step to change the percentage labeled versus unlabeled PEG-lipid. We analyzed 25% and 6.3% labeled groups (biotinylation) and in alignment with our results on varying the enzyme and linker concentration found the higher surface density (25%) to have a stronger response to glucose across the tested...
range. With 25% biotinylation, the ELiNS showed no response to 0 and 0.1 M glucose and a small response to 1 mM glucose. 10, 20, 30, and 50 mM glucose all showed approximately the same response, indicating that concentrations above 10 mM glucose are above the dynamic range of this ELiNS formulation. With just 6.3% biotinylated lipid, the shape of the response curves across the glucose concentrations tested closely approximates a linear trend, indicating a much weaker response to glucose that is similar to the low BGOx and low avidin data. With 6.3% biotinylation, the sensor shows no response to 0, 0.1, and 1 mM glucose, and a small but differentiable response between 10, 20, and 30 mM glucose (see Figure A.5B), indicating that the dynamic range of this ELiNS formulation is shifted toward higher glucose concentrations. These responses are also shown as a dose response curve in Figure A.6.

Figure 2.11 Effect of surface biotinylation on response of NSB-A-BGOx particles. Higher surface biotinylation (A) leads to a stronger response to glucose than a lower amount of surface biotinylation (B).

We can also control the glucose response of our nanosensors with our second ELiNS structure (NSB-AGOx sensors). While the initial step of controlling surface biotinylation is the same, sensor fabrication involves simply mixing AGOx conjugate with the biotinylated nanosensor rather than the multiple linkage steps. Figure 2.6 shows the nanosensor response to a range of glucose for 90%, 25%, 1%, and 0% biotinylation. Surprisingly, with NSB-AGOx sensors, the control sample (0% biotinylation) still responded to glucose. Figure A.7 shows a dose/response curve for each of the four sensor formulations, generated by plotting the initial slope of the response curve against glucose concentration. The 90% biotinylation sample shows the fastest response to glucose across the range tested. These sensors show a distinctly different response to
4 and 8 mM glucose in terms of initial slope, while the response curves to 12-28 mM glucose mostly overlap each other. The 0% biotinylation sample has the shallowest initial slopes across the glucose range tested, indicating the lowest amount of enzyme association as expected. The 1% and 25% samples have shallower initial slopes than the 90% sample, but steeper than the 0% sample. For the 0%, 1%, and 25% samples, there was an increase in signal upon addition of higher glucose concentration, for each glucose concentration tested, all the way up to 28 mM. Since the response to glucose for the 90% sample was overlapping at the higher glucose range, the 90% sample likely has a dynamic range shifted toward lower concentrations than the other three.

Figure 2.12 Response tuning with NSB-AG Ox sensors based on surface biotinylation. Kinetic traces of sensor response to a range of glucose for 100% (A), 25% (B), 1% (C), and 0% (D) biotinylation.
2.4 Discussion

One unexpected result from this work was the observation of decreased enzymatic activity at high levels (0.88mg / batch and greater) of avidin with the NSB-A-BGOx sensors (observed in Figures A.3 and 4). However, the literature indicates that this is likely due to excess avidin inhibiting the activity of BGOx, known as a ‘hook effect.’ Another possibility is that the higher concentrations of avidin lead to avidin consuming enough biotin sites on both the BGOx and the nanosensor surface that the number of possible enzyme/nanosensor linkages were reduced. Another confounding result was the retention of enzyme activity even with 0% biotinylation when using the NSB-AGOx method. This could be due to non-specific binding from the avidin group in AGOx. Since avidin is known to participate in a variety of unwanted non-specific interactions, it may be forming a weak interaction with the charged lipid surface of the nanosensor despite the complete absence of biotin groups.

Efforts were made to keep all synthesis, reaction, separation, and test conditions as consistent as possible, but a limitation of this work was the batch to batch reproducibility of incorporating iron oxide nanoparticles into nanosensors. TEM images indicate that iron oxide nanoparticles were not evenly distributed in the nanosensors and that some nanosensors contained no iron oxide nanoparticles at all (Figure A.8), leading to some batches with a higher concentration of iron oxide-containing nanosensors than others. When the sensors were put through a 3-step separation process, some batches would become more concentrated than others, leading to a confounding variable during response testing. This was especially troublesome when an experiment necessitated a different batch of nanosensors for each test condition, such as with response tuning with surface lipid biotinylation. Alternative nanosensor synthesis techniques are currently being explored to improve the reliability of iron oxide loading and corresponding sensor purification.

Imperfect control over nanosensor concentration presents additional complications for enzyme-coupled nanosensors when compared with the more traditionally studied ionophore-based optical nanosensors due to the differences in response mechanism. Ionophore-based optical nanosensor response is based on a combined thermodynamic equilibrium expression with no reaction kinetics to consider and fast transport steps due to the high surface area to volume ratio of the miniaturized sensors. Ionophore-based optical sensors in bulk optode form, however, take
longer to respond due to analyte diffusion in the hydrophobic sensor environment. With ionophore-based nanosensors, a secondary dye and a ratiometric signal can easily control for fluctuations in sensor concentration during signal transduction, and the amount of analyte consumed is small enough relative to the number of nanosensors that buffering does not impact the response. Therefore, sensor response becomes independent of sensor concentration. However, this strategy is insufficient for ELiNS. When ELiNS are analyzed in a well plate, the sensor concentration will affect the total amount of enzyme per well, and thus the observed rate of reaction that the sensors are monitoring. Therefore, the sensor response is dependent on sensor concentration even with ratiometric measurements. This reinforces the need for more consistent iron oxide nanoparticle loading for future studies.

The two main issues that this work aimed to address were 1) a lack of conjugation techniques for attaching enzymes to polymeric nanosensors 2) a lack of methods to control the response characteristics of enzyme-coupled nanosensors. The control experiment (Figure A.1) and the tuning experiments demonstrate that this approach for conjugating enzymes to our nanosensors is effective at yielding conjugated sensors. We can control the response of our sensors through tuning multiple steps in the fabrication process, including the concentration of enzyme, the concentration of the avidin linker, or the surface labeling site density. However, the proposed methods for range control did not provide as reliable of a tool for fine tuning the response characteristics as has been demonstrated with cation-selective nansosensors. This may be improved in the future with more reproducible iron oxide loading in nanosensors and better control of nanosensor concentration through separation steps, as described above. However, the biotin/avidin conjugation technique and response tuning through control of surface lipid biotinylation has a lot of promise for the future due to its versatility. Oxidase enzymes can be easily tagged with biotin molecules, or, with more effort, conjugated to avidin, making the method extendable for targets such as lactic acid and histamine by exchanging biotinylated glucose oxidase for biotinylated lactate oxidase or diamino oxidase. Another future extension of this work is building multi-layer enzyme structures on the nanosensor surface to further increase the reaction rate near each nanosensor and therefore decrease the detection limit of the ELiNS. Such structures would only be possible with the NSB-A-BGOx particles and may be necessary to achieve sufficiently high reaction rates with other oxidase enzymes that have much lower activity than GOx (i.e. lactate oxidase and diamino oxidase).
2.5 Conclusion

Nanosensors are an advanced sensing platform that is miniaturized, biocompatible, and non-invasive. Enzyme coupling has been demonstrated with nanosensors to extend the available analyte targets, but enzyme-nanosensor conjugation and dynamic range control are needed for the continued development of enzyme-coupled nanosensors. In this work, we demonstrate a biotin/avidin-based approach for forming Enzyme-Linked NanoSensors (ELiNS). In addition, we show control over the nanosensor response characteristics with multiple avenues during sensor synthesis, including control over the enzyme concentration, avidin linker concentration, and control over the surface attachment site density. While limited by batch reproducibility, we have demonstrated that this approach is a valuable way to combine the positive attributes of our nanosensors with the versatile recognition and tunable response based on enzymes. Future work should be aimed at reducing the batch to batch variability of nanosensors, which is critical for a reproducible enzyme/nanosensor scheme and for fine control over response characteristics.

2.6 Experimental Section

2.6.1 Reagents and Materials

Poly(vinyl chloride), high molecular weight (PVC), bis(2-ethylhexyl) sebacate (BEHS), tetrahydrofuran (THF), dichloromethane (DCM), mineral oil, avidin from egg white, glucose oxidase (GOx), D-(+)-Glucose were purchased from Sigma Aldrich (St. Louis, MO, USA). 1,2-Disteroyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-550] ammonium salt in chloroform (PE-PEG-750) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] ammonium salt (DSPE-PEG(2000)-Biotin) were purchased from Avanti polar lipids, Inc. (Alabaster, AL, USA). Dulbecco’s phosphate buffered saline (PBS, pH = 7.4) was purchased from Life Technologies (Grand Island, NY, USA). Biotin glucose oxidase conjugate (BGOx) and avidin glucose oxidase conjugated (AGOx) was purchased from Rockland Immunochemicals Inc. (Limerick, PA, USA). 5,10,15,20-Tetrakis(pentafluorophenyl)-21H,23H-porphine, platinum(II) (PtTPFPP) was purchased from Frontier Scientific (Logan, UT, USA). Iron oxide nanoparticles (Fe$_3$O$_4$NP) were synthesized at the National Institute of Standards and Technology (Boulder, CO, USA).
2.6.2 Nanosensor Synthesis

Oxygen nanosensors were fabricated similar to previously established method for ion selective nanosensors. Briefly, an optode cocktail was formulated by dissolving 30mg PVC and 33μL BEHS (1:2 by mass) along with 4.4 mg PtTPFPP and Fe₃O₄ NP in 250μL before adding 250μL DCM. Next, 2 mg PEG-Lipid (80μL of a 25mg/mL solution in chloroform)) was dried and resuspended in 5 mL PBS with a probe tip sonicator for 30 s at 20% intensity (Branson, Danbury CT). 100 μL of the optode cocktail solution was mixed was added to the PBS/ PEG-lipid solution while under probe tip sonication (3 min, 20% intensity). The nanosensor solution was filtered with a 0.8 μm syringe filter to remove excess polymer (Pall Corporation, Port Washington, NY).

2.6.3 Enzyme-Linked Nanosensor Synthesis

O₂NS were synthesized by replacing a percentage of PE-PEG-750 with DSPE-PEG(2000)-Biotin. The O₂NS were then mixed with either (i) Avidin and BGOx suspended in PBS to create NSB-A-BGOx sensors or (ii) AGOx suspended in AGOx to create NSB-AGOx sensors. To remove unbound enzyme, the sensor solution was placed on a magnet (N-52, Nickel Plated, 3.8 cm x 3.8 cm x 3.8 cm from K&J Magnetics, Inc.) to attract ELiNS to the bottom of the vial, the supernatant (containing unbound enzyme) was removed, and sensors were resuspended in fresh PBS. This process was repeated three times to remove all unbound enzyme. The sensor batch was placed on the magnet for 24 hours for the first separation step and 4 hours for subsequent separation steps.

2.6.4 Nanosensor Characterization

Sensor fluorescence was measured with a Synergy H1 microplate reader using Nunc™ 384-Well Optical Bottom Plates with polymer base (Nalge Nunc International, Roskilde, Denmark). Wells were filled with 26μL of sensors and covered with 39 μL of mineral oil. 26 μL of glucose solution was injected below the mineral oil layer prior to fluorescence measurements. Nanosensors were excited at 390 nm and emission was collected at 650 nm.

To extract initial slope values in Figures 2.3A, 2.3B, 2.5A, and A.5, the data was fit to a one-phase association equation,

\[ Y = Y_0 + (Plateau - Y_0) \times \left(1 - e^{(-k \times k)}\right) \] (2.1)
With GraphPad Prism version 7.03 (GraphPad, La Jolla, CA, USA) where $Y_0$ is the initial fluorescence intensity value, Plateau is the final fluorescence intensity value, and $k$ is a rate constant in units of $1/s$. The slope is then determined by taking the first derivative which simplifies to $(\text{Plateau} - Y_0)k$. To extract initial slope values in Figures 2.3C and 2.5B, the data was fit to a linear regression. To determine the midpoint of the dynamic range of the ELiNS in Figure 2.4A, the data sets were fit to a four-parameter logistic response equation,

$$Y = \text{Bottom} + \frac{(\text{Top} - \text{Bottom})}{1 + 10^{((\log EC_{50} - X) \cdot \text{HillSlope})}}$$  \hspace{1cm} (2.2)

with GraphPad Prism where Top and Bottom represent the maximum and minimum sensor signals, LogEC$_{50}$ is the analyte concentration corresponding to half-maximal response, and HillSlope is the slope of the tangent line drawn at the LogEC$_{50}$. The two data sets in Figure A.6 were also fit with Equation 2.2.

### 2.7 Notation

- **EnzNS** – Enzyme-coupled nanosensors (a solution of enzymes and polymeric nanosensors with no specific interaction between the two entities)
- **ELiNS** – Enzyme-linked nanosensors (a structure that consists of enzymes conjugated to a polymeric nanosensor with a specific interaction holding them together)
- **GOx** – Glucose oxidase
- **AGOx** – Avidin/glucose oxidase conjugates
- **BGOx** – Biotin/glucose oxidase conjugates
- **NSB-A-BGOx sensors** – ELiNS consisting of polymeric nanosensors with biotin groups on the surface and an avidin linker connecting BGOx to the nanosensors
- **NSB-AGOx sensors** – ELiNS consisting of polymeric nanosensors with biotin groups on the surface to which AGOx is bound
2.8 References Cited


CHAPTER 3

A DUAL-INDICATOR STRATEGY FOR CONTROLLING THE RESPONSE OF IONOPHORE-BASED OPTICAL NANOSENSORS

A paper published in Sensors and Actuators: B

Mark Ferris, Aakash Katageri, Greta Gohring, Kevin Cash

Figure 3.1 Graphical Abstract. The dynamic range of ionophore-based nanosensors is controllable through changes to the indicator composition.

3.1 Abstract

Optical nanosensors are used to detect a wide range of ions and molecules by changing their fluorescent properties in response to the local analyte concentration. Practical methods to adjust the sensor response characteristics of optical nanosensors are needed to match the sensor dynamic range with the expected analyte fluctuation for a given application. For ionophore-based optical sensors, the linear range is determined by three simultaneous equilibria, including the acid dissociation of a pH indicator. In this work, we add a second pH indicator to typical ionophore-based optical sensor formulations. We show that pH indicator acid-dissociation is fundamentally different when two indicators are loaded within the same nanoparticle, effectively coupling their

---

1 Reproduced with permission of Sensors and Actuators: B Chemical 256, 674-681. Copyright (2018) Elsevier
2 Primary author and editor, Department of Chemical and Biological Engineering, Colorado School of Mines
3 Co-author, graduate researcher, Department of Chemical and Biological Engineering, Colorado School of Mines
4 Co-author, high-school researcher, Conifer High School
5 Co-author, Assistant Professor, Department of Chemical and Biological Engineering, Colorado School of Mines
equilibria, as opposed to being housed in separate sensor nanoparticles that simultaneously interact with the sample. We demonstrate that these two methods of dual-indicator sensor design give control over the response range of ionophore-based optical sensors and can extend the linear range span over what is possible with a single-indicator nanosensor.

3.2 Introduction

While a range of optical sensor methods have been developed for various applications, in vivo and in situ biological imaging requires sensors that can be miniaturized and made biocompatible. Unlike their electrochemical counterparts, optical sensors are minimally invasive for in vivo use. They also have the ability to create spatial maps of analyte concentrations, a useful tool with varied applications such as biofilm monitoring, cellular analysis, and in vivo pharmacokinetics. The response of an optical sensor can be characterized by its linear or dynamic range and its EC_{50}, which is the concentration of an analyte corresponding to half-maximal signal from the sensor. For practical application, it is critical that the range of possible fluctuations in analyte concentration fall within the sensor’s dynamic range. It is also important for the sensor’s EC_{50}, where the sensor is the most sensitive to fluctuations, to match the physiological analyte concentration.

The basis of any optical sensor is a molecule that can change its optical properties (e.g. fluorescence) in response to a perturbation, like addition of another chemical species. There is an abundance of dyes that can detect hydrogen ions (pH) and oxygen via a direct mechanism, where one molecule serves as both the analyte receptor and indicator. Indirect sensing methods, where separate chemicals are used for analyte recognition and reporting, are used to expand the range of detectable analytes. An indirect approach is advantageous because changing the analyte receptor will alter the sensor specificity to a different analyte without needing new synthetic molecules, such as with direct binding sensors. Ionophore-based optical sensors (IBOS) are a class of indirect sensors that have proven to be a robust and versatile tool for measuring ionic concentration by reporting the ratio of ionic activities (i.e. sodium activity divided by hydrogen activity). IBOS are typically formulated with a pH indicator (chromoionophore), an analyte-specific ligand (ionophore), and a charge-balancing additive, all contained in a hydrophobic plasticized-polymer matrix. In this setting, the target ion (analyte) binding to an ionophore is indirectly coupled to the protonation state of the pH indicator via maintaining charge balance in
the hydrophobic phase. The IBOS components are dispersed in an organic medium to form an optode cocktail that can be further processed into biocompatible nanosensors. This class of sensors has been applied toward in vitro\textsuperscript{13,22,23} and in vivo\textsuperscript{24,25} imaging applications.

Recent research has focused on dynamic range control for a variety of sensor classes. Range control strategies for aptamer-based sensors have included experimentally controlling the binding conformation of the aptamer\textsuperscript{26} and incorporating multiple aptamers with varied binding strength toward the intended analyte.\textsuperscript{27} A similar strategy to the latter has been applied in DNA hybridization sensors by incorporating multiple probe sequences of different lengths, and therefore binding affinities, into a single sensor system.\textsuperscript{28} In optical pH sensors, the protonation equilibrium of the fluorescent molecule controls the linear range. Reports of combining multiple pH indicators with different pK\textsubscript{a} values to extend the detection range began as early as 1989 when Posch et al.\textsuperscript{29} immobilized the pH sensitive dyes fluorescein and eosin on cellulose supports to produce a fiber-optic sensor capable of detecting the full acidity range (pH 0 to pH 7). de Silva et al.\textsuperscript{30,31} later combined four members of a “fluorophore-spacer-receptor” type to create a fluorescent pH sensor with an extended range from pH 0 to pH 10. More recently, Qi et al.\textsuperscript{32} showed that the entire range of pH 1 to pH 14 can be detected with a single optical sensor platform by attaching a single fluorophore to 6 different receptors, each with a different pK\textsubscript{a}. Chauhan et al.\textsuperscript{31} developed a dual-fluorophore pH nanosensor with a tunable EC\textsubscript{50} by incorporating two pH indicators into a single particle and later used the nanosensors to create real-time images of the pH distribution in the pharyngeal and intestinal lumen of \textit{Caenorhabditis elegans}.\textsuperscript{33} The approaches outlined above control the response of different classes of sensors through judicious changes to the underlying thermodynamics of both the recognition and reporting events. For IBOS, the sensor response is dictated by three equilibria: pH indicator/hydrogen binding, ionophore/analyte binding, and ion exchange between the surrounding aqueous phase and the organic phase of the sensor. Dynamic range control, yielding large shifts in dynamic range, has been demonstrated by substituting the pH indicator for one with a different pK\textsubscript{a} in ionophore-based sensors, e.g. potassium,\textsuperscript{34} sodium,\textsuperscript{35} nitrite,\textsuperscript{36} and thiocyanate,\textsuperscript{36} or by substituting other components, such as the additive.\textsuperscript{37} Suzuki et al. analyzed the response of tandem pH-indicators in an ionophore-based optode film which was suitable for analysis with visual colorimetry, a useful method for single data point environmental field assays and household clinical assays.\textsuperscript{38} They showed a dynamic range of $10^{-6} – 10^{-2}$ M with a single indicator and extended it to $10^{-6} – 10^{-1}$ M with two indicators.
Figure 3.2 Mechanism of IBOS response to increasing analyte (I⁺) concentration with two pH indicators (Ind1 and Ind2) co-loaded into a sensor matrix with additive (R-), and ionophore (L). At low analyte concentration, both pH indicators are protonated and both give off a fluorescent signal. At a higher concentration, the analyte begins to diffuse into the sensor and binds to the ionophore that in turn deprotonates the pH indicator with the lower pKₐ. At high analyte concentrations, both pH indicators begin to deprotonate until a point where there is no signal from either molecule at the selected wavelength.

In this work, we demonstrate that a pair of pH indicators with different pKₐs can work in tandem to control and extend the linear range of lithium- and calcium-selective nanosensors, a miniaturized and biocompatible form of IBOS. The dynamic range of a sensor is the concentration range that causes any change in the measurable output whereas the linear range is restricted to the concentration range that causes a linear response. We employed two methods to test the dual-response of two chromoionophores to analyte concentration. After synthesizing single-pH indicator optode cocktails, we either combined the optode cocktails before nanosensor synthesis or produced nanosensors from each optode cocktail and combined the resulting nanosensor batches. We refer to these strategies as the ‘mixed optode method’ and the ‘mixed nanosensor method’, respectively. The mixed optode method therefore loads the two different chromoionophores into the same nanoparticle (Figure 3.2). As with typical IBOS, the analyte is extracted into the hydrophobic core where it binds with the ionophore. With two pH indicators present in the same phase, the response mechanism is analogous to the description in Suzuki et al.’s work,³⁸ where two pH sensitive dyes with different pKₐ values and colors were loaded into a
color-changing film sensor. The pH indicator with the lower $pK_a$ begins deprotonation first and as the local analyte concentration further increases, this pH indicator further deprotonates while the second pH indicator begins to deprotonate. After the first pH indicator has fully deprotonated, the second still deprotonates further with higher analyte concentration, yielding a sensor with a wider dynamic range than one with a single pH indicator. With the mixed nanosensor method, two pH indicators are enclosed in different nanosensors and do not directly interact with each other. The total combined fluorescent response is therefore a weighted average of the individual sensor responses. For both mixed optode and mixed nanosensor methods, adjusting the ratio of the two pH indicators dictates sensor response and allows for precise control over the EC$_{50}$.

### 3.3 Theory

#### 3.3.1 Derivation

Previous reports have summarized the underlying theory for predicting ionophore-based optical sensor response.\textsuperscript{2,39} There are a few variations of this derivation based on the pH indicator charge and for anion vs. cation sensing, but the following will focus only on cation sensing with a neutrally charged pH indicator. Briefly, the sensor mechanism is based on three simultaneous equilibriums: ion exchange between the aqueous and organic phase (hydrophobic core of the sensor) of the sensor, the ion/ionophore binding, and the acid dissociation of the pH indicator. Assuming electroneutrality is satisfied in the organic phase, the total amount of charge from the additive equals the total amount of charge from the pH indicator when fully protonated. With this assumption and others detailed in the literature\textsuperscript{2,39}, the protonation state of single-pH indicator IBOS is predicted by

\begin{equation}
    a_I = \frac{(a_H)^z}{zK_{overall}} \left[ \frac{R_T - (1 - \alpha)\text{Ind}_T}{L_T - \frac{n}{z} [R_T - (1 - \alpha)\text{Ind}_T]} \right]^n \left( \frac{\alpha}{1 - \alpha} \right)^z \tag{3.1}
\end{equation}

\begin{equation}
    K_{overall} = K_{exch}^{H^+\text{z+}} \beta_{Ln\text{z+}}(K_a)^z
\end{equation}

where $a_H$ and $a_I$ are the hydrogen and analyte activities, respectively; $z$ is the analyte charge; $R_T$, $\text{Ind}_T$, and $L_T$ are the sensing components (concentrations of additive, pH indicator, and ionophore, respectively); $K_a$, $\beta_{Ln\text{z+}}$, and $K_{exch}$ are equilibrium constants (pH indicator acid dissociation

53
constant, ionophore binding constant, and the ratio of ionic partition coefficients, respectively) with $K_{\text{overall}}$ being the overall equilibrium constant; and $\alpha$ is the degree of deprotonation of the pH indicator. The protonation degree $(1-\alpha)$ is directly related to the protonated and deprotonated fluorescence output of the pH indicator through

$$F = (1 - \alpha)(F_{\text{prot}} - F_{\text{deprot}}) + F_{\text{deprot}} \quad (3.2)$$

For the mixed nanosensor method, the individual response of each sensor is unchanged from traditional theory, since the underlying equilibria for each sensor are the same. Each sensor composition fluoresces according to Equation 3.1 and Equation 3.2 and, assuming the two sensor formulas have the same molar concentration of pH indicator, the combined fluorescence readout is represented by

$$F = \theta[(1 - \alpha_1)(F_{1\text{prot}} - F_{1\text{deprot}}) + F_{1\text{deprot}}] + (1 - \theta)[(1 - \alpha_2)(F_{2\text{prot}} - F_{2\text{deprot}}) + F_{2\text{deprot}}] \quad (3.3)$$

where $\theta$ is the molar fraction of indicator 1 (Ind1) in the total pH indicator composition $(\text{Ind1}_T/(\text{Ind1}_T + \text{Ind2}_T))$. For convention in this work, indicator 1 is defined as the pH indicator that begins deprotonation at a lower analyte concentration. To account for the inclusion of a second pH indicator in the same sensor matrix (mixed optode method), we expanded the derivation to account for this fourth equilibrium as well as the related supporting equations (detailed fully in Supporting Information). The analogous equation to Equation 3.1 for the mixed optode method is given by

$$a_1 = \frac{a_{H^z}^*}{zK_{\text{overall}}} \left[ \frac{R_T - (1 - \alpha_1)\text{Ind1}_T - (1 - \alpha_2)\text{Ind2}_T}{(L_T - \frac{n}{z}(R_T - (1 - \alpha_1)\text{Ind1}_T - (1 - \alpha_2)\text{Ind2}_T))^n} \left[ \frac{\alpha_1}{1 - \alpha_1} \left( \frac{\alpha_2}{1 - \alpha_2} \right)^{1-\theta} \right]^z \right]$$

$$\alpha_2 = \frac{(K_{a2})}{K_{a1}(1 - \alpha_1) + (K_{a2})} \quad (3.4)$$

$$K_{\text{overall}} = K_{\text{exch}}^{H^+I^{z+}} \beta_{ln,I^{z+}}(K_{a1})^{\theta}(K_{a2})^{(1-\theta)z}$$

where $\alpha_1$ and $\alpha_2$ represent the deprotonation state of the two pH indicators, $K_{a1}$ and $K_{a2}$ represent
their respective acid dissociation constants, and Ind1\(_T\) and Ind2\(_T\) are the molar concentration of each pH indicator. The degree of deprotonation of each pH indicator can be determined with Equation 3.4 and related to the fluorescence of a mixed optode nanosensor with Equation 3.3.

Figure 3.3 Theoretical normalized protonation dose-response curves for lithium-sensitive IBOS. (A) Mixed nanosensor method: changing \(\theta\) does not impact how the pH indicators deprotonate. (B) Mixed optode method: By mixing the pH indicators together in one sensor, the thermodynamics of deprotonation change based on \(\theta\). The linear range and EC\(_{50}\) shifts toward higher concentrations as Ind2 is replaced with Ind1.

### 3.3.2 Response Prediction

Using the theory described above, we generated pH indicator protonation curves and fluorescence response curves for the mixed optode and mixed nanosensor methods. For plotting purposes, we determined \(K_{\text{overall}}\) by fitting the \(\theta = 1\) data to Equation 3.1 and then aligned the pK\(_a\)s of Ind1 and Ind2 so that theoretical response aligned with experimental results for single-indicator nanosensors. Experimental studies were carried out with Chromoionophore II (ChII) and Chromoionophore VII (ChVII) in lithium-selective nanosensors to demonstrate the dual-indicator concept. With the mixed nanosensor method, theory shows that, as expected, the pH indicator composition in the overall sensor batch doesn’t affect the individual pH indicator protonation curve (Figure 3.3A). The inclusion of the second pH indicator in the same sensor matrix, as with the mixed optode method, affects the analyte concentration ranges where the different pH indicators deprotonate (Figure 3.3B), indicating that the underlying thermodynamics of optical detection are altered through the coupled equilibria. The LogEC\(_{50}\) of the ChII deprotonation curve increases as
more ChVII is added (increasing $\theta$) while the LogEC$_{50}$ of the ChVII deprotonation curve decreases as more ChII is added (decreasing $\theta$). This means that the mixed optode method (combining two pH indicators in the same formulation cocktail) pushes apart the pH indicator deprotonation curves compared to the mixed nanosensor method. Despite this difference in individual pH indicator response, the theory predicts similar response between the two methods for the combined fluorescent output (Figure 3.4). The larger response deviation near the top of each curve compared to the bottom of each curve is due to the difference in the maximum fluorescence of the two pH indicators (ChVII is approximately three times brighter than ChII). If the two indicators have the same maximum fluorescence, this deviation in response is minimized (Figure B.1). As expected, at $\theta = 0$ or 1, mixed optode and mixed nanosensor theory predict the same response curves, as the formulations are identical between the two methods.

Figure 3.4 Theoretical normalized dose-response curves. Solid red curves represent the mixed optode method while dashed black curves represent the mixed nanosensor method. $\theta$ increases from 0 to 1 at an increment of 0.2 from right to left. While the deprotonation of the individual pH indicators is very different, the detectable combined-fluorescent output is similar between the two methods at any value of $\theta$.

### 3.3.3 pK$_a$ Separation Analysis

Theoretical modeling also helps predict the extent of linear range extension with these methods. The greater the separation of pK$_a$ between the pH indicators, the more the linear range can be extended, up to a point where the response becomes non-linear and eventually biphasic.
Theory indicates the pKₐ separation of the two indicators can be greater for the mixed nanosensor method than with the mixed optode method before a non-linear response is observed (Figures B.2 and B.3). Due to the limitations in theory discussed above, this analysis is only instructive to elucidate the fundamental difference in the cooperativity between the pH indicators with the mixed optode and mixed nanosensor methods. A better understanding of the theoretical response of nanosensors and any differences from traditional optode theory is needed before useful values can be obtained with modelling.

3.4 Experimental

3.4.1 Reagents and Materials

9-dimethylamino-5-[4-(15-butyl-1,13-dioxo-2,14-dioxanondecyl)phenylimino]benzo[a]-phenoxazine (chromoionophore VII; ChVII), Poly(vinyl chloride), high molecular weight (PVC), 9-(Diethylamino)-5-(octadecanoylimino)-5H-benzo[a]phenoxazine (chromoionophore I; ChI), bis(2-ethylhexyl) sebacate (BEHS), tetrahydrofuran (THF), dichloromethane (DCM), Sodium tetrakis[3,5-bis(trifluoromethyl)phenyl] borate, Selectophore (NaBARF), 6,6-dibenzyl-1,4,8,11-tetraoxacy- clotetradecane (lithium ionophore VI; LiI VI), trioctylphosphine oxide (TOPO), 9-Dimethylamino-5-[4-(16-butyl-2,14-dioxo-3,15-dioxaeicosyl)phenylimino]benzo[a]phenoxazine (chromoionophore II; ChII), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), and lithium chloride were purchased from Sigma Aldrich (St. Louis, MO). 1,2-Disteroyl-sn-glycero-3-phosphoethanolamine-N- [methoxy(polyethylene glycol)-550] ammonium salt in chloroform (PEG-lipid) was purchased from Avanti Polar Lipids (Alabaster, AL). Phosphate buffered saline (PBS, pH = 7.4) was purchased from Life Technologies (Grand Island, NY). 2-Amino-2-hydroxymethylpropane-1,3-diol, 2M solution (TRIS, 2M), was purchased from Fisher Scientific (Waltham, MA). Calcium Ionophore I was purchased from Santa Cruz Biotechnology (Dallas, TX).

3.4.2 Optode cocktail and Nanosensor Formulation

3.4.2.1 Synthesis of Lithium Optode Cocktail

306 mmol/kg LiI VI, 146 mmol/kg NaBARF, 305 mmol/kg TOPO, and 16 mmol/kg of either ChII or ChVII in PVC/BEHS (1:2 by mass), with a total of mass of 117 mg, were dissolved
3.4.2.2 Synthesis of Lithium Nanosensor

Similar to prior publications, \(^{40}\) 2 mg PEG-Lipid (80 \(\mu L\) of a 25 mg/mL solution in chloroform) was dried in a 4 dram scintillation vial and then resuspended in 5mL of PBS or HEPES/TRIS with a probe tip sonicator for 30 s at 20\% intensity. 100 \(\mu L\) of optode cocktail formulation was injected into the PBS/PEG-Lipid or HEPES/TRIS/PEG-Lipid solution under probe tip sonication (3 min, 20\% intensity). The resultant nanosensor solution was filtered with a 0.8 \(\mu m\) syringe filter to remove excess polymer (Pall Corporation, Port Washington, NY, USA).

3.4.2.3 Synthesis of Calcium Optode Cocktail

137 mmol/kg CaI VI, 59 mmol/kg NaBARF, and 28 mmol/kg of either ChII or ChVII in PVC/BEHS (1:2 by mass), with a total of mass of 62 mg, were dissolved in 500 \(\mu L\) THF, followed by dilution with 500 \(\mu L\) DCM.

3.4.2.4 Synthesis of Calcium Nanosensor

1.25 mg PEG-Lipid (50 \(\mu L\) of a 25 mg/mL solution in chloroform) was dried in a 4-dram scintillation vial and then resuspended in 4 mL of HEPES/TRIS solution (buffered at pH=7.4) with a probe tip sonicator for 30 s at 20\% intensity. 100 \(\mu L\) of optode cocktail formulation was injected into the HEPES/TRIS/PEG-Lipid solution under probe tip sonication (3 min, 20\% intensity). The resultant nanosensor solution was filtered with a 0.8 \(\mu m\) syringe filter to remove excess polymer (Pall Corporation, Port Washington, NY, USA).

3.4.3 Procedures

3.4.3.1 Dual-Indicator Methods

Separate optode cocktails were prepared with either ChII or ChVII. For the ‘mixed nanosensor’ method, separate nanosensor batches were synthesized from each optode cocktail and then mixed together in the desired ratio. For the ‘mixed optode’ method, the two optode cocktails were first mixed together in the desired ratio and a nanosensor batch was synthesized with the optode cocktail mixture.
3.4.3.2 Nanosensor Response Characterization

Analyte solutions were prepared at double the test concentration in either HEPES/TRIS (buffered at pH = 7.4) or PBS to match the nanosensor medium. 100 μL of nanosensors and 100 μL of analyte solution were mixed in each well of a row in a 96 well plate to obtain a mixture of nanosensors and analyte at the desired analyte concentration. One well in each row also contained 0.1N sodium hydroxide (NaOH) and one contained 0.1 N hydrochloric acid (HCl) to obtain the maximum and minimum fluorescence response of the sensor. A Synergy H1 microplate reader (BioTek, Winooski, Vermont, USA) was used to collect fluorescence data. During pH indicator screening, absorbance spectra were collected from 300-700 nm with 10 nm resolution. Emission spectra were collected from 30 nm above the excitation peak to 700 nm with a resolution between 2 and 5 nm. Response curves were generated by collecting fluorescence intensity with an excitation of 660 nm and an emission of 700 nm and normalizing all values between the response to 0.1 N HCl and 0.1 N NaOH. The data was then fit to a four-parameter logistic response curve with Prism 7 software (GraphPad software Inc.) to determine the Log of the EC\textsubscript{50} (LogEC\textsubscript{50}). In this work, the linear range is defined as the range on the x-axis (log transform of concentration in molar) where a tangent line from the LogEC\textsubscript{50} deviates less than 5% from the sigmoidal response curve.

Log(K\textsubscript{overall, apparent}) was determined by fitting each dose-response curve to Equation 3.1 with Ind\textsubscript{T} = 16 mmol/kg, R\textsubscript{T} = 16 mmol/kg (due to the electroneutrality condition), L\textsubscript{T} = 307 mmol/kg, and n = z = 1. The value α was then set to equal the normalized response of the sensor at each value of a\textsubscript{i}/a\textsubscript{H} to calculate Log(K\textsubscript{overall, apparent}). The derived equation for IBOS response with two indicators (Equation 3.4) could not be used because α\textsubscript{1} and α\textsubscript{2} are unknown quantities due to spectral overlap.

3.4.3.3 Determination of Lithium Content in Real Sample

Two capsules of lithium orotate (Nutraceutical Corporation, 5 mg/capsule) were dissolved in 40 mL HEPES/TRIS. Insoluble species were separated with an 8 μm syringe filter. A series of five dilutions were made from this solution, each at one tenth the concentration of the previous one. A dose/response curve was generated by mixing nanosensors with the series of dilutions along with an acid and base reading. The same sample of nanosensors was also used to generate a dose/response curve with a series of dilutions from a lithium solution of known concentrations.
Data points were collected in triplicate and by following the methodology described in Section 3.3.2 and this data was fit to the data obtained with the calibration curve to determine the concentration. The concentration of unknown dilutions could then be determined, leading to a simple calculation for the lithium content in one capsule.

3.4.3.4 Statistical Analysis

LogEC$_{50}$ values were determined from a curve fit of 27 data points (9 levels with 3 triplicates) using Prism 7 software (GraphPad Software Inc.). Statistical differences in best-fit LogEC$_{50}$ values between samples were determined using a two-way t-test with $P$ value less than 0.05 considered significant.

3.5 Results and Discussion

3.5.1 Dual-Indicator Lithium Nanosensors

We tested dual-pH indicator range control and extension with lithium-sensitive ionophore-based nanosensors. After screening the chromoionophore series of pH indicators, chromoionophore II (ChII) and chromoionophore VII (ChVII) were chosen because they have overlapping absorbance and emission peaks of 660 and 700 nm (Figures B.4 and B.5) and partially overlapping linear ranges. While overlapping spectra is not necessary for this method, it simplifies experimental data collection. ChVII deprotonates within a lower analyte range in the context of lithium-selective nanosensors and so it is defined as indicator 1 (Ind1) while ChII is defined as indicator 2 (Ind2). Therefore, $\theta = 1$ corresponds to 100% ChVII and 0% ChII.

Experimental results from the mixed optode method confirm that increasing $\theta$ leads to the response range of the sensors shifting toward lower analyte concentrations (Figure 3.5). The LogEC$_{50}$ of each composition is indicated as the center line of each box in Figure 3.5B, and the LogEC$_{50}$ of the nanosensors was determined to change significantly with each shift of $\theta = 0.1$, except for between $\theta = 0.5$ and $\theta = 0.6$ and between $\theta = 0.9$ and $\theta = 1$. The mixed nanosensor method experiments showed the same trend (Figure B.6), and each shift of $\theta = 0.1$ produced a significantly different LogEC$_{50}$, except for the shift between $\theta = 0.7$ and $\theta = 0.8$. Figure 3.6A shows fine tuning of the LogEC$_{50}$ with pH indicator composition in both the mixed optode and mixed nanosensor methods. The mixed nanosensor method closely follows the predicted LogEC$_{50}$
from theory while the mixed optode method deviates more from the expected trend. Increased deviation is expected since an extra mixing step is necessary for the mixed optode method during synthesis, introducing more chance for error. Figure 3.6B shows extension of the linear range with both methods. Though the theoretical $\text{LogEC}_{50}$ of either single pH indicator sensor was set to match experimental results, Equation 3.4 predicted a shallower hill slope and therefore a wider linear range than was observed experimentally, as shown in Figure 3.6B. This is a limitation of the theory that explains why the linear range was consistently lower than expected at every $\theta$ value. However, the percent increase of linear range achieved with dual-pH indicator sensors over a single-pH indicator sensor often exceeded what was expected from theory with both methods (Figure B.7). Whereas theory predicted a maximum linear range extension over $\theta = 0$ of 40% with the mixed optode method and 23% with the mixed nanosensor method, experimental results showed improvements of 47% and 40%, respectively. Linear range extension with this method comes at the expense of sensitivity. This loss of raw sensitivity can be reclaimed with the use of brighter dyes which will result in steeper fluorescence response curves from pH indicator deprotonation.

Figure 3.5 Mixed optode lithium-sensitive IBOS response. (A) Normalized fluorescence dose-response curves. $\theta$ increases from 0 to 1 as the color shifts from blue to pink. (B) The linear range and $\text{LogEC}_{50}$ shifts toward lower concentrations as ChII is replaced with ChVII.

Since the linear range span of both single-pH indicator sensors are roughly the same, the brighter intensity of ChVII dictates that a higher composition of ChII in dual-indicator sensors is
necessary for maximum range extension. Theoretical predictions indicate the maximum range span should be obtained at $\theta = 0.2$ and that compositions with more ChVII should generally have wider ranges than compositions with more ChII. This trend was observed with the mixed nanosensor method but the mixed optode method showed better range extension with higher compositions of ChII. Again, this deviation with the mixed optode method is plausible due to an additional mixing step during synthesis. Regardless, both methods consistently produced sensors with a wider linear range than possible with either single-pH indicator sensor.

The apparent overall equilibrium constant for sensor response, $K_{\text{overall, apparent}}$, was also determined by fitting dose-response curves to Equation 3.1. The data fit methodology is detailed above in Section 3.3.2. Both the mixed optode and mixed nanosensor methods show $\log(K_{\text{overall, apparent}})$ changing directly proportional to $\theta$ (Figure B.8). With bulk optodes, the composition in the film is easily controlled, but it is not clear that components are incorporated into nanosensors in the same ratio they exist in the optode cocktail solution. Further analysis of $K_{\text{overall, apparent}}$ has limited value since the component composition of nanosensors is unknown.

![Figure 3.6 Control of LogEC50 with sensor composition. More deviation from theory observed with the mixed optode method than with the mixed nanosensor method.](image)

ChII and ChVII were the best candidates that we found in our screening of 11 chromoionophores due to their overlapping absorbance and emission ranges and overlapping linear ranges, but other pH indicators with wider $pK_a$ separation may combine for a dual-pH indicator
ion sensor for an additionally extended linear range. Furthermore, use of three or more pH indicators in IBOS could lead to an ion sensor with greatly expanded range and is the subject of further study. Experimental results and theoretical predictions indicate that the mixed nanosensor method is preferable for fine-tuning of the LogEC$_{50}$ while the mixed optode method is preferable for maximal range extension.

3.5.2 Single-Indicator Control

To verify that LogEC$_{50}$ control is due to the combined effect of two pH-indicators, rather than from changing a single indicator concentration in the sensor formulation, a series of nanosensors were formulated with ChVII as the only indicator. The ChVII concentration was ranged from its equivalent concentration from $\theta = 0$ to $\theta = 1$ with the mixed optode method. This experiment shows that the single-indicator composition affects only the brightness of the sensors and does not affect the LogEC$_{50}$ (Figure B.9). This observation is not in agreement with Equation 3.1, which predicts that indicator concentration should affect the dynamic range of the sensor. One possible explanation is that the composition of the nanosensors may differ from the composition of the optode cocktail solution, as mentioned above in Section 3.5.1. Furthermore, changing the concentration of indicator in the optode cocktail solution may actively change the concentration of other components that get incorporated into nanoparticles during nanosensor synthesis due to the charge of the indicator and the assumed electroneutrality condition.

3.5.3 Particle Sizing

It is known that nanosensor size can affect the ratio of ionic partition coefficients in IBOS and can therefore affect the LogEC$_{50}$. With dynamic light scattering we determined the mean particle diameter to be 140 nm with a polydispersity of 0.23. The polydispersity is small enough that nanosensor size should be a negligible factor towards response. Filtering nanosensors with a 0.8 micron filter also ensured no large aggregates existed that would skew dynamic light scattering results.

3.5.4 Selectivity and Real Sample Testing

We demonstrated that dual-pH indicator sensors show good selectivity to lithium over potentially interfering cations (Figure B.10) and we determined that lithium nanosensor response
can be controlled with the mixed optode method, even with a high background of sodium as with phosphate-buffered saline (Figure B.11). Additionally, we analyzed the lithium content in a capsule of lithium orotate, an over-the-counter drug used as a mood stabilizer. Lithium-selective nanosensors at $\theta = 0.5$ determined the soluble lithium content of one capsule of lithium orotate to be 4.9 mg, which is in good agreement with the lithium content reported by the manufacturer, 5 mg (Figure B.12).

### 3.5.5 Dual-Indicator Calcium Nanosensors

As IBOS are modular sensors, the dual-pH indicator approach should function for any similar system – demonstrated in the Supporting Information with calcium selective nanosensors (Figures B.12 and B.13). In the context of calcium-selective nanosensors, the LogEC$_{50}$ of ChII and ChVII sensors were closer to each other than they were in the context of lithium-selective nanosensors, limiting the ability for range extension from dual-pH indicator composition. Regardless, the dose/response trend for both the mixed optode method (Figure B.13) and mixed nanosensor method (Figure B.14) show that the response range can be controlled with dual-pH indicator use. Tuning of the LogEC$_{50}$ (Figure B.15) with calcium-selective nanosensors demonstrates the generalizability of this technique in IBOS. Dual-pH indicator calcium-selective nanosensors also showed an extended linear range over single-pH indicator nanosensors (Figure B.16).

### 3.6 Conclusion

We have demonstrated a facile method for finely tuning the response and extending the linear range of ionophore-based optical nanosensors. This method can easily be applied toward the detection of various ions by selecting an appropriate ionophore for the sensor formulation. The requirements for dual-pH indicator use are that the two dyes have sufficiently different pK$_a$s in the hydrophobic phase of a given sensor, but not by so much that a biphasic response is obtained. While data analysis is more straightforward when the two indicators have overlapping excitation and emission peaks, the number of potential indicator combinations is greatly expanded when considering all indicators regardless of matching peaks. The use of multiple indicators during the design of optical sensors can produce a class of ion sensors with a tunable LogEC$_{50}$ so that the sensor can be most sensitive at the desired analyte level for a given application. The realization of
a set of pH indicators matching the criteria outlined above and spanning a wide range of \( pK_a \)s theoretically could produce IBOS for a variety of target ions with a substantially wider linear ranged than possible with current, single pH indicator sensors.

### 3.7 Abbreviations

ChII, Chromoionophore II; ChVII, Chromoionophore VII; Ind1, Indicator 1; Ind2, Indicator 2; ULR, upper linear range; LLR, lower linear range; L, analyte-binding ligand; R-, charge-balancing additive; \( I^+ \), analyte

### 3.8 References Cited

8. F.M. Abdel-Haleem, Highly selective thiourea-based bulk optode for determination of salicylate in spiked urine samples, Aspirin (R) and Aspocid (R), Sens. Actuators B-Chem. 233 (2016) 257–262.
tomographic fluorescence imaging of pH microenvironments in microbial biofilms by use

12. T.R. Neu, J.R. Lawrence, Innovative techniques, sensors, and approaches for imaging

16150.

14. V. Singh, A.K. Mishra, Green and cost-effective fluorescent carbon nanoparticles for the
selective and sensitive detection of iron (III) ions in aqueous solution: mechanistic insights


16. K.J. Cash, H.A. Clark, Phosphorescent nanosensors for in vivo tracking of histamine levels,

17. B. Awqatty, S. Samaddar, K.J. Cash, H.A. Clark, J.M. Dubach, Fluorescent sensors for the
basic metabolic panel enable measurement with a smart phone device over the

2709–2728.

19. M. Quaranta, S.M. Borisov, I. Klimant, Indicators for optical oxygen sensors, Bioanal.

20. P. Buhlmann, E. Pretsch, E. Bakker, Carrier-based ion-selective electrodes and bulk
optodes. 2. Ionophores for potentiometric and optical sensors, Chem. Rev. 98 (1998) 1593–
1688.

21. X.J. Xie, Renovating the chromoionophores and detection modes in carrier-based ion-

22. M. Brasuel, R. Kopelman, T.J. Miller, R. Tjalkens, M.A. Philbert, Fluorescent nanosensors
for intracellular chemical analysis: decyl methacrylate liquid polymer matrix and ion
exchange-based potassium PEBBLE sensors with real-time application to viable rat C6

23. L.X. Xie, Y. Qin, H.Y. Chen, Direct fluorescent measurement of blood potassium with
2617–2622.


11932.


31. V.M. Chauhan, G.R. Burnett, J.W. Aylott, Dual-fluorophore ratiometric pH nanosensor with tuneable pK(a) and extended dynamic range, Analyst 136 (2011) 1799–1801.


CHAPTER 4

AN IONOPHORE-BASED PERSISTENT LUMINESCENT ‘GLOW SENSOR’ FOR SODIUM DETECTION

A paper submitted to RSC Advances

Mark S. Ferris, Madeline Behr, and Kevin J. Cash

Figure 4.1 Graphical Abstract. The brightness of the ‘glow’ emanating from the sensor decreases as the sodium concentration increases

4.1 Abstract

Optical sensors have numerous optimal features such as low invasiveness, miniaturizability, biocompatibility, and ease of signal transduction. Recently, there has been a strong research focus on using phosphorescent readout mechanisms, specifically from long-lifetime phosphorescent or ‘persistent luminescence’ particles, for *in vitro* and *in vivo* sensors. Phosphorescent readouts can avoid cellular autofluorescence and light scattering during biological monitoring, leading to an improved signal-to-noise ratio over a more traditional fluorescence readout. In this study, we show for the first time an ionophore-based optical bulk optode sensor

---

1 Submitted to *RSC Advances*
2 Primary author and editor, Department of Chemical and Biological Engineering, Colorado School of Mines
3 Co-author, undergraduate researcher, Department of Chemical and Biological Engineering, Colorado School of Mines
4 Co-author, Assistant Professor, Department of Chemical and Biological Engineering, Colorado School of Mines
that utilizes persistent luminescence microparticles for ion detection. To achieve this, we combined long-lifetime strontium aluminate-based ‘glow-in-the-dark’ microparticles with a non-fluorescent pH-responsive dye in a hydrophobic plasticized polymer membrane along with traditional ionophore-based optical sensor components to create a phosphorescent ‘Glow Sensor’. The non-fluorescent pH indicator dye gates the strontium aluminate luminescence signal so that it decreases in magnitude with increased sodium concentration. We characterized the Glow Sensor in terms of emission lifetime, dynamic range, response time, reversibility, selectivity, and stability.

4.2 Introduction

Optochemical sensors, where a target analyte triggers an optically detectable change in the device through absorbance or fluorescence readings, have advantages over electrochemical techniques that include low-invasiveness, spatial imaging, spectral multiplexing, and offline monitoring that make them ideal for many advanced biological applications. Of the many classifications of optochemical sensors, polymer-based ‘bulk optode’ sensors have proven to be robust devices capable of detecting analytes such as creatinine, sodium, potassium, calcium, lead, and lithium. Bulk optode sensors have a low cost of manufacturing and can be easily miniaturized into polymeric nanosensors for spatially-resolved sensing and imaging with low invasiveness since they remove the need for surgical implantation.

For cation sensing, ionophore-based optical sensors (IBOS) offer a facile and tunable approach that can be utilized in both polymer-based bulk optodes and bulk optode-based nanosensors. IBOS typically consist of an ion-binding molecule (ionophore) for analyte recognition, a fluorescent pH indicator, and a charge-balancing additive, all contained within a hydrophobic plasticized polymer matrix. The sensor response is based on well-established ion exchange theory that involves extracting the target cation from the sample to the sensor matrix to bind with the ionophore, causing deprotonation of the pH indicator and therefore a change in the sensor optical properties. The target analyte is determined by the choice of ionophore and the dynamic range of the sensor can be finely tuned to match a chosen application by using multiple pH indicators with appropriately spaced pKₘₐₚs.
Among the remaining issues to be addressed with polymer-based bulk optode sensors and nanosensors are signal attenuation and interference from biological autofluorescence and light scattering. Typically, organic fluorescent indicators have served as the pH indicators in these sensors, but organic indicators tend to suffer from poor tissue penetration and photobleaching from repeated stimulation with light. As an alternative to organic fluorescent indicating molecules, a few reports have demonstrated that brighter and more stable indicators can be used for signal transduction. These indicators tend to be chemically inert toward target molecules, but their signal can be gated by a non-fluorescent pH sensitive dye with a wide absorption spectrum, such as blueberry-C6-ester-652 (Blueberry dye) a concept demonstrated by Sahari et al.\textsuperscript{16} with quantum dots. In this setting, the target cation binds to the ionophore, causing deprotonation of the Blueberry dye, which in turn gates the luminescence of the quantum dot signaling element. This concept has been demonstrated with quantum dots\textsuperscript{17-19} and has been extended to carbon dots.\textsuperscript{20}

Signal interference from biological autofluorescence and light scattering has been addressed in other classes of sensors\textsuperscript{21-25} and in imaging techniques\textsuperscript{26-29} by using phosphorescent materials for signal transduction. Fluorescent indicators and background autofluorescence in biological samples have emission lifetimes in the range of $10^{-9} – 10^{-7}$ seconds,\textsuperscript{30} while phosphorescent indicators have longer emission lifetimes, ranging anywhere from microseconds to many hours.\textsuperscript{31} Therefore, by using a phosphorescent indicator and programming a delay between sensor excitation and emission collection, signal transduction from the sensor can avoid these background signals. Among a variety of materials that can display phosphorescent emission, of particular interest is ‘long-lifetime phosphorescent’ or ‘persistent luminescence’ particles, which come in the form of an ion-doped inorganic matrix and display exceptionally long luminescent lifetimes, typically on the order of hours or even days.\textsuperscript{32} In 2011, Wu et. al. developed an assay for α-Fetoprotein based on the modulation of Fluorescence Resonance Energy Transfer (FRET) between persistent luminescence particles and gold nanoparticle conjugates.\textsuperscript{33} Since then, persistent luminescence particles have been used as reporters in lateral flow assays,\textsuperscript{34} in immunoassays,\textsuperscript{35} for tumor imaging\textsuperscript{26} and therapy,\textsuperscript{36, 37} and in sensors for select
analytes such as ascorbic acid, avidin, 2,4,6- DNA molecular hybrids, TNP, and cyanide through a variety of mechanisms.

Figure 4.2 Glow Sensor mechanism. A charge balancing additive holds the Blueberry dye in a protonated state in the absence of sodium. Sodium from the test sample is extracted into the sensor core where it binds with the ionophore. Charged sodium ions force the deprotonation of the Blueberry dye to maintain electroneutrality in the organic phase. When deprotonated, the Blueberry dye absorbs photon emission from the persistent luminescence microparticles at a higher rate, minimizing the observed phosphorescence.

In this paper, we formulate and characterize a persistent luminescence ‘Glow Sensor’ that is, to the best of our knowledge, the first use of persistent-luminescence particles as the signal transduction element in ionophore-based optical sensors. We use bulk optode sensors for sodium detection as a proof-of-concept instead of nanosensors due to the size of the persistent luminescence microparticles (15-35 μm) being larger than typical nanosensor diameters (~180 nm), although the sensor mechanisms are the same. The sensor incorporates alkaline rare earth metal silicate-aluminate oxide europium doped microparticles and Blueberry dye into a plasticized polymer matrix along with the remaining IBOS components to create a sodium-selective sensor via the IBOS mechanism described earlier, but with the added step of Blueberry dye gating the persistent
luminescence microparticle signal (see Figure 4.2). The Glow Sensor is reversible and highly selective against other potentially interfering cations such as potassium and lithium and has a steady response range over 14 days.

### 4.3 Results and Description

To characterize response to sodium, we exposed Glow Sensor spots (Figure 4.3B, inset images) to a variety of solutions with different NaCl concentrations while recording luminescent emission from the spots with a fluorescence microscope. An automated program was run to open and close a shutter between the excitation source and the sample three times over the course of 150 seconds. We also exposed the Glow Sensor spots to 0.1N HCl and 0.1N NaOH to test the maximum and minimum response of the spots (i.e. fully protonated and deprotonated states of the Blueberry dye). Initially, four Glow Sensor spots were analyzed for Na\(^+\) responsiveness. The luminescence intensity emitted from the four spots during this automated program under different test conditions is shown in the left column of Figure C.1. This figure also shows that these Glow Sensor components are well optimized since the fluorescence of the spots under the lowest NaCl test condition (10\(^{-7}\) M Na) are roughly the same as the fluorescence of the spots when the Blueberry dye is fully protonated under acidic conditions. Furthermore, when the spots are fully deprotonated under basic conditions, they have a luminescence only slightly above the background noise of the system (Figure C.2). The Glow Sensor signal, when exposed to HEPES/TRIS buffer, persists above background levels on the minute scale (Figure C.3).

Figure 4.3A shows the phosphorescent decay curves for spot A (see the right column of Figure C.1 for the spots B, C, and D phosphorescent decay curves). The error bars in Figure 4.3A show the variability in the three decay curves collected for each test condition. Most error bars are smaller than the size of the data point symbol and therefore are not visible, indicating that the decay dynamics are consistent when the glow sensor is fully excited. For signal transduction, we integrated under the curve of each phosphorescent decay between one and two seconds after blocking excitation, shown as the average of the normalized response of all four spots in Figure C.2B. The Glow Sensors respond to Na\(^+\) with a linear range from 2.4 mM to 414 mM Na\(^+\) and an \(a_{0.5}\) of 52 mM Na\(^+\), where \(a_{0.5}\) represents the Na\(^+\) concentration at the midpoint of the sensor’s dynamic range and the
concentration of maximum sensitivity. The Glow Sensor is more suitable for physiological monitoring than for trace detection, with a linear range encompassing the physiologically relevant concentrations of Na⁺ (135-150 mM).\textsuperscript{38}

![Figure 4.3 Signal analysis for Glow Sensor spots. The Blueberry dye in the sensor turns from clear to blue upon deprotonation, increasing the absorbance of the glow from the persistence luminescence microparticles, thereby decreasing the amount of measured luminescence in basic solution. Due to the presence of sodium ionophore, the addition of sodium causes the same deprotonation of the Blueberry dye. (A) Phosphorescent decay curves (n=3) from a single sensor spot averaged together at increasing sodium concentrations. Dotted lines show area of integration used to calculate sensor response. (B) Dose/response curve showing average response to sodium of the four individual sensor spots. This shows that the sensor phosphorescence decreases as a function of sodium concentration. (B, inset) images of the phosphorescent spots under acidic (bright) and basic (dim) conditions.

To confirm the response mechanism, we made two control optodes - one lacking the Blueberry dye, and one lacking the persistent luminescence microparticles. With no Blueberry dye, the spots have a luminescence on the same order of magnitude as the Glow Sensor spots (See Figure C.4) but have no response to sodium (Figure C.5, left panel) as expected with no optical gating in the system. With no persistent luminescence microparticles, the optode has no phosphorescence, as expected (see Figure C.5, right panel). Only with both Blueberry dye and persistent luminescence microparticles present in the optode (along with the remaining sensing components) does the optode shows a
distinct decrease in luminescence and phosphorescence in response to increasing Na\(^+\) concentration. Energy coupling is possible between Blueberry dye and persistent luminescence microparticles because the emission phosphorescent peak of the persistent luminescence microparticles overlaps with the wide absorbance spectrum of the Blueberry dye, as shown in Figure C.6. Figure C.6 also shows that optode spots made without Blueberry dye do not change in their phosphorescence spectra between acidic and basic conditions, further confirming that Blueberry dye is necessary to change the phosphorescence intensity of the Glow Sensor spots. Energy coupling is likely due to either resonance transfer or the inner filter effect.

We also characterized the Glow Sensor in terms of response time, reversibility, selectivity, and stability (Figure 4.4). The response time was investigated by equilibrating a single spot in 0 mM NaCl, then switching to a 100 mM NaCl solution and beginning an extended shutter program that alternately opens and closes the shutter for 15 minutes (see Figure C.7 for full luminescence intensity over the course of this experiment). The sensor signal was plotted against time and fit with a one phase exponential decay model to determine a \(t_{95}\) (i.e. the time it takes to reach 95% of equilibrium) of 9.6 minutes (Figure 4.4A). While this response is slower than ideal for some biological applications, it is controlled by diffusion of the analyte into the optode membrane. This means that the response time will improve drastically with miniaturization into nanosensors – the subject of future study. A key advantage of the equilibrium-based mechanism of IBOS is that it leads to a reversible sensor which can be used to monitor analyte fluctuations in either direction. We tested the reversibility of four new Glow Sensor spots by exposing each to alternating solutions of 0 and 100 mM NaCl for a total of 10 measurements (steps). The sensor signal reliably decreased in the presence of Na\(^+\) and increased in 0 mM NaCl solution for 5 cycles, shown in Figure 4.4B. The selectivity of the Glow Sensor toward the potentially interfering cations K\(^+\) and Li\(^+\) were also investigated, resulting in selectivity coefficients of -2.2 and -3.3, respectively. However, due to the minimal response to the off-target analytes (Figure 4.4C), these are poorly constrained. The high selectivity of the Glow Sensor may be attributable to the binding strength of the ionophore, though the selectivity appears to be better than other ionophore-based sensors utilizing the same ionophore (NaIX).\(^{20}\) Finally, the stability of the Glow Sensor was investigated by analyzing the
response of four spots over a period of two weeks (Figure 4.4D). Minimal drift of the sensor response was as seen in this time frame. A Welch’s t-test concluded that a significant decrease in the $\alpha_{0.5}$ occurred only between day 2 and 3 (see Figure C.8).

![Graphs](image)

Figure 4.4 Full glow sensor characterization. (A) Sensor signal after addition of 100 mM sodium. Response time of the sensor is 9.6 minutes ($T_{95}$). (B) Reversibility of the sensor analyzed by exposing sensor to alternating solutions of 0 mM and 100 mM sodium. (C) The sensor is highly selective against the potentially interfering ions Li$^+$ and K$^+$. (D) The sensor response to sodium is stable over 14 days.

The brightness of the Glow Sensor spots during analysis varied considerably from spot to spot throughout this work, likely because the size of the spots were not well controlled due to two factors. Tetrahydrofuran (THF), the medium of the optode cocktail solution, is highly volatile, leading to difficulty in reproducibly using small volumes to fabricate the spots (2 μL for all spots in this work). Also, the persistent luminescence
microparticles used were 15-35 μm in size and were not small enough to be colloidally stable in solution, making the optode cocktail/microparticle ratio uneven during the spotting process. However, when the response of each sensor spot is normalized between the response to 10^{-7} M Na and 0.1N NaOH, the difference between the spots is minimal (see Figure C.9). Furthermore, the datasets from collections of spots used for different experiments (Figure 4.2B, 4.3C, 4.3D) have dose/response curves that are consistent with each other (see Figure C.10).

While the optode spots in this work serve as a crucial proof-of-concept for persistent luminescence detection, the mechanism will more useful for biological sensing and imaging with miniaturization into polymer-based nanosensors, the subject of future study. This requires nanosized persistent luminescence particles, which are not available commercially but have several reported synthesis routes. Nanosized persistent luminescent particles will have shorter emission lifetimes, but should still be long enough to avoid background noise from biological autofluorescence and scattering. An integration time of 1-2 seconds post-excitation was arbitrarily chosen in this work, but can be shortened to accommodate shorter lifetime persistent luminescence nanoparticles while still remaining long enough to avoid background signal.

4.4 Conclusions

In this work, we developed an ion selective ‘Glow Sensor’ by using persistent luminescence microparticles as an optical reporter with an ionophore-based detection mechanism for the first time. We show that the persistent luminescence microparticles couple with a pH-sensitive Blueberry dye so that the luminescence of persistent luminescent microparticles is increasingly quenched as Blueberry dye deprotonates in response to increasing Na^+ concentrations. The optode spots are sensitive to sodium and highly selective against potentially interfering K^+ and Li^+ ions, but the selectivity can easily be changed toward a different ion target by choosing a different ionophore. The Glow Sensor is also reversible and the response is stable for 14 days. The response time for the sensor is 10 minutes which is less than ideal for most advanced applications, but this can be drastically improved with miniaturization of the spots into nanosensors, which is the focus of ongoing work.
4.5 References


35. Wu, B. Y.; Yan, X. P., Bioconjugated persistent luminescence nanoparticles for Foster resonance energy transfer immunoassay of prostate specific antigen in serum and cell extracts without in situ excitation. Chemical Communications 2015, 51 (18), 3903-3906.

36. Su, F. X.; Zhao, X.; Dai, C.; Li, Y. J.; Yang, C. X.; Yan, X. P., A multifunctional persistent luminescent nanoprobe for imaging guided dual-stimulus responsive and
triple-synergistic therapy of drug resistant tumor cells. Chemical Communications 2019, 55 (36), 5283-5286.


42. Srivastava, B. B.; Kuang, A. X.; Mao, Y. B., Persistent luminescent sub-10 nm Cr doped ZnGa2O4 nanoparticles by a biphasic synthesis route. Chemical Communications 2015, 51 (34), 7372-7375.
CHAPTER 5

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS FOR FUTURE WORK

5.1 Summary and Conclusions

This work presents an advancement in bulk optode and polymeric nanosensor technology in three separate research projects. A summary of the key findings from each chapter is listed below.

5.1.2 Summary and Conclusions from Chapter 2

- A biotin/avidin-based approach for forming enzyme-linked polymeric nanosensor is proposed.
- Fe₃O₄NPs are incorporated into polymeric nanosensors, giving magnetic control over sensor separation, but batch-to-batch nanoparticle loading is inconsistent.
- A control experiment demonstrates successful synthesis of nanosensor/enzyme conjugates.
- Nanosensor response characteristics are controlled through multiple avenues during synthesis by altering the final ratio of enzyme/nanosensor.

5.1.2 Summary and Conclusions from Chapter 3

- Dual pH indicator use is proposed to control the response of ionophore-based optical nanosensors.
- Adjusting the ratio of the two indicators is shown to control the sensor LogEC₅₀.
- Dual-indicator use is shown to extend the total dynamic range span by 47%.
- A theoretical response equation is derived for a dual pH indicator nanosensor.
- Dual-indicator coupled equilibria affects deprotonation and sensor response.

5.1.3 Summary and Conclusions from Chapter 4

- Persistent luminescent microparticles are proposed for use as optical transducers with an ionophore-based response mechanism.
- A bulk optode sensor incorporating persistent luminescent microparticles, sodium ionophore, blueberry dye, and a charge balancing additive is constructed as a proof-of-concept.
- Control experiments suggest coupling between the static phosphorescent microparticles and the optically silent, pH sensitive Blueberry dye.
- The persistent luminescence of the sensor is shown to last on the minute scale.
- Signal transduction comes from an integration of the phosphorescent decay curves from the persistent luminescence microparticles.
- The sensor is shown to be reversible, highly selective against potentially interfering cations, and have a stable response range for up to 14 days.

5.2 Recommendations for Future Research

5.2.1 Recommendations for Future Work Based on Chapter 2

Chapter 2 demonstrates a method for enzyme conjugation on the surface of polymeric nanosensors that gives control over the response characteristics. The main focus for future work should be improving the batch-to-batch reproducibility of the Fe$_3$O$_4$NP loading in polymeric nanosensors – the key weakness limiting broader application. If Fe$_3$O$_4$NPs can be loaded consistently, then comparison of the response characteristics between batches will become much more valuable. If consistent loading can be achieved, it will open up several avenues of follow up investigations. One possible way to achieve this goal is functionalization of the Fe$_3$O$_4$NP to make them more stable in the environment of the nanoparticle core, or by covalently conjugating the Fe$_3$O$_4$NPs to the polymeric synthesis before use in synthesis of the optode cocktail solution. With a more consistent synthesis method, strategies for building multilayer enzyme structures on nanosensors could be explored through repeated conjugation and wash steps alternating between avidin and BGOx.

In chapter 2, response of the EliNS were characterized by analyzing the slope of the sensor response after the addition of glucose in a small volume. As explained in Section 2.4, this is a convenient way of analyzing and developing the sensors, but does not showcase the continuous monitoring ability of the sensors. Instead of analyzing the slope as the sensors deplete all oxygen in the well, it would be ideal for them to operate in a setting where they only partially deplete the local oxygen and therefore give a steady state signal that is somewhere between its minimum and maximum and will increase or decrease based on real time glucose concentrations. In order to achieve this, a bulk oxygen supply must continually replenish oxygen that is consumed by the
enzyme. One possibility is sealing the sensors in a microdialysis and adhering to the bottom of a well plate so that the sensors are limited to one area of the well while analyte can diffuse in and out of the microdialysis tube. Analyte solutions can also be changed without affecting the sensor concentration. This method was effective for analyzing EnzNS, as shown in Figure 5.1. Each incremental addition of glucose caused a rise in the sensor signal that remained steady for 6 minutes at a time. However, the method was much less effective with EliNS, likely due to the enzyme activity being too low. If the enzyme activity of ELiNS can be increased, such as through multilayer structures as described earlier in this section, then they should be analyzed in a continuous, ‘steady-state’ manner as described here.

Figure 5.1 EnzNS under ‘steady-state’ operation. Sensors are sealed in a microdialysis tube, adhered to the bottom of a well plate, and HEPES/TRIS solution is added on top. (A) A 3x7 area scan is taken of the center of the well every minute for 6 minutes before the glucose solution is changed and the scan is repeated. (B) The 7 points of interest from the brightest (middle) row of the area scan are processed into a dose/response curve.

Theoretical response models for enzyme-based mechanisms are limited to electrochemical sensors in the literature. Enzyme-based response is derived more from kinetics and transport phenomena and doesn’t rely on thermodynamic equilibria like ionophore-based response. This makes the difference in shape between planar, macrosized electrochemical sensors and the spherical nanosensors a more significant factor for a derived response equation. An important question for the response of enzyme-based polymeric nanosensors is whether their response can be calculated from diffusion boundary conditions around a single particle or from the collection of
particles in a defined volume as a single unit. Presumably, there exists some critical concentration above which the theoretical response switches from the former to the latter. Future experiments could be aimed at determining this critical concentration.

5.2.2 Recommendations for Future Work Based on Chapter 3

Chapter 3 demonstrates a method for dynamic range control and extension in ionophore-based optical nanosensors by adjusting the ratio of two pH indicators with separated pKₐ values and overlapping excitation and emission spectra. While this work shows extension of the dynamic range by 57%, polymeric nanosensors still have limited ranges compared to their electrochemical counterparts. In the future, use of three or more pH indicators could be explored to further increase the dynamic range of polymeric nanosensors. Use of multiple ionophores could be explored for extension as well, though preliminary experiments show that controlling the single-ionophore composition is sufficient for fine-tuning the logEC₅₀, as shown in Figure 5.2. However, even if not necessary for LogEC₅₀ fine-tuning, dual-ionophore use could allow for range extension without loss of signal strength. Both dual-indicator and dual-ionophore methods should also be extended to non-fluorescent pH indicators such as Blueberry dye so that brighter and more stable signal transducers, such as the persistent luminescence microparticles from chapter 4 and other elements such as quantum dots and carbon dots, can be utilized with this range control method.

Figure 5.2 Dual-ionophore use for dynamic range tuning with (A) mixed optode method and (B) mixed nanosensor method. A single ionophore tuning experiments suggests that the dynamic range can be adjusted without dual-ionophore use, but at the loss of signal-strength.

85
5.2.3 Recommendations for Future Work Based on Chapter 4

Chapter 4 presents an ionophore-based bulk optode film that utilizes persistent luminescence for signal transduction. The sensor has an appropriately long ‘glow’ to utilize for signal transduction that can avoid all background noise as well as excellent selectivity, reversibility, and stability traits. The main weakness is the long response time (~10 minutes), and future work should be aimed at improving this characteristic. The solution is miniaturization of the sensor platform into polymeric nanosensors, which will render the response time to near-instantaneous and allow the device to be used for advanced imaging and \textit{in vivo} applications where the bulk optode film is not suitable. This will require the synthesis of nanosized persistent luminescence particles that are compatible with the plasticized polymer matrix, of which there are several options reported in the literature.\textsuperscript{1-4}

![Graph.png](attachment:Graph.png)

Figure 5.3 (A) The silicon nanocrystal sensor is selective for sodium against potentially interfering cations (B) and is responsive for at least one week.

The Blueberry dye used in this report has an impressive history of being able to gate luminescent from many different indicator elements such as quantum dots,\textsuperscript{5} carbon dots,\textsuperscript{6} and now persistent luminescence microparticles. Silicon nanocrystals\textsuperscript{7} are another possible indicating agent with excellent characteristics in terms of brightness and photostability. We have collected preliminary results demonstrating the validity of using the same sensing scheme shown in Figure
4.2 but replacing the persistent luminescence microparticles with silicon nanocrystals and using polymeric nanosensor instead of bulk optodes. The silicon nanocrystal sensor is selective against potentially interfering cations (Figure 5.3A) and a stable response for up to 1 week (Figure 5.3B). However, the silicon nanocrystal sensor currently is not very bright, likely due to passivation of the silicon nanocrystal fluorescent properties from THF during nanosensor synthesis. Ongoing work is focused on finding a new polymer/plasticizer combination that will allow the nanosensors to be synthesized in toluene, in which they are stable. After that, the sensors will undergo a full characterization similar to what is shown for the Glow Sensor in Figure 4.3.

5.3 Final Thoughts on Polymeric Nanosensor Development

Polymeric nanosensors are a next-generation sensor class that is still in a developmental stage but already has many reported applications where the previous-generation bulk optode membranes and electrochemical sensors fall short. In the near future, a greater emphasis should be placed deploying these sensors for practical applications. So far, most polymeric nanosensor reports are for cation targets and small bio-molecules. Currently, cation-responsive polymeric nanosensors are better candidates for real-world applications than bio-molecule responsive polymeric nanosensors, thought development should still be addressed for both mechanisms. This is because cation-responsive nanosensors can operate in a ‘steady-state’ manner in free solution due to their heavier reliance on thermodynamics instead of transport and kinetic considerations in the response mechanism. The issues with ‘steady-state’ response, as discussed in Section 5.2 need to be addressed before bio-molecule responsive polymeric nanosensors are deployed more for practical application.

While the long-term goal should be in vivo use, a more manageable short-term goal is in vitro applications such as cell-culture analysis. In vitro analysis can make use of many of the advantages that polymeric nanosensors have over bulk optodes such as their ability to create 3D spatial maps before addressing some of the larger burdens associated with in vivo testing. Our lab has used polymeric nanosensors to study O₂ penetration and distribution as well as antibiotic efficacy in biofilms, and are working to deploy cation-responsive polymeric nanosensors next. Multiplexed detection, where several analyte targets are monitored simultaneously with a suite of polymeric nanosensors is also possible in the near future. Multiplexed polymeric nanosensor platforms will benefit from utilizing signaling agents with narrower luminescent peaks to prevent
signal interference across the sensor platform, a concept which has support from the literature. Of particular value will be co-monitoring pH along with cation targets and deconvoluting the dependency of ionophore-based mechanisms on pH. In preparation for future in vivo applications, research should also focus on functionalizing the outside of polymeric nanosensors with specific receptor targets to get the sensor to adhere to certain cell types or cellular structures for more focused monitoring. These types of developments can be demonstrated in vitro with the goal of expanding to in vivo application in the future. Targeted tumor metabolic analysis and non-invasive blood glucose monitoring systems for diabetics are two possible long-term in vivo goals for polymeric nanosensor application.

5.3 References Cited


4. Srivastava, B. B.; Kuang, A. X.; Mao, Y. B., Persistent luminescent sub-10 nm Cr doped ZnGa$_2$O$_4$ nanoparticles by a biphasic synthesis route. *Chemical Communications*, 2015, 51 (34), 7372-7375.


APPENDIX A

SUPPORTING INFORMATION FOR CHAPTER 2

A.1 Supplementary Figures

Figure A.1 Demonstration of enzyme/nanosensor separation scheme. ELiNS and EnzNS undergo three rounds of magnetic separation and purification to remove unbound GOx from the magnetic nanosensors. EnzNS formulations (without avidin) have no attachment between the magnetic nanosensors and enzyme, so all enzyme is removed and the nanosensors lose their responsiveness to glucose. ELiNS formulations (with avidin) contain linkages between enzyme and the magnetic nanosensors, and so they retain their responsiveness to glucose after separation.

Figure A.2 Triplicate data for NSB-A-BGOx sensors with highest enzyme loading (1 mg).
Figure A.3 Kinetic traces for three separate experiments that explored the effect of seven avidin levels on NSB-A-BGOx sensor response. The first experiment explored low levels of avidin (A, B, and C), the second explored intermediate levels of avidin (D, E, and F), and the third explored high levels of avidin (G, H, and I). Low enzyme activity is observed with low amounts of avidin (0.02 and 0.05 mg), and slightly diminished enzyme activity is observed with high avidin amounts (0.88 and 1.32 mg).
Figure A.4 Response to 4mM in the low avidin experiment (A), the intermediate avidin experiment (B), and the high avidin experiment (C). Summary of response to 4 mM glucose across the three experiments, with error bars showing standard deviation between overlapping levels of avidin (0.11 mg and 0.44 mg) tested between the two experiments (D).

Figure A.5 Effect of surface biotinylation on response of NSB-A-BGOx particles. Replica of Figure 4 with a narrow y-axis range in panel B showing that the sensors are still slightly responsive to glucose at 6.3% biotinylation.
Figure A.6 Dose response curve generated by plotting initial slope of kinetic trace vs. glucose concentration for NSB-A-BGOx particles with different amounts of surface biotinylation.

Figure A.7 Dose/response curves for NSB-AGOx sensors.
Figure A.8 TEM image of Fe$_3$O$_4$ nanoparticles in nanosensors. The darker shaded circles show nanosensors with no Fe$_3$O$_4$ nanoparticles encapsulated (blue, top), a low amount of Fe$_3$O$_4$ nanoparticles encapsulated (red, lower left), and a high amount of Fe$_3$O$_4$ nanoparticles encapsulated (green, lower right).
APPENDIX B

SUPPLEMENTARY INFORMATION FOR CHAPTER 3

B.1 Mathematical Derivation

The derivation of the theoretical response of dual-pH indicator nanosensors is obtained by expanding a previously determined derivation for optode response theory.\textsuperscript{1,2} Four equilibrium statements can be written for species in the organic phase: cation exchange between the aqueous and organic phase, analyte complexation with ionophore, and acid dissociation of both indicators, displayed in Equation B.1, where $\theta$ is the mole fraction of the first indicator in the total indicator concentration. The acid dissociation equations are weighted by the charge of the analyte and the fraction of the respective indicator in the total indicator composition.

\begin{align*}
  & zH^+(org) + I^{z+}(aq) \xrightleftharpoons[K^{H^+,I^{z+}}_{\text{exch}}]{K^{H^+,I^{z+}}_{\text{exch}}} zH^+(aq) + I^{z+}(org) \\
  & nL(org) + I^{z+}(org) \xrightleftharpoons[\beta_{LnI^{z+}}]{\beta_{LnI^{z+}}} L_nI^{z+}(org) \\
  & z\theta Ind1H^+(org) \xrightleftharpoons[K_{a1}]{K_{a1}} z\theta Ind1^0(org) + z\theta H^+(org) \\
  & z(1 - \theta) Ind2H^+(org) \xrightleftharpoons[K_{a2}]{K_{a2}} z(1 - \theta) Ind2^0(org) + z(1 - \theta) H^+(org) \quad (B.1)
\end{align*}

From these equations, the equilibrium constants can be expressed as

\begin{align*}
  K^{H^+,I^{z+}}_{\text{exch}} &= \frac{(a_H)^z[I^{z+}]}{(a_I)[H^+]^z} \\
  \beta_{LnI^{z+}} &= \frac{[L_nI^{z+}]}{[I^{z+}][L]^n} \\
  (K_{a1})^{\theta z} &\equiv \frac{[Ind1^0]^{\theta z}[H^+]^{\theta z}}{[Ind1H^+]^{\theta z}} \\
  (K_{a2})^{(1-\theta)z} &\equiv \frac{[Ind2^0]^{(1-\theta)z}[H^+]^{(1-\theta)z}}{[Ind2H^+]^{(1-\theta)z}} \quad (B.2)
\end{align*}

where bracketed terms represent species in the organic phase and ionic species in the aqueous phase are represented by their activities. By combining the expressions in Equation B.1 we obtain
We can now make a statement for the overall exchange constant $K_{\text{Overall}}$, as shown in Equation B.4:

$$\begin{align*}
K_{\text{Overall}} &= K_{\text{exch}}^{H^+,I^z} \beta_{L,T}^{L,T} (K_{a1})^{\theta z} (K_{a2})^{(1-\theta)z} = \\
&= \frac{(a_I)^2 [L]^{n} [L]^n} {(a_I) [H^+]^2 [I^z+]^2 [Ind1H^+]^{\theta z} [Ind2H^+]^{(1-\theta)z} [Ln^+]^{\theta z} [Ind1^0]^{(1-\theta)z} [Ind2^0]^{(1-\theta)z}} 
\end{align*}$$

(B.4)

In order to predict the protonation state of the two indicators based on analyte activity ($a_I$), a series of substitutions must be made. First, the protonation degree of each indicator is defined as

$$\begin{align*}
1 - \alpha_1 &= \frac{[\text{Ind1H}^+]}{\text{Ind1}_T} \\
1 - \alpha_2 &= \frac{[\text{Ind2H}^+]}{\text{Ind2}_T} 
\end{align*}$$

(B.5)

where $\alpha_1$ and $\alpha_2$ are the deprotonation degree of indicators 1 and 2, respectively, and $\text{Ind1}_T$, $\text{Ind2}_T$, and $L_T$ are the total amounts of indicator 1, indicator 2, and ionophore, respectively. They are defined here as the sum of all compounds containing the respective species:

$$\begin{align*}
\text{Ind1}_T &= [\text{Ind1H}^+] + [\text{Ind1}^0] \\
\text{Ind2}_T &= [\text{Ind2H}^+] + [\text{Ind2}^0] \\
L_T &= [L] + n[L_n^+] 
\end{align*}$$

(B.6)

To maintain electroneutrality in the organic phase, the total additive concentration must equal the concentration of positive charges:

$$R_T = [\text{Ind1H}^+] + [\text{Ind2H}^+] + z[L_n^+]$$

(B.7)

The expressions in Equations B.6 and B.7 can be rearranged to

$$[\text{Ind1H}^+] = (1 - \alpha_1) \text{Ind1}_T$$
\[ [\text{Ind2H}^+] = (1 - \alpha_2)\text{Ind2}_T \]

\[ [\text{L}_n\text{I}^{z+}] = \frac{R_T - [\text{Ind1H}^+] - [\text{Ind2H}^+]}{z} \]

\[ [\text{L}] = L_T - n[\text{L}_n\text{I}^{z+}] = L_T - \frac{n}{z}(R_T - [\text{Ind1H}^+] - [\text{Ind2H}^+]) \]  

The expressions in Equations B.5 and B.8 can now be inserted in Equation B.4 to yield

\[
a_1 = \frac{a_H z}{K_{\text{Overall}} z} \left[ \frac{R_T - (1 - \alpha_1)\text{Ind1}_T - (1 - \alpha_2)\text{Ind2}_T}{(L_T - \frac{n}{z}(R_T - (1 - \alpha_1)\text{Ind1}_T - (1 - \alpha_2)\text{Ind2}_T))^n} \right] \left[ \left( \frac{\alpha_1}{1 - \alpha_1} \right)^\theta \left( \frac{\alpha_2}{1 - \alpha_2} \right)^{1 - \theta} \right]^z 
\]

\[ K_{\text{Overall}} = K_{\text{exch}}^{+} \beta \text{L}\text{I}^{+} (K_{a1})^\theta (K_{a2})^{(1 - \theta)z} \]  

(B.9)

Finally, a relationship between \( \alpha_1 \) and \( \alpha_2 \) can be obtained by combining the acid dissociation constants of the two indicators, Equation B.5, and the first two expressions of equation B.6:

\[ \alpha_2 = \frac{(K_{a2})}{K_{a1}(1 - \alpha_1) + (K_{a2})} \]  

(B.10)

B.2 Supplementary Figures

Figure B.1 Theoretical predictions of mixed optode (red solid lines) and mixed nanosensor (black dashed lines) dose-response curves when both pH indicators have the same maximum fluorescence.
Figure B.2 Nanosensor response curves using theoretical predictions from the mixed optode theory at various pH indicator pKₐ separations. Blue curves show the response of sensors made with indicator 1, pink curves show the response of sensors made with indicator 2 and solid purple lines are the response of the mixed optode approach with \( \theta = 0.5 \).
Figure B.3 Nanosensor response curves using theoretical predictions at various pH indicator pKₐ separations. Blue curves show the response of sensors made with indicator 1, pink curves show the response of sensors made with indicator 2 and solid purple lines are the response of the mixed nanosensor approach with \( \theta = 0.5 \).
Figure B.4 Absorbance spectra of lithium-selective IBOS with (A) ChII or (B) ChVII.

Figure B.5 Emission Spectra of lithium-selective IBOS with (A) ChII or (B) ChVII after 660 nm excitation.

Figure B.6 Mixed nanosensor lithium-sensitive IBOS response. (A) Normalized fluorescence dose-response curves. \( \theta \) increases from 0 to 1 as the color shifts from blue to pink. (B) The linear range and LogEC\(_{50}\) shifts toward lower concentrations as ChII is replaced with ChVII. A * represents a significant difference in LogEC\(_{50}\) with \( p < 0.05 \); **\( p < 0.01 \); ***\( p < 0.001 \); ****\( p < 0.0001 \).
Figure B.7 Dynamic range extension of lithium-selective IBOS.

Figure B.8 Apparent overall equilibrium constant vs. θ.

Figure B.9 Single-Chromoionophore control experiment. Changing the concentration of chromoionophore VII changes only the intensity of the nanosensor without affecting the LogEC₅₀.
Figure B.10 Response of single-pH indicator nanosensors and dual pH indicator nanosensors made with the mixed optode method to lithium, sodium, and potassium.

Figure B.11 Control of nanosensor response with mixed optode method in PBS solution.

Figure B.12 (A) Dose/response curve obtained from sample with unknown lithium content. Two capsules of lithium orotate were dissolved in 40 mL HEPES/TRIS and then diluted by a factor of ten, five times. (B) Dose/response curve obtained from dilutions of lithium at known concentration. The x-axis value of ‘test sample’ (-1) was shifted by the difference in LogEC\textsubscript{50}s between the two curves, and then the lithium content of one pill was back-calculated.
Figure B.13 Mixed optode method for calcium-sensitive IBOS.

Figure B.14 Mixed nanosensor method for calcium-sensitive IBOS.

Figure B.15 LogEC$_{50}$ tuning of calcium-sensitive nanosensors.
Figure B.16 Dynamic range extension of calcium-sensitive nanosensors.

B.3 References Cited


APPENDIX C

SUPPLEMENTARY INFORMATION FOR CHAPTER 4

C.1 Materials

Poly(vinyl chloride), high molecular weight (PVC), bis(2-ethyl-hexyl) sebacate (BEHS), tetrahydrofuran (THF), dichloro-methane (DCM), sodium tetrakis[3,5-bis(trifluoromethyl)phenyl]borate (NaBARF; Selectophore™), 4-tert-Butylcalix[4] arene-tetraacetic acid tetraethyl ester (sodium ionophore X (NaI X); Selectophore™), sodium chloride (NaCl), and Trimethoxy(octyl)silane were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-[4-(2-Hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES; Molecular Biology grade), 2-amino-2-hydroxymethylpropane-1,3-diol (TRIS; 2 M), hydrochloric acid concentrate (HCl; 10 N, ACS certified), and sodium hydroxide concentrate (NaOH; 10 N, ACS certified) were purchased from Fisher Scientific (Waltham, MA, USA). Blueberry-C6-ester-652 (Blueberry dye) was purchased from Berry & Associates, Inc (Dexter, MI, USA). The persistent luminescent microparticles used in this work are the Coated Ultra Green V10 Glow in the Dark Powder (15-35μm) from Glow Inc. (Severn, MD, USA).

C.2 Glow Sensor Synthesis

To create glow sensor spots, an optode cocktail containing all sensing components except the phosphorescent microparticles was dissolved in organic solvent. To do this, 15 mg PVC was mixed with 30 mg BEHS and separately 3 mg NaI X, 0.5mg NaBARF, and 4 mg blueberry dye are combined in 500 µL THF. The latter solution is added to the PVC/BEHS suspension and immediately vortexed until all PVC particles are dissolved. This solution is referred to as an optode cocktail. Then, 50 µL of optode cocktail solution is added to 12.5 mg of phosphorescent microparticles. Sensor spots are created by vigorously mixing the optode cocktail/phosphorescent microparticle suspension and then quickly pipetting a 2 µL spot on a silane-modified glass-bottomed petri dish. The petri-dish was silane-modified by spreading a small amount of trimethoxy(octyl)silane on the glass surface and allowing it to dry over several hours. The spotting process is usually repeated to create at least four spots for analysis.
C.3 Glow Sensor Data Collection with Modified Fluorescence Microscope

Glow sensors were analyzed with an Olympus IX81 fluorescence microscope, using the following settings for all experiments: integration time 50 ms, gain 50, binning 4, resolution 16-bit, excitation filter 475 nm, and emission filter 525 nm. Before analysis, the sensor spots were conditioned by submerging them in 2 mL of HEPES/TRIS buffer (pH=7.2), 0.1N NaOH, and 0.1N HCl in that order for 30 minutes each before changing back to HEPES/TRIS and allowing to equilibrate overnight. The sensor spots were washed 3x with Millipore H2O in between each solution change. The next day, 2 mL of the desired test solution is added to the petri dish and allowed to equilibrate for 30 minutes followed by data collection with the microscope and switching to the next test solution. During data collection, cellSens (Shinjuku, Tokyo, Japan) was used to control a shutter program to open and close a shutter blocking the excitation light source while HCImage (Sewickley, PA, USA) was used to operate the microscope. Data collection was started right before beginning the shutter program and ended immediately following the completion of the shutter program. For all experiments except for response time and phosphorescence lifetime, the shutter program consisting of the following: Open for 60 s (collecting fluorescence and phosphorescence), closed for 10 s (collecting only phosphorescence), open for 30 s (F&P), closed for 10 s (only P), open for 30 s (F&P), closed for 10 s (only P). This data collection process was followed for all test conditions, as well as the acid and base conditions during optode conditions for each experiment. To test the background signal of the system, this program was run on a glass-bottomed petri dish containing 2 mL HEPES/TRIS and no Glow Sensor spots. For the initial dose/response curve regeneration, four spots were cycled through test solutions of 10^-7, 10^-6, 10^-5, 10^-4, 10^-3, 10^-2, 10^-1, and 1 M NaCl, before washing 3x with millipore H2O and storing in 2 mL HEPES/TRIS. Several days later, the solution was changed to 100 mM Na and a modified shutter program was used to analyze response time (open for 60 s, closed for 10 s, and then 15 cycles of open for 30 s and closed for 10 s). For reversibility, the test solution was alternated between 0 mM and 100 mM Na for 5 total cycles, washing the petri dish 3x with millipore H2O in between 100 mM and 0 mM readings. For selectivity, the sensor spots were tested by cycling through solutions of 10^-7, 10^-4, 10^-3, 10^-2, 10^-1, and 1 M NaCl on day 1, before washing 3x with millipore H2O and storing in 2 mL of HEPES/TRIS overnight. On day 2, the spots were tested in solutions of 10^-7, 10^-4, 10^-3, 10^-2, 10^-1, and 1 M KCl before washing 3x with millipore H2O and storing in 2 mL of HEPES/TRIS overnight. On day 3, the spots were tested in solutions of 10^-
7, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, and 1 M LiCl. For stability, the sensors were tested in 10^{-7}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, and 1 M NaCl on days 1, 2, 3, 7, and 14. After data collection, the images were analyzed for mean intensity using ImageJ. The sensor signal for each test condition was determined by integrating the average of the three phosphorescence decay curves from 1 – 2s for each test condition. All luminescence values in the first and last 0.2s of each decay curve were excluded to account for the time it takes for the shutter to completely shut and open.

C.4 Spectrometer Phosphorescence Spectra Collection

The glow sensor phosphorescence spectra, as well as the phosphorescence spectra of optode spots made without Blueberry dye, were characterized with an AvaSpec-ULS2048 Starline Versatile fiber-optic spectrometer (Avantes, Apeldoorn, Netherlands). First, 5 mm circular glass slides were adhered to the wells of a 96-well plate using a small dab of vacuum grease. Optode spots were then placed on the glass slides using the same method described above. 200 μL of test solution were added to each optode and allowed 30 minutes to equilibrate before testing. A 200 μM, 0.22 NA bifurcated fiber-optic cable (ThorLabs, Inc., Newton, New Jersey, United States) was coupled to an RPH-SMA Holder Block for Fiber Optic Probes with SMA Connectors (ThorLabs, Inc., Newton, New Jersey, United States) and taped to the top of a Nunc MicroWell 96-Well Optical-Bottom Plate with Polymer Base (Nalge Nunc International, Roskilde, Denmark) so that the spots would be excited by an LED through one cable while the luminescence output would be recorded by the spectrometer through the other cable. Optode spots were analyzed by illuminating for two minutes with a 405 nm LED (ThorLabs) at 85 mA, removing the excitation for one second, and finally collecting an emission spectra from the optode with an integration time of one second.

C.5 Well Plate Absorbance Collection

The absorbance spectra of the Blueberry dye was analyzed with a Synergy H1 microplate reader using Nunc MicroWell 96-Well Optical-Bottom Plate with Polymer Base (Nalge Nunc International, Roskilde, Denmark). 5 mm circular glass slides were adhered to the bottom of a well plate using a small dab of vacuum grease. Optode spots were then placed on the glass slides using the same method described above. 200 μL of test solution were added to each optode and allowed 30 minutes to equilibrate before testing.
C.6 Glow Sensor Analysis

Upon initial excitation, each spot shows a sharp increase in luminescence intensity for the first 15-20s before leveling off to a consistent signal. For the second and third excitations (at time = 70s and time = 110s), the spots showed a sharp increase in luminescence for only the first 7-8s before reaching a consistent signal. Glow Sensor luminescence takes the form of fluorescence when the shutter is open and phosphorescence when the shutter is closed. For phosphorescence decay plots, time zero is defined as the time when the shutter is closed to stop excitation.

The glow sensor dynamic range was determined by first normalizing the response to the range of sodium concentrations between fully protonated (0.1N HCl) and fully deprotonated (0.1N NaOH) test conditions followed by fitting the sodium response to a four-parameter logistic response curve,

\[ Y = Bottom + \frac{(Top - Bottom)}{1 + 10^{((\alpha_{0.5} - X) \times HillSlope)}} \]  

with GraphPad Prism Software version 7.03, where Top and Bottom represent the maximum and minimum sensor signals, \( \alpha_{0.5} \) is the sodium concentration corresponding to half-maximal response, and HillSlope is the slope of the tangent line drawn at the \( \alpha_{0.5} \). The linear range was then defined by the x-axis range when a tangent line at \( \alpha_{0.5} \) deviates less than 5% from the non-linear fit to sodium response. The glow sensor selectivity was determined by the Nicolskii-Eisenman model for a fixed interfering ion,

\[ logK_{ij}^{opt} = \alpha_{0.5}^i - \alpha_{0.5}^j \]  

where \( \alpha_{0.5}^i \) and \( \alpha_{0.5}^j \) are the \( \alpha_{0.5} \) constants for the interfering ion (potassium or lithium) and the target ion (sodium), respectively. The glow sensor response time was determined by first fitting the response to 100mM Na over time to a one-phase decay equation,

\[ Y = (Y_0 - Plateau) \times e^{(-k \times X)} + Plateau \]

where \( Y_0 \) is the initial sensor signal, plateau is the final sensor signal, and \( k \) is a rate constant in units of the reciprocal of the X axis units. The response time was then determined to be the time for the curve to decay 95% of the way from the \( Y_0 \) value to the Plateau value.
C.7 Supplementary Figures

Figure C.1 (Left Column) Glow Sensor luminescence (fluorescence and phosphorescence when the shutter is open, phosphorescence only when the shutter is closed) during shutter program for four spots: A, B, C, and D. (Right Column) The average of the three phosphorescent decay curves collected while the shutter is closed for spots A, B, C, and D.
Figure C.2 Spot A under basic conditions compared to background (no sensor) signal. This shows that the phosphorescence is predominantly quenched by full deprotonation of the blueberry dye, although some residual signal remains.

Figure C.3 Luminescent signal from the Glow Sensor compared to background noise over time after ending excitation. The trend is described by a two-phase exponential decay with a fast half-life of 1.6 seconds and a slow half-life of 14.08 seconds.
Figure C.4 (Left column) Luminescence during shutter program for Glow Sensor spots without Blueberry dye. (Right Column) Averaged phosphorescent decay curves for Glow Sensor spots without Blueberry dye.
Figure C.5 (Left) Dose/Response curve for Glow Sensor spots made without Blueberry Dye. Minimal response to Na\(^+\) is observed. (Right) Glow Sensor spots made without persistent luminescence microparticles show no phosphorescence under acid and base conditions (see Fig S2 red curve for comparison of background signal). Time zero in this panel is the time when the shutter is closed and the excitation source blocked from the sample.
Figure C.6 (Top) Absorbance of an optode spot made without phosphorescence microparticles under acidic and basic conditions, showing a change in absorption over a wide range. (Bottom) Phosphorescent spectra of Glow Sensor (Blue) and no-blueberry dye control spot made without blueberry dye (Red) under acidic (solid circles) and basic (hollow circles) conditions. This demonstrates that the glow sensor phosphorescence is greatly reduced in basic conditions, which corresponds with a rise in absorbance from the blueberry dye at the same range of wavelengths. Without blueberry dye, however, the Glow Sensor does not change its phosphorescence between acidic and basic conditions. The energy coupling between the blueberry dye and phosphorescent microparticles is likely either due to the inner filter effect or from resonance energy transfer, both of which would require a rise in absorbance in the blueberry dye to correspond to a decrease in phosphorescence from the phosphorescent microparticles.
Figure C.7 Luminescence during extended shutter program for Glow Sensor spots during response time experiment.

Figure C.8 Drift of Glow Sensor $\alpha_{50}$ over the course of the experiment. ** represents a significant difference in $\alpha_{50}$ with $p < 0.01$.

Figure C.9 (Left) Dose/Response curve and (Right) dynamic range for spots A, B, C, and D.
Figure C.10 Compilation of all Glow Sensor dose/response curves demonstrating excellent reproducibility between sensor batches.
APPENDIX D

REUSE AND REPRINT PERMISSIONS

Permission to Reproduce Two Figures from *Ionophore-Based Optical Sensors*
Permission to reprint one figure from Carrier-Based Ion-Selective Electrodes and Bulk Optodes.

1. General Characteristics
Permission to reprint two images from *Principles of Fluorescence Spectroscopy*

<table>
<thead>
<tr>
<th>SPRINGER NATURE LICENSE TERMS AND CONDITIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jul 14, 2019</td>
</tr>
<tr>
<td>This Agreement between 906 24th St. (&quot;You&quot;) and Springer Nature (&quot;Springer Nature&quot;) consists of your license details and the terms and conditions provided by Springer Nature and Copyright Clearance Center.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>License Number</th>
<th>4627840205247</th>
</tr>
</thead>
<tbody>
<tr>
<td>License date</td>
<td>Jul 14, 2019</td>
</tr>
<tr>
<td>Licensed Content Publisher</td>
<td>Springer Nature</td>
</tr>
<tr>
<td>Licensed Content Publication</td>
<td>Springer eBook</td>
</tr>
<tr>
<td>Licensed Content Title</td>
<td>Introduction to Fluorescence</td>
</tr>
<tr>
<td>Licensed Content Date</td>
<td>Jan 1, 2006</td>
</tr>
<tr>
<td>Type of Use</td>
<td>Thesis/Dissertation</td>
</tr>
<tr>
<td>Requestor type</td>
<td>academic/university or research institute</td>
</tr>
<tr>
<td>Format</td>
<td>print and electronic</td>
</tr>
<tr>
<td>Portion</td>
<td>figures/tables/illustrations</td>
</tr>
<tr>
<td>Number of figures/tables/illustrations</td>
<td>2</td>
</tr>
<tr>
<td>Will you be translating?</td>
<td>no</td>
</tr>
<tr>
<td>Circulation/distribution</td>
<td>&lt;501</td>
</tr>
<tr>
<td>Author of this Springer Nature content</td>
<td>no</td>
</tr>
<tr>
<td>Title</td>
<td>ADVANCES IN NANOSENSOR TECHNOLOGY FOR BIOLOGICAL ANALYSIS</td>
</tr>
<tr>
<td>Institution name</td>
<td>Colorado School of Mines</td>
</tr>
<tr>
<td>Expected presentation date</td>
<td>Jul 2019</td>
</tr>
<tr>
<td>Portions</td>
<td>1.5, 1.12</td>
</tr>
<tr>
<td>Requestor Location</td>
<td>906 24th St.</td>
</tr>
<tr>
<td></td>
<td>906 24th St.</td>
</tr>
<tr>
<td></td>
<td>GOLDEN, CO 80401</td>
</tr>
<tr>
<td></td>
<td>United States</td>
</tr>
<tr>
<td></td>
<td>Attn: 906 24th St.</td>
</tr>
<tr>
<td>Total</td>
<td>0.00 USD</td>
</tr>
</tbody>
</table>

**Springer Nature Customer Service Centre GmbH Terms and Conditions**

This agreement sets out the terms and conditions of the licence (the Licence) between you and Springer Nature Customer Service Centre GmbH (the Licensor). By clicking 'accept' and completing the transaction for the material (Licensed Material), you also confirm your acceptance of these terms and conditions.
Permission to Reproduce A Dual-Indicator Strategy for Controlling the Response of Ionophore-Based Optical Nanosensors

Title: A dual-indicator strategy for controlling the response of ionophore-based optical nanosensors
Author: Mark S. Ferris, Aakashi G. Katageri, Greta M. Gehring, Kevin J. Cash
Publication: Sensors and Actuators B: Chemical
Publisher: Elsevier
Date: March 2018
© 2017 Elsevier B.V. All rights reserved.

Please note that, as the author of this Elsevier article, you retain the right to include it in a thesis or dissertation, provided it is not published commercially. Permission is not required, but please ensure that you reference the journal as the original source. For more information on this and on your other retained rights, please visit: https://www.elsevier.com/about/our-business/policies/copyright#Author-rights

Copyright © 2019 Copyright Clearance Center, Inc. All Rights Reserved. Privacy statement. Terms and Conditions. Comments? We would like to hear from you. E-mail us at customerservice@copyright.com
Permission to reproduce *Enzyme-Linked Nanosensors with Tunable Detection Limits for Small Biomolecule Determination*

This Agreement between 906 24th St. ("You") and John Wiley and Sons ("John Wiley and Sons") consists of your license details and the terms and conditions provided by John Wiley and Sons and Copyright Clearance Center.

License Number: 4630930682313
License date: Jul 16, 2019
Licensed Content Publisher: John Wiley and Sons
Licensed Content Publication: AIChE Journal
Licensed Content Title: Enzyme-conjugated nanosensors with tunable detection limits for small biomolecule determination
Licensed Content Author: Mark S. Ferris, Makayla K. Elms, Kevin J. Cash
Licensed Content Date: Jun 28, 2019
Licensed Content Volume: 0
Licensed Content Issue: 0
Licensed Content Pages: 9
Type of use: Dissertation/Thesis
Requestor type: Author of this Wiley article
Format: Print and electronic
Portion: Full article
Will you be translating?: No
Title of your thesis / dissertation: Advances in Polymeric Nanosensor Technology for Biological Analysis
Expected completion date: Jul 2019
Expected size (number of pages): 1
Requestor Location: 906 24th St.
GOLDEN, CO 80401
United States
Attn: 906 24th St
Publisher Tax ID: EU826007151
Total: 0.00 USD

**TERMS AND CONDITIONS**

This copyrighted material is owned by or exclusively licensed to John Wiley & Sons, Inc. or one of its group companies (each a "Wiley Company") or handled on behalf of a society with which a Wiley Company has exclusive publishing rights in relation to a particular work (collectively "WILEY"). By clicking "accept" in connection with completing this licensing transaction, you agree that the following terms and conditions apply to this transaction (along with the billing and payment terms and conditions established by the Copyright
Co-author permissions

Greta Goebring
Mon, Jul 22, 9:53 AM (3 days ago)
Hi Mark!
It is going really well here at CSU. I am currently in a stem cell research lab working on regenerating cartilage. It's been an amazing experience so far. I am getting ready for senior year, however since my program is 5 years, I still have 2 more to go. Congrats for getting ready to finish up at mines! That is fantastic! I absolutely give you my permission to re-use the dual-indicator paper. What's the plan after Mines?
Best,
Greta

Makayla Elms
Sat, Jul 20, 9:17 PM (5 days ago)
Hello Mark,

This is exciting! So close to being done. Congrats! I am enjoying my job. I'll be back in the fall to do a ME non-thesis MS while working at LM to help with my current role.

Yes, of course you can have permission to reprint the enzyme paper.

Good luck!
Makayla