The Vault

https://prism.ucalgary.ca

Open Theses and Dissertations

2018-03-26

Lead Interactions Affect the Fluidity and Lateral Organization of Complex Lipid Membranes

Mundle, Robyn Louise

Mundle, R. L. (2018). Lead Interactions Affect the Fluidity and Lateral Organization of Complex Lipid Membranes (Master's thesis, University of Calgary, Calgary, Canada). Retrieved from https://prism.ucalgary.ca. doi:10.11575/PRISM/31755 http://hdl.handle.net/1880/106463 Downloaded from PRISM Repository, University of Calgary

UNIVERSITY OF CALGARY

Lead Interactions Affect the Fluidity and Lateral Organization of Complex Lipid

Membranes

by

Robyn Louise Mundle

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

GRADUATE PROGRAM IN BIOLOGICAL SCIENCES

CALGARY, ALBERTA

March, 2018

© Robyn Louise Mundle 2018

Abstract

Elevated levels of the heavy metal lead are associated with various toxic effects which may also be due to interactions with lipid membranes. Bilayer and monolayer techniques were used to monitor lead interactions with biomimetic and polar lipid extracts. Anionic and zwitterionic lipids as well as saturated and monounsaturated fatty acids were compared.

Permeabilization of model membranes by lead was assessed by quantifying fluorescent dye release, whereby leakage readily occurred upon Pb²⁺ incubation. Fluidity and phase transition changes were investigated by laurdan generalized polarization and dynamic light scattering. Electrostatic lipid targeting by lead induced membrane rigidification which varied with the negative charges within lipid structures.

Lead effects on the lateral organization of monolayers were characterized by surface pressure- and surface potential – area isotherms and Brewster angle microscopy. Pb – induced domain formation depends on the number and localization of the phosphate groups on various phosphatidylinositols.

Acknowledgements

First and foremost, I want to thank my supervisor Dr. Elmar J. Prenner, for his mentorship, guidance, support, and patience. This degree would not have been possible without your contribution. From assisting me with getting the degree started to convincing me that my thesis was strong enough to stop experiments and finally begin writing. Thank you for the incredible opportunities to attend conferences in Budapest, Parry Sound and Toronto as well as the work internship with Flurotech Ltd. I am greatly appreciative of your recommendation of me for an internship with ESR in New Zealand.

To my committee members, Dr. Ray Turner and Dr. Jürgen Gailer, thank you for your insight, suggestions, and balanced critique. I would also like to thank Dr. Sergei Noskov, for his input on my thesis as the internal examiner, as well as Dr. Marie Fraser, for her time investment as the neutral chair in my thesis defense.

I would like to thank all the past and present members of the Prenner Lab for their support. Research will continue as usual, but you will no longer be required to laugh at my jokes. Thank you to Mark Mahadeo for his wisdom in troubleshooting my problems and addressing my endless questions. Thank you to Weiam Daear for her assistance, encouragement, and support of me wholeheartedly. The friendship we have formed extends beyond the lab and I am grateful for the opportunity to work directly with you. To Jenelle Umbsaar, thank you for working alongside me and I aspire to be as driven and knowledgeable in my experiments as you are. I will always be grateful for the many friends I have made along the way and the wonderful memories I take with me.

And lastly, I extend the utmost appreciation to my mother, Carolle Sutter Mundle, and the remainder of my family as I cannot thank you enough for your love and support.

iii

Abstract	ii
Acknowledgements	iii
Table of Contents	iv
List of Tables	X
List of Figures	xi
Abbreviations	XXX
Chapter One: Introduction	
1.1 Human Exposure of Lead	1
1.2 Speciation of Lead in Aqueous Solutions	
1.3 Lead Targets in the Body	7
1.4 Membrane Lipid Bilayers	9
1.4.1 Membrane Lipids	9
1.4.2 Phospholipid Nomenclature	
1.4.3 Cholesterol	
1.5 Lipid Bilayer Asymmetry	
1.6 The Effect of Lead on Membrane Permeability	
1.7 The Effect of Lead on Membrane Fluidity	
1.8 The Effect of Lead on Membrane Size	
1.9 The Effect of Lead on Membrane Packing	
1.10 Project Goals	
1.11 Hypothesis	
Charter Trues Meterials and Methods	20
Chapter Two: Materials and Methods	
Chapter Two: Materials and Methods 2.1 Lipid System Preparations	
Chapter Two: Materials and Methods 2.1 Lipid System Preparations 2.1.1 Materials	20 20 20 20 22
Chapter Two: Materials and Methods 2.1 Lipid System Preparations 2.1.1 Materials 2.1.2 Lipid Structures 2.1.3 Large Unilemellar Vasiele (LUV) Proparations	20 20 20 20 22 24
Chapter Two: Materials and Methods 2.1 Lipid System Preparations 2.1.1 Materials 2.1.2 Lipid Structures 2.1.3 Large Unilamellar Vesicle (LUV) Preparations 2.1.4 Lipid Concentration Determination	20 20 20 20 22 22 24 24
Chapter Two: Materials and Methods 2.1 Lipid System Preparations 2.1.1 Materials 2.1.2 Lipid Structures 2.1.3 Large Unilamellar Vesicle (LUV) Preparations 2.1.4 Lipid Concentration Determination 2.1.5 Large Unilamellar Vesicle Systems Examined	20 20 20 20 22 24 24 26 28
Chapter Two: Materials and Methods 2.1 Lipid System Preparations	20 20 20 22 22 24 24 26 28 34
Chapter Two: Materials and Methods 2.1 Lipid System Preparations 2.1.1 Materials 2.1.2 Lipid Structures 2.1.3 Large Unilamellar Vesicle (LUV) Preparations 2.1.4 Lipid Concentration Determination 2.1.5 Large Unilamellar Vesicle Systems Examined 2.1.6 Red Blood Cell Total Extract Preparation	20 20 20 22 22 24 24 26 28 34 37
Chapter Two: Materials and Methods	20 20 20 20 22 24 24 26 28 34 37 37
Chapter Two: Materials and Methods 2.1 Lipid System Preparations	20 20 20 22 24 24 24 26 28 34 37 37 37
 Chapter Two: Materials and Methods. 2.1 Lipid System Preparations	20 20 20 22 24 24 26 28 34 37 37 37 37 38
 Chapter Two: Materials and Methods. 2.1 Lipid System Preparations 2.1.1 Materials 2.1.2 Lipid Structures 2.1.3 Large Unilamellar Vesicle (LUV) Preparations 2.1.4 Lipid Concentration Determination 2.1.5 Large Unilamellar Vesicle Systems Examined 2.1.6 Red Blood Cell Total Extract Preparation 2.2 Fluorescence Leakage Assays 2.2.1 Introduction to Leakage Assays 2.2.2 Large Unilamellar Vesicle Preparations 2.2.3 Encapsulation Volume Calculations 2.2 4 Experimental Procedure 	20 20 20 22 24 24 26 28 34 37 37 37 37 38 38
 Chapter Two: Materials and Methods. 2.1 Lipid System Preparations	20 20 20 20 22 24 26 28 34 37 37 37 37 37 37 37 38 38 40
 Chapter Two: Materials and Methods. 2.1 Lipid System Preparations	20 20 20 20 22 24 26 28 34 37 37 37 37 37 37 37 37 38 40 40
 Chapter Two: Materials and Methods	20 20 20 20 22 24 26 28 34 37 37 37 37 37 38 40 40 42
 Chapter Two: Materials and Methods	20 20 20 22 24 26 28 34 37 37 37 37 37 38 40 40 42
 Chapter Two: Materials and Methods	20 20 20 22 24 26 28 34 37 37 37 37 37 37 38 40 40 42 44
 Chapter Two: Materials and Methods	20 20 20 20 22 24 26 28 34 37 37 37 37 37 37 37 37 37 38 40 40 42 44 45 45
Chapter Two: Materials and Methods. 2.1 Lipid System Preparations 2.1.1 Materials 2.1.2 Lipid Structures. 2.1.3 Large Unilamellar Vesicle (LUV) Preparations 2.1.4 Lipid Concentration Determination 2.1.5 Large Unilamellar Vesicle Systems Examined. 2.1.6 Red Blood Cell Total Extract Preparation 2.2 Fluorescence Leakage Assays. 2.2.1 Introduction to Leakage Assays. 2.2.2 Large Unilamellar Vesicle Preparations 2.2.3 Encapsulation Volume Calculations 2.2.4 Experimental Procedure 2.3.1 Introduction to Laurdan 2.3.2 Generalized Polarization 2.3.3 Experimental Procedure 2.3.4 Tm Determination 2.4 1 Introduction to DI S	$\begin{array}{c} \begin{array}{c} \begin{array}{c} \begin{array}{c} 20 \\ 20 \\ 20 \\ 20 \\ 22 \\ 24 \\ 26 \\ 28 \\ 28 \\ 34 \\ 37 \\ 37 \\ 37 \\ 37 \\ 37 \\ 37 \\ 37$
 Chapter Two: Materials and Methods	$\begin{array}{c} \begin{array}{c} \begin{array}{c} 20 \\ 20 \\ 20 \\ 20 \\ 22 \\ 24 \\ 26 \\ 28 \\ 34 \\ 37 \\ 37 \\ 37 \\ 37 \\ 37 \\ 37 \\ 38 \\ 40 \\ 40 \\ 40 \\ 40 \\ 40 \\ 40 \\ 45 \\ 45$
 Chapter Two: Materials and Methods	$\begin{array}{c} 20 \\ 20 \\ 20 \\ 20 \\ 22 \\ 24 \\ 26 \\ 28 \\ 34 \\ 37 \\ 37 \\ 37 \\ 37 \\ 37 \\ 37 \\ 38 \\ 40 \\ 40 \\ 40 \\ 40 \\ 40 \\ 40 \\ 45 \\ 45$

Table of Contents

2.4.4 T _m Determination	
2.5 Langmuir Trough	
2.5.1 Introduction to the Langmuir Trough	
2.5.2 Metals in the Subphase	
2.5.3 Monolayer Lipid Systems Examined	
2.5.4 Surface Pressure – Area Isotherms	
2.5.5 Experimental Procedure	
2.5.6 Surface Pressure-Area Isotherm Analysis	
2.5.7 Compression Modulus	
2.5.8 Surface Potential – Area Isotherms	
2.5.9 Experimental Procedure	
2.6 Brewster Angle Microscopy (BAM)	59
2.6.1 Visualization of Lateral Domain Formation	59
2.6.2 Experimental Procedure	
Chapter Three: Primary Lipid Compositions	61
3.1 Speciation of Pb ²⁺ in Experimental Conditions	
3.2 Interactions of Pb ²⁺ with ANTS/DPX	
3.3 Interactions of Pb^{2+} with Laurdan	
3.4 Interactions of Pb^{2+} with Phosphatidylcholine (PC) Membranes.	
3.4.1 Leakage Assays of POPC LUVs with Pb^{2+}	
3.4.2 Laurdan GP of POPC with Pb^{2+}	
3.4.3 DLS of POPC with Pb^{2+}	
3.4.4 Laurdan GP of DMPC with Pb^{2+}	
3.4.5 DLS of DMPC with Pb^{2+}	
3.4.6 Laurdan GP of PCplasm with Pb^{2+}	
3.4.7 DLS of PCplasm with Pb^{2+}	
3.4.8 Discussion of Pb^{2+} Interactions with PC Membranes	
3.5 Interactions of Pb^{2+} with Sphingomyelin (SM) Membranes	
3.5.1 Laurdan GP of PSM with Pb^{2+}	
3.5.2 DLS of PSM with Pb^{2+}	
3.5.3 Laurdan GP of Brain SM with Pb^{2+}	
3.5.4 DLS of Brain SM with Pb^{2+}	
3.5.5 Discussion of Pb ^{-/} Interactions with SM Membranes	
3.6 Interactions of PD ²⁺ with Phosphatidylserine (PS) Membranes	88
3.6.1 Leakage Assays of POPS LUVs with PD^{2+}	88
3.6.2 Laurdan GP of POPS with Pb^{2+}	
3.6.3 DLS OF POPS with PO^{-1}	
3.6.4 Laurdan GP of DMPS with Pb^{2+}	
3.6.5 DLS OF DMPS with PD^{2+}	
3.6.0 Discussion of PD ⁻¹ interactions with PS Membranes	
5./ Interactions of PD ⁻ With Phosphatidic Acid (PA) Membranes	
5./.1 Laurdan GP OF POPA WITH PD^{-1}	
5.7.2 DLS OI FUFA WITH PD^{-1}	100
5.7.5 Lauruan GP OI DIVIPA WITH PD^{-1}	101 104
5.7.4 DLS OI DIVIPA WILL PO ⁻¹	
5.7.5 Discussion of Po ⁻⁺ interactions with PA Membranes	

3.8 Interactions of Pb ²⁺ with Cardiolipin (CL) Membranes	106
3.8.1 Laurdan GP of TOCL with Pb ²⁺	107
3.8.2 DLS of TOCL with Pb^{2+}	109
3.8.3 Laurdan GP of TMCL with Pb ²⁺	110
3.8.4 DLS of TMCL with Pb^{2+}	112
3.8.5 Discussion of Pb^{2+} Interactions with CL Membranes	113
3.9 Interactions of Pb^{2+} with Phosphatidylglycerol (PG) Membranes	114
3.9.1 Laurdan GP of POPG with Pb^{2+}	114
3.9.2 DLS of POPG with Pb^{2+}	
3.9.3 Laurdan GP of DMPG with Pb ²⁺	117
3.9.4 DLS of DMPG with Pb^{2+}	119
3.9.5 Discussion of Pb^{2+} Interactions with PG Membranes.	120
3.10 Summary of Pb^{2+} Interactions with Primary Lipid Compositions	120
Chapter Four: Simple Lipid Mixtures	122
4.1 Interactions of Pb ²⁺ with Membranes Containing Cholesterol (CHOL)	125
4.1.1 Leakage Assays of POPC and CHOL LUVs with Pb ²⁺	126
4.1.2 Laurdan GP of POPC and CHOL with Pb ²⁺	127
4.1.3 DLS for LUVs composed of POPC and CHOL with Pb ²⁺	130
4.1.4 Laurdan GP of PSM and CHOL with Pb ²⁺	132
4.1.5 DLS for LUVs composed of PSM and CHOL with Pb ²⁺	134
4.1.6 Laurdan GP of PSM and POPC with Pb ²⁺	135
4.1.7 DLS for LUVs composed of PSM and POPC with Pb ²⁺	137
4.1.8 Discussion of Pb ²⁺ Interactions with Membranes Containing CHOL	137
4.2 Interactions of Pb ²⁺ with Phosphatidylethanolamine (PE) Membranes	141
4.2.1 Leakage Assays of POPE and POPC LUVs with Pb ²⁺	142
4.2.2 Laurdan GP of POPE and POPC with Pb ²⁺	143
4.2.3 DLS for LUVs composed of POPE and POPC with Pb ²⁺	146
4.2.4 Laurdan GP of PEplasm with Pb ²⁺	148
4.2.5 DLS for LUVs composed of PEplasm and POPC with Pb ²⁺	151
4.2.6 Discussion of Pb ²⁺ Interactions with PE Membranes	152
4.3 Interactions of Pb ²⁺ with Mixed PS Membranes	154
4.3.1 Leakage Assays of POPS and POPC LUVs with Pb ²⁺	155
4.3.2 Discussion of Pb ²⁺ Interactions with PS Membranes	155
4.4 Interactions of Pb ²⁺ with Brain Ganglioside and Sulfatides Membranes	157
4.4.1 Laurdan GP of Total Brain Ganglioside Extract and POPC with Pb ²⁺	158
4.4.2 DLS for LUVs composed of Total Brain Ganglioside Extract and POPC	C with
Pb ²⁺	160
4.4.3 Laurdan GP of Brain Sulfatides and POPC with Pb ²⁺	161
4.4.4 DLS for LUVs composed of Brain Sulfatides and POPC with Pb ²⁺	162
4.4.5 Discussion of Pb ²⁺ Interactions with Ganglioside and Sulfatide Brain Ex	tracts
-	163
4.5 Interactions of Pb ²⁺ with Ceramide and Cerebroside Membranes	164
4.5.1 Laurdan GP of Palmitoyl Ceramide and POPC with Pb ²⁺	165
4.5.2 DLS for LUVs composed of Palmitoyl Ceramide and POPC with Pb ²⁺ .	167
4.5.3 Laurdan GP of Galactosyl Ceramide and POPC with Pb ²⁺	167
4.5.4 DLS for LUVs composed of Galactosyl Ceramide and POPC with Pb ²⁺	. 170

4.5.5 Laurdan GP of Hydroxylated Galactosyl Ceramide and POPC with Pb ²⁺	171
4.5.6 DLS for LUVs composed of Hydroxylated Galactosyl Ceramide and PC	OPC
with Pb^{2+}	172
4.5.7 Discussion of Pb ²⁺ Interactions with Ceramide and Cerebroside Membra	anes
$A \in \Omega$ (D) ²⁺ L (A) ²⁺ L	173
4.6 Summary of Pb ²⁺ Interactions with Simple Lipid Mixtures	1/4
Chapter Five: Biomimetic Compositions and Polar Lipid Extracts	176
5.1 Interactions of Pb ²⁺ with Red Blood Cell Total Extracts	178
5.1.1 Laurdan GP of RBC Extract LUVs with Pb ²⁺	180
5.1.2 DLS with RBC Extract LUVs with Pb ²⁺	182
5.1.3 Discussion of Pb ²⁺ Interactions with RBC Total Extracts	182
5.2 Interactions of Pb ²⁺ with Brain Polar Lipid Extracts	184
5.2.1 Laurdan GP of Brain Polar Extracts with Pb ²⁺	186
5.2.2 DLS of Brain Polar Extracts with Pb ²⁺	188
5.2.3 Discussion of Pb^{2+} Interactions with Polar Brain Extracts	188
5.3 Interactions of Pb^{2+} with the Myelin Sheath – Cytoplasmic Leaflet	189
5.3.1 Laurdan GP of the Inner Leaflet of the Myelin Sheath with Pb^{2+}	192
5.3.2 DLS of the Inner Leaflet of the Myelin Sheath with Pb^{2+}	194
5.3.3 Discussion of Pb ²⁺ Interactions with Myelin Sheath Inner Leaflet	195
5.4 Interactions of Pb ²⁺ with the Myelin Sheath – Extracellular Leaflet	196
5.4.1 Laurdan GP of Mixed Myelin Compositions with Pb ²⁺	199
5.4.2 DLS of Mixed Myelin Components with PD^{-1}	205
S.4.5 Discussion of Po Interactions with Prenninary Outer Myenn Mi	200
5.4.4 Laurdan GP of Myelin Sheath Outer Leaflet with Ph ²⁺	209
5.4.5 DI S of Myelin Sheath Outer Leaflet with Pb^{2+}	211
5.4.6 Discussion of Ph^{2+} Interactions with Myelin Sheath Outer Leaflet	212
5.5 Summary of Pb^{2+} Interactions with Biomimetic Compositions and Polar Extr	acts
	214
Chapter Six: Bilayer Study of PI and PC lipids	216
6.1 Interactions of Pb ²⁺ with Phosphatidylinositol (PI) LUVs	218
6.1.1 Laurdan GP of LUVs with DOPI and POPC	218
6.1.2 DLS of LUVs with DOPI and POPC	220
6.1.3 Discussion of Pb ²⁺ Interactions with PI and PC LUVs	220
6.2 Interactions of Pb ²⁺ with Phosphatidylinositol-phosphate (PIP) LUVs	221
6.2.1 Laurdan GP of LUVs with DOPI(3)P and POPC	221
6.2.2 DLS OI LUVS WIIII DOPI(5)P and POPC	223
6.2.4 DI S of LUVs with DOPI(4)P and POPC	223
6.2.5 Leurden GP of LUVs with DOPI(5)P and POPC	225
6.2.6 DLS of LUVs with DOPI(5)P and POPC	220
6.2.7 Discussion of Pb ²⁺ Interactions with PIP and PC LUVs	227
6.3 Interactions of Pb^{2+} with Phosphatidylinositol-hisphosphate (PIP ₂) LUVs	22.9
6.3.1 Laurdan GP of LUVs with DOPI(3.4)P ₂ and POPC.	220
	230

6.3.3 Laurdan GP of LUVs with DOPI(4,5)P ₂ and POPC	233
6.3.4 DLS of LUVs with DOPI(4,5)P ₂ and POPC	234
6.3.5 Laurdan GP of LUVs with DOPI(3,5)P ₂ and POPC	235
6.3.6 DLS of LUVs with DOPI(3,5)P ₂ and POPC	236
6.3.7 Discussion of Pb^{2+} Interactions with PIP ₂ and PC LUVs	236
6.4 Interactions of Pb ²⁺ with Phosphatidylinositol-trisphosphate (PIP ₃) LUVs	239
6.4.1 Laurdan GP of LUVs with DOPI(3.4.5)P ₃ and POPC	
6.4.2 DLS of LUVs with DOPI(3.4.5)P ₃ and POPC	
6.4.3 Discussion of Pb^{2+} Interactions with PIP ₃ and PC LUVs	
6.5 Summary of Pb ²⁺ Interactions with PI Lipids	
6.6 Implications of Pb^{2+} Interactions with PI Lipids	
···	
Chapter Seven: Monolayer Studies of PC, PS, and PI lipids	246
7.1 Interactions of Pb ²⁺ with PC Monolayers	247
7.1.1 Isotherms of POPC Monolayers with Pb ²⁺	248
7.1.2 Brewster Angle Microscopy of POPC Monolayers with Pb ²⁺	249
7.1.3 Isotherms of DMPC Monolayers with Pb ²⁺	251
7.1.4 Brewster Angle Microscopy of DMPC Monolayers with Pb ²⁺	252
7.1.5 Discussion of Pb Interactions with PC Monolayers with Pb ²⁺	254
7.2 Interactions of Pb ²⁺ with PS Monolayers	256
7.2.1 Isotherms of POPS Monolayers with Pb ²⁺	257
7.2.2 Brewster Angle Microscopy of POPS Monolayers with Pb ²⁺	258
7.2.3 Isotherms of DMPS Monolayers with Pb ²⁺	261
7.2.4 Brewster Angle Microscopy of DMPS Monolayers with Pb ²⁺	262
7.2.5 Discussion of Pb Interactions with PS Monolayers with Pb ²⁺	265
7.3 Interactions of Pb ²⁺ with 1% PI Monolayers	267
7.3.1 Isotherms of DOPI Monolayers with Pb ²⁺	267
7.3.2 Brewster Angle Microscopy of DOPI Monolayers with Pb ²⁺	269
7.3.3 Discussion of Pb Interactions with PI Monolayers with Pb ²⁺	271
7.4 Interactions of Pb ²⁺ with 1% PIP Monolayers	272
7.4.1 Isotherms of DOPI(3)P Monolayers with Pb ²⁺	273
7.4.2 Brewster Angle Microscopy of DOPI(3)P Monolayers with Pb ²⁺	274
7.4.3 Isotherms of DOPI(4)P Monolayers with Pb ²⁺	276
7.4.4 Brewster Angle Microscopy of DOPI(4)P Monolayers with Pb ²⁺	277
7.4.5 Isotherms of DOPI(5)P Monolayers with Pb ²⁺	279
7.4.6 Brewster Angle Microscopy of DOPI(5)P Monolayers with Pb ²⁺	280
7.4.7 Discussion of Pb ²⁺ Interactions with PIP Monolayers	282
7.5 Interactions of Pb ²⁺ with 1% PIP ₂ Monolayers	284
7.5.1 Isotherms of DOPI(3,4)P ₂ Monolayers with Pb^{2+}	285
7.5.2 Brewster Angle Microscopy of DOPI(3,4)P ₂ Monolayers with Pb ²⁺	286
7.5.3 Isotherms of DOPI(4,5)P ₂ Monolayers with Pb^{2+}	289
7.5.4 Brewster Angle Microscopy of DOPI(4,5)P ₂ Monolayers with Pb ²⁺	289
7.5.5 Isotherms of DOPI(3,5)P ₂ Monolayers with Pb^{2+}	291
7.5.6 Brewster Angle Microscopy of DOPI(3,5)P2 Monolayers with Pb ²⁺	292
7.5.7 Discussion of Pb ²⁺ Interactions with PIP ₂ Monolayers	294
7.6 Interactions of Pb ²⁺ with 1% PIP ₃ Monolayers	296
7.6.1 Isotherms of DOPI(3,4,5)P ₃ Monolayers with Pb^{2+}	296

7.6.2 Brewster Angle Microscopy of DOPI(3,4,5)P ₃ Monolayers with Pb ²⁺	297
7.6.3 Discussion of Pb ²⁺ Interactions with PIP ₃ Monolayers	299
7.7 Summary of Pb ²⁺ Interactions with PI Lipids	299
7.8 Implications of Pb ²⁺ Interactions with PI Lipids	300
Chapter Eight: Summary and conclusions	301
8.1 Future Directions	304
References	307
Appendix A	325
Appendix B	326
Appendix C	327
Appendix D	329
Poster Presentations	329
Oral Presentations	329

List of Tables

Table 1. The predominant species of Pb present under physiological conditions of pH 7.4,100mM NaCl, 37°C used in this study325
Table 2. The carboxyl reference, IUPAC, and common naming systems for fatty acids components of lipids used in this thesis. The abbreviated letter code by which they will be referred with has been included. 12
Table 3. The lipid systems used for fluorescent leakage, laurdan GP, DLS size and phase transition experiments. 28
Table 4. The lipid systems used for surface-pressure isotherms, surface-potentialisotherms, and Brewster angle microscopy experiments.50
Table 5. Lipid compositions of LUVs formulated in this study for the outer leaflet of the myelin sheath. 198

List of Figures

Figure 1. Speciation of lead as a function of the concentration of NaCl (mM) <i>(left)</i> and pH <i>(right)</i> in aqueous solutions of 100µM Pb(NO ₃) ₂ fixed at 37°C as determined from Visual Minteq 3.1 software ³² . pH curves were determined at 100mM NaCl while chloride curves were at pH 7.4
Figure 2 . The general structure of glycerol and sphingosine-based phospholipids. The dotted blue box highlights the ester, vinyl ether and amide linkages present in the lipids studied within this thesis. <i>Sn1</i> , <i>sn2</i> , and <i>sn3</i> positions on the glycerol backbone has been highlighted in red. A phosphocholine head group is shown on all structures
Figure 3. Example head group structures of monoglycosylated cerebrosides (galactosyl ceramide) and gangliosides (representative structure of GM1)
Figure 4. The thermotropic phase behaviour of bilayer lipids. The melting temperature (T_m) corresponds to the halfway point between these two phases
Figure 5. Glycerophospholipid, sphingolipid and cholesterol structures from this study.
Figure 6. The structures of phosphatidylinositol lipids from this study
Figure 7. Schematic of the formation of large unilamellar vesicles (LUVs)
Figure 8. (A) The structure of 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and (B) the structure of <i>p</i> -xylene-bis-pyridinium bromide (DPX)
Figure 9 . The structure of laurdan. Dipole moment across the naphthalene moiety is depicted by a grey arrow
Figure 10. Fluorescence intensity (a.u.) of the emission wavelength (nm) scan of laurdan in DMPG liposomes in 100mM NaCl pH 7.4 at 13°C (blue), 23°C (green) and 37°C (red). Excitation of laurdan set to 340nm, arrows indicate 440 and 490nm peaks 41
Figure 11. Schematic representation of laurdan (green) incorporated in a lipid membrane (black) when in the gel phase of tighter packing (left) and the liquid-crystalline phase of increased fluidity (right)
Figure 12. Generalized polarization as a function of temperature (°C) of laurdan in 300µM DMPC LUVs in 100mM NaCl pH 7.4. Fluorescence intensities measured at 440 and 490nm upon excitation at 340nm. Arrow depicts phase transition (T _m) of 24°C 43
Figure 13 . Schematic of DLS phase transition experiments. Lipids in gel phase (blue) reflect uniformly corresponding to higher count rates at lower temperatures. Increasing temperature for lipids in liquid-crystalline phase (orange) results uniformly in a lower photon count rate. Graph of photon count rate over temperature yields phase transition temperature (T_m)

Figure 14. Schematic of the Langmuir trough (components highlighted in black) used for isotherm and imaging experiments. Essential components for obtaining surface pressure isotherms (purple), surface potential isotherms (green) and Brewster angle microscopy (red) have also been highlighted
Figure 15. Schematic illustrating the forces acting on Wilhelmy filter paper plate used in calculating surface tension
Figure 16. Surface pressure-area isotherm of POPS to highlight the transition between the different lipid monolayer phases observed throughout this study
Figure 17. Schematic of the orientation of dipole moments found in the subphase (μ_1) , polar interface region (μ_2) and the alkyl chains (μ_3) of the lipid molecule
Figure 18. Surface potential-area isotherm of POPC
Figure 19. Brewster Angle Microscopy principle. Presence of a lipid film changes the interfacial properties of the air-water interface from no reflection of p-polarized light at the Brewster angle of 53° to reflection off the film into a camera
Figure 20. Structure of Phen Green SK
Figure 21. Overlay of the fluorescence intensity (a.u.) of 2μ M PGSK (primary axis) under NaCl concentrations (0-100mM) with increasing concentrations of Pb ²⁺ (0-1000ppb, line curves) with the percentage of total lead species (secondary axis) expected at these concentrations of NaCl (mM). Experimental and Visual Minteq software has fixed pH of 7.4 and temperature 37°C. $\lambda_{Ex} = 508 \pm 5$ nm, $\lambda_{Em} = 533 \pm 5$ nm
Figure 22. Overlay of the fluorescence intensity (a.u.) of 2µM PGSK (primary axis) at different pH with increasing concentrations of Pb ²⁺ (0-1000ppb, line curves) with the percentage of total lead species (secondary axis) expected at each pH. Experimental and Visual Minteq software has fixed 100mM NaCl and 37°C temperatures. $\lambda_{Ex} = 508 \pm 5$ nm, $\lambda_{Em} = 533 \pm 5$ nm
Figure 23. The fluorescence intensity (a.u.) of 10.1µM ANTS (black) and 10.1µM ANTS with 40.6µM DPX (red) in 100mM NaCl pH 7.4 over increasing concentrations of Pb ²⁺ (µM). $\lambda_{Ex} = 347 \pm 5$ nm, $\lambda_{Em} = 518 \pm 5$ nm. Fluorescence intensity was corrected for dilution. 67
Figure 24. The fluorescence intensity of 4.4μ M laurdan from 1mM DMSO in 100mM NaCl pH 7.4 over an emission wavelength spectrum (nm) in the absence (black) or presence of 2.1mM Pb ²⁺ (red) at 25°C. Each dataset is the average of 3 replicates

- **Figure 27.** (*top*) GP values of laurdan in LUVs of 0.3mM POPC (black) with 0.5 μ M (green), 0.3mM (blue), and 2.1mM (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM POPC LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).... 72
- **Figure 29.** (*top*) GP values of laurdan in LUVs of 0.3mM DMPC (black) with 0.5 μ M (green), 0.3mM (blue), and 2.1mM (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM DMPC LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M). . 75
- **Figure 31.** (*top*) GP values of laurdan in LUVs of 0.3mM PC plasmalogen (black) with 0.5µM (green), 0.3mM (blue), and 2.1mM (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM PCplasm LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (µM).

- **Figure 34.** (*top*) GP values of laurdan in LUVs of 0.3mM PSM (black) with 0.5 μ M (green), 300 μ M (blue), and 2.1mM (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM PSM LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M)...... 84
- **Figure 36**. (*top*) GP values of laurdan in LUVs of 0.3mM brain SM (black) with 0.5 μ M (green), 300 μ M (blue), and 2.1mM (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM brain SM LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).

- **Figure 40.** (*top*) GP values of laurdan in LUVs of 0.3mM POPS (black) with 0.5 μ M (green), 30 μ M (blue), and 75 μ M (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM POPS LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M)....90

- **Figure 45.** Contour plot of the change in GP for 0.3mM POPA LUVs at pH 7.4 (left) and 6.4 (right) across increasing temperature (°C) and concentrations of Pb^{2+} (μ M). 99

- **Figure 48.** Contour plot of the change in GP for 0.3mM DMPA LUVs at pH 7.4 (left) and 6.4 (right) across increasing temperature (°C) and concentrations of Pb^{2+} (μ M). 103

- **Figure 50.** (*top*) GP values of laurdan in LUVs of 0.3mM TOCL (black) with 0.5 μ M (green), 75 μ M (yellow), 300 μ M (blue) and 2.1Mm (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM TOCL LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).
- **Figure 52.** (*top*) GP values of laurdan in LUVs of 0.3mM TMCL (black) with 0.5 μ M (green), 75 μ M (red), and 300 μ M (blue) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM TMCL LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M). 111
- **Figure 54.** (*top*) GP values of laurdan in LUVs of 0.3mM POPG (black) with 0.5 μ M (green), 300 μ M (blue), and 2.1mM (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± standard deviation. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM POPG LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).
- **Figure 56.** (*top*) GP of laurdan in LUVs of 0.3mM DMPG (black) with 0.5 μ M (green), 15 μ M (blue), 45 μ M (yellow) and 75 μ M (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. 15 75 μ M Pb²⁺ are statistically significant (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM DMPG LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).117

- **Figure 58.** The change in laurdan GP at a temperature within the gel phase (blue) or liquidcrystalline phase (red) when exposed to different concentrations of Pb^{2+} as shown for each lipid system within Chapter 3. Results are the average of 3 replicates \pm SD. ... 121
- Figure 59. Pb²⁺-induced leakage (%) of liposome contents from 300μM LUVs of POPC (black), 15% CHOL 85% POPC (blue) and 40% CHOL 60% POPC (red) encapsulating 11.8mM ANTS and 47.4mM DPX in 100mM NaCl pH 7.4. Results are the average of 3 replicates ± SD.
- **Figure 60.** (*top*) GP values in LUVs of 300 μ M 15% CHOL 85% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 15% CHOL 85% POPC LUVs across increasing temperature (°C) and Pb²⁺ (0-2100 μ M).

- **Figure 64.** (*top*) GP values in LUVs of 300 μ M 40% CHOL 60% PSM (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 40% CHOL 60% POPC LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

- **Figure 66.** (*top*) GP values of laurdan in LUVs of 300µM 50% PSM 50% POPC (black) with 0.5µM (green), 300µM (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 50% PSM 50% POPC LUVs across increasing temperature (°C) and Pb²⁺ (µM). ... 136

- **Figure 70.** (*top*) GP values of LUVs of 300 μ M 30% POPE 70% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the GP change for 30% POPE 70% POPC LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

- **Figure 74.** (*top*) GP values in LUVs of 300 μ M 29% PEplasm 32% CHOL 39% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M). 150
- **Figure 76.** DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 29% PEplasm 32% CHOL 39% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ size is statistically significant, asterisks denote count rate curve (p < 0.05).
- Figure 77. Pb²⁺-induced leakage (%) of aqueous liposomal contents of 300μM LUVs of POPS (black), 85% POPS 15% CHOL (blue) and 15% POPS 85% POPC (red) encapsulating 11.8mM ANTS and 47.4mM DPX in 100mM NaCl pH 7.4. Results are the average of 3 replicates ± SD.
- **Figure 79.** (*top*) GP values of LUVs of 300 μ M 10% Total Gangliosides (Brain) 90% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentration (μ M).

- **Figure 82.** DLS determination of the count rate $(x10^4 \text{ kcps})$ (solid) and vesicle diameter size (nm) (dashed) for 0.3mM 10% Brain Sulfatides 90% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ size curve is statistically significant, asterisks denote count rate curve (p < 0.05).
- **Figure 84.** DLS determination of the count rate $(x10^4 \text{ kcps})$ (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 5% C16 Ceramide 95% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ size is statistically significant, asterisks denote count rate curve (p < 0.05).
- **Figure 85.** (*top*) GP values of LUVs of 300 μ M 3.5% Galactosyl Ceramide 96.5% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).
- **Figure 86.** DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 3.5% Galactosyl Ceramide 96.5% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05). 170
- **Figure 87.** (*top*) GP values of LUVs of 300 μ M 21% (2R)-OH Galactosyl Ceramide 79% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

- **Figure 88.** DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 21% (2R) Hydroxylated Galactosyl Ceramide 79% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).
- **Figure 89.** The change in laurdan GP at a low (blue) or high (red) temperatures within the range tested for each lipid system within Chapter 4 when exposed to different concentrations of Pb^{2+} as shown. Results are the average of 3 replicates ± SD. 175

- Figure 92. The average vesicle diameter of 0.3mM RBC Extract LUVs in the absence (black) and presence of 2.1mM Pb²⁺ (red) at 25 and 37°C. Results are the average of 3 replicates \pm SD. Asterisks denote statistical significance (* = p < 0.05, ** = p < 0.01). 182

- **Figure 95.** Contour plot of the change in GP for LUVs of 300 μ M Brain Polar Extract across increasing temperature (°C) and Pb²⁺ concentrations (0 300 μ M only). 187
- **Figure 96.** The average vesicle diameter of 0.3 mM Brain Polar Extract LUVs in the presence of 0 to 2.1mM Pb²⁺. Results are the average of 3 replicates \pm SD. Asterisks denote statistical significance (** = p < 0.01, **** = p < 0.00001).....188
- **Figure 97.** Percent composition of the inner myelin leaflet as adapted from literature^{95–97}. 191

- **Figure 111.** The change in laurdan GP at a low (blue) or high (red) temperatures within the range tested for each lipid system within Chapter 5 when exposed to different concentrations of Pb^{2+} as shown. Results are the average of 3 replicates ± SD. 215

Figure 112. Head group structures of PC and PI structures used in this study...... 217

- **Figure 113.** (*top*) GP values of LUVs of 300 μ M 1% DOPI 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).
- **Figure 115.** (*top*) GP values of LUVs of 300 μ M 1% DOPI(3)P 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).
- **Figure 117.** (*top*) GP values of LUVs of 300 μ M 1% DOPI(4)P 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).
- **Figure 119.** (*top*) GP values of LUVs of 300 μ M 1% DOPI(5)P 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

- **Figure 121.** (*top*) GP values of LUVs of 300µM 1% DOPI(3,4)P₂ 99% POPC (black) with 30µM (green), 300µM (blue), 1200µM (yellow) and 2100µM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (µM).
- **Figure 123.** (*top*) GP values of LUVs of 300 μ M 1% DOPI(4,5)P₂ 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).
- **Figure 125.** (*top*) GP values of LUVs of 300 μ M 1% DOPI(3,5)P₂ 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).
- **Figure 127.** (*top*) GP values of LUVs of 300 μ M 1% DOPI(3,4,5)P₃ 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

Figure 151. 3D images of lipid clusters found in control (**A**) and Pb²⁺ (**B**) trials from 1% DOPI(3,4)P₂ 99% POPC films at collapse from the highlighted red insert regions of Figure 150.

- **Figure C.1.** The change in molecular area (Å²/mol) when isotherms are held at 30mN/m in the presence of 86.2 μ M Pb²⁺ (red), Cd²⁺ (green), Co²⁺ (purple), Ni²⁺ (blue) or Mn²⁺ (orange) for each lipid system relative to control scans with no metal. Data is the average of 3 replicates ± SD. Asterisks denote statistical significance (p<0.05). 327

Abbreviations

³¹ P-NMR	³¹ P – Nuclear Magnetic Resonance
ANTS	8-aminonaphthalene-1,3,6-trisulfonic acid
BAM	Brewster Angle Microscopy
CHOL	Cholesterol
CL	Cardiolipin
DLS	Dynamic Light Scattering
DM-	Dimyristoyl
DMPA	1, 2-dimyristoyl-sn-glycero-3-phosphate
DMPC	1, 2-dimyristoyl-sn-phosphocholine
DMPG	1, 2-dimyristoyl-sn-phosphoglycerol
DMPS	1, 2-dimyristoyl-sn-glycero-3-phospho-L-serine
DOPI	1, 2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol)
DOPI(3)P	1, 2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3'-phosphate)
DOPI(4)P	1, 2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-4'-phosphate)
DOPI(5)P	1, 2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-5'-phosphate)
DOPI(3,4)P ₂	1, 2-dioleoyl- <i>sn</i> -glycero-3-phospho-(1'-myo-inositol-3', 4'-bisphosphate)
$DOPI(4,5)P_2$	1, 2-dioleoyl- <i>sn</i> -glycero-3-phospho-(1'-myo-inositol-4', 5'-bisphosphate)
DOPI(3,5)P ₂	1, 2-dioleoyl- <i>sn</i> -glycero-3-phospho-(1'-myo-inositol-3', 5'-bisphosphate)
DOPI(3,4,5)P ₃	1,2-dioleoyl- <i>sn</i> -glycero-3-phospho-(1'-myo-inositol-3',4',5'-trisphosphate)
DPX	<i>p</i> -xylene-bis-pyridinium bromide
DSC	Differential Scanning Calorimetry
GP	Generalized Polarization

Galactosyl Ceramides
High Performance Thin Layer Chromatography
Inductively Coupled Plasma Mass Spectroscopy
6-dodecanoyl-2-dimethyl-aminonaphthalene
Liquid Condensed
Liquid Expanded
Large Unilamellar Vesicles
Multilamellar Vesicles
Phosphatidic Acid
Inorganic Lead (II)
Phosphatidylcholine
1-(1Z-octadecenyl)-2-oleoyl-sn-glycero-3-phosphocholine
Phosphatidylethanolamine
1-(1Z-octadecenyl)-2-oleoyl-sn-glycero-3-phosphoethanolamine
Phen Green SK
Phosphatidylinositol
Phosphatidylinositol-3-phosphate
Phosphatidylinositol-4-phosphate
Phosphatidylinositol-5-phosphate
Phosphatidylinositol-3,4-bisphosphate
Phosphatidylinositol-4,5-bisphosphate
Phosphatidylinositol-3,5-bisphosphate
Phosphatidylinositol-3,4,5-trisphosphate

PIP	Phosphatidylinositol phosphate
PIP ₂	Phosphatidylinositol bisphosphate
PIP ₃	Phosphatidylinositol trisphosphate
PO-	Palmitoyl – Oleoyl
POPA	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol
POPS	1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine
PS	Phosphatidylserine
PSM	N-palmitoyl-D-erythro-sphingosylphosphorylcholine
RBC	Red Blood Cells
SM	Sphingomyelin
TLC	Thin Layer Chromatography
T _m	Melting Temperature
TMCL	1, 3-bis [1, 2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol
TOCL	1, 3-bis [1, 2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol

Chapter One: Introduction

1.1 Human Exposure of Lead

Due to industrialization and anthropogenic activities, lead (Pb²⁺) is a widely distributed and highly abundant heavy metal in the environment¹. Lead has been ranked second on the 2017 ATSDR Priority List of Hazardous Substances² and first for substances of greatest public health concern upon exposure on the 2017 ATSDR Completed Exposure Pathway Site Count Report³. Although lead ores occur naturally as minerals in the earth's crust, prehistoric Antarctic ice deposits indicate that the natural background level of lead is only 0.5 ng/L ⁴. Deposits typically find lead associated with other metals, particularly silver and zinc, and with counter ions such as sulphide, sulfate or carbonate¹. The annual lead emissions from on-road vehicle sources exceeded the natural rates by nearly 2000% before its elimination from gasoline¹. Approximately 220,000 tons were emitted in the 1970s in the USA which has been reduced to ~1,000 tons in 2011, originating from aircraft engines and metals industrial processing⁵.

Lead has become a common industrial contaminant due to the broad-based and long-standing utilization in industrial processing over the past millenia^{6–8}. One of the primary origins of lead production was as a by – product of silver-lead mining for silver coinage⁹ as leaded artifacts date back to 4,000 $_{B.C.}$ ¹. The soft and ductile properties of lead promoted its use in manufactured products in the ancient Roman Empire time for their water-transport systems of lead pipes, giving rise to its Latin name *plumbum*, and the leaded tableware of richer families^{1,7,10,11}.

Lead underwent rapid exploitation in the Industrial Revolution and during the mid-20th Century predominantly due to its incorporation into commercially sold products such as a petrol organic tetraethyllead(IV) additive. Exposure to inorganic PbBrCl from gasoline exhaust as well as deteriorating inorganic lead(II) paint causes a widespread dispersal of airborne residues in respirable form which remains suspended in air for long periods of time and penetrates deeply into the lungs upon inhalation ^{12,13}. Leaded gasoline accounts for the largest increase in environmental lead levels occurring between 1950 and 2000 despite attempts to phase lead out of general production and goods originating in the 1970s¹⁴. Similarly, although lead should no longer be added to paints, most houses built before the late 1970s contain lead paint in the exterior and interior walls. Toys containing lead paint continue to be inadvertently imported from overseas manufacturers and were subject to several worldwide product recalls in 2007⁶.

Lead-derived residues due to the leaching of lead plumbing systems is usually attributed to the hardness or pH of the water as soft, acidic waters will dissolve lead as the water passes through¹. The recent tragedy in Flint, USA has brought lead poisoning to the center stage when the city switched its drinking water source without properly treating with corrosion control resulting in the leaching of lead plumbing systems^{15,16}. At lead concentrations as low as 20 parts per billion ($20 \mu g/L$), infants consuming 1.2L of drinking water per day would consume sufficient levels of lead to raise blood lead levels beyond the safe threshold of $5\mu g/dL$ without any additional input of lead from other sources¹⁵. Modern industrial applications of lead is heavily concentrated in the battery industry whereas less common sources include pigments, ammunition, cable sheathing and the soldering in canned foods^{6,7,13}.

The growing concern for research on lead is the degree of toxicity associated with exposure levels. Only a finite number of lead poisoning symptoms and signs can be associated with certain blood lead levels¹⁰. The Centre for Disease Control (CDC) defines lead poisoning as the blood lead level (BLL) exceeding $10\mu g/dL$ however, a normal and healthy BLL is lower than $5\mu g/dL^{10,14,17,18}$. BLL as well as serum and urine lead levels are analyzed to assess how much lead enters the body through all routes of exposure and how it is distributed in the body, metabolized and eliminated.¹⁰ BLLs are an accurate representation of current lead exposure but fail to acknowledge chronic lead exposure which has been distributed away from the blood, throughout the body. The CDC has continually lowered the threshold BLLs over the past 50 years from 60 to $10\mu g/dL^{10,17}$. Children experience higher toxic effects than adults at BLLs as low as $5\mu g/dL$, suggesting that there may be no safe threshold for developmental effects at all^{14,18,19}. Treatment by DMPS or DMPA chelation is designed to decrease the body burden of lead however, it is only sought out for more severe cases^{6,18}. The primary treatment is removal of the sources of contamination to reduce exposure.

The degree of absorption from different sources is dependent on several factors including the physical and chemical states of the lead^{1,14}. For most individuals, the major route of absorption is the gastrointestinal tract due to ingestion of lead-contaminated food or water^{12,20,21}. Absorption of ingested lead can be as much as five times greater in children than adults^{8,14,21,22}, reflecting a higher vulnerability of young growing tissues and greater variation in gastrointestinal pH ranges to dissolve and hence, absorb lead ²³. The typical dietary input of lead amounts to approximately 10µg per day⁴. Lead absorption and retention can be influenced by fasting or nutritional deficiencies of Ca²⁺, Fe²⁺, or Zn²⁺ causing the upregulation of divalent metal ion transport protein pathways which result in
increased Pb²⁺ uptake and the role of nutrition may account for increased risk of continual poisoning for people of low socioeconomic status^{13,14,20,24}.

Organic lead(IV) and inorganic lead(II) is also respirable and lead-contaminated dust particles or aerosols can be nearly completely absorbed through the respiratory tract¹⁴. Effective lung retention halftime is approximately 10-14 hours whereby clearance rates from the respiratory tract depend on the particle size deposited in the lungs.^{14,25–27} Dermal contact is considered a less significant mode of absorption for inorganic lead compounds however, high exposure studies have observed absorption through human and rat skin followed by redistribution through the body^{28–31}. The hand-to-mouth activities of children in polluted environments results in the ingestion of considerable amounts of lead, a more significant source of poisoning than direct skin absorption¹⁴. Organic alkyllead(IV) compounds however, are readily absorbed across skin, lungs or any biological membrane due to their lipid-soluble characteristics^{4,29}.

1.2 Speciation of Lead in Aqueous Solutions

Metal speciation is the existence of different chemical forms under different environmental conditions. With no biological function, the toxicity of lead strongly depends on its speciation, resulting in physiological and biochemical dysfunctions⁸. Under physiological conditions of pH 7.4, 100mM NaCl, and 37°C, stock solutions of Pb(NO₃)₂ do not completely dissociate into Pb²⁺; the predominant species as determined by the Visual Minteq modelling software³² are shown in Table 1. **Table 1.** The predominant species of Pb present under physiological conditions of pH 7.4,100mM NaCl, 37°C used in this study³².

Metal Species	Percentage of Total Species
PbCl ⁺	47.4
Pb ²⁺	31.9
PbOH ⁺	13.8
PbCl ₂	6.3
PbCl ₃ -	0.5

Lead bioavailability is influenced by physical factors, such as temperature and sequestration with proteins or other small molecules, or chemical factors like thermodynamic equilibrium and complexation kinetics^{8,21}. The additional role of water interactions with Pb²⁺ cannot be determined through these speciation calculations or the bilayer and monolayer techniques discussed within this study but must also be considered for further Pb²⁺ coordination. The main factors to affect Pb speciation under experimental conditions are the concentrations of metal, chloride (Cl⁻) and the pH of the solution as demonstrated in Figure 1.



Figure 1. Speciation of lead as a function of the concentration of NaCl (mM) (*left*) and pH (*right*) in aqueous solutions of 100μ M Pb(NO₃)₂ fixed at 37°C as determined from Visual Minteq 3.1 software³². pH curves were determined at 100mM NaCl while chloride curves were at pH 7.4.

In the presence of 100mM NaCl, pH 7.4 and 37°C, the overall average estimated charge of metal in bulk solution would be +1.25 based on the distribution of charged species reported in Table 1. Another important physical characteristic of Pb^{2+} in solution is the size of the hydrated radii around the metal ions. Pb^{2+} is the second smallest divalent metal at 4.01 Å³³. The average electronegativity of Pb(II) has been reported at 1.87³⁴. As a member of Group 14, the carbon family of the Periodic Table, lead has naturally stable +2 and +4 oxidation states. This allows for the formation of organic lead compounds with tetravalent lead (Pb(IV)), which is best known for its wide use as an anti-knocking additive to gasoline, and bivalent inorganic lead (Pb(II)) compounds, such as those found in

manufactured goods³⁵. It is important to note that the abbreviation Pb^{2+} used in this thesis refer to the inorganic form of Pb(II) and the ionic Pb^{2+} species formed under the experimental conditions of 100 mM NaCl and pH 7.4.

1.3 Lead Targets in the Body

Although blood only carries a fraction of the total body burden of lead, it does serve as the initial receptacle of absorbed lead, providing its distribution throughout the body. When in blood, 99% of the lead will reside in the red blood cells (RBCs), leaving the remaining 1% with the plasma^{36,37}. Pb²⁺ has been found to interfere with heme biosynthesis, resulting in an accumulation of zinc protoporphyrin (ZPP)^{38–41}, and increased erythrocyte loss leading to anemia^{14,40–44}. The biological half-life of lead in blood is approximately 1 month, in most soft tissue up to 1–1.5 months and in the brain it can be extended upwards of 2 years.^{18,19} Lead will travel in the blood to soft tissues and organs such as the liver^{45,46}, kidneys^{42,47}, lungs²⁶, brain^{48–50}, muscles^{28,51}, and reproductive organs or the developing fetus^{52–58}.

Blood and bone stable isotope analyses indicated that bone Pb^{2+} stores contribute 40-70% of the lead found in the blood, providing evidence for exchange of bone lead with soft tissues and blood.⁵⁹ The bones and teeth have biological half-lives, reaching upwards of 30 years, as a result of lead following Ca²⁺ to become incorporated into the mineral¹⁴. The skeleton contains the majority of the total body burden of lead, approximately 73% in children compared to 94% in the bones of adults^{4,14}. Broken bones, old age, pregnancy and chronic diseases such as osteoporosis will also increase bone-to-blood mobilization of $Pb^{2+}.^{1,6,14,18}$

Lead already is a potent neurotoxin at very low exposure levels. Neurobehavioural

symptoms include headaches, impaired hearing and speech, anti-social behaviour and poor attention spans among other indicators^{6,14,19,24}. Epidemiological studies show detrimental effects on childhood intelligence quotient (IQ) as a result of lead exposure^{16,60,61}. Lead has been linked to inhibited neurotransmitter releases causing dysfunction of learning and memory similar to Alzheimer's disease^{14,29,36,61,62} and cognitive deficits similar to Parkinson's disease^{13,48,63,64}. A large part of Pb²⁺ toxicity is the ability to mimic divalent cations such as Ca²⁺, Fe²⁺ and Zn^{2+ 16,60,65}. Alterations to their homeostasis^{48,66} or substitution of Pb²⁺ in their place in the active or cofactor sites cause widespread changes to protein functions. Examples of improper protein activation for intracellular Ca²⁺ receptors include calmodulin^{14,19} or protein kinase C^{13,14,48,67} while substitutions for zinc occur primarily in zinc-finger motifs and the cofactor position of enzymes due to the coordination of thiol groups⁶⁸.

There is no single mechanism by which lead propagates its toxic effects. Research has shown the high level of Pb^{2+} – associated adverse health effects through a broad range of biochemical and abnormal behaviours. It has been identified as a potent neurotoxin with additional toxicity on cardiovascular, gastrointestinal, renal, and reproductive systems. Accordingly, studies that aim to increase our understanding of the interactions of the metal's biological targets are of great importance. However, to understand how Pb^{2+} functions in a complex biological environment, it is first necessary to study a reduced, controlled environment. Therefore, the initial goal of this thesis is to investigate the interactions of Pb^{2+} on simpler, well-defined biomimetic membranes of initial lipid compositions before more complicated lipid profiles or polar extracts are investigated.

1.4 Membrane Lipid Bilayers

Biological membranes separate the cellular content from the surrounding environment while internally compartmentalizing biomolecules and biological processes in distinct organelles⁶⁹. Membranes are comprised of lipids and proteins that are necessary for cellular functioning. First described in 1925, biochemical and biophysical studies have worked to construct detailed models of the composition and structure of the membrane^{70–74}.

1.4.1 Membrane Lipids

In addition, membranes provide an electrochemical barrier function and lipids have been identified as signaling molecules and energy storage molecules^{69,72,74}. The term lipids encompasses different amphiphilic and hydrophobic molecules, which spontaneously selfassociate into larger structures in an aqueous environment^{69,74,75}. The three main lipid classes present in biological membranes which were used in this thesis are the glycerolbased lipids, sphingosine-based lipids, and sterols. Both glycerol and sphingosine-based lipids are predominantly bilayer-forming and are characterized by common structural features (see Figure 2).

Firstly, they have fatty acid side chain(s) of varying hydrocarbon lengths which create the hydrophobic portion of the membrane. These side chains are linked to the backbone which differs for glycerol and sphingosine lipids. The 3 carbons in the glycerol backbones are labeled *sn1*, *sn2* and *sn3* as shown in Figure 2. The *sn1* and *sn2* carbons contain different fatty acid linkages which can be divided into subclasses. The most prevalent is the 1,2-diacyl groups while 1-alkenyl-2-acyl groups, commonly referred to as plasmalogens^{76–78}, are also investigated in this study. The sphingosine backbone typically

contains 18 carbons, 2 found in the backbone region whereas the other 16 forms one of the molecule's chains. A double bond is present within the sphingosine moiety and an amide linkage is used for the attachment of the other side chain⁷⁹. These backbones can be attached to additional polar and hydrophilic head groups, making the molecules amphiphilic.



Figure 2. The general structure of glycerol and sphingosine-based phospholipids. The dotted blue box highlights the ester, vinyl ether and amide linkages present in the lipids studied within this thesis. Sn1, sn2, and sn3 positions on the glycerol backbone has been highlighted in red. A phosphocholine head group is shown on all structures.

Lipid structures that also contain a phosphate group as a linker between the head group and backbone regions are termed glycerophospholipids and sphingophospholipids. Sphingomyelin (SM) is a common sphingophospholipid containing a phosphocholine head group attached to the sphingosine backbone⁸⁰. Sphingolipids without a phosphate group include ceramides, cerebrosides and gangliosides. The latter two are also classified as glycosphingolipids composed of the ceramide backbone and a glycosylated head group.

Cerebrosides are typically monoglycosylated with galactose or glucose⁸¹ while gangliosides contain multiple sugar groups with characteristic terminal sialic acids^{82,83} as depicted in Figure 3.



Figure 3. Example head group structures of monoglycosylated cerebrosides (galactosyl ceramide) and gangliosides (representative structure of GM1)

Biological membranes have a diverse lipid composition whereby the head groups are divided into major and minor classes based on their abundance. Major lipid head groups of glycerophospholipids include phosphocholine (PC), phosphoserine (PS), and phosphoethanolamine (PE)^{69,75}. Minor head groups include phosphoglycerol (PG), phosphoinositol (PI), and phosphate (PA).

The hydrophobic section of the lipids consists of fatty acyl tails of varying lengths and degrees of unsaturation. Longer and saturated fatty acyl chains result in a more rigid membrane due to increased hydrophobic van der Waals interactions between neighboring acyl chains for tighter lipid – lipid packing⁸⁴. Unsaturation in membrane lipids is most often present as *cis* double bonds in the acyl chain. This results in kinks in the side chain(s) which disrupt the close packing between the fully extended saturated acyl tails⁸⁴. A common

nomenclature is used to describe the phospholipid fatty acid chains.

1.4.2 Phospholipid Nomenclature

The carboxyl reference system utilizes two numbers representing the "number of carbons in the fatty acyl chain": "number of double bonds present". The carboxyl carbon is always counted as the first carbon using this system. Saturated fatty acids have no double bonds within the hydrocarbon chain, whereas monounsaturated fatty acids contain one, and polyunsaturated lipids contain multiple double bonds. The position of the double bond on the acyl chain is described using a delta (Δ) symbol followed by the carbon number at which the double bond starts as a superscript (18:1 Δ ⁹). A comparison of the fatty acids common names, IUPAC names and carboxyl references are outlined in Table 2.

Table 2. The carboxyl reference, IUPAC, and common naming systems for fatty acids components of lipids used in this thesis. The abbreviated letter code by which they will be referred with has been included.

Carboxyl Reference	CarboxylIUPAC ChemicalReferenceName		Abbreviation
14:0	14:0 Tetradecanoyl		М
16:0	Hexadecanoyl	Palmitoyl	Р
18:0 Octadecanoyl		Stearoyl	S
18:1 Δ ⁹	cis-9-Octadecanoyl	Oleoyl	0

The headgroup with an attached phosphate is referred to as the phospho-head group (e.g. phosphocholine) while the entire phospholipid is referred to as the phosphatidyl-name (e.g. phosphatidylcholine). The abbreviated letters for attached fatty acid tails are

combined with the abbreviated head group, corresponding to the phosphate and adjacent molecule (e.g. PC), to generate the shorthand name of each lipid tested in this thesis. In this regard, if a glycerol-based phosphocholine head group has a *sn1* chain of 16:0 and *sn2* chain of 18:1 Δ^9 , then it can be referred to as 16:0-18:1 PC or POPC. When the *sn1* and *sn2* positions are the same, then the IUPAC prefix of di- is used instead. If both *sn1* and *sn2* positions are 14:0 then the lipid is 14:0-14:0 PC or DMPC. In the case of cardiolipin (CL) lipids which contain 4 fatty acid chains, the letter T in TOCL or TMCL refers to the tetra- prefix.

1.4.3 Cholesterol

In addition to the glycerol and sphingosine-based lipids, sterols are another major class of lipids present in biological membranes whereby cholesterol is the most abundant^{85–88}. Sterols are structurally comprised of four fused rings with a small polar hydroxyl head group on one side and a short hydrocarbon chain on the other. The presence of cholesterol is mostly recognized for its effect on membrane fluidity throughout this study. The bulky, predominantly hydrophobic cholesterol structure modulates the fluidity of other lipids through restricted acyl tail mobility. In the lipid gel phase, this reduces the packing density when interspersed between ordered lipids thereby increasing fluidity while the opposite is found for lipids in the liquid – crystalline phase⁸⁹.

1.5 Lipid Bilayer Asymmetry

Eukaryotic membranes have a non-random distribution of different lipids in the bilayer. The asymmetry between the intracellular and extracellular leaflets provides different cellular functions on either side of the membrane^{69,90}. An example of the importance in asymmetric lipid distribution is the externalization of PS lipids as an

initiation signal for phagocytosis and other signal cascades^{69,91–93}. The context of asymmetry throughout this study is the understanding of relative exposure that any specific lipid may have to Pb^{2+} as well as the asymmetric lipid profiles of the myelin sheath bilayers. Lipid composition studies have reported that PEplasm, PC, PS and SM are enriched in the inner leaflets and different cerebrosides are more prevalent on the outer leaflet while cholesterol is distributed evenly between myelin leaflets^{94–98}. As very few Pb^{2+} – lipid studies have been completed, interactions for every component will need to be characterized to understand any metal effects observed on the complex biological membranes.

Asymmetric leaflets of biomimetic liposomes are possible however, the irregular liposome sizes and limited stability of the asymmetry restricts the study of physical and chemical properties⁹⁹. Therefore, experiments in this thesis were conducted with symmetric leaflet composition in the bilayer studies. Utilization of lipid monolayers allows the characterization of lipid lateral interactions within the plane of one leaflet comprised of an exact composition^{100,101}.

1.6 The Effect of Lead on Membrane Permeability

One of the primary functions of lipids within the membrane is to establish a barrier between the cytosol and external environment. This compartmentalization enables the segregation of ions and proteins required for specific cellular reactions^{69,102}. The interaction and binding of Pb²⁺ may destabilize the lipid bilayer causing membrane rupture or the formation of pores with the potential leakage of internal cellular components. Diaz and Monreal reported that Pb²⁺ exposure did not induce leakage from liposomes of myelin extracts however, PbOH⁺ was believed to passively permeate through the bilayer¹⁰³. Other reports have stated that membrane permeability increased as Pb^{2+} moved into the membranes^{48,50,104,105}. Thus, the effect of Pb^{2+} on membrane permeability may differ with the lipid composition. A preliminary investigation of Pb^{2+} with some of the major lipid classes is presented in this thesis to help understand the consequences of Pb^{2+} on the physical properties of biological membranes.

1.7 The Effect of Lead on Membrane Fluidity

The fluidity of the membrane depends on the relative fluidity/rigidity exerted by any given lipid^{106–110}. The lipids transition from a gel phase to liquid – crystalline phase upon increasing temperatures (Figure 4). The gel phase is characterized as a more rigid phase with tighter packing of head groups and acyl tails. The liquid – crystalline phase however, is less ordered with more motion of the acyl tails and head groups in the lateral and vertical dimensions. The temperature corresponding to this phase transition is defined as the melting temperature (T_m). This parameter is an indicator of the bilayer phase where composition of the fatty acid tails and head groups affect the biophysical and chemical properties of the lipids, including the $T_m^{107,111,112}$.



Figure 4. The thermotropic phase behaviour of bilayer lipids. The melting temperature (T_m) corresponds to the halfway point between these two phases.

 Pb^{2+} – lipid binding is expected to destabilize the membrane. Altered membrane fluidity has consequences on the activities of integral or peripheral proteins due to their association with lipids for insertion and proper functioning as well as the dynamic role of lipids in signaling pathways^{60,69,73,98}. Assessing Pb²⁺ induced packing changes will help to understand the potential impact of specific Pb²⁺ – lipid interactions within complex biomimetic membranes representing different cells within the body.

1.8 The Effect of Lead on Membrane Size

In addition to understanding the effects on membrane permeability, fluidity, and lipid phase transitions, changes of liposome sizes were also studied. Metal-induced size changes are a possible consequence of altered membrane fluidity which may result from the binding of a positively charged metal species with negatively charged head groups. This neutralizes the electrostatic repulsion between lipids causing increased interactions between neighbouring lipids. If preferential Pb²⁺ binding occurs between neighbouring lipid molecules, microdomains enriched in specific lipid targets may emerge to induce further aggregation as well. Liposome aggregation has been observed for other divalent metals: 15mM Ca²⁺and 0.5mM Cd²⁺ or Zn²⁺ with 50% PS 50% PC in 10mM HEPES 100mM NaCl pH 7.4¹¹³, 2mM Cd²⁺ with PG or 0.5mM Cd²⁺ with PS and PA in unbuffered 100mM NaCl pH 7.4¹¹⁴, 0.3mM Co^{2+} and Ni²⁺ for PA and PS in 20mM HEPES 100mM NaCl pH 7.4¹¹⁵. Pb²⁺ has a smaller hydrated radius³³ than all other metals, a similar electronegativity as Co^{2+34} and a multivalent metal speciation similar to Cd^{2+114} which suggests that some Pb²⁺ species observed under these conditions may behave like these other metals to induce liposome size changes.

1.9 The Effect of Lead on Membrane Packing

Localization of specific lipids occurs between different cell membranes, across the inner and outer leaflets of a bilayer and across the lateral plane within each leaflet. Monolayer studies allow detecting changes of lamellar packing as the lipids are compressed^{100,116}. Petelska and Naumowicz observed the effect of Pb²⁺ alongside Sr²⁺, Cd²⁺ and Ba²⁺ on egg PC monolayers, whereby the metal bound to the monolayer and shifted the surface pressure – area isotherms as lipids required a higher molecular area at equivalent surface pressures on the membrane with exposure of 50 μ M to 5 mM Pb^{2+ 117}. This suggests that metal binding events induce altered packing organization of lipids in the membrane.

Additionally, surface potential measurements were used to detect the orientation of molecular dipoles and correlate these values to membrane stability, ion adsorption on the membrane and transmembrane potentials^{116,118}. Studies using other divalent ions, Mg²⁺, Ca²⁺, and Mn²⁺, with PS monolayers showed the binding of the ions which neutralized some of the net negative charge on the membrane¹¹⁸. As Pb²⁺ is also positively charged, it may behave as these other metals and interfere with membrane electrostatics to offset the natural permeation of ions across the bilayer.

1.10 Project Goals

The goal of this thesis is to better understand the mechanism of Pb²⁺ binding to biological membranes. The objective are as follows:

 To assess Pb²⁺ – induced changes of the permeability of liposomes with a simple lipid composition under physiological conditions (100 mM NaCl, pH 7.4). The effect was assessed by fluorescence spectroscopy using a membrane impermeable dye, ANTS, and quencher, DPX, encapsulated within large unilamellar vesicles (LUVs).

- To assess Pb²⁺ induced fluidity and phase transition (T_m) changes in membranes of increasing complexity: individual compositions, simple lipid mixtures, complex biomimetic lipid profiles and polar lipid extracts. These tests were carried out in LUVs under physiological conditions using the probe laurdan, and a fluorescence spectroscopy method called generalized polarization (GP).
 - a. To investigate differences in Pb²⁺ effects on the fluidity and phase transition (T_m) of diverse zwitterionic and anionic lipids.
 - b. To investigate differences in Pb^{2+} effects on the fluidity and phase transition (T_m) in lipids with different acyl tails and the same head group structures.
 - c. To determine if the phosphate linker is a binding partner of Pb²⁺ and the relative affinity for binding when additional negative charges are present in the lipid structures.
- To assess Pb²⁺ induced LUV size and phase transition (T_m) changes in the same membrane systems as laurdan GP by using LUVs with dynamic light scattering (DLS) under physiological conditions.
- To assess Pb²⁺ induced changes of the lateral organization of simple lipid compositions under physiological conditions using monolayer experiments on the Langmuir trough.
 - a. To investigate differences in the surface pressure area isotherms and compression modulus as lipids are compressed in the presence of Pb^{2+} .
 - b. To investigate differences in the surface potential area isotherms as the lipids are compressed in the presence of Pb²⁺.

5. To visualize the lateral organization and domain or cluster formations of simple lipid systems under physiological conditions as the lipids are compressed in the presence of Pb²⁺. Experiments are also conducted on the Langmuir trough and visualized by Brewster Angle Microscopy (BAM).

1.11 Hypothesis

The calculated thermodynamic speciation of Pb²⁺ under 100 mM NaCl and pH 7.4 conditions found predominantly positively charged species³² which leads to the prediction that Pb²⁺ will electrostatically target the negatively charged membranes of PA, CL, PG, PS and PI. Previous studies have shown some interactions with PC^{105,119} and PS⁶⁰ lipids which generates the prediction that Pb²⁺ is able to access the negative phosphate linker within the polar interface of the membrane backbones in addition to more readily-accessible charges.

When Pb²⁺ is incubated with anionic membranes of different acyl chains, tighter packing between saturated lipids is hypothesized to better facilitate head group bridging than unsaturated lipids due to the small hydrated radii and preferred hemi-directed coordination of Pb²⁺. In addition, a stronger interaction is predicted between Pb²⁺ and PA membranes compared to CL membranes due to the higher surface charge density.

Chapter Two: Materials and Methods

2.1 Lipid System Preparations

2.1.1 Materials

Synthetic lipids such as 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1, 2-dimyristoyl-sn-phosphocholine (DMPC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-L-serine (POPS), 1, 2-dimyristoyl-sn-glycero-3-phospho-L-serine (DMPS), 1-palmitoyl-2-oleoylsn-glycero-3-phosphate (POPA), 1, 2-dimyristoyl-sn-glycero-3-phosphate (DMPA), 1palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol (POPG), 1. 2-dimyristoyl-snphosphoglycerol (DMPG), 1, 3-bis [1, 2-dioleoyl-sn-glycero-3-phospho]-sn-glycerol (TOCL), 1, 3-bis [1, 2-dimyristoyl-sn-glycero-3-phospho]-sn-glycerol (TMCL), 1-(1Zoctadecenyl)-2-oleoyl-sn-glycero-3-phosphocholine (PCplasm), 1-(1Z-octadecenyl)-2oleoyl-sn-glycero-3-phosphoethanolamine (PEplasm), 1. 2-dioleovl-sn-glycero-3phospho-(1'-myo-inositol) (DOPI), 1, 2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3'-phosphate) (DOPI(3)P),1. 2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-4'-2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-5'phosphate) (DOPI(4)P),1. phosphate) (DOPI(5)P), 1, 2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3', 4'bisphosphate) (DOPI(3,4)P2), 1, 2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-4', 5'bisphosphate) (DOPI(4,5)P2), 1, 2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3', 5'bisphosphate) (DOPI(3,5)P2),1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3',4',5'-trisphosphate) (DOPI(3,4,5)P3),N-palmitoyl-D-erythrosphingosylphosphorylcholine (PSM), Sphingomyelin (Brain) (Brain SM), Total Ganglioside Extract (Brain), Cholesterol (CHOL), N-palmitoyl-D-erythro-sphingosine

20

(Ceramide), Sulfatides (Brain), D-galactosyl-B1-1'-N-[2"(R)-hydroxystearoyl]-D-*erythro*sphingosine (2R-OH Galactosyl Ceramide), and D-galactosyl-B-1,1'-N-stearoyl-D*erythro*-sphingosine (Galactosyl Ceramide) as well as the Polar Lipid Extracts (Brain, Heart, Liver and *E. coli*) were purchased as lyophilized dry powders from Avanti Polar Lipids (Alabaster, AL). Pb(NO₃)₂ and 6-dodecanoyl-2-dimethyl-aminonaphthalene (laurdan) was purchased from Sigma-Aldrich (Oakville, ON). Phen Green SK (PGSK), 8aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and *p*-xylene-bis-pyridinium bromide (DPX) were purchased from Thermo Fisher Scientific (Waltham, MA). All reagents and lipid solutions were made using doubly distilled water (ddH₂O) filtered by a Millipore Synergy 185 water purification system (Billerica, MA). All chemicals were used without further purification.







2.1.3 Large Unilamellar Vesicle (LUV) Preparations

The formation of LUVs is illustrated in Figure 7. All lipid preparations were performed in 3mL glass vials which were cleaned with detergent and ddH₂O then a 4-solvent wash: acetone, methanol, hexanes, and chloroform and left to dry overnight. Synthetic, lyophilized lipids purchased from Avanti Polar Lipids (Alabaster, AL) were weighed using a Sartorius Microbalance MC 5 (Göttingen, Germany) and transferred into a clean glass vial. The powdered lipids were then dissolved in 7:3 (ν/ν) chloroform: methanol, vortexed and then sonicated for 30 seconds each to ensure complete dissolution of lipid in the solvent. When lipid mixtures were required, aliquots of each lipid solutions were mixed at appropriate molar ratios using known lipid concentrations. For experiments requiring laurdan, a 500 µM stock solution was prepared by dissolving dry laurdan powder in chloroform. The volume of laurdan was calculated using a final ratio of 1 mole laurdan: 550 moles lipid and added to the vial with lipids in organic solution. The organic solvent was then evaporated under argon gas and left overnight under vacuum to ensure complete solvent removal resulting in the formation of a lipid film on the glass vial.

The lipid film was rehydrated in 1-2mL of 100 mM NaCl (pH 7.4). For leakage experiments, stock solutions of dye (and quencher) were made using 100 mM NaCl pH 7.4 and used in the rehydration step. The rehydrated lipid films were vortexed using a Vortex Genie 2 (Scientific Industries, Bohemia, NY) for 30 seconds, sonicated using an Elmasonic S10/H sonicator (Singen, Germany) for 30 seconds and submitted to freeze/thaw cycles using a -80°C freezer and a water bath. This cycle was repeated twice to form a homogenous suspension of multilamellar vesicles (MLVs).

LUVs were formed using the mini-extruder apparatus (Avanti Lipids, Alabaster,

AL) which involved passing the MLV suspension 21 times across two Whatman Nucleopore polycarbonate membranes of 100 nm pore size (Maidstone, UK) and two supporting filters to form LUVs of a monodisperse size distribution. The lipid solutions were heated during extrusion to keep the temperature above the T_m of the lipid.

If the lipid film was rehydrated in dye containing solutions, further steps were required to remove the free dye. The LUVs were loaded onto a 14 x 1 cm Sephadex G-25 size exclusion column with 8 cm packed beads and eluted with 100 mM NaCl pH 7.4 at the void volume previously determined using 500 μ L of 1mg/mL blue dextran.



Figure 7. Schematic of the formation of large unilamellar vesicles (LUVs).

2.1.4 Lipid Concentration Determination

The process of extrusion and, if applicable, size exclusion chromatography reduces the lipid concentrations. Potential loss of lipids arises from interactions with the glass vials or membrane filters or leakage from the extruder apparatus. Dilution also occurs when eluting LUVs from the column with 100 mM NaCl pH 7.4. Thus, the concentration of lipids after LUV formation must be determined to appropriately study Pb²⁺ to lipid ratios in solution.

The Ames assay is a total phosphate assay used to determine the concentration of inorganic phosphate in solution¹²⁰. The assay is performed in small, 10 mm x 75 mm, 20mL pyrex glass test tubes which were cleaned with detergent and ddH₂O then a 4-solvent wash: acetone, methanol, hexanes, and chloroform and left to dry overnight to ensure no residual phosphate was present. All sample controls were repeated in triplicate. 5 μ L of the lipid sample, negative control (100 mM NaCl pH 7.4) or positive controls (2 mM sodium phosphate buffer) were added to separate test tubes.

To each tube, 30 μ L of 10% (*w/v*) Mg(NO₃)₂•6H₂O in 95% ethanol was added and the tubes were mixed by 10 seconds of vortexing. The tubes were ashed using gentle heating and shaking over the flame of a Bunsen burner. The ethanol was evaporated off as noted by a colour change from clear to dark brown to a white dry ash. Some silicate may be formed during ashing however it cancels out when read against the negative control which will contain the same amount of silicate. Once the tubes were cool, 300 μ L of 0.5M HCl_(aq) was added to each tube followed by vigorous vortexing to redissolve the white organic phosphate ash. The tubes were covered with aluminum foil and boiled for 15 minutes. The reaction of Mg(NO₃)₂ and HCl cleaves the inorganic phosphate from phospholipids as well as hydrolyzes any pyrophosphate formed during ashing into phosphate. After the tubes cooled down, 600 μ L of 0.42% (*w*/*v*) (NH₄)₆Mo₇O₂₄•4H₂O in 0.5 M H₂SO_{4(aq)} and 100 μ L of 10% (*w*/*v*) ascorbic acid in ddH₂O were added to each tube and thoroughly mixed. The tubes were incubated at 37°C for one hour in the Fischer Scientific IsoTemp 500 Series incubator (Ottawa, ON). The reaction of phosphate with molybdate creates the phosphomolybdate complex which is reduced by ascorbic acid to form a blue chromophore that can be quantified by absorbance at 820 nm. After one-hour incubation, the solutions were transferred into quartz cuvettes and the absorbance at 820nm was measured using the Shimadzu UV-1700 UV-VIS Spectrophotometer (Shimadzu Corporation, Kyoto, Japan).

The average absorption across all replicates for the negative control was subtracted from the positive and lipid sample averages to account for silicate formation. 5 μ L of 2mM phosphate (positive control) has 0.01 μ mol phosphate and results in an absorbance of approximately 0.260 for inorganic phosphate¹²⁰. The concentration of phospholipids from the lipid sample was then calculated using the ratio of absorbance determined for the positive control and lipids and the known concentration of the phosphate standard.

The Ames assay accurately determines the concentration of phospholipids in solution however, the concentration of non-phospholipids (cholesterol, ceramide, cerebrosides, gangliosides, etc.) cannot be determined. Instead, the determined lipid concentration is scaled to the molar ratio of phospholipids present in solution. For example, if 10% Brain Sulfatides 90% POPC was determined to be 1.5mM, the 1.5mM corresponds to 90% of the lipids present. Including the sulfatides the total lipid concentration is

1.67mM. This concentration correction assumes that phospho- and non-phospholipids changed equally during extrusion. For example, if the original lipid concentration (determined by known mass weighed and volume in rehydration) of 10% Brain Sulfatides 90% POPC was 1.75mM then it was assumed that the 5% decrease occurred equally between sulfatides and POPC to maintain their respective molar ratios.

For experiments using polar lipid extracts, the lipid composition analysis provided by the supplier, Avanti Lipids, was used to estimate phospho- and non-phospholipid percentages for correction to the total lipid concentrations. Additionally, for cardiolipin 2 phosphates needed to be considered in the calculation by dividing by 2.

2.1.5 Large Unilamellar Vesicle Systems Examined

The complete list of lipid systems prepared as LUVs have been summarized in Table 3. A brief rationale for each system is provided as well:

Table 3. The lipid systems used for fluorescent leakage, laurdan GP, DLS size and phase transition experiments.

#	Lipid Composition	Rationale
1	100% POPC	Major phospholipid in membranes, zwitterionic, positive choline may block binding to the negative phosphate
2	100% DMPC	Allows comparison of acyl tail saturation in membrane packing and metal binding to PC lipids
3	100% PCplasm	Comparison of PC lipids with different side chain linkage.

#	Lipid Composition	Rationale
4	100% PSM	Sphingomyelin is an important sphingolipid in cell membranes and only differs from PC lipids in
5	100% Brain SM	backbone structure Sphingomyelin is an important sphingolipid in the myelin sheath and this natural lipid mixture contains a distribution of fatty acid tail lengths/saturation present in the brain.
6	100% POPS	Negatively charged lipid for electrostatic interactions with Pb ²⁺ . Also, the negative charge is extended further out than phosphate at polar interface
7	100% DMPS	Allows comparison of acyl tail saturation in membrane packing and metal binding to PS lipids
8	100% POPA	Negatively charged target lipid for electrostatic interactions with Pb ²⁺ with no headgroup interference to phosphate at polar interface
9	100% POPA pH 6.4 adjusted	Adjustment in pH to reduce relative charge of PA from -1.2 (at pH 7.4) to -1.0 and observe any changes in Pb ²⁺ binding
10	100% DMPA	Comparison of Pb ²⁺ binding to tighter packed saturated PA membranes

Lipid Composition Ħ

11	100% DMPA pH 6.4 adjusted	Adjustment in pH to reduce relative charge of PA from -1.2 (at pH 7.4) to -1.0 and observe any changes in Pb binding
12	100% TOCL	CL lipids are large, bulky molecules with two potential phosphate binding targets and no head groups to block Pb binding
13	100% TMCL	Comparison of Pb ²⁺ binding to tighter packed saturated CL membranes
14	100% POPG	PG lipids are important components in the mitochondria, also structurally relevant to PA lipids with a bulky glycerol as head group that could interfere with Pb binding
15	100% DMPG	Comparison of Pb ²⁺ binding to tighter packed saturated PG membranes
16	15% CHOL: 85% POPC	Examine if packing alteration by cholesterol affect Pb ²⁺ binding
17	40% CHOL: 60% POPC	Examine if a further increase in cholesterol affects Pb ²⁺ binding
18	40% CHOL: 60% PSM	Comparison of the impact of cholesterol in different phosphocholine - containing systems

		Comparison of backbone/acyl tail function in Pb ²⁺
19	50% PSM: 50% POPC	binding between these phosphocholine -containing
		lipids
20	15% POPE: 85% POPC	15% POPE mimics outer leaflet content of RBC
		membranes
21	30% POPE: 70% POPC	30% POPE allows for 15% PE content on inner and
		outer leaflets in LUVs
		PEplasm allows for comparison to POPE for the
22	29% PEplasm: 71% POPC	same headgroup with a change from acyl to alkenyl
22		chain linkage. It is commonly found in RBCs and
		the myelin sheath
23	29% PEplasm: 32% CHOL:	Examine if cholesterol induced packing alters Pb ²⁺
23	39% POPC	binding
24	15% POPS: 85% POPC	15% POPS used for comparison to 15% POPE
21		system
25	10% Total Gangliosides	To consider a large, complex lipid present
23	(Brain): 90% POPC	throughout the brain.
26	10% Brain Sulfatides:	Complex cerebrosides with sulfate as potentially
	90% POPC	binding target of Pb ²⁺ , potential role in
		neurotoxicity

		Ceramides are hydrophobic sphingolipids with a
27	5% C16 Ceramide:	strong hydrogen bonding network in the polar
27	95% POPC	interface of the membrane ¹²¹ which may affect Pb ²⁺
		– PC interactions.
		Galactosyl ceramide is a type of cerebroside
28	3.5% Gal Cer: 96.5% POPC	enriched in the brain grey and white matter and the
		myelin sheath ⁹⁵ .
		Hydroxylated galactosyl ceramides are a large
20	21% (2R-OH) Gal Cer:	constituent of the outer leaflets of myelin sheaths, a
29	79% POPC	potential target of Pb2+. Comparison to non-
		hydroxylated galactosyl ceramides.
		To observe the effects of Pb ²⁺ on complex lipid
30	RBC Total Lipid Extract	profiles with varying headgroup and fatty acid tail
		compositions
		To observe the effects of Pb ²⁺ on complex lipid
31	Brain Polar Lipid Extract	profiles with varying headgroup and fatty acid tail
		compositions
32	29% PEplasm: 32% CHOL:	
	26% POPC: 7% POPS:	"Inner Myelin Mimic" ⁹⁷ .
	6% Brain SM	

33	 10% Brain Sulfatides: 33% CHOL: 42.5% POPC: 9% PEplasm: 4.5% Brain SM: 1% POPS 	Consideration of sulfatides in the presence of other lipids and cholesterol which add to the outer leaflet composition of the myelin sheath
34	3.5% Gal Cer: 33% CHOL: 49% POPC: 9% PEplasm:	Consideration of galactosyl ceramide in the presence of other lipids and cholesterol which add
	4.5% Brain SM: 1% POPS	to the outer leaflet composition of the myelin sheath
35	21% (2R-OH) Gal Cer:	Consideration of hydroxylated galactosyl ceramide
	33% CHOL: 31.5% POPC:	in the presence of other lipids and cholesterol which
	9% PEplasm:	add to the outer leaflet composition of the myelin
	4.5% Brain SM: 1% POPS	sheath
	21% (2R-OH) Gal Cer:	Combination of hydroxylated and non-
26	3.5% Gal Cer: 33% CHOL:	hydroxylated cerebrosides in the presence of other
50	28% POPC: 9% PEplasm:	lipids and cholesterol which add to the outer leaflet
	4.5% Brain SM: 1% POPS	composition of the myelin sheath
	21% (2R-OH) Gal:	
37	3.5% Gal Cer: 33% CHOL:	
	10% Brain Sulfatides:	"Outer Myelin Mimic" ⁹⁷
	18% POPC: 9% PEplasm:	
	4.5% Brain SM: 1% POPS	

38	1% DOPI: 99% POPC	PI lipids are prevalent in signaling pathways, tested Pb ²⁺ binding to phospholipids with a bulky sugar group extending outwards at a 1% exposure
		expected for most membranes
39	1% DOPI (3) P: 99% POPC	Comparison of the different location of a phosphate
40	1% DOPI (4) P: 99% POPC	group attached to the inositol ring of the PI lipid
41	1% DOPI (5) P: 99% POPC	containing a net negative charge of -2^{122} .
42	1% DOPI (3,4) P ₂ :	PIP_2 lipids such as PI (4,5) P_2 are well characterized
	99% POPC	for interactions with membrane proteins and
43	1% DOPI (4,5) P ₂ :	signaling pathways. Net negative charge of PIP_2
45	99% POPC	lipids ranging -3 to -4 containing 3 potential
11	1% DOPI (3,5) P ₂ :	phosphate targets of the metals in different
44	99% POPC	positions
		PIP ₃ is the most negative (-5) lipid considered for
45	1% DOPI (3,4,5) P ₃ :	metal binding. Allows comparisons for phosphate
	99% POPC	binding preferences from PI up to PIP ₃ and the
		respective varying locations of these groups.

2.1.6 Red Blood Cell Total Extract Preparation

To understand the interactions of Pb^{2+} with an important metal target in the blood stream^{123,124}, red blood cells were investigated by performing a total lipid extraction to isolate the complex lipid fraction of RBCs. Hemoglobin, which would interfere with the

fluorescence signal and has been identified as a potential target of Pb^{2+} once it entered the RBCs^{40,41,125-127}, was first removed to generate RBC ghosts as described by Dodge *et al.*¹²⁸. 50mL of rabbit blood was evenly divided into 4 tubes containing heparin, an anticoagulant. Tubes were centrifuged at 3,000 rpm for 10 min at 4°C using a Sorvall Legend X1R centrifuge (Thermo Scientific, Ottawa, ON) to pellet the red blood cells. The supernatant fraction of blood plasma was decanted, and the RBC pellet was resuspended using a low vortex speed in 4 washes of 3mL Krebs-Ringer Buffer (Sigma Aldrich, Oakville, ON) which contained 1.26 g/L sodium bicarbonate and pH was adjusted to 7.40. After the first 4 washes, the tubes were centrifuged at 1,600 rpm for 10 min at 4°C to separate the platelets and white blood cells into the supernatant while the RBCs remained in the pellet. This washing step was repeated 4 times and the supernatant was decanted and discarded each time.

3mL of washed RBCs were then transferred into 40mL centrifuge tubes and 27mL of 5mM phosphate buffer (pH 7.40) was added. The osmotic difference with the low-salt phosphate buffer causes lysis of the RBCs and the leakage of hemoglobin. Each tube was centrifuged at 20,000x g for 40 min at 4°C using a Sorvall RC SC Plus centrifuge (Thermo Scientific, Ottawa, ON). The supernatant fraction was decanted, and the absorbance spectra was obtained using the Shimadzu UV-1700 UV-VIS Spectrophotometer (Shimadzu Corporation, Kyoto, Japan) between 400-700nm to monitor hemoglobin content. Washes were repeated 6 times until the pellet was white in appearance and the absorbance spectra of the supernatant no longer showed characteristic hemoglobin peaks at 425, 550 and 575nm.

Lead is known to complex with phosphate buffers leading to precipitation thus 3

subsequent washes of 30mL of 100mM NaCl pH 7.40 followed by centrifugation at 20,000x g for 40 min at 4°C were required to remove phosphate from the RBC ghosts. Each pellet following the last centrifugation step was resuspended in 6mL of 100mM NaCl pH 7.4 and evenly divided amongst four 20mL glass tubes. In each tube, 3mL of a 2:1 (ν/ν) chloroform: methanol mixture was added to the 3mL RBCs which were mixed by vortex and sonication. The emulsion was separated by centrifugation at 2,500 rpm for 4 min at 4°C using the Sorvall Legend X1R centrifuge. The tube contents exhibited an upper less-dense aqueous layer, a thin white protein layer called buffy coat and a lower, denser organic layer. 1mL Hamilton glass syringes were carefully inserted to extract the organic layer which was pooled in a 20mL glass vial. The extraction process was repeated 4 more times by pooling all organic layers which contained the lipids of interest. The pooled organic fractions were then washed with an equal volume of deionized water to remove residual salt from the lipid extract.

Tubes were centrifuged at 1,000 rpm for 4 min at 4°C and the aqueous layer was aspirated off and discarded. Residual water was removed by sodium sulfate as a drying agent until no aqueous emulsion layer was visible. 2 min centrifugation at 1,000 rpm was used to pellet the sodium sulfate and the organic solvent was transferred into a 4mL glass vial. The organic solvent was evaporated off using argon gas forming a dry lipid film. LUVs were prepared as described in Section 2.1.3 Large Unilamellar Vesicle (LUV) Preparations. After the Ames assay, laurdan was incorporated at the 1:550 molar ratio of laurdan/lipids using a 1mM laurdan stock in DMSO.

2.2 Fluorescence Leakage Assays

2.2.1 Introduction to Leakage Assays

Leakage experiments examine the effect of Pb^{2+} on the membrane permeability by quantifying the rate at which Pb^{2+} binding causes a molecule to diffuse through the membrane. A water-soluble fluorescent dye was co-encapsulated within liposomes alongside a quencher molecule forming a non-fluorescent pair which is released upon dilution, allowing the dye to fluoresce. The chosen dye cannot directly be quenched by Pb^{2+} , as the leakage fluorescence increase would be directly opposed by Pb^{2+} , and the dye should be cell-impermeant as to avoid false-positive leakage. To quantify the amount of dye which has leaked, each trial is ended by the addition of a strong detergent which disrupts the membrane to cause full leakage of all internal components.

2.2.2 Large Unilamellar Vesicle Preparations

Liposomes were created as described in Section 2.1.3 Large Unilamellar Vesicle (LUV) Preparations. Lipid films were rehydrated in 450μ L of 25mM 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) in 100mM NaCl pH 7.40 and 500μ L of 90mM *p*-xylene-bispyridinium bromide (DPX) (Thermo Fisher Scientific, Waltham, MA) in NaCl pH 7.40 resulting in a ratio of 1:4 (11.8mM ANTS: 47.4mM DPX) inside the liposomes. The structures of these molecules are shown in Figure 8. At this ratio, DPX will completely quench ANTS causing only basal levels of fluorescence to be obtained while encapsulated inside the vesicles. Following extrusion, the liposomes were applied to the Sephadex G-25 column as previously described and eluted in the void volume. The Ames assay was also completed as previously described.



Figure 8. (**A**) The structure of 8-aminonaphthalene-1,3,6-trisulfonic acid (ANTS) and (**B**) the structure of *p*-xylene-bis-pyridinium bromide (DPX).

2.2.3 Encapsulation Volume Calculations

The mathematical model provided by Xu et al. was used in determining the final concentration of ANTS dye after complete leakage from the vesicles during each experiment¹²⁹. Accordingly, for a 300 μ M sample of LUVs of approximate size 100nm, assuming 10% size distribution, the total volume inside all vesicles in the 2mL cuvette sample is 0.97 μ L. The LUVs are encapsulating 11.84mM ANTS and 47.4mM DPX. Utilizing an average bilayer thickness of 5.1nm and lipid molecular surface area of 0.4nm², we observe a final dilute concentration of 5.72 μ M ANTS upon complete leakage into the surrounding cuvette volume.

2.2.4 Experimental Procedure

Fluorescent leakage experiments were conducted on a Varian Cary Eclipse Spectrofluorimeter (Agilent Technologies, Santa Clara, CA) by excitation at 356 nm and emission scan at 512 nm with 5 nm bandpasses each for an average of 3 measurements. Lipid solutions were diluted to 300μ M in 2mL plastic (methacrylate) UV cuvettes with a 10mm pathlength. All experiments were completed at room temperature. After recording the initial fluorescence intensity over time, injections from 0 to 10μ M Pb²⁺, with 0.5μ M intervals, were completed with a 1-minute incubation at each step to allow any metal-

induced release of dye. 100mM NaCl pH 7.4 injections of the same volume and repetition as Pb^{2+} injections were performed as a control to assess dilution and mechanical sheering of liposomes as cause of potential dye leakage. Another set of liposomes were scanned over time without injection to assess self-leakage without Pb^{2+} binding. After all injections were complete, 10µL of 1% Triton X-100 was added to the cuvette for vesicle lysis. The dye leakage experiment is quantified as a percentage increase in fluorescence by setting the initial intensity to 0% and maximum leakage after Triton X-100 addition as 100%.
2.3 Laurdan Generalized Polarization

2.3.1 Introduction to Laurdan

The effect of Pb²⁺ on the fluidity of membranes was determined with the fluorescent dye 6-dodecanoyl-2-dimethylaminonaphthalene (laurdan). Laurdan was first synthesized by Gregorio Weber in 1979 as a solvatochromic dye, a probe responding to changes in solvent polarity¹³⁰. As seen in Figure 9, laurdan is composed of a 12-carbon (lauroyl) hydrophobic tail which allows for incorporation into the lipid bilayer whereby a naphthalene moiety and dimethylamino headgroup is positioned near the lipid backbone at the polar interface of the membrane (Figure 11).



Figure 9. The structure of laurdan. Dipole moment across the naphthalene moiety is depicted by a grey arrow.

The naphthalene moiety allows for the detection of dipolar relaxation due to a partial charge separation between the electron rich 2-dimethylamino and the electron poor 6-carbonyl residues¹³¹. This dipole moment is increased during excitation and the polarity of the probe's environment may cause solvent relaxation and reorientation leading to a red-shift of the fluorescence emission spectrum^{109,130–133}. When the lipids are in a gel phase, the emission maximum of Laurdan is centered on 440 nm whereas when the lipids are in a liquid-crystalline phase, the emission maximum is centered at 490 nm as illustrated in Figure 10.



Figure 10. Fluorescence intensity (a.u.) of the emission wavelength (nm) scan of laurdan in DMPG liposomes in 100mM NaCl pH 7.4 at 13°C (blue), 23°C (green) and 37°C (red). Excitation of laurdan set to 340nm, arrows indicate 440 and 490nm peaks.

This spectral shift has been attributed to an increased level of water molecules in the bilayer at the glycerol backbone in the liquid-crystalline phase whereas reorientation of the solvent along the fluorophore dipole is unlikely to occur in the gel phase¹³¹. Figure 11 depicts the change in water penetration on laurdan across the lipid phases.

The phase change between the gel and liquid crystalline phases occurs at a characteristic temperature, the phase transition temperature (T_m) , which was determined by using laurdan as explain below in Section 2.3.2 Generalized Polarization.



Figure 11. Schematic representation of laurdan (green) incorporated in a lipid membrane (black) when in the gel phase of tighter packing (left) and the liquid-crystalline phase of increased fluidity (right).

2.3.2 Generalized Polarization

The shift in emission spectra can be measured by using the generalized polarization (GP) parameter, defined in Equation 1 where I_{440} and I_{490} correspond to the fluorescence emission intensities at 440 and 490nm, respectively^{109,131}:

Equation 1 $GP = \frac{I_{440} - I_{490}}{I_{440} + I_{490}}$

GP values range from a maximum +1 corresponding to maximum fluorescence at 440nm to a minimum of -1 corresponding to maximum fluorescence at 490nm although membrane GP values typically only range from +0.6 to -0.4.



Figure 12. Generalized polarization as a function of temperature (°C) of laurdan in 300μ M DMPC LUVs in 100mM NaCl pH 7.4. Fluorescence intensities measured at 440 and 490nm upon excitation at 340nm. Arrow depicts phase transition (T_m) of 24°C.

Laurdan detects changes to membrane fluidity as a function of temperature as the lipids transition from gel, rigid and ordered, to liquid-crystalline, fluid, and disordered. The largest shift in GP occurs at the phase transition temperature (T_m) which is often a sharp, sudden decrease over a short temperature increase as illustrated in Figure 12. Lipid systems with a T_m above 0°C result in the characteristic sigmoidal curve with the inflection point corresponding to its T_m and designated gel and liquid-crystalline sections at lower and higher temperatures respectively (see Figure 12). The tight packing of lipids in the gel phase provides a highly rigid state that limits water access to laurdan resulting in minimal GP changes.

Lipids with a T_m below 0°C (such as POPC at -2°C) limit only observing the change in membrane fluidity across the liquid-crystalline phase with increasing temperature. Fluorescence measurements below 0°C cannot be completed due to the formation of ice crystals and water condensation on the cuvettes which scatter light during the experiment.

2.3.3 Experimental Procedure

 $500 \ \mu$ L of $300 \ \mu$ M LUVs containing laurdan were loaded into small volume quartz cuvettes (Starna Scientific Ltd, Atascadero, CA) with a 5 mm path length. LUVs were combined with 0 to 2.1mM Pb(NO₃)₂ in the cuvette at room temperature and allowed to incubate for 5 minutes prior to experiments. Fluorescence measurements were performed on a Varian Cary Eclipse fluorescence spectrophotometer (Agilent Technologies, Santa Clara, CA) by excitation at 340 nm and dual emission scans at 440 and 490 nm with 5 nm bandpasses each for an average of 4 measurements. The temperature within the cuvette holder was maintained within $\pm 0.1^{\circ}$ C using a Cary circulating water bath temperature controller (Agilent Technologies, Santa Clara, CA). Each sample was equilibrated at the lowest temperature for 10 minutes before measurements were taken.

Lipid systems with a T_m below 0°C were started at 5°C whereas systems with higher T_m were incubated at a temperature at least 10°C below the T_m whereby 5°C was the lowest possible temperature used. The temperature was increased until the onset of the phase transition where measurements were recorded every 0.5 – 2°C depending on the cooperativity of the T_m . More data points within this region yield a better sigmoidal fit for determining the T_m . LUVs were equilibrated at each temperature for 1 minute per 1°C change to ensure accurate laurdan fluorescence readings.

2.3.4 T_m Determination

The generalized polarization versus temperature data was fitted to a non-linear Boltzmann function using Origin Pro 8.0 software¹³⁴ to determine the T_m of the sample which is x_0 as per Equation 2:

Equation 2
$$Y = \frac{A_1 - A_2}{1 + e^{(x_{1/2} - x_0)}/\Delta x} + A_2$$

Equation 2 describes the Boltzmann regression curves of GP (y) as a function of temperature (x) where A_1 and A_2 are the maximum and minimum GP values, respectively, $x_{1/2}$ is the temperature at which GP is halfway between A_1 and A_2 , x_0 is the centre of the distribution (T_m) and Δx describes the width of the slope¹¹¹.

2.4 Dynamic Light Scattering (DLS)

2.4.1 Introduction to DLS

DLS is a non-invasive technique providing information on size, mass, and charge of a particle sample. Its measurements are dependent on Brownian motion, the random motion of molecules in solution, whereby the distance travelled is inversely related to particle size: larger molecules move slower than small molecules. A laser is directed onto the sample and time dependent fluctuations in the scattering intensity of light is recorded by the detector. The rate of intensity fluctuations is dependent on its size where larger molecules move slower resulting in less scattered light fluctuations¹³⁵.

Additionally, DLS reports on the count rate which is the number of light photons detected from the scattering off the molecules. This is useful for determining sample quality, stability and modifications in the optical properties of the system^{111,136}.

2.4.2 Vesicle Sizing Determination

Size determination was important in ensuring uniformity during the preparation of LUVs via extrusion. The desired solution was a monodisperse population of LUVs with a diameter of ~100 nm based on the pore size of the polycarbonate membrane that was used. This technique also allowed for observations of lead-induced vesicle size changes or aggregations.

Liposome sizes and size distribution were analyzed by dynamic light scattering using the Zetasizer Nano ZSP (Malvern Instruments, Malvern, UK) with a 633 nm laser. Lipid solutions were degassed for 5 minutes to reduce air bubbles that scatter light and interfere with measurements. Quartz cuvettes of a 5mm path length were filled with 500 μ L of 300 μ M lipids and placed into the instrument. Cuvettes were set at a 4.20 mm distance from the detector which was positioned at 173° relative to the laser source allowing for backscatter detection¹¹¹. Each sample was measured at 25°C in triplicate with 10 runs of 5 seconds per measurement.

Samples were determined to be monodisperse based on the polydispersity index (PDI) value which describes the width of an assumed Gaussian distribution to the single calculated particle size¹³⁵. This index number is calculated using a simple 2-parameter fit to the correlation data collected by the instrument. PDI is scaled such that values as low as 0.05 are rarely seen, except for highly monodisperse standards, whereas values greater than 0.70 indicate a very broad distribution of size populations in solution. A % polydispersity of 20% indicates that the sample is monodisperse so a cut-off for PDI of 0.15 was selected for well-defined liposomes for further testing¹³⁶.

2.4.3 Temperature Phase Transitions

The gel (ordered) to liquid-crystalline (disordered, fluid) phase transition can also be measured by DLS allowing for a comparison of Pb^{2+} binding effects on the membrane phase behaviour and the phase transition temperature (T_m) between DLS and laurdan GP experiments. Another common technique used to measure lipid T_m is differential scanning calorimetry (DSC) which was not feasible because heavy metals may impact the calorimetric cell alloys. Thus DLS allows concurrent measurements of vesicle size and T_m determination in the absence and presence of metals such as Pb^{2+111} .

Michel *et al.* reported on changes to the optical properties of vesicles as they undergo phase transitions from gel to liquid-crystalline¹¹¹. The count rate is a function of light scattering from the surface of the particle into the detector. Lipids in the gel phase are highly ordered however as the temperature increases, up to and past the T_m , lipids in the liquid-crystalline phase are more fluid and disordered resulting in more random light scattering and less photons reflected into the detector. A schematic of the DLS phase transition detection is illustrated in Figure 13.



Figure 13. Schematic of DLS phase transition experiments. Lipids in gel phase (blue) reflect uniformly corresponding to higher count rates at lower temperatures. Increasing temperature for lipids in liquid-crystalline phase (orange) results uniformly in a lower photon count rate. Graph of photon count rate over temperature yields phase transition temperature (T_m).

2.4.4 T_m Determination

The derived count rate over temperature data is fit to a non-linear Boltzmann function using Origin Pro 8.0 software¹³⁴ to determine the T_m of the sample as described in Section 2.3.4 Tm Determination.

2.5 Langmuir Trough

2.5.1 Introduction to the Langmuir Trough

All monolayer experiments were performed on a 200cm² Teflon coated Langmuir trough from Nima (Medium Trough model, Espoo, Finland). A schematic of the trough and accessory experimental components is illustrated in Figure 14.



Figure 14. Schematic of the Langmuir trough (components highlighted in black) used for isotherm and imaging experiments. Essential components for obtaining surface pressure isotherms (purple), surface potential isotherms (green) and Brewster angle microscopy (red) have also been highlighted.

2.5.2 Metals in the Subphase

The trough was filled with a subphase consisting of 100 mM NaCl pH 7.4. Metals at a 500:1 metal/lipid molar ratio were supplemented to the subphase prior to lipid deposition. Based on the configuration of the trough set-up, metals could not be injected into the subphase after forming the lipid monolayer due to unequal dispersion, localized metal deposition and potentially uneven binding. A high molar ratio of 500:1 metal/lipid was used to overcome the large subphase volume of 125mL whereby the metal can reside anywhere within the bulk solution or at the air – water interface where the lipids are present. This ratio corresponds to 86μ M Pb²⁺.

2.5.3 Monolayer Lipid Systems Examined

Lipids were weighed using the Sartorius Microbalance MC 5 (Göttingen, Germany) and dissolved in 6:4 (v/v) chloroform: methanol for 1mM concentrations for use on the Langmuir trough. The complete list of lipid systems prepared as monolayers is summarized in Table 4. A brief reasoning on why these systems were chosen is also provided:

Table 4. The lipid systems used for surface-pressure isotherms, surface-potentialisotherms, and Brewster angle microscopy experiments.

#	Lipid Composition	Rationale
1	100% POPC	Major phospholipid in membranes with possible inaccessibility of phosphate target
2	100% DMPC	Allows comparison of acyl tail saturation in membrane packing and metal binding to PCs.
3	1% DOPI: 99% POPC	PI lipids are prevalent in signaling pathways, tested Pb ²⁺ binding to phospholipids with a bulky sugar group extending outwards at a 1% exposure expected for most membranes

#	Lipid Composition	Rationale
4	1% DOPI (3) P: 99% POPC	Comparison of the different location of a
5	1% DOPI (4) P: 99% POPC	phosphate group attached to the inositol ring
6	1% DOPI (5) P: 99% POPC	of the PI lipid containing a net -2 charge.
7	1% DOPI (3,4) P ₂ : 99% POPC	PIP_2 lipids such as PI (4,5) P_2 are well characterized for interactions with membrane
8	1% DOPI (4,5) P ₂ : 99% POPC	proteins and signaling pathways. PIP_2 with a -3 charge contains 3 potential phosphate
9	1% DOPI (3,5) P ₂ : 99% POPC	targets of the metals in different positions
10	1% DOPI (3,4,5) P ₃ : 99% POPC	PIP ₃ is the most negative lipid considered for metal binding. Allows comparisons for phosphate binding preferences from PI up to PIP ₃ and the respective varying locations of these groups.
11	100% POPS	Negatively charged lipid for electrostatic interactions with Pb ²⁺ . Also, the negative charge is extended further out than phosphate at polar interface
12	100% DMPS	Allows comparison of acyl tail saturation in membrane packing and metal binding to PS lipids.

2.5.4 Surface Pressure – Area Isotherms

Isotherms at the air-water interface were recorded using a pressure sensor equipped with a Wilhelmy plate dipped approximately 1mm into an aqueous subphase. Several forces including the weight of the plate, gravity and surface tension are pulling down on the sensor while buoyancy pushes it up as displayed in Figure 15.



Figure 15. Schematic illustrating the forces acting on Wilhelmy filter paper plate used in calculating surface tension.

The surface tension (γ) is calculated using the forces and contact angle from Figure 15, as shown in Equation 3:

Equation 3
$$\gamma = \frac{F}{2(w+d)\cos\theta}$$

Where F is the sum of forces (*N*) acting on the Wilhelmy plate, *w* is the width of the plate and *d* is the thickness of the plate (measured in metres). The contact angle (θ) equals 0 when the plate is correctly positioned perpendicular to the water surface such that $\cos(0) = 1$. The Langmuir trough reports surface pressure (π) (mN/m) which is calculated by the difference in the surface tension of the pure subphase (γ_0) and when a monolayer has been deposited (γ) as seen in Equation 4:

Equation 4 $\pi = \gamma_0 - \gamma$

Equation 3 and Equation 4 highlights the inverse relationship between surface tension and pressure. Water molecules at the surface of the air-water interface are limited to hydrogen bonding towards the bulk subphase and the magnitude of the downward pull is reflected by the surface tension, which consists of units of force per unit length (mN/m) (Equation 3). The air-water interface has a large free energy per unit area of approximately 72 mN/m¹⁰¹ which is equivalent to the surface pressure at which surface tension is zero. The addition of a lipid monolayer on the air-water interface lowers the surface tension which corresponds to an increase in surface pressure¹⁰¹ (Equation 4).

2.5.5 Experimental Procedure

Before each trial, the trough and gastight Hamilton syringe were thoroughly cleaned using the same organic solvent described in Section 2.1.3 Large Unilamellar Vesicle (LUV) Preparations. The Wilhelmy plate was cleaned by washing twice with 1mM EDTA pH 8 and six times with boiling water. Once cleaned, the trough was filled with 125mL 100mM NaCl pH 7.4 plus 86.2 μ M Pb²⁺ for the metal scans. A water isotherm was conducted to ensure that the trough was clean resulting in no surface pressure increases across the entire compression. After reopening the barrier, 21.5 μ L of 1mM lipid samples were deposited drop-wise onto the subphase. After a 10-minute delay for solvent evaporation, the lipid film was compressed to until collapse while recording the surface pressure as the lipid molecules are packed tighter once the area is reduced. Each lipid system was repeated in triplicate with Pb²⁺ and control scans without metals. After each isotherm, the subphase and lipid film was removed by vacuum and the trough was

subsequently cleaned with EDTA and the 4-solvent wash again. A new, clean Wilhelmy plate was used for each lipid system or metal in the subphase.

2.5.6 Surface Pressure-Area Isotherm Analysis

The molecular area (Å²/molecule) of each isotherm was calculated by the Nima software using the lipid sample's molecular weight, concentration, volume deposited and Avogadro's number. The surface pressure (π) (mN/m) is recorded as the area decreases thus the isotherm graph is read from right to left as demonstrated in Figure 16.



Figure 16. Surface pressure-area isotherm of POPS to highlight the transition between the different lipid monolayer phases observed throughout this study.

When lipids occupy a large area prior to compression, no surface pressure is observed. The lipids are in a "gaseous" or gas-like phase with limited intermolecular interactions and random orientations of the fatty acid tails. As the lipids are further compressed to smaller molecular areas, they are in closer contact where van der Waal's interactions amongst the tails result in an increase in surface pressure¹⁰⁰. The lipids transition into a liquid-expanded (LE) phase where they continue to exert more intermolecular forces as the lipids become more ordered while remaining fluid and loosely packed.

Upon further compression, some lipid classes transition into a liquid-condensed (LC) phase which has tighter packing, more rigidity, and stronger interactions at the tails, however this phase was not notable in most of the lipid systems tested in this study. In some of these monolayers, LE/LC phase coexistence may occur, but the lipids predominantly remain in the LE phase upon continued compression until the collapse pressure was reached representing the maximum packing density between lipids. Upon further compression multilayers are formed, and some lipids are expelled into the subphase. Collapse is observed as a horizontal slope or drop in pressure at the smallest molecular area (Figure 16). The equivalent surface pressure between lipid monolayers and bilayers is in the region of $30 - 35 \text{ mN/m}^{137}$.

The shape of π -A isotherms is strongly influenced by experimental conditions and any chemical modifications to the molecule's structure¹¹⁶. Changes in the shape of the isotherm in the presence of metals suggest changes in the organization and lipid packing arrangement of the monolayer. A shift to smaller areas corresponds to the binding or incorporation of ions in the monolayer by reducing the molecular areas of the lipids whereas right shifts to larger areas correspond to opposing effects, less tight packing, and larger molecular areas. Isotherm can also shift along the y-plane whereby upwards shifts correspond to increased surface pressure that could be due to enhanced and metal induced lipid-lipid interactions while downward shifts indicate destabilization.

2.5.7 Compression Modulus

Lipid packing and fluidity also define the mechanical properties of the monolayer in terms of elasticity and compressibility. The lateral surface compressibility (C_s) is a first derivative function obtained from the π -A isotherms for monolayers at any given surface pressure:

Equation 5
$$C_S = -\frac{1}{A} \times \frac{\partial A}{\partial \pi}$$

Where *A* is the molecular area at the indicated surface pressure $(\pi)^{100,138}$. The derivative was calculated in blocks of 50 data points using the slope function within the Microsoft Office Excel 2016 program (Redmond, WA). The compression modulus (β) is the reciprocal of the compressibility which is reported in literature as it provides insight into lateral packing elasticity and analyses information from the slopes of the isotherms. High compression modulus values correlate to low elasticity of lipids forming a monolayer¹⁰⁰.

2.5.8 Surface Potential – Area Isotherms

The deposition of a lipid monolayer generates an electrical potential perpendicular to the plane of the subphase interface due to the asymmetric orientation of lipid and water molecules¹⁰⁰. The dipole potential aids in the determination of the size and shape of lipid rafts and is measured as the difference in potential (ΔV) between an interface containing a monolayer and the pure subphase as seen in Equation 6:

Equation 6 $\Delta V = V - V_0$

The electrical potential of a lipid monolayer (V) is the sum of several dipole

moments at the surface of the monolayer due to the reorientation of water molecules and the double-layer formation of ionized monolayers between charged headgroups and the electrolytic subphase¹¹⁶ as depicted by μ_1 in Figure 17. A second dipole moment (μ_2) exists within the monolayer from the headgroups and polar interface region while the third (μ_3) arises from the hydrophobic tails for which the terminal methyl group has the largest influence.



Figure 17. Schematic of the orientation of dipole moments found in the subphase (μ_1), polar interface region (μ_2) and the alkyl chains (μ_3) of the lipid molecule.

The surface potential was measured using the vibrating capacitor method in which the potential difference between two parallel plate of a capacitor was measured. The bottom platinum counter-electrode was inserted into the subphase below the monolayer while the KSV NIMA Surface Pressure Sensor (SPOT) (Espoo, Finland) was positioned approximately 2-3mm above the subphase. The upper electrode oscillates to generate a displacement current which is nulled by an external bias such that the potential difference for a pure subphase is zero^{116,139}.

When a deposited film is being compressed, the surface potential changes due to

the alteration in the head group and tail orientations. The surface potential (ΔV) (mV) is recorded as the molecular area decreases thus the isotherm graph is read from right to left as demonstrated in Figure 18.



Figure 18. Surface potential-area isotherm of POPC.

2.5.9 Experimental Procedure

The surface potential isotherms were carried out concurrently to surface pressure experiments as described in Section 2.5.5 Experimental Procedure. The SPOT and counterelectrodes were cleaned with chloroform before placing the counter-electrode into the subphase prior to lipid deposition. SPOT was lowered down to the subphase within the final compressed region near the Wilhelmy plate and a water isotherm was conducted to ensure that the trough was clean resulting in no surface potential increases during the entire compression. Each lipid system was repeated in triplicate with Pb²⁺ and no metal conditions to ensure reproducibility. After each isotherm, the electrodes were removed and cleaned with 1mM EDTA pH 8 and chloroform.

2.6 Brewster Angle Microscopy (BAM)

2.6.1 Visualization of Lateral Domain Formation

Brewster Angle Microscopy (BAM) is a technique used for *in situ* visualization of lateral film organization in real time. BAM eliminates the need for labels, avoiding potential artifacts in the analysis of membrane organization. When a 658nm laser of p-polarized light hits the air-water interface at a Brewster angle of 53.1°, there is no reflection of light due to the different refractive indices of air and water (Figure 19). This refractive index of the interface changes upon addition of a lipid monolayer, modifying the Brewster angle conditions, and light that is now reflected can be used for imaging¹¹⁶.



Figure 19. Brewster Angle Microscopy principle. Presence of a lipid film changes the interfacial properties of the air-water interface from no reflection of p-polarized light at the Brewster angle of 53° to reflection off the film into a camera.

BAM images are dark in the absence of a film while images of a lipid film are grey and can show regions of lighter grey representing clusters or domains. Any structures protruding from the film appear brighter due to enhanced reflection of light. The lipid systems presented in this study did not undergo phase separation under the metal –free buffer of defined ionic strength. However, in the presence of different metals, some lipid systems showed metal induced clusters. The formation of these aggregates may be due to increased hydrogen bonding amongst the headgroups and/or metals, van der Waal's interactions within the acyl tails, or metal-induced attractive or repulsive forces to coordinate with preferential binding targets.

2.6.2 Experimental Procedure

Imaging of lipids on the Langmuir trough was performed using an EP3 Brewster Angle Microscope (Accurion, Germany). The Langmuir trough was positioned on a Halyconics active vibration isolation table (Accurion, Germany) to minimize vibrational interference with the imaging. The EP3 V3.20 software (Accurion, Germany) was used to operate the microscope and capture images of the air-water interface. BAM imaging was completed concurrently or during a second compression of the π - and Δ V-A isotherms. The cleaning procedure for the trough and the tools used were the same as described for the isotherm experiments. A black lens plate was inserted into the subphase near the Wilhelmy plate and platinum counter-electrode as seen in Figure 14. The compression was stopped at approximately every 5mN/m surface pressures along the π -A isotherm for at least 3 images. Representative images were recorded from take-off at 0.5mN/m until film collapse.

Chapter Three: Primary Lipid Compositions

Upon uptake of heavy metals such as lead, one of the primary points of contact will be the cellular membranes and their lipid constituents. The purpose of this study is to consider physiological consequences of Pb^{2+} binding to lipids and help identify different lipid targets within the eukaryotic membrane. The effect of Pb^{2+} on lipids has been minimally studied so far with very limited understanding of preferential or specific binding affinity for individual lipid classes within complex biological membranes.

Suwalsky *et al.*, observed increased rigidity as detected by GP at 37°C with 0-5mM Pb²⁺ on DMPC membranes, highlighting the binding of Pb²⁺ to negatively charged phosphates of the polar lipid head groups¹¹⁹. To better understand biological lipid targets of lead that could contribute to the widespread toxicity observed in lead poisoning; simple biomimetic mixtures were first analyzed of the most common lipids^{1,98}.

The capability of Pb^{2+} to order zwitterionic PC systems lead to the hypothesis that Pb^{2+} is electrostatically driven to anionic regions of the membrane. Perturbations to the phosphate group within the backbone region of lipids affects membrane organization, permeability and lipid packing¹¹⁹. The impact of Pb^{2+} on these membrane properties was investigated with LUV leakage of the fluorophore/quencher pair ANTS/DPX, the solvatochromic fluorophore laurdan and DLS to assess size changes and to characterize lipid phase transitions. Common zwitterionic and anionic lipids were formulated into LUVs to screen for potential targets for Pb^{2+} binding.

The phosphocholine headgroup was the first zwitterionic target as PC lipids are the most common and structurally important lipids in most eukaryotic membranes⁶⁹. Glycerol based PCs included POPC, DMPC and PC-plasmalogen for different acyl tail lengths,

61

degrees of saturation and side chain architecture by comparing acyl and vinyl ether linkages to analyze the strength of $Pb^{2+} - PC$ interactions. Sphingosine based lipids included sphingomyelin by comparing synthetic lipid with defined palmitoyl acyl tails with natural extracts of porcine brain containing a broad fatty acid tail distribution.

The negatively charged glycerol-based lipids used in this thesis which were amenable to liposome formulation on their own included POPS, DMPS, POPA, DMPA, TOCL, TMCL, POPG, and DMPG. PS lipids were selected due to their important role in lipid signaling and their externalization from the inner leaflet which is an early indicator of apoptosis, signals for the blood clotting initiation with erythrocytes, and several other cellular functions ^{91,92,140}. The carboxylic group of the serine headgroup extending deeper into the surrounding aqueous phase provides an additional negative binding target for Pb²⁺, the that is further removed from the hydrophobic core of the bilayer.

PA, CL, and PG lipids contain a phosphate group like PC or PS that provides the overall anionic charge. Moreover, they differ in the accessibility of the charges and the surface charge density. PAs are structurally the simplest anionic lipid with no additional head group to block Pb²⁺ binding to the phosphate. The cardiolipins (CL) were studied next as this class is structurally related to the PAs. Their structure is achieved by combining two PAs with a glycerol linker, which decreases the surface charge density compared to PAs. Finally, PGs were studied to observe how a large, uncharged polar head group extending outwards from the liposome would affect Pb²⁺ binding.

3.1 Speciation of Pb²⁺ in Experimental Conditions

While positively charged Pb²⁺ species are not expected to penetrate into the lipid membrane, Diaz and Monreal determined that PbOH⁺ was capable of passive diffusion

through myelin lipid bilayer¹⁰³. This was also confirmed when Suwalsky *et al.*, observed Pb²⁺ adhering to the external and internal leaflets of erythrocytes as well as within the core of the membrane¹¹⁹. The expected speciation provided by Visual Minteq¹⁴¹ as described in Section 1.2 Speciation of Lead in Aqueous Solutions, suggests predominantly Pb²⁺, PbCl⁺ or PbOH⁺ under the physiologically relevant experimental conditions; however this theoretical program cannot consider potential complexation of Pb²⁺ with other proteins or water that could change the ratio of charged species *in vivo*.

A fluorescent dye, Phen Green SK (PGSK) (Figure 20) was used to characterize the role of pH and ionic strength for Pb^{2+} speciation under the conditions tested for metal-LUV interactions. The biomimetic membrane tests used to assess permeability, fluidity, LUV size and phase transition cannot identify the nature of the Pb^{2+} species binding to the membrane. Thus, speciation was altered by changing pH or ionic strength and the spectroscopic response of the dye was recorded to estimate the degree of Pb^{2+} – dye binding. Results are shown in Figure 21 and Figure 22.



Figure 20. Structure of Phen Green SK.



Figure 21. Overlay of the fluorescence intensity (a.u.) of 2µM PGSK (primary axis) under NaCl concentrations (0-100mM) with increasing concentrations of Pb²⁺ (0-1000ppb, line curves) with the percentage of total lead species (secondary axis) expected at these concentrations of NaCl (mM). Experimental and Visual Minteq software has fixed pH of 7.4 and temperature 37° C. $\lambda_{Ex} = 508 \pm 5$ nm, $\lambda_{Em} = 533 \pm 5$ nm.

The PGSK fluorescence intensity at any concentration of NaCl is decreased with increasing concentrations of Pb^{2+} (line curves, Figure 21). This quenching is non-linear as noted by the changing slopes of the trend lines across increasing concentrations of NaCl and different Pb^{2+} concentrations. When we compare the fluorescence within each set of Pb^{2+} concentrations against increasing NaCl, the change in dominant species at the higher salt reduces the quenching of Pb^{2+} as noted by the upward curve of each trendline as NaCl is increased.

This change in ionic strength alters the dominant Pb^{2+} species as displayed in the bar graphs in Figure 21. We observe a large shift from Pb^{2+} and $PbOH^+$ to $PbCl^+$ species with increasing chloride. This suggests that $PbCl^+$ has a lower affinity and is a weaker quencher of PGSK. Thus, we observe less fluorescence quenching when it is the dominant species in solution. Since the percentage of Pb^{2+} and $PbOH^+$ are both decreasing, we cannot determine which species acts the strongest on PGSK.



Figure 22. Overlay of the fluorescence intensity (a.u.) of 2µM PGSK (primary axis) at different pH with increasing concentrations of Pb²⁺ (0-1000ppb, line curves) with the percentage of total lead species (secondary axis) expected at each pH. Experimental and Visual Minteq software has fixed 100mM NaCl and 37°C temperatures. $\lambda_{Ex} = 508 \pm 5$ nm, $\lambda_{Em} = 533 \pm 5$ nm.

In Figure 22, the ionic strength was held at 100 mM NaCl whereby the column of Pb²⁺ species at pH 7.4 corresponds to the same speciation obtained for 100mM NaCl in Figure 21. Altering the pH changes the PbOH⁺ species with the appearance of more positive, neutral, and even negative lead species at higher pH values (pink $Pb_3(OH)_4^{+2}$, green Pb(OH)_{2 (aq)} and yellow Pb(OH)₃⁻⁻, Figure 22). Experiments at each pH revealed an overall reduction of PGSK fluorescence as the concentration of Pb²⁺ was increased, a similar trend as obtained with NaCl in Figure 21. Without Pb²⁺, there is a maximal fluorescence above pH 6.4, suggesting non-optimal dye conditions at pH 5.4. The intensity of PGSK was reduced with increasing Pb²⁺ up to 600ppb however, minimal changes occurred between as a function of pH between 6.4 - 9.4. At higher lead concentrations (700) -1000 pbb Pb²⁺), the quenching of PGSK is strongest below pH 8.4. This decrease in fluorescence may be a result of increasing PbOH⁺, which was suggested to have a stronger quenching impact on PGSK than PbCl⁺ during the NaCl experiments (Figure 21). The new species above pH 8.4, $Pb_3(OH)_4^{+2}$ and $Pb(OH)_2$ (aq), may interact with PGSK differently than PbOH⁺ to cause the increase in fluorescence signals for pH 9.4.

Figure 21 and Figure 22 highlight how experimental conditions change the speciation of Pb²⁺ which can alter the response of PGSK. Since it was not possible to use buffered solutions, it was very important to frequently check for pH drifts during the measurements. Frequent pH adjustments were a priority for the unbuffered salt solutions.

3.2 Interactions of Pb²⁺ with ANTS/DPX

The quenching of PGSK by Pb²⁺ prevented an unequivocal interpretation of leakage experiments. Several other fluorescent dyes were also quenched with Pb²⁺ including calcein, HPTS, PBFI, PGSK, Rhodamine-6G, and Fluozin-3 (data not shown).

Concomitant Pb^{2+} – dye quenching interferes with the expected increase in fluorescence seen when encapsulated dye leaked from LUVs. Dye screening for interactions with Pb^{2+} exhibited no interference for the dye ANTS and its quencher DPX as shown in Figure 23.



Figure 23. The fluorescence intensity (a.u.) of 10.1µM ANTS (black) and 10.1µM ANTS with 40.6µM DPX (red) in 100mM NaCl pH 7.4 over increasing concentrations of Pb²⁺ (µM). $\lambda_{Ex} = 347 \pm 5$ nm, $\lambda_{Em} = 518 \pm 5$ nm. Fluorescence intensity was corrected for dilution.

Based on published protocols of LUV encapsulation volumes, ANTS is diluted to 10.1μ M ANTS upon leakage alongside DPX, co-encapsulated at a 1:4 molar ratio¹²⁹. There is a very small negative slope as Pb²⁺ is titrated into the solution of ANTS which is reduced further in the presence of DPX. These results show that the increase in fluorescence obtained when ANTS leaks out of LUVs and is released from DPX, will not be impacted by Pb²⁺. Additionally, if minor quenching is present in this range, it should not affect the percentage of leakage as the calculations use a ratio of intensities from pre- and post – Pb²⁺.

3.3 Interactions of Pb²⁺ with Laurdan

To avoid misinterpreting metal-induced changes of laurdan fluorescence as GP changes, control experiments with laurdan in dimethyl sulfoxide (DMSO) were conducted in 100mM NaCl pH 7.4 in the absence of lipids. The long, acyl tails of laurdan (structure shown in Figure 9) are hydrophobic and interact with the detergent while the dimethylamino head group of the molecule is a potential target for Pb^{2+} in solution. To amplify any potential Pb^{2+} interactions, laurdan was added at a much higher concentration (4.4µM) than the 1:550 molar ratio later used in 500µL of 300µM LUVs. Results are shown in Figure 24.



Figure 24. The fluorescence intensity of 4.4μ M laurdan from 1mM DMSO in 100mM NaCl pH 7.4 over an emission wavelength spectrum (nm) in the absence (black) or presence of 2.1mM Pb²⁺ (red) at 25°C. Each dataset is the average of 3 replicates consisting of 10 scans each.

Results in Figure 24 show that 2.1mM Pb^{2+} does not affect the intensity of 4.4µM laurdan in DMSO in 100mM NaCl pH 7.4 across most of the emission spectrum when excited at 340nm and especially at the emission maximum that is used for GP analysis. These results show that laurdan GP changes presented in this thesis are not artifacts of Pb^{2+} – dye interactions.

3.4 Interactions of Pb²⁺ with Phosphatidylcholine (PC) Membranes

The phosphocholine headgroup of DMPC lipids has already been addressed as a potential target of Pb^{2+} binding through electrostatically driven interactions with the negative phosphate group despite the positive choline extending outwards from the liposomes¹¹⁹. PC lipids have not been targeted by other metals such as Cd^{2+114} , $Hg^{2+142,143}$, Co^{2+} or Ni²⁺¹¹⁵. This distinction of Pb²⁺ may be the result of its small hydrated radius $(4.01\text{ Å})^{33}$ allowing for deeper penetration into the lipid backbone. Additionally, Pb²⁺ is highly electronegative at 2.33 compared to the other metals ranging from 1.69 to 2.00³⁴ as well as unique in the widespread speciation previously discussed in Chapter 1.2 Speciation of Lead in Aqueous Solutions. Targeting a very common lipid within most eukaryotic membranes could explain the widespread toxicity associated with lead poisoning as well.



Figure 25. Fluorescence intensity (a.u.) of 300µM POPC LUVs in 100mM NaCl pH 7.4 encapsulating 11.8mM ANTS and 47.4mM DPX with no additions ("Baseline", black dashed), additions of 100mM NaCl pH 7.4 ("Buffer", blue) or additions of 0 - 10µM Pb²⁺, every 0.5µM/min (red) over time (min). Addition of 10µL 1% Triton X-100 at 21min corresponds to full leakage. Results are the average of 3 replicates \pm SD. $\lambda_{Ex} = 347 \pm 5$ nm, $\lambda_{Em} = 518 \pm 5$ nm.

The "buffer" trials of Figure 25 confirm that any increase in fluorescence obtained through out the titration is not an artifact of liposome shearing. We observe an increase of fluorescence upon Pb^{2+} injections of $0.5\mu M$ every 1min additions. Quantitative results of this increase in fluorescence relative to the initial and final intensities are shown in Figure 26 as the percentage of dye leaked.



Figure 26. Pb^{2+} -induced leakage (%) of aqueous liposomal contents of 300µM POPC LUVs encapsulating 11.8mM ANTS and 47.4mM DPX in 100mM NaCl pH 7.4. Results are the average of 3 replicates ± SD.

The minimal interactions between Pb^{2+} and ANTS from Figure 23 suggest the increase in fluorescence observed upon Pb^{2+} injections is due to membrane perturbations that allow the dye to leak out of the liposomes, not the permeation of PbOH⁺ in. In pure POPC LUVs, 10µM Pb²⁺ causes the leakage of 9% of internal components (Figure 26).



Figure 27. (*top*) GP values of laurdan in LUVs of 0.3mM POPC (black) with 0.5 μ M (green), 0.3mM (blue), and 2.1mM (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM POPC LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M)

Results from POPC showed GP increases when Pb^{2+} was in excess (2.1 mM) which became progressively larger as a function of temperature as illustrated in the contour plot (Figure 27). The contour plots are visual representations of the changing generalized polarization and subsequent membrane fluidity across temperature and Pb^{2+} concentrations. Increased membrane rigidity is shown as red, no changes correspond to green shades and increasing fluidity is demonstrated as blue at the scale given for each lipid system (i.e. -0.010 for POPC). This gradual reduction of the GP curve is indicative of a membrane in the liquid – crystalline phase as POPC has already passed its phase transition of -2°C. The increases in GP are not statistically significant until 30°C, suggesting the membrane must be quite fluid for Pb^{2+} to access the phosphate group and induce LUV rigidification.

3.4.3 DLS of POPC with Pb^{2+}

DLS results for POPC LUVs are shown in Figure 28. DLS size data of Pb²⁺ at 2.1mM suggest statistical significant size changes in the presence of Pb²⁺, especially at higher temperatures. The scale of these changes only suggests a small swelling of the LUVs induced by Pb²⁺. This may be the result of Pb²⁺ interacting with the phosphate group, an increase in water molecular dynamics or enhanced water penetration at the polar interface of the membrane¹¹⁹. Significantly increased count rates are observed throughout the entire curve, suggesting that Pb²⁺ has an ordering effect on the lipids as they typically become more fluid at higher temperatures. This agrees with the rigidification observed with laurdan GP, Pb²⁺ binds POPC and changes the packing arrangement causing a higher DLS photon count into the detector throughout the temperature scale.



Figure 28. DLS determination of the count rate $(x10^4 \text{ kcps})$ (solid) and vesicle diameter size (nm) (dashed) of 0.3mM POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Data normalized using non-linear Boltzmann regression. Asterisks denote statistical significance (p < 0.05).



Figure 29. (*top*) GP values of laurdan in LUVs of 0.3mM DMPC (black) with 0.5 μ M (green), 0.3mM (blue), and 2.1mM (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM DMPC LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).
A direct comparison of Figure 27 and Figure 29 shows that the effects of Pb^{2+} are stronger in DMPC than POPC. The structural difference between these LUVs are the acyl tails and the monounsaturated PO- lipids are more fluid than the fully saturated DM- lipids due to the presence of the double bond causing steric hindrance for tight packing. DMPC likely provides a tighter coordination pocket for the small Pb^{2+} ions to interact with the phosphate group for preferential binding. Statistically significant rigidification of DMPC is observed at ratios as low as 1:1 Pb^{2+} /lipid (0.3 mM) (Figure 29).

Suwalsky *et al.*, observed a minor fluidization of this membrane below the T_m (18°C) with 1 mM Pb²⁺ (Δ GP of -0.006) which was not observed in Figure 29. During the gel phase, predominantly 2.1 mM Pb²⁺ induced rigidification which continued into the liquid – crystalline phase which is defined as a more fluid, disordered state of the membrane. Increased GP above the T_m (37°C) was also noted by Suwalsky *et al.*, for all Pb²⁺ concentrations above 0.1mM¹¹⁹. The T_m at 24.4°C observed here with 100mM NaCl pH 7.4 is in agreement with phase transitions observed by differential scanning calorimetry (DSC) in 10mM sodium phosphate buffer, pH 7.0 at 23.9°C¹⁴⁴. Chapman *et al.*, observed an increase in the transition temperature of DMPC with 1M lead acetate to 24.6°C compared to 23.6°C in water alone¹⁴⁵. The T_m of 2.1mM Pb²⁺ obtained in Figure 29 was 24.9°C. The contour plot of Figure 29 displays increased GP rigidification predominantly taking place at higher Pb²⁺ concentrations and lower temperatures.

3.4.5 DLS of DMPC with Pb^{2+}



Figure 30. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM DMPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All curves of Pb²⁺-treated LUVs were statistically significant (p < 0.05) from untreated.

We continue to observe enhanced effects of Pb^{2+} on DMPC over POPC by DLS when we compare Figure 30 to Figure 28. The larger swelling of LUV size from approximately 116 to 124nm indicates a structural disturbance of the lamellar organization of the membrane throughout the gel to liquid – crystalline phases.

The ordering effect of Pb^{2+} is also precedent throughout the count rate curve however we are seeing an increased T_m shift in with Pb^{2+} to 26.3°C as opposed to 24.9°C with laurdan GP. Two potential reasons could explain this difference: laurdan fluorescence is determined largely by water access causing a red shift in its emission spectra. If Pb^{2+} increases water penetration for DMPC, the emission spectra of laurdan may shift towards 490nm providing a drop in GP corresponding to the sigmoidal phase transition prior to the intrinsic transition induced by Pb^{2+} directly. A second possible explanation considers how significant changes to the optical properties correspond to a change in the membrane packing where well-organized gel phases scatter less light than fluid membranes. Since GP and DLS results suggest Pb^{2+} binding at temperatures as low as 18° C, there may be a Pb^{2+} – lipid complex coordination which holds the lipids in a preferential Pb^{2+} – bound orientation. This would delay the phase transition until the temperature is high enough for the system to overcome the bound state and melt into a more fluid phase. As a result, we observe a larger shift in the T_m to higher temperatures.

3.4.6 Laurdan GP of PCplasm with Pb^{2+}

PC plasmalogens are the final glycerol-based PC lipid studied. It has a unique enol – ether linkage instead of the esters found in DM- or POPC lipids. Plasmalogens are common in complex lipid profiles such as the heart or brain membranes^{78,95,98}. Diaz and Monreal compared the ability of Pb²⁺ to target the enol-ether bond of plasmalogens to mercury, a known interaction by Hg²⁺ resulting in the irreversible cleavage of the lipid¹⁰³. High performance thin layer chromatography (HPTLC) showed no metal induced alterations in the mobility of any lipids or additional spots relative to metal free controls indicating that Pb²⁺ did not induce break down of the lipids¹⁰³. PC plasmalogens were formulated into LUVs to screen for altered Pb²⁺ interactions.



Figure 31. (*top*) GP values of laurdan in LUVs of 0.3mM PC plasmalogen (black) with 0.5 μ M (green), 0.3mM (blue), and 2.1mM (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM PC plasm LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).

In agreement with Diaz and Monreal¹⁰³, GP results did not show any damage to the plasmalogen structure as observed for Hg^{2+} under similar experimental conditions¹¹⁴. There appears to be a sharp increase in GP between 5 - 15°C suggesting there may be binding of Pb²⁺ to the gel phase and shifting the phase transition. The expected T_m of this PC plasmalogen based on published DSC measurements is $5.1^{\circ}C^{146}$ however fluorimeter scans are restricted to a 5°C minimum temperature. No change in GP is detected in the liquid – crystalline phase.

3.4.7 DLS of PCplasm with Pb^{2+}

DLS results for PCplasm LUVs are shown in Figure 32. PC plasmalogen LUVs exhibited an average 20nm increase in diameter size in the presence of 2.1mM Pb²⁺. The count rate data contained a lot of variance between replicates from $5 - 30^{\circ}$ C, but the general trend of the curve shows Pb²⁺ interactions with the lipids to induce tighter packing, and thus a higher count rate signal.



Figure 32. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM PCplasm LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Data normalized using non-linear Boltzmann regression. Results are the average of 3 replicates \pm SD. All the size Pb²⁺ curve is statistically significant, asterisks denote significance for the count rate Pb²⁺ curve (p < 0.05).

3.4.8 Discussion of Pb^{2+} Interactions with PC Membranes

PC lipids account for more than 50% of the phospholipids in most mammalian cell membranes⁶⁹. Their ability to spontaneously assemble into stable bilayer structures allows for the inclusion of non-bilayer forming lipids into simple and complex biomimetic mixtures which will be addressed in Chapter Four: Simple Lipid Mixtures.

Several other metals do not interact with PCs including Cd^{2+114} , $Hg^{2+142,143}$, Co^{2+} and Ni²⁺¹¹⁵. Suwalsky *et al.*, reported a main disordering effect of Pb²⁺ on DMPC by laurdan GP as well as X-ray diffraction studies that noted a remarkable decrease in phospholipid low angle reflection intensities suggesting Pb^{2+} induced disordering of the polar head group region of the lipids¹¹⁹. The capability of Pb^{2+} to bypass the positively charged choline located in the head group can likely be explained by its small hydrated radius of 4.01Å³³. Another parameter to consider is speciation, Pb^{2+} does not remain entirely divalent¹⁴¹. It cannot be discounted that the chloride and hydroxide species formed by Pb^{2+} may have unique effects on their own and potentially allow for the coordination with water molecules at the surface of the membrane. It was previously mentioned that the average electronegativity of Pb^{2+} is high at 2.33 which may explain its targeting of the phosphate group in the polar interface despite choline or other bulky headgroups limiting access.

Within the PCs studied, Pb^{2+} had the strongest interactions with DMPC in the liquid – crystalline phase inducing a total shift in T_m by 0.5°C. While the acyl tails are not direct Pb^{2+} targets, they affect the fluidity and lamellar organization of the membrane which changes the packing density around the phosphate linker.

Pb²⁺ does not induce cleavage of plasmalogens as observed for the heavy metal mercury¹⁴². Increased GP and DLS count rate data suggests binding to this lipid does occur, especially at lower temperature, comparable with the other PC lipids.

3.5 Interactions of Pb²⁺ with Sphingomyelin (SM) Membranes

Sphingomyelin was the next zwitterionic lipid selected for testing due to the continued presence of a phosphocholine headgroup. The sphingosine backbone imparts a large degree of membrane rigidity due to the strong hydrogen bonding network induced by the amide linkage within the polar interface of the membrane⁸⁴. Differences in the fatty

acid tail of these lipids will be compared between a defined palmitoyl (16:0) SM to a natural lipid brain SM mixture with a fatty acid distribution as shown in Figure 33. As the glycerol-based PCs and SM share the phosphocholine head group, differences in Pb²⁺-induced fluidity may arise from targeting to the sphingosine backbone.



Figure 33. The fatty acid distribution found in the natural lipid mixture of Brain SM from Avanti Polar Lipids¹⁴⁷.

3.5.1 Laurdan GP of PSM with Pb^{2+}

GP results of palmitoyl sphingomyelin are shown in Figure 34. Exposure of LUVs containing PSM to 2.1mM Pb^{2+} resulted in an increase in membrane rigidity compared to the control throughout the entire temperature scale. The largest increase occurred at the T_m which was shifted from 40.2°C to 41.0°C.



Figure 34. (*top*) GP values of laurdan in LUVs of 0.3mM PSM (black) with 0.5 μ M (green), 300 μ M (blue), and 2.1mM (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM PSM LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).



Figure 35. Dynamic light scattering (DLS) determination of the derived count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM PSM LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Data normalized using non-linear Boltzmann regression. Results are the average of 3 replicates \pm SD. Asterisks denote statistical significance (p < 0.05).

DLS experiments conducted on PSM LUVs exhibited reduced count rates at 2.1mM Pb^{2+} compared to control tests. The lack of statistically relevant size change during the entire temperature region suggests that Pb²⁺ interactions are disordering the membrane without swelling LUV size.



Figure 36. *(top)* GP values of laurdan in LUVs of 0.3mM brain SM (black) with 0.5 μ M (green), 300 μ M (blue), and 2.1mM (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). *(bottom)* Contour plot of the change in GP for 0.3mM brain SM LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).

The phase transition of brain SM is very broad due to the large distribution of lipid fatty acyl tails within the LUVs. The high T_m of 39.2°C allowed the observation of Pb²⁺-induced rigidification throughout the gel phase region. As a result, there is a slight reduction in T_m to 38.1°C whereas the liquid – crystalline phase of the membrane was unaffected.

3.5.4 DLS of Brain SM with Pb^{2+}



Figure 37. Dynamic light scattering (DLS) determination of the derived count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM Brain SM LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Data normalized using non-linear Boltzmann regression. Results are the average of 3 replicates \pm SD. Asterisks denote statistical significance (p < 0.05).

DLS results support the observed rigidification of Brain SM LUVs throughout the gel phase. Pb²⁺ binding appears to be enhanced for ordered lipids as less interactions can

be detected after the phase transition as shown in Figure 36 and Figure 37.

3.5.5 Discussion of Pb^{2+} Interactions with SM Membranes

It is important to assess the impact of Pb^{2+} on SM membranes as these lipids also are main components of eukaryotic membranes such as erythrocytes and the myelin sheath^{69,95,98,138}.

 Pb^{2+} induced changes in membrane fluidity and lipid packing were higher for SM lipids relative to glycerol-based PC lipids. This general increase in GP can be explained by the tighter packing amongst sphingosine backbones due to an intrinsic hydrogen bonding network which may induce a higher affinity for Pb^{2+} interactions.

3.6 Interactions of Pb²⁺ with Phosphatidylserine (PS) Membranes

The first anionic lipid class that was studied were PS lipids to assess how the positioning of the serine carboxyl group affects Pb^{2+} – lipid interactions. The serine carboxyl group is separated from the phosphate linker by the presence of a positively charged primary amine within the head group giving this lipid class an overall charge of - 1.0. The monounsaturated POPS is also the second lipid class that was included in fluorescence leakage assays in addition to laurdan GP and DLS experiments with the fully saturated DMPS.

3.6.1 Leakage Assays of POPS LUVs with Pb²⁺

The control trials of "buffer" and baseline fluorescence scans were also completed for POPS leakage and showed no change to ANTS fluorescence over time, comparable with Figure 25 for POPC (POPS data not shown). The % leakage was calculated, and results are shown in Figure 38.



Figure 38. Pb^{2+} -induced leakage (%) of aqueous liposomal contents of 300µM POPS LUVs encapsulating 11.8mM ANTS and 47.4mM DPX in 100mM NaCl pH 7.4. Results are the average of 3 replicates ± SD.

Pb²⁺ induced up to 18% leakage of the internal contents of POPS liposomes. The significant increase from 9% leakage for POPC (Figure 26) clearly suggests additional Pb²⁺ targeting of the serine head group.

3.6.2 Laurdan GP of POPS with Pb^{2+}

The interactions between POPS LUVs and Pb^{2+} were strong enough to induce aggregation of vesicles starting at 100µM Pb^{2+} (1:3 molar ratio with 300µM LUVs in solution). An example of this aggregation is shown in Figure 39. Pb^{2+} incubations were only completed up to 75µM and GP results are shown in Figure 40.



Figure 39. 300μ M POPS LUVs in 100mM NaCl pH 7.4 with 600μ M Pb²⁺ and photographed immediately after Pb²⁺ addition to the cuvette.



Figure 40. (*top*) GP values of laurdan in LUVs of 0.3mM POPS (black) with 0.5 μ M (green), 30 μ M (blue), and 75 μ M (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM POPS LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).

The T_m of POPS LUVs at 14.7°C agrees with DSC experiments in 5mM phosphate 5mM EDTA pH 7.0 reported as 13.9°C¹⁴⁸. The shift to a slightly higher temperature is likely due to the presence of monovalent Na⁺ under these salt conditions as ion-induced shielding of the negatively charged PS surface depends on ionic strength¹⁴⁸. The T_m upon addition of 75µM Pb²⁺ was increased up to 17.6°C. At Pb²⁺ – lipid ratio as low as a 1:10, 30µM and 75µM Pb²⁺ induced significant GP changes at 0.062 and 0.10 at 18°C, which is significantly higher than any changes observed for PCs in Section 3.4 Interactions of Pb2+ with Phosphatidylcholine (PC) Membranes. The rigidification induced by Pb²⁺ only occurs once the lipids enter the phase transition when the phosphate linker may become more accessible for Pb²⁺ binding at the membrane interface as there is a higher probability of Pb²⁺ coordinating the extended negative carboxyl group from the serine head group. As the lipids become more fluid at higher temperatures, the phosphate within the polar interface of membranes becomes more accessible and Pb²⁺ induces considerable structural disturbances to the membrane.

3.6.3 DLS of POPS with Pb^{2+}

DLS results for POPS LUVs are shown in Figure 41. DLS results of POPS LUVs agree with the laurdan GP data. POPS lipids exhibited a Tm of 14.2°C which was increased to 18.3°C in the presence of 75μ M Pb²⁺. Minimal changes in fluidity or lipid packing were observed until the lipids began melting into the liquid – crystalline phase. In terms of LUV sizing, a shift upwards of a 20% size increase was observed throughout the temperature scale, confirming Pb²⁺ binding pre- and post-transition.



Figure 41. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM POPS LUVs (black) with 75 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Data normalized using non-linear Boltzmann regression. Results are the average of 3 replicates ± SD. All the size Pb²⁺ curve is statistically significant, asterisks denote significance for the count rate curve (p < 0.05).

3.6.4 Laurdan GP of DMPS with Pb^{2+}

The interactions of Pb^{2+} with DMPS were even stronger than with POPS. Noticeable aggregation of LUVs caused immediate precipitation from solution when 75µM Pb^{2+} were added to the cuvette. Significant changes to the T_m of DMPS were detected at a Pb^{2+} – lipid molar ratio of 1:600 thus determining to test lower concentrations of Pb^{2+} down to 1:3000 Pb^{2+} per lipid molecule.

GP data for DMPS LUVs are shown in Figure 42.



Figure 42. (*top*) GP values of laurdan in LUVs of 0.3mM DMPS (black) with 0.1 μ M (green) and 0.5 μ M Pb²⁺ (blue) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM DMPS LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).

DMPS membranes have a sharp T_m at 37.6°C which agreed with DSC values of 40.2°C in 5mM Tris-HCl + 10mM CaCl₂ pH 7.0¹⁴⁸. The GP results seem to suggest Pb²⁺ may bind but shows no effect in the gel phase however delayed the DMPS melting to a higher temperature of 39.7°C which also maintained the sharp sigmoidal transition. Changes to GP are centered around the T_m and no effect is detected before or after.





Figure 43. DLS determination of the count rate $(x10^4 \text{ kcps})$ (solid) and vesicle diameter size (nm) (dashed) of 0.3mM DMPS LUVs (black) with 0.5µM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Data normalized using non-linear Boltzmann regression. Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05).

The phase transition of DLS results agree with laurdan GP T_m of 37.9°C and 40.5°C with 0.5µM Pb²⁺. Interestingly, no detectable change in LUV size was noted at any temperature scanned. The Pb²⁺ concentration may be too low to induce size changes.

3.6.6 Discussion of Pb^{2+} Interactions with PS Membranes

PS lipids contain two negative charges, one close to the membrane backbone on the phosphate linker and a second at the serine carboxyl group that extends outwards and is more removed from the hydrophobic core. The analysis of membrane fluidity becomes complicated due to potentially different binding preferences for one charge site over the other due to differences in charge localization and Pb^{2+} accessibility.

The interactions of Pb^{2+} on PS lipids were significantly more pronounced than with PC or SM. There was also an increased effect on DMPS after the phase transition compared to the more fluid POPS lipids. The tighter packing of fully saturated lipids continues to provide a better coordination for Pb^{2+} binding over monounsaturated lipids.

The lack of perturbations of PS lipids during the gel phases suggests that Pb^{2+} binding to the serine head group does not affect membrane fluidity or the lipid packing arrangements. In both POPS and DMPS, changes were observed upon the onset of the phase transition into the liquid-crystalline phase and T_m 's were significantly shifted upwards. The binding of Pb^{2+} to PS lipids will also be compared to phosphatidylethanolamine (PE) lipids that have structural similarity to PS but lack the serine carboxyl group in Chapter Four: Simple Lipid Mixtures.

PS is an important structural lipid found preferentially in the inner membrane of eukaryotic membranes that is important in different signaling pathways^{40,91,92,140}. PS directs proteins to the membrane to carry-out their function, such as protein kinase C (PKC). Several studies have reported Pb²⁺ causing improper activation of PKC^{60,67,149} due to a higher binding affinity than its natural cofactor, Ca²⁺. These studies also include Morales *et al.*, who reported Pb²⁺ – PS associations which competed for membrane binding sites of

the peripheral membrane binding domain of PKC, $C2\alpha^{60}$. Other reports of protein – mediated lipid oxidation studies have linked enhanced Pb^{2+} induced effects to the PS concentration^{36,150}.

3.7 Interactions of Pb²⁺ with Phosphatidic Acid (PA) Membranes

Screening the zwitterionic phosphocholine-containing lipids has suggested the targeting of Pb^{2+} to the phosphate group at the membrane polar interface. These effects were increased when the attached headgroup also contained a negative charge as seen for PS. To better understand the effect of the charge localization in negatively charged lipids, the structurally most simple class was investigated, which only contained a phosphate group. This results in a negative binding target for Pb^{2+} that is exposed and readily accessible from the bulk solution.

3.7.1 Laurdan GP of POPA with Pb^{2+}

GP results for LUVs composed of POPA are shown at the top of Figure 44. In contrast to earlier systems, extensive fluidization was obtained throughout the gel phase. The sigmoidal phase transition centering around 23° C was abolished for 0.3 and 2.1mM Pb²⁺ incubations. The relative GP indicated strong rigidification throughout the liquid – crystalline phase. Other metals such as Cd²⁺ under 100mM NaCl pH 7.4¹¹⁴ and Co²⁺ or Ni²⁺ with 20mM HEPES 100mM NaCl pH 7.4¹¹⁵ have also induced large increases in GP and T_m of POPA with increasing temperatures and metal concentrations. Two potential reasons could explain this fluidization: the small Pb²⁺ hydrated radius allows for deep penetration into the backbone, potentially reducing the packing order of the membrane. As the temperatures increases past the T_m of controls, preferential Pb²⁺ binding maintains the order of the lipids which holds the level of GP as seen in Figure 44. A second reason to

distinguish Pb^{2+} from these other metals would be to consider the metal speciation, of which Pb^{2+} is the only metal to contain hydroxide species.

A comparison was performed with POPA LUVs formulated in 100mM NaCl at pH 6.4. The PA head group has two ionizable protons with $pKa_1 = 3.0$ and $pKa_2 = 8.0$ in 100mM NaCl^{151,152}. Therefore, the relative charge of PA head groups drops to -1.00 at pH 6.4 from -1.20 at pH 7.4. Additionally, the percent ionization of the PA head group second proton changes from 20% to approximately 5%¹⁵¹. As discussed in Section 3.1 Speciation of Pb²⁺ in Experimental Conditions, Pb²⁺ species will also change with pH adjustments. At pH 6.4 PbOH⁺ is reduced from 13.8% to 1.6% in favour of Pb²⁺ (36.4% from 31.9%) and PbCl⁺ (54.2% from 47.4%). Additional small hydroxide species are completely reduced to zero. GP results for LUVs composed of POPA at pH 6.4 are shown at the bottom of Figure 44.



Figure 44. GP values of laurdan in LUVs of 0.3mM POPA (black) with 0.5 μ M (green), 75 μ M (yellow), 300 μ M (blue), or 2.1mM (red) Pb²⁺ in 100mM NaCl pH 7.4 (*top*) or pH 6.4 (bottom) as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05).

The effects of Pb^{2+} on POPA were enhanced at pH 6.4. Most notably, Pb^{2+} -induced aggregation and LUV precipitation occurred at concentrations exceeding 300µM. A direct comparison of the change in GP is well illustrated in the contour plots of Figure 45.



Figure 45. Contour plot of the change in GP for 0.3mM POPA LUVs at pH 7.4 (left) and 6.4 (right) across increasing temperature (°C) and concentrations of Pb^{2+} (μ M).

POPA LUVs at pH 7.4 did not exhibit rigidification until 2.1mM Pb²⁺ which has been omitted from the contour plots for equal comparison across the same concentration range. The upper concentration of 300μ M induced stronger fluidization at colder temperatures and rigidification above 30° C.

The enhanced effect of Pb²⁺ at pH 6.4 suggests PbOH⁺ is not the dominant Pb²⁺ species causing these membrane disruptions. This correlates with the study of Pb²⁺ permeation in myelin lipid liposomes by Diaz and Monreal determining that PbOH⁺ can undergo protein-independent, passive diffusion across membranes¹⁰³. Instead, Pb²⁺ or PbCl⁺ species are targeting the phosphate groups of PA lipids, and likely other systems as well.



Figure 46. Dynamic light scattering (DLS) determination of the derived count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM POPA LUVs (black) with 0.3mM Pb²⁺ (red) in 100mM NaCl pH 7.4 (top) or pH 6.4 (bottom) as a function of temperature (°C). Data normalized using non-linear Boltzmann regression. Results are the average of 3 replicates \pm SD. All Pb²⁺ curves are statistically significant (p < 0.05).

Results from DLS experiments with POPA at pH 7.4 and 6.4 indicated significant increases in LUV sizes and complete abolishment of the phase transition seen in the control runs. Laurdan GP and DLS results both report on a strong interaction between Pb²⁺ and the negatively charged PA headgroup, which resulted in the complete change of the lamellar organization within the membrane.

3.7.3 Laurdan GP of DMPA with Pb^{2+}

As fully saturated lipids have displayed stronger effects within the same lipid type over monounsaturated lipids, DMPA LUVs were tested at pH 7.4 and 6.4 to perform the same comparisons of the impact of Pb²⁺ on membrane fluidity, size, and phase transitions. GP results of DMPA LUVs at pH 7.4 and 6.4 are shown in Figure 47.



Figure 47. GP values of laurdan in LUVs of 0.3mM DMPA (black) with 0.5 μ M (green), 75 μ M (blue), and 300 μ M (red) Pb²⁺ in 100mM NaCl pH 7.4 (top) or pH 6.4 (bottom) as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05).

The fluidization of DMPA LUVs at lower temperatures and rigidification above the T_m was also enhanced for pH 6.4 over 7.4 as seen with POPA (Figure 44). The comparison of the change in GP is best described by the contour plots in Figure 48.



Figure 48. Contour plot of the change in GP for 0.3mM DMPA LUVs at pH 7.4 (left) and 6.4 (right) across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).

Interestingly, Pb²⁺ affects POPA stronger in the gel phase whereby the scale of Δ GP approached -0.15 for both pH 7.4 and 6.4 experiments. DMPA exhibited weaker fluidization in favour of rigidification past the T_m with Δ GP scales of +0.135 for pH 7.4 and +0.18 for 6.4. Acyl tail saturation continues to play a role in Pb²⁺ binding however a different trend arises. In previous systems, DM- lipids have provided a tighter packing coordination for Pb²⁺ in the gel phase but for PAs, the negative charge is readily accessible. The fact that Pb²⁺ can fluidize the gel phase of POPA and not DMPA suggests a spatial limitation where the larger area provided by monounsaturated POPA is better suited for metal complexation. DMPA lipids begin to spread out upon heating and may enter the required spatial arrangement for improved coordination at temperatures throughout the liquid – crystalline phase.

The lower relative charge of -1.20 compared to -1.00 for PA at pH 6.4 reduced the lipid repulsion amongst head groups. The enhanced binding of Pb^{2+} at pH 6.4 may occur due to reduced ion-shielding by Na⁺ in 100mM NaCl conditions at the surface of the membrane.

3.7.4 DLS of DMPA with Pb^{2+}

DLS results for DMPA LUVs at pH 7.4 and 6.4 are shown in Figure 49. Larger increases in LUV sizes were present for DMPA than POPA at both pH values. Once again, DLS results suggest complete abolishment of a phase transition.



Figure 49. Dynamic light scattering (DLS) determination of the derived count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM DMPA LUVs (black) with 0.3mM Pb²⁺ (red) in 100mM NaCl pH 7.4 (top) or pH 6.4 (bottom) as a function of temperature (°C). Data normalized using non-linear Boltzmann regression. Results are the average of 3 replicates \pm SD. All Pb²⁺ curves are statistically significant (p < 0.05).

3.7.5 Discussion of Pb^{2+} Interactions with PA Membranes

With no positive or bulky head group blocking access of Pb^{2+} to the phosphate group found in PAs resulted in significant binding. The phosphate group is considered a strong target as Pb^{2+} confirmed by that fact that this metal forms an insoluble precipitate with phosphate¹⁵³.

Pb²⁺ clearly affected DMPA more than POPA as documented by the enhanced fluidization and rigidifications obtained across the temperature range. Furthermore, larger changes in the size and fluidity occurred across all systems tested.

PA lipids are minor components of different membranes but are critical for signaling pathways^{69,154}. Strong binding of Pb²⁺ will have consequences on several PA secondary messenger activities such as the activation of PI(4)P-5-kinases¹⁵⁵ although no literature data are available on the interactions of Pb²⁺ with pure PA bilayers or direct PA interactions.

3.8 Interactions of Pb²⁺ with Cardiolipin (CL) Membranes

Cardiolipin is a major constituent of mitochondrial membranes⁹² at 10 - 20 mol% of the total lipids¹⁵⁶ and preferentially associated with proteins that conduct oxidative phosphorylation¹⁵⁷ but is absent from most other organelles. The mitochondria has been found as a target site for Pb²⁺ through binding to the internal metal binding site of the mitochondrial permeability transition pore, which initiates a cascade of apoptotic events¹⁴.

This lipid class is also structurally-similar to two PA lipids linked by a glycerol molecule. The defined phosphate to phosphate distance within each CL lipid reduces the surface charge density as they cover a larger area per lipid molecule with its four acyl chains. The interactions of Pb^{2+} with the more fluid TOCL and the more rigid TMCL were

studied using laurdan GP and DLS to compare phosphate accessibility against surface charge density between CL and PA membranes.

3.8.1 Laurdan GP of TOCL with Pb^{2+}

GP results for TOCL LUVs are shown in Figure 50. The T_m of TOCL is below 0°C thus comparisons can only be drawn on the binding throughout the liquid – crystalline phases. Pb^{2+} induces significant rigidification across the temperature scale. No apparent saturation of Pb^{2+} has been reached for binding TOCL at the concentrations measured. The maximum change in GP in this liquid – crystalline phase is much higher with TOCL (+0.25) than POPA (+0.0875, 2.1mM Pb^{2+} at pH 7.4).



Figure 50. (*top*) GP values of laurdan in LUVs of 0.3mM TOCL (black) with 0.5 μ M (green), 75 μ M (yellow), 300 μ M (blue) and 2.1Mm (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM TOCL LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).



Figure 51. Dynamic light scattering (DLS) determination of the derived count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 300 μ M TOCL LUVs (black) with 75 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Data normalized using non-linear Boltzmann regression. Results are the average of 3 replicates \pm SD. All the Pb²⁺ curves are statistically significant (p < 0.05).

Despite the significant increase in GP, running DLS at 75μ M Pb²⁺ indicated a significant decrease in LUVs size. The offset in count rate seen for TOCL was also present in the liquid – crystalline phases of POPA and DMPA trials. While we cannot observe the gel phase, Pb²⁺ might be binding in a similar pattern to PA lipids. TOCL contains 4 monounsaturated acyl chains in addition to defined phosphate – phosphate surface charge density causing a fluid membrane. Pb²⁺ binding preferences from previous lipid studies have described tighter packing suggesting coordination of Pb²⁺ with the phosphates of CL

resulting in closer lipid interactions. This explains the significantly reduced LUV size observed by DLS. Additionally, tighter packing of CL lipids limits the water penetration to the fluorescent moiety of laurdan, restricting the red-shift of the emission, which is reflected in the GP values as shown in Figure 50.

3.8.3 Laurdan GP of TMCL with Pb^{2+}

GP results for TMCL LUVs are shown in Figure 52. TMCL lipids observed a T_m of 39.9°C which was significantly shifted up to 52.5°C at 300µM Pb²⁺. The rigidification observed throughout the liquid – crystalline phase can be compared to Cd where a similar T_m and GP increase was reported¹¹⁴. Pb²⁺ induced over 3-fold higher shifts in T_m at equivalent concentrations and the total increase in GP for 300µM Pb²⁺ is comparable to 2.0mM Cd. Additionally, there is a significant fluidization detected in the gel phase of TMCL by 300µM Pb²⁺ suggesting similar interactions as with PAs.



Figure 52. (*top*) GP values of laurdan in LUVs of 0.3mM TMCL (black) with 0.5 μ M (green), 75 μ M (red), and 300 μ M (blue) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM TMCL LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).
3.8.4 DLS of TMCL with Pb^{2+}



Figure 53. Dynamic light scattering (DLS) determination of the derived count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 300 μ M TMCL LUVs (black) with 75 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Data normalized using non-linear Boltzmann regression. Results are the average of 3 replicates \pm SD. All the count rate Pb²⁺ curve is statistically significant, asterisks denote significance for the size Pb²⁺ curve (p < 0.05).

Since TMCL has a detectable T_m , we can observe changes to the size starting in the gel phase. Interestingly, no change in LUV size was detected during the temperature region of the phase transitions of the controls but there is an increase before and after this range. The entire phase transition is profoundly shifted and broadened as seen in TOCL and PA curves. The T_m induced by 75µM Pb²⁺ that was recorded by DLS was 43.2°C, which is comparable to the 44.1°C for the same Pb²⁺ concentration investigated by laurdan GP.

The interactions of 300 μ M Pb²⁺ were stronger with TMCL than 2.1 mM metal and TOCL as reported by laurdan GP (results shown in Figure 50 and Figure 52, respectively). Following the logic provided previously for TOCL, TMCL contains fully saturated acyl tails which can pack tighter due to stronger van der Waals interactions⁸⁴. The small Pb²⁺ radii might facilitate their penetration into the backbone at low temperatures causing the LUVs to swell in size and potentially induce fluidization at the highest Pb²⁺ concentrations tested during the gel phase (Figure 52). As the temperature increases, Pb²⁺ binding maintains the lipid ordering causing a broadening in T_m and a delay in the overall phase transition. At higher temperatures, as the Pb²⁺-bound CL lipids begin to melt into the liquid – crystalline phase which is defined as a more fluid, disordered state of the membrane.

3.8.5 Discussion of Pb^{2+} Interactions with CL Membranes

The separation of two phosphatidyl groups by a glycerol within the CL structures (structures shown in Figure 5) lessens adjacent negative charges and reduces the overall surface charge density when compared to PA membranes which may play a role in Pb^{2+} binding. The overall effect of Pb^{2+} on CL lipids was stronger than for PA lipids in the liquid – crystalline phases after T_m however little to no fluidization was noted in the gel phases. This is consistent with the larger molecular area per lipid for CL, Pb^{2+} cannot disturb the structural packing of CL as much as in the tighter packed PA.

Smaller changes in LUV sizes for CL compared to the striking increases with PA lipids suggest that CLs are more resistant to aggregation due to the larger area and reduced surface charge density. These two properties effectively limit the ability of Pb²⁺ to complex multiple CL head groups at once.

3.9 Interactions of Pb²⁺ with Phosphatidylglycerol (PG) Membranes

PG lipids were the last pure anionic lipid studied in this Chapter. They were selected to evaluate if steric hindrance by a bulky, polar glycerol headgroup interfered with Pb²⁺ binding to the phosphate group. Experiments were conducted with the more fluid PO- and the rigid DM- forms of PG lipids to elucidate Pb²⁺'s ability to affect membrane fluidity, LUV size and the phase transition of the lipid system.

3.9.1 Laurdan GP of POPG with Pb^{2+}

GP results for POPG LUVs are shown in Figure 54. As seen for POPC and TOCL, no phase transition is detectable for POPG due to a T_m at $-3^{\circ}C^{158}$. Significant rigidification was observed throughout the liquid – crystalline phase of POPG comparable to TOCL and POPA suggesting similar accessibility for Pb²⁺ (Δ GP at 50°C: +0.17 for POPG to +0.235 with TOCL and +0.19 for POPA).



Figure 54. (*top*) GP values of laurdan in LUVs of 0.3mM POPG (black) with 0.5 μ M (green), 300 μ M (blue), and 2.1mM (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± standard deviation. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM POPG LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).

3.9.2 DLS of POPG with Pb^{2+}



Figure 55. Dynamic light scattering (DLS) determination of the derived count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM POPG LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Data normalized using non-linear Boltzmann regression. Results are the average of 3 replicates \pm SD. All Pb²⁺ curves are statistically significant except for the size at 5°C (p < 0.05).

Large increase in the count rate of POPG is very similar to the results obtained for CL and PA lipids. Collectively, the increased GP rigidification, count rates, and LUV sizing suggested that the glycerol headgroup did not block Pb²⁺ binding.

3.9.3 Laurdan GP of DMPG with Pb^{2+}



Figure 56. (*top*) GP of laurdan in LUVs of 0.3mM DMPG (black) with 0.5 μ M (green), 15 μ M (blue), 45 μ M (yellow) and 75 μ M (red) Pb²⁺ in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. 15 - 75 μ M Pb²⁺ are statistically significant (p < 0.05). (*bottom*) Contour plot of the change in GP for 0.3mM DMPG LUVs across increasing temperature (°C) and concentrations of Pb²⁺ (μ M).

The T_m of DMPG was determined at 23.4°C which agrees with previous DSC studies reporting 23.5°C using 10 mM HEPES 2 mM NaCl pH 7.4¹¹⁰. The T_m with 75 μ M Pb²⁺ was already completely abolished whereas 45 μ M already induced shifts to 28.1°C. The strongest rigidification for any zwitterionic or anionic lipid occurred for DMPG whereby the maximum concentration of Pb²⁺ tested was a molar ratio of 1:4 Pb²⁺ – lipid. Unlike TMCL, the GP of DMPG was increased in the gel phase and the liquid – crystalline phase.

The shift of +4.6°C for the T_m of DMPG was induced by 45 μ M Pb²⁺ at only a 0.15 metal – lipid molar ratio, which can be compared to a +5.8°C increase by Cd¹¹⁴, +5.7°C by Co and +2.0°C by Ni at 1:1 ratios¹¹⁵.

3.9.4 DLS of DMPG with Pb^{2+}



Figure 57. Dynamic light scattering (DLS) determination of the derived count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 300 μ M DMPG LUVs (black) with 75 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Data normalized using non-linear Boltzmann regression. Results are the average of 3 replicates \pm standard deviation. All the count rate Pb²⁺ curve is statistically significant, asterisks denote significance for the size Pb²⁺ curve (p < 0.05).

Strong Pb^{2+} interactions with DMPG lipids induced significant rigidification at low metal concentrations without liposome aggregation. Therefore, the glycerol head group does not block Pb^{2+} interactions, it may instead provide additional coordination targets of Pb^{2+} with the oxygens present in the polar glycerol head group. An optimal binding pocket with Pb^{2+} inside the membrane polar interface would explain strong metal interactions.

The initially raised count rate throughout the gel phase was expected based on DLS

results for TMCL and no fluidization was reported in the gel phases by GP. At the temperature where the control DMPG begins to melt, DMPG with Pb^{2+} exhibited an evident drop in count rates that was maintained at all higher temperatures. Detection of phase transitions by measuring count rates are based on changes of the optical properties corresponding to membrane packing. Well organized gel phases scatter less light then less organized fluid membranes. Pb^{2+} coordination of both phosphate and glycerol regions could induce membrane disorder at higher temperatures.

3.9.5 Discussion of Pb^{2+} Interactions with PG Membranes

In PCs, we observed some Pb²⁺ binding to the phosphate group despite a positively charged choline head group potentially blocking access. When this phosphate group was freely accessible in PA lipids, Pb²⁺ strongly interacted with this negative charge causing fluidizing and rigidifying effects on the surrounding membrane. The PG lipids were tested to observe if a different head group would also potentially block access to the negatively charged phosphate.

Results for PG lipids definitively showed that the polar glycerol head group did not prevent binding of Pb^{2+} to the phosphate. The high shifts in T_m suggest the formation of tight binding pockets and coordination of multiple phosphates. No other studies of Pb^{2+} and PG interactions have been reported to date.

3.10 Summary of Pb²⁺ Interactions with Primary Lipid Compositions

Figure 58 provides a comparison of the effect of different Pb²⁺ concentrations on each lipid system within this chapter at temperatures corresponding to the gel or liquid-crystalline phases of each specific lipid.



Figure 58. The change in laurdan GP at a temperature within the gel phase (blue) or liquid-crystalline phase (red) when exposed to different concentrations of Pb^{2+} as shown for each lipid system within Chapter 3. Results are the average of 3 replicates \pm SD.

Chapter Four: Simple Lipid Mixtures

The study of the most common zwitterionic and anionic lipids from Chapter Three: Primary Lipid Compositions identified several lipid targets with preferential affinity of Pb^{2+} . Building on these direct Pb^{2+} – lipid interactions, we can begin to mimic the physiological compositions of mammalian cell membranes to assess if complex lipid mixtures can affect these individual Pb^{2+} targets. The focus of this chapter are relevant lipid mixtures that will help to understand interactions with more complex biomimetic membranes and polar lipid extracts. The impact of Pb^{2+} on membrane permeability, fluidity and organization will be further investigated with LUV leakage of the fluorophore/quencher pair ANTS/DPX, the solvatochromic fluorophore laurdan and finally by DLS to assess changes of LUV size and lipid phase transitions.

The first mixed lipid system was the addition of cholesterol to PC and SM liposomes since it is well known for its role of regulating membrane fluidity depending on the phase of the bilayer⁸⁹. Pb²⁺ was reported to have affinity for the phosphate group within the backbone region of PC and SM lipids (Sections 3.4 Interactions of Pb²⁺ with Phosphatidylcholine (PC) Membranes and 3.5 Interactions of Pb²⁺ with Sphingomyelin (SM) Membranes). An increasing percentage of cholesterol (15% and 40%) will be compared to sterol-free systems to assess the impact of cholesterol on Pb²⁺ interactions.

The phosphatidylethanolamine (PE) headgroup is potentially another zwitterionic target like PC however, PEs are not able to form well defined LUVs. Their overall conical geometry and the negative curvature induced within membranes are consequences of the small head group size^{69,137,159}. The roles of this non-bilayer lipid include the induction of curvature stress for budding or fission as well as the accommodation of membrane

proteins⁹³. As an aminophospholipid, the polar PE head groups also create a strong hydrogen bonding network within the membrane¹⁵⁹. Despite the importance of the lipid in the plasma and mitochondrial membranes⁹³, the focus of this study is on erythrocytes and the myelin sheath, common targets for Pb²⁺ poisoning, which contain PEs enriched in the inner leaflets. These membranes are mimicked by using PE/PC mixtures⁶⁹. The selected PEs include POPE and PE-plasmalogen since the different side chain architecture allows a comparison of the role of acyl and vinyl ether linkages for Pb²⁺ – PE interactions. Plasmalogens make up approximately two-thirds of the PE content in erythrocytes and nearly all PE lipids in the myelin are plasmalogens^{76,78,95,160}.

The negative carboxyl group of PS lipids was identified as a Pb^{2+} binding target in Chapter 3 as it extends more into the surrounding aqueous phase than the phosphate linker. PE lipids are structurally similar to PS but lack the carboxyl group⁹³. Reduced binding of Pb^{2+} to PE would confirm the importance of the carboxyl group for membrane perturbations such as leakage (Figure 40). Thus PS/PC lipid mixtures were tested at the same concentrations as PE/PC systems for direct comparisons. Since $Pb^{2+} - PS$ LUVs exhibited significantly increased membrane permeability, the incorporation of cholesterol into PS was also carried out to assess the impact of enhanced packing for the membrane interactions of the metal.

 Pb^{2+} has exhibited high affinity for LUVs containing different glycerol and sphingosine-based lipids throughout Chapter 3 suggesting metal targeting of the phosphate linker despite the overall lipid charge, zwitterionic or anionic. The next lipid classes tested were negatively charged sphingolipids including cerebroside sulfate (sulfatides) and gangliosides. Neither lipid contains the phosphate linkage to test Pb^{2+} affinity for different negatively charged regions of the membrane.

Gangliosides are glycosphingolipids containing an oligosaccharide head structure with multiple sialic acids and are enriched in the brain^{64,82,94}. While minimal amounts of gangliosides are found within the myelin sheath, they constitute 10-12% of the total lipid content within neuronal membranes⁹⁴. A 10% total ganglioside extract from porcine brain consisting of more than 100 different species, was added to 90% POPC to reflect the complex ganglioside profile as the brain develops with differing acyl tails and degrees of sialylation^{82,94}

Sulfatides, alongside other cerebrosides, are among the most common myelin lipids at 10% of the total lipid content^{94,95}. Sulfatides are known to assist long term stability of the myelin sheath structure⁹⁸ and the porcine brain extract of cerebroside sulfates with various acyl tail distributions were also formulated into LUVs with 90% POPC.

Ceramides are structurally the simplest sphingolipid lacking any phosphate linkage or headgroup and were the only uncharged lipid tested. Ceramides are an important precursor in the sphingolipid metabolism¹⁶¹. The ceramides tested contained a defined palmitoyl (C16) acyl tail for comparison to the experiments with palmitoyl-sphingomyelin conducted in Chapter 3. Cerebrosides are a general name for monoglycosylated ceramides which typically contain glucose or galactose attached as head group and are also referred to as glucosyl or galactosyl ceramides, respectively. The cerebrosides used in this study were galactosyl ceramides (Gal Cer) due to their prevalence in the myelin sheath and importance for myelin development, stabilization and maintenance.^{94,98}

Hydroxylation at the 2-C atom of the amide linked fatty acid generates a chiral center, creating two possible stereoisomers: 2R- and 2S- hydroxy Gal Cer. Interestingly,

124

mammalian 2'-hydroxy sphingolipids are exclusively in the 2R configuration¹⁶². 2R-OH Gal Cer are the most abundant cerebrosides in the CNS and peripheral nervous system myelin at 21% in the extracellular leaflet^{96,97,162}. Comparably, non-hydroxylated Gal Cer was found to constitute only 3.5% of the total lipid content of the myelin sheath^{96,97}. These lipids aid in the stability of the myelin structure because the 2'-hydroxyl group is located near the polar region of the membrane thereby strengthening the network of hydrogen bonding found between sphingolipids^{94,162}.

4.1 Interactions of Pb²⁺ with Membranes Containing Cholesterol (CHOL)

Chapter 3 results for Pb^{2+} and different zwitterionic and anionic lipids confirmed that Pb^{2+} mainly induces increased fluidity via electrostatic interactions with the negative regions of the membrane. The addition of cholesterol modulates the behaviour of these lipids which can alter Pb^{2+} interactions. The hydroxyl group of cholesterol, located near the polar interface of the membrane, is not expected to be a Pb^{2+} target. Instead, the bulky sterol rings intercalating between the lipids may change the accessibility of the negative phosphate linker. Overall, an increase in membrane rigidity of the liquid-crystalline phase is expected compared to sterol-free systems.

4.1.1 Leakage Assays of POPC and CHOL LUVs with Pb²⁺



Figure 59. Pb^{2+} -induced leakage (%) of liposome contents from 300µM LUVs of POPC (black), 15% CHOL 85% POPC (blue) and 40% CHOL 60% POPC (red) encapsulating 11.8mM ANTS and 47.4mM DPX in 100mM NaCl pH 7.4. Results are the average of 3 replicates ± SD.

The leakage results for POPC LUVs (black) have been included for reference with the CHOL-included trials (Figure 59). The 9% overall leakage of internal components for POPC is reduced to just over 3% with the inclusion of 15% CHOL while 40% CHOL only induced 2.5% leakage (Figure 59).



Figure 60. (*top*) GP values in LUVs of 300 μ M 15% CHOL 85% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 15% CHOL 85% POPC LUVs across increasing temperature (°C) and Pb²⁺ (0-2100 μ M).

Results from 15% CHOL 85% POPC showed stronger GP increases than POPC LUVs when Pb²⁺ was in excess (2.1mM) however this rigidification was not exclusive to higher temperatures only (Figure 60). At 15% CHOL, the starting GP values are much higher with +0.4 for 5°C compared to +0.1 for POPC suggesting a strong overall rigidifying effect that was seen across the entire temperature range (Figure 27).

Results for 40% CHOL 60% POPC are shown in Figure 61 whereby the starting GP of ~+0.45 at 5°C indicates additional rigidification. Nevertheless, Pb^{2+} in excess continued to further induce rigidity of the membranes to the same degree as observed for the 15% sterol system (Figure 60).



Figure 61. (*top*) GP values of LUVs of 300 μ M 40% CHOL 60% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs versus increasing temperature (°C) and Pb²⁺ concentrations (μ M).

4.1.3 DLS for LUVs composed of POPC and CHOL with Pb^{2+}



Figure 62. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 15% CHOL 85% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).

DLS size data for Pb^{2+} at 2.1 mM showed significant changes and substantial swelling of the membrane at lower temperatures (Figure 62). This may be the result of cholesterol altering the packing arrangement between POPC lipids causing a different lipid organization and higher accessibility to the phosphate groups than Pb^{2+} – POPC alone (Figure 27). The raised count rates are based on significant changes to the optical properties corresponding to this tighter membrane packing where well-organized gel phases scatter less light than fluid membranes.



Figure 63. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 40% CHOL 60% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).

In agreement with GP results, 40% CHOL increases membrane rigidity of POPC as observed in the higher count rate across the entire temperature scale (Figure 63). Pb^{2+} at 2.1mM induces significant ordering of the lipids, predominantly at lower temperatures when lipid packing is less fluid alike 15% CHOL as seen in Figure 62. The scale of the size changes induced by Pb^{2+} is less pronounced than 15% CHOL though similarly suggestive of LUV swelling which is reduced upon increasing temperatures.

4.1.4 Laurdan GP of PSM and CHOL with Pb^{2+}

Sphingomyelin was tested in Chapter 3 to compare the importance of the sphingosine versus glycerol backbones in the presence of the same phosphocholine head group. Stronger interactions were observed for PSM over PC glycerophospholipids, one possible explanation may be that the hydrogen bonding network found within sphingolipids provides a tighter coordination for Pb²⁺ binding (Figure 34). Cholesterol has a strong interaction with SMs due to the ability of hydrogen bonding mediation, but the deeper insertion of the sterol within the membrane will change the packing of the interface⁸⁷. Laurdan GP results for 40% CHOL 60% PSM LUVs with Pb²⁺ are shown in Figure 64.

Exposure of 40% CHOL 60% PSM to 2.1mM Pb²⁺ resulted in less pronounced membrane rigidification than pure PSM LUVs (Figure 64). Cholesterol increased the initial GP value to +0.6 from +0.17 in PSM at 5°C. The shift in T_m observed for Pb²⁺ – PSM was weaker with cholesterol and the entire phase transition was less cooperative. PSM with a T_m of 40.2°C and a transition occurring across a 7°C temperature range was broadened across 60.7°C as indicative of the much more rigid membrane induced by cholesterol. The induced rigidity by Pb²⁺ occurs across the broad temperature range (Figure 64).



Figure 64. (*top*) GP values in LUVs of 300 μ M 40% CHOL 60% PSM (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 40% CHOL 60% POPC LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).



Figure 65. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 40% CHOL 60% PSM LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).

Interestingly, DLS results exhibited reduced count rates for 2.1mM Pb²⁺ across the entire temperature scale, unlike any previous trial. PSM LUVs with 2.1mM Pb²⁺ also resulted in this drop however only at higher temperatures in the liquid-crystalline phase. Since CHOL is known to rigidify the membrane, it may lead to maximal reflection of the laser light. In contrast, the binding of Pb²⁺ to PSM can reduce the packing order associated with strong SM-CHOL interactions and induce a higher scattering and thus overall lower count rates relative to control. The significant swelling of the LUV size induced by Pb²⁺

supports this theory of reduced packing order between SM and CHOL in the liposomes.

4.1.6 Laurdan GP of PSM and POPC with Pb^{2+}

Lastly, PSM and POPC were combined at equimolar concentrations in LUVs to directly compare these phosphocholine-containing lipids. Laurdan GP results are shown in Figure 66.

As POPC transitions at -2° C and PSM at 40°C, we observed a steady decrease in GP over temperature as these different lipids melted within the mixed LUVs. The total change in GP of +0.032 is larger than POPC alone (+0.01) but does not compare to the change in GP observed across the phase transition of PSM (+0.098). This would suggest that the mixed PSM and POPC are still rigidified by Pb²⁺ and targeting of the negative phosphate linker is possible.



Figure 66. (*top*) GP values of laurdan in LUVs of 300 μ M 50% PSM 50% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for 50% PSM 50% POPC LUVs across increasing temperature (°C) and Pb²⁺ (μ M).



Figure 67. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 50% PSM 50% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).

Pb²⁺ binding appears to be enhanced for ordered lipids as less interactions were detected at higher temperatures, similarly seen for GP results of the pure PSM LUV system (Figure 34). Significantly increased count rates observed throughout the curve suggest Pb²⁺ is ordering lipids to alter packing arrangements within the membrane causing less DLS light scattering. LUV size increases also decrease as a function of higher temperatures.

4.1.8 Discussion of Pb^{2+} Interactions with Membranes Containing CHOL

As cholesterol is abundant in mammalian membranes, it was included in this study

to observe if this sterol could affect Pb^{2+} induced lipid rigidification. Cholesterol is uncharged and mainly hydrophobic, and the lone polar hydroxyl group was not expected to interact with Pb^{2+} . The impact of the sterol on lipid packing was thought to affect Pb^{2+} binding to the phosphate linker of phosphocholine-containing lipids.

 Pb^{2+} -induced rigidification in the presence of CHOL was stronger at lower temperatures and more closely resembles the changes in GP observed for DMPC over POPC (Figure 27). Laurdan results of POPC LUVs showed maximal increases in GP of only +0.01 in the presence of Pb^{2+} whereas the incorporation of CHOL saw a noticeable change in the overall membrane rigidity as well as a 2-fold increase in the rigidity induced by Pb^{2+} (Figure 60 and Figure 61).

As the intrinsic POPC phase transition temperature is -2°C, LUVs are in the liquidcrystalline phase throughout the entire temperature range of the experiments. Cholesterol increases the ordering of lipids in this phase through restricted acyl tail mobility and enhances the packing density throughout the membrane⁸⁹. Additionally, the presence of CHOL in PC bilayers is suggested to significantly increase the absorption of water¹⁶³. CHOL raised the overall membrane rigidity, as observed in Figure 60 and Figure 61, potentially changing the accessibility of the phosphate linker for a tighter coordination by Pb²⁺, comparable to the increased interactions observed for the more ordered DMPC over POPC from Chapter 3 (Figure 29 versus Figure 27). This was predominantly noticed during lower temperatures for both 15 and 40% CHOL (Figure 60 and Figure 61). However, these significant size changes and pronounced membrane rigidity was reduced upon increasing temperatures. This leads to the speculation that Pb²⁺ may be coming off the membrane. At lower temperatures, organization of cholesterol and PC lipids may induce defects or other lipid packing problems that enhance the coordination of Pb^{2+} . Potentially, cholesterol induced a less fluid phase upon increasing temperatures which disrupts these Pb^{2+} interactions. This would explain the loss of LUV swelling and reduction in count rate signal corresponding to looser lipid packing observed in the DLS experiments (Figure 62 and Figure 63). An appropriate test for this theory would be to compare the concentration of Pb^{2+} in solution after being exposed to liposomes containing cholesterol content at 4°C or room temperature. This would help understanding if the affinity of Pb^{2+} to PC/CHOL membranes is temperature dependent.

The increased sterol content also explains the notable decreases in membrane permeability. Pb^{2+} -binding that resulted in POPC membrane leakage was reduced by the sterol from 9% to 2.6% (Figure 59). As the biological membranes become more complex, the mixing of lipid classes reduces the risk of membrane disruptions causing permeability despite the presence of Pb^{2+} lipid targets. A higher membrane order due to cholesterol resulted in less Pb^{2+} -induced membrane permeability⁸⁷.

The GP and DLS results agree that the entire PSM and CHOL membrane was highly rigidified by cholesterol causing a significant broadening of the phase transition. The intercalation of cholesterol between PSM lipids is important in membranes to support the functions of rafts and other membrane structures⁸⁷. Cholesterol adds to the hydrogen bonding network within the polar interface of the sphingolipids, potentially promoting tight lipid packing as well as restricted acyl tail mobility as the membrane melted into the fluid phase⁸⁷. These strong SM-CHOL interactions disrupted the Pb²⁺ binding pockets seen for pure PSM LUVs. Instead, DLS results suggest increased water penetration into the membrane interface causing swelling of the LUVs. Pb²⁺ binding appeared to disorder the

membrane, counteract the tight ordering, and reduce the maximal light reflection induced by cholesterol in the control.

As cholesterol caused enhanced Pb²⁺ binding and rigidification in the more fluid POPC while it decreased the interactions with PSM membranes, POPC and PSM were combined in LUVs for a direct comparison of these two phosphocholine-containing lipids. POPC is a more fluid lipid and exhibited +0.01 GP rigidification in the liquid-crystalline phase with Pb^{2+} at higher temperatures (Figure 27). PSM lipids, however, saw significantly higher Pb^{2+} rigidification (+0.098) throughout the gel phase and a higher phase transition shift (Figure 34). As SM lipids have stronger lipid – lipid interactions and tighter membrane packing, Pb²⁺ was suggested to coordinate with the hydrogen bonding network through PbOH⁺ or water-mediated species. Data for this lipid mixture containing the most common mammalian lipids are in between results for the single lipids, but closer to PC. DLS results suggest strongest rigidification at the lower temperatures where the membrane is more ordered which promotes Pb^{2+} binding (Figure 67). The sigmoidal curve of the count rate curves suggests a downshifted phase transition of SM, potentially due to localized demixing of SM and PC (Figure 67). This would induce packing defects that can allow for better alignment with POPC. Stronger interactions between Pb²⁺ and PC would explain the ability of SM to exhibit the downshifted phase transition at 25.4°C. A DSC study of 50% egg SM and 50% DMPC in 100mM sodium phosphate, 150mM NaCl, 10mM EDTA, and pH 7.2 observed a broad phase transition at 31°C, indicating a less ideal lipid packing arrangement¹⁶⁴. This transition is comparable to the melting of POPC/PSM despite different lipid structures.

4.2 Interactions of Pb²⁺ with Phosphatidylethanolamine (PE) Membranes

PE is the second most abundant phospholipid representing 25 mol % of the overall mammalian phospholipids content in cell membranes and upwards of 45 mol % in nervous tissue^{69,93,94,97,159}. Typically abundant in cytoplasmic membrane leaflets, approximately 15% PE content is also found on the surface of erythrocytes which take up 99% of Pb²⁺ from the blood^{75,93}. A significant portion of the PE content and approximately 20% of all phospholipids are ether-linked plasmalogens⁹³. The cytoplasmic leaflet of the myelin contains 29% PE plasmalogens whereas the opposing leaflets contain only approximately 9%^{96,97}.

The head group of PE and PC phospholipids have similar structures, except for the methylation of the amine¹⁵⁹. This allows PE to partake in hydrogen bonding within the membrane or ions and molecules from bulk solution¹⁵⁹. Therefore, the inclusion of PE in PC LUVs will assess the differences in Pb²⁺ binding when the positive amine changes from quaternary to primary within the head group structure. Although PE is zwitterionic alike PC, this hydrogen bonding capability as well as the small head group inducing negative curvature of the membrane reduces its propensity to form bilayers^{69,106,159}. One of the functions of PE molecules is inducing curvature stress for protein insertion and modulation of membrane curvature⁹³.

4.2.1 Leakage Assays of POPE and POPC LUVs with Pb²⁺



Figure 68. Pb^{2+} -induced leakage (%) of aqueous liposomal contents of 300µM 15% POPE 85% POPC LUVs encapsulating 11.8mM ANTS and 47.4mM DPX in 100mM NaCl pH 7.4. Results are the average of 3 replicates ± SD.

The addition of 15% POPE resulted in nearly 3.5% leakage. This reduction in membrane permeability is similar to the addition of 15% CHOL to POPC LUVs which also leaked just over 3% while pure POPC LUVs saw 9% leakage with 10μ M Pb²⁺ (Figure 59).



Figure 69. (*top*) GP values in LUVs of 300 μ M 15% POPE 85% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for LUVs across increasing temperature (°C) and Pb²⁺ concentration (μ M).

A phase transition temperature of POPE at 25°C cannot be detected when only 15% is present. The decreasing GP results shown in Figure 69 are indicative of the liquid-crystalline phase. Pb^{2+} rigidification is more pronounced with POPE present and the total GP change of +0.014 is comparable to +0.01 for POPC alone (Figure 27).

Since 15% PE content is reported for the outer leaflet of erythrocytes, LUVs with 30% POPE 70% POPC were also made assuming equal distribution between the leaflets upon liposome formation. This also allows for assessing Pb^{2+} – PE interactions with continued increasing PE content. Laurdan GP results for 30% POPE 70% POPC LUVs are shown in Figure 70 and the rigidification of the membrane by Pb^{2+} is further pronounced for 30% PE. The membrane appears to be undergoing a phase transition at 10.8°C, potentially a downshifted transition of POPE which is expected at 24.4°C, as reported in several different DSC experiments^{159,165}, and the effects of Pb²⁺ are seen across the entire temperature scale. The total change to GP was nearly 4-fold higher than POPC alone (+0.039) (Figure 70).



Figure 70. (*top*) GP values of LUVs of 300 μ M 30% POPE 70% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the GP change for 30% POPE 70% POPC LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

4.2.3 DLS for LUVs composed of POPE and POPC with Pb^{2+}



Figure 71. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 15% POPE 85% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. Pb²⁺ count rate curve is statistically significant (p < 0.05).

Interestingly, no statistical size difference is noted with 15% POPE 85% POPC LUVs however, count rate data suggests rigidification and membrane packing changes in the presence of 2.1mM Pb²⁺ (Figure 71).



Figure 72. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 30% POPE 70% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).

Similar trends for laurdan were observed and more rigidification was seen for 30% POPE with Pb²⁺ than for the 15%. Statistically significant size changes were detected, especially at higher temperatures when the control LUVs shrunk and Pb²⁺ maintained the LUV sizes. A similar phase transition can be observed at 11.9°C which was slightly shifted by Pb²⁺ to 14.2°C (Figure 72). This transition agrees with the GP sigmoidal inflection results. The appearance of a downshifted POPE phase transition is suspected based on the compilation by Koynova and Caffrey of the average POPE phase transition at 24°C¹⁶⁵.


Figure 73. (*top*) GP values of LUVs of 300 μ M 29% PEplasm 79% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for LUVs across increasing temperature (°C) and Pb²⁺ concentration (μ M).

In drastic contrast to 30% POPE LUVs, 29% PEplasm 71% POPC did not exhibit any PE-facilitated rigidification (Figure 73). While these two systems do not have equal PE content, 29% PEplasm was chosen due to its mol % composition in the outer leaflet of the myelin sheath⁹⁷. The total change in GP (+0.008) is on par with POPC interactions around +0.01 with Pb²⁺.

GP Results for the addition of CHOL (29% PEplasm 32% CHOL 39% POPC), an important component in the myelin sheath as well as most other mammalian membranes, are shown in Figure 74.

The addition of cholesterol changes the shape of the LUV phase transition as the membrane has a broadened transition from the gel into liquid-crystalline phases unlike the sharp transition observed in Figure 73. The only significant interactions with Pb²⁺ occurred at 5°C. Since sufficient phosphate targets are present it is not likely that Pb²⁺ cannot bind but rather that the membrane is already very rigid and may not be amenable to further rigidification by Pb²⁺.



Figure 74. (*top*) GP values in LUVs of 300 μ M 29% PEplasm 32% CHOL 39% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

4.2.5 DLS for LUVs composed of PEplasm and POPC with Pb^{2+}



Figure 75. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 29% PEplasm 79% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).

DLS results for 29% PEplasm similarly resemble the reduced count rates of 40% CHOL 60% PSM from Figure 65. Along with the significant size changes for the 29% PEplasm LUVs, Pb²⁺ binding appears to be disordering the membrane which scatters more light from the laser, resulting in lower count rates.



Figure 76. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 29% PEplasm 32% CHOL 39% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ size is statistically significant, asterisks denote count rate curve (p < 0.05).

Similar size changes as in Figure 75 are observed for the addition of cholesterol in the system: 29% PEplasm, 32% CHOL and 39% POPC and Pb²⁺ (Figure 76). There was an increase in the count rate of LUVs above 20°C unlike the cholesterol-free trials.

4.2.6 Discussion of Pb^{2+} Interactions with PE Membranes

Suwalsky *et al.*, performed X-ray diffraction with DMPE and observed that a concentration of Pb^{2+} 50-times higher than DMPC was required to induce any significant structural perturbations on the membrane. They suggested that DMPE molecules pack tighter and water does not impact the bilayer structure due to weak water interactions for

PE lipids as the presence of hydrogen bonds lower the hydration of the membrane^{77,119,163}. DLS results for 15% POPE also exhibited no size changes upon incubation with Pb²⁺ suggesting LUV swelling does not happen due to this tight packing. The small head group of PEs may intercalate amongst PC lipids dictating the membrane water penetration capabilities. This tighter membrane packing helps explain the reduced leakage observed for PE/PC LUVs compared to POPC alone.

At 15% PE content, no size changes were observed in the absence or presence of Pb^{2+} across all temperatures. However, the control 30% PE liposomes exhibited a decreasing size with increasing temperatures which was counteracted by Pb^{2+} . PE forms hydrogen bonds with neighbouring PEs with weak water binding in the presence of PC. This is due to intra-intermolecular hydrogen bonding in PE which is reduced in the presence of methyl groups on the amine group, as seen for PC^{159,163}. Thus, the higher PE content may induce more packing defects and PE clustering allowing for hydrogen bonding. Increasing temperatures in the control trials reduced hydrogen bonding which reduced water penetration and LUV size. With Pb²⁺ coordination within this region, PEs may not be free to hydrogen bond, water penetration may have remained the same and no size changes were seen (Figure 71). Laurdan and DLS are also in agreement that Pb²⁺ binds the membrane as it becomes more ordered in the presence of Pb²⁺.

As large size changes and a disordering of the membrane surface were detected, PEplasm lipids potentially interact with Pb²⁺ and water better than POPE. The replacement of the carboxyl ester to an enol ether in position 1 of plasmalogens reduces the polarity of the membrane interface, resulting in a less hydrophilic surface area of the lipids^{77,166}. The weak water hydration due to hydrogen bonding between PEs would be reduced causing a higher capability of water penetration with PE plasmalogens than POPE. As GP is a consequence of both solvent polarity and water penetration within the membrane, the laurdan results may not report the same degree of interactions with PEplasm as detected by DLS. With the incorporation of cholesterol, a similar initial membrane rigidification was observed for PEplasm as seen before for PC/SM and CHOL trials. This fact restricted Pb²⁺ binding to higher temperatures where a phase transition may be occurring due to the demixing of PEplasm and PCs. As suggested by PCplasm and PEplasm results, Pb²⁺ does not target the vinyl ether linkage of plasmalogens to induce phospholipid cleavage as seen for another toxic heavy metal, Hg^{2+ 103,142}.

4.3 Interactions of Pb²⁺ with Mixed PS Membranes

The rationale for the further PS leakage experiments were two-fold: leakage of nearly 18% internal contents seen in pure LUVs would have serious ramifications for every cellular membrane consisting of PS lipids. However, this lipid is often only a minor component in most mammalian cells and the lipid is more important for signaling pathways^{92,93,97,112}. Thus, the incorporation of 15% POPS in 85% POPC is a better representation of actual PS concentrations. Additionally, PS and PE lipids are structurally similar, PE lacking the serine carboxyl group⁹³, thus equivalent mol % contents can help to understand the role of the additional metal target presented by the carboxyl group. And the potential of Pb²⁺ induced permeability in PS containing membranes.

Since 15% CHOL greatly reduced the leakage observed for pure POPC LUVs, it was interesting to consider the sterol's impact on membranes when POPS is the dominant component. Thus 15% CHOL 85% POPS LUVs were formed in direct comparison with CHOL/POPC tests.



Figure 77. Pb^{2+} -induced leakage (%) of aqueous liposomal contents of 300µM LUVs of POPS (black), 85% POPS 15% CHOL (blue) and 15% POPS 85% POPC (red) encapsulating 11.8mM ANTS and 47.4mM DPX in 100mM NaCl pH 7.4. Results are the average of 3 replicates ± SD.

The leakage results of POPS LUVs (black) have been included for reference in the POPS mixtures (Figure 77). Significant reduction in membrane leakage is observed for 15% POPS (nearly 2.5%) compared to 100% POPS (nearly 18%). Interestingly, 15% CHOL lowers POPS leakage down to 3%, equivalent to 15% CHOL 85% POPC at 3.2% (Figure 59).

4.3.2 Discussion of Pb^{2+} Interactions with PS Membranes

The additional negative charge of the serine head group of POPS was used to explain why pure POPS LUVs exhibited nearly 18% leakage over 9% for POPC. The mixture of 15% POPS 85% POPC did not combine each individual lipid effect but rather a reduction of leakage down to nearly 2.5% (Figure 77). The intercalation of PC and PS lipids may induce packing defects due to different head group sizes and charges. The difference in the lipid packing defects may also be concentration dependent where Pb²⁺ on 15% POPS 85% POPC may have caused aggregation of PS molecules through subsequent clustering of PC molecules. The mixing of PC and PS, or potentially amino and carboxylate groups between PS lipids, changes the overall surface charge, charge density, and packing arrangements which would alter the ability of Pb²⁺-induced membrane permeability^{167,168}. A monolayer study on increasing PS content for 15 to 45% POPS with POPC observed tighter packing of the monolayers with 15% and 45% PS than 30% in respect to POPC alone¹⁶⁷. Moreover, the presence of varying headgroup sizes and orientations likely affected the binding coordination of Pb²⁺ that was provided by each of the pure systems. Further testing with increasing PS content would confirm the role of lipid composition on lipid packing and accessibility of Pb²⁺ to induce membrane leakage.

When comparing 15% POPS to POPE, the POPS leakage was similar (2.5% versus 3.5%). The lipid packing of smaller PE head groups may pack tighter than PS lipids with PC. This could allow for better Pb^{2+} coordination as its hydrated radii is very small³³ and it has been shown to prefer tighter coordination pockets for binding (Chapter 3). Additionally, the negative carboxyl group of PS will likely coordinate a potentially significant fraction of the available Pb^{2+} species over binding to the phosphate linker (Figure 40) suggesting less metal penetration into the polar interface of the membrane than for PE/PC LUVs.

The addition of CHOL had comparable results between PS and PC LUVs (3% and

3.2%, respectively). At ambient temperature, both POPS and POPC lipids are in the fluid phase suggesting cholesterol rigidifies the membrane thereby reducing permeability.

4.4 Interactions of Pb²⁺ with Brain Ganglioside and Sulfatides Membranes

Gangliosides are predominantly located in the grey matter of the brain and participate in the molecular interactions between axons and glia ⁹⁴. Their bulky oligosaccharide and sialic acids extend out of axonal membranes and are recognized by different proteins by sialic acid binding⁸³. Gangliosides are anionic however the negative charges are much further away from the hydrophobic core of the bilayer. A total ganglioside extract from porcine brains was used at a 10 mol % with 90% POPC.

Testing the interactions of Pb^{2+} to gangliosides and sulfatides will help to understand Pb^{2+} targeting to different anionic lipids based on charge localization and to assess the consequences of Pb^{2+} binding to ganglioside or sulfatide containing membranes.

Sulfatides, cerebroside sulfates, are also natural lipids in porcine brain with many possible side chains in the extract. The fatty acid distribution suggested by the manufacturer is presented in Figure 78 whereby the unknown content may contain hydroxylated fatty acids¹⁴⁷. Sulfatides are present in the myelin at much higher molar percentages with other cerebrosides than most sphingo- and glycerolipids⁹⁵. The sulfate was expected to interact with Pb²⁺ given the reactivity of Pb²⁺ with sulfhydryl groups of various proteins and sulfhydryl-containing molecules such as glutathione^{13,18,169} or metallothionein^{36,46,170,171}.



Figure 78. The fatty acid distribution found in the natural lipid mixture of Brain Sulfatides from Avanti Polar Lipids¹⁴⁷.

4.4.1 Laurdan GP of Total Brain Ganglioside Extract and POPC with Pb²⁺

GP Results for 10% Brain Total Ganglioside Extracts with 90% POPC are shown in Figure 74. As seen for POPC, no phase transition is detectable with 10% gangliosides 90% POPC due to a T_m below 0°C. Pb²⁺ induces significant rigidification across the temperature range, in fact 6-fold greater than POPC alone (Δ GP of +0.06 versus +0.01) (Figure 27).



Figure 79. (*top*) GP values of LUVs of 300 μ M 10% Total Gangliosides (Brain) 90% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentration (μ M).

4.4.2 DLS for LUVs composed of Total Brain Ganglioside Extract and POPC with Pb²⁺



Figure 80. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 10% Total Gangliosides (Brain) 90% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ count rate is statistically significant, asterisks denote size curve (p < 0.05).

DLS results agree with laurdan GP, Pb²⁺ incubation causes the rigidification of the membrane as noted by the higher count rate corresponding to tighter membrane packing but no relevant LUV size changes were observed (Figure 80).





Figure 81. (*top*) GP values of LUVs of 300 μ M 10% Brain Sulfatides 90% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

Laurdan results show GP increases when Pb^{2+} concentrations were equimolar and higher than the concentration of LUVs and the values became progressively larger as a function of temperature as illustrated in the contour plot (Figure 81). The increases in GP are not statistically significant until 20 – 25°C, suggesting the membrane must be quite fluid for Pb²⁺ binding. The effects of Pb²⁺ occur at lower concentrations with higher GP increases than in POPC alone, suggesting Pb²⁺ – sulfatide interactions.

4.4.4 DLS for LUVs composed of Brain Sulfatides and POPC with Pb²⁺



Figure 82. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) for 0.3mM 10% Brain Sulfatides 90% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ size curve is statistically significant, asterisks denote count rate curve (p < 0.05).

DLS size data of Pb²⁺ at 2.1mM exhibited a uniform statistically significant size

change throughout the temperature range. The scale of these changes suggests a limited Pb^{2+} induced swelling of the LUVs. In contrast, the Pb^{2+} -induced changes to the count rate are temperature dependent. The membrane packing is ordered at 5-20°C but becomes disordered with increasing temperatures.

4.4.5 Discussion of Pb²⁺ Interactions with Ganglioside and Sulfatide Brain Extracts

 Pb^{2+} is associated with several toxic effects on the central nervous system (CNS) such as reduced cell viability^{169,172}, disrupted neurotransmitter control^{19,62}, inhibited brain barriers^{19,49,169} and overall cell apoptosis^{19,172}. Meng *et al.*, reported the protective effect of gangliosides for Pb^{2+} -induced neurotoxicity of the spatial reference memory through inhibition of autophagic pathways⁶⁴. They showed that ganglioside supplementation alleviated Pb^{2+} -induced impairment of the cognitive function, cytotoxicity, apoptosis-related protein expression, and autophagy in infant mice. They described gangliosides as a neuroprotective agent against Pb^{2+} injuries to neurons. Their extensive study investigated the molecular mechanisms of Pb^{2+} neurotoxicity and the protective effects of gangliosides on neuronal damage⁶⁴ however it does not address the possibility of direct Pb^{2+} – ganglioside interactions as examined in this study. The results obtained here likely provide an explanation for their observed neuroprotective effects and support further interactions of gangliosides with Pb^{2+} to reduce neurotoxicity.

Gangliosides with POPC (10:90 mol ratio) exhibited rigidification upon Pb²⁺ binding which was much stronger than for POPC LUVs alone. The large head group size of gangliosides likely protrudes from the membrane and overarches on surrounding PC lipids which may scatter DLS to limit any detectable size changes. The addition of gangliosides in LUVs exhibited higher GP values when compared to POPC throughout the

entire temperature range. This is likely due to packing defects due to significant differences in lipid sizes causing a more rigid membrane. Tighter packing can promote enhanced Pb²⁺ interactions with the phosphate linker of PCs while potentially interacting with the negative components of the bulky ganglioside.

GP and DLS data for brain sulfatides and POPC (10:90 mol ratio) are not in agreement for Pb^{2+} interactions at higher temperatures. GP increases at a higher level than in POPC suggesting rigidification of the membrane with 10% sulfatides. DLS count rates however, show membrane disordering at higher temperatures. The larger head group structure of this lipid may change the surface characteristics of the LUV, a less smooth surface would reflect more light and thus affect the DLS results. There may also be a reduction of sulfatide interactions with Pb^{2+} as a function of temperature. A consistent size increase throughout may suggest Pb^{2+} binding occurs at all temperatures although the interaction originating with sulfatides or PCs due to packing defects remains unknown. As the temperature increases, Pb^{2+} may also alter the water penetration of the membrane causing GP increases relative to the solvent penetration whilst inducing significant disorder to the membrane.

4.5 Interactions of Pb²⁺ with Ceramide and Cerebroside Membranes

Ceramides are the simplest two-chained sphingolipid containing a hydroxyl group in position 1 of the sphingosine backbone^{80,121}. Similar to cholesterol, ceramide associates with SM and other large head group lipids to minimize unfavourable water exposure¹²¹. The small polar head group is not expected to be targeted by Pb²⁺ however the extensive hydrogen bonding network formed among ceramides¹²¹ may promote direct Pb²⁺ interactions at the membrane backbone.

164

Ceramides play an essential role in sphingolipid metabolism as the source or degradation pathways for SM and glycosphingolipids⁸⁰. The glycosphingolipid, or cerebroside, tested in this study was galactosyl ceramides (Gal Cer) which are enriched in the extracellular leaflet of the myelin and oligodendrocytes^{94,97,98}. Gal Cer lipids with long saturated fatty acids as well as a hydroxylated 2-C on the amide linked side chain act as myelin stabilizers⁹⁴. This 2-OH group strengthens the network of hydrogen bonding between sphingolipids⁹⁴. Comparisons will be made between saturated and hydroxylated Gal Cer lipids at the compositions reported for the myelin outer leaflet^{96,97}.

4.5.1 Laurdan GP of Palmitoyl Ceramide and POPC with Pb^{2+}

GP results for 5% Ceramides (C16) 95% POPC are shown in Figure 83. At only a 5 mol % inclusion, 2.1mM Pb²⁺ induced higher GP of up to +0.018 starting at 10°C rather than values seen at 30°C for POPC alone (Figure 27).



Figure 83. *(top)* GP values in LUVs of 300 μ M 5% C16 Ceramides 95% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). *(bottom)* Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentration (μ M).



Figure 84. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 5% C16 Ceramide 95% POPC LUVs (black) with 2.1mM Pb^{2+} (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Data normalized using non-linear Boltzmann regression. All Pb^{2+} size is statistically significant, asterisks denote count rate curve (p < 0.05).

The interactions of Pb²⁺ with 5% Ceramides suggest much stronger interactions than for POPC alone, inducing membrane ordering and LUV size increases at lower temperatures (Figure 84).

4.5.3 Laurdan GP of Galactosyl Ceramide and POPC with Pb^{2+}

2R-OH Gal Cer are the most abundant cerebrosides in the CNS and PNS myelin at 21% in the extracellular leaflet^{96,97,162}. Comparably, non-hydroxylated Gal Cer was found to constitute only 3.5% of the total lipid content of the myelin sheath^{96,97}. LUVs were

formulated with each Gal Cer at their respective mol % to understand the impact of Pb^{2+} at physiological compositions. Comparisons made directly between the lipid systems to elucidate the role of the 2'-hydroxylation are in recognition of the different lipid percentages within each LUV system.

GP results for 3.5% Gal Cer 96.5% POPC are shown in Figure 84. 3.5% Gal Cer showed decreased interactions with Pb²⁺ relative to the pure PC LUVs. Increases in GP became progressively smaller as a function of temperature (Figure 85). For POPC LUVs, GP data exhibited a rigidification of the membrane (+0.01) at higher temperatures which is completely undetected with 3.5% Gal Cer (Figure 27 and Figure 85, respectively).



Figure 85. (*top*) GP values of LUVs of 300 μ M 3.5% Galactosyl Ceramide 96.5% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).





Figure 86. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 3.5% Galactosyl Ceramide 96.5% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).

The interactions of Pb²⁺ induce significant ordering to the membrane and size increases which also became progressively smaller as a function of temperature (Figure 86). DLS results display stronger interactions in the presence of 2.1mM Pb²⁺ relative to the GP curves in Figure 85. DLS size and count rate effects with Pb²⁺ on 3.5% Gal Cer are significantly larger than pure POPC tests from Chapter 3.





Figure 87. (*top*) GP values of LUVs of 300 μ M 21% (2R)-OH Galactosyl Ceramide 79% POPC (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

The GP curve of control trials was higher than POPC LUVs alone suggesting a more rigid base membrane in the presence of 21% (2R-OH) Gal Cer (Figure 87). Interactions with Pb^{2+} were stronger at higher temperatures inducing rigidification as the membrane typically becomes more fluid. Total change in GP is 50% higher than POPC trials (+0.015 versus +0.01, Figure 27).

4.5.6 DLS for LUVs composed of Hydroxylated Galactosyl Ceramide and POPC with Pb²⁺



Figure 88. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 21% (2R) Hydroxylated Galactosyl Ceramide 79% POPC LUVs (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).

While insignificant, size changes were observed with 2.1mM Pb²⁺, especially at higher temperatures suggesting a small swelling of LUVs (Figure 88). Increased count rates

are observed throughout the entire temperature range agreeing with GP results indicating Pb^{2+} induced ordering of the lipids. A phase transition for the 2R-OH Gal Cer appears to occur at 27.3°C and 30.0°C for the control and 2.1 mM Pb²⁺ trials, respectively.

4.5.7 Discussion of Pb^{2+} Interactions with Ceramide and Cerebroside Membranes

DLS results suggest strong changes when 5% ceramides were added to POPC. Ceramides typically associate closely with other lipids to limit unfavourable water interactions¹²¹. The hydroxyl head group of ceramides are small, potentially fitting closely between PC lipids. LUV size increases and lipid ordering upon Pb²⁺ incubations suggest better coordination and accessibility of the phosphate target within POPC. Unlike other metals such as cadmium or mercury, Pb²⁺ speciates as PbOH⁺ and also likely interacts with water molecules directly explaining the more direct and extensive interactions within the polar interface of these membranes compared to the other metals¹⁴². At higher temperatures, the loss in LUV swelling may suggest a reduction in water penetration relative to control while Pb²⁺ was bound as indicated through increased GP results. The formation of ceramide domains would cause weaker water hydration to explain the sudden change in DLS data. DSC heating scans of anhydrous powder C16 ceramides showed a broad endothermic transition at 95.4°C which shifts lower to 90.0°C upon increasing hydration¹⁷³. At 5 mol %, the sigmoidal drop in count rate may be indicative of a shifted phase transition for the ceramide and PC mixture. When egg-Cer was mixed with DPPC in increasing ceramide content, an increase in the main transition temperature of DPPC was induced by egg-Cer¹⁰⁷. Pb²⁺ interactions may have induced this ceramide clustering which altered the optical properties of the LUVs due to structural changes on the surface.

The comparable reduction in GP when 5% ceramides was replaced with 3.5%

galactosyl ceramides may indicate the inability of Pb²⁺ species and water to effectively coordinate the phosphates and hydrogen bonding network within the POPC backbone in the presence of larger, bulky galactose sugars. The large head group would protrude from the membrane and potentially hinder access to the phosphate linker of POPC. Tighter lipid packing and larger LUV sizes with Pb^{2+} indicated by DLS at lower temperatures agree with GP results. Gal Cer was reported by DSC to increase the phase transition temperature of DEPE by 0.2°C per mol % of the cerebroside added⁸¹. Optimal orientations which promote Pb²⁺ binding to this phosphate may be enhanced at lower temperatures where the membrane is tighter. Possible clustering or cerebroside domain formation may decrease Pb²⁺ coordination at higher temperatures where the Gal Cer lipids undergo a phase transition at 30.2°C, not unlike the shifted T_m seen with the Gal Cer and DEPE study. The addition of a hydroxyl group on the second carbon of these galactosyl ceramides is expected to add to the sphingosine hydrogen bonding network explaining their role as myelin stabilizers 94,98 . More prominent rigidification is observed for Pb²⁺ incubations than for 3.5% Gal Cer systems or POPC alone. PbCl⁺ and PbOH⁺ species may allow for improved coordination at the surface of the membrane whereby the small Pb²⁺ radii and higher electronegative properties of the metal may help to overcome the steric hindrance of the bulky head groups.

4.6 Summary of Pb²⁺ Interactions with Simple Lipid Mixtures

Figure 89 provides a comparison of the effect of different Pb²⁺ concentrations on each lipid system within this chapter at temperatures corresponding to the beginning or end of each specific lipid temperature range tested.



Figure 89. The change in laurdan GP at a low (blue) or high (red) temperatures within the range tested for each lipid system within Chapter 4 when exposed to different concentrations of Pb^{2+} as shown. Results are the average of 3 replicates \pm SD.

Chapter Five: Biomimetic Compositions and Polar Lipid Extracts

Following exposure, Pb²⁺ enters the bloodstream where it can be transported to other tissues within the body. Plasma Pb²⁺ concentrations of 103 subjects including lead workers and not occupationally-exposed participants showed that 99% of the Pb^{2+} is taken up by the erythrocytes leaving only the remaining 1% of Pb^{2+} with the plasma³⁷. Among other studies^{174,175}, this was further confirmed by inductively coupled plasma mass spectroscopy (ICP-MS) measurements of Pb²⁺ in the plasma and whole blood of 43 lead workers and 7 controls, which also reported average plasma-Pb²⁺ levels of 1.2 μ g/L Pb²⁺ in workers and 0.15 μ g/L for controls. The corresponding blood-Pb²⁺ levels of 281 μ g/L and 40 μ g/L demonstrated a preference of Pb²⁺ uptake in blood cells¹²⁴. Hematological effects of Pb²⁺ poisoning include interference with heme and hemoglobin synthesis^{40,41,125-} ¹²⁷, changes to erythrocyte morphology and ion transport^{42,105,125,176}, reduced electrophoretic mobility¹²⁵, and reductions to cell survivability associated with anemia^{44,127,177}. The effects of Pb²⁺ on membrane proteins^{13,123,178} as well as Pb²⁺ toxicity associated with protein-mediated alterations of the fatty acid composition and lipid peroxidation effects^{36,150,179}, have been extensively studied. However, direct metal interactions with the lipids of erythrocyte membranes remain unknown. A total lipid extraction was performed as described in Chapter Two: Materials and Methods in collaboration with a colleague, Jenelle Umbsaar. A similar approach can also be used to prepare so called erythrocyte ghosts, where hemoglobin has been removed, prior to the total lipid extraction used in this study¹²⁸. Unfortunately, sample freezing caused too much hemolysis and coagulation to perform experiments with intact and asymmetric leaflets. The lipids isolated from rabbit red blood cells were formulated into LUVs and analyzed

identically to LUVs from Chapter Three: Primary Lipid Compositions Chapter Four: Simple Lipid Mixtures.

As Pb²⁺ is distributed by the blood throughout the body, the brain is one of several "soft tissues" and organs exhibiting extensive Pb²⁺-induced effects. Neurobehavioural symptoms include headaches, poor memory, fatigue, impaired hearing and speech, anti-social behaviours, and poor attention spans among other indicators^{6,14,19,24}. Epidemiological studies on children show detrimental decreases in their intelligence quotient (IQ) due to Pb²⁺ exposure^{6,16,61}.

The neurotoxic effects of Pb²⁺ cannot be linked to a single unifying mechanism. Previous studies have observed apoptosis^{19,64}, neurotransmitter interference^{48,61,62}, impaired synaptic functioning^{13,61,62,64} and improper protein activation^{16,60,65} due to chronic and acute lead exposure. No threshold for toxicity is apparent suggesting blood lead levels assumed to be harmless may be a risk for brain injury.

Alike erythrocytes, no interactions with lipids have been studied. The ability of Pb²⁺ to pass through the blood brain barrier (BBB) may be suggestive of the negative effects produced with low Pb²⁺ concentrations¹⁹. Deane and Bradbury⁵⁰ suggest that Pb²⁺ transport across the BBB is via passive diffusion of PbOH⁺ which was later confirmed by Diaz and Monreal by protein-independent permeation through myelin liposomes¹⁰³. Pb²⁺ interactions have also been shown to induce protein inactivations¹⁴ which further impairs the development and maintenance of the blood brain barrier (BBB)^{169,180}. This may explain pronounced neurodevelopmental damage and higher susceptibility to lead neurotoxicity in children due to the immaturity of the BBB²⁴.

To study the effects of Pb^{2+} on brain lipids, two different experiments were

undertaken. The first used a polar lipid extract from porcine brains, purchased from Avanti Polar Lipids, which represents a complex biological brain lipid profile. LUVs were formulated by Jenelle Umbsaar and in acknowledgement of her work, Pb²⁺-induced effects on membrane fluidity and LUV size were measured. The second was a comprehensive study of the lipid composition associated with the myelin sheath. Lead neuropathy has been observed to induce demyelination and remyelination of the sheaths to induce "onion bulb" formations in the morphology of the myelin sheath bilayers of Schwann cells^{172,181}. The effect of Pb²⁺ on the myelin has also been listed as a possible cause for impaired synaptic functioning^{19,180}. The lipid compositions pertaining to the outer and inner leaflets of the multilamellar structure of myelin sheaths were determined by Min *et al.*⁹⁷, Inouye and Kirschner⁹⁶, and O'Brien and Sampson⁹⁵. Complex brain lipids including sulfatides and cerebrosides were investigated in Chapter 4 in simple mixtures with POPC. These lipids were then mixed in LUVs mimicking brain leaflet composition and finally combined into complex biomimetic profiles of myelin sheath membrane.

5.1 Interactions of Pb²⁺ with Red Blood Cell Total Extracts

Few studies report on the interactions of Pb²⁺ with lipids directly. Caspers and Siegel observed ²¹⁰ Pb²⁺ tightly bound to fragmented human erythrocyte membranes¹²⁶ while Suwalsky *et al.*, observed the adherence of Pb²⁺ to both leaflets of the erythrocytes as well as within the membrane bilayer¹¹⁹. Ong and Lee, 1980a, found a high proportion of Pb²⁺ (79.9%) had penetrated the erythrocyte membranes to bind to haemolysate fractions while 14.1% of ²⁰³Pb²⁺ remained attached to the membrane fraction¹²³. Upon extraction of the lipids, the protein fractions contained 74% of total Pb²⁺ present, 22% was found in the organic, lipid-containing phase while about 3% remained unassociated, possibly in the free ionic form¹²³.

The total extract of rabbit RBCs has a more complex lipid composition than any previously investigated membranes from Chapters 3 and 4 in terms of lipid head groups and acyl tail lengths or saturation¹⁸². These LUVs are a better representation of *in vivo* lipid membranes however, they lack asymmetry, a cytoskeleton or the membrane proteins which may alter true Pb²⁺ interactions. Ong and Lee, 1980b, examined Pb²⁺ binding sites in the stromal membrane and indicated binding to identical molecular groups as calcium, predominantly to carboxyl over thiol or amino groups¹⁷⁸.

The lipid composition of the rabbit erythrocytes, used based on the average of for the different literature studies^{75,76,88,183–185} is averaged presented in Figure 90. Thin layer chromatography (TLC) experiments confirmed the presence of each lipid class in the RBC extract post-extraction used in this study. The cholesterol to phospholipid molar ratio of rabbit erythrocytes membranes is approximately 1 to 1 (0.93)¹⁸⁵. As suggested in Figure 90, plasmalogens represent approximately two-thirds of the PE content in erythrocytes while only 10% of PC and 8% of PS total lipids are found in this form⁷⁶.



Figure 90. Average percentage distribution of lipid classes in rabbit erythrocytes as adapted from literature^{75,76,88,183–185}.

Concerning the anionic lipids, very small amounts of PA and PI are reported while PS are an important constituent on the inner leaflet and the main anionic lipid in the membrane^{75,88,93}. This asymmetry is important for RBCs as PS externalization is involved in the degrading and recycling of the cells by the macrophages in the bloodstream^{91,92,186}. Low Pb²⁺ (~0.1 μ M) was found to induce PS exposure in isolated human erythrocytes through ATP depletion and flippase inhibition, a protein involved in maintaining PS asymmetry¹⁷⁷. Strong Pb²⁺ – PS interactions were observed in Chapter 3 which may have implications on the binding to erythrocyte membranes and the uptake of Pb²⁺ *in vivo*.

In addition, all individual lipid components of erythrocytes have shown varying degrees of interactions with Pb²⁺. Therefore, experiments with RBC extracts will further elucidate Pb²⁺ effects on the fluidity and packing of the complex, biological membranes. Interestingly, the strength of rigidification induced by Pb²⁺ as seen in Chapters 3 and 4 is approximately ranked in the order PA > PS >> SM > PE > PC which is inversely related to the percentage of the phospholipid abundance found in rabbit RBC extracts.

5.1.1 Laurdan GP of RBC Extract LUVs with Pb²⁺

GP results for the RBC extract LUVs are shown in Figure 91. GP values for this membrane were comparable to the rigidity observed in membranes containing cholesterol from Section 4.1 Interactions of Pb^{2+} with Membranes Containing Cholesterol (CHOL). Trials with Pb^{2+} at equimolar and higher concentrations showed significantly induced rigidification which increased as a function of temperature for a total change of up to +0.023.



Figure 91. (*top*) GP values of LUVs of 300 μ M Red Blood Cell Total Extract (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).



Figure 92. The average vesicle diameter of 0.3mM RBC Extract LUVs in the absence (black) and presence of 2.1mM Pb²⁺ (red) at 25 and 37°C. Results are the average of 3 replicates \pm SD. Asterisks denote statistical significance (* = p < 0.05, ** = p < 0.01).

The increase in LUV size between control samples from 25 to 37°C can be attributed to a more fluid membrane as the temperatures increase. At both temperatures, LUV sizes were increased by Pb²⁺. The polydispersity index of all samples was on the upper limit of a monodisperse sample, but there is no indication of Pb²⁺ induced liposome aggregation.

5.1.3 Discussion of Pb^{2+} Interactions with RBC Total Extracts

The lipids in the RBC total extract LUVs represent a complex distribution of head groups and acyl tail lengths or saturations found within either leaflet of rabbit erythrocyte membranes (Figure 90). When considering ~50% cholesterol¹⁸⁵ as well, there are

potentially up to 8% anionic lipids available for electrostatic interactions in addition to the major zwitterionic lipids PC, PE and SM which have been identified as Pb²⁺ targets before (Chapter Three: Primary Lipid Compositions and 4.1 Interactions of Pb²⁺ with Membranes Containing Cholesterol (CHOL)).

Metal-induced GP changes occurred across the entire temperature scale and were similar in terms of rigidification to the cholesterol-containing membranes presented in Chapter Four: Simple Lipid Mixtures. As cholesterol raises the overall rigidity of membranes due to head group spacing, charge separation and reduced acyl tail mobility⁸⁹, the additional metal-induced rigidity is only possible until a maximum threshold is reached. This can explain the concentration independent Pb²⁺ rigidification where the higher 2.1 mM Pb²⁺ concentration may be saturating the total membrane rigidity changes possible.

LUVs formed with an average diameter of 155 and 180 nm for 25 and 37°C, respectively (Figure 92). The high content of cholesterol and PE may have increased resistance to size reduction and bilayer rupture during the LUV extrusion process. This could be responsible for a significantly larger LUV size than expected. The trials with 2.1mM Pb²⁺ was found to induce significant size increases of the LUVs, indicating significant membrane interactions. That is consistent with previous studies reporting abnormally large erythrocyte cells and a dose-dependent increase in the number of echinocytes¹⁰⁵.

Results for Pb^{2+} and the RBC extract LUVs suggest direct metal interactions with RBC lipids. Suwalsky *et al.*, was able to visualize these interactions by the adherence of Pb^{2+} masses extending 20 to 200 Å from both internal and external membrane surfaces¹¹⁹. The implications of these interactions may assist in explaining the extensive uptake of Pb^{2+}
into RBCs. The known uptake of Pb^{2+} through calcium^{51,65} and other metal transporters^{42,63,187}, including the anion exchange protein in RBCs¹⁸⁸, has been used to explain the extent of hematological dysfunction caused by Pb^{2+} uptake^{1,39}. Nevertheless, Ong and Lee, 1980a, found Pb^{2+} bound to the lipids fraction¹²³ and Diaz and Monreal have observed protein-independent permeation of PbOH⁺ through lipid membranes¹⁰³. The capability of Pb^{2+} to interact with each individual component as well as a total lipid extract of red blood cells suggest Pb^{2+} affinity to lipids within the membrane will contribute to the overall uptake of Pb^{2+} onto the membrane and into the erythrocytes.

5.2 Interactions of Pb²⁺ with Brain Polar Lipid Extracts

Lead uptake mechanisms have been studied in both excitable and non-excitable cells, predominantly occurring through Pb²⁺ imitation of calcium in protein channels^{13,63,65,169,180}. Lipids constitute up to 70% of the total mass of brain unlike most other cell membranes⁸⁶ which are more enriched in proteins. As Pb²⁺ has been described as a potent neurotoxin, it is important to better understand the effects of this metal on membrane fluidity and lipid packing. LUVs were formed from the brain polar extract by Jenelle Umbsaar and the Pb²⁺ data presented in this subsection was completed by her for this investigation. The composition of the brain polar extract provided by Avanti Polar Lipids is illustrated in Figure 93A. Due to a high composition of unknown components unidentified in the Avanti percent distribution, a ³¹P-NMR composition study on combined brain tissue from Pettegrew *et al.*, has been included to supplement our understanding of the brain lipids in these samples (Figure 93B).



Figure 93. (*A*) Average percentage distribution of lipid classes in the brain polar extract provided by Avanti Polar Lipids¹⁴⁷. (*B*) ³¹P-NMR phospholipid levels of brain tissue adapted from Pettegrew *et al.*, 2001¹⁸⁹.

In addition to phospholipids and sphingolipids, there is also an abundance of cholesterol which is reported at a 4:4:2 molar ratio with cholesterol, phospholipids and glycosphingolipids⁸⁶. Approximately 25% of the total cholesterol found in humans is localized to the brain, predominantly in the myelin⁸⁶. A large portion of PEs in the brain are plasmalogens⁹⁵, nearly entirely in the myelin while less so in the nerve plasma membranes¹⁸⁹.

Concerning anionic lipids, PS was first identified in whole brain lipid extracts by Folch in the $1940s^{190}$ and is present as 14 - 19% of brain lipids making this the most abundant negative lipid^{93,147,189}. It is worth noting that any non-phosphate containing lipids are not included in either percent composition listed in Figure 93.



Figure 94. (*top*) GP values of LUVs of 300 μ M Brain Polar Extract (black) with 30 μ M (green), 300 μ M (blue) and 1.2mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

Like RBC extracts, the GP of brain polar extracts were high, starting at +0.43 at 15°C, likely due to the large percentages of cholesterol and PE. Rigidity induced by Pb²⁺ occurred at concentrations as low at 30 μ M and became progressively larger with increasing temperature. The strongest rigidifying effects were noted at the highest temperatures with 1.2 mM Pb²⁺ when the membrane would be the most fluid in the control. As a result, the large changes in GP for 1.2 mM at 45 – 50°C increases the scale of the Δ GP contour plots causing no noticeable statistical increase of the GP for the lower Pb²⁺ concentrations. A secondary Δ GP contour plot displaying Pb²⁺ concentrations of 0 – 300 μ M only has been illustrated in Figure 95. A +0.05 increase in GP at 0.3 mM Pb²⁺ is larger than some interactions of 2.1 mM Pb²⁺ with systems such as POPC (+0.01), DMPC (+0.035), 30% POPE (+0.039), and each PC/SM mix with CHOL (up to +0.035).



Figure 95. Contour plot of the change in GP for LUVs of 300 μ M Brain Polar Extract across increasing temperature (°C) and Pb²⁺ concentrations (0 – 300 μ M only).

5.2.2 DLS of Brain Polar Extracts with Pb²⁺



Figure 96. The average vesicle diameter of 0.3 mM Brain Polar Extract LUVs in the presence of 0 to 2.1mM Pb²⁺. Results are the average of 3 replicates \pm SD. Asterisks denote statistical significance (** = p < 0.01, **** = p < 0.00001).

Highly significant increases to LUV sizes were observed from equal concentrations of Pb^{2+} to LUVs up to metal excess at 2.1 mM Pb^{2+} . The polydispersity index of every replicate in these results was all below 9% suggesting very monodisperse populations. In the presence of Pb^{2+} , the size increases can be attributed to LUV swelling and there is no indication of liposome aggregation.

5.2.3 Discussion of Pb²⁺ Interactions with Polar Brain Extracts

The lipids included in these brain polar extract LUVs represent a complex distribution of head groups and acyl tail lengths or saturations found within either leaflet of brain membranes (Figure 90). When including ~40% cholesterol⁸⁶, there are up to 15% anionic phospholipids as well as the gangliosides, sulfatides and other glycosphingolipids within the brain at unknown amounts available for electrostatic interactions. Additionally,

the major zwitterionic lipids PC, PE, and SM, which have been identified as Pb²⁺ targets, are present in high percentages.

Laurdan GP results in Figure 94 exhibited significant rigidification at low Pb²⁺ concentrations of one-tenth of the molar ratio of LUVs in solution. Effects at such low concentrations have only been seen for pure PS systems. These results suggest that even very low amounts of Pb²⁺ may induce membrane effects in this complex lipid profile. The increasing GP for 1.2 mM at higher temperatures suggests that the binding of Pb²⁺ restricts the increased mobility of more fluid membranes and is potentially ordering the lipids for preferential coordination for this metal. DLS results agree with the rigidification seen by GP experiments. Very significant size increases to LUVs were detected at increasing Pb²⁺ concentrations without any signs of aggregation.

The interactions reported here and the concomitant ordering of brain polar extract lipids membranes by Pb^{2+} have never been reported before. Implications of these direct interactions may supplement the current knowledge of the extensive neurotoxicity of Pb^{2+} poisoning. Other polar lipid extracts including heart, liver, yeast, and *E. coli* have also been scanned and are included in Appendix A.

5.3 Interactions of Pb²⁺ with the Myelin Sheath – Cytoplasmic Leaflet

The myelin uniquely contains a much higher content of lipids than other biological membranes, approximately 70% by weight⁸⁶. Myelin sheaths are created through extensions of oligodendrocyte cells wrapping around $axons^{191}$. The bilayers wrapped within the few tens of micrometers thick sheaths have significantly different lipid and protein content in opposing leaflets. It is often referred to as repeat units of alternating cytoplasmic and extracellular leaflets with 3 – 4 nm of aqueous layers between these

bilayers¹⁹¹. Cerebrosides, originally rich in the extracellular leaflet of oligodendrocytes, are maintained during the formation of the myelin multilamellar structure in the same leaflet while the cytoplasmic leaflet contains more PE and PC⁹⁷. The asymmetry of myelin bilayers is important for the formation and maintenance of the sheath to act as a capacitor on axons, allowing for faster conduction of nerve impulses¹⁹².

Present studies of Pb²⁺ show metal–induced demyelination and remyelination events¹⁷² causing regionally disturbed morphologies and loosely arranged myelin membranes¹⁸¹. This has significant consequences for the speed of nerve conduction, potentially causing impairments of motor, sensory and possibly cognitive skills¹⁹². Additionally, Pb²⁺ utilizes store-operated and voltage-sensitive calcium channels causing intracellular calcium depletion^{51,65}. These interactions between Pb²⁺ and the lipids of the myelin membranes are vital to understanding the overall consequences of Pb²⁺ neurotoxicity.

The lipid compositions of the outer and inner leaflets was determined by Min *et al.*⁹⁷, Inouye and Kirschner⁹⁶, and O'Brien and Sampson⁹⁵. Brain lipids compositions presented by O'Brien and Sampson included isolated grey, white and myelin lipids from 10 months up to 55 years old frontal lobes of humans. This addressed the lipid content across these different regions and its changes with age⁹⁵. However, up to 9% of the lipid dry weight remained unidentified. Inouye and Kirschner added calculations of surface charge density and data to determine the distribution of specific lipid fractions between cytoplasmic and extracellular sides of the myelin bilayer⁹⁶. Finally, Min *et al.*, applied this distribution of the myelin bilayer asymmetry to the total lipid composition to create model myelin monolayers.

The lipid composition of the inner myelin leaflets is illustrated in Figure 97. Phosphatidylinositol (PI) lipids were not included in the model myelin monolayers by Min *et al.*, ⁹⁷ however they are present at approximately 1 mol % in most cell plasma membranes including the cytoplasmic leaflet of the myelin⁹⁴. The inner myelin mimic presented in this study also omits 1% PI lipid content as the phosphorylation of the inositol head group of PI lipids at one of three possible positions gives rise to eight different lipid species¹⁹³. Phosphatidylinositol-4,5-bisphosphate (PI(4,5)P₂) is the most abundant PI lipid as it is well known to interact with different proteins that regulate cellular processes such as the myelin basic protein (MBP)^{94,180,193}. A full investigation of the binding of Pb²⁺ to each PI lipid at 1 mol % within a POPC matrix will be investigated in bilayer studies in Chapter 6 and monolayer studies in Chapter 7.



Figure 97. Percent composition of the inner myelin leaflet as adapted from literature^{95–97}.

Starting with the cytoplasmic myelin leaflet, the largest constituents are PEplasm, PC and cholesterol. A simple mixture of these lipids was investigated in Chapter 4 (29% PEplasm, 32% CHOL and 39% POPC) and observed to have significant disordering to the

membrane packing arrangements upon Pb^{2+} incubations (Figure 76) which was not detected by GP (Figure 74).

5.3.1 Laurdan GP of the Inner Leaflet of the Myelin Sheath with Pb^{2+}

GP results for LUVs formulated with the biomimetic lipid composition of the inner leaflet of myelin sheaths as 29% PEplasm, 32% CHOL, 26% POPC, 7% POPS, and 6% Brain SM are shown in Figure 98. The addition of PS and brain SM to this mixture has completely changed the GP outcome of Pb^{2+} interactions from Figure 74 whereby significant increases in GP up to +0.08 were detected. Rigidification was observed as low as 0.3mM Pb²⁺ and increased as a function of temperature.



Figure 98. (*top*) GP values of LUVs of 300 μ M Myelin Sheath Inner Leaflet (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

5.3.2 DLS of the Inner Leaflet of the Myelin Sheath with Pb^{2+}



Figure 99. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM Myelin Sheath Inner Leaflet (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).

Significant size increases to the myelin sheath inner leaflet mimic were observed with 2.1mM Pb²⁺ (Figure 99). The polydispersity index for the Pb²⁺ trial was on the upper limit of 0.2 and higher across the entire temperature range suggesting Pb²⁺-induced aggregation. The DLS count rate data shows increased ordering of lipids with 2.1 mM resulting in a higher reflection of light into the detector. This change in membrane packing relative to the control is reduced at higher temperatures where GP data showed the highest rigidification. No equivalent changes to size were detected at the higher temperatures.

5.3.3 Discussion of Pb²⁺ Interactions with Myelin Sheath Inner Leaflet

In Chapter 4, three of the major components of the myelin inner leaflet were combined (29% PEplasm, 32% CHOL, and 39% POPC) into a lipid mixture. GP results did not indicate any membrane fluidity changes in the presence of Pb²⁺ (Figure 74) while DLS results exhibited an increase in order of the lipids at higher temperatures and large LUV size increases across the entire temperature range (Figure 76). The rigidification at higher temperatures was suggested to occur due to Pb²⁺ coordination in regions of demixed lipids allowing for a shift in the phase transition of PEplasm. The significant size increases of the LUVs, which was also seen for the inner myelin leaflet mimic, may be a result of increased water penetration of PEplasm lipids. The change of the carboxyl ester to an enol ether in plasmalogens reduces the membrane interface polarity causing a stronger water adsorption than diacyl-linked PE lipids^{77,166}.

GP results of Pb²⁺ interactions with the myelin sheath inner leaflet (29% PEplasm, 32% CHOL, 26% POPC, 7% POPS, and 6% Brain SM) however, indicated changes of membrane fluidity. The incorporation of PS and brain SM into this biomimetic profile greatly enhanced the rigidification of the membrane by Pb²⁺ species. The high content of cholesterol and PE lipids promoted a very rigid membrane which was tightly ordered through Pb²⁺ coordination on the membrane surface (Figure 98). The contrast between higher GP rigidification and lower DLS count rates at higher temperatures may suggest that binding of Pb²⁺ to the membrane in the more fluid phase restricts this fluidity through a less structured and organized membrane resulting in a lower reflection of light into the detector.

5.4 Interactions of Pb²⁺ with the Myelin Sheath – Extracellular Leaflet

The last tests for Pb²⁺-associated interactions with brain lipids to causing widespread neurotoxicity in humans is the extracellular leaflet of the myelin sheath. The biological composition reflects the intercellular signaling and glycosylated lipids present on the outside of oligodendrocyte cells⁹⁸. Lipids such as cerebrosides and sulfatides have a major function in packing the membrane into stable, long-lived, insulating sheaths for axons⁹⁴.

The lipid composition of the outer myelin leaflets has been illustrated in Figure 100. Each of the components have been investigated in pure LUVs in Chapter 3 or simple mixtures with POPC at relevant mol % concentrations in Chapter 4. The overview of the previous results indicated ordering of the membrane based on laurdan GP and swelling of the LUVs as reported by DLS. The degree of these Pb²⁺ interactions ranged from strong interactions with 100% POPS (+0.10) to weaker, less significant interactions for 3.5% Gal Cer 96.5% POPC (+0.009).



Figure 100. Percent composition of the outer myelin leaflet as adapted from literature^{95–97}.

To better understand each component of this complex myelin leaflet composition, each of the lipids enriched in the brain (cerebrosides and sulfatides) will first be formulated into LUVs with the remaining lipid components (33% CHOL, 9% PEplasm, 4.5% Brain SM, 1% POPS and POPC to make up the remainder mol % of the system). Hydroxylated and non-hydroxylated Gal Cer will then be added to this mixture to directly compare these structurally similar lipids. Finally, the complete lipid profile of the outer myelin leaflet was formulated into LUVs as 21% (2R-OH) Gal Cer, 3.5% Gal Cer, 10% Brain Sulfatides, 33% CHOL, 18% POPC, 9% PEplasm, 4.5% Brain SM and 1% POPS⁹⁷ as depicted in Figure 100. Each of these lipid systems is described in Table 5.

Table 5.	. Lipid	composition	s of LUVs	formulated	in this	study f	for the out	ter leaflet	t of the
myelin s	heath.								

#	System Names	Lipids				
		10% Brain Sulfatides, 33% CHOL,				
1	10% Sulfatides Mix	42.5% POPC, 4.5% Brain SM, 9%				
		PEplasm, 1% POPS				
2		3.5% Gal Cer, 33% CHOL, 49% POPC,				
	3.5% Gal Cer Mix	4.5% Brain SM, 9% PEplasm, 1%				
		POPS				
		21% (2R-OH) Gal Cer, 33% CHOL,				
3	21% (2R-OH) Gal Cer Mix	31.5% POPC, 9% PEplasm, 4.5% Brain				
		SM, 1% POPS				
	Cerebrosides Mix or	21% (2R-OH) Gal Cer, 3.5% Gal Cer				
4	21% (2R-OH) Gal Cer and	33% CHOL, 28% POPC, 9% PEplasm,				
	3.5% Gal Cer Mix	4.5% Brain SM, 1% POPS				
		21% (2R-OH) Gal Cer, 10% Brain				
5	Muelin Outer Leoflet Mimie	Sulfatides, 3.5% Gal Cer, 33% CHOL,				
	Wyenn Outer Leanet Winnie	18% POPC, 9% PEplasm, 4.5% Brain				
		SM, 1% POPS				



Figure 101. (*top*) GP values of 300µM 10% Sulfatides, 33% CHOL, 42.5% POPC, 4.5% Brain SM, 9% PEplasm, 1% POPS (black) with 0.5µM (green), 300µM (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (µM).

GP results for 10% Brain Sulfatides 90% POPC from Chapter 4 showed rigidification by Pb²⁺ (+0.016 Δ GP), potentially through the accessibility of the PC phosphate by packing defects between lipids or coordination with the sulfate groups in the bulky head groups of the sulfatides (Figure 81). The inclusion of 33% CHOL, 9% PEplasm, 4.5% Brain SM, and 1% PS lipids into 10% Sulfatides and the remaining 42.5% POPC prompted larger rigidification (+0.03) by Pb²⁺ at 2.1 mM which increased as a function of temperature (Figure 101).

GP results for LUVs formulated with 3.5 % Gal Cer, 33% CHOL, 49% POPC, 9% PEplasm, 4.5% Brain SM, and 1% POPS are shown in Figure 102. When Gal Cer was tested with POPC alone, GP results showed little to no significant interactions with Pb²⁺, suggesting the protruding galactosyl head group potentially hinders the accessibility of the PC phosphate linker (Figure 85). In the myelin Gal Cer Mix, Pb²⁺ is exhibiting much stronger rigidification with a +0.023 change in GP which appears to be temperature dependent for equimolar concentrations and higher (Figure 102).



Figure 102. (*top*) GP values of 300 μ M 3.5% Gal Cer, 33% CHOL, 49% POPC, 4.5% Brain SM, 9% PEplasm, 1% POPS (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).



Figure 103. (*top*) GP values of 300 μ M 21% (2R-OH) Gal Cer, 33% CHOL, 31.5% POPC, 9% PEplasm, 4.5% Brain SM, 1% POPS (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

The hydroxylated Gal Cer mix with 21% (2R-OH) Gal Cer, 33% CHOL, 31.5% POPC, 9% PEplasm, 4.5% Brain SM, and 1% POPS exhibited a rigidification very similar to the 3.5% Gal Cer mix (+0.024 versus +0.023) however the effect by Pb²⁺ appeared to saturate when Pb²⁺ was in excess of the molar concentration of the LUVs in solution and less temperature dependence is illustrated by the contour plots of Figure 103. Regarding 21% (2R-OH) Gal Cer 79% POPC, a similar lack of temperature dependence was noted however the maximal change in GP was only +0.015.

Lastly, the two cerebroside lipids were combined in the mixed myelin LUVs with 21% (2R-OH) Gal Cer, 3.5% Gal Cer, 33% CHOL, 28% POPC, 9% PEplasm, 4.5% Brain SM, and 1% POPS. GP results for these LUVs are shown in Figure 104. The absence of temperature independent Pb^{2+} interactions with 21% (2R-OH) Gal Cer present is maintained after the addition of 3.5% Gal Cer. At colder temperatures, significant rigidification was noted for low Pb^{2+} concentrations (0.5µM) however the total change in GP was not as effective as higher Pb^{2+} concentrations and higher temperatures (Figure 104).



Figure 104. (*top*) GP values of 300 μ M 21% (2R-OH) Gal Cer, 3.5% Gal Cer 33% CHOL, 28% POPC, 9% PEplasm, 4.5% Brain SM, 1% POPS (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP across increasing temperature (°C) and Pb²⁺ (μ M).



Figure 105. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 10% Brain Sulfatides, 33% CHOL, 42.5% POPC, 9% PEplasm, 4.5% Brain SM, 1% POPS (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).

GP results for the mixed system with sulfatides noted the largest increases in GP induced by Pb²⁺ for these initial mixed systems (+0.03, Figure 101). DLS results with Pb²⁺ exhibited similar membrane ordering throughout the temperature range corresponding to the swelling of the LUVs.



Figure 106. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 3.5% Gal Cer, 33% CHOL, 49% POPC, 9% PEplasm, 4.5% Brain SM, 1% POPS (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).

A similar trend in the DLS count rate data emerged in the presence of 3.5% Gal Cer with a change in the surface characteristics upon increasing temperatures which caused a loss in the count rate and LUV size increases. Figure 86 of 3.5% Gal Cer and 96.5% POPC showed a similar trend, packing deficiencies and potentially a lower phase transition of Gal Cer was observed for both the simple and mixed LUV systems.



Figure 107. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 21% (2R-OH) Gal Cer, 33% CHOL, 31.5% POPC, 9% PEplasm, 4.5% Brain SM, 1% POPS (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ size is statistically significant, asterisks denote count rate curves (p < 0.05).

The DLS polydispersity index of the LUVs in the absence of Pb^{2+} were on the upper limit for a monodisperse size distribution while the Pb^{2+} trials exhibited high polydispersity and possibly liposomal aggregation due to Pb^{2+} binding. Structural perturbations on the membrane are represented by the reduced count rates of this lipid mixture at higher temperatures whereby Pb^{2+} interactions disorder the membrane organization.



Figure 108. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM 21% (2R-OH) Gal Cer, 3.5% Gal Cer, 33% CHOL, 28% POPC, 9% PEplasm, 4.5% Brain SM, 1% POPS (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).

Comparable with DLS results for 21% (2R-OH) Gal Cer mixed system, LUVs consisting of 21% (2R-OH) Gal Cer, 3.5% Gal Cer, 33% CHOL, 28% POPC, 9% PEplasm, 4.5% Brain SM, and 1% POPS were on the upper limit of a monodisperse LUV population whilst 2.1 mM Pb²⁺ trials induced complete liposomal aggregation with PDI values averaging 100 % polydispersity across the entire temperature range (monodisperse limit is 20%¹³⁶). The reduced count rate is likely the result of Pb²⁺-induced disordering of the membrane.

5.4.3 Discussion of Pb²⁺ Interactions with Preliminary Outer Myelin Mixed Components

GP and DLS data for 10% Brain Sulfatides with 90% POPC exhibited contradicting results for Pb²⁺ interactions at higher temperatures (Section 4.4.3 Laurdan GP of Brain Sulfatides and POPC with Pb). Packing defects, altered water penetration, steric hindrance due to bulky head groups, and sulfate-Pb²⁺ coordination were considered as potential explanations of how Pb²⁺ could affect these membranes. The incorporation of CHOL, PEplasm, SM and PS lipids into Sulfatides/PC liposomes caused moderate rigidification (GP of +0.03) which was supported by DLS observations of significant membrane ordering and LUV size increases across the entire temperature range.

As Gal Cer and POPC LUVs did not exhibit these rigidifying effects upon Pb^{2+} incubations, the change in GP seen for the more complex mixed system (+0.023) was likely due to the presence of the minor components such as PS which was shown to strongly interact with Pb^{2+} (Figure 40). In comparison with the 10% sulfatides mixture (+0.03), a favourable interaction with sulfatides would cause this additional rigidification in the LUVs containing mixed Gal Cer. DLS results for simple and mixed Gal Cer LUVs demonstrate a temperature-dependent change of membrane organization (Figure 85 and Figure 106). Optimal orientations which promote Pb^{2+} binding to the membrane may be enhanced at colder temperatures and tighter packing. Increasing temperatures in the presence of Pb^{2+} may induce lipid demixing where Gal Cer lipids undergo a upshift of their phase transition⁸¹, as suggested by the sigmoidal nature of the count rate data.

The presence of the 2' hydroxylation was suggested to coordinate Pb²⁺ species to overcome the steric hindrance of the bulky head groups as an explanation of the higher membrane order with 2R-OH Gal Cer over Gal Cer in POPC LUVs (Section 4.5

209

Interactions of Pb²⁺ with Ceramide and Cerebroside Membranes). In the presence of additional lipids, changes to the packing arrangements are substantially larger causing rigidification of up to +0.024 and aggregation of the LUVs with 2.1 mM Pb²⁺. Cholesterol may have stronger associations with the hydroxylated sphingolipids and sphingomyelin present due to the enhanced hydrogen bonding network allowing for the formation of lipid rafts *in vivo*^{194,195}. This interaction could induce demixing and lipid domain formation thereby altering Pb²⁺ interactions with glycerophospholipids. The total rigidification of the membranes containing non-hydroxylated and hydroxylated Gal Cer mixtures are very similar however the disordering of the membrane detected by DLS for the hydroxylated lipids would suggest that the hydroxylation alters the lipid packing profile of the membrane.

The combination of these cerebrosides in the mixed LUVs with 21% (2R-OH) Gal Cer, 3.5% Gal Cer, 33% CHOL, 28% POPC, 9% PEplasm, 4.5% Brain SM, and 1% POPS continued to show temperature independent interactions of Pb²⁺ with the membrane. Unlike the 2R-OH Gal Cer mixture, Pb²⁺ induced such large LUV size changes that the polydispersity index values were averaging 1.0 across the entire temperature range where values greater than 0.7 indicate a very broad size distribution¹³⁶ (Figure 108). Pb²⁺-induced aggregation of the mixed cerebroside and myelin mimic models highly suggest increased water penetration and packing defects due to clustering of lipid molecules.





Figure 109. (*top*) GP values of 300 μ M 21% (2R-OH) Gal Cer, 10% Brain Sulfatides, 3.5% Gal Cer, 33% CHOL, 18% POPC, 9% PEplasm, 4.5% Brain SM, 1% POPS (black) with 0.5 μ M (green), 300 μ M (blue) and 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP across increasing temperature (°C) and Pb²⁺ (μ M).

GP results of LUVs formulated with the biomimetic lipid composition of the outer leaflet to myelin sheaths with 21% (2R-OH) Gal Cer, 10% Brain Sulfatides, 3.5% Gal Cer, 33% CHOL, 18% POPC, 9% PEplasm, 4.5% Brain SM, 1% POPS are shown in Figure 109. The inclusion of brain sulfatides in this lipid composition when compared to the mixed cerebroside myelin LUVs further increases rigidification with GP increases of +0.034 (from +0.027) and interactions with Pb²⁺ appears to be temperature and Pb²⁺ concentration dependent.



5.4.5 DLS of Myelin Sheath Outer Leaflet with Pb^{2+}

Figure 110. DLS determination of the count rate (x10⁴ kcps) (solid) and vesicle diameter size (nm) (dashed) of 0.3mM Myelin Sheath Outer Leaflet (black) with 2.1mM Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Data normalized using non-linear Boltzmann regression. All Pb²⁺ curves are statistically significant (p < 0.05).

The total change in LUV size for 21% (2R-OH) Gal Cer, 10% Brain Sulfatides, 3.5% Gal Cer, 33% CHOL, 18% POPC, 9% PEplasm, 4.5% Brain SM, and 1% POPS was not as polydisperse as seen for the mixed cerebroside and 2.1mM Pb²⁺ trials in Figure 108. Control trials of the outer myelin mimic were also on the upper limit of a monodisperse size population while Pb²⁺ caused a higher polydispersity to approximately 0.35 across the entire temperature range. This would suggest some Pb²⁺-induced aggregation is occurring and the count rate data corresponds to higher surface changes causing scattering of the light on the surface of the membrane.

5.4.6 Discussion of Pb²⁺ Interactions with Myelin Sheath Outer Leaflet

In Section 5.4.3 Discussion of Pb²⁺ Interactions with Preliminary Outer Myelin Mixed Components mixed Gal Cer liposomes exhibited rigidification of the membrane predominantly through the incorporation of CHOL, PEplasm, SM and PS as simple Gal Cer/PC LUVs had little to no GP increases (Figure 85). The relative increase in rigidification seen for mixed sulfatide LUVs suggested a direct Pb²⁺-interaction to promote binding at the membrane. The sulfate was expected to interact with Pb²⁺ given the reactivity of Pb²⁺ for sulfhydryl groups of various proteins and sulfhydryl-containing molecules such as glutathione^{13,18,169} or metallothionein^{36,46,170,171}. Direct comparison of the cerebroside mixed LUVs to the final outer myelin sheath leaflet mimic can be made where 10% brain sulfatides was the final lipid component added to the profile. The total increase in GP was higher than any preliminary trial of outer leaflet model systems (+0.034). The rigidification demonstrated a temperature and Pb²⁺ concentration dependent manner unlike other 2R-OH Gal Cer liposomes. DLS results also exhibited significant LUV swelling, Pb²⁺-induced aggregation, and altered packing of the membrane causing decreased count rates (Figure 110). Pb²⁺ binding may be triggering larger packing defects in the membrane through lipid demixing. The strong hydrogen bonding interactions found between sphingolipids, cholesterol and especially hydroxylated cerebrosides may facilitate domain formation^{94,98,121}. These lipids contain very large head groups which likely protrude from the membrane and scatter light as well as reported by DLS. Overall, 2.1 mM Pb²⁺ induced membrane packing disturbances causing significant LUV swelling and rigidification of the myelin sheath outer leaflet biomimic of 21% (2R-OH) GalCer, 10% Brain Sulfatides, 3.5% Gal Cer, 33% CHOL, 18% POPC, 9% PEplasm, 4.5% Brain SM, and 1% POPS^{95–97}.

The interactions of Pb^{2+} with the myelin sheath have been reported in literature as rapid myelin degeneration, regionally disturbed morphologies and loosening of the myelin membranes^{172,181}. As Diaz and Monreal reported protein-independent PbOH⁺ permeation through myelin lipid liposomes¹⁰³, the observation of direct Pb^{2+} – leaflet binding by this biomimetic study helps explain how Pb²⁺ associates with several micrometers thick sheaths of lipid bilayers. The myelin is by nature a fluid membrane, lipids must be able to move laterally unless endocytosis or vesicle transport is initiated⁹⁴. Additionally, the myelin is metabolically-inert with limited ability to activate channels for ion transport to maintain homeostasis⁹⁴. Thus, the rigidification induced by Pb²⁺ and subsequent alterations to proton and ion concentrations may be the root cause of myelin decompaction and instability seen with Pb²⁺ poisoning^{172,181}.

5.5 Summary of Pb²⁺ Interactions with Biomimetic Compositions and Polar Extracts

Figure 111 provides a comparison of the effect of different Pb²⁺ concentrations on each lipid system within this chapter at temperatures corresponding to the beginning or end of each specific lipid temperature range tested.



Figure 111. The change in laurdan GP at a low (blue) or high (red) temperatures within the range tested for each lipid system within Chapter 5 when exposed to different concentrations of Pb^{2+} as shown. Results are the average of 3 replicates \pm SD.

Chapter Six: Bilayer Study of PI and PC lipids

Phosphatidylinositol (PI) lipids constitute a minor component of most plasma membranes but play a critical role in signaling pathways. PIs control several aspects of a cell's regular functioning as the source of secondary messengers from cell surface receptor pathways such as the G-protein-coupled receptor, receptor tyrosine kinases and activated phospholipase C enzymes^{155,193,196}. Availability of small PI pools is necessary for cell growth, division, homeostasis and membrane organization^{69,94,155,197,198}. PI signaling pathways operate through the interconversion of specific PI lipids whereby electrostatic properties of the PIs and surrounding lipids drive these protein-lipid associations¹⁹⁹.

PIs vary in their structure in the position and extent of the phosphorylation at the inositol head group at the 3', 4' and/or 5' positions giving rise to eight different lipid species^{193,196} as depicted in Figure 112. The PIs investigated in this study include commercially available structural variants of major signaling lipids and precursors: DOPI, DOPI(3)P, DOPI(4)P, DOPI(5)P, DOPI(3,4)P₂, DOPI(4,5)P₂, DOPI(3,5)P₂, and DOPI(3,4,5)P₃. The presence of PIs results in regions of enhanced charge density in the membrane¹⁹⁹. Kooijman *et al.*, reported that PI(3,5)P₂, where the two phosphomonoester groups are separated by the 4' hydroxyl group, has an overall charge of -3. The two vicinal phosphomonoester groups in PI(3,4)P₂ and PI(4,5)P₂ lipids share a proton, bringing the total lipid charge to -4. PIP₃ lipids also have complex ionization behaviour giving a lower charge on the P4 group leading to an overall charge of -5^{122} .

In the bilayer, orientation of the head group will determine the accessibility of PI lipids for metal binding¹⁹⁸. There is uncertainty about the true conformational characteristics of PIs in bilayers as reported by molecular dynamic simulations. Li *et al.*,

indicated that the inositol rings of PI(4,5)P₂ and PIP₃ in POPC bilayers are tilted at ~40° with respect to the bilayer plane as compared to ~17° for the PC head groups¹⁹⁷. They showed a small depression in the z-height of the POPC phosphates, allowing for PI(4,5)P₂ and PIP₃ to protrude slightly into the solvent, ~5.5 Å for the P4 atoms above the average PC phosphate plane¹⁹⁷. Lupyan *et al.*, investigated the 2' and 6' positions of the inositol ring which cannot be phosphorylated for hydrogen bonding. They observed the 2-hydroxyl and 6-hydroxyl groups to hydrogen bond with phosphate groups of neighbouring lipids ~61% and ~81% of simulation time, respectively²⁰⁰. Lupyan *et al.*, also calculated that PI(4,5)P₂ head groups are tilted at ~42° from the bilayer plane with protrusion of the P4 and P5 atoms at ~6 and 5 Å from the membrane backbone, respectively²⁰⁰.



Figure 112. Head group structures of PC and PI structures used in this study.

Bradshaw *et al.*, 1997 found the orientation of the inositol ring in PI(4)P to extend the 5-hydroxyl group out into the aqueous solution making the 5' position the most accessible site within the structure²⁰¹. This tilt of the P4 group is suggested due to an electrostatic attraction between the negative phosphate and positive PC choline²⁰¹. Neutron diffraction studies by Bradshaw *et al.*, 1999 however, demonstrated that a 50:50 molar mixture of DMPI and DMPC resulted in orientations of the inositol ring being close along the bilayer normal, projecting directly out into the water. This orientation may be a result of coordinating 2' and 6' hydroxyl group hydrogen bonding while minimizing the steric hindrance of the inositol ring and maximizing hydration of the head group when no phosphorylation is present²⁰².

The structural features of PIs are crucial to understand the effects of metals at the membrane surface. Liposomes composed of 1 mol % PI and 99 mol % POPC were made to investigate interactions between Pb^{2+} and PI lipids at physiologically – relevant compositions.

6.1 Interactions of Pb²⁺ with Phosphatidylinositol (PI) LUVs

6.1.1 Laurdan GP of LUVs with DOPI and POPC

GP results for LUVs formulated with 1% DOPI and 99% POPC are shown in Figure 113 below. With 2.1 mM Pb²⁺, rigidification of pure PC LUVs was only observed at higher temperatures (Figure 27). Experiments performed with 1 mol % DOPI, which is the simplest PI lipid containing an unphosphorylated inositol ring, show insignificant changes to GP throughout most of the temperature range. Statistical rigidification is noted with 2.1mM Pb²⁺ at 15 and 50°C however the relative change in GP likely does not have any physical meaning to the enhanced binding of Pb²⁺ at these temperatures.



Figure 113. (*top*) GP values of LUVs of 300 μ M 1% DOPI 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).


Figure 114. The average vesicle diameter of 300μ M 1% DOPI 99% POPC LUVs in the presence of 0 to 2.1mM Pb²⁺. Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05).

DLS results confirmed the minimal interactions between Pb^{2+} and DOPI/POPC LUVs as reported by GP. While 75 and 900 μ M Pb^{2+} concentrations induced statistically significant changes of the vesicle diameter, no large changes to LUV sizes were observed across the entire Pb^{2+} concentration range.

6.1.3 Discussion of Pb^{2+} Interactions with PI and PC LUVs

As the structurally simplest PI, the lack of phosphorylation on the inositol ring limits the only negative charge to be in the phosphate linker to the glycerol backbone. This lipid is a precursor of all signaling PIs and served as a control for Pb²⁺ interactions to determine if the large, neutral inositol ring would reduce or prevent Pb²⁺ binding. Reduced interactions of 2.1 mM Pb²⁺ with 1 mol % DOPI 99% POPC compared to pure POPC

LUVs suggests that the accessibility of the negative charge from the aqueous bulk is lowered due to steric hindrance of the head group. Bradshaw *et al.*, reported that DMPI lipids orientated perpendicular to the plane of the lipids, projecting directly out of the membrane²⁰².

6.2 Interactions of Pb²⁺ with Phosphatidylinositol-phosphate (PIP) LUVs

As the backbone phosphate of PIs were not accessible by Pb²⁺, PI lipids containing a single phosphate group at the 3', 4' or 5' position were studied. These lipids are expected to carry an overall charge of -2 due to the presence of one phosphate group in the head group and another connected to the glycerol backbone. The first lipid studied was PI(3)P which is phosphorylated at the 3' position on the inositol ring. PI(3)P are important for vesicle transport in endosomal compartments ^{203,204} through interactions with proteins containing FYVE and PX domains¹⁹³.

6.2.1 Laurdan GP of LUVs with DOPI(3)P and POPC

GP results for 1% DOPI(3)P 99% POPC LUVs are shown in Figure 115. GP results for LUVs composed of 1% DOPI(3)P indicated rigidification of the membrane at lower temperatures for 1.2 and 2.1mM Pb²⁺ (Figure 115). There appears to be a concentration – dependent GP increase throughout the temperature range however values are only significant below 25°C.



Figure 115. (*top*) GP values of LUVs of 300 μ M 1% DOPI(3)P 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).



Figure 116. The average vesicle diameter of 300 μ M 1% DOPI(3)P 99% POPC LUVs in the presence of 0 to 2.1mM Pb²⁺. Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05).

Concentrations of Pb^{2+} of 75 μ M and higher induce swelling of the liposome diameter up to 17 nm suggesting Pb^{2+} binding spreads the head groups apart.

6.2.3 Laurdan GP of LUVs with DOPI(4)P and POPC

The second PIP lipid studied was PI(4)P which is phosphorylated at the 4' position of the inositol ring. PI(4)P are the predominant precursor of the most abundant $PI(4,5)P_2$ signaling lipids⁹⁴.



Figure 117. (*top*) GP values of LUVs of 300 μ M 1% DOPI(4)P 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

In contrast to PI(3)P, 1 mol % DOPI(4)P exhibited significant rigidification indicated by a GP of +0.036 at Pb²⁺ concentrations up to 1.2 mM. Excess Pb²⁺ at 2.1 mM resulted in less rigidification compared to 1.2 mM. The largest increases are occurring in the middle of the temperature range suggesting optimal Pb²⁺ – lipid coordination.



6.2.4 DLS of LUVs with DOPI(4)P and POPC

Figure 118. The average vesicle diameter of 300 μ M 1% DOPI(4)P 99% POPC LUVs in the presence of 0 to 2.1mM Pb²⁺. Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05).

The initial incubation of a low concentration of Pb^{2+} (15 μ M) appeared to reduce LUV size while the progressive addition of Pb^{2+} up to 2.1 mM increased LUV swelling. The effects of Pb^{2+} on PI(4)P was greater in magnitude over PI(3)P, with a total change in diameter of ~25 nm.



Figure 119. (*top*) GP values of LUVs of 300 μ M 1% DOPI(5)P 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

Lastly, PI(5)P was tested, which is phosphorylated on the 5' position of the inositol ring and removed further from the backbone than PI(3)P²⁰¹. GP results of 1% DOPI(5)P 99% POPC showed Pb²⁺-induced two-fold higher rigidification than pure POPC LUVs (+0.02 to +0.01). The effects of Pb²⁺ were significant as low as 30 μ M at higher temperatures but the largest increases to GP occurred at excess Pb²⁺ (Figure 119).

6.2.6 DLS of LUVs with DOPI(5)P and POPC



Figure 120. The average vesicle diameter of 300 μ M 1% DOPI(5)P 99% POPC LUVs in the presence of 0 to 2.1mM Pb²⁺. Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05).

Increases to vesicle diameter occurred at all Pb^{2+} concentrations. Similar to the trend seen for PI(4)P, Pb^{2+} caused LUV swelling from 125 nm up to 149 nm.

6.2.7 Discussion of Pb^{2+} Interactions with PIP and PC LUVs

The monophosphorylated PIP lipids, PI(3)P, PI(4)P, and PI(5)P, were considered to understand the binding preferences of Pb^{2+} for phosphates across the inositol ring. The

distance of the phosphates relative to the membrane backbone and thus relative metal accessibility of the positions is suggested to be the best for 5', 4' then 3'. Molecular dynamic simulations of PI(3)P indicated the tilt of the head group relative to the normal of the membrane plane as ~44° with P3 atoms tilted down towards the membrane¹⁹⁹. This may be due to favourable hydrogen bonding between the axial 2-hydroxyl groups hydrogen bonding with neighbouring lipid phosphates while all other hydroxyl groups are in equatorial positions²⁰⁰. Neutron diffraction studies also suggested an orientation of PI(4)P with the 3-hydroxyl group positioned down to allow intermolecular hydrogen bonds with neighbouring phosphates while the charged 4-phosphate is available for favourable electrostatic interactions with the positive choline groups of PC lipids²⁰¹. In this orientation, the 5-hydroxyl group is most accessible as it extends into the aqueous phase, available for the frequent conversion from PI(4)P to PI(4,5)P₂ for signaling pathways *in vivo*¹⁹³.

Results of Pb²⁺ binding appears to favour PI(4)P over PI(5)P with PI(3)P having the least metal accessibility and lowest rigidity effects. Rigidification was noted at lower temperatures only for excess Pb²⁺ with PI(3)P (+0.021) whereas PI(4)P and PI(5)P exhibited stronger interactions across the entire temperature range. For only 1 mol % composition of PI content, Pb²⁺ showed significant binding to PI(5)P (+0.018) and even stronger binding with PI(4)P (+0.036). The enhanced rigidification relative to 100% POPC (+0.01) suggests strong Pb²⁺ affinity for PI(4)P, PI(5)P and to a lesser extent, PI(3)P.

In terms of size changes, similar binding trends were observed for LUV swelling as seen for GP results. Higher concentrations of Pb^{2+} were required for significant vesicle size increases for PI(3)P. Pb^{2+} induced swelling of PI(4)P and PI(5)P diameters nearly twice that of PI(3)P (~16 nm vs ~25 nm).

228

6.3 Interactions of Pb²⁺ with Phosphatidylinositol-bisphosphate (PIP₂) LUVs

The next class of PI lipids were phosphorylated at two of the three possible positions on the inositol ring. Kooijman *et al.*, reported that $PI(3,5)P_2$, where the two phosphomonoester groups are separated by the 4' hydroxyl group, has an overall negative charge of -3 due to pK_a values of ~7.0 and ~6.6 for the 3- and 5-phosphates, respectively. The two vicinal phosphomonoester groups within $PI(3,4)P_2$ and $PI(4,5)P_2$ show a biphasic pH-dependent behaviour due to sharing of the last remaining proton. At physiological pH, the total charge for these PIP_2 lipids is -4^{122} .

The first lipid of this class studied was $PI(3,4)P_2$, phosphorylated at the 3' and 4' positions. $PI(3,4)P_2$ lipids are involved in the recruitment of signaling effectors such as pleckstrin homology (PH) domain-containing proteins^{193,205}. This lipid also represents the least-affected (3P) and most-affected (4P) positions from the PIP experiments of Pb²⁺ induced rigidification. Next, $PI(4,5)P_2$ was studied, containing two of the single positions at the 4' and 5' phosphates that resulted in the strongest rigidification. This PIP₂ molecule is the major polyphosphoinositide in mammalian cells for signal transduction pathways as the source of two second messengers, diacylglycerol (DAG) and inositol(1,4,5)trisphosphate (IP₃), for the development of signaling cascades in the plasma membranes^{122,155,193,197}. Molecular dynamic simulations of PI(4,5)P₂ in PCs suggest an average tilt of 41°C from the bilayer surface^{197,200}, whereas the head groups PIP lipids exhibited a 44° tilt from the bilayer normal²⁰¹ and non-phosphorylated PI lipids extend perpendicularly out of the membrane²⁰². Hydrogen bonding and phosphate to choline interactions between PI and PC may explain this closer orientation of the head group phosphates to the membrane surface.

Lastly for this series, PI(3,5)P₂ was considered, which is phosphorylated at the 3' and 5' positions. The separation by the 4' hydroxyl group makes this PIP₂ lipid only -3 in charge¹²². This PIP₂ lipid is a low-abundance phosphoinositide that predominates in endolysosomes for regulation of morphology, trafficking, autophagy and ion transport²⁰⁶. The orientation of this head group is unknown, the 3' and 5' positions represent opposite sides of the inositol ring which may incur lipid packing and metal accessibility issues. The prevalence of 2- and 6-hydroxyl hydrogen bonding²⁰⁰ may promote a perpendicular protrusion of this PIP₂ from the membrane alike PI lipids²⁰². This would allow space for 3' and 5' phosphates to interact with neighbouring PC choline groups however, this molecular orientation is speculative without molecular dynamic simulations.

6.3.1 Laurdan GP of LUVs with DOPI(3,4)P₂ and POPC

GP results for LUVs of 1% DOPI(3,4)P₂ in 99% POPC are shown in Figure 121. Pb²⁺ induced GP rigidification throughout the entire temperature scale. Like PI(3)P – Pb²⁺ trials (Figure 116), the largest GP increases (+0.025) also occurred at lower temperatures (Figure 121). The inclusion of the 4' phosphate to PI(3,4)P₂ caused rigidification at lower concentrations of Pb²⁺ across the temperature range as well.



Figure 121. (*top*) GP values of LUVs of 300 μ M 1% DOPI(3,4)P₂ 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).



Figure 122. The average vesicle diameter of 300μ M 1% DOPI(3,4)P₂ 99% POPC LUVs in the presence of 0 to 2.1 mM Pb²⁺. Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05).

Significant LUV size changes up to 20 nm were detected by DLS for 1% $DOPI(3,4)P_2$ LUVs at all Pb^{2+} concentrations.



Figure 123. (*top*) GP values of LUVs of 300 μ M 1% DOPI(4,5)P₂ 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

PI(4)P and PI(5)P lipids observed strong rigidification upon Pb^{2+} incorporation across the entire temperature range while the presence of 1% PI(4,5)P₂ exhibited fluidization of the membrane at low to equimolar concentrations from 20°C and higher. The changes in GP for higher Pb^{2+} concentrations were small increases at low temperatures while the remainder of the curve showed little to no changes of GP.

6.3.4 DLS of LUVs with DOPI(4,5)P₂ and POPC



Figure 124. The average vesicle diameter of 300μ M 1% DOPI(4,5)P₂ 99% POPC LUVs in the presence of 0 to 2.1mM Pb²⁺. Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05).

DLS results observed LUV swelling in 1% $DOPI(4,5)P_2$ up to 24 nm with increasing concentrations of Pb^{2+} .



Figure 125. (*top*) GP values of LUVs of 300 μ M 1% DOPI(3,5)P₂ 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

Like PI(4,5)P₂, 1 mol % DOPI(3,5)P₂ GP shows reduced interactions between Pb²⁺ and the lipids compared to GP results. Moderate rigidification occurs for equimolar concentrations of Pb²⁺ and higher from $40 - 50^{\circ}$ C.





Figure 126. The average vesicle diameter of 300μ M 1% DOPI(3,5)P₂ 99% POPC LUVs in the presence of 0 to 2.1 mM Pb²⁺. Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05).

Less significant changes to LUV sizes were detected for Pb^{2+} and 1% $PI(3,5)P_2$ LUVs. Despite the variance in sizing, the general trend suggests lower concentrations of Pb^{2+} cause vesicle diameter increases which is lost upon increasing concentrations.

6.3.7 Discussion of Pb^{2+} Interactions with PIP_2 and PC LUVs

The next class of PIs considered were the PIP₂ lipids which are larger and carry a charge of roughly -3 to -4 ¹²². Based on the known head group orientations^{197,199,200,205}, phosphate accessibility considerations and binding preferences seen through PIP lipid

interactions with Pb^{2+} , it was expected that the strongest interactions would occur with $PI(4,5)P_2$, $PI(3,4)P_2$ over $PI(3,5)P_2$. GP and DLS results, however, were not as expected.

There are no reports in the literature on the head group orientations of $PI(3,4)P_2$. The PI(3,4)P₂ inositol headgroup may also average a similar $\sim 41^{\circ}$ tilt of the inositol head group found in $PI(4,5)P_2$ studies^{197,200}. The respective tilt of the ring would likely orient the 3' position into the membrane for optimal electrostatic interactions between phosphate and choline, as seen for the 5' position of $PI(4,5)P_2$. Pb^{2+} appears to target $PI(3,4)P_2$ with moderate affinity. The overall -4 charges^{122,198} likely attract Pb²⁺ for binding and the higher Δ GP at lower temperatures seen for PI(3,4)P₂ and PI(3)P suggests an affinity of Pb²⁺ towards the 3' position for tighter packed membranes (Figure 121). This 3' position is reported as the closest position to the membrane surface when phosphorylated^{199,201}. The small hydrated radii and high electronegativity of Pb²⁺, when compared to other metals, may promote stronger coordination with the phosphates present in the membrane. DLS results confirm this interpretation of the Pb²⁺ lipid interactions as noted by the increased LUV sizes at all Pb²⁺ concentrations (Figure 122). A previous study using calcium found that $PI(3,4)P_2$ was a determining factor in Ca binding to liposomes of mixed compositions, suggesting a relatively high affinity as well²⁰⁷.

The planar organization of PC lipids may also be altered upon introduction of PIP₂. PC head groups have a natural tilt of 17° at the membrane surface which would likely reorient the positively charged choline groups towards the neighbouring negativelycharged phosphates. This may result in an altered conformation of lipids in the membrane as packing defects emerge when PI lipids are present compared to pure PC lipid compositions.

 $PI(4,5)P_2$ is the most abundant PI in the cell^{94,155,193,196}. It is an important second messenger and precursor molecule in signaling pathways and is thus a highly-studied lipid²⁰⁰. Interestingly, the metal effects on $PI(4,5)P_2$ assessed by GP showed fluidization at lower Pb²⁺ concentrations and higher temperatures (Figure 123) despite the results for single phosphorylations at the 4' or 5' positions indicated the most rigidification by Pb²⁺ (Figure 117 and Figure 119). However, DLS results showed LUV swelling at all Pb²⁺ concentrations. A molecular dynamics simulation of $PI(4,5)P_2$ reported that the average distance of P4 and P5 atoms were 5.99 Å and 5.10 Å from the phosphate linker (P1) of PCs. This suggests protrusion of these negative charges from the membrane surface which would make them highly accessible. Two molecular dynamic studies agree on an $\sim 41^{\circ}$ tilt from the membrane surface for PI(4,5)P₂ in PC and 100 mM NaCl¹⁹⁷ or 150 mM KCl²⁰⁰. A third study which incorporates PC, PS, PE, CHOL and 150 mM NaCl found the average angle only $\sim 13.9^{\circ}$ tilted from the membrane surface¹⁹⁸. The difference between these studies suggests that the association of PI head groups varies in the presence of different neighbouring lipids, such as a potential hydrogen bonding interaction of PE with the 2- or 6-hydroxyl groups. If the head group lies flatter along the membrane surface, fluidization of the membrane because of Pb^{2+} binding could be explained by destabilization of the PI hydrogen bonding network.

The orientation of $PI(3,5)P_2$ lipids are also more debatable due to the flanking 3' and 5' positions of the inositol ring. This may incur further lipid packing issues with a bulkier head group which may further affect metal accessibility. The reduced ΔGP values may reflect reduced accessibility as equimolar and higher concentrations only exhibited rigidification at higher temperatures. If the 2- and 6-hydroxyl group hydrogen bonding stabilizes these PIP₂ lipids to sit perpendicular to the membrane, neighbouring PC choline groups could turn inwards to facilitate electrostatic interactions with the phosphates. This would cause changes to the lipid packing and minimize lipid availability for Pb²⁺ binding. Additionally, the outward orientation of one or both phosphates within the PI head group may incur Pb²⁺ binding at a further distance from the membrane surface. This would cause reduced effects on the membrane surface properties such as fluidity or liposome size.

6.4 Interactions of Pb²⁺ with Phosphatidylinositol-trisphosphate (PIP₃) LUVs

The final lipid to consider was the fully phosphorylated PI(3,4,5)P₃, representing all positions with phosphate groups on the inositol ring. This lipid is also the largest and most negatively charged tested in this study at -5.05¹²². PIP₃ lipids are also secondary messengers controlling a broad range of membrane trafficking events²⁰⁸, cell proliferation and cytokinesis^{122,209}. The P4 atom of PIP₃ lipids was calculated to extend 5.5 Å further into the aqueous surroundings, ~1 Å beyond the other ring phosphates¹⁹⁷. The analysis of PIP₃ head group orientation suggests that the tilt of the inositol ring is also ~40° with respect to the bilayer surface. There is an oscillating twist angle between C3 to C5 molecules of the ring where 3' and 5' positions may be at equal height from the membrane (~0°) or twisted with either ring phosphate higher than the other ($\pm 35^{\circ}$)¹⁹⁷.



Figure 127. (*top*) GP values of LUVs of 300 μ M 1% DOPI(3,4,5)P₃ 99% POPC (black) with 30 μ M (green), 300 μ M (blue), 1200 μ M (yellow) and 2100 μ M Pb²⁺ (red) in 100mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm SD. Asterisks denote statistical significance (p < 0.05). (*bottom*) Contour plot of the change in GP for the LUVs across increasing temperature (°C) and Pb²⁺ concentrations (μ M).

The rigidification induced by Pb^{2+} occurs predominantly when the metal is in excess throughout the temperature range. Slight fluidization was observed at low Pb^{2+} concentrations and temperatures. The overall trend of ΔGP resembles the results for PI(3)P (+0.021, Figure 115), PI(5)P (+0.018, Figure 119) and PI(3,4)P₂ (+0.025, Figure 121) which found increased membrane rigidity at higher Pb²⁺ concentrations and lower temperatures. The ΔGP for 1% PIP₃ was +0.02 within the middle of the temperature range (Figure 127). Minor fluidization was detected at low Pb²⁺ concentrations and temperatures (ΔGP -0.012), similar to the effect of low Pb²⁺ concentrations on 1% DOPI(4,5)P₂ membranes (-0.015, Figure 123).





Figure 128. The average vesicle diameter of 300μ M 1% DOPI(3,4,5)P₃ 99% POPC LUVs in the presence of 0 to 2.1mM Pb²⁺. Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05).

Small increases in LUV size were detected by DLS across the concentration range. Significance of this slight swelling was lost between 75 and 300 μ M Pb²⁺. The largest size change was induced by 2.1 mM Pb²⁺ at 9 nm.

6.4.3 Discussion of Pb²⁺ Interactions with PIP₃ and PC LUVs

The final lipid tested was the fully phosphorylated PI(3,4,5)P₃, the largest and most negative PI lipid. In the cell, the PIP₃ pool is dynamic and its availability is important for membrane trafficking pathways^{122,208} or the interconversion to PI(4,5)P₂ through specific phosphatases^{193,203,204}, thus its regulation is tightly controlled¹⁹⁷. The orientation of this phosphorylated head group was reported as a 40° tilt off the membrane surface. A GP monitored rigidification was observed across 1% PIP₃ membranes when Pb²⁺ was in excess (1.2 mM and 2.1 mM) which reflects possible binding sites are available in the presence of multiple phosphate groups. The similarity of GP and DLS results between PI(5)P, PI(3,4)P₂ and PIP₃ suggest that the presence of each individual phosphate group is less impactful when multiple positions are phosphorylated. Additionally, the larger increase in GP rigidification seen for PIP₃ relative to PI(3,5)P₂ confirms the binding of Pb²⁺ to the 4' position, as supported by strong interactions with PI(4)P.

6.5 Summary of Pb²⁺ Interactions with PI Lipids

Pb²⁺ was observed to bind and induce similar changes in membrane fluidity and LUV size for many types of PIs in liposomes composed of 1 mol % PI lipids with 99 mol % POPC. The phosphorylated PI head group was confirmed as a major target, especially when monophosphorylated at the 4' position (Figure 117). Pb²⁺ interactions depended on specific head group structures giving remarkable effects with only 1% PI content, surpassing the previously determined Pb²⁺-PC interactions within the membrane (Figure 27). Figure 129 provides a comparison of the effect of different Pb²⁺ concentrations on each 1% PI lipid system within this chapter for laurdan GP at 5, 25 and 50°C and changing vesicle diameters.

PIP lipids served to understand preferential binding and metal accessibility at each position of the inositol ring, identifying PI(4)P as the favourable binding target over PI(5)P or PI(3)P. PIP₂ lipids demonstrated very unique interactions based on phosphate positions: PI(3,4)P₂ was moderately rigidified but more than PI(3,5)P₂ whereas PI(4,5)P₂ became fluidized by Pb²⁺ when the membrane was packed the tightest. Lastly, PIP₃ appeared to have increased membrane rigidification for the entire temperature range, similarly seen with PI(5)P, although the highest rigidification occurred in the middle of the temperature range. The fluidization at low temperatures and Pb²⁺ concentration for 1% DOPIP₃ membranes also resembles the effects by Pb²⁺ on PI(4,5)P₂ membranes suggesting that Pb²⁺ binding with each region of the 3', 4' and 5' positions is different. Lipids containing phosphorylation at each site (PIP₃) may be exhibiting an additive effect of each coordination event.



Figure 129. (A) The change in laurdan GP at a 5°C (blue), 25°C (green) or 50°C (red) temperature within the range tested and (B) the change in vesicle diameter for each lipid system within Chapter 4 when exposed to different concentrations of Pb^{2+} as shown. Results are the average of 3 replicates ± SD.

6.6 Implications of Pb²⁺ Interactions with PI Lipids

This diverse group of PI lipids are critical to signaling pathways in different membranes despite being a minor component of all lipids present^{193,210}. The PIs control vesicular trafficking^{206,208}, organelle biology through lipid distribution and metabolism^{211,212}, regulate ion channels and transporters^{213,214} and interact with several different proteins through PI-specific binding domains^{193,196,205,208,215}. The observed coordination of Pb²⁺ with specific PI classes will have implications on the membrane functioning for these signaling pathways. As expected for regulators of such diverse pathways, dysfunction of the PI metabolism and regulation is reported to cause diseases ranging from rare genetic disorders²¹⁶ to cancer^{217,218} or diabetes¹⁹³. The resulting detrimental effects on the cell may help explain the widespread toxicity reported for Pb²⁺ poisoning. Further understanding of PI conformations and metal properties are needed to fully explain the results of this study, nevertheless significant interactions with only 1% PI warrants further interest.

A multi-metal study was also completed in parallel to the interactions tested for Pb²⁺ with each PI lipid. The complete profiling for all lipids from PI to PIP₃ was performed with Pb²⁺, Cd²⁺, Co²⁺, Ni²⁺ and Mn²⁺ using GP to assess membrane fluidity and DLS to test LUV sizes against increasing concentrations of metal. A summary of these additional experiments has been included in Appendix B.

Chapter Seven: Monolayer Studies of PC, PS, and PI lipids

The use of monolayers allow for a biophysical characterization of lipid or protein based films, their interactions with each other and their modulation by biologically relevant molecules such as ions or drugs^{116,117,219}. These systems allow for a strict control of their lipid composition without any assumptions of lipid distribution between bilayer leaflets¹⁰¹. Additionally, the lipid lateral-packing density and surface area exposed to potential binding partners such as metals can be defined¹⁰¹. The work presented in this chapter was completed in collaboration with a colleague, Weiam Daear. Langmuir monolayers of PC, PS and PI were examined based on their interesting affinity for Pb^{2+} determined in the bilayer studies (Chapters 3 - 6). DMPC and DMPS were added to POPC and POPS to allow for the comparison of acyl tail saturation in terms of membrane packing and metal binding to the lipid films. PI monolayers continued the 1 mol % compositions of each PI type with 99 mol % POPC. All experimental subphases contained 100 mM NaCl pH 7.4 while metal trials included a 500:1 molar ratio of Pb^{2+} to lipids which corresponds to 86.2 μ M Pb²⁺. A high Pb²⁺ concentration in great excess of the lipids was used to overcome the large subphase volume compared to the much smaller film area reducing the partitioning of Pb²⁺ species into the monolayer. Langmuir monolayers were studied by the Wilhelmy method (see Section 2.5 Langmuir Trough). The surface pressure (π) versus molecular area (A) isotherms plot the change in surface pressure as the area available to each lipid molecule is compressed on the aqueous subphase^{116,219}. π -A isotherms provide information on monolayer formation, film compressibility, the interaction of subphase species with the monolayer and collapse behaviour¹¹⁶. From the slope of the π -A isotherms, the compression modulus (β) was calculated to better understand the packing of lipid within each system

tested⁸⁵.

Surface potential (*V*) was also recorded simultaneously as a function of the molecular area during the compression. This technique provides information concerning the orientation of the molecular dipoles, the association of molecules at early stages of compression and the interaction of metals from the subphase with the monolayer¹¹⁶. Lastly, Brewster angle microscopy (BAM) images were taken to observe morphological features of the monolayer during compression such as domain formation or lipid clustering¹¹⁶.

7.1 Interactions of Pb²⁺ with PC Monolayers

The zwitterionic lipids POPC and DMPC contain the same head group (phosphocholine) but different acyl chain lengths and degrees of saturation. The change from fully saturated (DMPC) to one unsaturated bond (POPC) in the lipids increases the area per molecule during compression due to looser packing which introduces fluidity.



Figure 130. (**A**) Surface pressure (π -A) (solid lines) and surface potential (V-A) (dashed lines)-area isotherms and (**B**) compression modulus curves (β) of POPC monolayers compressed over 100 mM NaCl pH 7.4 subphases (black) with 86.2 μ M Pb²⁺ (red). Results are the average of 3 replicates.

At high molecular areas, the lipids are in the gaseous phase with limited intermolecular interactions (Figure 130A). During this phase, the control π -A isotherm does not detect any lipid – lipid interactions while the *V*-A isotherm notes an immediate increase in potential as the monolayer begins compression. When compression reached ~122 Å²/mol, the control π -A isotherm takes off the zero-pressure line and lipid packing changes to a liquid-expanded (LE) phase characterized by increasing interactions. The addition of Pb²⁺ to the subphase resulted in an earlier takeoff at ~145 Å²/mol, indicating that the molecules in the film require a larger surface area. At collapse, the monolayer cannot be further compressed, and multilayers are formed^{100,116} which occurs at a surface pressure of 42.5 mN/m and an area of 59 Å²/mol for both control and Pb²⁺ systems.

The Pb²⁺ V-A isotherm distinctly begins at a higher potential and does not increase dramatically with early compression, suggesting no reorientation of lipid molecules is occurring. The increase in potential as the monolayer is compressed is notably reduced for the Pb²⁺ trial and reaches only 203 mV at collapse as compared to 236 mV for the control. In Figure 130B, the β for Pb²⁺ trials is significantly lowered at lower surface pressures suggesting a more compressible or less tightly packed monolayer.

7.1.2 Brewster Angle Microscopy of POPC Monolayers with Pb²⁺

BAM imaging for each lipid film was conducted at the air – water interface under the same compression conditions used in the π -A isotherm experiments. The representative images of POPC monolayers on a metal – free or Pb²⁺ – containing subphase presented in Figure 131 were taken at various surface pressures of 0.5, 5, 10, 15 and 30 mN/m during compression including collapse.



Figure 131. BAM images of POPC monolayers + 86.2 μ M Pb²⁺ in 100 mM NaCl pH 7.4 during compression at the air-water interface. Each image is 218 x 271 microns; the scale bar is 50 microns. Inset image at 30 mN/m for Pb²⁺ trials is a magnification of the red box.

POPC monolayers did not show any distinct lateral organization or domain formation and appeared as a homogenous film throughout the entire compression therefore only the images from 15 mN/m and higher were included. The only notable change in these images was the corresponding greyscale intensity within the liquid-expanded (LE) membrane due to the film compression. The greyscale between Pb^{2+} and control trials also appears slightly lighter for Pb^{2+} with small pinhole defects appearing at 30 mN/m (highlighted by the red insert).



Figure 132. (**A**) Surface pressure (π -A) (solid lines) and surface potential (*V*-A) (dashed lines)-area isotherms and (**B**) compression modulus curves (β) of DMPC monolayers compressed over 100mM NaCl pH 7.4 subphases (black) with 86.2µM Pb²⁺ (red). Results are the average of 3 replicates.

There is no sign of a gaseous phase for DMPC monolayers under the conditions tested here prior to compression as demonstrated by π -*A* isotherms take off areas of 133 Å²/mol for control and Pb²⁺ trials indicating the onset of film pressure. The addition of Pb²⁺ to the subphase caused a minor difference in the π -*A* isotherm is noted between 5 and 15 mN/m. The upward shift of this region suggests that lipids exhibit a higher surface pressure under the same molecular area as the control. Control DMPC surface pressure increased steadily upon compression until collapse at an area of ~52 Å²/mol and surface pressure of ~45 mN/m. Another difference in the π -*A* isotherm was a slightly reduced collapse surface pressure of ~42 mN/m at 52 Å²/mol (Figure 132A).

V-A isotherms also showed a lack of molecular reorientation during these higher

molecular areas. Throughout the entire compression, the surface potential of $Pb^{2+} - DMPC$ monolayers was raised by 14 mV indicating immediate metal binding during film deposition. The surface potential at collapse was 268 mV for the control and 282 mV for the Pb^{2+} system.

The monolayer was least compressible at 18 mN/m as determined by the peak of the compression modulus (Figure 132B). The π -A isotherm shift corresponds to a decrease in β between 5 – 15 mN/m relative to the control isotherm. The film in the presence of Pb²⁺ was also least compressible at 18 mN/m however, the remainder of the curve was downshifted and thus more compressible compared to the control trial (Figure 132B).

7.1.4 Brewster Angle Microscopy of DMPC Monolayers with Pb²⁺

The representative BAM images of DMPC monolayers on a control or Pb²⁺containing subphase are presented in Figure 133.



Figure 133. BAM images of DMPC monolayers + 86.2μ M Pb²⁺ in 100mM NaCl pH 7.4 during compression at the air-water interface. Each image is 218 x 271 microns; the scale bar is 50 microns. Red arrows highlight black pinholes. Inserts are magnifications of the red boxes within each BAM image.

Upon compression of control DMPC monolayers, small defects in lipid packing were detectable as black pin hole defects (red arrows) and lighter grey clusters (red boxes), especially at 15 and 30 mN/m (top of Figure 133). At collapse, these defects differentiated into small bead-like liquid-condensed (LC) domains in a mostly LE membrane. The addition of Pb²⁺ to the subphase strongly altered the lateral organization of the monolayer. Each image from takeoff (0.5 mN/m) to collapse is lighter, suggesting a more condensed monolayer compared to the control. At 30 mN/m we observe Pb²⁺-induced phase separation with domains of predominantly LC (lighter grey) and LE (darker grey) circles of varying sizes. Interestingly, striations appeared across the membrane, changing regions that are mostly LE in an LC phase to LC circular domains within an LE monolayer were observed at 30 mN/m (see red box). At collapse, most of the monolayer appeared more condensed, regions of less packed LE lipids remained circular but coalesced into fewer, larger regions. Additionally, sharp white peaks of more tightly packed rigid clusters were detected across the collapse images (red box insert, Figure 133).

7.1.5 Discussion of Pb Interactions with PC Monolayers with Pb^{2+}

The bilayer study of pure POPC LUVs indicated rigidification by GP values at higher temperatures when the lipids would be furthest apart. In this monolayer study, such higher temperature conditions would correspond to larger molecular areas within the physiological relevant range where monolayers are equivalent to surface pressures of ~35 mN/m¹³⁷. The affinity of Pb²⁺ to loosely packed POPC (Figure 27) may explain the enhanced binding of Pb²⁺ throughout higher molecular area regions as observed in Figure 130 as well.

The immediate takeoff of POPC monolayers in the presence of Pb²⁺ suggest a loss

of the gaseous phase due to metal binding. This binding would restrict the reorientation of molecular dipoles during the initial compression as noted by the *V*-*A* isotherm. The binding of Pb²⁺ caused an overall rightward shift of the π -*A* isotherm which signifies a more fluid membrane by increasing the area per lipid molecules at equivalent surface pressures relative to the control. Pb²⁺ also reduced the compression modulus until ~35 mN/m, where the curves rejoined the control isotherms up to collapse. This downward shift suggests a higher compressibility due to the increased fluidity of the membrane. The lowered potential throughout the compression and at collapse suggests that Pb²⁺ binding likely alters the orientation of the PC head group and water dipoles in the aqueous phase. The binding of Pb²⁺ was less detectable by BAM imaging. The Pb²⁺ – lipid interactions which impact the isotherms does not induce lateral lipid domain formations as the predominant interactions occurred during higher molecular areas of POPC monolayers. The overall greyscale of Pb²⁺ trials suggested a higher packing order of lipids compared to the controls.

For DMPC, the results for controls and metal-containing π -*A* and *V*-*A* isotherms were less pronounced (Figure 132) while BAM visualized significant changes of the lateral domain organization in the presence of Pb²⁺ (Figure 133). The binding of Pb²⁺ was detected through π -*A* isotherms as a slight rightward shift and reduced collapse pressure. The additional increase in *V*-*A* isotherms across the entire compression would suggest Pb²⁺ binding which changes the lateral organization. Surface potential is very sensitive to the difference in potential induced by lipid dipoles within the electrical field thus the Pb²⁺ – induced increase of lipid density increases the ΔV . BAM images visualized this change in lipid ordering as a phase coexistence of LE and LC phases. The presence of Pb²⁺ caused clusters which were not present in metal-free controls. These brighter clusters confirm a
Pb^{2+} – DMPC interaction and protrude from the monolayer, which may explain the lower collapse pressure seen for $Pb^{2+} \pi$ -*A* isotherms.

7.2 Interactions of Pb²⁺ with PS Monolayers

Bilayer studies of Pb^{2+} and PS displayed substantial binding preference for the carboxyl group of the serine head group. POPS LUVs exhibited significant rigidification with 30μ M Pb²⁺, LUV swelling and leakage up to 18% (3.6 Interactions of Pb²⁺ with Phosphatidylserine (PS) Membranes). Furthermore, DMPS LUVs exhibited stronger Pb²⁺ binding at a concentration as low as 0.1μ M Pb²⁺ (Figure 42). Monolayer studies on the interactions of Pb²⁺ with these PS lipids were performed to understand the impact of Pb²⁺ binding on the membrane lateral organization.



Figure 134. (**A**) Surface pressure (π -A) (solid lines) and surface potential (*V*-A) (dashed lines)-area isotherms and (**B**) compression modulus curves (β) of POPS monolayers compressed over 100mM NaCl pH 7.4 subphases (black) with 86.2µM Pb²⁺ (red). Results are the average of 3 replicates.

During the gas phase, the control π -*A* isotherm does not detect any lipid – lipid interactions, while *V*-*A* isotherms note an increase in potential as the monolayer begins compression. The control π -*A* isotherm took off at ~115 Å²/mol while the Pb²⁺ take off was delayed to ~105 Å²/mol. The π -*A* isotherm for Pb²⁺ trials is slightly left-shifted from the control throughout the compression from takeoff until ~20 mN/m. The monolayer is becoming more rigid due to Pb²⁺ as the area per lipid molecules is lowered for equivalent surface pressures relative to the control. This is confirmed by the larger compression modulus in Figure 134B, signifying a resistance to lateral compression. Above 20 mN/m the π -*A* isotherm appears comparable with the control and the monolayer has increased packing. Both monolayers collapse at a surface pressure of 39 mN/m and an area of 59 $Å^2/mol.$

V-A isotherms for Pb²⁺ trials begin 15 mV higher than the control potential before undergoing a similar sigmoidal increase as compression of the monolayer continues. Both surface potential curves continue to increase upon further compression until collapse. The Pb²⁺ trials have a much larger ΔV (+213 mV) than the control (+171 mV) (Figure 134A).

7.2.2 Brewster Angle Microscopy of POPS Monolayers with Pb²⁺

The representative BAM images of POPS monolayers on control or Pb^{2+} containing subphases are presented in Figure 135.



Figure 135. BAM images of POPS monolayers + 86.2μ M Pb²⁺ in 100mM NaCl pH 7.4 during compression at the air-water interface. Each image is 218 x 271 microns; the scale bar is 50 microns. Red arrows highlight black pinholes. Inserts are magnifications of the coloured boxes within each BAM image.

Compression of the control POPS monolayer indicated several small pinhole defects in the lipid packing from 10 mN/m until collapse (as noted by red arrows). At 30 mN/m, POPS lipids begin to form small LC domains across the LE monolayer with several bright clusters (highlighted by orange box). This effect is dramatically enhanced at collapse where the monolayer appears to be predominantly in the LC phase with large regions of PS clusters protruding from the monolayer (red insert).

The addition of Pb²⁺ further increased the cluster formation of POPS monolayers. PS clusters were detected as low as 5 mN/m alongside black pinhole defects in the film (red arrows). The clustering was often shaped in long striations throughout the membrane (see inserts). At 15 mN/m, the formation of LC within the LE phases appeared as well. At collapse, larger and elongated PS clustering with protruding ridges was observed (red box, Figure 135).

The greyscale intensity of these images correlates to changes in molecular topography whereby brighter spots indicate areas that protrude from the film due to enhanced laser reflections. Using the EP3 software, the red box inserts which were highlighted in the BAM images were transformed into domain topology images for control and Pb^{2+} trials at collapse (Figure 136). The 3D surface topology images verified what was observed in the box inserts from collapse BAM images of POPS films. Monolayers exposed to Pb^{2+} displayed larger, brighter domain ridges compared to the metal – free control.



Figure 136. 3D images of domains found in control (**A**) and Pb^{2+} (**B**) trials from POPS films at collapse from highlighted red insert regions of Figure 135.

7.2.3 Isotherms of DMPS Monolayers with Pb^{2+}



Figure 137. (**A**) Surface pressure (π -A) (solid lines) and surface potential (*V*-A) (dashed lines)-area isotherms and (**B**) compression modulus curves (β) of DMPS monolayers compressed over 100 mM NaCl pH 7.4 subphases (black) with 86.2 μ M Pb²⁺ (red). Results are the average of 3 replicates.

As seen for DMPC (Figure 132A), the tighter packing of fully saturated acyl tails eliminated any notable gas phase in DMPS controls (Figure 137A). A shoulder in the π -A isotherm was observed at a molecular area of ~60 Å²/mol and π of 15 mN/m. Collapse of the control DMPS occurred at 34 Å²/mol and 50 mN/m. The leftward shift in Pb²⁺ – trials is significantly larger for DMPS (Figure 137) than seen for POPS (Figure 134). Takeoff of the π -A isotherm with Pb²⁺ occurs immediately upon the onset of compression however, the slow increase in π causes a very different isotherm than for DMPS controls. The collapse pressure of the Pb²⁺ – DMPS monolayers is equivalent however the molecular area is lower at 31 Å²/mol.

 β is lower during this surface pressure due to the change in the slope of the π -A isotherm. No plateau is present in Pb²⁺ – trials and the point of highest rigidity occurs at 7 mN/m when β is at its maximum (Figure 137B).

As the compression continues after the π -*A* isotherm plateau region, a change in the increasing slope of the control *V*-*A* isotherm was seen. Interestingly, the *V*-*A* isotherm shows a unique pattern for Pb²⁺ – trials compared to the control. The change in relative slope occurs at the molecular area which corresponds to the beginning of the phase coexistence in the control trials. The ΔV of the Pb²⁺ – trial is higher throughout the entire compression, reaching 288 mV at collapse compared to ~243 mV for the control.

7.2.4 Brewster Angle Microscopy of DMPS Monolayers with Pb²⁺

The representative BAM images of POPS monolayers on a control or Pb²⁺containing subphase are presented in Figure 138.



Figure 138. BAM images of DMPS monolayers + 86.2μ M Pb²⁺ in 100mM NaCl pH 7.4 during compression at the air-water interface. Each image is 218 x 271 microns; the scale bar is 50 microns. Red arrows highlight black pinholes. Inserts are magnifications of the coloured boxes within each BAM image.

The formation of LC domains begins at 15 mN/m for the control DMPS monolayers which directly corresponds to the beginning of the plateau observed in the π -A and β isotherms (red box, Figure 137). The film appears to immediately coalesce into the LC phase as noted by the lighter greyscale of the images at 30 mN/m and at collapse. Few defects (red arrows) and clusters were imaged for these films at higher surface pressures.

Immediate binding of Pb²⁺ to DMPS occurred as a significant change in the lateral organization can be visualized at takeoff of 0.5 mN/m (purple box). Phase coexistence of LE, LE/LC and LC can all be detected as an increasingly lighter grey. Regions of LC clusters line the perimeter of LE to LE/LC phases which each contain defects in the lipid packing. As compression begins, the clusters become more condensed into irregularly – shaped domains in the LC phase (blue box). As the film is further compressed, the boundaries of these domains coalesce (green and yellow boxes) into a homogenous LC phase by 30 mN/m with the appearance of PS aggregates protruding from the membrane (orange box). At collapse, the lateral organization of the monolayer is completely disrupted with evidently multilayers forming (red box).

The domain formation with Pb^{2+} indicates a pronounced impact on the lateral organization of DMPS lipids. 3D surface topology images were generated to analyze the formation of these domains within each of the coloured box highlights from the BAM images. Lipid clusters are observed for DMPS at take – off (0.5 mN/m, purple box) within a dark image background due to a lack of reflection where the lipids are in the gas phase and unstructured. Small circular domains are observed at 5 mN/m (blue box), populating the entire image. These domains begin to coalesce upon further compression by creating a more uniform film with the formation of a few spikes at 15 mN/m (yellow box). A few

visible bright spots are found at 30 mN/m (orange box) and confirmed to be protrusions from the surface by the 3D imaging which indicates Pb²⁺ insertion or interactions with the monolayer. At collapse (red box), the loss of these protrusions suggest that Pb²⁺ has been squeezed out of the monolayer or no longer at the lipid surface to induce these spikes. Instead, the presence of darker grey voids indicates the formation of multilayers.



Figure 139. 3D images of domains found during the compression of Pb²⁺ trials from DMPS films. Box colours correspond to highlighted insert regions of Figure 138.

7.2.5 Discussion of Pb Interactions with PS Monolayers with Pb^{2+}

The leftward shift in the π -A isotherm for Pb²⁺ – trials with POPS indicates a more rigid membrane whereby the lipids occupy a smaller molecular area to exert the same level of surface pressure. The coordination of Pb²⁺ may reduce lipid – lipid repulsion which would decrease the surface pressure exerted between lipids. The increase in ΔV throughout the entire compression is also in agreement with an ordered film for Pb^{2+} – trials as a smaller molecular area results in more lipid dipoles are present across the electrical field. BAM images visualized this change of lipid packing as Pb^{2+} – induced PS clustering was significantly enhanced from as low as 5 mN/m. At collapse, full ridges of PS clusters were protruding from the membrane (Figure 135 and Figure 136).

The bilayer studies of Pb²⁺ with PS found stronger binding with DMPS over POPS. The tighter packing of fully saturated acyl tails potentially provides a better coordination pocket for Pb²⁺ within the PS head groups. In the monolayer studies, the similar trend of Pb²⁺ – binding was observed. Indeed, a significant change in the π -A isotherm for Pb²⁺ – trials was observed. The coordination of Pb²⁺ to the DMPS film caused significant reduction of the molecular areas with minimal increases in surface pressure. This isotherm did not exhibit any plateau regions, unlike the pronounced shoulder of the control isotherm at 60 Å²/mol and 15 mN/m.

The plateau range in surface pressure corresponds to a local minima in β values in the control DMPS, which represents LE/LC phase coexistence during the transition from LE to LC¹⁰⁰. This was directly recorded by the transitions of the LC phase at 15 mN/m into a homogenous film at higher surface pressures (red inserts, Figure 138 top). The inflection of surface potential at the beginning of this plateau also confirms the beginning of a more condensed phase. The steeper surface potential increase upon further compression is a consequence of the tighter lipid packing in the LC phase.

The similar inflection of surface potential for Pb^{2+} – DMPS trials occurs at the pressure take-off area of the π -A isotherm. This suggests that Pb^{2+} induces reordering of the lipids which shifts the LC phase formation to much higher molecular areas. This

266

reorientation of the molecules as detected by *V-A* isotherms occurs during the compression between the BAM and 3D images recorded at 0.5 and 5 mN/m. A significant transition from LC clustering within phases dominated by LE/LC and LE to LC domain formation was directly observed (as highlighted by the purple – yellow inserts, Figure 138 and Figure 139). These domains continue to grow into a homogenous LC film and significant PS clusters protruded from the monolayer at 30 mN/m (see orange box). The steep decrease in β after 7 mN/m suggests an ease of compressibility with Pb²⁺ bound which accounts for the larger ΔV of +45 mV and multilayer formation at collapse (see red box).

7.3 Interactions of Pb²⁺ with 1% PI Monolayers

Unlike the pure lipid systems, the inability to control the distribution of lipids mixtures between leaflets must be considered in bilayer studies. The composition of lipids and area exposed to the surrounding aqueous medium may vary between LUV preparations¹⁰¹. The monolayer allows for evaluating the lateral interactions of lipids in each leaflet of a biomembrane in a defined lipid composition¹⁰⁰. PIs comprise only a minor component (~1%) of the phospholipids in the plasma membranes of many cells^{94,155,193,220} as tight regulation of PIs is important in the balance of signaling cascades²⁰⁹ and metabolic dysfunctions²¹⁸.

7.3.1 Isotherms of DOPI Monolayers with Pb²⁺

Surface pressure– and surface potential – area isotherms and the compression modulus for 1% DOPI 99% POPC monolayers on a control or Pb²⁺ – containing subphase are shown in Figure 140.



Figure 140. (**A**) Surface pressure (π -*A*) (solid lines) and surface potential (*V*-*A*) (dashed lines)-area isotherms and (**B**) compression modulus curves (β) of 1% DOPI 99% POPC monolayers compressed over 100 mM NaCl pH 7.4 subphases (black) with 86.2 μ M Pb²⁺ (red). Results are the average of 3 replicates.

The take-off pressure for control π -*A* isotherms was ~124 Å²/mol which occurred earlier at ~138 Å²/mol with the addition of Pb²⁺ to the subphase. This Pb²⁺ – trial take-off is a slightly lower molecular area than the takeoff for Pb²⁺ – POPC trials (145 Å²/mol). During compression, the π -*A* isotherm for Pb²⁺ – trials appears to be shifted to larger molecular areas at equivalent surface pressures as the control 1% DOPI monolayers. This shift is less than seen for the Pb²⁺ – POPC trials (Figure 130). No change in π -*A* isotherm collapse was detected. In Figure 130B, the β for Pb²⁺ trials is reduced throughout the entire film compression.

At high molecular areas, the lipids are in the gaseous phase as seen for the pure POPC monolayer (Figure 130A). During this phase, the control π -A isotherm does not indicate any lipid – lipid interactions while V-A isotherms note a significant increase in potential from 41 to 152 mV at the take-off, *V*-*A* continues to increase upon further compression until film collapse at 42.5 mN/m, 59 Å²/mol, and 229 mV. The Pb²⁺ *V*-*A* isotherm however, distinctly begins at a higher potential and does not change with early compression. The slope of the surface potential increase upon compression is reduced for the Pb²⁺ trial and reaches an equivalent potential at collapse.

7.3.2 Brewster Angle Microscopy of DOPI Monolayers with Pb²⁺

The representative BAM images of 1% DOPI 99% POPC monolayers on control or Pb²⁺-containing subphase are presented in Figure 141.

1% DOPI containing monolayers did not show any domain formation throughout the entire compression however at 10 mN/m, lipid packing defects were visualized (red box, Figure 141 top). Small defects in the lipid packing and a few lipid clusters can be seen at collapse pressures. The corresponding greyscale intensity of these images was lighter for more condensed films.

The greyscale between Pb^{2+} and control trials also appears lighter for Pb^{2+} during the compression. Long regions of interspersed lipid clusters were detected between 10 and 15 mN/m (as seen in orange and red inserts, respectively). The film became more homogenous at 30 mN/m with the appearance of some smaller clusters at collapse.



Figure 141. BAM images of 1% DOPI 99% POPC monolayers + 86.2μ M Pb²⁺ in 100mM NaCl pH 7.4 during compression at the air-water interface. Each image is 218 x 271 microns; the scale bar is 50 microns. Inserts are magnifications of the coloured boxes within each BAM image.

7.3.3 Discussion of Pb Interactions with PI Monolayers with Pb²⁺

The larger increase in *V-A* isotherms during the initial compression of the control films demonstrate the importance of such experiments to assess dipole reorientations due to molecular interactions¹¹⁶. This increase is much larger than seen for POPC controls (Figure 130) as simulations by Bradshaw *et al.*, 1999 showed that the bulky inositol ring protrudes perpendicularly from the membrane plane²⁰². This induces a higher molecular dipole within the head group region and surrounding water molecules. The higher ΔV for Pb²⁺ – trials suggest interaction with Pb²⁺ in the subphase as dipole ordering has already occurred because of Pb²⁺ coordination.

The reduction of the surface compression modulus of 1% DOPI 99% POPC monolayers in the presence of Pb²⁺ suggests an increase of lateral compressibility due to the metal coordination, as expected based on previous results from POPC monolayers. The shift in the π -*A* isotherm documented that Pb²⁺ induced higher surface pressure of the lipids at equivalent molecular areas of the control. This shift is actually larger with Pb²⁺ – POPC than the 1% DOPI containing monolayers (Figure 130 versus Figure 140) and the compression modulus indicates a higher β value for 1% DOPI monolayers (91 versus 85 m/mN) signifying that the DOPI film is less compressible than POPC alone.

The BAM images for pure POPC monolayers showed no Pb^{2+} – induced domain or cluster formations. The addition of 1% DOPI suggested a stronger interaction with the lipids to induce small aggregates protruding from the membrane, thus appearing as brighter white spots (see orange and red inserts, Figure 141). The lipid clustering induced by Pb^{2+} may be a result of lipid packing defects that maximize interactions with the phosphate linker, explaining the more rigid membrane detected through the π -A isotherm.

271

7.4 Interactions of Pb²⁺ with 1% PIP Monolayers

In this section, monolayers of 1% mixtures of DOPI(3)P, DOPI(4)P and DOPI(5)P with 99% POPC were analyzed using the Langmuir techniques in the absence and presence of Pb²⁺ at a 500:1 molar ratio of metals to lipids. As mentioned in Chapter Six: Bilayer Study of PI and PC lipids, molecular dynamic simulations of PIP lipids found that the inositol headgroups with single phosphates are fit over a large inclined angle range of ~0 – 80° to the bilayer normal^{199,201,221}. This angle deviance from perpendicular orientation with DOPI lipids is caused by an electrostatic attraction of the negative phosphates on the inositol towards the positive trimethylammonium of the PCs^{199,221}. The phosphocholine head groups are known to lie close to the membrane surface (~17°)^{197,221,222}. The tilting of the ring brought about by this interaction may result in reduced hydration and intramolecular hydrogen bonding²²¹ as the perpendicular extension of non-phosphorylated PI lipids maximizes the 2' and 6' positions for hydrogen bonding²⁰⁰. Monolayer studies will provide more information on how the orientation of the inositol head groups changes the packing of lipids within membranes.

The strength of Pb^{2+} interactions with the PIP lipids were PI(4)P > PI(5)P > PI(3)Pas determined by membrane rigidity and LUV swelling from the Chapter Six: Bilayer Study of PI and PC lipids.



Figure 142. (**A**) Surface pressure (π -*A*) (solid lines) and surface potential (*V*-*A*) (dashed lines)-area isotherms and (**B**) compression modulus curves (β) of 1% DOPI(3)P 99% POPC monolayers compressed over 100mM NaCl pH 7.4 subphases (black) with 86.2µM Pb²⁺ (red). Results are the average of 3 replicates.

The π -A isotherm of 1% DOPI(3)P 99% POPC follows a very similar pattern as 1% DOPI monolayers for control and Pb²⁺ trials (Figure 140). In metal trials, a rightward shift in the π -A isotherm was observed starting with an earlier take off for Pb²⁺ trials at 131 Å²/mol compared to ~124 Å²/mol for the control. This corresponds to a sharp drop-off in the compression modulus above 13 mN/m. Both control and Pb²⁺ 1% DOPI(3)P isotherms exhibited collapse at 57 Å²/mol, reaching a surface pressure of 42 mN/m, and potential of 208 mV.

The initial compression of the film displayed a sharper increase in ΔV from -22 to +127 mV at an area where the π -A isotherm takeoff also occurred at ~124 Å²/mol. The surface potential for Pb²⁺ trials started higher with a larger change from 50 to ~140 mV

unlike 1% DOPI monolayers which did not exhibit any *V*-*A* isotherm changes during the initial compression of the monolayer.

7.4.2 Brewster Angle Microscopy of DOPI(3)P Monolayers with Pb^{2+}

The representative BAM images of 1% DOPI(3)P 99% POPC monolayers on a control or Pb²⁺-containing subphase are presented in Figure 143.

1% DOPI(3)P monolayers exhibited defects in the lipid packing at the takeoff pressure of 0.5 mN/m (see orange insert). Minor defects in the film were observed from 5 mN/n until collapse (red arrows). The increased lipid – lipid interactions in this mixture were clearly evident in the BAM images of control subphases. Lipid clusters in the absence of any metal were prevalent at 30 mN/m until film collapse (highlighted in red inserts, Figure 143 top).

The formation of these clusters was enhanced in the presence of Pb^{2+} . Regions of protruding lipids were evident above 15 mN/m (see red inserts, Figure 143 bottom). The relative brightness of these spots is stronger with Pb^{2+} suggesting increased protrusions from the membrane lateral organization than the control trials.

A comparison of these clusters present for 1% DOPI(3)P 99% POPC films at collapse was presented in 3D images in Figure 144. The brighter colour in the 3D image with Pb²⁺ denotes an increased height of the clusters while the control image has an overall lighter grey surrounding film, indicating a more uniform lateral organization of the monolayer.



Figure 143. BAM images of 1% DOPI(3)P 99% POPC monolayers + 86.2μ M Pb²⁺ in 100mM NaCl pH 7.4 during compression at the air-water interface. Each image is 218 x 271 microns; the scale bar is 50 microns. Red arrows highlight black pinholes. Inserts are magnifications of the coloured boxes within each BAM image.



Figure 144. 3D images of domains found in control (A) and Pb^{2+} (B) trials from 1% DOPI(3)P 99% POPC films at collapse from highlighted red insert regions of Figure 143.

7.4.3 Isotherms of DOPI(4)P Monolayers with Pb^{2+}



Figure 145. (**A**) Surface pressure (π -*A*) (solid lines) and surface potential (*V*-*A*) (dashed lines)-area isotherms and (**B**) compression modulus curves (β) of 1% DOPI(4)P 99% POPC monolayers compressed over 100mM NaCl pH 7.4 subphases (black) with 86.2µM Pb²⁺ (red). Results are the average of 3 replicates.

Unlike previous PI lipids, the ΔV in the presence of Pb²⁺ was reduced at the beginning of compression to -6 mV whereas the control monolayer started at 88 mV. The potential of the monolayer for Pb²⁺ trials remained lower than the control through the compression to collapse at 227 mV compared to 248 mV. In contrast to these different Pb²⁺ effects, minimal differences were detected for the π -*A* isotherms. The take off for Pb²⁺ was slightly sooner than the control (123 versus 118 Å²/mol) and the compression modulus β maxima demonstrates that the membrane is most rigid between 10 – 15 mN/m with Pb²⁺ trials.

7.4.4 Brewster Angle Microscopy of DOPI(4)P Monolayers with Pb²⁺

The representative BAM images of 1% DOPI(4)P 99% POPC monolayers on a control or Pb^{2+} – containing subphase are presented in Figure 146. The 1% DOPI(4)P monolayer exhibited a homogenous film with minimal packing defects appearing as black pinholes in the grey LE lipid phase (red arrows). Lipid clustering and the occurrence of an LC phase within the LE film can be seen at the collapse pressure of the control trials (red insert). The greyscale of the Pb²⁺ trials was lighter above 15 mN/m suggesting a more compressed monolayer than the control. At 30 mN/m there appears to be an increase in the packing defects and lipid clusters which continues into collapse where the clusters are brighter aggregates than seen in the control (as highlighted by red box inserts, bottom of Figure 146).



Figure 146. BAM images of 1% DOPI(4)P 99% POPC monolayers + 86.2μ M Pb²⁺ in 100mM NaCl pH 7.4 during compression at the air-water interface. Each image is 218 x 271 microns; the scale bar is 50 microns. Red arrows highlight black pinholes. Inserts are magnifications of the red boxes within each BAM image.

7.4.5 Isotherms of DOPI(5)P Monolayers with Pb^{2+}



Figure 147. (**A**) Surface pressure (π -A) (solid lines) and surface potential (*V*-A) (dashed lines)-area isotherms and (**B**) compression modulus curves (β) of 1% DOPI(5)P 99% POPC monolayers compressed over 100 mM NaCl pH 7.4 subphases (black) with 86.2 μ M Pb²⁺ (red). Results are the average of 3 replicates.

A similar effect of Pb²⁺ on the π -A and V-A isotherms for 1% DOPI(5)P was detected compared to DOPI(4)P. The π -A isotherms for control and Pb²⁺ trials are nearly identical from take-off to collapse however the trend within the compression modulus is reversed from DOPI(4)P (Figure 145B). Pb²⁺ – DOPI(5)P monolayers are most rigid at the β maxima at a surface pressure of ~ 15 mN/m while control monolayers are most rigid at ~22.5 mN/m.

As the surface potential for Pb²⁺ trials was also lower than the control, the ΔV at the beginning of compression was only -45 mV compared to -94 mV for DOPI(4)P. The control DOPI(5)P potential was lower at higher molecular areas and throughout compression, the levels of ΔV for Pb²⁺ and control trials were very similar (only a difference of 10 mV at collapse).

7.4.6 Brewster Angle Microscopy of DOPI(5)P Monolayers with Pb^{2+}

The representative BAM images of 1% DOPI(5)P 99% POPC monolayers on a control or Pb²⁺-containing subphase are presented in Figure 148. The control monolayers did not exhibit a homogenous film, the monolayer appears to have a phase coexistence causing bead-like defects throughout the monolayer at takeoff areas (see purple insert). The monolayer appeared to coalesce by 5 mN/m. Small pinholes (red arrows) and the origin of an LC phase clustering can be detected in a long-banded pattern at 10 mN/m (see blue insert). From 15 mN/m up to collapse, the quantity of lipid clusters protruding within the lighter grey LC phases increases with compression (green, orange and red inserts, respectively).

1% DOPI(5)P and 99% POPC monolayers that were spread onto a Pb^{2+} – containing subphase did not exhibit the same level of lipid – lipid interactions as seen for the control trials as minor defects are present in every image. Only images recorded at 30 mN/m and collapse showed small condensed regions (see highlighted orange and red inserts).



Figure 148. BAM images of 1% DOPI(5)P 99% POPC monolayers + 86.2µM Pb in 100mM NaCl pH 7.4 during compression at the air-water interface. Each image is 218 x 271 microns; the scale bar is 50 microns. Red arrows highlight black pinholes. Inserts are magnifications of the coloured boxes within each BAM image.

7.4.7 Discussion of Pb²⁺ Interactions with PIP Monolayers

Fuller *et al.*, indicated that the orientation of the PI(3)P inositol was 44° from the bilayer normal and an internal tilt of the ring pointed the 3' phosphate down towards the membrane plane¹⁹⁹. This 3' position is reported as the closest position to the membrane surface when phosphorylated^{199,201}. Thus, the inaccessibility of the 3P was suggested to explain reduced Pb²⁺ – LUV interactions for 1% DOPI(3)P relative to the other PIPs. The rigidification of LUVs as detected by GP at lower temperatures indicating that tighter packing promoted Pb²⁺ interactions.

In the monolayer studies, as the membrane was compressed to a smaller area, a significant increase in lipid clustering was visualized by BAM. The shift in π -A isotherms and increased ΔV also represented an increase in the packing or film structure of the membrane. Lipids exerted a higher surface pressure and potential for Pb²⁺ – trials than the control at equivalent molecular areas (Figure 142). Therefore, combining the bilayer and monolayer studies on mixtures of 1% DOPI(3)P and 99% POPC further implies that Pb²⁺ binding to the 3P occurs through a tight coordination thus inducing an increase in lipid packing.

The interactions of Pb²⁺ with DOPI(4)P in the bilayer study demonstrated significant rigidification of the membrane, stronger than any other PI lipid tested (Chapter Six: Bilayer Study of PI and PC lipids). The initial results of π -A isotherms demonstrated little change in the presence of Pb²⁺ relative to controls (Figure 145). Considering the rightward shifts found for isotherms of POPC (Figure 130) and DOPI monolayers (Figure 140) with Pb²⁺ trials, the observed overlap of Pb²⁺ and control curves with the 1% DOPI(4)P monolayers suggests a different type of Pb²⁺ coordination. Surface potential –

282

area isotherms directly signify that interactions must be occurring between this lipid mixture and Pb²⁺ as a significant reduction in the potential was recorded (Figure 146). Surface potential is proportional to the change in dipole density with respect to a control subphase with no films^{116,223}. The effective dipole strength of the hydrated polar groups will affect the electrostatic properties of the monolayer such that if the conformation or orientation of the lipid and polarized water remain constant, the dipole moment of these molecules would also remain constant^{138,223}.

The reduced potential at the beginning of Pb²⁺ isotherms with 1% DOPI(4)P monolayers suggests that Pb^{2+} reduces the dipole density. This could potentially be through metal coordination which alters the directional electrostatic attraction of these negative phosphate groups towards the positive choline head groups at the membrane surface found within the PI(4)P groups relative to the control. This interaction does not appear to affect the monolayer formation or phases detected in π -A isotherms. On the other hand, they may induce a change in the hydration and water penetration within the polar interface resulting in the increased GP values and LUV swelling detected in the bilayer studies. Limited changes to the lateral organization of the 1% DOPI(4)P monolayers was visualized by BAM experiments. The greyscale of Pb^{2+} to control trials appeared lighter, suggesting tighter lipid packing. The white clusters also did not accumulate such that Pb²⁺ induces better mixing for 1% DOPI(4)P monolayers and acts as an emulsifier on the different phase regions within the membrane which occurred at collapse for the control trials. Thus, the binding of Pb^{2+} to the 4P of PI(4)P was completely unique from the alterations of membrane lateral organization as seen for PI and PI(3)Ps.

The binding of Pb²⁺ to DOPI(5)P lipids appears to follow the same trends as

DOPI(4)P but there seems to be a lower affinity for the 5P over 4P. Bilayer studies exhibited similar rigidification patterns across the temperature range with lower Δ GP than PI(4)P. The π -A isotherms of DOPI(5)P monolayers also recorded no change in takeoff π , collapse π , or the shape of the curve. As previously suggested, the lack of a shift in an isotherm of 99 mol % POPC, which causes higher molecular areas at constant surface pressures in Pb²⁺ trials, may be indicative of the demixing and lateral packing defects reduced by Pb²⁺ – PI(5)P binding. The compression modulus was notably opposite to Pb²⁺ – PI(4)P trials where the monolayer exhibited a higher β at higher surface pressures suggesting a more rigid membrane than 1% DOPI(5)P with Pb²⁺ (Figure 147B). The range of these compressibility values however, may not be indicative of relevant changes between systems.

The lowering of ΔV at the beginning of compression advocates that Pb²⁺ binding to PI(5)P occurred immediately upon deposition of the lipids onto the subphase which altered the dipole density within the polar head groups and surrounding water molecules. Most notably, 1 mol % PI(5)P lipids appear to cluster within the pure monolayers, stronger than seen for PI(3)P or PI(4)P. The binding of Pb²⁺ abolishes these different phases and lipid clusters which agrees with the proposal that Pb²⁺ would coordinate amongst the PI lipids for preferential binding to the 5P causing this emulsification. This reduces the electrostatic attraction of PI phosphates towards the PC choline head groups, thus altering the potential found across the dipoles of these monolayers.

7.5 Interactions of Pb^{2+} with 1% PIP₂ Monolayers

Monolayers composed of 1% DOPI(3,4)P₂, DOPI(4,5)P₂ and DOPI(3,5)P₂ with 99% POPC were analyzed next in the absence and presence of Pb^{2+} . The molecular

dynamic simulations of PIP₂ lipids found the inositol headgroups with two phosphates lie closer to the surface of the membrane, reported at a tilt between 13 and 41° ^{197,198,200,220}. This ring orientation is also driven by an electrostatic attraction of the negative inositol phosphates towards the positive choline^{199,221}.

The strength of Pb^{2+} rigidification for the bilayer study in Chapter Six: Bilayer Study of PI and PC lipids for PIP₂ lipids were PI(3,4)P₂ >> PI(3,5)P₂ > PI(4,5)P₂ where PI(4,5)P₂ membranes exhibited fluidization at low Pb²⁺ concentrations (Figure 123).





Figure 149. (A) Surface pressure (π -A) (solid lines) and surface potential (V-A) (dashed lines)-area isotherms and (**B**) compression modulus curves (β) of 1% DOPI(3,4)P₂ 99% POPC monolayers compressed over 100 mM NaCl pH 7.4 subphases (black) with 86.2 μ M Pb²⁺ (red). Results are the average of 3 replicates.

The π -A and V-A isotherms of 1% DOPI(3,4)P₂ and 99% POPC monolayers closely resemble the shift seen for Pb²⁺ with DOPI(3)P (Figure 142). When compression reached

~116 Å²/mol, the control π -A isotherm took off and lipid interactions increased. The addition of Pb²⁺ to the subphase resulted in an earlier takeoff at ~135 Å²/mol. During compression, the π -A isotherm for Pb²⁺ – trials was shifted to larger molecular areas at equivalent surface pressures compared to controls. No change in π -A isotherm collapse was detected at 43 mN/m and 57 Å²/mol.

During the gaseous phase, the control π -A isotherm did not detect any lipid – lipid interactions while the V-A isotherm notes a significant increase in potential from -9 to 142 mV at the take – off area. The Pb²⁺ V-A isotherm distinctly begins at a higher potential and small changes are recorded with initial compression. The slope of the increasing surface potential upon compression is reduced for the Pb²⁺ trial and reaches equivalent potential values for both systems at collapse. V-A continues to increase upon further compression until film collapse at 236 mV.

In Figure 149B, the β for Pb^{2+} trials is reduced throughout the entire film compression.

7.5.2 Brewster Angle Microscopy of DOPI(3,4)P₂ Monolayers with Pb^{2+}

The representative BAM images of 1% DOPI(3,4)P₂ 99% POPC monolayers on a control or Pb²⁺-containing subphase are presented in Figure 150. The control and Pb²⁺ trials both exhibited the formation of lipid clusters upon compression (see inserts). Pb²⁺ appeared to induce more lipid clustering. At collapse, the formation of a more condensed LC phase was detected for Pb²⁺ trials due to the higher ordering for Pb²⁺ lipid interactions.



Figure 150. BAM images of 1% DOPI(3,4)P₂ 99% POPC monolayers + 86.2 μ M Pb²⁺ in 100mM NaCl pH 7.4 during compression at the air-water interface. Each image is 218 x 271 microns; the scale bar is 50 microns. Inserts are magnifications of the coloured boxes within each BAM image.



Figure 151. 3D images of lipid clusters found in control (**A**) and Pb^{2+} (**B**) trials from 1% DOPI(3,4)P₂ 99% POPC films at collapse from the highlighted red insert regions of Figure 150.

The 3D surface topology images for 1% $DOPI(3,4)P_2$ 99% POPC monolayers indicate that Pb^{2+} induces the formation of a higher number of larger spikes, different from those found in the controls on the left.



Figure 152. (**A**) Surface pressure (π -A) (solid lines) and surface potential (*V*-A) (dashed lines)-area isotherms and (**B**) compression modulus curves (β) of 1% DOPI(4,5)P₂ 99% POPC monolayers compressed over 100 mM NaCl pH 7.4 subphases (black) with 86.2 μ M Pb²⁺ (red). Results are the average of 3 replicates.

The π -A isotherms show no difference in the take-off and collapse pressures, but a very small leftward shift was recorded for Pb²⁺ trials up to pressures of ~20 mN/m. The compression modulus demonstrates that the membrane is more rigid with Pb²⁺ in the subphase between 5 mN/m and 30 mN/m (Figure 152B).The ΔV in the presence of Pb²⁺ was reduced at the beginning of compression but aligned with the control through the compression until a slight lowering of potential collapse (244 mV compared to 251 mV).

7.5.4 Brewster Angle Microscopy of DOPI(4,5)P₂ Monolayers with Pb^{2+}

The representative BAM images of 1% $DOPI(4,5)P_2$ 99% POPC monolayers on a control or Pb^{2+} -containing subphase are presented in Figure 153.



Figure 153. BAM images of 1% DOPI(4,5)P₂ 99% POPC monolayers + 86.2 μ M Pb²⁺ in 100mM NaCl pH 7.4 during compression at the air-water interface. Each image is 218 x 271 microns; the scale bar is 50 microns. Red arrows highlight black pinholes. Inserts are magnifications of the red boxes within each BAM image.

BAM images of the control monolayers of DOPI(4,5)P₂ and POPC mixtures demonstrate that lipid – lipid interactions induce clustering in the absence of any metals (see highlighted insert). Minor defects in the homogenous film are seen through compression leading up to brighter white clusters which further protrude from the lateral monolayers.

While lipid clusters and pinhole defects were recorded throughout the compression of monolayers on subphase with Pb^{2+} , the overall number of clusters formed was less than the control.



7.5.5 Isotherms of DOPI(3,5)P₂ Monolayers with Pb^{2+}

Figure 154. (**A**) Surface pressure (π -A) (solid lines) and surface potential (*V*-A) (dashed lines)-area isotherms and (**B**) compression modulus curves (β) of 1% DOPI(3,5)P₂ 99% POPC monolayers compressed over 100mM NaCl pH 7.4 subphases (black) with 86.2µM Pb²⁺ (red). Results are the average of 3 replicates.

The control π -A isotherms demonstrate the most unique curves than any other PI
lipid. The larger head group consisting of an inositol ring with flanking phosphates attached on either side appears to be occupy larger lipid molecular areas, thus inducing reduced lipid packing at the beginning of compression as no gaseous phase was detected. Instead, π -*A* isotherms took off immediately as the monolayer lipids occupied higher molecular areas, collapsing at 65 Å²/mol and a surface pressure of 44 mN/m. As a result, the compression modulus shape shows a more pronounced peak than other PI lipid systems (Figure 154B). The β peak at a pressure of 15 mN/m indicates when the monolayer was most resistant to compression.

The lack of any sharply changing surface potential for the control and Pb²⁺ trials indicated that the monolayer had already transitioned into the LE phase at the start of compression. A higher surface potential was maintained for Pb²⁺ trials throughout the film compression. DOPI(3,5)P₂ monolayers with Pb²⁺ also demonstrated immediate takeoff but a small rightward shift in the isotherm shape correlated to a lower β maximum and a more compressible film than the control. A drop in the collapse pressure to ~40 mN/m from ~44 mN/m was detected.

7.5.6 Brewster Angle Microscopy of DOPI(3,5)P₂ Monolayers with Pb^{2+}

The representative BAM images of 1% DOPI(3,5)P₂ 99% POPC monolayers on a control or Pb²⁺-containing subphase are presented in Figure 155. Minor pinhole defects can be seen in the otherwise homogenous control films (red arrows). At collapse, a large region of condensed lipid clusters was observed (red insert). The monolayer with Pb²⁺ exhibited a dramatic increase in the level of defects and clusters throughout the entire compression (see red box at collapse).



Figure 155. BAM images of 1% DOPI(3,5)P₂ 99% POPC monolayers + 86.2 μ M Pb²⁺ in 100 mM NaCl pH 7.4 during compression at the air-water interface. Each image is 218 x 271 microns; the scale bar is 50 microns. Red arrows highlight black pinholes. Inserts are magnifications of the red boxes within each BAM image.

7.5.7 Discussion of Pb²⁺ Interactions with PIP₂ Monolayers

The additional phosphorylation at the 4' position did not impact the lateral organization and degree of Pb²⁺ interactions seen for 1% DOPI(3,4)P₂ monolayers over 1% DOPI(3)P. As the membrane was compressed to a smaller area, BAM and 3D imaging visualized an increase in lipid clustering with Pb²⁺. The shift in π -A isotherms and increased ΔV also represented an increase in the Pb²⁺ interactions on the membrane. This directly agrees with the Pb²⁺ – induced membrane rigidification and LUV swelling detected from Chapter 6 for 1% DOPI(3,4)P₂ liposomes.

While it was previously suggested that the 4P position would be the preferential target for Pb^{2+} binding, being more accessible at the surface of the membrane, the 3P position appears to dictate the orientation of the inositol ring due to preferential electrostatic attractions at the membrane surface¹⁹⁹ and fulfillment of hydrogen bonding for the 2' hydroxyl group²⁰⁰. The small hydrated radius of Pb^{2+} could explain why a tighter coordination with 3P is preferred. Interactions with the 4P are still occurring between Pb^{2+} and DOPI(3,4)P₂ lipids, the *V*-*A* isotherms demonstrate a higher binding of Pb^{2+} to the monolayer at the start of compression compared to DOPI(3)P and the membrane rigidification recorded by GP experiments also showed enhanced effects for DOPI(3,4)P₂.

Bilayer studies cannot fully explain the small fluidization of the membrane which was detected upon Pb²⁺ binding LUVs of 1% DOPI(4,5)P₂ versus the significant Pb²⁺ – induced rigidification of LUVs for PI(4)P and PI(5)P lipids. The disordering of the lateral organization by Pb²⁺ which was observed for 1% PI(4)P and 1% PI(5)P monolayers does provide insight on the binding of Pb²⁺ to DOPI(4,5)P₂ lipids. Rightward shifts observed for the π -A isotherms of POPC (Figure 130) and DOPI monolayers (Figure 140) demonstrated the binding of Pb^{2+} at the membrane surface. DOPI(4)P (Figure 145) and DOPI(5)P (Figure 147) monolayers exhibited no notable shifts with Pb^{2+} and control curves as a different alteration of the lateral organization caused reduced dipole density, possible due to the fact that the negative phosphates extended further out into the aqueous subphase.

The observed results of DOPI(4,5)P₂ showed an overall leftward shift in the π -A isotherm and increased compression modulus indicating that the binding of Pb²⁺ reduced the lateral elasticity of the monolayers (Figure 152). The lower potential at the beginning of compression demonstrates that Pb²⁺ is interacting with the monolayer to alter the molecular dipole orientations but a reordering with the initial compression occurs to match the lipid behaviour seen in the control. This could explain the loss of early phase coexistence seen for PI(4,5)P₂ lipids in the control BAM images at 0.5 mN/m (Figure 153) while lipid clustering was still recorded throughout compression. The binding of Pb²⁺ to the 4P and 5P positions seems to primarily reduce the lipid – lipid interactions through coordination that changes the electrostatic attractions. These effects may be less dependent on the penetration of Pb²⁺ into the polar interface of the backbones due to the exposed phosphates from the inositol ring. This helps explaining reduced GP changes to the membrane as less changes to the polar environment occur around the laurdan molecule.

Following the trend of important $Pb^{2+} - 3P$ interactions, additional phosphorylation at the 5' position did not impact the lateral organization and degree of Pb^{2+} interactions seen for DOPI(3,5)P₂ films over DOPI(3)P. The monolayer study on 1% DOPI(3,5)P₂ mixtures showed an increased lipid density causing a higher surface potential and more lipid clusters in BAM images throughout the compression.

The presence of the 5P changes the lateral packing of these lipids within the

monolayer compared to the other PIP₂ and PIP lipids. No molecular dynamic simulations have reported the orientation of this head group. Driving forces of 2' and 6' hydroxyl group intramolecular hydrogen bonding²⁰⁰ and a downward twist angle of the $3P^{197,199}$ to the membrane due to electrostatic attractions would suggest that the 5P is left on the opposite side of the inositol ring, more accessible for interactions with the subphase directly. As a result, this would increase the surface area required for these lipids with a bulkier head group as confirmed by π -A isotherms in this monolayer study (Figure 154).

7.6 Interactions of Pb²⁺ with 1% PIP₃ Monolayers

The final PI lipid to consider was the fully phosphorylated $DOPI(3,4,5)P_3$, representing all positions phosphorylated at the inositol ring.





Figure 156. (A) Surface pressure (π -A) (solid lines) and surface potential (*V*-A) (dashed lines)-area isotherms and (**B**) compression modulus curves (β) of 1% DOPI(3,4,5)P₃ 99% POPC monolayers compressed over 100mM NaCl pH 7.4 subphases (black) with 86.2µM Pb²⁺ (red). Results are the average of 3 replicates.

For trials in the presence of Pb^{2+} , a slight shift to lower molecular areas at equivalent surface pressures in the π -A isotherm was observed causing an increased compression modulus above surface pressures of 10 mN/m.

Initial compression of the film displayed a sharp increase in ΔV from 31 to 133 mV when the π -A isotherm takeoff occurred at 128 Å²/mol. Both control isotherms increase throughout the compression until collapse at 57 Å²/mol, reaching a surface pressure of 40 mN/m, and potential of 216 mV. Additionally, a higher surface potential was recorded for Pb²⁺ during the entire compression, reaching collapse at 57 Å²/mol, 42 mN/m and 235 mV.

7.6.2 Brewster Angle Microscopy of DOPI(3,4,5)P₃ Monolayers with Pb²⁺

The representative BAM images of 1% DOPI(3,4,5)P₃ 99% POPC monolayers on a control or Pb²⁺-containing subphase are presented in Figure 157. Pinhole packing defects are evident at the takeoff pressure of 0.5 mN/m which was significantly enhanced in the presence of Pb²⁺ (see purple inserts). The control monolayers exhibited minor defects in the lateral organization up to 30 mN/m when lipid clusters formed (red arrows). At collapse, the strongest lipid – lipid interactions of any 1% PI mixtures were detected in large regions of clusters protruding from the membrane plane (red box inserts). The addition of Pb²⁺ prompted the formation of these lipid clusters at lower surface pressures (10 mN/m and up) which did not exhibit the same degree of cluster sizes or projection from the lateral membrane at collapse (as highlighted by the red boxes).



Figure 157. BAM images of 1% DOPI(3,4,5)P₃ 99% POPC monolayers + 86.2 μ M Pb²⁺ in 100mM NaCl pH 7.4 during compression at the air-water interface. Each image is 218 x 271 microns; the scale bar is 50 microns. Red arrows highlight black pinholes. Inserts are magnifications of the coloured boxes within each BAM image.

7.6.3 Discussion of Pb²⁺ Interactions with PIP₃ Monolayers

The PI(3,4,5)P₃ are the most negatively charged lipids with an overall charge of -5 due to a complex ionization behaviour promoting the sharing of protons between phosphate groups on the inositol ring¹²². The conformation of the inositol ring is believed to lie closely to the membrane surface^{197,198}. Laurdan GP results of LUVs with 1% DOPIP₃ and 99% POPC showed significant membrane rigidification without consistent changes to the LUV sizes (Chapter Six: Bilayer Study of PI and PC lipids). The positive increase in surface potential for the monolayers that were exposed to Pb²⁺ in the subphase compared to the controls suggests that metal interactions from the subphase increased lipid density. The change in the monolayer packing was directly visualized by BAM to show the formation of lipid clusters during monolayer compression.

7.7 Summary of Pb²⁺ Interactions with PI Lipids

Pb²⁺ induced distinct effects on the lateral organization of PI lipids depending on the location of additional phosphorylation at each of the three positions on the inositol ring. The 3' position was previously reported as the closest position to the membrane surface upon phosphorylation^{199,201} due to favourable electrostatic attractions and hydrogen bonding between the 2' hydroxyl groups and neighbouring lipids^{200,220}. Pb²⁺ interactions were observed with the 3P position inducing membrane ordering in both bilayer and monolayer studies for DOPI(3)P, DOPI(3,4)P₂ and DOPI(3,5)P₂ lipids through a tight coordination at the membrane surface.

The interactions of Pb²⁺ with the 4P and 5P positions induced a very different change on the lateral organization of the lipids compared to the 3P position. Monolayer studies indicated a reduction in lipid density causing a dispersion of lipid clusters. This

299

metal binding is believed to interfere with the electrostatic attraction of PI phosphates towards the PC choline head groups, thus also altering the potential found across the dipoles of DOPI(4)P, DOPI(5)P and $DOPI(4,5)P_2$ monolayers.

The combination of each of these unique Pb^{2+} interactions appeared to occur for 1% PIP₃ mixtures when all positions were phosphorylated. The surface potential increased for Pb^{2+} trials suggesting a higher lipid packing density, as seen with other 3' phosphorylated lipids, which was evident in BAM images throughout the compression. However, the degree of clusters protruding from the monolayer at collapse was reduced and the lower shift in π -A isotherms closely resembles the altered monolayer formations seen with 4P and 5P binding by Pb²⁺.

7.8 Implications of Pb²⁺ Interactions with PI Lipids

Differences in the binding of Pb^{2+} were observed for each PI lipid depending on the location of the phosphorylation between bilayer and monolayer studies. Each PI lipid has a unique role in a broad range of signaling pathways throughout the compartments of eukaryotic cells^{193,211}. Since differences to the coordination of the metal can occur with each lipid at only 1 mol % composition, Pb^{2+} will affect the association of PI lipids with other lipids or proteins within the membrane. Dysfunction of the PI metabolism may explain the widespread toxicity observed for Pb^{2+} poisoning.

A multi-metal study was also completed in parallel with Pb^{2+} for each PI lipid. The complete profiling for all lipids from PI to PIP₃ was performed with Pb^{2+} , Cd^{2+} , Co^{2+} , Ni^{2+} and Mn^{2+} using the Langmuir trough to assess surface pressure– and potential – area isotherms and BAM to visualize the lateral organization of each monolayer in the absence and presence of Pb^{2+} . A summary of these experiments is included in Appendix C.

Chapter Eight: Summary and conclusions

The impact of Pb²⁺ on membrane properties such as permeability, lateral film organization, and lipid packing were investigated by LUV leakage of the fluorophore/quencher pair ANTS/DPX, the solvatochromic fluorophore laurdan using generalized polarization and dynamic light scattering to assess liposome size changes and characterize lipid phase transitions.

One of the key elements of the fluid-mosaic model is the fact that lipids have the freedom to diffuse in the plane. Moreover, the can adopt localized domains to facilitate cellular transport, signal transduction and other metabolic processes at the membrane surface^{69,87}. Disruptions to the lipid organization would therefore influence the biological structure and functioning of the cell.

The speciation of Pb²⁺ to adopt positively charged species under 100 mM NaCl and pH 7.40 conditions led to the hypothesis that they would target negative lipids. In addition, the metal even exhibited effects on zwitterionic lipids including phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, PC and PE plasmalogens and cerebrosides. As expected, the impact of Pb²⁺ was stronger with anionic lipids including phosphatidylserine, phosphatidic acids, cardiolipin, phosphatidylglycerol, phosphatidylinositol, gangliosides, and cerebroside sulfates.

The extent of the Pb²⁺ interactions depended on the location of the negative charge, including exposure and proximity to other charged groups, as well as the ratio of metal – lipid in solution. Pb²⁺ was observed to disturb the membrane order which induced leakage of nearly 18% of liposomal internal contents of PS lipids (Figure 38). In most cases, Pb²⁺ also increased membrane rigidity and sizing of liposomes except for the gel phase of PAs which exhibited significant fluidization of the membrane (3.7 Interactions of Pb²⁺ with Phosphatidic Acid (PA) Membranes). The largest rigidification observed occurred with DMPG membranes (Δ GP +0.56, Figure 56) suggesting that the polar glycerol head group provides optimal coordination for Pb²⁺ species.

When lipids with structurally identical head groups but different acyl chain composition were compared, Pb²⁺ consistently targeted the more saturated lipids better. As the membranes became more complex, Pb²⁺ continued to induce significant rigidity when present in excess of lipids. Tighter lipid packing due to the presence of cholesterol and lipid mixtures appeared to enhance the effects of Pb²⁺, potentially in the latter through higher accessibility due to lipid packing defects in the membrane organization. Results for the red blood cell and brain polar lipid extracts showed that Pb²⁺ can target these complex lipid profiles and is the first step to understanding why substantial uptake of Pb²⁺ occurs in these different cell types. These extracts have a higher biological relevance due to the diverse lipid compositions considering head groups, acyl tail lengths and degrees of saturations despite the limitations of using non-asymmetric membranes.

Attempts to model the asymmetry of the lipid bilayers were made with separate preparations of the outer and inner leaflets of the myelin sheath. The lipids present are distinct as the outer leaflets are enriched in glycosylated cerebrosides and sulfatides while the inner leaflets have more phospholipids with PEplasm, PC, PS and SM. Despite the vast difference in potential Pb²⁺ targets, Pb²⁺ was observed to significantly induce membrane rigidification and LUV size increases for both biomimetic lipid membranes, with stronger effects occurring with the inner leaflet (Δ GP of 0.082 compared to 0.034, Figure 109 and Figure 98).

Considering additional biological relevant effects, the interactions of Pb²⁺ with the PI lipids that are important signaling molecules were carried out at a 1 mol % within 99% POPC. This low concentrations reflects their minimal content in cellular membranes^{94,193,224}. The number and position of the phosphates on the inositol ring altered the interactions of Pb²⁺. For monophosphorylated lipids, preferential targeting of PI(4)P > PI(5)P > PI(3)P was determined for Pb²⁺ – LUV binding. The orientation of the head groups was largely considered to explain the differences between PIP or PIP₂ lipids for the degree of Pb²⁺ binding.

In addition to bilayer studies with 1% PI molecules, the impact of Pb^{2+} on lipid packing was assessed by using monolayer experiments on the Langmuir trough using the Wilhelmy method, surface potential measurements and visualization by Brewster angle microscopy. Once again, differences in the binding of Pb^{2+} were observed for each PI lipid. The positioning of the phosphate at 4' and 5' positions caused Pb^{2+} to act as an emulsifier which reduced lipid – lipid interactions and altered the electrostatic dipoles attracting negative phosphates to the neighbouring positive choline head groups within the monolayer. Opposite effects were observed with Pb^{2+} and the 3' position phosphate which saw the induction of membrane ordering for 1% DOPI(3)P, 1% DOPI(3,4)P₂ and 1% DOPI(3,5)P₂ lipid systems.

Other monolayer studies included PC and PS lipids to confirm interactions with Pb^{2+} and the differences in affinity between head group structures and acyl tail saturations. Significant increases of the lateral organization by lipid clustering of PS lipids was imaged, which helped to explain the Pb^{2+} – induced aggregation of LUVs observed in bilayer studies.

8.1 Future Directions

Since Pb^{2+} can form aggregates through coordination which induces formation of domains enriched in the anionic lipids, fluorescently labelled synthetic lipids or specific lipid – dye interactions could monitor the phase separation in lipid mixtures upon Pb^{2+} exposure. This could be paired with leakage experiments to assess the extent of dye release for systems exhibiting Pb^{2+} -induced phase separation.

Another aspect of leakage assays as a direct follow – up of the experiments presented in this thesis, would be increasing the PS content between 0 to 100% PC. This would help understanding the effect of packing defects as a function of lipid composition on the strength of Pb^{2+} interactions compared to the large membrane leakage seen with POPC and POPS LUVs individually. Since Pb^{2+} – PA interactions introduced fluidization in the gel phase then rigidification to the membrane in the liquid – crystalline phase and Pb^{2+} – PG lipids exhibited the strongest rigidification of any lipid tested, leakage assays with Pb^{2+} should also be done to assess how membrane fluidity correlates with the leakage rate.

As Pb^{2+} affects numerous systems in the body, biomimetic lipid mimicking other cell and organelle types such as other brain tissues, kidneys, where Pb^{2+} is transported to for detoxification and elimination^{225,226}, or the mitochondria as Pb^{2+} is known to localize in this membrane resulting in the opening of the mitochondrial permeability transition pore²²⁷ would be valuable information. Lipid extracts of these membranes could be used to formulate LUVs to perform laurdan and DLS experiments. Real – time fluorescence could also be conducted *in vivo* using laurdan and two – photon microscopy to visualize changes in fluorescence as the systems are exposed to Pb^{2+} . Red blood cell ghosts¹²⁸ could be used instead of lipid extracts, whereby the asymmetry should be retained through the activation of integral membrane proteins responsible for lipid ordering, flippases and floppases¹⁷⁷, by including Mg-ATP²²⁸. This would better mimic the targeting of Pb²⁺ to biological cells maintaining the asymmetric lipid composition.

This thesis included the interactions of Pb^{2+} with the most common sphingo- and phospholipids in mammalian cells. Further Pb^{2+} – lipid studies should also consider the presence of proteins or other molecules within the cells to understand the affinity for the interactions with the lipids in respect to other biomolecules. While lipids are a major constituent of cellular membranes, proteins are also present and may compete for the coordination of Pb^{2+} ions. There is a high probability that Pb^{2+} also has a high affinity for proteins moving throughout the body. This could change the coordination available for lipid binding or even the speciation profile of Pb^{2+} *in vivo*. The first tests which could be run for Pb^{2+} – protein/peptide – lipid experiments might be metallothionein or glutathione chelating molecules known for the detoxification and removal of Pb^{2+} and other metals through high affinity sulfate coordination^{42,46,170,229,230}. Additional experiments might include protein transporters, since Pb^{2+} has been reported to use for cellular entry^{51,65,188}, or calcium-binding proteins like calmodulin²³¹ and protein kinase C⁶⁰, which can be bound and improperly activated by Pb²⁺.

The affinity of Pb²⁺ to the lipids can be further investigated by isothermal titration calorimetry (ITC) experimentation. This technique would allow for the thermodynamic characterization of binding constants, the stoichiometry of binding, and enthalpy, entropy and free-energy changes associated from contact between the metal and lipids¹⁴³. These

parameters may help distinguish differences in how Pb^{2+} binds lipids of similar charge but different accessibilities to the target groups. Some ITC parameters have already been determined for Pb^{2+} and proteins such as metallothionein^{170,171} and protein kinase C⁶⁰ which could be utilized in comparison to such lipid data to increase the understanding of Pb^{2+} interaction and localization within cell membranes.

References

- (1) Al-Saleh, I. A. S. The Biochemical and Clinical Consequences of Lead Poisoning. *Med Res Rev* **1994**, *14* (4), 415–486.
- (2) ATSDR. Summary Data for 2017 Priority List of Hazardous Substances https://www.atsdr.cdc.gov/spl/index.html (accessed Feb 12, 2018).
- (3) ATSDR. Summary Data for 2017 Completed Exposure Pathway (CEP) Site Count Report https://www.atsdr.cdc.gov/cep/index.html (accessed Feb 12, 2018).
- (4) Baxter, D. C.; Frech, W. Speciation of Lead in Environmental and Biological Samples. *Pure Appl. Chem.* **1995**, 67 (4), 615–648.
- (5) EPA. Lead Emissions https://www.epa.gov/roe/indicator.cfm?i=13 (accessed Feb 13, 2018).
- (6) Rossi, E. Low Level Environmental Lead Exposure--a Continuing Challenge. *Clin. Biochem. Rev.* **2008**, *29* (2), 63–70.
- (7) Mao, J. S.; Dong, J.; Graedel, T. E. The Multilevel Cycle of Anthropogenic Lead. I. Methodology. *Resour. Conserv. Recycl.* 2008, 52 (8–9), 1058–1064.
- (8) Tchounwou, P. B.; Yedjou, C. G.; Patlolla, A. K.; Sutton, D. J. Heavy Metals Toxicity and the Environment. *EXS* **2012**, *101*, 133–164.
- (9) Nriagu, J. Tales Told in Lead. *Science* (80-.). **1998**, 281 (5383), 1622–1623.
- (10) CDC. Fourth National Report on Human Exposure to Environmental Chemicals; 2012.
- (11) Lide, D. R. Hardness of Minerals and Ceramics. *CRC Handb. Chem. Phys.* 2005, 2313–2314.
- (12) UNEP. Leaded Gasoline in Context; 1998.
- (13) Martinez-Finley, E. J.; Chakraborty, S.; Fretham, S. J. B.; Aschner, M. Cellular Transport and Homeostasis of Essential and Nonessential Metals. *Metallomics* 2012, 4, 593–605.
- (14) Abadin, H.; Ashizawa, A.; Stevens, Y.-W.; Llados, F.; Diamond, G.; Sage, G.; Citra, M.; Quinones, A.; Bosch, S. J.; Swarts, S. G. Toxicological Profile for Lead. U.S Public Heal. Serv. Agency Toxic Subst. Dis. Regist. 2007, No. August, 582.
- (15) Kennedy, C.; Yard, E.; Dignam, T.; Buchanan, S.; Condon, S.; Brown, M. J. Blood Lead Levels Among Children Aged < 6 Years Flint, Michigan, 2013 2016. *Morb. Mortal. Wkly. Rep.* 2016, 65 (25), 650–654.

- (16) Ordemann, J. M.; Austin, R. N. Lead Neurotoxicity: Exploring the Potential Impact of Lead Substitution in Zinc-Finger Proteins on Mental Health. *Metallomics* 2016, 8, 579–588.
- (17) Shin, J. H.; Lim, K. M.; Noh, J. Y.; Bae, O. N.; Chung, S. M.; Lee, M. Y.; Chung, J. H. Lead-Induced Procoagulant Activation of Erythrocytes through Phosphatidylserine Exposure May Lead to Thrombotic Diseases. *Chem. Res. Toxicol.* 2007, 20 (1), 38–43.
- (18) ATSDR. Lead Toxicity: What is the Biological Fate of Lead? http://www.atsdr.cdc.gov/csem/csem.asp?csem=7&po=9.
- (19) Lidsky, T. I.; Schneider, J. S. Lead Neurotoxicity in Children: Basic Mechanisms and Clinical Correlates. *Brain* **2003**, *126* (1), 5–19.
- (20) James, H.; Hilburn, M.; Blair, J. Effects of Meals and Meal Times on Uptake of Lead from the Gastrointestinal Tract in Humans. *Hum. Toxicol.* **1985**, *4*, 401–407.
- (21) Weis, C. P.; Lavelle, J. M.; Wels, C. P.; Lavelle, J. M. Characteristics to Consider When Choosing an Animal Model for the Study of Lead Bioavailability Characteristics to Consider When Choosing an Animal Model for the Study of Lead Bioavailability. *Chem. Speciat. Bioavailab.* **1991**, *3* (314), 113–119.
- (22) Pounds, J.; Mariar, R.; Allen, J. Metabolism of Lead-210 in Juvenile and Adult Rhesus Monkeys (Macaca Mulatta). *Bull. Environm. Contam. Toxicol.* **1978**, *19*, 684–691.
- (23) Goyer, R. A.; Mahaffey, K. R. Susceptibility to Lead Toxicity. *Environ. Health Perspect.* **1972**, *2* (October), 73–80.
- (24) Mason, L. H.; Harp, J. P.; Han, D. Y. Pb Neurotoxicity: Neuropsychological Effects of Lead Toxicity. *Biomed Res. Int.* **2014**, 2014, 1–8.
- (25) Chamberlain, A.; Heard, M.; Little, P.; Newton, D.; Wells, A.; Wiffen, R. Investigations into Lead from Motor Vehicles. *Philos. Trans. R. Soc. Lond. A* 1978, 290, 557–589.
- (26) Morrow, P. E.; Beiter, H.; Amato, F.; Gibb, F. R. Pulmonary Retention of Lead: An Experimental Study in Man. *Environ. Res.* **1980**, *21* (2), 373–384.
- (27) Heard, M.; Wells, A.; Newton, D. Human Uptake and Metabolism of Tetra Ethyl and Tetramethyl Lead Vapour Labelled with 203Pb. In *International Conference on Management and Control of Heavy Metals in the Environment*; 1979; pp 103–108.
- (28) Rastogi, S. C.; Clausen, J. Absorption of Lead through the Skin. *Toxicology* **1976**, *6*, 371–376.

- (29) Florence, T. M.; Lilley, S. G.; Stauber, J. L. Skin Absorption of Lead. *Lancet* **1988**, *332* (8603), 157–158.
- (30) Lilley, S. G.; Florence, T. M.; Stauber, J. L. The Use of Sweat to Monitor Lead Absorption through the Skin. *Sci. Total Environ.* **1988**, *76* (2–3), 267–278.
- (31) Filon, F. L.; Boeniger, M.; Maina, G.; Adami, G.; Spinelli, P.; Damian, A. Skin Absorption of Inorganic Lead (PbO) and the Effect of Skin Cleansers. *J. Occup. Environ. Med.* **2006**, *48* (7), 692–699.
- (32) Gustafsson, J. P. Visual MINTEQ 3.1. KTH Department of Land and Water Resources Engineering: Stockholm, Sweden 2011.
- (33) Nightingale, E. R. Phenomenological Theory of Ion Solvation. Effective Radii of Hydrated Ions. J. Phys. Chem. **1959**, 63 (9), 1381–1387.
- (34) Allred, A. L. Electronegativity Values from Thermochemical Data. J. Inorg. Nucl. Chem. **1961**, 17, 215.
- (35) Baxter, D. C.; Frech, W. Speciation of Lead in Environmental and Biological Samples Technical Report. *Pure Appl. Chem.* **1995**, *67* (4), 615–648.
- (36) Rehman, S. Effect of Lead on Lipid Peroxidation, Phospholipids Composition, and Methylation in Erythrocyte of Human. *Biol. Trace Elem. Res.* 2013, 154 (3), 433–439.
- (37) deSilva, P. E. Determination of Lead in Plasma and Studies on Its Relationship to Lead in Erythrocytes. *Br. J. Ind. Med.* **1981**, *38* (3), 209–217.
- Meissner, P. N.; Corrigall, A. V; Hift, R. J. Fifty Years of Porphyria at the University of Cape Town. *South African Med. Journal. Suid-Afrikaanse Tydskr. Vir Geneeskd.* 2012, *102* (6), 422–426.
- (39) Bergdahl, I.; Sheveleva, M.; Schutz, A.; Artamonovca, V.; Skerfving, S. Plasma and Blood Lead in Humans: Capacity-Limited Binding to Delta-Aminolevulinic Acid Dehydratase and Other Lead-Binding Components. *Toxicol. Sci.* 1998, 46, 247–253.
- (40) Warren, M. J.; Cooper, J. B.; Wood, S. P.; Shoolingin-Jordan, P. M. Lead Poisoning, Haem Synthesis and 5-Aminolaevulinic Acid Dehydratase. *Trends Biochem. Sci.* 1998, 23 (6), 217–221.
- (41) Erskine, P. T.; Norton, E.; Cooper, J. B.; Lambert, R.; Coker, A.; Lewis, G.; Spencer, P.; Sarwar, M.; Wood, S. P.; Warren, M. J.; et al. X-Ray Structure of 5-Aminolevulinic Acid Dehydratase from *Escherichia Coli* Complexed with the Inhibitor Levulinic Acid at 2.0 Å Resolution. *Biochemistry* 1999, 38, 4266–4276.

- (42) Gugliotta, T.; De Luca, G.; Romano, P.; Rigano, C.; Scuteri, A.; Romano, L. Effects of Lead Chloride on Human Erythrocyte Membranes and on Kinetic Anion Sulphate and Glutathione Concentrations. *Cell. Mol. Biol. Lett.* **2012**, *17* (4), 586–597.
- (43) Boudene, C.; Despaux-Pages, N.; Comoy, E.; Boguon, C. Immunological and Enzymatic Studies of Erythrocytic Delta-Aminolevulinate Dehydratase. Comparison of Results Obtained in Normal and Lead-Exposed Subjects. *Int. Arch. Occup. Environ. Heal.* **1984**, 55 (1), 87–96.
- (44) Osterode, W.; Barnas, U.; Geissler, K. Dose Dependent Reduction of Erythroid Progenitor Cells and Inappropriate Erythropoietin Response in Exposure to Lead: New Aspects of Anaemia Induced by Lead. Occup. Environ. Med 1999, 56, 106– 109.
- (45) Bolanowska, W. Distribution and Excretion of Triethyllead in Rats. *Br. J. Ind. Med.* **1968**, *25* (3), 203–208.
- (46) Eaton, D.; Stacey, N.; Wong, K.; Klaassen, C. Dose Response Effects of Various Metal Ions on Rat Liver Metallothionein, Glutathione, Heme Oxygenase, and Cytochrome P-450. *Toxicol. Appl. Pharmacol.* **1980**, *55*, 393–402.
- (47) Fowler, B.; Kimmel, C.; Woods, J.; McConnell, E.; Grant, L. Chronic Low-Level Lead Toxicity in the Rat: III. An Integrated Assessment of Long-Term Toxicity with Special Reference to the Kidney. *Toxicol. Appl. Pharmacol.* **1980**, *56*, 59–77.
- (48) Clarkson, T. W. Metal Toxicity in the Central Nervous System. *Environ. Health Perspect.* **1987**, *75* (15), 59–64.
- (49) Hertz, M. M.; Bolwig, T. G.; Grandjean, P.; Westergaard, E. Lead Poisoning and the Blood-Brain Barrier. *Acta Neurol. Scand.* **1981**, *63* (5), 286–296.
- (50) Deane, R.; Bradbury, M. Transport of Lead-203 at the Blood-Brain Barrier. J. Neurochem. 1990, 54 (3), 905–914.
- (51) Kerper, L. E.; Hinkle, P. M. Cellular Uptake of Lead Is Activated by Depletion of Intracellular Calcium Stores. *J. Biol. Chem.* **1997**, *272* (13), 8346–8352.
- (52) Bellinger, D. C. Teratogen Update: Lead and Pregnancy. *Birth Defects Res. Part A* - *Clin. Mol. Teratol.* **2005**, *73* (6), 409–420.
- (53) Semczuk, M.; Semczuk-Sikora, A. New Data on Toxic Metal Intoxication (Cd, Pb, and Hg in Particular) and Mg Status during Pregnancy. *Med. Sci. Monit.* 2001, 7 (2), 332–340.
- (54) Vigeh, M.; Smith, D. R.; Hsu, P. C. How Does Lead Induce Male Infertility? *Iran. J. Reprod. Med.* **2011**, *9* (1), 1–8.

- (55) Chuang, H.; Schwartz, J.; Gonzales-Cossio, T.; Lugo, M.; Palazuelos, E.; Aro, A.; Hu, H.; Hernandez-Avila, M. Interrelations of Lead Levels in Bone, Venous Blood, and Umbilical Cord Blood with Exogenous Lead Exposure through Maternal Plasma Lead in Peripartum Women. *Environ. Health Perspect.* 2001, *109* (5), 527– 532.
- (56) Gulson, B. L.; Pounds, J. G.; Mushak, P.; Thomas, B. J.; Gray, B.; Korsch, M. J. Estimation of Cumulative Lead Releases (Lead Flux) from the Maternal Skeleton during Pregnancy and Lactation. *J. Lab. Clin. Med.* **1999**, *134* (6), 631–640.
- (57) Oliver, T. A Lecture on Lead Poisoning and the Race. Br. Med. J. 1911, 1096–1098.
- (58) Tabacova, S.; Balabaeva, L. Environmental Pollutants in Relation to Complications of Pregnancy. *Environ. Health Perspect.* **1993**, *101* (SUPPL. 2), 27–31.
- (59) Smith, D.; Osterloh, J.; Flegal, A. Use of Endogenous, Stable Lead Isotopes to Determine Release of Lead from the Skeleton. *Environ. Heal. Perspect.* 1996, 104 (1), 60–66.
- Morales, K. A.; Lasagna, M.; Gribenko, A. V.; Yoon, Y.; Reinhart, G. D.; Lee, J. C.; Cho, W.; Li, P.; Igumenova, T. I. Pb²⁺ as Modulator of Protein-Membrane Interactions. *J. Am. Chem. Soc.* 2011, *133* (27), 10599–10611.
- (61) Neal, A. P.; Guilarte, T. R. Molecular Neurobiology of Lead (Pb²⁺): Effects on Synaptic Function. *Mol. Neurobiol.* **2010**, *42* (3), 151–160.
- (62) Kostial, K.; Vouk, V. B. Lead Ions and Synaptic Transmission in the Superior Cervical Ganglion of the Cat. *Br. J. Pharmacol. Chemother.* **1957**, *12* (2), 219–222.
- (63) Garrick, M. D.; Dolan, K. G.; Horbinski, C.; Ghio, A. J.; Higgins, D.; Porubcin, M.; Moore, E. G.; Hainsworth, L. N.; Umbreit, J. N.; Conrad, M. E.; et al. DMT1: A Mammalian Transporter for Multiple Metals. *BioMetals* **2003**, *16* (1), 41–54.
- Meng, H.; Wang, L.; He, J.; Wang, Z. The Protective Effect of Gangliosides on Lead (Pb)-Induced Neurotoxicity Is Mediated by Autophagic Pathways. *Int. J. Environ. Res. Public Health* 2016, *13* (4).
- (65) Chang, Y. F.; Teng, H. C.; Cheng, S. Y.; Wang, C. T.; Chiou, S. H.; Kao, L. Sen; Kao, F. J.; Chiou, A.; Yang, D. M. Orai1-STIM1 Formed Store-Operated Ca²⁺ Channels (SOCs) as the Molecular Components Needed for Pb²⁺ Entry in Living Cells. *Toxicol. Appl. Pharmacol.* **2008**, 227 (3), 430–439.
- (66) Papadia, S.; Soriano, F. X.; Léveillé, F.; Martel, M.-A.; Dakin, K. a; Hansen, H. H.; Kaindl, A.; Sifringer, M.; Fowler, J.; Stefovska, V.; et al. Synaptic NMDA Receptor Activity Boosts Intrinsic Antioxidant Defenses. *Nat. Neurosci.* 2008, *11* (4), 476– 487.

- (67) Vijverberg, H. P. M.; Westerink, R. H. S. Sense in Pb²⁺ Sensing. *Toxicol. Sci.* **2012**, *130* (1), 1–3.
- (68) Austin, R. N.; Freeman, J. L.; Guilarte, T. R. Neurochemistry of Lead and Manganese. *Metallomics* 2016, 8 (6), 561–562.
- (69) van Meer, G.; Voelker, D.; Feigenson, G. Membrane Lipids Where They Are and How They Behave. *Nat. Rev. Mol. Cell. Biol.* **2008**, *9* (2), 112–124.
- (70) Gorter, E.; Grendel, F. On Bimolecular Layers of Lipoids on the Chromocytes of the Blood. J. Exp. Med. **1925**, 41 (4), 439–443.
- (71) Singer, S. J.; Nicolson, G. L. The Fluid Mosaic Model of the Structure of Cell Membranes. *Science (80-.).* **1972**, *175* (4023), 720–731.
- (72) Vereb, G.; Szöllosi, J.; Matkó, J.; Nagy, P.; Farkas, T.; Vigh, L.; Mátyus, L.; Waldmann, T. A.; Damjanovich, S. Dynamic, yet Structured: The Cell Membrane Three Decades after the Singer-Nicolson Model. *Proc. Natl. Acad. Sci. U. S. A.* 2003, *100* (14), 8053–8058.
- (73) Vigh, L.; Escribá, P. V.; Sonnleitner, A.; Sonnleitner, M.; Piotto, S.; Maresca, B.; Horváth, I.; Harwood, J. L. The Significance of Lipid Composition for Membrane Activity: New Concepts and Ways of Assessing Function. *Prog. Lipid Res.* 2005, 44 (5), 303–344.
- (74) Peetla, C.; Stine, A.; Labhasetwar, V. Biophysical Interactions with Model Lipid Membranes: Applications in Drug Discovery and Drug Delivery. *Mol Pharm.* 2009, 6 (5), 1264–1276.
- (75) Zachowski, A. Phospholipids in Animal Eukaryotic Membranes: Transverse Asymmetry and Movement. *Biochem. J.* **1993**, *294* (Pt 1), 1–14.
- (76) Farquhar, J. W. Human Erythrocyte Phosphoglycerides I. Quantification of Plasmalogens, Fatty Acids and Fatty Aldehydes. *Biochim. Biophys. Acta* 1962, 60 (1), 80–89.
- (77) Lohner, K. Is the High Propensity of Ethanolamine Plasmalogens to Form Non-Lamellar Lipid Structures Manifested in the Properties of Biomembranes? *Chem. Phys. Lipids* **1996**, *81* (2), 167–184.
- (78) Maulik, N.; Bagchi, D.; Ihm, W. J.; Cordis, G. A.; Das, D. K. Fatty Acid Profiles of Plasmalogen Choline and Ethanolamine Glycerophospholipids in Pig and Rat Hearts. *J. Pharm. Biomed. Anal.* **1995**, *14* (1–2), 49–56.
- (79) Ohler, B.; Revenko, I.; Husted, C. Atomic Force Microscopy of Nonhydroxy Galactocerebroside Nanotubes and Their Self-Assembly at the Air-Water Interface, with Applications to Myelin. *J. Struct. Biol.* **2001**, *133* (1), 1–9.

- (80) Fanani, M. L.; Maggio, B. The Many Faces (and Phases) of Ceramide and Sphingomyelin II Binary Mixtures. *Biophys. Rev.* **2017**, *9* (5), 601–616.
- (81) Sáez-Cirión, A.; Basáñez, G.; Fidelio, G.; Goñi, F. M.; Maggio, B.; Alonso, A. Sphingolipids (Galactosylceramide and Sulfatide) in Lamellar-Hexagonal Phospholipid Phase Transitions and in Membrane Fusion. *Langmuir* 2000, 16 (23), 8958–8963.
- (82) Svennerholm, L.; Boström, K.; Fredman, P.; Månsson, J. E.; Rosengren, B.; Rynmark, B. M. Human Brain Gangliosides: Developmental Changes from Early Fetal Stage to Advanced Age. *Biochim. Biophys. Acta* **1989**, *1005* (2), 109–117.
- (83) Schengrund, C. L. Gangliosides: Glycosphingolipids Essential for Normal Neural Development and Function. *Trends Biochem. Sci.* **2015**, *40* (7), 397–406.
- (84) Seelig, J.; Waespe-Šarĉević, N. Molecular Order in Cis and Trans Unsaturated Phospholipid Bilayers. *Biochemistry* **1978**, *17* (16), 3310–3315.
- (85) Jurak, M. Thermodynamic Aspects of Cholesterol Effect on Properties of Phospholipid Monolayers: Langmuir and Langmuir-Blodgett Monolayer Study. J. Phys. Chem. B 2013, 117 (13), 3496–3502.
- (86) Björkhem, I.; Meaney, S.; Fogelman, A. M. Brain Cholesterol: Long Secret Life behind a Barrier. *Arterioscler. Thromb. Vasc. Biol.* **2004**, *24* (5), 806–815.
- (87) Ohvo-Rekilä, H.; Ramstedt, B.; Leppimäki, P.; Peter Slotte, J. Cholesterol Interactions with Phospholipids in Membranes. *Prog. Lipid Res.* 2002, 41 (1), 66– 97.
- (88) Nelson, G. J. Composition of Neutral Lipids from Erythrocytes of Common Mammals. J. Lipid Res. 1967, 8 (4), 374–379.
- (89) Demel, R. A.; De Kruyff, B. The Function of Sterols in Membranes. *BBA Rev. Biomembr.* **1976**, 457 (2), 109–132.
- (90) Fadeel, B.; Xue, Di. Membrane : Roles in Health and Disease. *Crit Rev Biochem Mol Biol* **2009**, *44* (5), 264–277.
- (91) Fadok, V. a; Bratton, D. L.; Frasch, S. C.; Warner, M. L.; Henson, P. M. The Role of Phosphatidylserine in Recognition of Apoptotic Cells by Phagocytes. *Cell Death Differ.* 1998, 5 (7), 551–562.
- (92) Leventis, P. A.; Grinstein, S. The Distribution and Function of Phosphatidylserine in Cellular Membranes. *Annu. Rev. Biophys.* **2010**, *39* (1), 407–427.
- (93) Vance, J. E.; Tasseva, G. Formation and Function of Phosphatidylserine and Phosphatidylethanolamine in Mammalian Cells. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 2013, 1831 (3), 543–554.

- (94) Schmitt, S.; Cantuti Castelvetri, L.; Simons, M. Metabolism and Functions of Lipids in Myelin. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **2014**, *1851* (8), 999– 1005.
- (95) O'Brien, J. S.; Sampson, E. L. Lipid Composition of the Normal Human Brain: Grey Matter, White Matter and Myelin. *J. Lipid Res.* **1965**, *6* (9), 537–544.
- (96) Inouye, H.; Kirschner, D. A. Membrane Interactions in Nerve Myelin: II. Determination of Surface Charge from Biochemical Data. *Biophys. J.* 1988, 53 (2), 247–260.
- (97) Min, Y.; Alig, T. F.; Lee, D. W.; Boggs, J. M.; Israelachvili, J. N.; Zasadzinski, J. A. Critical and off-Critical Miscibility Transitions in Model Extracellular and Cytoplasmic Myelin Lipid Monolayers. *Biophys. J.* **2011**, *100* (6), 1490–1498.
- (98) Aureli, M.; Grassi, S.; Prioni, S.; Sonnino, S.; Prinetti, A. Lipid Membrane Domains in the Brain. *Biochim. Biophys. Acta* **2015**, *1851* (8), 1006–1016.
- (99) Pautot, S.; Frisken, B. J.; Weitz, D. A. Engineering Asymmetric Vesicles. *Proc. Natl. Acad. Sci.* **2003**, *100* (19), 10718–10721.
- (100) Brown, R. E.; Brockman, H. L. Using Monomolecular Films to Characterize Lipid Lateral Interactions. *Methods Mol. Biol.* **2007**, *398*, 41–58.
- (101) Brockman, H. Lipid Monolayers: Why Use Half a Membrane to Characterize Protein-Membrane Interactions? *Struct. Biol.* **1999**, *9*, 438–443.
- (102) Weiss, V. U.; Bilek, G.; Pickl-Herk, A.; Subirats, X.; Niespodziana, K.; Valenta, R.; Blaas, D.; Kenndler, E. Liposomal Leakage Induced by Virus-Derived Peptides, Viral Proteins, and Entire Virions: Rapid Analysis by Chip Electrophoresis. *Anal. Chem.* 2010, 82 (19), 8146–8152.
- (103) Diaz, R. S.; Monreal, J. Protein-Independent Lead Permeation Through Myelin Lipid Liposomes. *Mol. Pharmacol.* **1995**, *47*, 766–771.
- (104) Slobozhanina, E. I.; Kozlova, N. M.; Lukyanenko, L. M.; Oleksiuk, O. B.; Gabbianelli, R.; Fedeli, D.; Caulini, G. C.; Falcioni, G. Lead-Induced Changes in Human Erythrocytes and Lymphocytes. J. Appl. Toxicol. 2005, 25 (2), 109–114.
- (105) Ahyayauch, H.; Sansar, W.; Rendón-Ramírez, A.; Goñi, F. M.; Bennouna, M.; Gamrani, H. Effects of Chronic and Acute Lead Treatments on the Biophysical Properties of Erythrocyte Membranes, and a Comparison with Model Membranes. *FEBS Open Bio* 2013, *3*, 212–217.
- (106) Rappolt, M.; Hickel, A.; Bringezu, F.; Lohner, K. Mechanism of the Lamellar/inverse Hexagonal Phase Transition Examined by High Resolution X-Ray Diffraction. *Biophys. J.* 2003, 84 (5), 3111–3122.

- (107) Carrer, D. C.; Maggio, B. Phase Behavior and Molecular Interactions in Mixtures of Ceramide with Dipalmitoylphosphatidylcholine. *J. Lipid Res.* **1999**, *40* (11), 1978–1989.
- (108) Vallejo, A. A.; Velázquez, J. B.; Fernández, M. S. Lateral Organization of Mixed, Two-Phosphatidylcholine Liposomes as Investigated by GPS, the Slope of Laurdan Generalized Polarization Spectra. Arch. Biochem. Biophys. 2007, 466 (1), 145–154.
- (109) Parasassi, T.; Gratton, E. Membrane Lipid Domains and Dynamics as Detected by Laurdan Fluorescence. J. Fluoresc. **1995**, 5 (1), 59–69.
- (110) Riske, K. A.; Amaral, L. Q.; Lamy-Freund, M. T. Thermal Transitions of DMPG Bilayers in Aqueous Solution: SAXS Structural Studies. *Biochim. Biophys. Acta - Biomembr.* 2001, 1511 (2), 297–308.
- (111) Michel, N.; Fabiano, A.-S.; Polidori, A.; Jack, R.; Pucci, B. Determination of Phase Transition Temperatures of Lipids by Light Scattering. *Chem. Phys. Lipids* 2006, *139* (1), 11–19.
- (112) Vaughan, D. J.; Keough, K. M. Changes in Phase Transitions of Phosphatidylethanolamine- and Phosphatidylcholine-Water Dispersions Induced by Small Modifications in the Headgroup and Backbone Regions. *FEBS Lett.* **1974**, 47 (1), 158–161.
- (113) Barfield, K. D.; Bevan, D. R. Fusion of Phospholipid Vesicles Induced by Zn2+, Cd2+, and Hg2+. *Biochem. Biophys. Res. Commun.* **1985**, *128* (1), 389–395.
- (114) Kerek, E. M.; Prenner, E. J. Inorganic Cadmium Affects the Fluidity and Size of Phospholipid Based Liposomes. *Biochim. Biophys. Acta - Biomembr.* 2016, 1858 (12), 3169–3181.
- (115) Umbsaar, J.; Kerek, E.; Prenner, E. J. Cobalt and Nickel Affect the Fluidity of Negatively-Charged Biomimetic Membranes. *Chem. Phys. Lipids* 2017, No. November, 1–11.
- (116) Dynarowicz-Łątka, P.; Dhanabalan, A.; Oliveira, O. N. Modern Physicochemical Research on Langmuir Monolayers. *Adv. Colloid Interface Sci.* 2001, 91 (2), 221– 293.
- (117) Petelska, A. D.; Naumowicz, M. The Effect of Divalent Ions on L-α-Phosphatidylcholine from Egg Yolk Monolayers at the Air/water Interface. J. Biol. Inorg. Chem. 2017, 22 (8), 1187–1195.
- (118) Ohki, S.; Sauve, R. Surface Potential of Phosphatidylserine Monolayers. I. Divalent Ion Binding Effect. *BBA Biomembr.* **1978**, *511* (3), 377–387.

- (119) Suwalsky, M.; Villena, F.; Norris, B.; Cuevas, Y. F.; Sotomayor, C. P.; Zatta, P. Effects of Lead on the Human Erythrocyte Membrane and Molecular Models. J. Inorg. Biochem. 2003, 97 (3), 308–313.
- (120) Ames, B. N. Assay of Inorganic Phosphate, Total Phosphate and Phosphatases. *Methods Enzymol.* **1966**, 8 (C), 115–118.
- (121) Lönnfors, M.; Laišngvik, O.; Björkbom, A.; Slotte, J. P. Cholesteryl Phosphocholine
 A Study on Its Interactions with Ceramides and Other Membrane Lipids. *Langmuir* 2013, 29 (7), 2319–2329.
- (122) Kooijman, E. E.; King, K. E.; Gangoda, M.; Gericke, A. Ionization Properties of Phosphatidylinositol Polyphosphates in Mixed Model Membranes. *Biochemistry* 2009, 48 (40), 9360–9371.
- (123) Ong, C. N.; Lee, W. R. Distribution of Lead-203 in Human Peripheral Blood in Vitro. *Br. J. Ind. Med.* **1980**, *37* (1), 78–84.
- (124) Schütz, A.; Bergdahl, I. A.; Ekholm, A.; Skerfving, S. Measurement by ICP-MS of Lead in Plasma and Whole Blood of Lead Workers and Controls. *Occup. Environ. Med.* **1996**, *53* (11), 736–740.
- (125) Terayama, K. Effects of Lead on Electrophoretic Mobility, Membrane Sialic Acid, Deformability and Survival of Rat Erythrocytes. *Ind. Health* **1993**, *31*, 113–126.
- (126) Caspers, M. Lou; Siegel, G. J. Inhibition by Lead of Human Erythrocyte (Na++ K+)-Adenosine Triphosphatase Associated with Binding of210Pb to Membrane Fragments. *Biochim. Biophys. Acta - Biomembr.* **1980**, *600* (1), 27–35.
- (127) Sakai, T. Biomarkers of Lead Exposure. Ind. Health 2000, 38 (2), 127–142.
- (128) Dodge, J. T.; Mitchell, C.; Hanahan, D. J. The Preparation and Chemical Characteristics of Hemoglobin-Free Ghosts of Human Erythrocytes. Arch. Biochem. Biophys. 1963, 100 (1), 119–130.
- (129) Xu, X.; Khan, M. A.; Burgess, D. J. Predicting Hydrophilic Drug Encapsulation inside Unilamellar Liposomes. *Int. J. Pharm.* **2012**, *423* (2), 410–418.
- (130) Weber, G.; Farris, F. J. Synthesis and Spectral Properties of a Hydrophobic Fluorescent Probe: 6-Propionyl-2-(Dimethylamino)naphthalene. *Biochemistry* 1979, *18* (14), 3075–3078.
- (131) Sanchez, S. A.; Tricerri, M. A.; Gunther, G.; Gratton, E. Laurdan Generalized Polarization: From Cuvette to Microscope. *Mod. Res. Educ. Top. Microsc.* 2007, 2, 1007–1014.
- (132) Parasassi, T.; Krasnowska, E. K.; Bagatolli, L.; Gratton, E. Laurdan and Prodan as Polarity-Sensitive Fluorescent Membrane Probes. *J. Fluoresc.* **1998**, *8* (4), 365–373.

- (133) Velázquez, J. B.; Fernández, M. S. GPS, the Slope of Laurdan Generalized Polarization Spectra, in the Study of Phospholipid Lateral Organization and Escherichia Coli Lipid Phases. *Arch. Biochem. Biophys.* **2006**, *455* (2), 163–174.
- (134) Origin Lab Corporation. Origin Pro 8.0 Software. Origin Lab Corporation: Northampton, Massachusetts 2008.
- (135) Nobbmann, U. Protein Sizing by Light Scattering, Molecular Weight and Polydispersity. *Int. J. Biol. Macromol.* **1996**, *19* (3), 213–221.
- (136) Instruments, M. Inform White Paper Dynamic Light Scattering. *Malvern Guid*. **2011**, 1–6.
- (137) Marsh, D. Lateral Pressure Profile, Spontaneous Curvature Frustration, and the Incorporation and Conformation of Proteins in Membranes. *Biophys. J.* 2007, 93 (11), 3884–3899.
- (138) Prenner, E.; Honsek, G.; Hönig, D.; Möbius, D.; Lohner, K. Imaging of the Domain Organization in Sphingomyelin and Phosphatidylcholine Monolayers. *Chem. Phys. Lipids* 2007, 145 (2), 106–118.
- (139) Spot, K. S. V. N. Surface Potential Sensor. 1-4.
- (140) Bevers, E. M.; Williamson, P. L. Getting to the Outer Leaflet: Physiology of Phosphatidylserine Exposure at the Plasma Membrane. *Physiol. Rev.* 2016, 96 (2), 605–645.
- (141) Gustafson, J. P. Visual Minteq 3.1 Beta. Royal Institute of Technology, Land and Water Resource Engineering: Stockholm, Sweden 2010.
- (142) Kerek, E.; Hassanin, M.; Zhang, W.; Prenner, E. J. Preferential Binding of Inorganic Mercury to Specific Lipid Classes and Its Competition with Cadmium. *Biochim. Biophys. Acta - Biomembr.* 2017, 1859 (7), 1211–1221.
- (143) Hassanin, M.; Kerek, E. M.; Chiu, M.; Anikovskiy, M.; Prenner, E. J. Binding Affinity of Inorganic Mercury and Inorganic Cadmium to Biomimetic Erythrocyte Membranes and Their Influence on Membrane Phase Transition. J. Phys. Chem. B 2016, 120, 12872–12882.
- (144) Mabrey, S.; Sturtevant, J. M. Investigation of Phase Transitions of Lipids and Lipid Mixtures by High Sensitivity Differential Scanning Calorimetry. *Proc. Natl. Acad. Sci. USA* 1976, 73 (11), 3862–3866.
- (145) Chapman, D.; Peel, W. E.; Kingston, B.; Lilley, T. H. Lipid Phase Transitions in Model Biomembranes: The Effect of Ions on Phosphatidylcholine Bilayers. *Biochim. Biophys. Acta* 1977, 464, 260–275.

- (146) Broniec, A.; Goto, M.; Matsuki, H. A Peculiar Phase Transition of Plasmalogen Bilayer Membrane under High Pressure. *Langmuir* **2009**, *25* (19), 11265–11268.
- (147) Avanti Polar Lipids Inc. Avanti Lipids http://avantilipids.com.
- (148) Mattai, J.; Hauser, H.; Demel, R. A.; Shipley, G. G. Interactions of Metal Ions with Phosphatidylserine Bilayer Membranes: Effect of Hydrocarbon Chain Unsaturation. *Biochemistry* **1989**, 28 (5), 2322–2330.
- (149) Xu, J.; Ji, L. D.; Xu, L. H. Lead-Induced Apoptosis in PC 12 Cells: Involvement of p53, Bcl-2 Family and Caspase-3. *Toxicol. Lett.* **2006**, *166* (2), 160–167.
- (150) Adonaylo, V. N.; Oteiza, P. I. Pb2+ Promotes Lipid Oxidation and Alterations in Membrane Physical Properties. *Toxicology* **1999**, *132* (1), 19–32.
- (151) Marsh, D. CRC Handbook of Lipid Bilayers, 2nd ed.; CRC Press, 2013.
- (152) Abramson, M.; Wilson, C.; Gregor, H.; Katzman, R. Ionic Properties of Aqueous Dispersions of Phosphatidic Acid. J. Biol. Chem. **1964**, 239 (12), 4066–4072.
- (153) Sauvé, S.; Mcbride, M.; Hendershot, W. Lead Phosphate Solubility in Water and Soil Suspensions. *Environ. Sci. Technol.* **1998**, *32* (3), 388–393.
- (154) Foster, D. A. Phosphatidic Acid Signaling to mTOR: Signals for the Survival of Human Cancer Cells. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 2009, 1791 (9), 949–955.
- (155) McLaughlin, S.; Wang, J.; Gambhir, A.; Murray, D. PIP 2 and Proteins: Interactions, Organization, and Information Flow. *Annu. Rev. Biophys. Biomol. Struct.* 2002, 31 (1), 151–175.
- (156) Sathappa, M.; Alder, N. N. The Ionization Properties of Cardiolipin and Its Variants in Model Bilayers. *Biochim. Biophys. Acta Biomembr.* **2016**, *1858* (6), 1362–1372.
- (157) Haines, T. H.; Dencher, N. A. Cardiolipin: A Proton Trap for Oxidative Phosphorylation. *FEBS Lett.* **2002**, *528* (1–3), 35–39.
- (158) Mansour, H. M.; Zografi, G. The Relationship Between Water Vapor Absorption and Desorption by Phospholipids and Bilayer Phase Transitions. J. Pharm. Sci. 2007, 96 (2), 377–396.
- (159) Sendecki, A. M.; Poyton, M. F.; Baxter, A. J.; Yang, T.; Cremer, P. S. Supported Lipid Bilayers with Phosphatidylethanolamine as the Major Component. *Langmuir* 2017, *33* (46), 13423–13429.
- (160) Brites, P.; Waterham, H. R.; Wanders, R. J. A. Functions and Biosynthesis of Plasmalogens in Health and Disease. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 2004, 1636 (2–3), 219–231.

- (161) Kosicek, M.; Hecimovic, S. Phospholipids and Alzheimer's Disease: Alterations, Mechanisms and Potential Biomarkers. *Int. J. Mol. Sci.* **2013**, *14* (1), 1310–1322.
- (162) Kota, V.; Hama, K. 2'-Hydroxy Ceramide in Membrane Homeostasis and Cell Signaling. *Adv. Biol. Regul.* **2014**, *0*, 223–230.
- (163) Jendrasiak, G. L. The Hydration of Phospholipids and Its Biological Significance. *J. Nutr. Biochem.* **1996**, 7 (11), 599–609.
- (164) Chiu, M. H.; Wan, C. P. L.; Weers, P. M. M.; Prenner, E. J. Apolipophorin III Interaction with Model Membranes Composed of Phosphatidylcholine and Sphingomyelin Using Differential Scanning Calorimetry. *Biochim. Biophys. Acta -Biomembr.* 2009, 1788 (10), 2160–2168.
- (165) Koynova, R.; Caffrey, M. Phases and Phase Transitions of the Hydrated Phosphatidylethanolamines. *Chem. Phys. Lipids* **1994**, *69* (1), 1–34.
- (166) Lohner, K.; Balgavy, P.; Hermetter, A.; Paltauf, F.; Laggner, P. Stabilization of Non-Bilayer Structures by the Etherlipid Ethanolamine Plasmalogen. BBA -Biomembr. 1991, 1061 (2), 132–140.
- (167) Le, M. T.; Hassanin, M.; Mahadeo, M.; Gailer, J.; Prenner, E. J. Hg- and Cd-Induced Modulation of Lipid Packing and Monolayer Fluidity in Biomimetic Erythrocyte Model Systems. *Chem. Phys. Lipids* **2013**, *170–171*, 46–54.
- (168) Alsina, M. A.; Mestres, C.; Valencia, G.; Antón, J. M. G.; Reig, F. Interaction Energies of Mixing Phosphatidylserine and Phosphatidylcholine Monolayers. *Colloids and Surfaces* **1988**, *34* (2), 151–158.
- (169) Zheng, W.; Aschner, M.; Ghersi-Egea, J. F. Brain Barrier Systems: A New Frontier in Metal Neurotoxicological Research. *Toxicol. Appl. Pharmacol.* 2003, 192 (1), 1– 11.
- (170) He, Y.; Liu, M.; Darabedian, N.; Liang, Y.; Wu, D.; Xiang, J.; Zhou, F. PH-Dependent Coordination of Pb2+ to metallothionein2: Structures and Insight into Lead Detoxification. *Inorg. Chem.* **2014**, *53* (6), 2822–2830.
- (171) Carpenter, M. C.; Shami Shah, A.; DeSilva, S.; Gleaton, A.; Su, A.; Goundie, B.; Croteau, M. L.; Stevenson, M. J.; Wilcox, D. E.; Austin, R. N. Thermodynamics of Pb(<scp>ii</scp>) and Zn(<scp>ii</scp>) Binding to MT-3, a Neurologically Important Metallothionein. *Metallomics* 2016, 8 (6), 605–617.
- (172) Lampert, P. W.; Schochet, S. S. Demyelination and Remyelination in Lead Neuropathy. J. Neuropathy Exp. Neurol. **1968**, 27 (4), 527–545.
- (173) Shah, J.; Atienza, J. M.; Duclos, R. I.; Rawlings, a V; Dong, Z.; Shipley, G. G. Structural and Thermotropic Properties of Synthetic C16:0 (Palmitoyl) Ceramide: Effect of Hydration. J. Lipid Res. 1995, 36 (9), 1936–1944.

- (174) Baloh, R. W. Laboratory Diagnosis of Increased Lead Absorption. *Environ. Heath* **1974**, *28* (4), 198–208.
- (175) Cavalleri, A.; Minoia, C.; Pozzoli, L.; Baruffini, A. Determination of Plasma Lead Levels in Normal Subjects and in Lead-Exposed Workers. *Br. J. Ind. Med.* **1978**, *35*, 21–26.
- (176) Hajem, S.; Moreau, T.; Hannaert, P.; Lellouch, J.; Huel, G.; Hellier, G.; Orssaud, G.; Claude, J. R.; Juguet, B.; Festy, B.; et al. Influence of Environmental Lead on Membrane Ion Transport in a French Urban Male Population. *Environ. Res.* 1990, 53 (2), 105–118.
- (177) Jang, W. H.; Lim, K. M.; Kim, K.; Noh, J. Y.; Kang, S.; Chang, Y. K.; Chung, J. H. Low Level of Lead Can Induce Phosphatidylserine Exposure and Erythrophagocytosis: A New Mechanism Underlying Lead-Associated Anemia. *Toxicol. Sci.* 2011, 122 (1), 177–184.
- (178) Ong, C. N.; Lee, W. R. Interaction of Calcium and Lead in Human Erythrocytes. *Br. J. Ind. Med.* **1980**, *37*, 70–77.
- (179) Donaldson, W. E.; Knowles, S. O. Is Lead Toxicosis a Reflection of Altered Fatty Acid Composition of Membranes? *Comp. Biochem. Physiol.* **1993**, *104* (3), 377– 379.
- (180) Bressler, J. P.; Goldstein, G. W. Mechanisms of Lead Neurotoxicity. *Biochem. Pharmacol.* **1991**, *41* (4), 479–484.
- (181) Dąbrowska-Bouta, B.; Sulkowski, G.; Walski, M.; Strużyńska, L.; Lenkiewicz, A.; Rafałowska, U. Acute Lead Intoxicationin Vivoaffects Myelin Membrane Morphology and CNPase Activity. *Exp. Toxicol. Pathol.* **2000**, *52* (3), 257–263.
- (182) Abbott, S. K.; Else, P. L.; Atkins, T. A.; Hulbert, A. J. Fatty Acid Composition of Membrane Bilayers: Importance of Diet Polyunsaturated Fat Balance. *Biochim. Biophys. Acta - Biomembr.* 2012, *1818* (5), 1309–1317.
- (183) Nouri-Sorkhabi, M. H.; Agar, N. S.; Sullivan, D. R.; Gallagher, C.; Kuchel, P. W. Phospholipid Composition of Erythrocyte Membranes and Plasma of Mammalian Blood Including Australian Marsupials; quantitative31P NMR Analysis Using Detergent. *Comp. Biochem. Physiol. - B Biochem. Mol. Biol.* **1996**, *113* (2), 221– 227.
- (184) van den Boom, M. A. P.; Wassink, M. G.; Westerman, J.; de Fouw, N. J.; Roelofsen, B.; Op den Kamp, J. A. F.; van Deenen, L. L. M. In Vivo Turnover of Phospholipids in Rabbit Erythrocytes. *Biochim. Biophys. Acta (BBA)/Lipids Lipid Metab.* 1994, 1215 (3), 314–320.

- (185) Kamber, E.; Kopeikina-Tsiboukidou, L. Alterations in the Activities of Rabbit Erythrocyte Membrane-Bound Enzymes Induced by Cholesterol Enrichment and Depletion Procedures. *Zeitschrift fur Naturforsch. Sect. C J. Biosci.* **1986**, *41* (3), 301–309.
- (186) Marquardt, D.; Geier, B.; Pabst, G. Asymmetric Lipid Membranes: Towards More Realistic Model Systems. *Membranes (Basel)*. **2015**, *5* (2), 180–196.
- (187) Shettihalli, A. K.; Gummadi, S. N. Biochemical Evidence for Lead and Mercury Induced Transbilayer Movement of Phospholipids Mediated by Human Phospholipid Scramblase 1. *Chem. Res. Toxicol.* **2013**, *26* (6), 918–925.
- (188) Bannon, D.; Olivi, L.; Bressler, J. The Role of Anion Exchange in the Uptake of Pb by Human Erythrocytes and Madin-Darby Canine Kidney Cells. *Toxicology2* **2000**, *147*, 101–107.
- (189) Pettegrew, J. W.; Panchalingam, K.; Hamilton, R. L.; Mcclure, R. J. Brain Membrane Phospholipid Alterations in Alzheimer's Disease. *Neurochem. Res.* 2001, 26 (7), 771–782.
- (190) Folch, J. Brain Cephalin, a Mixture of Phosphatides. Separation from It of Phosphatidyl Serine, Phosphatidyl Ethanolamine and a Fraction Containing an Inositol Phosphatide. *J. Biol. Chem.* **1942**, *146*, 35–44.
- (191) Hu, Y.; Doudevski, I.; Wood, D.; Moscarello, M.; Husted, C.; Genain, C.; Zasadzinski, J. A. A.; Israelachvili, J. Synergistic Interactions of Lipids and Myelin Basic Protein. *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101* (37), 13466–13471.
- (192) Seidl, A. H. Regulation of Conduction Time along Axons. *Neuroscience* **2014**, 276, 126–134.
- (193) Balla, T. Phosphoinositides: Tiny Lipids With Giant Impact on Cell Regulation. *Physiol. Rev.* **2013**, *93* (3), 1019–1137.
- (194) ECKHARDT, M.; YAGHOOTFAM, A.; FEWOU, S. N.; ZÖLLER, I.; GIESELMANN, V. A Mammalian Fatty Acid Hydroxylase Responsible for the Formation of α-Hydroxylated Galactosylceramide in Myelin. *Biochem. J.* 2005, 388 (1), 245–254.
- (195) Brown, R. E. Sphingolipid Organization in Biomembranes: What Physical Studies of Model Membranes Reveal. J. Cell Sci. **1998**, 111 (Pt 1, 1–9.
- (196) Gennis Robert B. Biomembranes: Molecular Structure and Function; 1989.
- (197) Li, Z.; Venable, R. M.; Rogers, L. A.; Murray, D.; Pastor, R. W. Molecular Dynamics Simulations of PIP2 and PIP3 in Lipid Bilayers: Determination of Ring Orientation, and the Effects of Surface Roughness on a Poisson-Boltzmann Description. *Biophys J* 2009, 97 (1), 155–163.

- (198) Slochower, D. R.; Huwe, P. J.; Radhakrishnan, R.; Janmey, P. A. Quantum and All-Atom Molecular Dynamics Simulations of Protonation and Divalent Ion Binding to Phosphatidylinositol 4,5-Bisphosphate (PIP2). J. Phys. Chem. B 2013, 117 (28), 8322–8329.
- (199) Fuller, J. C.; Martinez, M.; Wade, R. C. On Calculation of the Electrostatic Potential of a Phosphatidylinositol Phosphate-Containing Phosphatidylcholine Lipid Membrane Accounting for Membrane Dynamics. *PLoS One* **2014**, *9* (8).
- (200) Lupyan, D.; Mezei, M.; Logothetis, D. E.; Osman, R. A Molecular Dynamics Investigation of Lipid Bilayer Perturbation by PIP₂. *Biophys. J.* **2010**, *98* (2), 240– 247.
- (201) Bradshaw, J. P.; Bushby, R. J.; Giles, C. C. D.; Saunders, M. R.; Saxena, A. The Headgroup Orientation of Dimyristoylphosphatidylinositol-4-Phosphate in Mixed Lipid Bilayers a Neutron Diffraction Study. *Biochim. Biophys. Acta - Biomembr.* **1997**, *1329* (1), 124–138.
- (202) Bradshaw, J. P.; Bushby, R. J.; Giles, C. C. D.; Saunders, M. R. Orientation of the Headgroup of Phosphatidylinositol in a Model Biomembrane as Determined by Neutron Diffraction. *Biochemistry* **1999**, *38* (26), 8393–8401.
- (203) Toker, A.; Cantley, L. C. Signalling through the Lipid Products of Phosphoinositide-3-OH Kinase. *Nature* **1997**, *387* (6634), 673–676.
- (204) Campa, C. C.; Martini, M.; De Santis, M. C.; Hirsch, E. How PI3K-Derived Lipids Control Cell Division. *Front. Cell Dev. Biol.* 2015, 3 (September), 1–9.
- (205) Li, H.; Marshall, A. J. Phosphatidylinositol (3,4) Bisphosphate-Specific Phosphatases and Effector Proteins: A Distinct Branch of PI3K Signaling. *Cell. Signal.* 2015, 27 (9), 1789–1798.
- (206) Ho, C. Y.; Alghamdi, T. A.; Botelho, R. J. Phosphatidylinositol-3,5-Bisphosphate: No Longer the Poor PIP2. *Traffic* **2012**, *13* (1), 1–8.
- (207) Mühleisen, M.; Probst, W.; Hayashi, K.; Rahmann, H. Calcium Binding to Liposomes Composed of Negatively Charged Lipid Moieties. *Jpn. J. Exp. Med.* **1983**, 53 (2), 103–107.
- (208) Lindmo, K. Regulation of Membrane Traffic by Phosphoinositide 3-Kinases. J. Cell Sci. 2006, 119 (4), 605–614.
- (209) Janetopoulos, C.; Devreotes, P. Phosphoinositide Signaling Plays a Key Role in Cytokinesis. J. Cell Biol. 2006, 174 (4), 485–490.

- (210) Delage, E.; Puyaubert, J.; Zachowski, A.; Ruelland, E. Signal Transduction Pathways Involving Phosphatidylinositol 4-Phosphate and Phosphatidylinositol 4,5-Bisphosphate: Convergences and Divergences among Eukaryotic Kingdoms. *Prog. Lipid Res.* 2013, 52 (1), 1–14.
- (211) Balla, T.; Szentpetery, Z.; Kim, Y. J. Phosphoinositide Signaling: New Tools and Insights. *Physiol.* 2009, 24, 231–244.
- (212) Gassama-Diagne, A.; Yu, W.; ter Beest, M.; Martin-Belmonte, F.; Kierbel, A.; Engel, J.; Mostov, K. Phosphatidylinositol-3,4,5-Trisphosphate Regulates the Formation of the Basolateral Plasma Membrane in Epithelial Cells. *Nat. Cell Biol.* 2006, *8* (9), 963–970.
- (213) Gamper, N.; Shapiro, M. S. Regulation of Ion Transport Proteins by Membrane Phosphoinositides. *Nat. Rev. Neurosci.* **2007**, *8* (12), 921–934.
- (214) Hilgemann, D. W.; Feng, S.; Nasuhoglu, C. The Complex and Intriguing Lives of PIP2 with Ion Channels and Transporters. *Sci. Signal.* **2001**, *2001* (111), re19-re19.
- (215) Cantrell, D. a. Phosphoinositide 3-Kinase Signalling Pathways. J. Cell Sci. 2001, 114 (Pt 8), 1439–1445.
- (216) Rawlings, D.; Saffran, D.; Tsukada, S.; Largaespada, D.; Grimaldi, J.; Cohen, L.; Mohr, R.; Bazan, J.; Howard, M.; Copeland, N.; et al. Mutation of Unique Region of Bruton's Tyrosine Kinase in Immunodeficient XID Mice. *Science (80-.).* **1993**, *261* (5119), 358–361.
- (217) Sun, Y.; Turbin, D. A.; Ling, K.; Thapa, N.; Leung, S.; Huntsman, D. G.; Anderson, R. A. Type I Gamma Phosphatidylinositol Phosphate Kinase Modulates Invasion and Proliferation and Its Expression Correlates with Poor Prognosis in Breast Cancer. *Breast Cancer Res.* **2010**, *12* (1), 1–11.
- (218) Bunney, T. D.; Katan, M. Phosphoinositide Signalling in Cancer: Beyond PI3K and PTEN. *Nat. Rev. Cancer* **2010**, *10* (5), 342–352.
- (219) Daear, W.; Mahadeo, M.; Prenner, E. J. Applications of Brewster Angle Microscopy from Biological Materials to Biological Systems. *Biochim. Biophys. Acta - Biomembr.* 2017, 1859 (10), 1749–1766.
- (220) Wang, Y. H.; Slochower, D. R.; Janmey, P. A. Counterion-Mediated Cluster Formation by Polyphosphoinositides. *Chem. Phys. Lipids* **2014**, *182*, 38–51.
- (221) Bradshaw, J. P.; Bushby, R. J.; Giles, C. C.; Saunders, M. R.; Reid, D. G. Neutron Diffraction Reveals the Orientation of the Headgroup of Inositol Lipids in Model Membranes. *Nat. Struct. Biol.* **1996**, *3* (2), 125–127.
- (222) Büldt, G.; Gally, H. U.; Seelig, J.; Zaccai, G. Neutron Diffraction Studies on Phosphatidylcholine Model Membranes. *J. Mol. Biol.* **1979**, *134* (4), 673–691.

- (223) Vogel, V.; Möbius, D. Local Surface Potentials and Electric Dipole Moments of Lipid Monolayers : Contributions of the Water / Lipid and the Lipid / Air Interfaces. J. Colloid Interface Sci. 1988, 126 (2), 408–420.
- (224) Peng, A.; Pisal, D. S.; Doty, A.; Balu-Iyer, S. V. Phosphatidylinositol Induces Fluid Phase Formation and Packing Defects in Phosphatidylcholine Model Membranes. *Chem. Phys. Lipids* **2012**, *165* (1), 15–22.
- (225) Smith, D.; Kahng, M.; Quintanilla-Vega, B.; Fowler, B. High-Affinity Renal Lead-Binding Proteins in Environmentally-Exposed Humans. *Toxicol. Appl. Pharmacol.* **1998**, *115*, 39–52.
- (226) Oskarsson, A.; Fowler, B. Effects of Lead Inclusion Bodies on Subcellular Distribution of Lead in Rat Kidney: The Relationship to Mitochondrial Function. *Exp. Mol. Pathol.* **1985**, *43*, 397–408.
- (227) He, L.; Poblenz, A. T.; Medrano, C. J.; Fox, D. A. Lead and Calcium Produce Rod Photoreceptor Cell Apoptosis by Opening the Mitochondrial Permeability Transition Pore. J. Biol. Chem. 2000, 275 (16), 12175–12184.
- (228) Manno, S.; Takakuwa, Y.; Mohandas, N. Identification of a Functional Role for Lipid Asymmetry in Biological Membranes: Phosphatidylserine-Skeletal Protein Interactions Modulate Membrane Stability. *Proc. Natl. Acad. Sci. U. S. A.* 2002, 99 (4), 1943–1948.
- (229) Jozefczak, M.; Remans, T.; Vangronsveld, J.; Cuypers, A. Glutathione Is a Key Player in Metal-Induced Oxidative Stress Defenses. *Int. J. Mol. Sci.* **2012**, *13* (3), 3145–3175.
- (230) Thirumoorthy, N.; Kumar, K. T. M.; Sundar, A. S.; Panayappan, L.; Chatterjee, M. Metallothionein : An Overview. *World J. Gastroenterol.* **2007**, *13* (7), 993–996.
- (231) Gorkhali, R.; Huang, K.; Kirberger, M.; Yang, J. J. Defining Potential Roles of Pb2+ in Neurotoxicity from a Calciomics Approach. *Metallomics* **2016**, *8* (6), 563–578.

Appendix A



The work presented below using laurdan GP was completed by Jenelle Umbsaar for this study on Pb²⁺:

Figure A.1. GP values for 300µM LUVs of heart, liver, yeast, and *E. coli* polar lipid extracts with increasing concentrations of Pb²⁺ (0 – 2.1mM) in 100 mM NaCl pH 7.4 as a function of temperature (°C). Results are the average of 3 replicates \pm standard deviation.

As seen with the brain polar lipid extract presented in Chapter 5, Section 5.2.1 Laurdan GP of Brain Polar Extracts with Pb, the GP of heart, liver, yeast, and *E. coli* extracts exhibited rigidity by Pb^{2+} across the entire temperature range for increasing concentrations of Pb^{2+} .

Appendix B





Figure B.1. The change in laurdan GP at 35°C when exposed to 1200 μ M Pb²⁺ (red), Cd²⁺ (green), Co²⁺ (purple), Ni²⁺ (blue) and 900 μ M Mn²⁺ (orange) for each 1% PI lipid in 99% POPC and 100% POPC systems relative to control scans with no metal. Results are the average of 3 replicates ± SD. Asterisks denote statistical significance (p < 0.05).

Multi – metal comparison of the effects of lead (Pb²⁺), cadmium (Cd²⁺), cobalt (Co²⁺), nickel (Ni²⁺), and manganese (Mn²⁺) on the membrane rigidity of phosphatidylinositol (PI) LUVs composed of 1:99 PI/PC. Overall, the system most strongly affected by all of the metals was PI(3,4)P₂. The most consistently impactful metal was Pb²⁺, while the largest effects in both GP (PI(4)P) were caused by Co²⁺. This study has demonstrated significant interactions of various metal ions with liposomes containing a physiologically-relevant PI level of 1%.

Appendix C

The work presented below was completed in collaboration with a colleague, Weiam Daear for a multi – metal (Pb^{2+} , Cd^{2+} , Co^{2+} , Ni^{2+} , and Mn^{2+}) study on PIs by using monolayers:



Figure C.1. The change in molecular area (Å²/mol) when isotherms are held at 30mN/m in the presence of 86.2 μ M Pb²⁺ (red), Cd²⁺ (green), Co²⁺ (purple), Ni²⁺ (blue) or Mn²⁺ (orange) for each lipid system relative to control scans with no metal. Data is the average of 3 replicates ± SD. Asterisks denote statistical significance (p<0.05).

Multi – metal comparison of the effects of lead (Pb^{2+}), cadmium (Cd^{2+}), cobalt (Co^{2+}), nickel (Ni^{2+}), and manganese (Mn^{2+}) on the lateral organization of phosphatidylinositol (PI) monolayers composed of 1:99 PI/PC. Figure C.1. is a comparison of the shift in molecular area at the physiologically relevant surface pressure of 30 mN/m¹³⁷. Similar to Appendix A, the system most strongly affected by all of the metals was PI(3,4)P₂. The most consistently impactful metal was Mn^{2+} , while different
interactions were observed for each metal and each PI system. These differences are highlighted by the BAM images of 1% $DOPI(3,4,5)P_3$ 99% POPC monolayers with each metal taken at collapse in Figure C.2.



Figure C.2. BAM collapse pressure images of 1% DOPI(3,4,5)P₃ 99% POPC monolayers + 86.2 μ M Pb²⁺, Cd²⁺, Co²⁺, Ni²⁺ or Mn²⁺ in 100mM NaCl pH 7.4 at the air-water interface. Each image is 218 x 271 microns; the scale bar is 50 microns.

Appendix D

Poster Presentations

- <u>Robyn Mundle</u>, Evan Kerek, and Elmar Prenner. (2016). Lead and Cadmium Toxicity: Their Effect on Biomimetic Lipid Membranes. 13th European Biological Inorganic Chemistry Conference (EuroBIC 13). Budapest, Hungary. August 28 – September 1, 2016.
- <u>Robyn Mundle</u>, Evan Kerek, and Elmar Prenner. (2017). Lead and Cadmium Toxicity: Their Effect on Biomimetic Lipid Membranes. 1st Biological Sciences Graduate Symposium. Calgary, Alberta, Canada. February 17, 2017.
- <u>Robyn Mundle</u>, Elmar Prenner. (2017). The Effect of Lead on the Fluidity of Phospholipid Membranes. 6th Georgian Bay International Conference on Bioinorganic Chemistry (CanBIC 6). Parry Sound, Ontario, Canada. May 24 – 26, 2017.
- Jenelle Umbsaar, <u>Robyn Mundle</u>, Elmar Prenner. (2017). Effects of Nickel and Cobalt on Biomimetic Lipid Membranes. 100th Canadian Chemistry Conference and Exhibition (CSC). Toronto, Ontario, Canada. May 28 – June 1, 2017.

Oral Presentations

<u>Robyn Mundle</u>. (2018). Lead Interactions Affect the Fluidity and Lateral Organization of Complex Lipid Membranes. Prairie University Biological Symposium. Calgary, Alberta, Canada. February 22 – 24, 2018.