NMR Sensing Strategies Based on Modulation of Chemical Exchange Rate With Small-Molecule Synthetic Sensors

by

Loïse Hélène Perruchoud

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Chemistry
University of Toronto

© Copyright by Loïse Hélène Perruchoud 2016
NMR Sensing Strategies Based on Modulation of Chemical Exchange Rate With Small-Molecule Synthetic Sensors

Loïse Hélène Perruchoud
Doctor of Philosophy
Department of Chemistry
University of Toronto
2016

Abstract

Nuclear magnetic resonance (NMR) spectroscopy is a powerful and versatile tool for monitoring molecular interactions. Dynamic processes, such as conformational changes or binding events, can induce drastic effects on NMR spectra in response to variations in chemical exchange rate. Molecular strategies in which the chemical exchange rate is deliberately controlled can lead to attractive opportunities in NMR sensor design. This thesis explores two main strategies involving structurally similar sensors in which modifications of the chemical exchange rate have been utilized to sense anions or detect changes in pH.

The first section of the thesis describes a novel and ultrasensitive approach to detect anions indirectly by NMR and is based on a small synthetic sensor (TUC) displaying conformational rigidity due to intramolecular hydrogen bonding. With an increase in chemical exchange rate, due to anion-induced conformational flexibility, detection and quantification of spherical and tetrahedral anions as low as 120 nM concentrations, which is more than 1000 fold lower than the detection limit of NMR, are possible.

The next part describes a slow proton exchange strategy for pH sensing. Monitoring pH change accurately and non-invasively by NMR has important implications in the detection and treatment
of pathologies such as cancer, ischemia or cystic fibrosis. Conventional NMR pH detection methods are mostly based on changes in chemical shift due to fast proton exchange between the different protonation states of the sensors. Slowing down proton exchange allows ratiometric pH measurement, which eliminates the errors from pH-independent factors such as artifacts caused by the chemical environment, ionic strength, etc. Therefore, pH can be measured by using the ratio of the different protonation states of the pH sensor. Detection of pH in vitro and in bacterial cultures was successfully demonstrated with the sensor SPE1 using this new method, and preliminary chemical exchange saturation transfer (CEST) experiments were carried out. Optimization of SPE1, through $^{13}$C-labeling ($^{13}$C-SPE1) and new design to decrease the pK$_a$ and increase the pH window of the sensor (SPE2), was also studied. Overall, both strategies demonstrate how modulation of chemical exchange rate can provide a platform for the development of new NMR sensors with high sensitivity and accuracy.
Acknowledgments

There are many people that I am grateful for and without whom the completion of this thesis would not have been possible. First of all, I want to thank my supervisor, Xiao-an Zhang for his guidance and support throughout the years, as well as my committee members, Chuck Cunningham and Scott Prosser who, in addition to providing much needed focus and help during our annual meetings, were kind enough to allow me to collaborate with them on different projects.

I would like to thank Andre Simpson for his flexibility concerning the NMR time during our collaborations, the wonderful things he taught me about NMR spectroscopy and for being part of my defense committee. Many thanks to Andre’s post-doctoral fellows, Andre, Ronald and Blythe whose tremendous help allowed me to complete my reports in a timely fashion. I also extend my thanks to Deborah Zamble and Micheal Jones, for teaching me about biological chemistry and bacterial cultures. Their insights and advice were invaluable.

Special thanks to all members of the Zhang lab, past and present, and particularly to Wayne and Inga who started the program with me in a brand new laboratory. Their support during difficult times and advice when all seemed lost allowed me to be where I am today. Wayne, thanks for being so calm and rational during all these years and helping me focus on what is important. Inga, your love and passion for organic chemistry really inspired me and your advice, both professional and personal, was invaluable. I wish you both all the best in your future endeavors.

I also want to thank the teaching faculty at UTSC, including Lana Mikhaylichenko, who was an inspiring lab coordinator and who showed me how much fun bioorganic chemistry could be, and Scott Ballantyne for his support and helpful discussions concerning my degree, my teaching and life in general.

I would like to acknowledge funding sources, including an NSERC Discovery Grant (XaZ), a Connaught New Researcher Award (XaZ), the University of Toronto Scarborough, Canada Foundation for Innovation and Ontario Research Fund (XaZ); a School of Graduate Studies Conference Grant, UTSC Travel Grant, University of Toronto International Student Scholarship a Euromar Travel Grant and a Doctoral Completion Award (LHP).
I give many thanks to the Toronto School of Circus Arts, a place where I have been able to decompress from hard lab days and where I could find some balance. I would especially like to thank my coaches, Bill and Joanne, who have pushed me further than I thought possible and taught me that great things can be achieved with perseverance: a lesson that I bring to the lab with me every day. I also thank my close friends in the “real” world, Natalie, Rouzbeh, Laura and Cornelia. Thanks for our discussions over skype or in person, which brought me comfort and ideas so I could always move forward.

Finally, I am immensely grateful to my entire family. To my parents, a huge thank for your financial help but most importantly your unconditional support of my dreams, which have been a little exuberant from time to time. I could not have done this without your help.
Contributions of Authors

The work presented in this thesis is based on manuscripts of which I am the first author, that have been published in peer-reviewed journals (chapters 2 and 3) or are in preparation (chapter 4). The introduction and chapter 5 are the result of my own interpretation of the work that I did during the past five years and how it fits into the current state of the field. Below is a detailed explanation of the roles of each author from chapters 2 to 4.

**Chapter 2:** The initial idea for the project described in this chapter is based on a compound described in Prof. Xiao-an Zhang’s PhD thesis. Prof. Zhang and I designed the project, I conducted the syntheses, characterizations, recrystallizations, $^1$H NMR experiments, Dr. Hadzovic conducted the crystallographic study and all contributed to data analysis and manuscript preparation.

**Chapter 3:** Prof Zhang and I designed the project, I conducted the syntheses, titrations and microinjection experiments. A discussion between myself, Prof. Zhang and Prof. Deborah Zamble led to the development of the pH sensing in bacteria application described in this chapter. Michael Jones and I performed the experiments involving *E. coli*, Dr. Andre Sutrisno and Prof Andre Simpson carried out the NMR experiments of oocytes and bacteria and all contributed to data analysis and manuscript preparation.

**Chapter 4:** Prof Zhang and I designed the project, I conducted the syntheses and titrations. Surgical removal of the oocytes from the frog as well as microinjection was done by Prof. Brad Hanna and the NMR experiments were done by Dr. Sasha Larda, Prof. Scott Prosser and myself. I prepared the hyperpolarization experiments, which were carried out by Dr. Albert Chen and Prof. Charles Cunningham at the Sunnybrook Research Institute.

**Chapter 5:** Prof. Zhang and I designed the project, Ryan Correa and I conducted the syntheses and I completed the titrations. Michael Jones and I performed the bacteria experiments, Dr. Blythe Fortier-McGill and Prof. Andre Simpson carried out the NMR experiments. I prepared the blueberries and Ioana, Dr. Ronald Soong, Prof. Andre Simpson and I performed the NMR experiments. All contributed to data analysis and manuscript preparation.
# Table of Contents

Acknowledgments.................................................................................................................. iv
Contributions of Authors ........................................................................................................ vi
Table of Contents .................................................................................................................. vii
List of Tables ........................................................................................................................ xii
List of Figures ......................................................................................................................... xiii
Abbreviations ......................................................................................................................... xix

**Chapter 1** Introduction..................................................................................................... 1
  1.1 Physical Basics of Magnetic Resonance ........................................................................ 1
  1.2 Effects of Chemical Exchange Rate in NMR ............................................................... 3
  1.3 MR based Sensing ......................................................................................................... 6
    1.3.1 Methods for MR Sensing ..................................................................................... 7
      1.3.1.1 NMR Sensing ............................................................................................... 7
      1.3.1.2 MRI Sensing ................................................................................................ 9
    1.3.1.2.1 Chemical Exchange Saturation Transfer .................................................... 9
    1.3.1.3 Hyperpolarization .......................................................................................... 11
      1.3.1.3.1 Dynamic Nuclear Polarization ............................................................... 12
      1.3.1.3.2 Spin-Exchange Optical Pumping ........................................................... 13
  1.4 Rationale ....................................................................................................................... 14

**Chapter 2** Ultrasensitive Anion Detection by NMR Spectroscopy: a Supramolecular Strategy Based on Modulation of Chemical Exchange Rate ....................................................... 16
  2.1 Introduction .................................................................................................................. 16
  2.2 Results and Discussion ............................................................................................... 18
    2.2.1. Design and Synthesis of **TUC** as an NMR Anion Sensor .............................. 18
    2.2.2. Dynamic Intramolecular Hydrogen Bonding in **TUC** ................................. 20
2.2.3. Anion Sensing Using Competition Between HB\textsubscript{intra} and HB\textsubscript{inter}........................... 23
2.2.4. Anion Selectivity .................................................................................................................... 28
2.3 Conclusions.............................................................................................................................. 30
2.4 Materials and Methods............................................................................................................ 30
2.4.1. General Experimental Procedures....................................................................................... 30
2.4.2. Synthesis of Tris-(2-isothiocyanate-ethyl)amine (ITC)................................................. 30
2.4.3. Synthesis of TUC ................................................................................................................ 31
2.4.4. X-Ray Crystallography....................................................................................................... 31
2.4.5. \textsuperscript{1}H NMR Titrations............................................................................................. 32

Copyright Acknowledgements........................................................................................................ 33

Chapter 3 An Accurate and Biocompatible Ratiometric NMR pH Sensing Strategy Based on Slow-Proton-Exchange (SPE) Mechanism ................................................................................... 34
3.1 Introduction.............................................................................................................................. 34
3.2 Principle and Design of the SPE pH Sensing Strategy .......................................................... 36
3.3 Results and Discussion .......................................................................................................... 38
3.3.1 Synthesis of SPE\textsubscript{1}.................................................................................................... 38
3.3.2 Measurement of pH.............................................................................................................. 38
3.3.3 Biological Applications of SPE\textsubscript{1}................................................................................ 41
3.3.3.1 Characteristics of SPE\textsubscript{1} for Biological Applications........................................ 41
3.3.3.2 Determination of pH by CEST-MRI .............................................................................. 42
3.3.3.3 In-Cell pH Detection .................................................................................................... 43
3.3.3.4 Monitoring pH Change in \textit{Escherichia coli} Cultures ................................................... 45
3.4 Summary and Conclusions .................................................................................................... 47
3.5 Materials and Methods.......................................................................................................... 48
3.5.1 General Experimental Procedures....................................................................................... 48
3.5.2 Synthesis of SPE\textsubscript{1}.................................................................................................... 48
Chapter 4 A Ratiometric and Hyperpolarized pH-Sensitive $^{13}$C NMR Probe Based on the Slow Proton Exchange (SPE) Method

4.1 Introduction

4.1.1 Principle and Design of the SPE Strategy

4.2 Results and Discussion

4.2.1 Synthesis of $^{13}$C-SPE1

4.2.2 Measurement of pH Based on the SPE Strategy

4.2.3 Applications of $^{13}$C-SPE1

4.2.3.1 Measurement of Intracellular pH

4.2.3.2 Hyperpolarization

4.3 Summary and Conclusions

4.4 Materials and Methods

4.4.1 General Experimental Procedures

4.4.2 Syntheses

4.4.2.1 Synthesis of $^{13}$C-Labelled Tris-(2-isothiocyanate-ethyl)amine ($^{13}$CITC)

4.4.2.2 Synthesis of $^{13}$CTUC

4.4.2.3 Synthesis of $^{13}$C-SPE1
4.4.3 NMR Monitored pH Calibration of $^{13}$C-SPE1

4.4.4 Measurement of Intracellular pH

4.4.5 Hyperpolarization

Chapter 5 Second Generation SPE Based pH Sensor with a Modified $pK_a$ and Extended pH Window

5.1 Introduction

5.1.1 Goal and Molecular Design

5.2 Results and Discussion

5.2.1 Synthesis of SPE2

5.2.2 Measurement of pH

5.2.3 Biological Applications of SPE2

5.2.3.1 Monitoring pH Change in Escherichia coli Cultures

5.2.3.2 Detecting pH of a Fermenting Solution

5.3 Summary and Conclusions

5.4 Materials and Methods

Chapter 6 Summary, Significance and Future Directions
6.1 Anion Sensing ........................................................................................................................................ 78
6.2 Sensing pH ........................................................................................................................................ 79
6.3 Future Directions .................................................................................................................................. 80
  6.3.1 Determination of pH of Organic Solvents .................................................................................. 80
  6.3.2 Development of Cell-Trappable and Regioselective SPE Based pH Sensors ...... 81
6.4 Significance and Conclusion.................................................................................................................. 83
References .................................................................................................................................................. 85
Appendices .................................................................................................................................................. 95
A1 Application of TUC: Detection of Fatty Acids in Milk................................................................. 95
A2 X-Ray Crystallization Data of TUC .................................................................................................. 97
A3 Titrations with Different Tetrabutylammonium Anions ................................................................. 102
A4 Measuring pH of Blueberries .............................................................................................................. 105
List of Tables

Table 1. T$_2$ of the 4 methylene peaks of SPE1 and SPE1H$_2^{2+}$ 43
List of Figures

Figure 1. Energy levels for a nucleus of spin quantum number ½ with increasing magnetic field. .......................................................... 3

Figure 2. An simple example of mutual chemical exchange involving a N,N-dimethyl group bonded to an aromatic ring, reproduced with permission from Annual Reports on NMR Spectroscopy. .............................. 4

Figure 3. Chemical exchange effect on NMR spectrum of an intermolecular reaction of a receptor (R) with and analyte (A). a) fast exchange: the peaks of the receptor and analyte show a chemical shift change upon increase in concentration of analyte. b) slow exchange: the peaks of free (R_f) and bound (R_b) form of the receptor are chemical shift independent but their ratio changes with increase in analyte. A_b = bound analyte and A_f = free analyte ........................................... 8

Figure 4. Chemical exchange saturation transfer (CEST) principle. a, b) Exchangeable protons (blue) are saturated at their specific resonance frequency (here 8.25 ppm for amide protons). This saturation is transferred to water at 4.75 ppm. After a period (t_sat), the partial saturation of the water peak becomes visible (b, right). c) Normalized water saturation (S_sat/S_0) measurement depending on the irradiation frequency creates a Z-spectrum (or CEST spectrum). The frequency of the water peak is assigned to 0 ppm in Z-spectra. d) Magnetization transfer ratio (MTR) asymmetry analysis of the Z-spectrum to remove direct saturation effects, reproduced with permission from Magnetic Resonance in Medicine. ................................. 10

Figure 5. Description of the orientation of the spins in a magnetic field at thermal equilibrium and in the hyperpolarized state. ........................................................................................................ 12

Figure 6. Structure and proposed binding modes of a neutral anion receptor that can bind a) sulfate anions using 9 HB_int slower than NMR timescale and b) spherical anions like acetate with 6 HB_int in the fast exchange regime, reproduced with permission from Supramolecular Chemistry. ................................................................. 18
Figure 7. The molecular structure of TUC. Thermal ellipsoids at 50% probability. a) Side view, methylene hydrogens omitted for clarity. b) View along N(2)-N(2)’ axis, methylene hydrogens omitted for clarity.

Figure 8. Synthesis of TUC.

Figure 9. $^1$H NMR spectra of TUC at 500 MHz demonstrating the effects of solvents and temperature and the assignment of NMR signals. a) in DMSO-d$_6$ at 25 ºC. b) in CDCl$_3$ at 25 ºC. c) in CDCl$_3$ at -40ºC. d) Structure of TUC with assignment of methylene hydrogen atoms. e) Newman projection from the methylene carbons of TUC, used for assigning the methylene hydrogen atoms to the corresponding NMR signals. Solvent peaks are labelled * for DMSO, ** for dichloromethane and *** for chloroform.

Figure 10. Partial $^1$H NMR of 1.7 mM TUC at -40 ºC and 500 MHz with coupling constants labelled.

Figure 11. Partial gDQCOSY of TUC at -40 ºC at 500 MHz.

Figure 12. Schematic mechanism of conformational chemical exchange between geminal hydrogens in TUC. Top view of TUC is shown in a dynamic average C$_3$ symmetry, with the dipole moment of three thiourido groups in a clockwise (a) or anti-clockwise (b) orientation. Only selected representative hydrogen atoms are shown for clarity.

Figure 13. $^1$H NMR titration of TUC in CDCl$_3$ at 500 MHz and 25 ºC (1.7 mM) with chloride (as a TBA salt).

Figure 14. Partial $^1$H NMR spectra of 1.7 mM TUC at 500 MHz and 25 ºC in the presence of trace amount of Cl$^-$. 

Figure 15. Variable temperature partial $^1$H NMR spectra of 1.7 mM TUC with 7 meq TBACl between -40 ºC and 35 ºC in CDCl$_3$ at 500 MHz.

Figure 16. Quantification of a) nM, b) µM to c) mM chloride concentration with TUC, using peak width and chemical shift change respectively.
Figure 17. Effect of different TBA anions (2.8 meq) on conformational rigidity of TUC monitored by $^1$H NMR................................................................................................................................. 28

Figure 18. Partial $^1$H NMR spectra of 1.7 mM TUC with different amount of selected anion species that generate effect similar to 28 meq. Cl$^-$................................................................. 29

Figure 19. Structure and protonation states of cage-shaped pH sensor SPE1. The protons attached to the bridgehead nitrogen atoms are trapped inside the cage due to hydrogen bonding with the ureido oxygen atoms, thereby allowing SPE between the two states. The $^1$H NMR signals of labelled methylene positions are used for pH sensing......................................................... 37

Figure 20. Synthesis of SPE1. Tren = (2-aminoethyl) amine, 3 = tris-(2-isothiocyanate-ethyl)amine .......................................................................................................................... 38

Figure 21. $^1$H NMR pH titration of SPE1 at 25 and 37 °C in phosphate buffer. a) Selected partial $^1$H NMR spectra of SPE1 at different pH values at 25 °C. Chemical shifts: A: 3.40 ppm, B: 3.56 ppm, A': 2.58 ppm, B': 3.13 ppm. b) Ratiometric curve of $^1$H NMR pH titrations derived from ratio of different protonation states of SPE1. .................................................................................... 39

Figure 22. Normalized local NMR spectra of SPE1 in phosphate buffer (pH = 7.90) with D$_2$O. Red: D$_2$O in sealed capillary (pH 7.896 as measured by NMR); Blue: D$_2$O directly added (10%) to the solution (pH 7.898 as measured by NMR). The similar ratio of neutral and protonated SPE1 indicates isotope impact from 10% D$_2$O or less does not significantly affect the accuracy of pH sensing. ......................................................................................................................... 40

Figure 23. Selected $^1$H NMR spectra showing the high accuracy of pH measurement by SPE1. Overlay of local $^1$H NMR spectra of SPE1 at pH 7.91 (blue) and 7.93 (red). The peak intensity was normalized to the signals of neutral SPE1 at 2.58 and 3.13 ppm. A difference in pH of 0.02 pH units can be detected. ......................................................................................................................... 41

Figure 24. Viability experiment of different concentrations of SPE1 on SH-SY5Y undifferentiated neuronal cells.......................................................................................................................... 42

Figure 25. Imaging pH with CEST MRI using exchangeable protons in SPE1. a) Protonation states of SPE1. b) Partial $^1$H NMR spectra of SPE1 at different pH values. c) Z spectrum of an
aqueous solution of **SPE1**. d-f) Phantom CEST images (MTR\textsubscript{asym}) of aqueous solutions of **SPE1** between pH 6.5 and 8.5. d) Saturation at 1 ppm, e) saturation at 2 ppm, f) ratiometric image (1/2 ppm).

**Figure 26.** Determination of intracellular pH of *Belonidae* oocytes with **SPE1** at 25 °C by \textsuperscript{1}H NMR. a) \textsuperscript{1}H NMR spectrum of an untreated oocyte. b) \textsuperscript{1}H NMR spectrum of an oocyte treated with 2 μl of 0.7 M **SPE1**. Chemical shifts: A: 3.40 ppm, B: 3.56 ppm, A’: 2.58 ppm, B’: 3.13 ppm. *Spinning sideband due to slow spinning at 1 KHz at 2.69 ppm. Percentage of neutral **SPE1** was 9%, corresponding to a pH value of 7.50.

**Figure 27.** Stacked \textsuperscript{1}H NMR spectra at 500 MHz of *E. coli* incubated for 6 h with 10 mM **SPE1**. a) Initial \textsuperscript{1}H NMR spectrum of *E. coli* cells with **SPE1**, after washing out the extracellular sensor. b) \textsuperscript{1}H NMR spectrum of *E. coli* cells taken after the cells were washed again with fresh buffer following the first NMR experiment in a).

**Figure 28.** Diffusion edited \textsuperscript{1}H NMR spectra of *E. coli* cells (OD\textsubscript{600} = 1) with 27 mM **SPE1**. Top: \textsuperscript{1}H DE with the gradient on, corresponding to bound species in the sample. Bottom: \textsuperscript{1}H DE with the gradient strength set to zero, representing all species in the sample. As no signal is detected with the diffusion gradient applied it demonstrates the pH sensor exhibits fast self-diffusion and is not in a bound state.

**Figure 29.** Monitoring of pH in *E. coli* (OD\textsubscript{600} = 1) using 1.8 mM solution of **SPE1** in phosphate buffer. a) Selected stacked \textsuperscript{1}H NMR spectra of *E. coli* cells in the presence of 1.8 mM **SPE1**. NMR measurements were taken continuously for 3 hrs using 256 scans (15 min intervals). b) Graph of decrease of pH over time of **SPE1** treated *E. coli* cells.

**Figure 30.** Structure and protonation states of cage-shaped \textsuperscript{13}C-labelled pH sensor \textsuperscript{13}C-**SPE1**. The protons attached to the bridgehead nitrogen atoms are trapped inside the cage due to hydrogen bonding with the ureido oxygen atoms, which produces two species that are in slow exchange in NMR.

**Figure 31.** Synthesis of \textsuperscript{13}C-**SPE1**.

**Figure 32.** \textsuperscript{13}C NMR pH titration of \textsuperscript{13}C-**SPE1** at 25 and 37 °C in phosphate buffer. a) Selected partial \textsuperscript{13}C NMR spectra of \textsuperscript{13}C-**SPE1** at different pH values at 25 °C. Chemical shifts: C: 161.90
ppm, C': 161.12 ppm. b) Ratiometric curve of $^{13}$C NMR pH titrations derived from ratio of different protonation states of $^{13}$C-SPE1. ............................................................................................................. 56

**Figure 33.** $^{13}$C NMR spectra of *Xenopus laevis* oocytes injected with $^{13}$C-SPE1. The peak of the urea carbon of the sensor ($^{13}$CSE1H$_2^{2+}$) is labelled C'. ............................................................................................................. 57

**Figure 34.** Hyperpolarization of $^{13}$C-SPE1 with pyruvic acid as solvent. Left, stack of spectra of $^{13}$C-SPE1/pyruvic acid mixture acquired every 2 seconds. The T$_1$s of each peak are shown below the spectra. Top right, blank spectrum of pyruvate. Bottom right, spectrum of $^{13}$C-SPE1/pyruvic acid mixture dissolved with D$_2$O. ............................................................................................................. 59

**Figure 35.** Structure and protonation states of the cage-shaped pH sensor SPE2. The bridgehead N-atoms are protonated and intramolecular hydrogen bonds are formed with the closest O-atoms from the oxalamide group trapping the incoming proton inside the molecular cavity longer than NMR timescale and allowing SPE to occur between the different protonation states of SPE2. $^1$H NMR methylene signals used for determination of pH are labelled. ................................................. 65

**Figure 36.** Synthesis of SPE2. ............................................................................................................. 66

**Figure 37.** $^1$H NMR pH titration of SPE2 at 25 and 37 °C in 10 mM phosphate buffer. a) Selected partial $^1$H NMR spectra of SPE2 at different pH values close to pK$_{a1}$. b) Ratiometric curve of SPE2 based on the methylene protons in the $^1$H NMR spectrum around pK$_{a2}$. The fit was done using only the 2 most upfield peaks (2.61 and 2.70 ppm) due to overlapping of other peaks. c) Selected partial $^1$H NMR spectra of SPE2 at different pH values close to pK$_{a2}$. d) Ratiometric curve of SPE2 based on the methylene protons in the $^1$H NMR spectrum close to pK$_{a2}$. ............................................................................................................. 67

**Figure 38.** Monitoring of pH in *E. coli* (OD$_{600}$ = 1) using a 0.9 mM solution of SPE2 in phosphate buffer. a) Selected stacked $^1$H NMR spectra of *E. coli* cells in the presence of 0.9 M SPE2. NMR measurements were taken continuously for 15 hours using 256 scans (15 min intervals). b) Graph of decrease of pH over time of SPE2 treated *E. coli* cells. ................................. 69

**Figure 39.** Monitoring pH in *E. coli* (OD$_{600}$ = 1) using a 0.9 mM solution of SPE2 in phosphate buffer in the presence of an added carbon source. NMR measurements were taken continuously for 28 hours using 256 scans (15 min intervals) ............................................................................................................. 70
**Figure 40.** Monitoring of pH of *E. coli* knockout strains (OD$_{600} = 1$) using a 0.9 mM solution of SPE$_2$ in phosphate buffer. a) selected stacked $^1$H NMR spectra of HD705 knockout *E. coli* cells in the presence of 0.9 M SPE$_2$. NMR measurements were taken continuously for 15 hours using 256 scans (15 min per experiment) and the peak of acetate has higher intensity than succinate which contrasts with the parent strain. b) Graph of decrease of pH over time of SPE$_2$ treated HYD723 and DHPB knockouts. ................................................................. 71

**Figure 41.** Chemical reactions for the conversion of ethanol to acetic acid. ADH: alcohol dehydrogenase, ALDH: aldehyde dehydrogenase. ................................................................. 72

**Figure 42.** Monitoring the course of vinegar production reactions using pH determined by ratio of SPE$_2$H$^+$ and SPE$_2$H$_2$$^{2+}$. $^1$H NMR spectrum of a semi-fermented solution of vinegar containing 3.6% alcohol. A ratio of 83/17 (83% SPE$_2$H$^+$) was obtained, corresponding to a pH of 2.35 which is in agreement with the pH reading from the electrode. The ratio of alcohol to acetic acid (0.32:1) also correlated to a 3.6% alcohol solution. ................................................................. 73

**Figure 43.** Carboxylation of the tertiary N-atoms of the sensor SPE$_1$ and its intermediate ITC. Ethyl bromoacetate was used as the carboxylating agent in chloroform but decomposed before it could react to form the carboxylated sensors................................................................. 81

**Figure 44.** Retrosynthesis of carboxylated SPEC$_1$ and SPEC$_2$. ................................................................. 82

**Figure 45.** Synthesis of trenC that could be used in the synthesis of the carboxylated sensors. 82
Abbreviations

$^{1}\text{H}$ proton
$^{13}\text{C}$ carbon 13
$^{15}\text{N}$ nitrogen 15
$^{17}\text{O}$ oxygen 17
$^{19}\text{F}$ fluorine 19
$^{23}\text{Na}$ sodium 23
$^{31}\text{P}$ phosphorus 31
$^{129}\text{Xe}$ xenon 129
A analyte
ADH alcohoh dehydrogenase
ALDH aldehyde dehydrogenase
$B_0$ strength of the external magnetic field
BPP-LED bipolar pulse pair longitudinal encode–decode
CDCl$_3$ deuterated chloroform
CE chemical exchange
CEST chemical exchange saturation transfer
CHCl$_3$ chloroform
Cl$^-$ chloride anion
CO$_2$ carbon dioxide
cryA cryptophane A
CS$_2$ carbon disulfide
D$_2$O deuterium oxide
DCC N,N’-dicyclohexylcarbodiimide
DCM dichloromethane
DE diffusion edited
DMSO dimethylsulfoxide
DMSO-d$_6$ deuterated DMSO
DNA desoxyribonucleic acid
DNP dynamic nuclear polarization
DS direct saturation
$E. \text{coli}$ Escherichia coli
EI electron impact
eq equivalent
FHL formate hydrogen lyase
FT Fourier transform
$\gamma$ gyromagnetic ratio
$h$ Planck’s constant
HB hydrogen bonding
HB\textsubscript{inter}  intermolecular hydrogen bonding
HB\textsubscript{intra}  intramolecular hydrogen bonding
HCl  hydrochloric acid
HCO\textsubscript{3}\textsuperscript{-}  bicarbonate
hetero-CEST  Heteronuclear CEST
hyperCEST  hyperpolarized CEST
\( l \)  nuclear spin quantum number
iCEST  ion CEST
IDE  inverse diffusion edited
IDP  intrinsically disordered proteins
IR  infrared
ITC  isothiocyanate
\( k \)  exchange rate
\( k_B \)  Boltzmann's constant
\( m \)  magnetic quantum number
M\textsubscript{0}  net magnetization
MAS  magmic angle spinning
MeOH  methanol
MR  magnetic resonance
MRI  magnetic resonance imaging
MRS  magnetic resonance spectroscopy
MRSI  magnetic resonance spectroscopic imaging
MS  mass spectrometry
MTR\textsubscript{asym}  magnetization transfer ratio asymmetry
NaOH  sodium hydroxide
NMR  nuclear magnetic resonance spectroscopy
NOE  nuclear overhauser effect
P  polarization
PHIP  parahydrogen-induced prolaziation
p-TSOH  paratoluenesulfonic acid
R  receptor
Rb  rubidium
rf  radiofrequency
S/N  signal to noise
SEOP  spin-exchange optical pumping
SPE  slow proton exchange
SPIO  superparamagnetic iron oxide
T  temperature
T\textsubscript{1}  spin-lattice relaxation
T\textsubscript{2}  spin-spin relaxation
TBAAc  tetrabutyl ammonium acetate
TBABF\textsubscript{4}  tetrabutyl ammonium tetrafluoroborate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBABr</td>
<td>tetrabutyl ammonium bromide</td>
</tr>
<tr>
<td>TBACl</td>
<td>tetrabutyl ammonium chloride</td>
</tr>
<tr>
<td>TBAI</td>
<td>tetrabutyl ammonium iodide</td>
</tr>
<tr>
<td>TBAP</td>
<td>tetrabutyl ammonium phosphate</td>
</tr>
<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>tren</td>
<td>(2-aminoethyl)amine</td>
</tr>
<tr>
<td>trenest</td>
<td>tris(2-(ethyl(oxy(oxamoyl)))ethyl)amine</td>
</tr>
<tr>
<td>$t_{\text{sat}}$</td>
<td>saturation time</td>
</tr>
<tr>
<td>TUC</td>
<td>thiourea cryptand</td>
</tr>
<tr>
<td>VT</td>
<td>variable temperature</td>
</tr>
<tr>
<td>$\omega_0$</td>
<td>Larmor frequency</td>
</tr>
<tr>
<td>WATERGATE</td>
<td>water suppression by gradient-tailored excitation</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction

Since its first observation in the 1940s, nuclear magnetic resonance (NMR or MR) spectroscopy has undergone major improvements, including Fourier transformation, the development of superconducting magnets and cryogenic probes, and the advent of multi-dimensional techniques. NMR and its spatially encoded counterpart, magnetic resonance imaging (MRI), play a significant role in chemical and biological research for structure determination or the study of supramolecular interactions. They are also involved in the medical field in diagnosis and treatment of pathologies. Portable low field NMRs are now able to increase the range of applications such as the types and sizes of objects that can be studied as well as the locations where NMR analysis can be performed. Development of nanoscale NMR spectrometers or NMR on a chip\(^1\) could improve personalized medicine, detect bacteria or cancer cells proteins, or they could be used in quality control of drug or chemical production. NMR is a powerful and versatile analytical tool that can investigate a wide variety of molecules and their interactions. New NMR sensing techniques are constantly being developed and are of general interest for chemists, biologists, physicists and physicians alike. This thesis focuses on two new techniques and their optimization in terms of sensors and biological applications.

1.1 Physical Basics of Magnetic Resonance

The following sections include some elementary principles of the MR phenomenon. All nuclei possess a nuclear spin quantum number, \(I\). Nuclei with a spin quantum number of a multiple of \(\frac{1}{2}\) such as protons \((^1\text{H}, I = 1/2)\), carbons \((^{13}\text{C}, I = 1/2)\), fluorine \((^{19}\text{F}, I = 1/2)\), phosphorus \((^{31}\text{P}, I = 1/2)\), sodium \((^{23}\text{Na}, I = 3/2)\) and oxygen \((^{17}\text{O}, I = 5/2)\) will be NMR active. Nuclei with spin of zero will not precess in a magnetic field and will therefore be NMR silent. The NMR active
nuclei possess an angular momentum $P$ and a charge. The movement of the charge provides the magnetic moment, $\mu$, according to the following equation:

$$\mu = \gamma P$$

where $\gamma$ is the gyromagnetic ratio, which is constant for each particle or nucleus. When placed in a magnetic field, the nuclei behave as tiny bar magnets such that they will precess at a specific resonance frequency defined by

$$\omega_0 = \gamma B_0$$

where $\omega_0$ is the Larmor frequency of precession, $\gamma$ is the gyromagnetic ratio that is specific for each nucleus and $B_0$ is the strength of the external magnetic field.

In a magnetic field, the spins can only adopt a limited set of spin states $(2I + 1)$ or energy levels. Each level is given a magnetic quantum number $m$ (Figure 1). The energy gap between the states ($\Delta E$), also called the Zeeman effect, is governed by the following equation

$$\Delta E = E_+ - E_- = h\omega_0 = h\gamma B_0/2\pi$$

where $h$ is Planck’s constant. The difference in energy between the number of spins aligned with ($E_+$) and against ($E_-$) the magnetic field is very small and accounts for only $1 \times 10^4$. In this case, more spins populate the lower energy state, according to the Boltzmann distribution:

$$N_+ / N_- = e^{\Delta E/k_B T}$$

where $N_+$ is the number of spins separated by $\Delta E$, $k_B$ is Boltzmann’s constant and $T$ is temperature (Figure 1). If $E_+ = E_-$, the effect would cancel out and no net polarization would be observed. This small difference in spin orientation explains why the sensitivity of NMR is low compared to other analytical techniques.
Figure 1. Energy levels for a nucleus of spin quantum number ½ with increasing magnetic field.

In equilibrium, all spins are oriented in the direction of the magnetic field, defined as the z-direction, and produce a net magnetization $M_0$, which needs to be tilted to obtain a signal. This is achieved by applying another external magnetic field perpendicular to $B_0$ at the Larmor frequency of the nuclei of interest, which for protons is in the radiofrequency (rf) range between 63.9 MHz and 1 GHz. The simplest NMR experiment consists of a $90^\circ$ rf pulse, which will bring the spins in the XY plane. Once the spins are in the XY plane, they will attempt to return to their equilibrium state (in the z-direction), a phenomenon called $T_1$ or spin-lattice relaxation. Another relaxation ($T_2$ or spin-spin relaxation) consists of the dephasing of the spins in the XY plane.

Both $T_1$ and $T_2$ relaxations are important NMR parameters that have implications in the practice of NMR including in chemical exchange.

1.2 Effects of Chemical Exchange Rate in NMR

Chemical exchange (CE) occurs when an NMR active nucleus exchanges between two or more environments with differences in chemical shift, and less frequently scalar coupling or relaxation. The exchange produces diverse and dramatic changes in the NMR spectrum, including line broadening, coalescence and lack of coupling to hydroxide or amine protons.

There are different sub-types of chemical exchange, including mutual vs non-mutual exchange, coupled vs uncoupled spin systems and intramolecular vs intermolecular interactions. In a mutual exchange, the molecule and its NMR parameters after the exchange are the same but the
nuclei have switched. This is the case of N,N-dimethyl groups bonded to a $\pi$ system, as in the simple uncoupled spin-system of dimethylformamide\textsuperscript{5} or azapropazone (Figure 2)\textsuperscript{6} or more complicated \textit{para-}nitrosodimethylaniline,\textsuperscript{7} which involves swapping of scalar coupling in addition to chemical shifts.

Figure 2. An simple example of mutual chemical exchange involving a N,N-dimethyl group bonded to an aromatic ring, reproduced with permission from Annual Reports on NMR Spectroscopy.\textsuperscript{8}

Non-mutual exchange occurs when a different molecule is created after the exchange process and the two forms exhibit different energies and Boltzmann populations. The cis-trans isomerization of 2-furanaldehyde (furfural) is a classic example of non-mutual exchange, in which the aldehyde exists in two unequally-populated conformations at room temperature.\textsuperscript{8} In
addition to exchange generated in the same molecule, it is also possible to encounter intermolecular exchange reactions. This kind of exchange is prevalent in biological NMR, such as amide protons on the surface of proteins exchanging with the aqueous environment\textsuperscript{9,10} or ion binding to large proteins.\textsuperscript{11-12} It is also found in chemistry\textsuperscript{13} in small molecules and their interactions with ions.\textsuperscript{14-15}

To observe the effects of chemical exchange in a spectrum, the system under study needs to display appropriate exchange rates that are related to the NMR timescale.\textsuperscript{16} The resonance frequency of a nucleus involved in an exchange process is related to the lifetime of that frequency according to the following equation:

\[
\Delta \omega = \frac{h}{2\pi \tau}
\]

where \(\Delta \omega\) is the energy difference between the resonance frequencies, \(h\) is Planck’s constant and \(\tau\) is the lifetime of the resonance frequency. The exchange process depends on the difference between the frequencies and their lifetime. The frequencies can only be observed if their lifetime is long enough to be detected by the spectrometer. As the lifetime decreases, the difference between the frequencies increases and line broadening is observed in the spectrum. As such, when the exchange rate approaches the NMR timescale of the experiment (intermediate exchange), it is difficult to obtain meaningful data, due to extreme line broadening. More suitable rates for NMR study are either faster (fast exchange) or slower (slow exchange) than the intermediate regime. Methods to determine the exchange rate for both slow\textsuperscript{13, 17-18} and fast\textsuperscript{19-20} exchange regimes are well-established. Fast exchange occurs well after coalescence when the frequencies in the NMR spectrum appear as one single narrow line that can be analyzed after deconvolution. In this case, line broadening is still produced by the exchange, but it is comparable to broadening due to field inhomogeneities and natural linewidth. In slow exchange, the difference between the lines is larger than the exchange rate, so the signals for each state can be detected separately. Therefore, a multitude of dynamic processes can be studied by NMR, depending on the exchange rate and the timescale of the exchange. The rest of this thesis will focus on exchanging systems involved in sensing of chemical processes.
1.3 MR based Sensing

Sensing, in chemical terms, involves a molecular recognition event that is translated into a measurable signal detected by an analytical instrument, in this case an NMR spectrometer. Molecular recognition occurs between two or more molecules through a noncovalent interaction such as hydrogen bonding, electrostatic effects, metal coordination, anion binding, π-π interactions, halogen bonding, etc. Due to the low intrinsic detection limit of NMR, which is on the order of low to sub-millimolar concentrations, NMR sensing lacks sensitivity compared to other analytical methods including isothermal calorimetry, UV-visible absorption and fluorescence spectroscopy, which can detect concentrations as low as picomolar. The initial NMR sensing methods were based on direct detection of NMR signals produced by the molecular recognition event, but recently developed indirect detection techniques such as chemical exchange saturation transfer (CEST, see section 1.3.1.2.1) can provide increased sensitivity due to signal amplification. NMR spectroscopy has specific advantages in that it can provide quantitative as well as qualitative information and is less affected by changes in concentrations and temperatures. NMR methods can also be combined with spatial encoding, which provides 3D information in methods such as MRI and magnetic resonance spectroscopy (MRS). Since NMR detects nuclei with a spin quantum number of ½, such nuclei intrinsic to living organisms, including $^1$H and $^{31}$P, are useful endogenous sensors particularly for sensing pH using the histidine NHs and inorganic phosphate peaks, or metabolism by observing the phosphorylated glucose breakdown products \textit{in vivo}. $^1$H NMR can easily be implemented for \textit{in vivo} sensing since protons are highly sensitive and present in 80 M concentrations in humans. However, signal overlap from different species \textit{in vivo} is a disadvantage. Phosphorus is less sensitive but allows for detection of more specific molecules. In addition to endogenous sensors, a plethora of synthetic sensors have also been developed based on $^1$H, $^{13}$C, $^{19}$F, $^{31}$P and $^{129}$Xe nuclei (see section 1.3.1 for more details). \textit{In vivo} exogenous sensors need to possess optimal parameters such as high aqueous solubility and stability to avoid disrupting physiological metabolism and to prevent a pathological response, low toxicity and targeted distribution and for intracellular sensors, membrane permeability and cell trappability. In addition, the sensors also need to produce a high signal-to-noise ratio in a short amount of time, be concentration independent and free of background interference.
1.3.1 Methods for MR Sensing

Different methods for MR sensing have been developed over the years. The following section

describes the major sensing strategies, starting with basic NMR approaches, followed by
spatially encoded MRI and high sensitivity hyperpolarization methods.

1.3.1.1 NMR Sensing

NMR based sensors can be classified into two major categories: fast exchanging and slow
exchanging sensors. When a sensor interacts with an analyte like a proton, cation, anion, a small
molecule, if the strength of the interaction is weak, the rate of exchange between the free form of
the sensor and the analyte-bound complex is fast and the interaction will generate an observable
chemical shift change (Figure 3a). This is the fast exchanging scenario. In this case, the signals
for each species do not appear on the spectrum. Only an average between the free and bound
form of the sensor can be seen. For example, most NMR pH sensors display fast exchanging
properties because protonation/deprotonation reactions occur quickly (see section 3.1 for specific
examples). Another example consists of a fluorinated derivative of the calcium-selective sensor
BAPTA that was used to selectively detect calcium with $^{19}$F NMR.21 With this fast exchanging
sensor, intracellular concentrations of calcium could be detected in mouse thymocytes with no
major effect from competing cations such as Mg$^{2+}$, Zn$^{2+}$, Fe$^{2+}$ or Mn$^{2+}$. Methods to study protein-
protein or protein-ligand interactions involving chemical shift changes upon binding or based on
relaxation times or transferred NOEs have also been developed and can be used for drug
screening.22 Fast exchanging sensors are the most common in NMR spectroscopy, but they
possess some disadvantages, including dependence on chemical shift, which can be affected by
other factors, such as magnetic field inhomogeneities, binding to other species in the sample, like
metal ions, or temperature. The low sensitivity of NMR itself can also hinder sensing since
detection of the sensor peaks is necessary to obtain meaningful data. In addition, factors causing
peak broadening such as interactions with a large macromolecule also produce spectra difficult
to interpret and prohibit accurate sensing.

If the sensor-analyte interaction is strong, the exchange rate between the two states will be slow
and distinct peaks for the free and bound form of the sensor will be observed in the NMR
spectrum (Figure 3b). In this case, rather than generating a chemical shift change, variations in
ratio between the free and bound forms of the sensor will occur. The rate of exchange is crucial,
since very slow binding or exchanging systems will require long mixing and experimental times because the peaks of the bound form of the sensor will not be readily detected. Since the slow exchanging sensors rely on changes in ratio to obtain a signal, they are generally more accurate than the fast exchanging sensors, because they provide a platform for ratiometric sensing, which includes important advantages such as chemical shift and concentration independence.

Ratiometric sensing has become important for fluorescence spectroscopy,\textsuperscript{23-25} but examples in NMR spectroscopy have also been reported (see section 2.1 and 3.1 for specific examples). The use of ratiometric sensing is beneficial in NMR spectroscopy and can easily be applied to spatially encoded MRI and MRS, particularly because many MR sensors are concentration dependent.

![Figure 3](image_url)

**Figure 3.** Chemical exchange effect on NMR spectrum of an intermolecular reaction of a receptor (R) with an analyte (A). a) fast exchange: the peaks of the receptor and analyte show a chemical shift change upon increase in concentration of analyte. b) slow exchange: the peaks of free (R\textsubscript{f}) and bound (R\textsubscript{b}) form of the receptor are chemical shift independent but their ratio changes with increase in analyte. A\textsubscript{b} = bound analyte and A\textsubscript{f} = free analyte
1.3.1.2 MRI Sensing

MRI is based on NMR technology with the added advantages of high spatial resolution and the ability to image physiological and pathological processes deep within tissues. For decades, gadolinium based contrast agents (GBCAs) and superparamagnetic iron oxide probes (SPIOs) were used as MRI sensors for both research and clinical applications. Recently, new methods for MRI were developed called CEST and hyperpolarization.

1.3.1.2.1 Chemical Exchange Saturation Transfer

In 2000, the CEST mechanism for MRI was proposed.$^{26}$ It is based on chemical exchange between two pools of protons, a large water protons pool and a smaller exchangeable (solute) proton pool, combined with rf irradiation, also called direct saturation (DS), at the exchangeable proton frequency (Figure 4a). If the saturation pulse is applied for a specific amount of time ($t_{\text{sat}}$) and the exchange rate ($k$) is appropriate, saturation of the exchangeable protons will transfer to the bulk water pool and will cause a detectable decrease in the water signal (Figure 4b). This reduction of the water peak is represented using a Z-spectrum also called CEST spectrum (Figure 4c) and provides an indirect and amplified signal, which allows detection of sensors in the milimolar to high micromolar range. Due to the short distance between the solute and bulk water peaks, it is possible that DS interferes with the water peak. To overcome this issue, magnetization transfer ratio (MTR) asymmetry is applied. It consists of applying a saturation pulse equidistant from the solute and bulk water peak ($\Delta\omega$) on the opposite side of the latter (i.e. $-\Delta\omega$):

$$MTR_{\text{assym}}(\Delta\omega) = MTR(\Delta\omega) - MTR(-\Delta\omega) = S_{\text{sat}}(-\Delta\omega)/S_0 - S_{\text{sat}}(\Delta\omega)/S_0.$$

Despite providing more accurate results, the MTR detection method assumes independent contribution of the solute and bulk signals which isn’t necessarily true. Another assumption that introduces errors in measurements is that non-CEST effects are symmetric around the water peak.
Figure 4. Chemical exchange saturation transfer (CEST) principle. a, b) Exchangeable protons (blue) are saturated at their specific resonance frequency (here 8.25 ppm for amide protons). This saturation is transferred to water at 4.75 ppm. After a period \( t_{\text{sat}} \), the partial saturation of the water peak becomes visible (b, right). c) Normalized water saturation \( S_{\text{sat}}/S_0 \) measurement depending on the irradiation frequency creates a Z-spectrum (or CEST spectrum). The frequency of the water peak is assigned to 0 ppm in Z-spectra. d) Magnetization transfer ratio (MTR) asymmetry analysis of the Z-spectrum to remove direct saturation effects, reproduced with permission from Magnetic Resonance in Medicine.

The CEST contrast possesses two main advantages. First, contrast can be turned on at will, when the selective radiofrequency saturation at a specific frequency is applied. The second advantage is simultaneous imaging of different mobile proton resonances. This can be used to specifically saturate those resonances to provide a “multi-color” image of different agents at the same time.

CEST has been applied to endogenous molecules, such as glutamate, creatine, glucose and others that possess suitable exchangeable protons. In addition, synthetic sensors designed specifically for CEST have been developed. For example, paramagnetic complexes (PARACEST agents) produce highly shifted protons, which increase the exchange rate suitable to obtain the CEST contrast. These agents, however, can be toxic due to the presence of the paramagnetic atom and the lack of biodegradability of the complexes.
The majority of CEST sensors developed so far are based on $^1$H MRI but other heteronuclei like $^{19}$F or $^{129}$Xe have also been studied (Hetero-CEST). Since both those nuclei are not present in biological tissues, better MRI images can be obtained due to the absence of background interferences. $^{19}$F MRI was used to detect calcium selectively and with high sensitivity using a fluorinated derivative of BAPTA (mentioned in section 1.3.1.1). $^{33}$ Exchange between the free and Ca$^{2+}$ bound form of the sensor was suitable to obtain a selective CEST contrast, called ion CEST (iCEST), for Ca$^{2+}$ over other ions like Mg$^{2+}$ and Zn$^{2+}$. $^{129}$Xe can also produce a CEST signal, but it is commonly used with a hyperpolarization method, which is presented in the next section.

### 1.3.1.3 Hyperpolarization

The intrinsically low sensitivity of MR experiments can be improved through a method called hyperpolarization. During an MR experiment, polarization (P) of nuclei occurs automatically when an external magnetic field is applied and is proportional to signal intensity, according to the following equation,

$$ P = \frac{N_+ - N_-}{N_+ + N_-}. $$

Under thermal equilibrium, the populations of ground and excited spin states are roughly identical and cancel each other to give low net magnetization, hence low signal intensity. For a nucleus with $I = \frac{1}{2}$, polarization is given by

$$ P = \tanh\left(\frac{\gamma \hbar B_0}{2k_B T}\right) $$

where tanh is the hyperbolic tangent. For a more intense signal, the population difference between the two spin states needs to be increased (Figure 5), which can be achieved by increasing the magnetic field and/or decreasing the temperature, according to the Boltzmann distribution equation ($N_+/N_- = e^{\Delta E/k_B T}$) described in section 1.1.
There are different ways to hyperpolarize a sample, like parahydrogen induced polarization (PHIP), but for the purpose of my thesis, I will only focus on two methods: dynamic nuclear polarization because of its versatility and spin-exchange optical pumping because it involves chemical exchange.

1.3.1.3.1 Dynamic Nuclear Polarization

Dynamic nuclear polarization (DNP) was first developed in 2003 to increase sensitivity of the MR signal by at least 10,000 fold for biological applications. DNP is the most versatile hyperpolarization methods since it can hyperpolarize every single nucleus in a sample. DNP is ideal for quaternary $^{13}$C-labelled carbons because hyperpolarization is not lost through spin-spin coupling of the hydrogen atoms. $^1$H and $^{15}$N can also be polarized with this method although loss of polarization through spin-spin coupling in $^1$H and the low natural abundance and gyromagnetic ratio of $^{15}$N limits their applications.

The mechanism involves unpaired electrons that need to be placed homogeneously in the sample. To do this, organic free radicals and a glassing agent are added to prevent crystallization and produce an amorphous solid. At low temperature and high magnetic field, electrons are highly polarized due to their high gyromagnetic ratio. Polarization is then transferred to the nuclei through microwave irradiation. $^1$H and $^{13}$C can be polarized in this way to almost 100 and 50% respectively. Once the nuclei are polarized, the sample is brought to 37 °C to be used in vivo. It
is important that the dissolution and injection process occurs in a timely fashion, since the \( T_1 \) of \(^{13}\text{C} \) nuclei is on the order of tens of seconds, and the increase in signal intensity due to hyperpolarization depends on \( T_1 \).

The major applications of DNP are in material science\(^{38-39}\) and most importantly in biological and biomedical imaging.\(^{40}\) Vascular imaging and angiography is now possible using hyperpolarized probes.\(^{41}\) Hyperpolarized \(^{13}\text{C}\)-pyruvate and \(^{13}\text{C}\)-labelled glucose among other metabolites have been used extensively in the study of healthy and pathological metabolism of the brain, skeletal and cardiac muscles in, mice,\(^{42}\) rats\(^{43}\) and pigs.\(^{44}\) Detection of pH using bicarbonate has also been studied for its applications in cancer diagnosis and treatment.\(^{45}\) Although signal amplification using DNP open many application possibilities, the short \( T_1 \) of the probes prohibits their detection over long periods of times and can affect measurement accuracy as well.

1.3.1.3.2 Spin-Exchange Optical Pumping

Other than the \(^{13}\text{C} \) nucleus, noble gases can be hyperpolarized by spin-exchange optical pumping (SEOP). SEOP has been used extensively with \(^3\text{He} \) and \(^{129}\text{Xe} \) gases to image void spaces like the lungs. Particularly, \(^{129}\text{Xe} \) has advantages such as a spin of \( \frac{1}{2} \), and no background interference from biological samples, reducing issues of dynamic range. It is also soluble in organic and aqueous solvents and possesses a large, polarizable electron cloud that provides a large chemical shift window of Xe in magnetically different environments, like in DMSO, water, blood, bound to hemoglobin or cryptophanes, which are synthetic cages developed to bind xenon selectively with high affinity.

The SEOP mechanism is similar to DNP in that it needs a source of electrons that can be easily polarized. The electrons come from the alkali metal rubidium (Rb) in vapor form, which is mixed with the xenon gas. Irradiation of the mixture using circularly polarized light generated by lasers polarizes the electrons of Rb and the polarization is then transferred to the noble gas via spin exchange. A variety of applications using hyperpolarized Xe have been performed,\(^{46-47}\) but the signal still suffers from low sensitivity (subnanomolar concentrations cannot be detected) compared to other imaging methods. Recently, hyperpolarization has been combined with CEST to provide an even greater signal, in a method called hyperCEST. Cryptophane A (CryA), a small hydrophobic cage like molecule that binds xenon with an ideal exchange rate for CEST,
has been used for hyperCEST measurements. CryA minimally relaxes xenon and produces a chemical shift when bound to xenon far enough to be used for CEST imaging. The $T_1$ of bound xenon is $\sim 150$ min, which allows for longer acquisition, as opposed to $^{13}$C-labelled compounds. HyperCEST was first developed in 2006\cite{48} and increased signal sensitivity into the submicromolar to nanomolar range with short acquisition times. In addition, the cryptophane cage can be attached to different ligands, which opens the doors for a plethora of applications including targetable responsive agents. Therefore, although hyperpolarized Xe might not provide crucial improvements in sensitivity, the combination with CEST produces signals detectable in the nanomolar range.

1.4 Rationale

NMR, as a versatile analytical tool, can provide information on dynamic processes involving chemical exchange, which is a very well-known NMR phenomenon. Drastic variations can be observed in NMR spectra in a system involving exchange, including chemical shift changes and line broadening. Those variations provide opportunities for NMR sensor design. Important MR methods using this CE concept have been developed, such as CEST or hyperpolarization, but sensing accuracy and sensitivity remain a challenge. To address this, we have focused on the development of small synthetic molecular cages with similar structure that use chemical exchange is very different ways to sense pH accurately or detect anions indirectly at unusually low concentrations for NMR.

To explore the possibility of ultrasensitive NMR detection, we synthesized a small thiourea-based cryptand, TUC, which, used a change in the chemical exchange rate from slow to fast to detect anions (indirectly) through anion-induced conformational flexibility ) as low as 120 nM concentrations (Chapter 2).

A new method for accurate pH sensing was developed based on a small synthetic cryptand, SPE1, which has a similar molecular structure as TUC, in that it possesses 3 ureido groups instead of the 3 thiouredio groups of TUC. SPE1 can bind protons selectively in slow exchange compared to the NMR timescale. This slow proton exchange (SPE) technique allows ratiometric sensing and provides high accuracy in pH measurements of 0.02 pH units. The technique and its applications in bacterial culture are presented in Chapter 3.
To investigate whether the SPE method could produce highly sensitive signals, a $^{13}$C-labelled version of SPE1 ($^{13}$C-SPE1) was synthesized. This sensor produced sensitive and accurate pH determination by $^{13}$C NMR due to $^{13}$C enrichment and decreased background noise. The possibility to DNP-hyperpolarize $^{13}$C-SPE1 to obtain more sensitive signals was also studied (Chapter 4).

Because the $pK_a$ of SPE1 was slightly basic and numerous biological applications occur in more acidic environments, optimization of the $pK_a$ of SPE1 was examined. Substitution of an oxalamide group instead of the urea group of SPE1 produced SPE2 that exhibited a lower $pK_a$, ideal for biological applications as well as a larger pH window (Chapter 5).
Chapter 2

Ultrasensitive Anion Detection by NMR Spectroscopy: a Supramolecular Strategy Based on Modulation of Chemical Exchange Rate

A significant portion of this chapter has been adapted from the article below. Changes have been made for clarity and consistency. Experiments not included in the manuscript have also been added.


2.1 Introduction

Monitoring and quantifying intermolecular non-covalent interactions, including hydrogen bonding (HB), ion pairing, dipole or electrostatic interactions, is of paramount importance in supramolecular chemistry and can be carried out using different analytical techniques such as isothermal calorimetry, UV-visible absorption, fluorescence or nuclear magnetic resonance (NMR) spectroscopy. These studies are commonly conducted by titration to obtain thermodynamic and kinetic parameters and have been increasingly applied for detection and quantification of analytes (sensing). NMR spectroscopy has the unique ability to provide detailed structural information at the atomic level, and versatile readout methods (i.e. pulse sequences), which are available to give information about both structure and dynamic processes. Therefore, NMR is widely used to characterize supramolecular interactions. This powerful
technique, however, is limited for sensing purposes, due to low intrinsic sensitivity for direct signal detection.

Anion sensing has been developing intensely in the past decades. Anions play an important role in biological systems. For example, DNA and RNA are polyanions and many enzyme substrates, co-factors and products of metabolism are anionic. For example, pyruvate, citrate and succinate are negatively charged products of the citric acid cycle, which is involved in cell respiration and energy production. Anions are also involved in catalysis, environmental science and medicine. Since anions are important in many recent scientific fields, having the ability to detect them is becoming a major need. However, the design of anion sensors is more challenging than cation sensors due to the larger size of isoelectronic anions. They have a lower charge to radius, which decreases the strength of non-covalent interactions involved in the molecular recognition event. Anions also have a variety of geometries, which forces chemists to design receptors for each specific geometry. Finally, anions only function within a certain pH window, as they can become protonated at low pH and therefore lose their negative charge. Solvent effects can also influence binding strength and affinity.

Conventional approaches to the design of supramolecular anion sensors typically require a specific receptor for the target anion. When intermolecular interactions between receptor and anion are studied by NMR spectroscopy, two classical scenarios must be distinguished depending on the chemical exchange rate of the binding equilibrium. If the non-covalent binding of analyte to the receptor is strong and the exchange rate is slow (slower than the NMR timescale), distinct NMR signals for free and bound species will be detected separately. On the other hand, if the interaction is weak and in the fast exchange regime, the interaction generates an observable chemical shift change which corresponds to the average signal between the peaks of the free and bound species. For example, the neutral receptor shown in Figure 6 was designed to mimic phosphate and sulfate binding proteins. It can bind anions using well-positioned hydrogen bond donor groups such as thioureas and amides, which, when combined, are known to provide higher binding affinity. In particular, this neutral anion receptor binds strongly sulfate anions in a 1:1 manner, using 9 intermolecular hydrogen bonds, with 6 bonds involved with the thiourea groups and 3 with the amide groups. This interaction occurs slower than NMR timescale so that the peaks of the free and bound form of the receptor can be seen independently and simultaneously. The same receptor also shows fast exchange binding with
acetate because the acetate anion only binds the thiourea moieties and does not interact with the amide groups. In this case, a chemical shift change can be seen upon increase of anion concentration in the NMR spectrum.65

Figure 6. Structure and proposed binding modes of a neutral anion receptor that can bind a) sulfate anions using 9 HBinter slower than NMR timescale and b) spherical anions like acetate with 6 HBinter in the fast exchange regime, reproduced with permission from Supramolecular Chemistry.65

In both scenarios, NMR spectroscopy can typically monitor complex formation with a host concentration in the mM to sub-mM range with regular NMR setup69-70 and is generally able to detect guest effects of 0.1 eq or more.53, 59, 70-72 Therefore, sensitivity is a major drawback of NMR spectroscopy, mainly due to its poor intrinsic detection limit. Sensing anions is generally more challenging as opposed to the well-established metal cation detection,73 in part because the supramolecular anion receptors with high selectivity and affinity are relatively rare and structurally more complicated for practical applications.57-58, 74-77

Herein, a fundamentally different and significantly more sensitive approach for anion detection by 1H NMR spectroscopy is reported, which does not rely on a specific anion receptor. This indirect anion sensing technique detects the changes of CE rate of the sensor NMR signals, based on anion-induced conformational flexibility. Trace amounts of anions can be detected by this novel method in a selective manner at very low concentrations (120 nM) due to the transient and catalytic nature of the anion-sensor interaction.

2.2 Results and Discussion

2.2.1. Design and Synthesis of TUC as an NMR Anion Sensor

Intramolecular HB (HBintra), whether in small synthetic molecules or in large proteins, can provide conformational stability that might be easily disrupted due to the weak and transient nature of this non-covalent interaction.78 Conformational fluctuations arising from competition between these different interactions can be detected by NMR in small compounds79-82 similar to
conformational changes observed in biomacromolecules such as proteins. Therefore analyte-induced conformational fluctuations can potentially be employed as an NMR readout for sensing purpose, in addition to the well-established approaches, such as monitoring the chemical shift change (fast exchange scenario) or emergence of new peaks from the complex (slow exchange scenario) aforementioned. To test this unexplored approach, a pre-organized thioureido cryptand, TUC (Figure 7), which was originally developed to study vanadate binding, was chosen for the current study because it displays an exceptional conformational rigidity due to the formation of HB$_{\text{intra}}$ between the thioureido moieties. As the smallest bicyclic thiourea cryptand known to date, TUC is composed of three thiourea containing arms in a $C_3$ symmetric fashion. Widely used in neutral anion receptor design, the thioureido group consists of one HB acceptor (C=S) and two HB donor (NH) sites, capable of forming bifurcated HBs with an anion intermolecularly or with another thiourea intramolecularily.

![Figure 7.](image) The molecular structure of TUC. Thermal ellipsoids at 50% probability. a) Side view, methylene hydrogens omitted for clarity. b) View along N(2)-N(2)' axis, methylene hydrogens omitted for clarity.

Exposing TUC to anions should in principle induce its conformational flexibility, since HB$_{\text{intra}}$ is competitively disrupted by formation of intermolecular hydrogen bonds (HB$_{\text{inter}}$) between the thioureido hydrogens and anions in the solution. In relation to the NMR spectrum, the competition between HB$_{\text{inter}}$ and HB$_{\text{intra}}$ produces significant changes, which can be used as a sensitive readout for anion detection.

TUC was prepared from tris-(2-aminoethyl)amine (1) which was converted to a tripodal isothiocyanate intermediate (2). Equimolar coupling of 1 and 2 under high dilution conditions
gave TUC, the tris-thiourea cryptand product (TUC, 3) in a quasi-quantitative yield (Figure 8 and Methods for synthetic details).

![Synthesis of TUC](image)

2.2.2. Dynamic Intramolecular Hydrogen Bonding in TUC

To confirm the existence of HB\textsubscript{intra} and to obtain conformational information, the structure of TUC was determined by X-ray crystallography (Figure 7). Single crystals of TUC can be obtained from CHCl\textsubscript{3} or DMSO/water solutions by solvent evaporation or vapor diffusion, respectively. Both procedures gave a comparable high quality structure. In the solid state, TUC displays a mirror plane symmetry and possesses only one pair of bifurcated HB\textsubscript{intra}, between the two thioureido hydrogens NH(1) and the neighbouring sulfur atom S(2) (Figure 7). The bond angle involved in HB (N1-H1-S2) is 161° and the N(1)-S(2) distance is 3.473(3) Å, suggesting a relatively weak hydrogen bond. The other two sulfur atoms, S(1) and S(3), are pointing outwards and involved in the formation of HB\textsubscript{inter} with NH of neighboring TUC molecules instead (see Tables A2 to A87 in the appendix for details). Because only one sulfur atom is involved in HB\textsubscript{intra}, the three thiourea arms are not identical and TUC does not show the expected $C_3$ symmetry. This is not surprising since the simultaneous formation of more than one pair of HB\textsubscript{intra} would cause significant increase in conformational ring strain in TUC, and therefore only one pair of HB\textsubscript{intra} can exist at one time point.

In solution, however, TUC appears to be a highly symmetrical cryptand possessing a $C_3$ symmetry axis as well as a mirror plane, as demonstrated in the NMR spectrum (Figure 9). The $^1$H NMR spectrum of TUC in DMSO-$d_6$ contains only 3 peaks at 7.09, 3.48 and 2.56 ppm with a 1:2:2 ratio corresponding to the 6 thioureido NH hydrogens, the 12 methylene hydrogens close to
the thioureido group and the 12 methylene hydrogens directly bonded to the bridgehead nitrogen, respectively (Figure 9a), consistent with expected high symmetry.

![Figure 9](image)

**Figure 9.** $^1$H NMR spectra of TUC at 500 MHz demonstrating the effects of solvents and temperature and the assignment of NMR signals. a) in DMSO-$d_6$ at 25°C. b) in CDCl$_3$ at 25°C. c) in CDCl$_3$ at -40°C. d) Structure of TUC with assignment of methylene hydrogen atoms. e) Newman projection from the methylene carbons of TUC, used for assigning the methylene hydrogen atoms to the corresponding NMR signals. Solvent peaks are labelled * for DMSO, ** for dichloromethane and *** for chloroform.

Interestingly, the $^1$H NMR spectrum of TUC in a less polar solvent such as CDCl$_3$ exhibits a drastically different pattern than in DMSO-$d_6$, containing 5 peaks instead of 3 at 6.66, 4.63, 3.00, 2.80 and 2.42 ppm with equal integrals (Figure 9b). The significantly broader peaks in CDCl$_3$ (average peak width at half height is ~30 Hz in CDCl$_3$ in contrast to 5-10 Hz in DMSO-$d_6$) indicate possible involvement of CE. All peaks become sharper at low temperature and their splitting patterns can be resolved at -40°C (Figure 9c). Based on the chemical shifts, coupling constants and 2D NMR data (Figure 10 and 11), all peaks can be reasonably assigned as shown in Figure 9d-e. The peaks at 4.63 ppm and 3.00 ppm can be assigned to the two geminal methylene hydrogen atoms closer to the thioureido group and the signals at 2.80 and 2.42 ppm belong to the two geminal methylene hydrogen atoms next to the bridgehead nitrogens. The NMR inequivalency of the geminal hydrogens is consistent with the X-ray structure which also reveals that these geminal hydrogen pairs in TUC are found in different chemical environments. The unusual splitting of the geminal –CH$_2$– NMR signals suggests a relatively higher conformational rigidity of TUC in CDCl$_3$ than in DMSO. The pair of bifurcated HB$_{\text{intra}}$ between the neighbouring thioureido groups most likely significantly contributes to this rigidity. Since TUC also exhibits $C_3$ symmetry in CDCl$_3$ solution, the HB$_{\text{intra}}$ must be shifting between the three thioureido arms faster than NMR timescale without causing dramatic conformational reorientation of the macrocycle.
Figure 10. Partial $^1$H NMR of 1.7 mM TUC at -40 °C and 500 MHz with coupling constants labelled.

$^1$H NMR (600 MHz, Chloroform-d) δ 6.69 (d, $J = 9.4$ Hz, 2H), 4.55 (q, $J = 12.5$ Hz, 2H), 3.00 (dd, $J = 13.8, 2.5$ Hz, 2H), 2.76 (td, $J = 13.6, 3.1$ Hz, 2H), 2.43 (d, $J = 13.4$ Hz, 2H).

Figure 11. Partial gDQCOSY of TUC at -40 °C at 500 MHz.

This dynamic $C_3$ symmetry is maintained even at very low temperature, as the three thioureido arms still exhibit identical signals at -40°C (Figure 9c), suggesting a low energy barrier for the rapid transfer of the HB$_{intra}$ pair among the three arms. This low barrier can be attributed to the head-to-tail circular orientation of the three thioureido groups as shown in the X-ray structure (Figure 7), which facilitates the rapid translocation of the single HB$_{intra}$ pair among the three
arms without major change in conformation. To maintain a fluctuating HB$_{\text{intra}}$ pair, the three thioureido groups must retain the same relative dipole orientation, either clockwise or anti-clockwise (Figure 12). Only switching the relative dipole orientation of the three thioureido groups simultaneously, i.e., from clockwise (Figure 12a) to anti-clockwise (Figure 12b), or vice versa, would cause the interconversion of chemical environments between the geminal hydrogen pairs (H$_a$ becomes H$_b$, H$_c$ becomes H$_d$, and vice versa). This mechanism enables the NMR CE between the geminal hydrogen pairs. In CDCl$_3$, this chemical exchange is slower than NMR time scale. Polar solvents such as DMSO are HB acceptors, thus can compete with and break HB$_{\text{intra}}$. Therefore, conformational rigidity is diminished in DMSO (Figure 9a) and the otherwise chemically inequivalent geminal hydrogen atoms become dynamically equivalent due to rapid CE through conformational reorientation (Figure 12). The NH signal in DMSO-$d_6$ (7.09 ppm) is significantly shifted to a higher frequency in contrast to that in CDCl$_3$ (6.66 ppm), supporting the occurrence of extra HB$_{\text{inter}}$ involving NH and solvent molecules.

![Figure 12](image)

**Figure 12.** Schematic mechanism of conformational chemical exchange between geminal hydrogens in TUC. Top view of TUC is shown in a dynamic average $C_3$ symmetry, with the dipole moment of three thiourido groups in a clockwise (a) or anti-clockwise (b) orientation. Only selected representative hydrogen atoms are shown for clarity.

### 2.2.3. Anion Sensing Using Competition Between HB$_{\text{intra}}$ and HB$_{\text{inter}}$

The drastic change observed in the NMR spectrum in response to alterations of CE rate induced by HB competition can be utilized for sensing purposes. To verify the feasibility of this novel approach, TUC was first exposed to chloride anion, a strong hydrogen bond acceptor. A solution of TUC (1.7 mM in CDCl$_3$) was titrated with tetrabutylammonium chloride (TBACl) and the titration was followed by $^1$H NMR (Figure 13). Adding a small quantity of Cl$^-$ (0.0014
equivalents, or 700:1 host-guest ratio) led the geminal resonances at 2.42 and 2.80 ppm to coalesce and produced one broad peak at 2.64 ppm. Meanwhile, the other geminal hydrogen pair at 4.63 and 3.00 ppm became significantly broader, but did not coalesce due to larger difference in their chemical shifts. These expected effects confirmed the increase of conformational flexibility of TUC induced by Cl−. Upon increase in Cl− concentration, the broad single peak at 2.64 ppm became sharper and the separate geminal –CH2– signals at 4.63 and 3.00 ppm became even broader, and eventually emerged into a single peak at 3.86 ppm with 0.028 eq. of Cl− added, consistent with a gradual increase in CE rate.

![Figure 13. 1H NMR titration of TUC in CDCl3 at 500 MHz and 25 °C (1.7 mM) with chloride (as a TBA salt).](image)

To further illustrate the high sensitivity of the current method, anion detection was carried out using only trace amounts of Cl−. As demonstrated in Figure 14, detectable peak broadening could be reliably observed at Cl− concentrations as low as 120 nM, corresponding to 1/14,000 equivalents of that of the sensor. Stepwise increase of the anion concentration continuously led to further broadening of the peaks. This catalytic effect of anion decreases the activation barrier of conformational interconversion by weakening of HB_{intra} and increases the kinetics of this
reversible process. Similar effects can also be produced with increase in temperature, as followed by the broadening effect on the NMR spectrum (Figure 15).

![Image](image_url)

**Figure 14.** Partial \(^1\)H NMR spectra of 1.7 mM TUC at 500 MHz and 25 °C in the presence of trace amount of Cl\(^-\).

Overall, this kinetic-related mechanism is different from the conventional anion sensing methods, which are mainly based on the thermodynamic molecular recognition of anions by the receptors. It should be noted that for practical purposes, all NMR titration experiments were carried out with regular NMR settings. Higher sensitivity in principle can be obtained by using lower concentration of sensor, changing acquisition parameters or optimizing the hardware.
Figure 15. Variable temperature partial $^1$H NMR spectra of 1.7 mM TUC with 7 meq TBACl between -40 °C and 35 °C in CDCl$_3$ at 500 MHz.

The unprecedented high sensitivity for NMR anion sensing demonstrated here relies on the detection of competition between HB$_{\text{intra}}$ and HB$_{\text{inter}}$. Due to the transient nature of these HBs, the competition effect of anions can be efficiently amplified in a catalytic manner. This novel approach is therefore significantly more sensitive than traditional NMR detection methods which are based on specific anion receptors. When a strong receptor is used, the anion is usually detected by directly monitoring the formation of anion-receptor complex, which is in slow exchange with the free receptor. To compare the sensitivity of the current method with direct NMR detection, the signal of the counter ion (TBA) was used as a surrogate measure of [Cl$^-$]. As shown in Figure 13, the N(CH$_2$)$_4$ methylene signal of TBA at 3.43 ppm (black arrow) only appeared at 28 meq (~ 50 μM) Cl$^-$. As a reference to 1 equivalent of chloride anion, this detectable $^1$H NMR signal corresponds to 8 equivalents of hydrogen atoms (~ 400 μM per hydrogen), more than three orders of magnitude higher than the low concentration (120 nM) detected by the current method using same settings.

The majority of weak anion receptors, which include most thiourea based receptors, bind anion in the fast exchange mode. In these cases, detection of anion typically relies the on chemical shift
change of receptor resonances induced by anions. For these receptors, the sensitivity of anion detection is determined by multiple factors, such as the magnitude of the chemical shift change ($\Delta \delta$) between free receptor and bound complex, the concentration of the sensor, and the binding constant. For weak receptors, the latter can be the limiting factor. These receptors generally can only detect high concentrations of anions (starting at ~ 100 meq). Interestingly, TUC possesses thioureido groups, which also display a high-frequency shift after addition of high concentrations of anions. The NH peak is shifted up to 0.53 ppm after addition of up to 15 eq chloride. Because TUC has a relatively low affinity for the chloride anion, $\Delta \delta_{NH}$ is rather insensitive for detecting trace amounts of anions. This signal, however, expands the range of anion concentrations that can potentially be quantified with TUC, when combined with the signal from geminal hydrogen atoms. Quantitative analysis can be carried out using peak width or chemical shift change (Figure 16). Peak width at 4.63 ppm allows quantification in the high nM range. Micro-molar concentrations of anions can be obtained using peak width at 2.64 and 3.86 ppm whereas mM concentrations can easily be calculated from the chemical shift change of the NH signal. Overall the combination of three sets of NMR signals, including two pairs of geminal -CH$_2$- signals and the NH signal, offers a large dynamic range for anion detection, covering concentrations from nM to mM, which is uniquely advantageous for analytical purposes.

Figure 16. Quantification of a) nM, b) $\mu$M to c) mM chloride concentration with TUC, using peak width and chemical shift change respectively.
2.2.4. Anion Selectivity

The selectivity of this new anion sensing approach for other anion species was then examined. Since our strategy is based on HB competition, it is expected that selectivity correlates to the HB acceptor ability of anions. To test this hypothesis, five anion species, bromide (Br⁻), iodide (I⁻), tetrafluoroborate (BF₄⁻), acetate (Ac⁻) and phosphate (H₂PO₄⁻), were chosen, and similar NMR titration studies with TUC were conducted (Figure 17 and section A3 in the appendix). The study focused on the three spherical anions (bromide, iodide and tetrafluoroborate) because they did not contain a water peak at 1.59 ppm. The response of TUC to the same amount of these spherical anions was analyzed. As shown in Figure 17, 2.8 meq of Br⁻ generated coalescence of the geminal hydrogen signals at 2.80 and 2.42 ppm in the ¹H NMR spectrum, similar to the effect of the same amount of Cl⁻. The difference between these two anions, however, could be detected using peak widths: while 2.8 meq Cl⁻ generated a peak at 2.64 ppm with peak width at half height of 100 Hz, Br⁻ produced an even broader peak (123 Hz). This is in agreement with chloride being a stronger HB acceptor and therefore more capable of competing with HB_{intra} to generate faster CE. The different selectivity of TUC for iodide is even more evident: 2.8 meq of I⁻ was not enough to cause peak coalescence, but induced significant peak broadening (~ 100 Hz) of all four methylene signals. As expected, the effects of BF₄⁻, a relatively inert anion, were the most modest among all anions tested, causing minimal peak broadening (~ 50 Hz).

![Figure 17. Effect of different TBA anions (2.8 meq) on conformational rigidity of TUC monitored by ¹H NMR.](image-url)
The anion selectivity of TUC can also be demonstrated in a different manner, by comparing the equivalents of anions needed to produce the same level of increase in CE rate, measured by the peak width (Figure 18). To obtain a comparable change in conformational flexibility of TUC equal to the effect of 28 meq of Cl\textsuperscript{-} (peak width at 2.64 ppm of 17 Hz), equal amount of acetate and phosphate (28 meq) and slightly more Br\textsuperscript{-} (70 meq) was needed. In contrast, a significantly higher amount of iodide (560 meq) and BF\textsubscript{4}\textsuperscript{-} (2000 meq) anions were required. Polar solvents such as DMSO are neutral and thus form even weaker HB with TUC, in contrast to anions. Small amounts of DMSO (up to 14 meq) did not cause observable peak broadening in NMR of TUC. Therefore, the impact of solvent impurity on anion sensing is rather small. These observations were consistent with the prediction that the magnitude of the anion-induced CE rate increase correlates with HB acceptor ability. Therefore the anion selectivity of TUC was demonstrated, according to the HB acceptor ability of the anion across a wide range of concentrations.

Figure 18. Partial \textsuperscript{1}H NMR spectra of 1.7 mM TUC with different amount of selected anion species that generate effect similar to 28 meq Cl\textsuperscript{-}.
2.3 Conclusions

A new NMR strategy to detect anions with unprecedented high sensitivity, predictable selectivity and a broad dynamic range of analyte concentrations was established. For the first time, anions can be detected by NMR in the nano-molar range with regular instrumental setup, using a synthetic anion sensor, TUC. Unlike the conventional NMR sensing methods, which either detect the appearance of new anion-receptor complex peaks (slow-exchange scenario) or follow the change of chemical shift (fast-exchange scenario), the current approach monitors the increase in CE rate of geminal hydrogen signals of TUC. Since anions can catalytically induce the conformational flexibility of the sensor through HB competition, they produce a drastic effect on NMR spectra. To the best of our knowledge TUC is the first NMR sensor based on this unique HB competition strategy.

2.4 Materials and Methods

2.4.1 General Experimental Procedures

All reactions were carried out under argon atmosphere, using dry glassware. Reagents were used as received from Alfa Aesar (Georgetown, Canada) and Caledon Laboratory Chemicals (Georgetown, Canada). Thin layer chromatography (TLC) was performed on aluminum-backed silica TLC plates (silica gel 60 F254) obtained from EMD Chemicals (Gibbstown, MA, USA) and the spots were located by UV light (254 nm). Column chromatography was performed on silica gel 60, 63-200 microns, obtained from Caledon Laboratory Chemicals (Georgetown, Canada). All nuclear magnetic resonance (NMR) experiments were recorded on a Varian Mercury 400MHz, a Bruker Avance-III 500MHz or an Agilent DD2-600MHz spectrometer. NMR solvents were obtained from Aldrich (St. Louis, MO, USA). Electron impact (EI) mass spectrometry (MS) were performed on an AB/Sciex QStarXL mass spectrometer. Infra-red (IR) spectra were recorded on a Bruker Alpha FT-IR spectrometer.

2.4.2 Synthesis of Tris-(2-isothiocyanate-ethyl)amine (ITC)

(2-aminoethyl) amine (tren, 0.60 g, 4.00 mmol) in 10 ml of THF was added dropwise to a solution of DCC (3.30 g, 16 mmol) and 1.2g CS2 (4.8 eq) in 10 ml THF at -10°C under nitrogen atmosphere. The reaction was stirred overnight. 25 ml diethyl ether were added and the mixture was filtered. Evaporation of the filtrate and purification by chromatography on silica gel gave 0.8673g (78%) ITC. The spectral characteristics of ITC were identical to the literature.91
2.4.3. Synthesis of **TUC**

A solution of tren (191.0 mg, 1.3 mmol) in 250 ml of CHCl₃ and a solution of ITC (355.9 mg, 1.3 mmol) in 250 ml CHCl₃ were added dropwise simultaneously to 300 ml CHCl₃ at 60°C under argon atmosphere. The mixture was refluxed for 30 min. After evaporation, the solid was washed with 20 ml MeOH to give 0.526 g **TUC** (97%) as white solid.

1H NMR (400 MHz, DMSO-d₆) δ: 7.09 (broad s, 6H), 3.48 (s, 12H), 2.51 (t, J= 5.0 Hz, 12H); (500 MHz, CDCl₃) δ: 6.72 (broad s, 6H), 4.63 (broad s, 6H), 3.00 (broad s, 6H), 2.80 (broad s, 6H), 2.42 (broad s, 6H); 13C NMR (75 MHz, DMSO-d₆) δ: 50.31, 41.24; IR: 2181, 1568 cm⁻¹. MS (EI) m/z 419.2 (M+1⁺).

2.4.4. X-Ray Crystallography

A colorless needle-like single crystal of C₁₅H₃₀N₈S₃ (**TUC**), grown from a DMSO solution infused with H₂O with approximate dimensions 0.150 mm x 0.280 mm x 0.600 mm, was used for the X-ray crystallographic analysis. The data were collected on a Bruker SMART X2S instrument equipped with Mo X-ray tube (λ = 0.71073 Å) The frames were integrated with the Bruker SAINT software package using a narrow-frame algorithm. The integration of the data using an orthorhombic unit cell yielded a total of 17722 reflections to a maximum θ angle of 25.00° (0.84 Å resolution), of which 1860 were independent (average redundancy 9.528, completeness = 98.1%, R_int = 10.16%, R_sig = 5.37%) and 1306 (70.22%) were greater than 2σ(F²). The final cell constants of a = 24.353(11) Å, b = 10.977(5) Å, c = 7.663(3) Å, volume = 2048.5(15) Å³, are based upon the refinement of the XYZ-centroids of 2492 reflections above 20 σ(I) with 4.989° < 2θ < 45.68°. Data were corrected for absorption effects using the multi-scan method (SADABS). The ratio of minimum to maximum apparent transmission was 0.712. The calculated minimum and maximum transmission coefficients (based on crystal size) are 0.8043 and 0.9453.

The structure was solved and refined using the Bruker SHELXTL Software Package, using the space group P n m a, with Z = 4 for the formula unit, C₁₅H₃₀N₈S₃. The final anisotropic full-matrix least-squares refinement on F² with 139 variables converged at R1 = 3.85%, for the observed data and wR² = 9.77% for all data. The goodness-of-fit was 1.019. The largest peak in the final difference electron density synthesis was 0.215 e/Å³ and the largest hole was -0.195 e⁻ /Å³ with an RMS deviation of 0.044 e⁻/Å³. On the basis of the final model, the calculated density was 1.357 g/cm³ and F(000), 896 e⁻. See appendix for details of **TUC** structure.
The data in cif format were deposited with CCDC (deposition number CCDC 990668).

2.4.5. $^1$H NMR Titrations

All solids were dried under vacuum for at least 24 hr prior to titration. Solutions of 1.7 mM TUC and 1.7-170 mM anions (in their tetrabutylammonium salt form) were prepared in CDCl$_3$, and dried over basic aluminium oxide. 700 $\mu$l of the TUC solution was placed in a 5 mm NMR tube and small aliquots of anions stock solution were added. $^1$H NMR spectra were recorded with 16 scans on a Bruker Avance-III 500 MHz spectrometer. VT NMR was performed on an Agilent DD2-600MHz spectrometer.
Copyright Acknowledgements

Parts of this chapter have been reproduced from Ref. Perruchoud, L. H., Hadzovic, A. & Zhang X.-a. 2015, Ultrasensitive anion detection by NMR: a supramolecular strategy based on modulation of chemical exchange rate. Chemistry – a European Journal, 21, 24, 8711-8715 with permission from Chemistry – a European Journal.49
Chapter 3
An Accurate and Biocompatible Ratiometric NMR pH Sensing Strategy Based on Slow-Proton-Exchange (SPE) Mechanism

A significant portion of this chapter has been adapted from the article below. Experiments not included in the manuscript have also been added here.


3.1 Introduction
As a measure of proton activity, pH is a universally important parameter of our aqueous environment and biological milieu, which is routinely measured in different research fields such as chemistry, environmental science, food science, biology, and medicine.94 In living organisms, acid-base homeostasis is essential for maintaining healthy physiological functions and therefore requires tight regulation.95-96 Disrupted pH balance is associated with various abnormal states in biological systems. For example, low pH in humans has been linked to pathological conditions such as cystic fibrosis, ischemia and cancer,97-99 whereas elevated pH (alkalosis) may lead to hyperphosphatemia and hypocalcemia.100 The development of in vivo pH detection methods is currently of great importance for understanding the physiological roles of pH homeostasis, as well as for disease diagnosis and therapeutic monitoring in cases where pH variation is a hallmark of abnormality. It is possible to measure the pH in tissues by using conventional pH
microelectrodes, but their invasiveness and lack of spatial resolution is a major limitation. In contrast, fluorescence and bioluminescence imaging with optical pH sensors can report on pH with high spatial and temporal resolution, but they are restricted to superficial imaging depths due to light scattering and absorption. Although elegantly designed proof-of-principle methods based on other detection techniques have emerged, noninvasive, accurate, and sensitive methods to measure the pH of living organisms remains an urgent challenge.

MR based techniques can offer unlimited tissue penetration in a truly non-invasive manner, and versatile MR read-out methods are established for both spectroscopy and imaging purposes. The recent development of MRI contrast agents based on pH-dependent relaxivity or CEST offer promise for in vivo pH mapping. These methods, however, often require specific calibration or external standards and high accuracy is difficult to achieve. The conventional and most widely used NMR and MR spectroscopic imaging (MRSI) methods for measuring pH rely on sensors that exhibit pH-dependent NMR chemical shift changes, which can be monitored by $^1$H, $^{13}$C, $^{19}$F or $^{31}$P NMR signals. These pH sensors are typically small molecule acids or bases, such as phosphate or imidazole derivatives, with a $pK_a$ compatible with physiological conditions. They exist as a mixture of protonation states but exhibit only one set of NMR signals because the chemical exchange between these states is faster than the NMR time scale. The protons are highly mobile and rapid protonation/deprotonation is greatly facilitated by the hydrogen bond network of hydrated proton in aqueous media, such that it exceeds the speed of diffusion. The observed average chemical shift of the conventional pH sensors is determined by the relative population of the protonated and unprotonated states and thus reflects the pH in solution. However, chemical shift is susceptible to artifacts caused by variations in ionic strength, local magnetic susceptibility, interactions with metal ions, etc. In addition, the proton binding site (lone pair) of regular pH sensors will unavoidably be involved in interactions with metal ions, which will also induce pH-independent chemical shift changes and therefore experimental errors. An innovative strategy involving hyperpolarized $^{13}$C NMR techniques based on the pH-dependent equilibrium between carbon dioxide (CO$_2$) and bicarbonate (HCO$_3^-$), which are in slow exchange in vivo, was recently explored. This approach, however, relies heavily on the carbonic anhydrase enzyme that catalyzes the interconversion between CO$_2$ and HCO$_3^-$. These species are also components of pH-independent biomolecular processes, and the CO$_2$ partial pressure is affected.
by the gas/solute equilibrium. In another strategy, a pilot study showed the possibility of $^{19}$F NMR pH sensing by ratio, when fast proton exchange is coupled with slow dissociation of intramolecular metal-ligand binding. The interaction of metal with other coordinative species in the aqueous media, such as HCO$_3^-$ perturbs the equilibrium between different protonation states. An ideal ratiometric MR pH sensor should have a slow proton exchange (SPE) on the NMR time scale, but still be fast enough for real time pH monitoring, and more importantly, its protonation equilibrium should not be affected by any factors other than pH.

In this chapter, we report the first ratiometric $^1$H NMR pH sensing strategy to meet these criteria, based on a synthetic pH sensor, SPE1. This novel sensor is a cage-shaped urea cryptand with high proton selectivity; it exhibits unusually slow interconversion rates between the different protonation states, which produce distinct NMR signals, allowing highly accurate ratiometric pH measurements. We demonstrate that this novel pH sensor is biocompatible and can be applied to monitor the pH in living biological systems, including fish oocytes and bacterial cultures.

3.2 Principle and Design of the SPE pH Sensing Strategy

The rapid chemical exchange between the non-protonated (B) and protonated (BH$^+$) states of conventional pH probes makes it difficult to accurately measure the ratio of [B]/[BH$^+$] directly by NMR, which is needed to calculate the pH value with the Henderson-Hasselbalch equation:

$$\text{pH} = pK_a + \log\frac{[B]}{[BH^+]}.$$  

In contrast, SPE in protein structures is well documented. While amide or alcohol protons on the surface of a protein are in fast exchange with the surrounding aqueous solution, protons from similar groups in the deep protein core have restricted mobility due to the hydrophobicity of the local environment as well as the involvement of intramolecular hydrogen bonds. It is in principle possible to slow down proton exchange in synthetic molecules by introducing a sterically hindered hydrophobic environment and neighbouring hydrogen bond acceptor groups that mimic protein structures. Small molecules with slow proton exchange however are rare and have only been sporadically reported in the literature as unexpected findings. No systematic study has been conducted to explore this unusual phenomenon. One molecule that displays such slow proton exchange properties is a tris-urea cryptand (1,4,6,9,12,14,19,21-Octaazabicyclo[7.7.7] tricosane-5,13,20-trione) that we named SPE1 (Figure 19). SPE1 possesses two bridgehead N-atoms that can be protonated. In small molecules with two protonation sites such as diamines, $pK_a$ is smaller than $pK_a$.

In other
words, it is more difficult to protonate the second site once the first site is occupied, and it becomes very difficult the closer the two sites are due to increased electrostatic repulsion. Interestingly, despite obeying this rule, the polyoxa-[2]-cryptands\textsuperscript{136} containing tertiary bridgehead amino groups display increasing pK\textsubscript{a2}s with decreasing cavity size. The small cryptand, SPE\textsubscript{1}, however, does not follow this trend and shows strong cooperative binding (pK\textsubscript{a2} > pK\textsubscript{a1}). Although some electrostatic repulsion occurs during protonation, the bridgehead N-atoms in SPE\textsubscript{1} adopt an *endo* conformation with the lone pair electrons pointing inside the molecular cavity. Upon the first protonation, the incoming proton is trapped inside the cage and stabilized in this position through intramolecular hydrogen bonding with the ureido oxygen atoms that are pointing inwards (Figure 19).\textsuperscript{135} This creates an efficient preorganization of the second protonation site with an electron rich pocket, which facilitates the second protonation step, thereby confirming the strong cooperativity properties of SPE\textsubscript{1}. The proton transfer is sufficiently slow to allow direct NMR observation of both the protonated and the neutral forms of SPE\textsubscript{1}. The ratio between these two forms can therefore be used for accurate pH sensing. In addition, the size of the cryptand cavity is too small to bind any ions larger than H\textsuperscript{+}, including Li\textsuperscript{+}, the smallest metal cation.\textsuperscript{23b} This minimalizes the interaction with ions, which can perturb the chemical shift of conventional NMR pH sensors previously mentioned. Other advantageous features of SPE\textsubscript{1} include a pK\textsubscript{a} close to physiological pH and good water solubility. Moreover, because the molecule exhibits mirror plane and C\textsubscript{3} symmetry, the NMR spectrum is simple and unambiguous for peak assignment. Only 3 peaks are detected in the \textsuperscript{1}H NMR spectrum of neutral SPE\textsubscript{1} in aqueous solution, one peak corresponding to the 6 urea protons and two peaks for 12 methylene protons each. Having more chemically identical protons contributing to the intensity of a single peak in the spectrum increases sensitivity, which is one of the most common limitations of NMR.

![Figure 19. Structure and protonation states of cage-shaped pH sensor SPE1.](image)

The protons attached to the bridgehead nitrogen atoms are trapped inside the cage due to hydrogen bonding with the ureido oxygen atoms, thereby allowing SPE between the two states. The \textsuperscript{1}H NMR signals of labelled methylene positions are used for pH sensing.
3.3 Results and Discussion

3.3.1 Synthesis of SPE1

To test the applicability of the SPE strategy for pH sensing, a novel synthetic route was implemented to generate SPE1 in 3 steps, with a 38% overall yield (Figure 20). SPE1 was synthesized from a tripodal amine, tren, which was readily converted into an isothiocyanate derivative (3) upon treatment with carbon disulfide and N,N'-dicyclohexylcarbodiimide (DCC) in 78% yield, according to our previously published procedure. Rapid coupling of isothiocyanate compound 3 with the trivalent amino counter partner, tren, under high dilution conditions generated the C3 symmetrical thiourea compound 2 in nearly quantitative yield. The thioureido groups in 2 were then converted to more water-soluble ureido analogs based on a reaction reported by Mikolajczyk in 1972, in which DMSO acts as the oxidant and solvent, in the presence of an acid catalyst. This convenient synthesis allows production of SPE1 in large scale, facilitating the following pH sensing studies.

![Figure 20. Synthesis of SPE1. Tren = (2-aminoethyl) amine, 3 = tris-(2-isothiocyanate-ethyl)amine](image)

3.3.2 Measurement of pH

Both bridgehead N-atoms of SPE1 can be protonated under acidic conditions. Due to slow chemical exchange, the neutral SPE1 and its bis-protonated form (SPE1H22+) are simultaneously detected by 1H NMR as distinct species in aqueous solution. Notably, the mono-protonated form of SPE1 (SPE1H+) was not observed by NMR, due to the strong positive cooperativity in protonation (pK_a2 > pK_a1). This property greatly simplifies the NMR spectrum, as both neutral and bis-protonated SPE1 are highly symmetrical, enhancing the sensitivity of NMR signal detection. For pH calculations, a modified Henderson-Hasselbalch equation, which takes into account both protonation steps of SPE1, was used based on the ratio of neutral and bis-
protonated \textbf{SPE1}: $\text{pH} = pK'_a + \frac{1}{2} \log[\text{SPE1}]/[\text{SPE1H}_2^{2+}]$, where $pK'_a$ is the apparent $pK_a$ and is defined as $pK'_a = \frac{1}{2}(pK_{a1} + pK_{a2})$.

In order to determine $pK'_a$ and further demonstrate that \textbf{SPE1} can be applied for accurate ratiometric pH sensing, a series of $^1$H NMR spectra of \textbf{SPE1} dissolved in phosphate buffer were collected at several pH values between 7 and 9 (Figure 21a). At room temperature, under basic conditions ($\text{pH} \geq 9$), \textbf{SPE1} is predominantly in the neutral form, producing two $^1$H NMR peaks at 2.58 and 3.13 ppm for both bridge methylene units (-CH$_2$-CH$_2$-). Upon gradual decrease in pH, the signals of \textbf{SPE1H}_2^{2+} emerge, as represented by new methylene peaks at 3.40 and 3.56 ppm, while the signals of neutral \textbf{SPE1} remain detectable at the same chemical shift. As the pH decreases, the intensity of the \textbf{SPE1} peaks diminishes with simultaneous increase of the \textbf{SPE1H}_2^{2+} peaks, and the latter become dominant at pH 7 and below, confirming the relation between solution pH and the \textbf{SPE1/SPE1H}_2^{2+} ratio.

\textbf{Figure 21.} $^1$H NMR pH titration of \textbf{SPE1} at 25 and 37 °C in phosphate buffer. a) Selected partial $^1$H NMR spectra of \textbf{SPE1} at different pH values at 25 °C. Chemical shifts: A: 3.40 ppm, B: 3.56 ppm, A': 2.58 ppm, B': 3.13 ppm. b) Ratiometric curve of $^1$H NMR pH titrations derived from ratio of different protonation states of \textbf{SPE1}.

During the titration, a capillary with D$_2$O was inserted into the NMR tube for deuterium lock. Alternatively, a small amount of D$_2$O can also be mixed directly with the NMR solution, since we observed that adding 10% D$_2$O to the buffer solution did not change the \textbf{SPE1/SPE1H}_2^{2+} ratio significantly (Figure 22), indicating that the isotope impact from 10% D$_2$O or less does not affect the accuracy of pH sensing.
Figure 22. Normalized local NMR spectra of SPE1 in phosphate buffer (pH = 7.90) with D$_2$O. Red: D$_2$O in sealed capillary (pH 7.896 as measured by NMR); Blue: D$_2$O directly added (10%) to the solution (pH 7.898 as measured by NMR). The similar ratio of neutral and protonated SPE1 indicates isotope impact from 10% D$_2$O or less does not significantly affect the accuracy of pH sensing.

For quantitative analysis, pH was plotted against the fraction of neutral SPE1 (SPE1/[SPE1+SPE1H$_2^{2+}$]), obtained from the NMR integrals (Figure 21). A similar titration curve was obtained at 37 °C. For pH calculations, an equation describing the percentage of neutral SPE1:

$$\left( \frac{[\text{SPE1}]}{[\text{SPE1}]+[\text{SPE1H}_2^{2+}]} \right) = \frac{10^{(2p\text{H}-p\text{K}_a1-p\text{K}_a2)}}{1+10^{(2p\text{H}-p\text{K}_a1-p\text{K}_a2)}}$$

was derived from the modified Henderson-Hasselbalch equation above and was used for nonlinear least square fitting of the experimental data. Using this equation, the apparent pK$_a$ for SPE1 was 8.00 ± 0.06 at room temperature and 7.72 ± 0.07 at 37 °C. These numbers are in agreement with the pK$_a$ determined by potentiometric titration,\textsuperscript{134} confirming that the pH measured by the current ratiometric approach is comparable to the conventional pH electrode. At body temperature, SPE1 can operate as a pH sensor between pH 6.7 and 8.7, covering slightly acidified to mildly basic conditions. In addition, this method is very sensitive. Within a pH window close to the pK$_a$ of SPE1, differences as small as 0.02 pH units could be experimentally observed (Figure 23).
Figure 23. Selected $^1$H NMR spectra showing the high accuracy of pH measurement by SPE1. Overlay of local $^1$H NMR spectra of SPE1 at pH 7.91 (blue) and 7.93 (red). The peak intensity was normalized to the signals of neutral SPE1 at 2.58 and 3.13 ppm. A difference in pH of 0.02 pH units can be detected.

3.3.3 Biological Applications of SPE1

3.3.3.1 Characteristics of SPE1 for Biological Applications

Before applying SPE1 in biological systems, solubility and stability of the sensor need to be tested. The presence of ureido groups instead of thioureido groups of the TUC intermediate increased aqueous solubility of SPE1 trememdously since TUC is not water soluble but SPE1 can be dissolved at a maximum aqueous solubility of 1.7 M, which makes it ideal for biological applications. In addition, SPE1 does not decompose under very acidic conditions (pH < 0), heat (> 140 °C) or extreme cold (3 K).

Before using SPE1 for in vivo pH measurements, it is also important to test its toxicity. Preliminary viability experiments were performed on SH-SY5Y undifferentiated neuronal cells with different concentrations of sensor. The goal of this assay was to determine whether different concentrations of SPE1 (100 nM to 2.5 mM) would affect cell viability. As shown in Figure 24, the highest sensor concentration (2.5mM) did not appear to affect cell viability. Interestingly, at that concentration, morphologically healthier cells were observed. Therefore, these preliminary results demonstrate that SPE1 does not seem to cause any toxic effects on neuronal cells. This
could be in part because SPE1 acts as a buffer and does not react with biomacromolecules in living systems (see section 3.5.6 for more details on prokaryotic cell culture viability).

Figure 24. Viability experiment of different concentrations of SPE1 on SH-SY5Y undifferentiated neuronal cells.

For in vivo studies, it is also crucial to determine whether the linewidth of the peaks of SPE1 are suitable, which is why a study of the transverse relaxation (i.e. T2) of SPE1 was carried out. T2 was measured with PURGE water suppression at pH 8.2 and 7.3 (Table 1). A solution of SPE1 in phosphate buffer at pH 8.2 was initially prepared in a 5 mm NMR tube with a D2O insert and used for T2 measurements and the pH was decreased with HCl to 7.3 for the second measurement. The average T2 for the SPE1 peaks was ~ 70 ms, which means that SPE1 has the potential to be used for in vivo pH measurements.

Table 1. T2 of the 4 methylene peaks of SPE1 and SPE1H22+

<table>
<thead>
<tr>
<th>Peaks (ppm)</th>
<th>3.56</th>
<th>3.40</th>
<th>3.13</th>
<th>2.58</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH = 7.3</td>
<td>75</td>
<td>75</td>
<td>60</td>
<td>48</td>
</tr>
<tr>
<td>pH = 8.2</td>
<td>76</td>
<td>64</td>
<td>80</td>
<td>73</td>
</tr>
</tbody>
</table>

3.3.3.2 Determination of pH by CEST-MRI

Since CEST can increase the sensitivity of MR experiments and SPE1 possesses exchangeable ureido NHs that are sufficiently far from the water signal with a suitable exchange rate, SPE1 was tested as a CEST pH sensor. As shown in Figure 25a-b, the NH chemical shift of neutral SPE1 is 1 ppm away from the water proton signal. This peak is shifted at even higher frequency (~2 ppm) in SPE1H22+. Both +1 ppm peak (C’) and +2 ppm (C) peak show a CEST effect as shown in the MTRasym spectrum (Figure 25c), which can be used to generate ratiometric pH-
dependent CEST images (Figure 25d-f). Unfortunately, the exchange rates of the NH signals in SPE1 and SPE1H\textsuperscript{2+} are pH-dependent, and therefore pH determination with SPE1 using ratiometric CEST imaging is not accurate.

![Diagram of SPE1 and SPE1H\textsuperscript{2+}]

**Figure 25.** Imaging pH with CEST MRI using exchangeable protons in SPE1. a) Protonation states of SPE1. b) Partial \textsuperscript{1}H NMR spectra of SPE1 at different pH values. c) Z spectrum of an aqueous solution of SPE1. d-f) Phantom CEST images (MTR\textsubscript{asym}) of aqueous solutions of SPE1 between pH 6.5 and 8.5. d) Saturation at 1 ppm, e) saturation at 2 ppm, f) ratiometric image (1/2 ppm).

### 3.3.3.3 In-Cell pH Detection

To demonstrate that this novel ratiometric approach is suitable for measuring pH in living biological systems by NMR, SPE1 was applied to measure the intracellular pH in a Belonidae oocyte. Live oocytes are widely used as model organisms for drug screening and to study reproduction and development.\textsuperscript{138-139} The popular platform, Xenopus laevis oocytes, require hundreds of cells for NMR acquisition.\textsuperscript{140-141} In contrast, the Belonidae oocyte chosen for this study has an average diameter of 3 mm. Using a 4 mm MAS NMR probe, pH detection of a single cell with low \(\mu\text{M}\) sensor concentration was achieved. A solution of 1.4 \(\mu\text{mol}\) SPE1 was administered via microinjection and the \textsuperscript{1}H NMR spectrum of the oocyte was obtained at 25 °C (Figure 26) with slow spinning (1000 Hz). In addition to the signals from endogenous species
visible in the control spectrum of the untreated oocyte (Figure 26), the peaks of both neutral and bis-protonated SPE1 were clearly resolved in the NMR of the treated oocyte with almost 100:1 S/N (Figure 26b). The chemical shifts were referenced relative to the peaks of neutral SPE1 at 2.58 and 3.13 ppm and the chemical shifts of SPE1H2<sup>2+</sup> were consistent with peaks from the calibration spectra acquired in phosphate buffer. Leakage of sensor was negligible, as the sensor was not detectable in the <sup>1</sup>H NMR spectrum of the extracellular solution alone and no phenol red (co-injected with SPE1) absorption was observed in the extracellular solution as measured by UV-visible absorption spectroscopy. A ratio of 9/91 (9% of SPE1) was obtained, corresponding to an intracellular pH of 7.50, which was confirmed by using a pH electrode in cell lysates. This intracellular pH is in line with previous measurements acquired by different methods on oocytes of other species. 142-143

![Figure 26. Determination of intracellular pH of Belonidae oocytes with SPE1 at 25 °C by <sup>1</sup>H NMR. a) <sup>1</sup>H NMR spectrum of an untreated oocyte. b) <sup>1</sup>H NMR spectrum of an oocyte treated with 2 µl of 0.7 M SPE1. Chemical shifts: A: 3.40 ppm, B: 3.56 ppm, A': 2.58 ppm, B': 3.13 ppm. *Spinning sideband due to slow spinning at 1 KHz at 2.69 ppm. Percentage of neutral SPE1 was 9%, corresponding to a pH value of 7.50.](image)
3.3.3.4 Monitoring pH Change in *Escherichia coli* Cultures

To demonstrate that the novel ratiometric pH sensing strategy can be applied to different biological systems, we used SPE1 to monitor real time pH changes in live bacterial culture. The biocompatibility of SPE1 was first tested on *Escherichia coli* (*E. coli*). A 1.8 mM solution of SPE1 in phosphate buffer was added to cultured *E. coli* MC4100 cells (OD<sub>600</sub> = 1) and incubated at 37 °C for 12 hours. The viability of the sensor-treated cells was not significantly different compared to non-treated cells (ca. 7.3 x 10<sup>7</sup> CFU/ml for both samples). The cells incubated with SPE1 were concentrated, washed and placed into a 4 mm rotor and subjected to NMR experiment. The observed NMR signals of the pH sensor indicate that SPE1 is cell permeable (Figure 27a). After the NMR experiment, the cells were washed again with phosphate buffer and the NMR signals of SPE1 disappeared, suggesting SPE1 can readily come out from the MC4100 cells (Figure 27b).

![Figure 27. Stacked 1H NMR spectra at 500 MHz of *E. coli* incubated for 6 h with 10 mM SPE1. a) Initial 1H NMR spectrum of *E. coli* cells with SPE1, after washing out the extracellular sensor. b) 1H NMR spectrum of *E. coli* cells taken after the cells were washed again with fresh buffer following the first NMR experiment in a).](image)

The diffusion editing method revealed that the sensor is freely diffusing after cell uptake (Figure 28), suggesting no specific binding of SPE1 to bio-macromolecules in *E. coli*. Overall SPE1 causes no observable toxicity in *E. coli* cells.
Various microorganisms, including *E. coli* cells can grow in both aerobic and anaerobic culture, and are known to increase production of acidic metabolites in response to low oxygen stress. To monitor this process in real time by NMR, we conducted a kinetic study of concentrated *E. coli* culture (1 ml aliquot at OD$_{600}$ = 1) in a sealed 4 mm NMR rotor at 37 °C and recorded the change in pH over time using SPE1 (1.8 mM, Figure 29).
An initial pH of 7.55 was determined from the intensity ratio 31/69 (31% for neutral SPE1). The solid NMR rotor insert remained sealed in the spectrometer and new $^1$H NMR spectra were acquired every 15 minutes. A continuous slow increase in intensity of the SPE1H$_2^{2+}$ peaks with a diminution of the peak intensities of SPE1 was observed. The high accuracy of the SPE based method allowed precise measurements of small pH changes over 3 hours from pH 7.55 to 6.95 (Figure 29). Interestingly, in conjunction with gradual decrease of pH, two new sharp peaks appeared in the $^1$H NMR spectra and increased in intensity over the course of the experiment. The chemical shifts of 2.40 and 1.92 ppm of these singlet peaks are consistent with succinate and acetate, which are common metabolites observed in bacterial cultures growing with limited oxygen availability.\cite{145-147} It is known that bacteria modify their metabolism upon switching from aerobic to micro-aerobic or anaerobic conditions, by increasing the glycolysis rate with a concomitant decrease of acetyl-CoA degradation by the citric acid cycle.\cite{148} This adjustment causes an overall increase in proton concentration as well as other acidic overflow metabolites such as acetate and succinate.\cite{149} Therefore our experiments confirmed that SPE1 was able to accurately monitor pH changes in real time in a biocompatible and reproducible manner and recorded the alteration of metabolism in live bacterial cultures deprived of oxygen. The current setup does not allow determination of the precise location of SPE1 within cells. Future work will involve development of new SPE based pH sensors with controllable cell-permeability and subcellular localization (see section 6.3.2).

### 3.4 Summary and Conclusions

We reported a novel and versatile strategy for ratiometric $^1$H NMR pH sensing based on a slow proton exchange (SPE) mechanism. A water-soluble small molecule cryptand SPE1, was prepared through a new synthetic route and was evaluated in vitro and in live cells for ratiometric NMR pH sensing. Slow chemical exchange between different protonation states and high proton selectivity of SPE1 was achieved by shielding the incoming protons inside the molecular cavity and trapping them with intramolecular hydrogen bonding. Unlike typical small molecule acids or bases, which exhibit a single set of average NMR signals, SPE1 displays distinct peaks for the neutral and protonated forms due to unusual slow chemical exchange. It is therefore possible to use the ratio of NMR peak intensities to provide highly precise pH values of the aqueous media. The new approach is more robust, sensitive and accurate than conventional chemical shift based methods, which are vulnerable to many pH-independent factors. SPE1 exhibits an apparent pK$_a$
value suitable for biological applications and shows no toxicity effects on cell cultures. Therefore the new method was applied to measure the pH in a single live fish oocyte, and to monitor the real time pH changes of a bacterial cultures. Overall, SPE1 has great potential for measuring and mapping pH and pH changes in living systems. Next generation pH sensors based on SPE mechanism are currently under development to cover different pH windows, which can further expand the scope of biological applications of this new strategy.

3.5 Materials and Methods

3.5.1 General Experimental Procedures

All reactions were carried out under argon atmosphere, using dry glassware. Reagents were used as received from Alfa Aesar (Georgetown, Canada) and Caledon Laboratory Chemicals (Georgetown, Canada). Thin layer chromatography (TLC) was performed on aluminum-backed silica TLC plates (silica gel 60 F254) obtained from EMD Chemicals (Gibbstown, MA, USA) and the spots were located by UV light (254 nm). Column chromatography was performed on silica gel 60, 63-200 microns and alumina basic standard gel, 50-200 microns, obtained from Caledon Laboratory Chemicals (Georgetown, Canada). All nuclear magnetic resonance (NMR) experiments were recorded on a Bruker Avance-III 500MHz or Varian Mercury 400MHz spectrometer. All 1H NMR spectra were manually corrected for phase and baseline distortion using TopSpin™ 3.1 and MestReNova 8.1.4 and integral ratios were obtained by taking ± 35 Hz around each peak. The chemical shifts were first calibrated to DSS as an internal standard, where the peaks of the neutral SPE1 appeared at 2.58 and 3.13 ppm. The chemical shifts were then referenced relative to the peaks of neutral SPE1. NMR solvents were obtained from Aldrich (St. Louis, MO, USA). Electron impact (EI) mass spectrometry (MS) and electron spray ionization (ESI) MS were performed on an AB/Sciex QStar mass spectrometer. Infra-red (IR) spectra were recorded on a Bruker Alpha FT-IR spectrometer.

3.5.2 Synthesis of SPE1

200 mg (0.5 mmol) of 2 and 547 mg (3 mmol) tosyllic acid (p-TsOH) were dissolved in 10 ml DMSO under argon atmosphere. The solution was heated to 110°C for 5 hrs and then distilled to dryness. The resulting water soluble solid was dissolved in 20 ml water, washed 3 times with 20 ml diethyl ether and the aqueous layer was neutralized with 500 mg Na₂CO₃. The solvent was
evaporated, and 20 ml CH$_2$Cl$_2$/MeOH (3:1) were added to the solid. The mixture was filtered, the filtrate was evaporated and purification by chromatography on aluminium oxide using a solution of 3% methanol in CH$_2$Cl$_2$ gave 91.1 mg (52%) compound 1. $^1$H NMR (500 MHz, D$_2$O) δ: 2.99 (t, J= 5.3Hz, 12H), 2.43 (t, 12H); $^{13}$C NMR (125 MHz, D$_2$O) δ: 161.09, 50.95, 37.40; IR: 3304, 1669, 1631, 1569 cm$^{-1}$; MS (ESI) m/z 371.2 (M+1$^+$).

3.5.3 NMR Monitored pH Calibration

A solution of SPE1 (2 mM) was dissolved in 10 mM phosphate buffer (pH = 7.4). Aliquots of 500 µl were prepared at different pH values by addition of HCl or NaOH, and the pH was measured using a calibrated pH electrode (Cole Parmer Thermo Scientific Orion pH microelectrode) and a VWR sympHony SB70P pH meter. The sample was placed in a 5 mm NMR tube with a sealed capillary filled with D$_2$O. $^1$H NMR spectra were obtained at 25°C or 37°C with 64 scans per sample on a 500 MHz Bruker Avance spectrometer and presaturation of the water signal with a recycle time of 50 sec and a 90° pulse width. The experimental data were fitted with MATLAB software using non-linear least square regression.

3.5.4 Transverse Relaxation (T$_2$) Measurements

T$_2$ of SPE1 was measured on a 400 MHz Varian spectrometer using PURGE$^{150}$ as the water suppression method and standard CPMG pulse sequence for T$_2$ determination at pH 8.2 and 7.3. A solution of 6.2 mM (1.6 mg in 700 ul) of SPE1 in PBS at pH 8.2 was initially prepared in a 3 mm NMR tube with a D$_2$O insert and used for T$_2$ measurements. HCl (7 ul at 1 M) was used to decrease the pH to 7.2 for the second measurement.

3.5.5 Measurement of Intracellular pH of Belonidae Oocytes

3.5.5.1 Sample Preparation

Freshly produced, unfertilized Belonidae oocytes (~3 mm in diameter) were washed with OR-2 buffer$^{151}$ and used within 2 days. Each oocyte was microinjected with 2 µl of a solution of 0.7 M SPE1 with 0.05% phenol red. A single oocyte was used for each measurement. One oocyte without sensor injection was scanned as a control.
3.5.5.2 NMR Experiments

The $^1$H NMR experiments were performed on a Bruker Avance III 500 MHz spectrometer, using a prototype CMP MAS 4 mm $^1$H-$^{13}$C-$^{19}$F-$^2$H probe fitted with an actively shielded Z gradient (Bruker BioSpin) at a spinning speed of 1000 Hz. The oocyte was placed into a 4 mm o.d. zirconium rotor with 10 µl D$_2$O and the experiments were locked using D$_2$O solvent. Water suppression was achieved using the purge pulse sequence$^{150}$. All spectra were recorded with 256 scans, recycle delay set at 5 x $T_1$ and ~ 4 µs 90° pulse widths for the blank and injected oocyte experiment respectively. 32768 time domain points were acquired for each spectrum with a spectral width of 20 ppm. Data were zero filled and multiplied by an exponential window function corresponding to a 1 Hz line broadening in the transformed spectrum.

3.5.6 Real Time pH Monitoring of E. coli Culture

3.5.6.1 Sample Preparation

E. coli MC4100 cells transformed with a pBAD24 plasmid (to confer ampicillin resistance) were plated on solid LB-agar medium supplemented with ampicillin and grown overnight at 37°C. LB media and agar were purchased from Bioshop Inc. and used as received. One colony was transferred from the plate into a 50 mL culture of LB-Amp liquid medium and grown for approximately 16 hrs. The overnight cultures were used to inoculate fresh liquid cultures, which were grown at 37°C to an OD$_{600}$ < 1. A 1 ml aliquot of the culture was centrifuged at 10,000 g and re-suspended in 30 µl of a 1.8 mM solution SPE$^1$ in 10 mM phosphate buffer pH 8.0 containing 10% D$_2$O. Another aliquot was collected and re-suspended in phosphate buffer to act as a blank for the NMR experiment and a control for cell viability over the course of the experiment. The sample was transferred to an NMR top insert made from Kel-F, sealed with a Kel-F sealing screw and cap, then inserted into a 4 mm o.d. zirconium rotor for the NMR experiment.

To test for viability of the sensor-free and sensor-treated cells, the cells were serially diluted $10^3$ - $10^9$ times in phosphate buffer after the experiment and plated on LB-Amp plates to determine cell survival during the experiment.
3.5.6.2 NMR experiments

The $^1$H NMR experiments were performed on a Bruker Avance III 500 MHz spectrometer, using a prototype CMP MAS 4 mm $^1$H-$^{13}$C-$^{19}$F-$^2$H probe fitted with an actively shielded Z gradient (Bruker BioSpin) at 37°C. The samples were all spun at a spinning speed of 6666 Hz and all experiments were locked using D$_2$O solvent. Water suppression was achieved using water suppression by gradient-tailored excitation (WATERGATE) and was carried out using a W5 pulse train$^{152-153}$. All spectra were recorded with 256 scans, recycle delay set at 5 x $T_1$, 5.8 µs 90° pulse widths and collected using 32768 time domain points with spectral widths of 20 ppm. Data were zero filled and multiplied by an exponential window function corresponding to a 1 Hz line broadening in the transformed spectrum.

Diffusion-edited (DE) proton spectra were produced using a bipolar pulse pair longitudinal encode–decode (BPP-LED) sequence$^{154}$ with inverse gated decoupling. Scans were collected using encoding/decoding gradients of 1.8 ms at 49 gauss/cm and a diffusion time of 180 ms. Inverse diffusion edited (IDE) spectrum was created via difference from the appropriate controls as previously described$^{155}$. The spectra were scaled until the dominant component being subtracted was nulled, leaving a difference spectrum containing positive peaks$^{155}$. 

Copyright Acknowledgements

Parts of this chapter have been reproduced from Ref. Perruchoud, L. H., Jones, M. D., Sutrisno, A., Zamble, D. B., Simpson, A. J. & Zhang, X.-a. 2015, A ratiometric NMR pH sensing strategy based on slow proton exchange (SPE) mechanism. Chemical Science, 6, 6305-6311 with permission from the Royal Society of Chemistry.93
Chapter 4

A Ratiometric and Hyperpolarized pH-Sensitive $^{13}$C NMR Probe Based on the Slow Proton Exchange (SPE) Method

4.1 Introduction

Measurement of pH is of particular importance in the biochemical and biomedical fields, since pH must be tightly regulated for normal functioning of cells and organisms and for disease prevention such as ischemia, inflammation or cancer. For this purpose, MR based methods have important advantages including non-invasiveness and deep penetration of soft tissues in high spatial resolution. (For more information on pH-sensitive MR methods, refer to section 3.1.)

Due to its high sensitivity, proton is the most commonly used nucleus in MR experiments and despite their lower sensitivities, $^{19}$F and $^{31}$P have also produced applications with decent pH accuracy. The $^{13}$C nucleus, however, is significantly less sensitive because its gyromagnetic ratio is less than $\frac{1}{4}$ of that of $^1$H and $^{19}$F and its natural abundance is only 1.1%. Fortunately, $^{13}$C MR has the advantage of possessing low background signals. Overall, measuring pH using $^{13}$C MR methods usually requires very long acquisition times to compensate for the lack of sensitivity, which prevents acquisition of data at high temporal resolution and is difficult to implement, unless $^{13}$C enrichment has been performed. Another way to increase sensitivity of the $^{13}$C MR signal is by hyperpolarization (see section 1.3.1.3 for more details on hyperpolarization). A well-known example in the field of $^{13}$C MR pH detection was developed in 2008 in which DNP-hyperpolarized $^{13}$C-labelled HCO$_3^-$ was injected in tumor-bearing mice and pH was obtained from the ratio of the slow exchanging H$^{13}$CO$_3^-$ and $^{13}$CO$_2$ in vivo. However, the major limitations of the method are that bicarbonate is involved in many
biochemical and metabolic pathways and the ratio of HCO$_3^-$ and CO$_2$ is dependent on the enzyme carbonic anhydrase, whose activity and concentration varies between tissues.

In this chapter, the first synthetic ratiometric $^{13}$C NMR sensor ($^{13}$C-SPE1) that can detect pH using the SPE strategy is presented. This sensor, which is based on SPE1 (chapter 3), has been $^{13}$C enriched to improved signal detection and allow very accurate ratiometric pH sensing using the $^{13}$C nucleus in Xenopus laevis oocytes. It was also hyperpolarized to further improve sensitivity.

### 4.1.1 Principle and Design of the SPE Strategy

The general principle of the SPE strategy is described in details in section 3.2. The following section summarizes the main points of the method and elaborates on its uses for $^{13}$C NMR specifically. The SPE strategy is a general ratiometric MR based method of detecting pH. Theoretically, any molecule which displays slow protonation/deprotonation behavior on the NMR time scale can be used as an SPE based pH sensor. In this method, each protonation state of the sensor displays distinct NMR signals and the ratio of these signals affords precise pH values. Because the method is ratiometric, it does not suffer from inaccuracies due to artifacts caused by pH-independent factors which affect chemical shift, such as variations in ionic strength, local magnetic susceptibility, etc. Therefore, it is more accurate than the conventional chemical shift based NMR pH sensing techniques.

SPE1 was the first $^1$H NMR sensor developed for the SPE strategy that successfully and accurately monitored pH in real time in a living bacterial culture. $^{13}$C-SPE1 is the $^{13}$C-labelled version of SPE1 in which all ureido carbons are $^{13}$C enriched (Figure 30).

![Figure 30. Structure and protonation states of cage-shaped $^{13}$C-labelled pH sensor $^{13}$C-SPE1. The protons attached to the bridgehead nitrogen atoms are trapped inside the cage due to hydrogen bonding with the ureido oxygen atoms, which produces two species that are in slow exchange in NMR.](image-url)
It possesses the same physical properties as SPE1, including the slow proton exchange. The enriched $^{13}$C NMR sensor has several advantages. First, sensitivity issues are reduced along with long experiment times. In addition, due to the low natural abundance of the $^{13}$C nucleus \textit{in vivo}, the background signals in a $^{13}$C NMR spectrum are diminished compared to $^1$H NMR signals which include lipids, sugars and other $^1$H containing molecules which can overlap with the sensor’s signals. Since the quaternary ureido carbons are $^{13}$C-labelled, this compound is also a good candidate for DNP-hyperpolarization.

4.2 Results and Discussion

4.2.1 Synthesis of $^{13}$C-SPE1

The synthesis of $^{13}$C-SPE1 is in all aspects identical to SPE1 synthesis (see chapter 3, section 3.3.1), except that $^{13}$C-labelled CS$_2$ ($^{13}$CS$_2$) was used as the starting material in the first step reaction as outlined in Figure 31.

[Figure 31. Synthesis of $^{13}$C-SPE1.]

4.2.2 Measurement of pH Based on the SPE Strategy

The mechanisms for pH detection using $^{13}$C-SPE1 are identical to SPE1 (see section 3.3.2). $^{13}$C-SPE1 displays slow proton exchange properties which allow detection of neutral ($^{13}$C-SPE1) and bis-protonated ($^{13}$C-SPE1H$_2^{2+}$) ureido sensor peaks simultaneously by $^{13}$C NMR. Similar to SPE1, the mono-protonated form of the $^{13}$C sensor is not observed in the NMR spectrum due to positive cooperative protonation. The same modified Henderson-Hasselbalch equation ($\text{pH} = pK_a - \log([^{13}\text{C-SPE1}]/[^{13}\text{C-SPE1H}_2^{2+}])$) was used for pH calculations and a similar pH titration of the sensor in phosphate buffer at 25 and 37 °C between pH 7 and 9 was carried out to determine the $pK_a$ of the ureido signals of $^{13}$C-SPE1 (Figure 32a). At room temperature and pH
> 9, only the neutral form of the sensor was present in solution with the peak of neutral ureido $^{13}$C occurring at 161.12 ppm in the $^{13}$C NMR spectrum. As pH decreased, this peak diminished in intensity while the peak of bis-protonated $^{13}$C-SPE1H$_2^{2+}$ appeared at 161.90 ppm. With variations in pH, the ratio of the peaks changed but the chemical shift remained the same, due to slow exchange, confirming the relation between solution pH and the $^{13}$C-SPE1/$^{13}$C-SPE1H$_2^{2+}$ ratio.

![Figure 32. $^{13}$C NMR pH titration of $^{13}$C-SPE1 at 25 and 37 °C in phosphate buffer. a) Selected partial $^{13}$C NMR spectra of $^{13}$C-SPE1 at different pH values at 25 °C. Chemical shifts: C: 161.90 ppm, C': 161.12 ppm. b) Ratiometric curve of $^{13}$C NMR pH titrations derived from ratio of different protonation states of $^{13}$C-SPE1.](image)

For quantitative analysis, a titration curve was obtained by plotting pH against the fraction of neutral sensor ($^{13}$C-SPE1/[$^{13}$C-SPE1+ $^{13}$C-SPE1H$_2^{2+}$]) obtained from NMR integrals (Figure 32b) using the same equation described in section 3.3.2., a similar curve was obtained at 37 °C. From the equation, the apparent $pK_a$ for $^{13}$C-SPE1 was 8.01 ± 0.04 at 25 °C and 7.70 ± 0.05 at 37 °C. These numbers are in agreement both with values determined from potentiometric titrations$^{134}$ and with the $pK_a$ values obtained from the $^1$H methylene signals of SPE1$^{93}$ (see section 3.3.2). Therefore $^{13}$C-SPE1 can cover a similar pH window to SPE1 (between pH 6.7 and 8.7) and can detect differences in pH as low as 0.02 pH units accurately.
4.2.3 Applications of $^{13}$C-SPE1

4.2.3.1 Measurement of Intracellular pH

To demonstrate that the novel ratiometric $^{13}$C-labelled sensor $^{13}$C-SPE1 can be applied to a biological system, we used $^{13}$C-SPE1 to measure the intracellular pH of *Xenopus laevis* oocytes. Live fish oocytes, including the zebrafish, have been used as a model organism to study reproduction and development and for drug screening\textsuperscript{138-139}. The frog *Xenopus laevis* was the first eukaryotic cellular model system that was implemented for in-cell NMR\textsuperscript{140-141, 160-161} and its applications include the study of conformation and/or functional properties of proteins under biological conditions, including intrinsically disordered proteins (IDPs).\textsuperscript{162-164} For this study, a solution of 450 mM $^{13}$C-SPE1 was microinjected into 200 *Xenopus laevis* oocytes (50 nl each). As a control, 200 oocytes were microinjected with SPE1. The eggs were then placed in a Shigemi NMR tube for NMR spectroscopy. A representative $^{13}$C NMR spectrum of sensor-injected oocytes is shown in Figure 33. Intrinsic peaks corresponding to sugars, lipids and other bio-macromolecules were clearly visible in the $^{13}$C NMR spectrum and were also present in the spectrum of SPE1-injected control oocytes. In addition, a large peak at 161.90 ppm, matching the chemical shift of the bis-protonated sensor, was clearly observable. Therefore, the ureido carbon of $^{13}$C-SPE1 can be detected inside *Xenopus laevis* oocytes under the current conditions.

![Figure 33](image)

**Figure 33.** $^{13}$C NMR spectra of *Xenopus laevis* oocytes injected with $^{13}$C-SPE1. The peak of the urea carbon of the sensor ($^{13}$CSE1H\textsubscript{z}\textsuperscript{2+}) is labelled C'.

The lack of decomposition products peaks suggests that the sensor is stable for at least 12 hours inside the oocytes. Unfortunately, the pH of the sensor solution was too acidic and decreased the pH of the eggs so that the final intracellular pH was outside the pH range of the sensor. This is
supported not only by the absence of a neutral sensor peak at 161.12 ppm but also by the acidic pH of lysed oocytes (pH = 5.68) obtained after the NMR experiments with a pH electrode. Although the intracellular pH of the oocytes could not be measured, it is in principle possible to use $^{13}$C-SPE1 for pH detection in oocytes. Further experiments, in which the pH of the sensor solution is carefully controlled, should be carried out to provide support for this claim.

### 4.2.3.2 Hyperpolarization

In addition to $^{13}$C-labelling, hyperpolarization is an efficient method to increase sensitivity of MR based methods. During an MR experiment, polarization of nuclei occurs naturally when they are placed in an external magnetic field. Under thermal equilibrium, the populations of ground and excited spin states are roughly identical and cancel each other to give low net magnetization and hence low signal intensity. For a more intense signal, the population difference between the two spin states needs to be increased which can be achieved by different methods including parahydrogen-induced polarization (PHIP) and dynamic nuclear polarization (DNP). A more complete explanation of hyperpolarization can in found in chapter 1, section 1.3.1.3.

To demonstrate how hyperpolarization can be used to increase sensitivity of a $^{13}$C nucleus, $^{13}$C-SPE1 was hyperpolarized using DNP. The sensor was initially protonated with acetic acid and dissolved in a water/DMSO mixture containing 15 mM trityl radical OX63. After hyperpolarization of the sensor solution, only the carbonyl peak of acetic acid and the methyl peak of DMSO were visible. A possible reason for the lack of hyperpolarization of the sensor itself is that its solubility in an aqueous solution is too low. Alternatively, it may be that hyperpolarization is achieved but lost quickly due to the short T1 of the carbonyl carbon (11 sec).

To increase solubility, pyruvic acid, which has been shown to stay amorphous when frozen, was used as the acid, glassing agent and solvent. The same source of unpaired electrons, OX63, was used. The sample was later diluted with D$_2$O before acquisition. The set of spectra obtained contained a variety of peaks, most of which came from pyruvic acid and its derivatives (Figure 34). However there were extra peaks that couldn’t be identified and did not correspond to the pH sensor. To minimize the effects of pyruvic acid derivatives, a D$_2$O-tris buffer was used as the dilution solvent. After hyperpolarization, a peak at 161 ppm was clearly visible in the $^{13}$C NMR spectrum. Unfortunately, a bubble formed in the syringe during the experiment which produced
broad MR signal lines. This last experiment should therefore be repeated in the future to determine whether $^{13}\text{C-SPE1}$ could be used to sense pH \textit{in vitro} and \textit{in vivo}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure34.png}
\caption{Hyperpolarization of $^{13}\text{C-SPE1}$ with pyruvic acid as solvent. Left, stack of spectra of $^{13}\text{C-SPE1}$/pyruvic acid mixture acquired every 2 seconds. The $T_1\text{s}$ of each peak are shown below the spectra. Top right, blank spectrum of pyruvate. Bottom right, spectrum of $^{13}\text{C-SPE1}$/pyruvic acid mixture dissolved with D$_2$O.}
\end{figure}

4.3 Summary and Conclusions

In this chapter, the first ratiometric $^{13}\text{C}$ NMR pH sensor, $^{13}\text{C-SPE1}$, based on the slow proton exchange (SPE) strategy is reported. This water soluble compound, which is the $^{13}\text{C}$-labelled derivative of SPE1, with $^{13}\text{C}$-labelled ureido carbons, was synthesized similarly to SPE1 using a $^{13}\text{C}$-labelled starting material, $^{13}\text{CS}_2$. Slow chemical exchange between the different protonation states was obtained by trapping the incoming protons inside the molecular cavity using intramolecular hydrogen bonds. Distinct signals were produced for the neutral and protonated forms due to unusual slow chemical exchange. The ratio of the different protonation states of the sensor could be used to produce highly precise pH values of the aqueous medium \textit{in vitro}. In
addition to possessing the advantages of SPE1, such as high pH accuracy, high proton selectivity and ratiometry, $^{13}$C-SPE1 can detect pH using $^{13}$C NMR which is not affected by background signals from hydrogen containing molecules, like lipids or sugars, and potentially allows higher signal detection using hyperpolarization. Hyperpolarization was performed on $^{13}$C-SPE1, but the fast decrease in polarization does not permit applications in biological samples yet. On the other hand, the apparent pH for $^{13}$C-SPE1 is similar to SPE1 and is suitable for pH measurement in Xenopus laevis oocytes. Overall, $^{13}$C-SPE1 has great potential for detecting and mapping pH and pH changes in living systems. Next generation pH sensors with a wide range of pHs are under investigation which could further increase the scope of biological applications of the SPE strategy.

4.4 Materials and Methods

4.4.1 General Experimental Procedures

See chapter 3 section 3.5.1 for details on experimental procedures.

4.4.2 Syntheses

4.4.2.1 Synthesis of $^{13}$C-Labelled Tris-(2-isothiocyanate-ethyl)amine ($^{13}$CITC)

(2-aminoethyl) amine (tren, 0.40 g, 3.00 mmol) in 10 ml of THF was added dropwise to a solution of DCC (2.23 g, 12 mmol) and 1.0 g $^{13}$CS$_2$ (4.8 eq) in 10 ml THF at -10°C under argon atmosphere. The reaction was stirred overnight. 15 ml diethyl ether were added and the mixture was filtered. Evaporation of the filtrate and purification by chromatography on silica gel with DCM/hexane (2:1) gave 0.595 g (81%) $^{13}$CITC. mp: 49-50°C (lit 48-49°C$^{166}$); $^1$H NMR (500MHz, CDCl$_3$): $\delta$ 3.61 (t, J = 6.2Hz, 6H), 2.98 (t, J = 6.2 Hz, 6H); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$ 133.4, 54.69, 44.51; IR (neat): 2071 cm$^{-1}$.

4.4.2.2 Synthesis of $^{13}$CTUC

A solution of tren (187.0 mg, 1.3 mmol) in 250 ml of CHCl$_3$ and a solution of $^{13}$CITC (350.0 mg, 1.3 mmol) in 250 ml CHCl$_3$ were added dropwise simultaneously to 300 ml CHCl$_3$ at 60°C under argon atmosphere. The mixture was refluxed for 30 min. After evaporation, the solid was washed with 20 ml MeOH to give 514.2 mg $^{13}$CTUC (96%) as white solid. $^1$H NMR (400 MHz,
DMSO-\(d_6\) \(\delta\): 7.09 (broad s, 6H), 3.48 (s, 12H), 2.51 (t, \(J= 5.0\) Hz, 12H); (500 MHz, CDCl\(_3\)) \(\delta\): 6.72 (broad s, 6H), 4.63 (broad s, 6H), 3.00 (broad s, 6H), 2.80 (broad s, 6H), 2.42 (broad s, 6H); \(^{13}\)C NMR (75 MHz, DMSO-\(d_6\)) \(\delta\): 50.31, 41.24; IR: 2181, 1568 cm\(^{-1}\).

4.4.2.3 Synthesis of \(^{13}\)C-SPE1

200 mg (0.5 mmol) of \(^{13}\)CTUC and 541.6 mg (3 mmol) toslyic acid (TsOH) were dissolved in 10 ml DMSO under argon atmosphere. The solution was heated to 110°C for 5 hrs and then distilled to dryness. The resulting solid was dissolved in 20 ml water, washed 3 times with 20 ml diethyl ether and the aqueous layer was neutralized with 500 mg Na\(_2\)CO\(_3\). The solvent was evaporated, and 20 ml CH\(_2\)Cl\(_2\)/MeOH (3:1) were added to the solid. The mixture was filtered, the filtrate was evaporated and purification by chromatography on aluminium oxide using a solution of 3% methanol in CH\(_2\)Cl\(_2\) gave 89.8 mg (51%) \(^{13}\)C-SPE1. \(^1\)H NMR (500 MHz, D\(_2\)O) \(\delta\): 2.99 (t, \(J= 5.3\)Hz, 12H), 2.43 (t, 12H); \(^{13}\)C NMR (125 MHz, D\(_2\)O) \(\delta\): 161.09, 50.95, 37.40; IR: 3304, 1669, 1631, 1569 cm\(^{-1}\); MS (ESI) \(m/z\) 374.26 (M+1\(^+\)).

4.4.3 NMR Monitored pH Calibration of \(^{13}\)C-SPE1

A solution of \(^{13}\)C-SPE1 (2 mM) was dissolved in 10 mM phosphate buffer (pH = 7.4). Aliquots of 500 \(\mu\)l were prepared at different pH values by addition of HCl or NaOH, and the pH was measured using a calibrated pH electrode (Cole Parmer Thermo Scientific Orion pH microelectrode) and a VWR symphony SB70P pH meter. The sample was placed in a 5 mm NMR tube with a sealed capillary filled with D\(_2\)O. \(^{13}\)C NMR spectra were obtained at 25°C or 37°C with 256 scans per sample on a 500 MHz Bruker Avance spectrometer. The experimental data were fitted with ORIGIN 9.0 software using non-linear least square regression.

4.4.4 Measurement of Intracellular pH

A Xenopus laevis frog was anesthetized in tricaine methane-sulfonate (MS-222) for 1 hour.\(^{141}\) Unfertilized oocytes were surgically removed to OR2 buffer (82.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl\(_2\), 1 mg MgCl\(_2\), 1 mM Na\(_2\)HPO\(_4\), 5 mM HEPES, pH 7.8). The oocytes were washed with OR2 buffer followed by collagenase solution (OR2 buffer supplemented with 1-2 mg/ml collagenase) for 10 min. The eggs were washed with OR2 buffer and divided into small plates in ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl\(_2\)•2 H\(_2\)O, 1 mM MgCl\(_2\)•6 H\(_2\)O, 5 mM
HEPES, pH 7.6). After 12 hrs, the oocytes were defolliculated, placed in OR2 buffer and microinjected (49 nl of a 450 mM pH sensor solution per oocyte per injection).

The $^{13}$C NMR experiments were performed on a 500 MHz Varian Inova spectrometer, equipped with an inverse cryogenic probe. 200 oocytes were placed in a Shigemi NMR tube in ND96 buffer with 7% D$_2$O. The spectrum was recorded with 32 scans and processed with MestReNova 10.0.1 software.

### 4.4.5 Hyperpolarization

Dynamic nuclear polarization (DNP) and dissolution was used to produce hyperpolarized $^{13}$C-SPE1/pyruvate solution. 50 mg $^{13}$C-SPE1 was dissolved in 83.6 µl pyruvic acid and 15 mM OX63 trityl radical. The solution was hyperpolarized using a HyperSense DNP polarizer and 4.54 mls tris/EDTA solution was used to dissolve the sample to a 30 mM sensor solution that was used for the MR experiments.
Chapter 5

Second Generation SPE Based pH Sensor with a Modified $pK_a$ and Extended pH Window

A significant portion of this chapter has been adapted from a manuscript in preparation.


5.1 Introduction

MR spectroscopy can provide information on environmental pollution, food production as well as physiological and pathological processes in living organisms and particularly humans. For example, pH, which is a measure of acidity of the aqueous medium and requires accurate monitoring can be analyzed using MR spectroscopy. When pH homeostasis fails, conditions such as in inflammation, cystic fibrosis or cancer can occur. Different MR methods for pH measurements in humans have been explored, but routine, non-invasive, accurate and sensitive pH detection techniques in living organisms remain a challenge. For example, MRI based methods involving sensors with pH-dependent relaxivity have successfully been applied for pH determination in vivo in research settings, but these probes produce concentration dependent signals that can interfere with pH accuracy. The emerging MRI based approach CEST, which uses saturation transfer between the bulk water and exchangeable protons to produce pH dependent signals, flourished in the past decade and, similar to pH-dependent relaxivity MRI, offers promise for pH mapping (see section 1.3.1.2.1 and 3.1 for more details). However, both methods require calibration or the use of external standards which reduces accuracy or sensitivity of pH measurements. A few pH-sensitive NMR methods have also been
designed for research purposes. These conventional methods use pH sensors with two or more protonation states which are in fast exchange on the NMR timescale (for more information on conventional pH sensors, see section 3.1). Because of fast exchange, most NMR pH sensors produce a pH-dependent signal which is a weighted average of the chemical shifts between the two protonation states. These sensors will however, be affected by pH-independent factors such as ionic strength, magnetic field inhomogeneities or interaction with metal ions. We recently developed a new approach called the slow proton exchange (SPE) method (section 3.2 and 4.1.1). Because proton exchange is fast, it is challenging to obtain pH from the ratio of the protonated (B) and non-protonated (BH⁺) states of a sensor which could otherwise easily be obtained by using the Henderson-Hasselbalch equation: \( \text{pH} = pK_a + \log \frac{[B]}{[BH^+]}. \) The SPE method is based on the premise that a small molecule is able to bind protons strongly and selectively in order to trap them inside their molecular cavity for a period longer than the NMR timescale, thereby artificially creating unusually slow proton exchange, in analogy to the protons found in the core of proteins in vivo. This sterically hindered hydrophobic environment was successfully created and reported previously with the pH-sensitive SPE1 molecule, which produced accurate pH readings in vitro and in bacterial culture at slightly basic pH. Because the ratiometric mechanism in SPE is not based on chemical shift change and the signals used for pH determination are not concentration dependent, the SPE method offers better accuracy than the conventional chemical shift based NMR pH detection methods.

In this chapter, a pH sensor with optimized \( pK_a \) which displays slow proton exchange and uses the SPE strategy to detect pH with high accuracy is reported. This synthetic sensor called SPE2 has a \( pK_a \) in the neutral range, more suitable for biological applications, as well as a wider pH window extending pH detection from pH 0.7 to 8.1. We demonstrate that SPE2 can monitor pH in real time in bacterial culture, not only by providing similar pH values as the first generation sensor but also by allowing detection of a wider pH range. In addition, the low pH of a solution of half-fermented vinegar was successfully detected with this sensor thereby demonstrating the possibility to detect a wide range of pHs with SPE2, which extends tremendously the potential applications of the SPE-based strategy.
5.1.1 Goal and Molecular Design

The $pK_a$ of the first generation sensor $\text{SPE1} \ (pK_a = 8.00)$ was fairly high for \textit{in vivo} pH detection. Sensors with $pK_a$s close to 7 are more suitable for this purpose. One such molecule is $1,4,7,10,13,16,21,24$-Octaazabicyclo$[8.8.8]$ hexacosan-5,6,14,15,22,23-hexone or $\text{SPE2}$ (Figure 35). Similar to $\text{SPE1}$, $\text{SPE2}$ displays slow proton exchange but its $pK_a$ is more suitable for biological applications. $\text{SPE2}$ possesses two bridgehead N-atoms that can be protonated and three oxalamide groups oriented in a C$_3$ symmetric fashion, which provide hydrogen bond acceptor sites. As opposed to $\text{SPE1}$ that only has three O-atoms involved in intramolecular hydrogen bonding, $\text{SPE2}$ possesses six O-atoms. Upon protonation of the first bridgehead N-atom, the O-atoms close to the added proton turn inward to form intramolecular hydrogen bonds. As a result, the other three O-atoms point away from the core of the structure. This creates an electron poor region near the non-protonated bridgehead N-atom which in turn causes the second $pK_a$ of $\text{SPE2}$ to be unusually low for a tertiary amine (Figure 35). Due to this negative cooperative protonation behavior, $\text{SPE2}$ has 2 distinct $pK_a$s, 5 pH units apart. The three protonation states of the cage (i.e. neutral, mono-protonated and bis-protonated) can be detected by $^1$H NMR. Both protonation steps occur slower than NMR timescale thus allowing the use of $\text{SPE2}$ for pH detection using the SPE strategy. This also increases the pH range of the second generation sensor from 2 to 4 pH units. Overall, the two major goals for the development of $\text{SPE2}$ were a lower $pK_a$ than $\text{SPE1}$, more appropriate for biological applications, and a wider pH window which broadens the potential applications of the sensor.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{structure.png}
\caption{Structure and protonation states of the cage-shaped pH sensor $\text{SPE2}$. The bridgehead N-atoms are protonated and intramolecular hydrogen bonds are formed with the closest O-atoms from the oxalamide group trapping the incoming proton inside the molecular cavity longer than NMR timescale and allowing SPE to occur between the different protonation states of $\text{SPE2}$. $^1$H NMR methylene signals used for determination of pH are labelled.}
\end{figure}
5.2 Results and Discussion

5.2.1 Synthesis of SPE2

SPE2 was synthesized from the tripodal amine \( \text{tris}(2\text{-aminoethyl})\text{amine (tren)} \) to \( \text{tris}(2\text{-ethyl(oxy(oxamoyl)))ethyl)amine (trenest)} \) using diethyloxalate, following by high dilution reaction of equimolar concentration of tren and trenest to form the C₃ and mirror plane symmetrical SPE2 (Figure 36).¹⁷⁸

![Synthesis of SPE2](image)

Figure 36. Synthesis of SPE2.

5.2.2 Measurement of pH

SPE2 possesses two bridgehead N-atoms which can be protonated under acidic conditions. Depending on the pH of the solution, the different protonation states of SPE2 display distinct signals in the \(^1\text{H} \) NMR spectrum due to slow chemical exchange. This allows determination of pH using the Henderson-Hasselbalch equation (\( \text{pH} = \text{pK}_a + \log[B]/[BH^+] \)), where B is the neutral form of the sensor and BH\(^+\) is the protonated form. This is in contrast to the conventional fast exchanging pH sensors that display a pH-dependent chemical shift and therefore make it difficult to obtain the [B]/[BH\(^+\)] ratio directly. To determine the pK\(_a\)s of SPE2, a series of \(^1\text{H} \) NMR spectra were collected at different pH values between 0.50 and 8.50. A capillary of D₂O was inserted into the NMR tubes during titration for deuterium lock. Under basic conditions (pH > 8.5) at room temperature, only two peaks for the neutral SPE2 at 2.61 and 3.35 ppm appeared in the NMR spectrum for both methylene units (-CH₂-CH₂-). When the pH gradually decreased and the first N-atom became protonated, the peaks of the mono-protonated form of SPE2 (SPE2H\(^+\)) appeared in the spectrum. This caused the mirror plane of symmetry of SPE2 to be broken, which produced four peaks in the \(^1\text{H} \) NMR spectrum at 2.70, 3.39, 3.61 and 3.78 ppm, while the peaks of neutral SPE2 remained at the same chemical shift. As pH decreased, the peaks of
neutral SPE2 gradually diminished in intensity while the peaks of SPE2H⁺ increased. After the first protonation step, an electron poor pocket was formed inside the cage, which dramatically decreased the second pKₐ of SPE2. Therefore, the mono-protonated form of the sensor was dominant until pH ~ 2.5, where the two peaks of the bis-protonated sensor (SPE2H₂²⁺) emerged at 3.72 and 3.83 ppm due to recovery of the mirror plane symmetry (see Figure 35 and 37).

Figure 37. ¹H NMR pH titration of SPE2 at 25 and 37 °C in 10 mM phosphate buffer. a) Selected partial ¹H NMR spectra of SPE2 at different pH values close to pKᵢ. b) Ratiometric curve of SPE2 based on the methylene protons in the ¹H NMR spectrum around pKᵢ. The fit was done using only the 2 most upfield peaks (2.61 and 2.70 ppm) due to overlapping of other peaks. c) Selected partial ¹H NMR spectra of SPE2 at different pH values close to pKᵢ. d) Ratiometric curve of SPE2 based on the methylene protons in the ¹H NMR spectrum close to pKᵢ.
For $pK_{a1}$ determination, a graph of pH against the fraction of neutral sensor
$(\text{SPE2}/(\text{SPE2}+\text{SPE2H}^+))$ from the NMR integrals was obtained (Figure 37), at 25 and 37 °C.
For $pK_{a2}$, a similar graph was created using the fraction of mono-protonated sensor
$(\text{SPE2H}^+/(\text{SPE2H}^++\text{SPE2H}_2^{2+}))$ at 25 and 37 °C as well. For pH calculations, Henderson-
Hasselbalch equations were used for nonlinear least square fitting of the data (see methods section 5.4.3). With these equations, the $pK_{a1}$ of SPE2 was 7.33 ± 0.02 at 25 °C and 7.10 ± 0.02 at 37 °C and the $pK_{a2}$ was 1.65 ± 0.02 at 25 °C and 1.64 ± 0.02 at 37 °C. These values are similar
to the $pK_a$ obtained by potentiometric titrations, which confirms the reliability of the method.

5.2.3 Biological Applications of SPE2

5.2.3.1 Monitoring pH Change in *Escherichia coli* Cultures

To demonstrate that SPE2 can accurately detect pH in biological systems and that the pH range
of the sensor is more suitable than the first generation sensor (i.e. closer to pH 7), we applied
SPE2 to live bacterial cultures consisting of wild type as well as *E. coli* knockouts to monitor pH
changes in real time. Many organisms, including bacteria, can grow using different oxygen
concentrations and they increase production of acidic metabolites, such as acetate, succinate and
formate, in the absence of sufficient oxygen.144Previously, we have shown that SPE1 could
monitor pH changes of an *E. coli* culture deprived of oxygen.93 Because the $pK_a$ of SPE1 was
slightly basic, the pH of the culture could no longer be measured after 3 hours. The same
experimental setup was used with the second generation sensor and a kinetic study of
concentrated *E. coli* cells (1 ml aliquot at OD$_{600}$ = 1) was carried out. The bacteria were placed in
a sealed 4 mm NMR rotor with a solution of SPE2 (0.9 mM, pH 7.6) at 37 °C and the pH was
recorded over time. The initial pH of the solution was determined to be 7.31 from a ratio of
63/37 (62% neutral SPE2) and mainly displayed the peaks of neutral SPE2. A series of $^1$H NMR
spectra were continuously acquired every 15 minutes for 15 hours. During this time, the signals
of neutral SPE2 gradually decreased while the peaks of mono-protonated SPE2 (SPE2H$^+$) in
creased, which gave a slow decrease in pH of 7.31 to 6.62 over 15 hours (Figure 38a).
Similarly to previous experiments with SPE1, the two sharp peaks of succinate (at 2.40 ppm)
and acetate (at 1.92 ppm) appeared gradually with acidification of the solution and the viability
of sensor-treated cells did not differ significantly compared to non-treated cells.
An experiment was carried out for a longer period of time (28 hours) and \textbf{SPE2} was able to reproducibly determine pH of the \textit{E. coli} solution for over 15 hours after which the pH plateaued at 6.57. These experiments demonstrate that the major disadvantage of \textbf{SPE1} (i.e. its inability to monitor pH for longer than 3 hours due to the high pK\textsubscript{a}) was mitigated using \textbf{SPE2}: the second generation sensor can measure pH based on the SPE mechanism similarly to \textbf{SPE1}, but its lower pK\textsubscript{a} allows pH determination for a longer period, until the pH of the solution plateaus.

A similar kinetic experiment was carried out in \textit{E. coli} with an added carbon source (0.5% glucose and 30 mM sodium formate). In addition to providing a nutrient source, the solution of glucose/formate is known to induce hydrogenase-III activity during anaerobic growth. The results showed that compare to MC4100, the non-linear pH decrease is much sharper initially with an added carbon source, as shown in Figure 39. This is consistent with the expectation that the bacterial culture is able to survive at lower pH provided that a food source is present in the sample.\textsuperscript{181}
In addition to monitoring pH in wild-type *E. coli*, three different knockout strains, which lack important genes and proteins involved in pH homeostasis, were also tested. The first strain is HYD723\textsuperscript{182} lacking the NikA nickel import protein specifically expressed for anaerobic growth. Hydrogenase proteins\textsuperscript{183-184} such as (HycE) and possibly HyaA and HybB, which are metalloenzymes that catalyse the reversible conversion of H\textsubscript{2} into protons and electrons, might be deactivated in the knockout. The second strain is DHPB,\textsuperscript{185} which lacks the HypB maturation protein resulting in all hydrogenases (HyaA, HybB and HycC) being deactivated. Finally, HD705\textsuperscript{182} was used in the study because it is an anaerobic hydrogenase III knockout (HycE) and HycE is part of the formate hydrogen lyase (FHL) pathway which is tightly related to pH homeostasis. Kinetic studies of *E. coli* knockout strains with the pH sensor were run similarly to the wild-type study. The experimental setup was the same as previously mentioned: SPE2 sensor solution (0.9 mM, pH 7.6) was added to the knockout strains (1 ml aliquot at OD\textsubscript{600} = 1) and the sensor/cell solution was placed in a sealed rotor for NMR experiments. The decrease in pH over time for the HD705 knockout strain did not significantly differ from the parent strain. However, in contrast to the parent strain, the peak of acetate was larger than succinate initially which might indicate that acetate production is upregulated in this knockout while under oxygen stress until the bacteria switch to anaerobic fermentation (Figure 40a). The pH in HYD723 decreased at a faster rate than the parent strain over the first two hours and at a much slower rate after that (Figure 40b). Rates of increase in succinate and acetate production were similar to the parent
strain. It is possible that this transition is related to a changeover in aerobic to anaerobic growth, where anaerobic nickel import, in the absence of NikA is sufficiently different to produce an observable effect on the overall pH of the solution. The pH drop in DHPB knockout was overall more rapid than the parent strain (Figure 40b). It is possible that overall hydrogenase activity is more impaired in DHPB versus HYD723, as HypB is necessary for the maturation of all three hydrogenases but NikA is mostly implicated in HycE activity alone. Given the links between pH regulation and hydrogenase activity in *E. coli*¹⁸⁶ this may account for the change in cytoplasmic pH, but the exact source of this observation requires further study. Similar experiments of knockout strains in the presence of an added carbon source were also carried out in two knockout strains (HD705 and DHPB) with similar results as the parent strain: a much faster pH decrease was observed.

**Figure 40.** Monitoring of pH of *E. coli* knockout strains (OD₆₀₀ = 1) using a 0.9 mM solution of SPE2 in phosphate buffer. a) selected stacked ¹H NMR spectra of HD705 knockout *E. coli* cells in the presence of 0.9 M SPE2. NMR measurements were taken continuously for 15 hours using 256 scans (15 min per experiment) and the peak of acetate has higher intensity than succinate which contrasts with the parent strain. b) Graph of decrease of pH over time of SPE2 treated HYD723 and DHPB knockouts.

The experiments presented in this section confirmed that SPE2 is able to monitor pH changes in real time in a reproducible and biocompatible manner in a bacterial culture deprived of oxygen, similarly to SPE1. Even though the current set does not allow determination of the sensor location within the cells, pH detection using SPE2 in wild-type and *E. coli* knockout strains occurred over a wider pH range due to the more suitable pKₐ₁ of SPE2 and displayed different rates of pH decrease and acidic metabolites increase depending on the strain.
5.2.3.2 Detecting pH of a Fermenting Solution

To demonstrate that SPE2 can also be used to measure acidic pH, the sensor was applied to a solution of half-fermented vinegar. Vinegar is produced using bacteria from the genus Acetobacter, which converts ethanol into the major component of vinegar: acetic acid. The progress of the conversion of ethanol to acetic acid that occurs at pH lower than 4 can be analyzed using SPE2. The most common way to produce vinegar is by a method called submerged fermentation.\textsuperscript{187} The chemical reactions involved in this aerobic process are shown in Figure 41.\textsuperscript{187} The first step of the reaction converts ethanol into acetaldehyde using the enzyme alcohol dehydrogenase, followed by the aldehyde dehydrogenase catalyzed oxidation of the aldehyde to form acetic acid. The process is almost quantitative and the bacteria are known to exhibit resistance to low pH and high concentrations of acetic acid.

\[ \text{CH}_3\text{CH}_2\text{OH} \xrightarrow{\text{ADH}} \text{CH}_3\text{CHO} + 2 \text{H}^+ \xrightarrow{\text{ALDH}} \text{CH}_3\text{COOH} + 2 \text{H}^+ \]

\textbf{Figure 41.} Chemical reactions for the conversion of ethanol to acetic acid. ADH: alcohol dehydrogenase, ALDH: aldehyde dehydrogenase.

Usually, the vinegar production process starts at a concentration of 5% alcohol in the presence of Acetobacter and ends at 0.05% alcohol. Because this process generates protons and produces a weak acid, it is possible to follow the course of the reactions using pH. For this purpose, a solution of half-fermented vinegar (3.6% alcohol) containing SPE2 was subjected to NMR (Figure 42). A ratio of 83/17 (83 \% $\text{SPE2H}^+$) was obtained from NMR integrals which corresponds to a pH of 2.35. This is in agreement with the pH obtained from pH electrode readings. From NMR peak intensities, the ratio of ethanol to acetic acid can also be determined.

At pH 2.35, this ratio is 0.32/1 which is in line with the values obtained from near-infrared (NIR) spectroscopy. Therefore, this experiment confirmed that SPE2 could accurately determine pH of an acidic solution and observe the progress of the conversion of ethanol into acetic acid. Future work will involve monitoring of the reaction over time to correlate pH with acetic acid production.
An optimized ratiometric $^1$H NMR pH sensor based on the SPE strategy, SPE2, was presented in this chapter. SPE2 possesses the advantages of the SPE method, such as high sensitivity and accuracy compared to the conventional NMR pH detection methods which are chemical shift-based and therefore susceptible to pH-independent factors. SPE2 was prepared through an optimized synthesis from a previously published procedure and was applied to live cells and a fermenting solution for ratiometric NMR pH sensing. The SPE strategy is similar in SPE1 and SPE2, in that the high proton selectivity and slow proton chemical exchange between the different protonation states was achieved by trapping the incoming protons inside the molecular cavity with intramolecular hydrogen bonds. Therefore, the different protonation states of the sensor could be detected simultaneously in the $^1$H NMR spectrum and pH was measured ratiometrically and accurately close to the $pK_a$ of SPE2. Accurate pH detection was then possible by using the ratio of the basic and acidic species obtained from NMR integrals. Unlike SPE1, SPE2 possesses two distinct $pK_a$s, due to the presence of an extra C=O double bond forming an oxalamide group which produces SPE2’s negative cooperative protonation behavior. This provides important advantages for the sensor, including a $pK_a$ more suitable for biological
applications ($pK_a = 7.10$ at $37^\circ C$) as well as a wider pH window due to an unusually low second $pK_a$ ($pK_{a2} = 1.65$). **SPE2** successfully monitored pH changes over time of *E. coli* live bacterial cultures as well as a fermenting *A. acetii* solution. Overall, **SPE2** has great potential for pH measurement and pH mapping in living systems. Its two $pK_a$s as well as the lower $pK_{a1}$ value also broadens the possible applications of the sensor. Next generations **SPE**-based pH sensors are currently being investigated to produce cell trappable and regioselective sensors.

5.4 Materials and Methods

5.4.1 General Experimental Procedures

All reactions were carried out under argon atmosphere, using dry glassware. Reagents were used as received from Alfa Aesar (Georgetown, Canada) and Caledon Laboratory Chemicals (Georgetown, Canada). Thin layer chromatography (TLC) was performed on aluminum-backed silica TLC plates (silica gel 60 F 254) obtained from EMD Chemicals (Gibbstown, MA, USA) and the spots were located by UV light (254 nm). Column chromatography was performed on silica gel 60, 63–200 microns and alumina basic standard gel, 50–200 microns, obtained from Caledon Laboratory Chemicals (Georgetown, Canada). All nuclear magnetic resonance (NMR) experiments were recorded on a Bruker Avance-III 500MHz or Varian Mercury 400MHz spectrometer. NMR solvents were obtained from Aldrich (St. Louis, MO, USA). All $^1H$ NMR spectra were manually corrected for phase and baseline distortion using TopSpinTM 3.1 and MestReNova 8.1.4 and integral ratios were obtained by taking $\pm 20$ Hz around each peak. The chemical shifts were first calibrated to DSS as an internal standard, where the peaks of the neutral **SPE2** appeared at 2.61 and 3.35 ppm. The chemical shifts were then referenced relative to the peaks of neutral **SPE2**. Electron impact (EI) mass spectrometry (MS) and electron spray ionization (ESI) MS were performed on an AB/Sciex QStar mass spectrometer. Infra-red (IR) spectra were recorded on a Bruker Alpha FT-IR spectrometer.

5.4.2 Syntheses

5.4.2.1 Synthesis of N,N,N-Tris(2-(ethyloxamate)ethyl)amine (**trenest**)

Tris(2-amoethyoxamly)amine (**tren**, 0.73g, 5 mmol) in 20 ml THF was added dropwise to the solution of 15 ml diethyl oxalate. The reaction mixture was stirred for 3 hours at room temperature. After evaporation of the slightly yellow solution on the rotary evaporator, 75 mL of
diethyl ether were added to the flask. After 24 hours, a yellow oil appeared in the flask and was extracted. Purification of the oil by chromatography on silica gel using a gradient of 5-10% ethanol in ethyl acetate gave trenest as a yellow oil. Purification of the remaining solution by chromatography on silica gel using the same gradient of 5-10% ethanol in ethyl acetate gave the remaining trenest (1.714.0 mg, 77%). TLC (5% ethanol in ethyl acetate): Rf = 0.30. IR: 3323.16 (N-H); 2982.17 (C-H sp³); 1679.45 (-C=O); 1524.94 (N-H bend); 1200.25 (C-O) cm⁻¹. ¹H NMR (500 MHz, CDCl₃) δ 1.38 (t, 9H); 2.73 (t, 6H), 3.40 (t, 6H), 4.33 (q, 6H), 7.55 (s, 3H). ¹³C NMR (125 MHz, CDCl₃) δ 160.58, 157.84, 63.10, 53.40, 37.69, 13.90. MS (ESI) m/z 447.2082 (M+1⁺).

5.4.2.2 Synthesis of SPE2

SPE2 was obtained using a high dilution technique: ethanolic equimolar solutions of tren and trenest (5 mM in 90 ml) were added dropwise simultaneously to a flask containing 350 ml ethanol and the solution was stirred overnight. The solution was rotary evaporated to until 10% solvent remained. After 5 hrs, a white precipitate appeared and was collected by vacuum filtration to give 153.2 mg (59%) white solid. TLC (20% methanol in ethyl acetate): Rf = 0.13; IR: 3355.52, 3226.74 (N-H), 2792.66 (C-H sp³), 1638.81 (-C=O), 1526.61 (N-H bend), 1169.02 (C-N) cm⁻¹; ¹H NMR (pH > 9, 500 MHz, D₂O) δ: 2.61 (broad t, 12H), 3.35 (broad t, 12H); (3 < pH < 6) δ: 2.70 (broad t, 6H), 3.39 (broad t, 6H), 3.60 (broad t, 6H), 3.78 (broad t, 6H); (pH < 0.5) δ: 3.71 (broad t, 12H), 3.83 (broad t, 12H). MS (ESI) m/z 455.2361 (M+1⁺).

5.4.3 NMR Monitored pH Calibration of SPE2

A solution of SPE2 (0.9 mM) was dissolved in 10 mM phosphate buffer (pH = 7.6). Aliquots of 500 µl were prepared at different pH values by addition of HCl or NaOH, and the pH was measured using a calibrated pH electrode (Cole Parmer Thermo Scientific Orion pH microelectrode) and a VWR sympHony SB70P pH meter. The sample was placed in a 5 mm NMR tube with a sealed capillary filled with D₂O. ¹H NMR spectra were obtained at 25 °C or 37°C with 64 scans per sample on a 500 MHz Bruker Avance spectrometer and presaturation of the water signal with a recycle time of 50 sec and a 90° pulse width. The experimental data were fitted with ORIGIN 9.0 software using non-linear least square regression.
5.4.4 Theoretical Model for NMR pH Titration Fitting

Two Henderson-Hasselbalch equations, one for each $pK_a$, were used based on the ratio of basic and acidic species of $SPE2$:

$$\text{pH} = pK_{a1} + \log\frac{[SPE2]}{[SPE2H^+]}, \text{ for } 6.1 < \text{pH} < 8.1 \quad (E1)$$

$$\text{pH} = pK_{a2} + \log\frac{[SPE2H^+]}{[SPE2H_2^{2+}]}, \text{ for } 0.7 < \text{pH} < 2.7 \quad (E2).$$

The equations for percentage of basic sensor shown below (E3 and E4) are derived from the Henderson-Hasselbalch equations (E1 and E2) and are used for nonlinear least square fitting of the experimental data using ORIGIN 9.0.

$$\frac{[SPE2]}{[SPE2] + [SPE2H^+]} = \frac{10^{(pH-pK_a)}}{1 + 10^{(pH-pK_a)}} \quad (E3)$$

$$\frac{[SPE2^+]}{[SPE2^+] + [SPE2H_2^{2+}]} = \frac{10^{(pH-pK_a)}}{1 + 10^{(pH-pK_a)}} \quad (E4).$$

5.4.5 Real Time pH Monitoring of *E. coli* Culture

5.4.5.1 Sample Preparation

*E. coli* MC4100 cells transformed with a pBAD24 plasmid (to confer ampicillin resistance) and knockout strains HD705, HYD723 and DHPB were plated on solid LB-agar medium supplemented with ampicillin and grown overnight at 37°C. LB media and agar were purchased from Bioshop Inc. and used as received. One colony was transferred from the plate into a 50 mL culture of LB-Amp liquid medium and grown for approximately 16 hrs. The overnight cultures were used to inoculate fresh liquid cultures, which were grown at 37°C to an OD$_{600}$ < 1. A 1 ml aliquot of the culture was centrifuged at 10,000 g and re-suspended in 30 µl of a 0.9 mM solution SPE2 in 50 mM phosphate buffer pH 7.6 containing 10% D$_2$O. Another aliquot was collected and re-suspended in phosphate buffer to act as a blank for the NMR experiment and a control for cell viability over the course of the experiment. The sample was transferred to an NMR top insert.
made from Kel-F, sealed with a Kel-F sealing screw and cap, then inserted into a 4 mm o.d. zirconium rotor for the NMR experiment.

To test for viability of the sensor-free and sensor-treated cells, the cells were serially diluted $10^3$ - $10^9$ times in phosphate buffer after the NMR experiment and plated on LB-Amp plates to control for cell survival during the experiment.

5.4.5.2 NMR Experiments

The $^1$H NMR experiments were performed on a Bruker Avance III 500 MHz spectrometer, using a prototype CMP MAS 4 mm $^1$H-$^{13}$C-$^{19}$F-$^2$H probe fitted with an actively shielded Z gradient (Bruker BioSpin) at 37°C. The samples were all spun at a spinning speed of 6666 Hz and all experiments were locked using D$_2$O solvent. Water suppression was achieved using water suppression by gradient-tailored excitation (WATERGATE) and was carried out using a W5 pulse train. All spectra were recorded with 256 scans, recycle delay set at 5 x $T_1$, 5.8 μs 90° pulse widths and collected using 32768 time domain points with spectral widths of 20 ppm. Data were zero filled and multiplied by an exponential window function corresponding to a 1 Hz line broadening in the transformed spectrum.

5.4.6 Detecting pH of a Fermenting *Acetobacter aceti* Solution

A solution of semi-fermented vinegar (3.6% alcohol and 9.68% acetic acid and *Acetobacter aceti*) was obtained from the Reinhart factory in Ontario. The pH of the solution was measured with a pH electrode (2.37) and the solution was diluted with PBS pH 2.35. 10% D$_2$O and 1.6 mg SPE2 were added to 0.3 μl vinegar solution in 1 ml phosphate buffer pH 2.35 and $^1$H NMR on a 500 MHz Bruker spectrometer was performed using 256 scans and presaturation of the water signal with a recycle time of 50 sec and a 90° pulse width.
NMR spectroscopy is a powerful analytical tool used for decades in the study of static and dynamic processes. Important changes in the NMR spectrum can be detected in dynamic systems involving the well-known phenomenon of chemical exchange in NMR.\(^6\) Using those variations of the NMR spectrum can provide new and exciting possibilities in the design of NMR sensors. Different strategies have already been successfully developed, including the MRI-based CEST and hyperpolarization techniques. However, sensitivity and accuracy remain the most important drawbacks of all NMR based sensing techniques. The purpose of this thesis was to develop new NMR strategies which are highly accurate and sensitive based on sensors with similar structures. Two new approaches were presented. First, indirectly sensing anions at very low concentrations (in the nano-molar range) through anion-induced conformational flexibility of a small caged-like synthetic molecule, TUC (chapter 2) was described. The second approach was developed to sense pH ratiometrically by NMR (chapter 3-5). Because the conventional NMR pH sensing methods are chemical shift based, they are sensitive to pH-independent factors that affect chemical shift and therefore reduce accuracy. A ratiometric pH sensing technique therefore produces inherently more accurate pH values. Two generations of sensors were designed, synthesized and successfully applied to biological systems to detect pH and monitor pH changes over time. These two strategies demonstrate the great potential of NMR spectroscopy in the design and implementation of novel sensing strategies as well as their applications in the fields of analytical chemistry, biology and biomedicine.

6.1 Anion Sensing

Selective recognition and sensing of ion by MR spectroscopy has attracted interest for decades.\(^{189-190}\) In the 1960s, cation sensing was predominant due to the small size of cations with
similar geometries and high charge to radius ratio which produce strong intermolecular interactions and binding.\textsuperscript{191-193} Only years later did chemists start to design anion sensors due to the important and ubiquitous nature of those ions in biological systems. Conventional anion sensors consist of a receptor that binds the anion of interest, ideally with high specificity and sensitivity. Sensing of those receptor-anion complexes in NMR occurs using appearance of peaks or changes in chemical shift, which require a high concentration of both receptor and anion, in the low millimolar to sub-millimolar range to obtain a signal. Indirect anion sensing can be used to improve sensitivity. Instead of detecting the appearance of peaks in the spectrum, the method focuses on observing changes in the linewidth of the receptor signals. To implement this method practically, a small synthetic cryptand, TUC was synthesized. TUC possesses HB\textsubscript{intra} which rigidify the structure. In non-polar solvents, the HB\textsubscript{intra} can be broken in the presence of competing HB\textsubscript{inter} from anions. This produces a change in the linewidth of the methylene peaks of TUC, which can be detected at 1000 fold lower concentration than the conventional anions sensors. Because anions catalytically induce conformational flexibility in TUC, the effects produced on NMR are drastic and the concentrations of anions to produce a small effect are minimal. This sensor can therefore detect anion concentrations as low as 120 nM.

6.2 Sensing pH

A new strategy based on slow chemical exchange was developed to sense protons and provide information on the pH of aqueous solutions called the SPE strategy. As opposed to the anion sensing technique which focused on an increase in chemical exchange upon addition of anions, the pH sensing technique used small synthetic molecules that can trap protons inside their molecular cavity to slow down proton exchange and allow detection of different protonation states of the sensor simultaneously in the NMR spectrum. If the pH is close to the pK\textsubscript{a} of the sensor, the ratio between the 2 protonation species can be entered into the Henderson-Hasselbalch equation directly to obtain pH. The conventional NMR pH detection methods are chemical shift based and can be affected by pH-independent factors, such as variation in ionic strength, binding with macromolecules or metal ions that affect chemical shift and decrease pH accuracy. By using ratios of the sensor’s different protonation states, pH can be detected with high accuracy, as low as 0.02 pH units. Two generation of sensors were developed for the SPE strategy: SPE\textsubscript{1} (chapter 3) and SPE\textsubscript{2} (chapter 5). The second generation sensor was investigated because the pK\textsubscript{a} of SPE\textsubscript{1} was slightly basic for biological applications. In addition, SPE\textsubscript{2}
possessed 2 distinct pKₐs (at 1.65 and 7.33) which broadened the possible applications of the sensor. Finally, a $^{13}$C-labelled version of SPE₁ ($^{13}$C-SPE₁) was also studied (chapter 4).

Biological applications using all sensors were performed, including pH monitoring over time of a live *E. coli* bacterial culture (wild-type as well as knockout strains) deprived of oxygen using SPE₁ and SPE₂. The pH of fish and frog oocytes was determined with SPE₁ and $^{13}$C-SPE₁ and a solution of very acidic semi-fermented vinegar with *A. aceti* was investigated with SPE₂.

Overall, pH sensing with SPE sensors produced very accurate results *in vitro* and in biological systems. Moreover, the development of the $^{13}$C-labelled hyperpolarized sensor ($^{13}$C-SPE₁) allowed for pH detection with higher sensitivity with and without hyperpolarization compared to $^1$H NMR sensors.

### 6.3 Future Directions

This thesis focused on the development of two new NMR sensing techniques. Based on those techniques, different sensors can be designed with improved parameters, such as increased solubility, better regioselectivity and selectivity that would increase the possible applications for each sensor. The following sections describe two directions for future research in anion and pH sensing respectively.

#### 6.3.1 Determination of pH of Organic Solvents

Measurement of pH by definition occurs in aqueous media. Determination of pH in non-aqueous or aqueous-organic solvents is not an easy task and requires standardization. $^{194-195}$ TUC, which was described and characterized in chapter 2 is only minimally soluble in aqueous solvents but has decent solubility in organic solvents. TUC is also structurally similar to SPE₁ (chapter 3) which is known to bind protons selectively. Therefore, it should be possible to use both TUC and SPE₁ to determine pH in organic solvents. One of the advantages of using both sensors for pH determination in non-aqueous solvents is that it would be easy to compare the values from both sensors. In addition, since SPE₁ is water soluble, the comparison could also be done between organic and aqueous solvents to create a standard or a pH scale that relates values in organic and aqueous environments.
6.3.2 Development of Cell-Trappable and Regioselective SPE Based pH Sensors

The first generations sensors possess some disadvantages, such as a pKₐ relatively high for *in vivo* application (SPE1) and low aqueous solubility (SPE2, chapter 5). Their most important drawback is the lack of sub-cellular localization during NMR experiment. To overcome these limitations, a new approach consisting of carboxylating the tertiary nitrogen of the sensors was designed. To test this hypothesis, direct carboxylation of the sensors (SPE1 (Figure 43a) and SPE2) was carried out using ethyl bromoacetate as the carboxylating agents in chloroform. No product was formed, even after > 48 hours of reaction. The reaction conditions were changed to the more reactive ethyl iodoacetate in DMSO at higher temperatures (up to 120 °C) as well as longer reaction times in an unsuccessful attempt to facilitate the nucleophilic substitution reaction. Carboxylation of the intermediate isothiocyanate (ITC) was also carried out and produced similar failed results (Figure 43b).

A different synthetic route was designed to produce carboxylated versions of both SPE1 and SPE2 and would simultaneously lower the pKₐ of the sensors due to the electron withdrawing ability of this group as well as increase the aqueous solubility of the cages. Instead of carboxylating the intermediate, the starting material, tren, is carboxylated after protection of the
primary amine groups (see Figure 44 for retrosynthesis of both cages and Figure 45 for the specific synthesis of the intermediate trenC).

Figure 44. Retrosynthesis of carboxylated SPEC1 and SPEC2.

Figure 45. Synthesis of trenC that could be used in the synthesis of the carboxylated sensors.

The successful synthesis of the carboxylated SPE sensors presented in this section would broaden the scope of potential applications of SPE sensors tremendously. High aqueous solubility is an important parameter for any in vivo sensor, since it allows administration of the sensor at high concentration to the targeted area of interest. With the addition of this extra carboxyl group, it would also be possible to further functionalize the molecule and thereby allowing specific and targeted pH sensing to mimic the applications of fluorescent sensors, like green fluorescent proteins or SNARFs sensors. In addition, adding acetoxyethyl ester
The group would create cell-permeable and cell-trappable sensors for intracellular pH sensing. The development of SPE based pH-sensitive reporter genes, similar to the lysine rich-protein reporter developed by van Zijl and colleagues for CEST imaging, is also an excellent example of a future direction with great potential. Applications such as the study of different physiological events, like respiration or change between aerobic metabolism to micro-aerobic metabolism in bacteria and pathological conditions, like cancer, could be further investigated. Due to the high sensitivity and accuracy of the current and future SPE sensors, mapping pH in small animals like mice or rats using MRSI might have crucial implications for human beings with potential to detect major diseases like cancer at earlier stages.

### 6.4 Significance and Conclusion

A variety of techniques have been designed for sensing ions by NMR spectroscopy but the major limitation remains the low sensitivity. In the search for novel and more sensitive NMR techniques, this thesis diverged from chemical shift based methods to focus on chemical exchange based approaches. In the NMR spectrum, the effects of chemical exchange can be observed as changes in chemical shift or changes in linewidth, which are more subtle. Since most conventional sensing techniques are chemical shift based, developing strategies which involved more dynamic processes, like chemical exchange, can overcome disadvantages of the chemical shift based methods and therefore produce more sensitive results.

In particular, the anion sensing strategy presented in chapter 2, which is based on conformational flexibility induced by competition between intramolecular and intermolecular hydrogen bonds, allows anion detection in the high nano-molar range. Imitating this strategy, new sensing techniques can be developed in which a small synthetic molecule or a larger protein, with intramolecular hydrogen bonds can become more flexible in the presence of different species. Of particular interest for biological applications, developing such a method for aqueous environments would have great potential.

The ratiometric pH sensing method presented in chapters 3-5 provides very accurate pH values and could possibly be applied in vivo. With the future development of targetable, cell-permeable and trappable sensors, this method could be applied in small animals and even in humans, for disease diagnostics, providing that the sensors are biocompatible and non-toxic. This is of particular importance since early disease detection correlates with survival rate. Therefore, the
new SPE method, in addition to advancing the field of NMR pH sensing could have a lasting impact on human lives.

Overall, both sensing techniques improve their respective fields by providing more sensitive and accurate measurements through the use of chemical exchange with similar small synthetic molecules. Those molecules have the potential to be modified synthetically to provide increased water solubility, better targeting or improved sensitivity thereby widening the potential applications of all sensors and sensing techniques.
References


63. Desvergne, J. P.; Czarnick, A. W. Proceedings of the NATO Advanced Research Workshops on Chemosensors of Ion and Molecule Recognition, Bonas, France, 1997.


175. Bryant, R. G. *Journal of Chemical Education* **1983**, *60* (11), 933.


Appendices

A1 Application of TUC: Detection of Fatty Acids in Milk

Milk which contains negatively charged fatty acids (FAs) was chosen to demonstrate how TUC can be used to detect anions. Cow milk contains approximately 4% fat, composed of essential FAs, such as linoleic, linolenic and arachidonic acid. Fats can be studied by different methods, such as IR and NMR\textsuperscript{205-206} whereas FAs can easily be analyzed as fatty acid methyl esters (FAMEs) by GC-MS\textsuperscript{207-208}, after esterification of the acids. FAs are rarely detected directly. A 2007 NMR study of whole milk was able to differentiate FAs specifically\textsuperscript{209}, but quantification was not attempted. Using TUC, milk of different fat concentrations can be studied and quantification of FAs can potentially be achieved, based on the degree of line broadening of the TUC $^1$H NMR peaks. For the purpose of this study, 1% and 2% milk was extracted three times with dichloromethane. The organic layer was dried over Na$_2$SO$_4$ and evaporated. 2% milk produced double the fatty acid content than 1% milk (28 mg vs 14 mg FAs in 100 ml milk). Quantification of FA in milk was then carried out. A 1.7 mM solution of TUC was prepared and FAs from milk (dissolved in 500 \mu l CDCl$_3$) were titrated into the solution. Upon addition of FAs, the unsaturated FAs peaks appeared in the spectrum, at 4.31 and 4.18 ppm, and their integrals increased compared to the methylene peaks of TUC (see Table A1 and Figure A1 and A2). In addition, the methylene peaks also increased in peak width: the more FAs added, the wider the peak (see Table A1). Interestingly, FAs obtained from 1% milk produced a smaller effect than 2% milk, consistent with lower amounts of FAs in 1% milk. Therefore, FAs are interacting with TUC in solution and affecting the CE rate. By looking at the integral ratio (of the methylene peaks of TUC and the unsaturated FA peaks) as well as TUC’s methylene peaks broadening it is in theory possible to quantify FAs with this method.

Table A1. Integrals and peak broadening of TUC and FAs during FA titration of milk.

<table>
<thead>
<tr>
<th>Volume of FAs added (ul)</th>
<th>1% milk Peaks (ppm)</th>
<th>2% milk Peaks (ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.68</td>
<td>4.31</td>
</tr>
<tr>
<td>0 ul</td>
<td>43.95</td>
<td>1</td>
</tr>
<tr>
<td>1 ul</td>
<td>49.86</td>
<td>1</td>
</tr>
<tr>
<td>5 ul</td>
<td>51.19</td>
<td>1</td>
</tr>
<tr>
<td>10 ul</td>
<td>53.7</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure A1. Partial $^1$H NMR spectra of 1.7 mM TUC at 500 MHz and 25 °C in the presence of trace amount of FAs from 1% milk.

Figure A2. Partial $^1$H NMR spectra of 1.7 mM TUC at 500 MHz and 25 °C in the presence of trace amount of FAs from 2% milk.
A2 X-Ray Crystallization Data of **TUC**

**Table A2.** Sample and crystal data for 1.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical formula</td>
<td>$\text{C}<em>{15}\text{H}</em>{30}\text{N}<em>{8}\text{S}</em>{3}$</td>
</tr>
<tr>
<td>Formula weight</td>
<td>418.65</td>
</tr>
<tr>
<td>Temperature</td>
<td>300(2) K</td>
</tr>
<tr>
<td>Wavelength</td>
<td>0.71073 Å</td>
</tr>
<tr>
<td>Crystal size</td>
<td>0.150 x 0.280 x 0.600 mm</td>
</tr>
<tr>
<td>Crystal habit</td>
<td>colorless needle</td>
</tr>
<tr>
<td>Crystal system</td>
<td>orthorhombic</td>
</tr>
<tr>
<td>Space group</td>
<td>P n m a</td>
</tr>
<tr>
<td>Unit cell dimensions</td>
<td>$a = 24.353(11)$ Å, $a = 90^\circ$</td>
</tr>
<tr>
<td></td>
<td>$b = 10.977(5)$ Å, $\beta = 90^\circ$</td>
</tr>
<tr>
<td></td>
<td>$c = 7.663(3)$ Å, $\gamma = 90^\circ$</td>
</tr>
<tr>
<td>Volume</td>
<td>2048.5(15) Å³</td>
</tr>
<tr>
<td>Z</td>
<td>4</td>
</tr>
<tr>
<td>Density (calculated)</td>
<td>1.357 Mg/cm³</td>
</tr>
<tr>
<td>Absorption coefficient</td>
<td>0.380 mm$^{-1}$</td>
</tr>
<tr>
<td>$F(000)$</td>
<td>896</td>
</tr>
</tbody>
</table>

**Table A3.** Data collection and structure refinement for 1.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theta range for data collection</td>
<td>1.67 to 25.00°</td>
</tr>
<tr>
<td>Index ranges</td>
<td>$-24 \leq h \leq 28$, $-13 \leq k \leq 13$, $-8 \leq l \leq 8$</td>
</tr>
<tr>
<td>Reflections collected</td>
<td>17722</td>
</tr>
<tr>
<td>Independent reflections</td>
<td>1860 [R(int) = 0.1016]</td>
</tr>
<tr>
<td>Coverage of independent reflections</td>
<td>98.1%</td>
</tr>
<tr>
<td>Absorption correction</td>
<td>multi-scan</td>
</tr>
<tr>
<td>Max. and min. transmission</td>
<td>0.9453 and 0.8043</td>
</tr>
<tr>
<td>Struc. solution technique</td>
<td>direct methods</td>
</tr>
<tr>
<td>Structure solution program</td>
<td>SHELXS-97 (Sheldrick, 2008)</td>
</tr>
<tr>
<td>Refinement method</td>
<td>Full-matrix least-squares on F^2</td>
</tr>
<tr>
<td>Refinement program</td>
<td>SHEXL-97 (Sheldrick, 2008)</td>
</tr>
<tr>
<td>Function minimized</td>
<td>$\Sigma w(F_o^2 - F_c^2)^2$</td>
</tr>
<tr>
<td>Data/restraints/parameters</td>
<td>1860 / 0 / 139</td>
</tr>
</tbody>
</table>
Goodness-of-fit on $F^2$ 1.019

Final R indices 1306 data; $R_1 = 0.0385$, $wR_2 = 0.0866$

$I > 2\sigma(I)$

all data $R_1 = 0.0666$, $wR_2 = 0.0977$

Weighting scheme $w = 1/[(\sigma(F_o^2)^2 + 0.0378P)^2 + 0.5108P]

where $P = (F_o^2 + 2F_c^2)/3$

Largest diff. peak and hole 0.215 and -0.195 eÅ$^{-3}$

R.M.S. deviation from mean 0.044 eÅ$^{-3}$

Table A4. Atomic coordinates and equivalent isotropic atomic displacement parameters (Å$^2$) for 1.

U(eq) is defined as one third of the trace of the orthogonalized $U_{ij}$ tensor.

<table>
<thead>
<tr>
<th>x/a</th>
<th>y/b</th>
<th>z/c</th>
<th>U(eq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.08288(3)</td>
<td>0.25</td>
<td>0.69315(12)</td>
<td>0.0415(3)</td>
</tr>
<tr>
<td>0.25466(3)</td>
<td>0.25</td>
<td>0.19873(12)</td>
<td>0.0413(3)</td>
</tr>
<tr>
<td>0.95215(4)</td>
<td>0.25</td>
<td>0.35667(15)</td>
<td>0.0589(3)</td>
</tr>
<tr>
<td>0.15751(9)</td>
<td>0.14712(19)</td>
<td>0.9012(3)</td>
<td>0.0389(5)</td>
</tr>
<tr>
<td>0.12051(8)</td>
<td>0.97518(18)</td>
<td>0.1682(2)</td>
<td>0.0362(5)</td>
</tr>
<tr>
<td>0.17922(9)</td>
<td>0.1471(2)</td>
<td>0.4056(3)</td>
<td>0.0410(6)</td>
</tr>
<tr>
<td>0.03875(9)</td>
<td>0.14748(19)</td>
<td>0.2044(3)</td>
<td>0.0455(6)</td>
</tr>
<tr>
<td>0.13545(13)</td>
<td>0.25</td>
<td>0.8392(4)</td>
<td>0.0315(8)</td>
</tr>
<tr>
<td>0.13973(11)</td>
<td>0.0240(2)</td>
<td>0.8597(3)</td>
<td>0.0462(7)</td>
</tr>
<tr>
<td>0.14992(11)</td>
<td>0.9382(2)</td>
<td>0.0109(3)</td>
<td>0.0438(7)</td>
</tr>
<tr>
<td>0.14895(10)</td>
<td>0.9408(2)</td>
<td>0.3287(3)</td>
<td>0.0437(7)</td>
</tr>
<tr>
<td>0.19677(10)</td>
<td>0.0230(2)</td>
<td>0.3713(3)</td>
<td>0.0422(6)</td>
</tr>
<tr>
<td>0.20190(13)</td>
<td>0.25</td>
<td>0.3445(4)</td>
<td>0.0326(8)</td>
</tr>
<tr>
<td>0.01286(13)</td>
<td>0.25</td>
<td>0.2528(4)</td>
<td>0.0376(9)</td>
</tr>
<tr>
<td>0.02750(10)</td>
<td>0.0267(2)</td>
<td>0.2704(4)</td>
<td>0.0474(7)</td>
</tr>
<tr>
<td>0.06233(11)</td>
<td>0.9377(2)</td>
<td>0.1683(4)</td>
<td>0.0494(7)</td>
</tr>
</tbody>
</table>

Table A5. Bond lengths (Å) for 1.

S1-C1 1.701(3) S2-C6 1.703(3) S3-C7 1.679(3) N1-C1 1.338(3) N1-C2 1.454(3) N1-H1 0.85(2) N2-C3 1.460(3) N2-C4 1.461(3)
N2-C9  1.476(3)  N3-C6  1.342(3)
N3-C5  1.451(3)  N3-H2  0.83(2)
N4-C7  1.342(3)  N4-C8  1.445(3)
N4-H4  0.85(3)  C1-N1#1  1.338(3)
C2-C3  1.513(4)  C2-H2A  0.97
C2-H2B  0.97  C3-H3A  0.97
C3-H3B  0.97  C4-C5  1.509(3)
C4-H4A  0.97  C4-H4B  0.97
C5-H5A  0.97  C5-H5B  0.97
C6-N3#1  1.342(3)  C7-N4#1  1.342(3)
C8-C9  1.512(4)  C8-H8A  0.97
C8-H8B  0.97  C9-H9A  0.97
C9-H9B  0.97

Symmetry transformations used to generate equivalent atoms:
#1  x, -y+1/2, z

**Table A6.** Bond angles (°) for 1.

<table>
<thead>
<tr>
<th>Bond</th>
<th>Angle</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-N1-C2</td>
<td>126.0(2)</td>
</tr>
<tr>
<td>C2-N1-H1</td>
<td>118.7(16)</td>
</tr>
<tr>
<td>C3-N2-C9</td>
<td>113.2(2)</td>
</tr>
<tr>
<td>C6-N3-C5</td>
<td>127.3(2)</td>
</tr>
<tr>
<td>C5-N3-H2</td>
<td>118.0(17)</td>
</tr>
<tr>
<td>C7-N4-H4</td>
<td>114.9(17)</td>
</tr>
<tr>
<td>N1-C1-N1#1</td>
<td>115.2(3)</td>
</tr>
<tr>
<td>N1#1-C1-S1</td>
<td>122.41(15)</td>
</tr>
<tr>
<td>N1-C2-H2A</td>
<td>109.4</td>
</tr>
<tr>
<td>N1-C2-H2B</td>
<td>109.4</td>
</tr>
<tr>
<td>H2A-C2-H2B</td>
<td>108.0</td>
</tr>
<tr>
<td>N2-C3-H3A</td>
<td>109.2</td>
</tr>
<tr>
<td>N2-C3-H3B</td>
<td>109.2</td>
</tr>
<tr>
<td>H3A-C3-H3B</td>
<td>107.9</td>
</tr>
<tr>
<td>N2-C4-H4A</td>
<td>108.9</td>
</tr>
<tr>
<td>N2-C4-H4B</td>
<td>108.9</td>
</tr>
<tr>
<td>H4A-C4-H4B</td>
<td>107.8</td>
</tr>
<tr>
<td>N3-C5-H5A</td>
<td>109.2</td>
</tr>
<tr>
<td>N3-C5-H5B</td>
<td>109.2</td>
</tr>
</tbody>
</table>
Table A7. Anisotropic atomic displacement parameters (Å²) for 1.

The anisotropic atomic displacement factor exponent takes the form: 

$$-2\pi^2 \left[ h^2 a^*^2 U_{11} + ... + 2hk a^* b^* U_{12} \right]$$

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$U_{11}$</td>
<td>$U_{22}$</td>
<td>$U_{33}$</td>
<td>$U_{23}$</td>
<td>$U_{13}$</td>
<td>$U_{12}$</td>
</tr>
<tr>
<td>S1</td>
<td>0.0332(5)</td>
<td>0.0541(6)</td>
<td>0.0371(6)</td>
<td>0</td>
<td>-0.0046(4)</td>
<td>0</td>
</tr>
<tr>
<td>S2</td>
<td>0.0284(5)</td>
<td>0.0525(6)</td>
<td>0.0430(6)</td>
<td>0</td>
<td>0.0037(4)</td>
<td>0</td>
</tr>
<tr>
<td>S3</td>
<td>0.0304(5)</td>
<td>0.0762(8)</td>
<td>0.0700(8)</td>
<td>0</td>
<td>0.0219(5)</td>
<td>0</td>
</tr>
<tr>
<td>N1</td>
<td>0.0397(12)</td>
<td>0.0343(13)</td>
<td>0.0426(14)</td>
<td>0.0021(11)</td>
<td>-0.0105(10)</td>
<td>-0.0036(10)</td>
</tr>
<tr>
<td>N2</td>
<td>0.0383(11)</td>
<td>0.0319(11)</td>
<td>0.0383(13)</td>
<td>-0.0006(9)</td>
<td>0.0043(9)</td>
<td>-0.0021(9)</td>
</tr>
<tr>
<td>N3</td>
<td>0.0397(13)</td>
<td>0.0419(14)</td>
<td>0.0413(14)</td>
<td>-0.0027(11)</td>
<td>0.0099(11)</td>
<td>-0.0025(11)</td>
</tr>
<tr>
<td>N4</td>
<td>0.0316(12)</td>
<td>0.0430(13)</td>
<td>0.0619(16)</td>
<td>0.0027(11)</td>
<td>0.0197(11)</td>
<td>-0.0026(10)</td>
</tr>
<tr>
<td>C1</td>
<td>0.0274(17)</td>
<td>0.039(2)</td>
<td>0.0279(19)</td>
<td>0</td>
<td>0.0066(14)</td>
<td>0</td>
</tr>
<tr>
<td>C2</td>
<td>0.0592(17)</td>
<td>0.0371(15)</td>
<td>0.0424(16)</td>
<td>-0.0028(13)</td>
<td>0.0020(13)</td>
<td>-0.0034(13)</td>
</tr>
<tr>
<td>C3</td>
<td>0.0548(16)</td>
<td>0.0301(14)</td>
<td>0.0466(17)</td>
<td>-0.0042(13)</td>
<td>0.0066(13)</td>
<td>0.0048(12)</td>
</tr>
<tr>
<td>C4</td>
<td>0.0552(17)</td>
<td>0.0323(15)</td>
<td>0.0437(17)</td>
<td>0.0086(12)</td>
<td>0.0044(13)</td>
<td>0.0031(12)</td>
</tr>
<tr>
<td>C5</td>
<td>0.0420(14)</td>
<td>0.0408(15)</td>
<td>0.0438(15)</td>
<td>0.0032(13)</td>
<td>0.0004(12)</td>
<td>0.0078(12)</td>
</tr>
<tr>
<td>C6</td>
<td>0.0276(17)</td>
<td>0.044(2)</td>
<td>0.0262(19)</td>
<td>0</td>
<td>-0.0085(14)</td>
<td>0</td>
</tr>
<tr>
<td>C7</td>
<td>0.0259(17)</td>
<td>0.053(2)</td>
<td>0.034(2)</td>
<td>0</td>
<td>0.0008(15)</td>
<td>0</td>
</tr>
<tr>
<td>C8</td>
<td>0.0349(14)</td>
<td>0.0513(17)</td>
<td>0.0560(18)</td>
<td>0.0062(14)</td>
<td>0.0087(13)</td>
<td>-0.0112(13)</td>
</tr>
<tr>
<td>C9</td>
<td>0.0508(16)</td>
<td>0.0377(16)</td>
<td>0.0598(18)</td>
<td>-0.0018(14)</td>
<td>0.0006(14)</td>
<td>-0.0155(12)</td>
</tr>
</tbody>
</table>
Table A8. Hydrogen atomic coordinates and isotropic atomic displacement parameters (Å²) for 1.

<table>
<thead>
<tr>
<th></th>
<th>x/a</th>
<th>y/b</th>
<th>z/c</th>
<th>U(eq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>0.1850(10)</td>
<td>0.157(2)</td>
<td>-0.032(3)</td>
<td>0.040(7)</td>
</tr>
<tr>
<td>H2</td>
<td>0.1530(9)</td>
<td>0.158(2)</td>
<td>0.473(3)</td>
<td>0.035(7)</td>
</tr>
<tr>
<td>H4</td>
<td>0.0718(11)</td>
<td>0.157(2)</td>
<td>0.171(3)</td>
<td>0.046(8)</td>
</tr>
<tr>
<td>H2A</td>
<td>0.1594</td>
<td>-0.0048</td>
<td>-0.2422</td>
<td>0.055</td>
</tr>
<tr>
<td>H2B</td>
<td>0.1009</td>
<td>0.0246</td>
<td>-0.1680</td>
<td>0.055</td>
</tr>
<tr>
<td>H3A</td>
<td>0.1384</td>
<td>-0.1432</td>
<td>-0.0219</td>
<td>0.053</td>
</tr>
<tr>
<td>H3B</td>
<td>0.1890</td>
<td>-0.0643</td>
<td>0.0355</td>
<td>0.053</td>
</tr>
<tr>
<td>H4A</td>
<td>0.1622</td>
<td>-0.1422</td>
<td>0.3173</td>
<td>0.052</td>
</tr>
<tr>
<td>H4B</td>
<td>0.1230</td>
<td>-0.0573</td>
<td>0.4247</td>
<td>0.052</td>
</tr>
<tr>
<td>H5A</td>
<td>0.2158</td>
<td>-0.0085</td>
<td>0.4729</td>
<td>0.051</td>
</tr>
<tr>
<td>H5B</td>
<td>0.2224</td>
<td>0.0229</td>
<td>0.2743</td>
<td>0.051</td>
</tr>
<tr>
<td>H8A</td>
<td>-0.0111</td>
<td>0.0074</td>
<td>0.2561</td>
<td>0.057</td>
</tr>
<tr>
<td>H8B</td>
<td>0.0365</td>
<td>0.0222</td>
<td>0.3936</td>
<td>0.057</td>
</tr>
<tr>
<td>H9A</td>
<td>0.0591</td>
<td>-0.1428</td>
<td>0.2197</td>
<td>0.059</td>
</tr>
<tr>
<td>H9B</td>
<td>0.0491</td>
<td>-0.0667</td>
<td>0.0491</td>
<td>0.059</td>
</tr>
</tbody>
</table>
A3 Titrations with Different Tetrabutylammonium Anions

**Figure A3.** Partial $^1$H NMR spectra of 1.7 mM TUC titrated with TBACl in CDCl$_3$.

**Figure A4.** Partial $^1$H NMR spectra of 1.7 mM TUC titrated with TBABr in CDCl$_3$. 
Figure A5. Partial $^1$H NMR spectra of 1.7 mM TUC titrated with TBAI in CDCl$_3$.

Figure A6. Partial $^1$H NMR spectra of 1.7 mM TUC titrated with TBABF$_4$ in CDCl$_3$. 
Figure A7. Partial $^1$H NMR spectra of 1.7 mM TUC titrated with TBAAc in CDCl$_3$.

Figure A8. Partial $^1$H NMR spectra of 1.7 mM TUC titrated with TBAP in CDCl$_3$. 
A4 Measuring pH of Blueberries

Blueberries are acidic fruits whose pH is between 2.90 and 3.15 depending on the variety.\textsuperscript{210} Ontario-grown blueberries were chosen to test the applicability of \textit{SPE2} to determine pH in a whole fruit by NMR. Those blueberries have an average diameter of 0.8 cm which is ideal for NMR analysis with the NMR probe. The berries were first homogenized. After filtration of the mixture and addition of 10% D\textsubscript{2}O, the sample was placed in a 5 mm NMR tube for NMR analysis. The \textit{\textsuperscript{1}H} NMR spectrum of the homogenate is shown in Figure A9. Due to the high concentration of intrinsic sugar peaks, it is not possible to obtain pH using the methylene groups of \textit{SPE2}. Only the peak of \textit{SPE2H}\textsuperscript{+} at 2.61 ppm can be detected. However, since the oxalamide NH peaks of \textit{SPE2H}\textsuperscript{+} and \textit{SPE2H}\textsubscript{2}\textsuperscript{2+} are discernable, they were used to determine pH.

\textbf{Figure A9} \textit{\textsuperscript{1}H} NMR spectrum of blended blueberries with 1.8 mM \textit{SPE2}. The pH obtained from the NH signals of \textit{SPE2} was at the end range of the pH sensor and did not provide accurate pH measurement as demonstrated by the disagreement between pH values determined by NMR (3.59) and pH electrode (3.04).

To do this, the apparent pK\textsubscript{a} from the NH signals was first determined using the same \textit{\textsuperscript{1}H} NMR spectra obtained for the titration from section 5.2.2 (Figure A10). Quantitative analysis consisted of plotting pH against the fraction of mono-protonated \textit{SPE2} (\textit{SPE2H}\textsuperscript{+}/[\textit{SPE2H}\textsuperscript{+}+\textit{SPE2H}\textsubscript{2}\textsuperscript{2+}])
from NMR integrals. The results of the titration were fitted to the Henderson-Hasselbalch equation. A $pK_a$ of 1.61 ± 0.02 was obtained, which is 0.04 pH units lower than the $pK_a$ obtained from the methylene peaks. This is because the NH protons exchange with the surrounded water, which causes slight discrepancies in the results.

![Figure A10](image)

**Figure A10** NMR pH titration of oxalamide NH peaks of **SPE2** at 25 °C in 10 mM phosphate buffer. a) Selected partial $^1$H NMR spectra of NH signals of **SPE2** at different pH values close to $pK_{a2}$. b) Ratiometric curve of **SPE2** based on the NH protons in the $^1$H NMR spectrum around $pK_{a1}$. The fit was done using only the peaks at 8.20, 8.77 and 8.97 ppm.

Using this newly obtained $pK_a$, the pH of blended blueberries was determined to be 3.59 which corresponded to a ratio of 99/1 or 99% **SPEH2+**. This value is almost out of the range of the pH sensor and is therefore not accurately measured by the SPE method using **SPE2**. It is also in disagreement with pH electrode reading, which was 3.04. In addition to blended blueberries, a single blueberry, injected with **SPE2** was also tested. Due to the berry’s lack of homogeneity and the low water solubility of **SPE2**, $^1$H NMR signals were not adequately resolved and **SPE2** was not detected. Future work could focus on NMR experimentation of very acidic fruits like lemons or key limes, which have a more suitable size for NMR experiments. In addition, the development of highly water soluble pH sensors with low $pK_a$ would be more beneficial and allow for a wider range of applications.