SILOXANE-CONTAINING PHOSPHOLIPIDS AS POTENTIAL DRUG DELIVERY SYSTEMS

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Abstract

Drug delivery systems (DDS) have become an important focus research over the past three decades with the emergence of new therapies based on biomolecules such as peptides, DNA, enzymes etc., with the aim of improving or optimising the potency of these biomolecules.

Liposomes are colloidal vesicles derived from amphiphilic phospholipid biomolecules and have the capacity to encapsulate a broad spectrum of molecules. The promise of liposomes in regard to drug delivery relates to an increase in the bioavailability of drugs, a decrease in drug toxicity, an increase in the efficacy of the drug, and targeted delivery to areas of pathology.

Different liposomes varying in their phospholipid composition present different characteristics in terms of entrapment capacity of drugs, stability of liposomes and drug release profile. Optimizing any of these characteristics will hence improve the efficiency of liposomes as DDS.

This study reveals the potential for siloxane-containing phospholipids to display a higher entrapment capacity than conventional liposomes related to 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) and controlled released when using the model compound, calcein. With the siloxane-phospholipids being studied, zero-order release was observed and in some instances appeared to be independent of the pH of the release media.
To my family
Acknowledgments

This work could have never come to an end without the contribution and assistance of several people. First and foremost, I would like to express my gratitude to my supervisor Dr. Paul Zelisko, for providing me with the opportunity to express myself as a researcher. His guidance, patience and moral support throughout this work as well as his pieces of advice to clarify various future goals and thoughts were greatly appreciated.

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Finally, I would like to thank my parents, my siblings, and friends who helped me to become an evolved human through all this work. A special thought for Hawa M. Conde and Emil Nelly Tchapchet for their genuine presence and help over the years.
“what really counts is not the value itself but how valuable it is to you”
Table of Content

List of Figures: ........................................................................................................ vii
List of Abbreviations .......................................................................................... xii

1 Introduction ........................................................................................................... 1

2 Historical .............................................................................................................. 4
  2.1 Drug delivery ..................................................................................................... 4
    2.1.1 Parenteral routes for drug delivery ............................................................... 5
    2.1.2 Transdermal drug delivery ........................................................................ 6
    2.1.3 Pulmonary drug delivery ........................................................................... 7
    2.1.4 Oral drug delivery ...................................................................................... 8
    2.1.5 Strategies for increasing bioavailability ..................................................... 10
  2.2 Colloidal drug delivery systems ...................................................................... 11
    2.2.1 Micelles ...................................................................................................... 11
    2.2.2 Emulsions .................................................................................................. 12
  2.3 Introduction to liposomal drug delivery .......................................................... 14
    2.3.1 Phospholipids and liposomes .................................................................... 15
    2.3.2 Liposomes stability and stabilization .......................................................... 21
    2.3.3 Liposomes as a drug delivery system ......................................................... 23
      Biological role .................................................................................................. 25
  2.4 Drug loading and drug release ...................................................................... 28
    2.4.1 Assay to study drug release ....................................................................... 32
  2.5 A brief introduction to silicon chemistry ....................................................... 33
    2.5.1 Biomedical silicones ................................................................................ 36
    2.5.2 Siloxane liposomes in drug delivery .......................................................... 37
  2.6 Phospholipid synthesis .................................................................................. 38
    2.6.1 Lipases ....................................................................................................... 39
    2.6.2 Chemoenzymatic synthesis of siloxane phospholipids ............................. 40
    2.6.3 Nomenclature of silicon-containing phospholipids ................................ 42
  2.7 Fluorescence spectroscopy .......................................................................... 45
    2.7.1 Calcein ....................................................................................................... 47
  2.8 Aim of the Thesis ............................................................................................ 48

3 Results and Discussion ....................................................................................... 49
  3.1 Hydrolysis of siloxane ester to siloxane acid ............................................... 49
    3.1.1 Chemoenzymatic synthesis of trisiloxane acid (3) .................................... 49
    3.1.2 Chemoenzymatic synthesis of disiloxane acid (8) .................................... 51
  3.2 Hydrosilylation of mono-unsaturated esters and mono-unsaturated acids .... 52
  3.3 sn-2 Substitution of lysophospholipids to synthesize phospholipids ............... 54
  3.4 Size analysis of liposomes before extrusion ................................................. 58
  3.5 Size analysis of liposomes after extrusion .................................................... 60
  3.6 Entrapment efficiency of liposomes ............................................................. 61
  3.7 Calcein release profiles .................................................................................. 64
    3.7.1 Proposed release mechanism(s) ................................................................. 77
  3.8 Size analysis of liposomes after release studies ............................................ 82
  3.9 Size analysis of liposomes after application of Triton X-100 ....................... 82
3.10 Specific rotation of lipids ................................................................. 83
3.11 Stability of liposomes with time ...................................................... 85

4 Conclusions .......................................................................................... 87

5 Experimental ............................................................................................ 90
  5.1 Materials ............................................................................................. 90
  5.2 General Experimental Procedures ...................................................... 92
  5.3 Detailed Experimental Methods .......................................................... 93
    5.3.1 Synthetic Experimental Procedures .............................................. 93
    5.3.2 Drug Delivery Experimental Methods .......................................... 108
      5.3.2.1 Liposome preparation and formulation with Calcein .................. 108
      5.3.2.1.1 POPC ............................................................................ 108
      5.3.2.1.2 PalValDSPC (10) ................................................................. 109
      5.3.2.1.3 16:0 LPC ....................................................................... 109
      5.3.2.1.4 LysoTSPC (4) .................................................................. 109
      5.3.2.1.5 1,2-SiPC (5) .................................................................... 110
      5.3.2.1.6 PalCpcTSPC (11) ................................................................. 110
      5.3.2.1.7 OleCpcTSPC (12) ............................................................... 110
    5.3.2.2 Liposome purification ..................................................................... 111
    5.3.2.3 Liposome release .......................................................................... 111
    5.3.3 Liposome Stability experiment ....................................................... 113
    5.3.4 Buffers preparation ........................................................................... 113

6 Appendices .................................................................................................. 115

7 Vita ............................................................................................................. 171

8 References ................................................................................................... 172
List of Figures:

Figure 1. Routes of drug administration. Reproduced with permission from Ref. 18. Copyright © 2017 by National Pain Centers LLC. ................................................................. 5
Figure 2. Illustration of transdermal pathway. Reprinted with permission from Ref. 27. Copyright © 2012 by Elsevier B.V. ................................................................. 7
Figure 3. Biopharmaceutical Classification System (BCS) of Drugs. Adapted from Ref. 45. Copyright © 2012 by Wiley Periodicals, Inc. .................................................. 9
Figure 4. Schematic representation of a micelle. ................................................................. 12
Figure 5. Some colloidal vesicles. Reprinted with permission from Ref. 62. Copyright © 2010 by Elsevier B.V. ................................................................. 14
Figure 6. Schematic representation of a liposome. Reprinted with permission from Ref. 86. Copyright © 2008 by Philip Chalmers. ................................................................. 16
Figure 7. Classification of glycerophospholipids with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine(POPC), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-Distearoyl-sn-glycero-3-phosphoglycerol (DSPG) as examples. ................................................................. 16
Figure 8. Relationship between geometrical packing and surfactant parameter. Reprinted with permission from Ref. 85. Copyright © 2014 by Royal Society of Chemistry. ................................................................. 19
Figure 9. Net interaction potential between particles based on DLVO theory. Reprinted from Ref. 96. Copyright © 2016 by Nguyen Phat JSC. ................................................................. 22
Figure 10. Illustration of active targeting of liposomes. Conjugation of ligand directly on the phospholipid head groups of conventional liposomes (a); conjugation directly on the phospholipid head groups of PEGylated liposomes (b); and conjugation on the free terminus of PEGylated chains (c). Reprinted with permission from Ref. 103. Copyright © 2012 by American Association of Pharmaceutical Scientists. ................................................................. 27
Figure 11. Preparation of Silicone. Adapted from Ref. 11. Copyright © 2009 by Scientific Information Database (SID). ................................................................. 34
Figure 12. Structure of polydimethylsiloxane. ................................................................. 36
Figure 13. Illustration of matrix and reservoir configuration in silicone drug delivery systems. Reproduced with permission from Ref. 145. Copyright © 2010 by Advantage Business Marketing. ................................................................. 38
Figure 14. Regioselective hydrolysis of a triacylglycerol to a diacylglycerol. Adapted from Ref. 164. Copyright © 2009 by Elsevier Inc. ................................................................. 40
Figure 15. General scheme of first-generation synthesis of 1,2-SiPC and 1,3-SiPC. (A) 10wt% Lipozyme RM, 40°C, 72h; (B) 2-chloro-1,3,2-dioxophospholane-2-oxide, NEt3, PhMe; (C) NEt3, AcCN, -40°C to RT M. B. Adapted with permission from Ref. 75. Copyright © 2017 John Wiley & Sons, Inc. ................................................................. 41
Figure 16. Unsymmetrical silicon-containing lipid. ................................................................. 42
Figure 17. Some named silicon-containing lipids. ................................................................. 45
Figure 18. Illustration of the Jablonski Diagram. Adapted from Ref. 168. Copyright © 2006 by Springer Science+Business Media, LLC. ................................................................. 46
Figure 19. Chemical structure of Calcein. ................................................................. 48
Figure 20. Structure of trisiloxane ester (2), trisiloxane acid (3) and LysoTSPC (4). ................................................................. 49
Figure 21. Proposed reaction scheme of lipase hydrolysis of a siloxane ester. 51
Figure 22. Proposed reaction scheme of platinum catalyzed hydrosilylation of an unsaturated moiety.

Figure 23. Karstedt's catalyst.

Figure 24. The chemical structures of PalValDSPC (10), PalCpcTSPC (11) obtained from the lysophospholipid 16:0 LPC and 1,2-SiPC (5) from LysoTSPC.

Figure 25 General reaction scheme for the second-generation total synthesis of 1,2-SiPC.

Figure 26 General reaction scheme for the total synthesis of PalValDSPC.

Figure 27 General reaction scheme for the total synthesis of PalCpcTSPC.

Figure 28 DLS measurements of vesicles before release experiments. Lysophospholipid 16:0 LPC after vortexing exhibiting 40nm in size, siloxane lysophospholipid LysoTSPC exhibiting ~100nm in size after vortexing and ~ 90 after extrusion, phospholipids PalValDSPC and POPC exhibiting ~100nm in size. Experiments were done in triplicates.

Figure 29 Release profiles of POPC at different pH values All points were measured in triplicate, and error bars represent the standard deviation in the measurements.

Figure 30 Release profiles of 1,2-SiPC at different pH values All points were measured in triplicate, and error bars represent the standard deviations in the measurements.

Figure 31 Release profiles of 16:0 LPC at different pH values. All points were measured in triplicates, and the error bars represent the standard deviation in the measurements.

Figure 32 Release profiles of LysoTSPC at different pH values. All points were measured in triplicate, and the error bars represent the standard deviation in the measurements.

Figure 33 Release profiles of extruded LysoTSPC at different pH values. All points were measured in triplicate, and the error bars represent the standard deviation in the measurements.

Figure 34 Release profiles of 1,2-SiPC at different pH measurements. All points were measured in triplicate, and the error bars represent the standard deviation in the measurements.

Figure 35 Release profile of PalValDSPC at different pH values. All points were measured in triplicate, and the error bars represent the standard deviation in the measurements.

Figure 36 Release profile of PalCpcTSPC at different pH values. All points were measured in triplicate, and the error bars represent the standard deviation in the measurements.

Figure 37 Release profile of OleCpcTSPC at different pH values. All points were measured in triplicate, and the error bars represent the standard deviation in the measurements.

Figure 38 Different protonation states of calcein during release. Calcein is always at pH 7.5 inside each vesicle.

Figure 39 DLS measurements of vesicles before release experiments.

Figure 40 DLS measurements of liposomes after Triton X-100.

Figure 41 Calcein absorbance calibration curve. Absorbance was measured at 495 nm. All points were measured in triplicate, and error bars represent the standard deviation.
List of Tables:

Table 1. List of some fatty acids with their structural formula. The lipid number indicates the carbon length of the lipid and the level of unsaturation, with n (∆n) specifying which carbon number is saturated. .......................................................... 17
Table 2. Liposomal formulations approved by the FDA. .......................................................... 25
Table 3. Carboxylic acids and their three letter abbreviations. .................................................. 44
Table 4. Entrapment efficiency obtained for various liposomes. Extrusion was done using a 100 nm membrane (Mean ± SEM, n=3). .......................................................... 64
Table 5. Illustrating the different rate constants of lipids as well as their corresponding permeability coefficient. Pm is the permeability coefficient of calcein, which is calculated in terms of k, k here being an average of each individual release profile. ................................. 76
Table 6. Specific rotation of GPC and some synthesized siloxane lipids. ................................. 84
Table 7. Stability experiment of PalValDSPC and POPC. Sizes were obtained at room temperature using DLS. .......................................................... 86
List of Appendix Figures

Appendix Figure 1 $^1$H NMR Spectrum of heptamethyltrisiloxanemethyldecanoate (2) in CDCl$_3$ ................................................................. 115
Appendix Figure 2 $^{13}$C NMR Spectrum of heptamethyltrisiloxanemethyldecanoate (2) in CDCl$_3$ ................................................................. 116
Appendix Figure 3 $^{29}$Si NMR Spectrum of heptamethyltrisiloxanemethyldecanoate (2) in CDCl$_3$ ................................................................. 117
Appendix Figure 4 FTIR spectrum of heptamethyltrisiloxanemethyldecanoate (2) ................................................................. 118
Appendix Figure 5 EI-MS spectrum of heptamethyltrisiloxanemethyldecanoate (2) ................................................................. 119
Appendix Figure 6 $^1$H NMR Spectrum of heptamethyltrisiloxanedecanoic acid (3) in CDCl$_3$ ................................................................. 120
Appendix Figure 7 $^{13}$C NMR Spectrum of heptamethyltrisiloxanedecanoic acid in CDCl$_3$ (3) ................................................................. 121
Appendix Figure 8 $^{29}$Si NMR Spectrum of heptamethyltrisiloxanedecanoic acid (3) in CDCl$_3$ ................................................................. 122
Appendix Figure 9 FTIR spectrum of heptamethyltrisiloxanedecanoic acid (3) ................................................................. 123
Appendix Figure 10 EI-MS spectrum of heptamethyltrisiloxanedecanoic acid (3) ................................................................. 124
Appendix Figure 11 ESI+/MS spectrum of heptamethyltrisiloxanedecanoic acid (3) ................................................................. 125
Appendix Figure 12 $^1$H NMR Spectrum of LysoTSPC (4) in CDCl$_3$ ................................................................. 126
Appendix Figure 13 $^{13}$C NMR Spectrum of LysoTSPC (4) in CDCl$_3$ ................................................................. 127
Appendix Figure 14 31P NMR Spectrum of LysoTSPC (4) in CDCl$_3$ ................................................................. 128
Appendix Figure 15 $^{29}$Si NMR Spectrum of LysoTSPC (4) in CDCl$_3$ ................................................................. 129
Appendix Figure 16 FTIR spectrum of LysoTSPC (4) ................................................................. 130
Appendix Figure 17 EI-MS spectrum of LysoTSPC (4) ................................................................. 130
Appendix Figure 18 ESI+/MS spectrum of LysoTSPC (4) ................................................................. 130
Appendix Figure 19 $^1$H NMR Spectrum of 1,2 Si-PC (5) in CDCl$_3$ ................................................................. 131
Appendix Figure 20 $^{13}$C NMR Spectrum of 1,2 Si-PC (5) in CDCl$_3$ ................................................................. 132
Appendix Figure 21 31P NMR Spectrum of 1,2 Si-PC (5) in CDCl$_3$ ................................................................. 133
Appendix Figure 22 $^{29}$Si NMR Spectrum of 1,2 Si-PC (5) in CDCl$_3$ ................................................................. 134
Appendix Figure 23 ESI+/MS Spectrum of 1,2 Si-PC (5) ................................................................. 134
Appendix Figure 24 $^1$H-NMR spectrum of Pentamethyldisiloxanemethylpentanoate (7) in CDCl$_3$ ................................................................. 135
Appendix Figure 25 $^{13}$C NMR Spectrum of Pentamethyldisiloxanemethylpentanoate (7) in CDCl$_3$ ................................................................. 136
Appendix Figure 26 $^{29}$Si NMR Spectrum of Pentamethyldisiloxanemethylpentanoate (7) in CDCl$_3$ ................................................................. 137
Appendix Figure 27 FTIR spectrum of Pentamethyldisiloxanemethylpentanoate (7) ................................................................. 138
Appendix Figure 28 EI-MS spectrum of Pentamethyldisiloxanemethylpentanoate (7) ................................................................. 139
Appendix Figure 29 ESI+/MS spectrum of Pentamethyldisiloxanemethylpentanoate (7) ................................................................. 140
Appendix Figure 30 $^1$H NMR Spectrum of Pentamethyldisiloxanepentanoic acid (8) in CDCl$_3$ ................................................................. 140
Appendix Figure 31 $^{13}$C NMR Spectrum of Pentamethyldisiloxanepentanoic acid (8) in CDCl$_3$ ................................................................. 141
Appendix Figure 32 $^{29}$Si NMR Spectrum of Pentamethyldisiloxanepentanoic acid (8) in CDCl$_3$ ................................................................. 142
Appendix Figure 33 FTIR spectrum of pentamethyldisiloxanepentanoic acid (8) ................................................................. 143
Appendix Figure 34 EI-MS spectrum of pentamethyldisiloxanepentanoic acid (8) .................. 144
Appendix Figure 35 ESI-/MS spectrum of pentamethyldisiloxanepentanoic acid (8) ............ 145
Appendix Figure 36 $^1$H NMR Spectrum of 16:0 LPC (9) in CDCl$_3$ ............................ 145-45
Appendix Figure 37 $^{13}$C NMR Spectrum of 16:0 LPC (9) in CDCl$_3$ ............................ 146
Appendix Figure 38 $^{31}$P NMR Spectrum of 16:0 LPC (9) in CDCl$_3$ ............................ 147
Appendix Figure 39 FTIR spectrum of 16:0 LPC (9) .................................................. 148
Appendix Figure 40 FAB-MS spectrum of 16:0 LPC (9) ............................................. 149
Appendix Figure 41 ESI+/MS spectrum of 16:0 LPC (9) ............................................. 150
Appendix Figure 42 $^1$H NMR Spectrum of PalValDSPC (10) in CDCl$_3$ ....................... 151
Appendix Figure 43 $^{13}$C NMR Spectrum of PalValDSPC (10) in CDCl$_3$ ....................... 152
Appendix Figure 44 $^{31}$P NMR Spectrum of PalValDSPC (10) in CDCl$_3$ ....................... 153
Appendix Figure 45 $^{29}$Si NMR Spectrum of PalValDSPC (10) in CDCl$_3$ ....................... 154
Appendix Figure 46 FTIR Spectrum of PalValDSPC (10) ........................................... 155
Appendix Figure 47 EI-MS Spectrum of PalValDSPC (10) .......................................... 156
Appendix Figure 48 ESI+/MS Spectrum of PalValDSPC (10) ....................................... 157
Appendix Figure 49 $^1$H NMR Spectrum of OleCpcTSPC (12) in CDCl$_3$ ....................... 158
Appendix Figure 50 $^{13}$C NMR Spectrum of OleCpcTSPC (12) in CDCl$_3$ ....................... 159
Appendix Figure 51 $^{31}$P NMR Spectrum of OleCpcTSPC (12) in CDCl$_3$ ....................... 160
Appendix Figure 52 $^{29}$Si NMR Spectrum of OleCpcTSPC (12) in CDCl$_3$ ....................... 161
Appendix Figure 53 FTIR Spectrum of OleCpcTSPC (12) .......................................... 162
Appendix Figure 54 FAB-MS spectrum of OleCpcTSPC (12) ....................................... 163
Appendix Figure 55 $^1$H NMR Spectrum of PalCpcTSPC (11) in CDCl$_3$ ....................... 164
Appendix Figure 56 $^{13}$C NMR Spectrum of PalCpcTSPC (11) in CDCl$_3$ ....................... 165
Appendix Figure 57 $^{29}$Si NMR Spectrum of PalCpcTSPC (11) in CDCl$_3$ ....................... 166
Appendix Figure 58 $^{31}$P NMR Spectrum of PalCpcTSPC (11) in CDCl$_3$ ....................... 167
Appendix Figure 59 FTIR spectrum of PalCpcTSPC (11) .......................................... 168
Appendix Figure 60 FAB-MS spectrum of PalCpcTSPC (11) ....................................... 169
Appendix Figure 61 Different rate constants of lipids as well as their corresponding permeability coefficient ............................................................... 170
Appendix Figure 69 Line of best fit for zero-order release profiles of OleCpcTSPC .............. 170
# List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATR</td>
<td>Attenuated total reflection</td>
</tr>
<tr>
<td>API</td>
<td>Active pharmaceutical ingredient</td>
</tr>
<tr>
<td>BCS</td>
<td>Biopharmaceutical classification system</td>
</tr>
<tr>
<td>CAL A</td>
<td><em>Candida antarctica</em> lipase A</td>
</tr>
<tr>
<td>CAL B</td>
<td><em>Candida antarctica</em> lipase B</td>
</tr>
<tr>
<td>CDCl$_3$</td>
<td>Chloroform-d</td>
</tr>
<tr>
<td>CHCl$_3$</td>
<td>Chloroform</td>
</tr>
<tr>
<td>CHEMS</td>
<td>Cholesteryl hemisuccinate cholesterol</td>
</tr>
<tr>
<td>CL</td>
<td>Conventional liposome</td>
</tr>
<tr>
<td>DCC</td>
<td><em>N</em>,<em>N</em>-Dicyclohexylcarbodiimide</td>
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<tr>
<td>DDAB</td>
<td>Dimethyl-dioctadecyl ammonium bromide</td>
</tr>
<tr>
<td>DDS</td>
<td>Drug delivery system</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DLVO</td>
<td>Derjaguin-Landau-Verwey-Overbeek</td>
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<td>DMAP</td>
<td>4-Dimethylaminopyridine</td>
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<td>DMRIE</td>
<td>1,2-Dimyristyloxypropyl-3- dimethyl-hydroxyethyl ammonium bromide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DODAC</td>
<td>Dioctadecyldimethyl ammonium chloride</td>
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<td>DOPE</td>
<td>Dioleoylphosphatidyl ethanolamine</td>
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<td>2,3-Dioleoyloxy-N-(2(spermine carboxamido)-ethyl)-N,N-dimethyl-1-propanaminium fluoroacetate</td>
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DOTAP  1,2-Dioleoyloxy-3-(trimethylammonio)-propane
DOTMA  \(N\{-1-(2,3\text{-Dioleoyloxy})\text{propyl}\}\) - N,N,N-trimethylammonium chloride
EC     Enzyme commission number
FA     Fatty acid
FDA    Food and drug administration
FTIR   Fourier-transform infrared spectroscopy
GI     Gastrointestinal
GM1    Monosialoganglioside
GPC    \(sn\)-3-Glycerophosphocholine
HPI    Hydrogenated phosphatidylinositol
ID     Intradermal
IM     Intramuscular
IV     Intravenous
IVR    \textit{In vitro} release
LCL    Long-circulating liposomes
LPC    1-Palmitoyl-2-hydroxy-\(sn\)-glycero-3-phosphocholine
Log P  Partition coefficient
LysoTSPC 1-decanoyl,1,1,3,3,5,5-heptamethyltrisiloxane-\(sn\)-glycero-3-phosphocholine
MeOH   Methanol
MLV    Multilamellar vesicle
N435   Novozym-435
NaCl   Sodium chloride
<table>
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<td>NaHCO₃</td>
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<td>Na₂SO₄</td>
<td>Sodium sulphate</td>
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<td>NIR</td>
<td>Near infrared</td>
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<td>NMR</td>
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<tr>
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<td>o/w</td>
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<td>Polydimethylsiloxane</td>
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</tr>
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<td>Retention factor</td>
</tr>
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<td>SAXS</td>
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<td>SC</td>
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<tr>
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<td>Transition temperature</td>
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<td>ULV</td>
<td>Unilamellar vesicle</td>
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<tr>
<td>UV-Vis</td>
<td>Ultraviolet-visible</td>
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</table>
1 Introduction

Liposome technology has expanded throughout the years since the discovery of liposomes in 1961 by Alec Bangham, a British haematologist [1]. The application of liposomes at that time mainly targeted the dynamic structure of biomembranes, but nowadays liposomes are of great interest in various fields such as the agro-food industry, ecology and physics, the cosmetics industry, and in the medical field [1], [2].

A liposome is defined as a closed phospholipid vesicle composed of an aqueous core surrounded by one or more lipid bilayer membranes. The word liposome is derived from two Greek words: “lipos” meaning fat and “soma” meaning body [1]. In general, liposomes can be classified as unilamellar or multilamellar, depending on the number of membranes surrounding the aqueous interior, and typically range in size from 20 nm to 10 μm [3]. Despite extensive clinical research since the discovery of liposomes structures, it was not until 1995 that the Food and Drug Administration (FDA) approved the first liposomal formulation Doxil® for cancer treatment. The liposome, which was made up of the phospholipid phosphatidylcholine, encapsulated the anti-cancer drug doxorubicin, improving the drug's therapeutic index (TI) by increasing its efficacy while minimizing its toxicity [4], [5]. Other beneficial properties of liposomes that make them advantageous for use in the pharmaceutical field as drug delivery systems include the increased solubility of lipophilic and amphiphilic drugs, active and passive targeting to cells (refer to Section 2.3.3 for more details), and sustained drug release [6]. The main reason that liposomes have broad applications is attributed to their physiochemical properties. They are made up of phospholipids, which, because of their amphiphilic nature, form vesicles when in an aqueous environment. These vesicles, or liposomes, are characterized by
lipid bilayer membrane(s) sequestering part of the aqueous system in which they float as an aqueous core, where the hydrophobic tails of phospholipids are shielded in the interior with the hydrophobic head exposed to the aqueous environment, thereby resulting to nearly spherical structures. The fact that liposomes are made up of phospholipids not only contributes to their nearly spherical forms in aqueous systems but also for their low to zero toxicity, biocompatibility and biodegradability, which are key factors favouring their application in the medical field [7], [8].

Silicones or siloxanes are polymeric chains and networks constructed around a Si-O-Si backbone [9]. Silicon itself does not exist as a pure element in nature, but it is found in practically all rocks (inclusive of sand, soils, and clays), which can be in the form of silica (SiO$_2$) when combined with oxygen, or silicates when combined with oxygen and other elements such as aluminum, magnesium, calcium, or iron. Silicon is isolated by carbo-thermically reducing sand, or silica in a furnace [10]. After obtaining silicon, the next step towards the preparation of silicones follows the direct process which involves the conversion of isolated silicon using a copper based catalyst to a chlorine-containing compound, dimethyl dichlorosilane, which is then further hydrolysed to yield a mixture of both linear and cyclic silicone [11]. Silicones have a long history of use in a variety of settings from the most strenuous to the most sensitive applications due to their unique material properties [12]. Silicones are ideal in biomedical applications due to their low surface tension, chemical and thermal stability, favourable biocompatibility and their hydrophobic nature which keeps the integrity of devices by preventing water from penetrating the interior [13].

This thesis explores the incorporation of siloxanes as a structural component of lipids and the capacity of these structures to behave as delivery vehicles. Part 1 of this thesis will discuss the
synthesis of various siloxane-containing lipids, while Part 2 will examine the use of synthetic, siloxane-lipids in terms of their entrapment capacity and release profiles using calcein as a model drug compound.
2 Historical

2.1 Drug delivery

The pharmaceutical industry, for the majority of its existence, consisted mainly of simple and fast-acting active pharmaceutical ingredients (APIs) administered orally or by injection [14], [15]. Over the past 30 years, modern pharmaceutical technology, in parallel with the growth of the biotechnology industry, has led to more advanced and complex drug formulations resulting in administration challenges for those compounds [15]. A drug delivery system (DDS) can be contextualised as any appropriate excipient or carrier put in place to improve the pharmacokinetics and pharmacodynamics of a drug. In a deeper sense, a DDS’s objective is typically to minimise drug degradation and loss, to optimise the drug’s bioavailability, and to enhance the drug’s therapeutic effect in a pathological area [16]. DDSs usually consist of particles in the nanometer size ranges that have unique combinations of capacities such as controlled release, biological site-specific targeting, and reduced toxicity. These DDSs can bring both therapeutic and commercial value to medications and can be classified based on the various routes of drug administration into the body [17], [18]. Different administration routes for drugs include: intravenous injection (into a vein), intramuscular injection (into a muscle), subcutaneous injection (beneath the skin), pulmonary (inhalation or nebulization), oral (taken by mouth), ocular (in the eye), otic (in the ear), buccal (between the gum and cheek), intrathecal (into the cerebral spinal fluid), sublingual (under the tongue), nasal (into the nose), vaginal (in the vagina), rectal (in the rectum), transdermal (via the skin), and implanted (inside a body cavity) [19]. An overview of the most frequently used routes is presented in Figure 1 below [20].
2.1.1 Parenteral routes for drug delivery

Parenteral drug delivery encompasses four main routes: intravenous (IV), intramuscular (IM), subcutaneous (SC), and intradermal (ID). These four routes are a good choice for the delivery of medicaments that are poorly bioavailable or ineffective when taken orally. Specifically, IV delivery is seen as a convenient method of delivery in situations where a patient is to receive a medication very quickly, as in emergencies such as a heart attack, stroke, or poisoning [18]. Delivery via the four routes listed above is also convenient for uncooperative and unconscious patients and can reach up to 100% bioavailability as the hepatic first-pass effect and enzymatic inactivation in the gut is avoided [21]. Some disadvantages with these methods of delivery include pain or discomfort for the patient, needle phobia, and the requirement of trained staff (as there are risks of infection from the injection site as well as phlebitis (i.e., blood vessel
inflammation) during injection) [18], [19]. In chronic diseases like insulin-dependent diabetes, these issues become more concerning due to the need for repetitive drug administration resulting in lower compliance by patients caused either by physical difficulties, demographic factors, or lack of accessibility to the drug [23]. Different measures, however, are being taken into consideration in order to improve parenteral delivery, one of which is to include an injectable depot system from biodegradable microspheres to reduce the injection rate and enable constant drug release. On the other hand, alternatives for parenteral delivery such as transdermal, pulmonary, and oral administration are being favoured for development as much as possible [24], [25].

2.1.2 Transdermal drug delivery

Transdermal drug delivery is a painless route for the systemic delivery of an active pharmaceutical ingredient that involves the application of a drug onto intact and healthy skin, often using a patch. The penetration pathway starts through the stratum (Figure 2) and then moves along through the deeper epidermis and finally the dermis avoiding drug accumulation in the dermal layer [26], [27]. At the dermal layer, the drug is then available for systemic absorption through the dermal microcirculation. This method of delivery bypasses drug degradation which usually occurs in the liver, and occasionally take place in the gastrointestinal (GI) tract, lungs or kidneys [28]. Transdermal delivery is a convenient method of delivery for unconscious or vomiting patients as well as for patients who rely on self-administration. However, due to the barrier nature of the skin to foreign substances, the number of drug molecules that can be administered using this route is limited. This includes hydrophilic drugs, ionic drugs, and macromolecules such as DNA and small-interfering RNA as permeation via the
skin favours small and lipophilic molecules (partition coefficient log P between 1 and 3) [29], [30]. Ongoing research aims to enhance the permeation of drugs across the skin using technologies such as sonophoresis, laser irradiation, electroporation, ionophoresis, jet injection, and microneedles. Insulin, parathyroid hormone, and influenza vaccine delivery were shown to be successful using these technologies [31]–[34].

2.1.3 Pulmonary drug delivery

Pulmonary drug delivery is very useful in illnesses such as asthma, chronic obstructive pulmonary disease, chronic bronchitis, and cystic fibrosis [35]. Pulmonary drug delivery is a needle-free technique primarily focusing on inhalation, which involves the channelling of drugs through the respiratory tract (Figure 1). Delivery of drugs through the lungs is advantageous in situations where the biological issue to be addressed is at the level of the lungs, such that the administered drug can be delivered directly to the region where its therapeutic effect is required. The drug, therefore, acts faster and side effects can be reduced via low dose administration [36].
Inhalation also offers an opportunity to address systemic diseases via the alveoli of the lungs, which provide an enormous surface area (80-140 m²/adult) and a high level of permeability for the absorption of drugs into the blood. Biomolecules such as proteins, which are susceptible to degradation and inactivation in the GI tract as well as elimination by the hepatic first-pass effect, can be delivered via the lungs [37]. However, challenges in pulmonary drug delivery include inefficient delivery systems, low drug mass per puff, poor formulation stability for the drug, and poor dosing reproducibility [38], [39].

2.1.4 Oral drug delivery

Despite the risk of lower patient compliance, exposure to the hepatic first-pass effect, risks of irritation of mucosal linings resulting in nausea and vomiting, oral drug delivery remains the most convenient, economic, and widely used drug delivery route in the world [40].

About 40% of newly synthesized APIs encounter formulation challenges and are rejected by the pharmaceutical industry [41]. Part of this 40% includes discoveries based on biomolecules such as peptides, proteins, oligonucleotides, and DNA, all of which are susceptible to enzymatic and acid-catalyzed degradation in the body thereby lowering the bioavailability necessary to achieve their therapeutic effect [24]. The bioavailability of these drugs is also reduced due to their physio-chemical properties such as their molecular weight, pKₐ, partition coefficient (i.e., their solubility in water), and/or their degree of permeability through membranes. The fact that the human body is predominantly made up of water (about 75% [42]) makes it necessary for a drug to possess a level of hydrophilicity for distribution via the bloodstream towards its target. Furthermore, the same drug must also exhibit a certain level of lipophilicity to be capable of
crossing cell membranes while transiting from the aqueous environment in the bloodstream into the target tissue [43], [44]. These two factors of solubility and permeability have led to a biopharmaceutical classification system of drugs, regardless of their route or mode of delivery as depicted in Figure 3 below [45].

![Figure 3: Biopharmaceutical Classification System (BCS) of Drugs.](image)

This biopharmaceutical classification system (BCS) illustrates four categories of drugs based on their solubility in water and their capacity to cross biological membranes. Class I drugs are ideal for delivery because of their high bioavailability. Class II and III drugs present some challenges due to their reduced bioavailability caused respectively by their low solubility and low permeability across membranes. Finally, Class IV drugs are hampered by their low permeability and solubility causing them to never successfully complete clinical trials on their own [46].
2.1.5 Strategies for increasing bioavailability

A drug delivery system, as mentioned earlier, is a method used to increase the concentration of a drug in the circulatory system [47]. One of the oldest strategies used in the pharmaceutical industry to improve the bioavailability of APIs is to increase the surface area-to-volume ratio per particle via particle size minimization. This approach subjects desired APIs to processes such as milling, grinding, mixing, and extrusion that not only reduce the potency of the API but still do not address the degradation issue encountered when the API is exposed to the gut [48].

Carrier-based drug delivery is an alternative option to enhance the bioavailability of drugs as well as to protect the drug. The main systems for these applications are: liposomes, formed by the self-assembly of phospholipids; niosomes, formed by uncharged single-chain surfactants; nano- and microparticulate carriers formed by various processes; and polymeric micelles formed through the self-assembly of charged or neutral amphiphilic block copolymers [49]. Liposomes represent the major focus of this thesis.
2.2 Colloidal drug delivery systems

One of the ongoing areas of research in the pharmaceutical industry for the past three decades is the design and development of new drug delivery systems that could optimise the efficacy of existing drugs.

A colloid, is a homogeneous system consisting of nanoscale (1–100nm) or mesoscale (from 100nm to hundreds of microns) molecules or particles dispersed through a medium (or second phase) [50].

Various drug delivery systems have been explored to protect the drug and slow its degradation, to optimize the drug’s targeting, to limit the drug’s accumulation in healthy organs, to reduce the API’s potential toxicity, and/or to control its release [51]. Among the library of drug delivery systems that have so far been exploited, the colloidal drug delivery systems, including micelles, microemulsions, macroemulsions (or just emulsions), niosomes and liposomes [52], have been found to have great potential for achieving all of the goals stated above.

2.2.1 Micelles

Micelles (Figure 4) are optically isotropic and thermodynamically stable liquid suspensions consisting of water and an amphiphile [53]. The amphiphile can be a fatty acid (FA) or a lysophospholipid (bearing a single hydrophobic chain), that in an aqueous medium rearranges to a more thermodynamically stable structure driven by the hydrophobic effect; a tendency of non-polar molecules or molecular segments in an aqueous solution to avoid contact with polar molecules [54]. The amphiphile, however, does not form micelles until it reaches a certain concentration known as the critical micelle concentration (CMC). Below the CMC, the
amphiphile partitions between the surface and the bulk system where it exists as a monomer and above the CMC, adding more amphiphile induces the formation of more micelles [55]. Micelles can be up to about 20 nm in diameter and can either be normal micelles or reverse micelles depending on which phase, aqueous or organic, is dominant over the other. In normal micelles where an aqueous medium makes up the bulk, the amphiphile tails orient into the hydrophobic inner core having the hydrophilic head interacting with the exterior aqueous phase via hydrogen bonding and electrostatic forces. In reverse micelles (Figure 5) where an organic phase makes up the bulk, the polar head groups are instead oriented towards the hydrophilic interior leaving the tails interacting with the hydrophobic exterior phase [56]. Micelles typically do not tolerate large amounts of non-polar solvents due to their extremely low capacity to solubilize oils [57].

**Figure 4. Schematic representation of a micelle.**

2.2.2 Emulsions

In cases where two immiscible liquids (usually an organic solvent and water) get mixed such that one liquid (the dispersed phase) is in the form of microscopic droplets dispersed in the other (continuous), an emulsion is produced (Figure 5). With an oil-in-water emulsion, the oil droplets are dispersed in water containing an oil-saturated emulsifier that concentrates at the interface between the two liquids to lower the interfacial tension but also to generate a repulsive force or physical barrier between the two phases to reduce the energy required to break the
dispersed phase and limit the possibility of flocculation and/or coalescence of the emulsion droplets [58]. The emulsion droplets are usually larger than 1 μm [59]. The mixing of water and oil is not thermodynamically favourable due to the high Gibbs free energy and decrease in entropy associated with this process and usually requires the addition of a surfactant; an amphiphilic compound that lowers the surface tension, to obtain a level of stability [60]. Compared to normal micelles, oil-in-water emulsions droplets are larger in diameter and typically have a large amount of oil sequestered, which favours the solubilisation of hydrophobic drugs in the dispersed phase. As with reverse micelles, we can also have water-in-oil emulsions where water droplets are dispersed in a continuous oil phase. In conditions where the droplets are so small that they do not refract light, the dispersion is referred to as a microemulsion [61]. Microemulsions are thermodynamically stable in association with surfactants and co-surfactants such as alcohols, amines, and sulphoxides which lower the interfacial tension. These microemulsions are generally < 100 nm in size, spontaneously assemble and can be either water-in-oil (w/o) microemulsions or oil-in-water (o/w) microemulsions (Figure 5) [57], [62].

The choice of surfactant, as well as its concentration, are crucial to obtain a sustainable colloidal system being it emulsion or microemulsion system. Among the variety of surfactants and emulsifiers, lipids, due to their amphiphilic properties, are considered ideal candidates [58]. Silicone polymers present dual characteristics due to their organic/inorganic hybrid nature. These silicones are also suitable for applications as emulsifiers as they are highly surface active due to their low surface tension caused by the large number of methyl groups and due to the small intermolecular attractions between the siloxane hydrophobes [63], [64] An ideal colloidal system in regards to drug delivery, however, should be able to have low viscosity, a long shelf-
life, small droplet size, high solubility of drugs, low toxicity to the patient, a controlled drug-release rate, a simple preparation method, and be target specific when administered [49], [54].

Figure 5. Some colloidal vesicles. Reprinted with permission from Ref. 62. Copyright © 2010 by Elsevier B.V.

2.3 Introduction to liposomal drug delivery

The desired outcome of a drug research project is to succeed in the development of a molecule that, when administered into the body, diffuses to reach the desired location. The fraction of the drug reaching the target site must be unmodified and of a sufficient quantity to produce the desired effect without an unacceptable level of toxicity caused by off-target activity [66]. The design of carrier delivery systems is primarily to enable the administration of drugs that are usually compromised by to their low therapeutic index (TI) [67]. Many anti-cancer drugs, for example, are found to be highly cytotoxic in vitro to tumor cells, but because of their lack of specificity in vivo and their low TI, they act upon the body’s normal cells in addition to
cancerous cells. This means that many anti-cancerous drugs are unable to reach the market and be therapeutically useful [68], [69]. The pharmaceutical industry, because of such bottlenecks among others, has reached a point of diminishing returns. While the investment in drug research projects increases exponentially each year, the number of approved drugs remains constant, a phenomenon referred to as “Eroom’s Law” [66], [70], [71]. In order to bring out the therapeutic usefulness of new drugs, which either have low solubility, low permeability (or both), high toxicity or low specificity to target tissues, and to improve the efficacy of existing drugs to increase patient compliance (50% of all patients do not take their medicines as prescribed [72]), one of the considerations has been the design and development of liposomal DDS (Figure 6) [73]. These liposomal formulations have been used to enhance the TI of drugs by modifying drug absorption, reducing catastrophic drug metabolism, prolonging the biological half-life of the drug, or reducing the drug’s toxicity. The drug distribution that results is then governed primarily by properties of the liposome, and no longer just by the physio-chemical properties of the drug itself [74].

2.3.1 Phospholipids and liposomes

Phospholipids (PLs) constitute the main component of biological membranes, are among the most abundant biomolecule in nature and are known to be essential for the absorption and transportation of nutrients and molecules, as well as act as a source of energy. They are also known to participate in the regulation of cellular and sub-cellular biochemical functions such as membrane trafficking, regulating and stabilizing membrane proteins, and creating sub-cellular compartments that contribute to overall cellular function [75], [76]. A phospholipid is an amphiphilic molecule made up of two acyl chains linked to a polar head group like phosphatidylcholine (PC) by means of a glycerol-backbone via ester linkages [77]. Figure 7
illustrates the structural formula of a phospholipid, where R1 and R2 are either saturated or unsaturated acyl chains and R3 is the polar head group. Some possible acyl chains are illustrated in Table 1. In addition to PC other head groups such as phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and phosphatidylserine (PS) are present as natural occurring phospholipids. These different head groups are used in the classification of various phospholipids [78].

Figure 6. Schematic representation of a liposome. Reprinted with permission from Ref. 86. Copyright © 2008 by Philip Chalmers.

Phosphatidylglycerol (PG)

\[
\begin{align*}
&\text{POPC} & R_1 = C_{16:0} & R_2 = C_{18:1} \\
&\text{DOPE} & R_1 = R_2 = C_{18:1} \\
&\text{DSPG} & R_1 = R_2 = C_{18:0}
\end{align*}
\]

Phosphatidylinositol (PI)

\[
\begin{align*}
R_6 & \quad \text{H}
\end{align*}
\]

Phosphatidylserine (PS)

\[
\begin{align*}
R_6 & \quad \text{N}^+\text{S}^- \\
R_5 & \quad \text{C}=\text{O} \quad \text{OH}
\end{align*}
\]

Figure 7. Classification of glycerophospholipids with 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-Distearoyl-sn-glycero-3-phosphoglycerol (DSPG) as examples.
Table 1. List of some fatty acids with their structural formula. The lipid number indicates the carbon length of the lipid and the level of unsaturation, with n (Δ^n) specifying which carbon number is saturated.

<table>
<thead>
<tr>
<th>Name</th>
<th>Lipid number</th>
<th>Δ^n</th>
<th>Structural formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentadecyl acid</td>
<td>C15:0</td>
<td>-</td>
<td>(\text{CH}_3(\text{CH}<em>2)</em>{13}\text{COOH})</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>C16:0</td>
<td>-</td>
<td>(\text{CH}_3(\text{CH}<em>2)</em>{14}\text{COOH})</td>
</tr>
<tr>
<td>Margaric acid</td>
<td>C17:0</td>
<td>-</td>
<td>(\text{CH}_3(\text{CH}<em>2)</em>{15}\text{COOH})</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>C18:0</td>
<td>-</td>
<td>(\text{CH}_3(\text{CH}<em>2)</em>{16}\text{COOH})</td>
</tr>
<tr>
<td>Palmitoleic acid</td>
<td>C16:1</td>
<td>Δ^9</td>
<td>(\text{CH}_3(\text{CH}_2)\text{CH=CH(CH}_2)\text{COOH})</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>C18:1</td>
<td>Δ^9</td>
<td>(\text{CH}_3(\text{CH}_2)\text{CH=CH(CH}_2)\text{COOH})</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>C20:4</td>
<td>Δ^5,8,11,14</td>
<td>(\text{CH}_3(\text{CH}_2)\text{CH=CHCH}_2\text{CH=CHCH}_2\text{CH=CHCH}_2\text{CH=CH(CH}_2)\text{COOH})</td>
</tr>
</tbody>
</table>

Throughout this thesis, PC is the biologically relevant lipid being used as a benchmark.

Phosphatidylcholine or PC-lipids are the most commonly used lipids in liposome work [79]. They have a pH-independent behaviour as they keep their zwitterionic or neutral property with pH values ranging from strongly acidic to strongly alkaline [80]. Within a particular lipid family, diverse molecules exist based on the different substitutions at the acyl chains. The non-polar hydrocarbon chain can vary in length and degree of saturation resulting in different degrees of membrane permeability and fluidity reflective on the lipid’s physical state. At low temperature or
at a high degree of saturation, the hydrocarbon chains exhibit a close packing conformation, which causes the bilayer to adopt a gel state. Increasing the temperature or substituting with unsaturated acyl chains, the bilayer becomes more fluid having a liquid crystalline state as the hydrocarbon chains are more anisotropic and have a degree of high mobility. A lipid phase transition temperature (Tm) is the temperature required to induce a change in the lipid physical state from the ordered gel phase to the disordered liquid crystalline phase (i.e., where the gel-to-liquid phase transition occurs) [81], [82]. PCs are water-insoluble lipids, which implies that they will self-assemble in aqueous media, with the hydrocarbon chains oriented away from the aqueous phase. This lipid bilayer arrangement is what is observed in liposomes. This self-assembly occurs due to the dual preference for solvent exhibited by the amphiphile. Having one part that is soluble in polar solvents and another part that is soluble in non-polar ones makes the amphiphile susceptible to self-assembly in a media containing a very non-polar solvent, or a very polar solvent like water [79]. This self-assembly results in an increase in entropy gained from the water molecules that are in an ordered structure when surrounding the hydrocarbon chain. This increase in entropy leads to an overall gain in free energy making the aggregation occur spontaneously [80], [83]. This spontaneous aggregation, influenced by the hydrophobic effect, results in structures that can be described by what is referred to as the surfactant parameter, which takes into account the molecular parameters of the amphiphile. These parameters, which include hydrophobic volume, chain length, and head group area are a good predictor of the aggregate structure [84]. The surfactant parameter S, can be obtained via the formula in equation (Eq.) 1 below;

\[
S = \frac{V}{a_0 l}
\]  

（Eq. 1）
where \( v \) is the volume of the lipid, \( l \) is the length of the hydrocarbon chain and \( a_0 \) is the effective area per head group. The value of the surfactant parameter can predict the geometrical shape of the liposome. Small \( S \) values indicate highly curved aggregates such as micelles, while \( S \) values \( \approx 1 \) indicate the formation of planar bilayers [79], [84], [85]. The relationship between the geometrical packing of lipids and the surfactant parameter is depicted in **Figure 8** below.

![Figure 8](image.png)

**Figure 8.** Relationship between geometrical packing and surfactant parameter. Reprinted with permission from Ref. 85. Copyright © 2014 by Royal Society of Chemistry.

A liposome (**Figure 6**) is defined as a closed phospholipid vesicle composed of an aqueous core sequestered by one or more lipid bilayer membranes [1], [86]. Liposomes can either be made up of naturally occurring phospholipids or synthetic ones and their formation does not require the use of surfactants or emulsifiers but can instead be generated when a lamellar crystal phase is shaken with water [87]. These vesicles usually range from 0.05 \( \mu \)m to
5.0 μm in diameter and usually consist of multiple PL membranes (up to 14 lipid layers) separated from one another by a layer of aqueous solutions in an onion-like pattern known as multilamellar vesicles (MLVs), which can be subjected to extrusion or sonication to form a single or unilamellar vesicle (ULV) membranes [88]. Liposomes are generally considered non-toxic, biodegradable, non-immunogenic, and can be classified based on their composition and delivery mode. In that sense, they can be conventional liposomes (CL) - composed of neutral or negatively charged PL; pH-sensitive liposomes – as in PE or dioleoylphosphatidyl ethanolamine (DOPE) in combination with either cholesteryl hemisuccinate (CHEMS) or oleic acid (OA); cationic liposomes - derived from cationic lipids such as 2,3-dioleoyloxy-N-(2(spermine carboxamido)-ethyl)-N,N-dimethyl-1-propanaminium fluoroacetate (DOSPA), N-{1 -(2,3-dioleoyloxy)propyl}-N,N,N-trimethyl ammonium chloride (DOTMA), 1,2-dimyristyoxypropyl-3- dimethyl-hydroxyethyl ammonium bromide (DMRIE), dimethyl-dioctadecyl ammonium bromide (DDAB), 1,2-dioleyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide (DORIE) and dioctadecyltrimethyl ammonium chloride (DODAC) with dioleoylphosphatidyl ethanolamine (DOPE) [69]. They can also be long-circulating liposomes (LCL); which are conventional liposomes optimized with polyethylene glycol (PEG) (which can also be conjugated with distearoyl PE (PEG-DSPE)), hydrogenated phosphatidylinositol (HPI) or monosialoganglioside (GMI) and finally immunoliposomes, which are basically a CL or a LCL conjugated with a desired antibody [69], [89]. Liposomes can also be classified based on their lamellarity, (uni-, oligo-, and multi-lamellar vesicles), size (small (20-100nm), intermediate (100-500nm), or large (>500nm)), and preparation method (for example, reverse phase evaporation vesicles, VETs) [90].
2.3.2 Liposomes stability and stabilization

The fact that liposomes are composed of phospholipids exposes them to various
degradation processes. The stability of liposomes can be compromised physically and/or
chemically.

Chemical degradation usually results from the hydrolysis of the ester bond linking the
acyl tails to the glycerol backbone or from the peroxidation of any unsaturated acyl chain
present. These two chemical degradation reactions may lead to the appearance of short-chain
lipids and compromise the quality of liposome products [91]. Factors responsible for chemical
degradation include exposure to oxygen, ionic strength, and pH of surrounding media. Therefore,
better ways to minimize chemical degradation are the use of argon or nitrogen environments
during storage of liposomes, the use of antioxidants such as α-tocopherol, betahydroxy toluene
(BHT) or nitrooxides, and the use of light-resistant containers for the storage of liposomes at
various temperatures [5], [92].

Physical degradation involves processes such as aggregation/flocculation and
fusion/coalescence. These processes affect the shelf life of liposomes, result in a change in
liposome size, and ultimately the loss of any liposome-encapsulated drug. In aggregation,
individual liposomes develop into larger units generally via reversible processes such as
temperature change or metal ion binding that induces aggregation. However, the beginning of
aggregation can accelerate the process of liposome fusion, which in turn produces new colloidal
structures [93]. The fact that fusion is irreversible makes it impossible for the original liposome
to be retrieved. When small particles fuse, only new, larger particles remain meanwhile with
aggregation, the small particles retain their identity and moves as a single unit [92], [93].
The Derjaguin-Landau-Verwey-Overbeek (DLVO) theory quantitatively explains the physical stability of liposomes [94]. According to this theory, the total interaction potential ($V_{tot}$) between two or more particles is a function of the repulsive component ($V_R$) of the interaction potential and the attractive component ($V_A$):

$$V_{tot} = V_R + V_A \quad (Eq. 2)$$

van der Waals interactions are the main attractive force between liposomes with electrostatic repulsion being the main repulsive force. Colloid stability is dependent on the balance between these two forces. As particles get closer to one another, the electronic forces increase exponentially and the attractive forces increase as an inverse power of separation (Figure 9) [95], [96].

![Net interaction potential between particles based on DLVO theory](image)

*Figure 9. Net interaction potential between particles based on DLVO theory. Reprinted from Ref. 96. Copyright © 2016 by Nguyen Phat JSC.*

*In vivo*, the stability of liposomes depends on external factors such as the presence of proteins that can interact with present vesicles. This implies that measures taken to improve the stability of liposomes will improve liposome-mediated drug delivery *in vivo* and therefore
increase not only liposomes’ but the drug’s longevity in the bloodstream. It was observed that stability towards plasma components was obtained after the incorporation of $N$-acyl-phosphatidylethanolamine into liposomes as the compound shifts the liposomal transition temperature to higher values [5], [97]. In terms of physical instability, it was observed that liposomes that lack a net electrical charge tend to be less stable with respect to aggregation than charged ones as the presence of charges on liposomes induces electrostatic repulsion among liposomes by creating a net potential, positive or negative, that prevents liposome aggregation and/or flocculation. Thus, to reduce the rate of aggregation, charge-carrying lipids can be incorporated into the liposomal formulation. The hydration medium was also observed to be a driving force towards aggregation when containing polyvalent cations. If polyvalent ions are present in the hydration medium used for the formation of liposomes, the use of chelating agents such as ethylenediaminetetraacetic acid (EDTA) is recommended [5], [92], [95]. Also, the permeability of a liposome is increased when the liposome is stored around its phase transition temperature. This causes an increase in the leakage of encapsulated drugs. Cholesterol, however, can be incorporated to decrease the bilayer permeability as the presence of cholesterol induces a dense packing of phospholipids [5], [91].

2.3.3 Liposomes as a drug delivery system

Liposomes have been applied as carrier vesicles for various APIs such as anti-cancer and antimicrobial drugs, chelating agents, steroids, vaccines, and genetic material [69]. Table 2 illustrates a list of some liposomal formulations that are currently on the market. Liposomes are considered good carrier candidates for drug delivery not only because various drugs can be encapsulated within them, but also due to the possibility of targeting specific cells or organs in the human body, thereby limiting or reducing harmful side effects caused in part by the
distribution of the drug to non-targeted tissues [98]. The amphiphilic characteristic of liposomes enables the encapsulation of drugs with various lipophilicities; where hydrophobic drugs can be incorporated in the lipid bilayer, hydrophilic drugs can be solubilized or encapsulated in the hydrophobic inner core and amphiphilic drugs can be encapsulated at the interface [99], [100]. The inner core of the liposome is relatively spacious, and biocompatible of lipid exterior permits the delivery of large macromolecules such as DNA, proteins, and imaging agents.
Table 2. Liposomal formulations approved by the FDA.

<table>
<thead>
<tr>
<th>Number</th>
<th>Clinical products</th>
<th>Biological role</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Doxil®</td>
<td>Anti-cancer</td>
</tr>
<tr>
<td>2</td>
<td>DaunoXome®</td>
<td>Anti-cancer</td>
</tr>
<tr>
<td>3</td>
<td>Mepact®</td>
<td>Anti-cancer</td>
</tr>
<tr>
<td>4</td>
<td>Depocyt®</td>
<td>Anti-cancer</td>
</tr>
<tr>
<td>5</td>
<td>Marqibo®</td>
<td>Anti-cancer</td>
</tr>
<tr>
<td>6</td>
<td>Onivyde™</td>
<td>Anti-cancer</td>
</tr>
<tr>
<td>7</td>
<td>Myocet®</td>
<td>Anti-cancer</td>
</tr>
<tr>
<td>8</td>
<td>Epaxal®</td>
<td>Viral vaccines</td>
</tr>
<tr>
<td>9</td>
<td>Inflexal® V</td>
<td>Viral vaccines</td>
</tr>
<tr>
<td>10</td>
<td>Visudyne®</td>
<td>Photodynamic therapy</td>
</tr>
<tr>
<td>11</td>
<td>Exparel®</td>
<td>Analgesics</td>
</tr>
<tr>
<td>12</td>
<td>DepoDur™</td>
<td>Analgesics</td>
</tr>
<tr>
<td>13</td>
<td>Amphotec®</td>
<td>Antifungal</td>
</tr>
<tr>
<td>14</td>
<td>Ambisome®</td>
<td>Antifungal</td>
</tr>
<tr>
<td>15</td>
<td>Abelect®</td>
<td>Antifungal</td>
</tr>
</tbody>
</table>

Note. Adapted from Ref. 92. Copyright © 2016 by U. Bulbake, S. Doppalapudi, N. Kommineni, and W. Khan.

The initial trials of conventional liposomes (CLs) in the pharmaceutical field were hampered for a long time by a poor stability in serum, low drug accumulation at a target site, an uncontrolled drug release from the vesicles, and a rapid clearance from the bloodstream [76]. The rapid clearance was the result of the removal of the liposomes by the reticuloendothelial
system (RES). This recognition causes an accumulation of the CL in RES-associated organs such as the liver, kidney, lungs, bone marrow, lymph nodes, and spleen. The liver, having the largest capacity for liposomal uptake (size clearance on the scale of 20 - 1000 nm [101]), eventually sequesters the majority of circulating CLs, which are then subjected to phagocytosis by macrophages and cleared from the system [102]. The only advantage the RES rapid clearance system could bring is in situations where a biological issue is present in these RES-associated organs; this is referred to as passive targeting. CL formulations are mainly used in passive targeting as they are easily recognized by opsonins which enhance recognition by the immune system and hence phagocytosis and clearance by the RES [69]. It is also found that the extent of opsonisation of liposomes by serum proteins and the rate of elimination from the liver depends on the size, charge, and stability of the liposomes, with larger liposomes being more susceptible to elimination than smaller ones as they are easily recognized by the RES [79].

PEGylated liposomes have been observed to have a significantly longer shelf-life than conventional liposomes (Figure 10). These PEGylated liposomes are conventional liposomes coated with the synthetic polymer polyethylene glycol (PEG). The grafting of PEG provides a shield for the liposomes such that recognition and removal by the RES is reduced [103]. This prolonged shelf-life provided by PEG is due to a reduction in electrostatic and hydrophobic interactions with the plasma proteins responsible for opsonisation and/or cells involved in phagocytosis [104]. This observed trend is due to the highly hydrated group created on the surface of PEGylated liposomes.

The next development in liposome technology involved conjugation of the liposomes with a targeting ligand (active targeting). The two approaches involve coupling of the ligand directly on the lipid bilayer membrane of conventional and PEGylated liposomes, or coupling of
the target ligand onto the distal end of surface-modified PEG-chains of PEGylated liposomes (Figure 10). The first approach, coupling the ligand directly to the lipid bilayer membrane was found to be the simplest, and it worked well with conventional liposomes [103].

Figure 10. Illustration of active targeting of liposomes. Conjugation of ligand directly on the phospholipid head groups of conventional liposomes (a); conjugation directly on the phospholipid head groups of PEGylated liposomes (b); and conjugation on the free terminus of PEGylated chains (c). Reprinted with permission from Ref. 103. Copyright © 2012 by American Association of Pharmaceutical Scientists.

With PEGylated liposomes, however, the flexible PEG-coat was a hindrance for the effective interaction of the conjugated ligand with its target as well as the conjugation to reactive groups on the liposomal surface. As far as PEGylated liposomes are concerned the second method of ligand conjugation (i.e., coupling ligands to the distal end of the PEG-chains) is preferred [105]. Antibodies and peptide ligands are the most commonly used types of targeting ligands for the conjugation to liposomes due to their unique in vivo properties and high target specificities [106]. It is important to note that having a ligand grafted to the surface of a liposome may increase recognition of the liposomes by the RES even if it is a PEGylated liposome. Also, there is a risk of an unwanted immune reaction towards the ligand or other parts of the ligand-conjugated liposome [107]. Considering these facts, choosing a target ligand and attaching it to a liposome must be well managed. The level of immunogenicity depends both on the type of ligand and the lipid component of the liposome. Small peptides, for example, are less immunogenic than a whole foreign antibody. The amount of conjugated ligand must hence be
perfectly balanced to provide successful binding to the target cell or organ while maintaining a long circulation time [98].

2.4 Drug loading and drug release

The pathway for liposomal drug delivery can be divided into four main steps: (i) encapsulation or adsorption of a drug into the colloidal particle; (ii) delivery of the drug-carrier complex to the desired location in the body; (iii) release of the drug from the carrier vehicle into the desired cell; and (iv) degradation of the carrier vehicle by the RES [108], [109]. Drug encapsulation into liposomes and its subsequent release after administration are two important factors that influence the efficacy of the DDS. The process of formulating a liposome with an API is known as drug loading. Desorption of the drug is the reverse process in which the drug is released from the colloidal vesicle and is delivered to exert a therapeutic effect. The in vitro release simulation of the drug is a good quality control for predicting the internal behaviour of the liposome, the interaction between the liposome and the drug, and its in vivo performance [109]. Drug loading and drug release depend on the formulation method, the physiochemical properties of both liposome and drug, and the surrounding environments that they are interacting with (pH, temperature, salt concentration, etc). Drug loading and drug release are also inter-related. The amount of drug loaded in a vesicle determines the rate and duration of release from the system [110].

Different approaches can be used for loading drugs and particles into liposomes. The two main approaches to drug loading are active and passive drug loading. Passive loading involves all techniques where the drug to be encapsulated and the lipid are co-dispersed in an aqueous buffer thereby achieving entrapment in the process of liposome formation. This technique is
ideal for drugs that are relatively soluble in an aqueous medium. Hydrophobic drugs nevertheless, can be co-solubilized in an organic solvent with the lipid, and subsequently dispersed in an aqueous buffer either after removing the solvent or by reverse-phase procedure [111].

Active loading, on the other hand, includes various techniques where the drug to be encapsulated is incorporated after the liposomes have been formed. In order to achieve entrapment using this approach, liposomes with a membrane potential or transmembrane pH gradient are generated such that they can accumulate many drugs. A main strategy to perform uptake in response to a pH gradient involves first preparing the liposomes in a low pH buffer such as pH 4.0 and then subsequently tuning the external pH to 7 or higher by adding a base to the liposome solution or by modifying the external medium to a high pH buffer [112]. Uptake of lipophilic cations is then simply achieved by addition of the drug and a short incubation period. The fact that the drugs can be incorporated after the formation of the liposome allows formulation to be performed immediately prior to use [113].

As mentioned earlier, drug release can be influenced by both the membrane composition of the liposome and the drug (surfactants can fluidize the membrane increasing the rate of release, whereas cholesterol can have the opposite effect; reducing release from unilamellar liposomes). In addition, environmental triggers such as pH, the presence of particular enzymes and external stimuli such as heat or ultrasound can result in different release rates from the system [114]. The release rate can either follow a zero-order release model, a first-order release model, the Hixson-Crowell release model, the Higuchui release model, or the Korsmeyer-Peppas release model [115], [116].
Zero-order release kinetics describe systems where the drug release rate is constant over a period of time even as the concentration of the drug decreases. The equation for zero-order release is shown in Eq. 3, where \( Q_t \) is the cumulative amount of drug released at time \( t \), \( Q_0 \) is the initial concentration of the drug at time \( t = 0 \), \( K \) is the release constant, and \( t \) is the time at which the drug release is calculated or measured. This characteristic indicates that the process progresses at the same speed regardless of the concentration of substance present until the substance is completely released [117].

Zero-order release kinetics are difficult to achieve but diffusion at a constant rate is the ultimate goal of all controlled-release mechanisms, wherein blood levels of drugs would remain constant throughout the delivery period [118].

\[
Q_t = Q_0 + K_0 t 
\]  
\( (Eq. 3) \)

In typical first-order release kinetics, the rate of dissolution of the drug is directly proportional to its concentration. The first-order release equation can be explained as shown in Eq. 4. This first-order rate law indicates that as the physical process progresses and the drug is depleted, the diffusion rate decreases with the drop in molecular concentration [119]. Many APIs in their base state exhibit first order kinetics, which is often referred to as “burst release”, and then comes a steady reduction in drug concentration.

\[
\log C = \log C_0 - \frac{kt}{2.303} 
\]  
\( (Eq. 4) \)

where \( C_0 \) is the initial concentration of the drug, \( k \) is the first order rate constant, and \( t \) is the time. The data obtained would yield a straight line with a slope of -\( K/2.303 \).

Hixson and Crowell proposed a correlation between drug release from the particle and surface area and diameter area of the particle. Their equation (Eq. 5) shows that the drug release from the particle is proportional to the cubic root of its volume [120].
\[(Q_t)^{1/3} = (Q_0)^{1/3} - K^{1/3} \]  \hspace{1cm} (Eq. 5)

Higuchi developed several theoretical models to study the release of water-soluble and low solubility drugs incorporated into semi-solid and/or solid matrices. Mathematical expressions were obtained for drug particles dispersed in a uniform matrix behaving as the diffusion media. To study the dissolution from a planar system having a homogeneous matrix, equation 6 was developed, where \(Q_\infty\) is the total amount of drug released.

Higuchi was the first to derive an equation to describe the release of a drug from an insoluble matrix as the square root of a time-dependent process based on Fickian diffusion [121].

\[Q_t = Q_\infty K \sqrt{t} \]  \hspace{1cm} (Eq. 6)

Korsmeyer and Peppas developed a simple, semi-empirical model that relates exponentially the drug release to the fractional release of the drugs. Hence, the final equation can be written as shown in Eq. 7 where \(n\) is the diffusional exponent. The \(n\) value is used to characterize different release mechanisms, which can either be Fickian diffusion \(n= 0.45\), anomalous (non-Fickian) diffusion \(0.45<n<0.89\), case-II transport \(n= 0.89\) or super case-II transport \(n> 0.89\) [122].

\[\frac{Q_t}{Q_\infty} = Kt^n \]  \hspace{1cm} (Eq. 7)

Sometimes, a drug following first-order kinetics may shift to zero-order if the process is carrier dependent and saturation takes place. Using plots drawn between concentration (C) vs. time and log C vs. time, we can determine which order of kinetics the drug release is following. If a C vs time plot gives a straight line, then the drug release is following a zero-order kinetics and if log C vs time plot gives a straight line then the drug release follows a first-order kinetics.
2.4.1 Assay to study drug release

There are various assays to study in vitro release (IVR) of liposomal formulations. Based on the literature, IVR methods can be classified into three main categories. First, “in situ” methods measure the desorption of a drug in real time using an analytical method capable of differentiating between encapsulated and released drug in the IVR system. With fluorescent drugs, the change in fluorescence intensity upon release from the liposomes can be determined and correlated with the drug release rate [123]. This IVR method is found to be limited to model compounds such as calcein and carboxyfluorescein but has also been useful for the drug doxorubicin [124]. One advantage of this technique is the production of rapid data combined with negligible sample manipulation. Unfortunately, it is not compatible with most drugs but other IVR methods can be considered if appropriate [125], [126].

A second category for assaying IVR involves membrane dialysis, which operates by physically separating the released drug from the encapsulated drug in the IVR system. This can be performed in the standard dialysis mode, the reverse dialysis mode, or using diffusion cells [127]. These dialysis-based methods are advantageous in conditions where the release rate from the liposome vesicle is relatively slow compared with the time frame for the free drug to diffuse across the membrane. However, even in the presence of a surfactant, the transfer of 90% of the free drug across the membrane can take up to 2 h. Thus, these dialysis methods are difficult to consider for IVR systems where the release rate of the API from the liposomes is comparable to or exceeds the rate of the drug transport via the dialysis membrane [124].

A third category of IVR methods proceeds by first sampling the IVR system and then using a second method to separate the free drug from the encapsulated drug, and finally quantifying the drug. The separation methods include chromatography (gel filtration or cation
exchange), centrifugation, or filtration. A disadvantage to these methods has historically been the additional sample manipulation, which can lead to artifacts including additional drug release during the separation step [128].

2.5 A brief introduction to silicon chemistry

Silicon is the second most abundant element on earth after oxygen. This element denoted Si is never found freely in nature but always in combination with other elements predominantly oxygen to produce various forms of silica, silicates, glasses, and sand [129]. Even though silicon falls under the same group as carbon in the periodic table, group 14, these atoms present different chemistries from each other due to differences in their properties. Some of these properties include longer bonds associated with silicon, which is due to its larger size compared to carbon. This larger atomic size displayed by silicon also causes a difficulty in its ability to form double bonds (i.e., Si=Si) as the larger atomic radius results in the inability for the p-orbitals to overlap [130]. In nature silicon in the form of silica is also seen to be present in marine organisms such as sea sponges, diatoms and terrestrial plants such as rice where silicon is involved in numerous important structural and functional roles [131].

Silicone or siloxanes are synthetic polymers made up of repeating unit of silicon and oxygen. This backbone unit (Si-O) exhibit unique properties that make silicones to date, one of the most widely used and researched biomaterial in the healthcare industry. The hydrophobic nature of silicones, their low surface tension, chemical and thermal stability, and biocompatibility constitute some of the reasons why highly used and developed in the healthcare industry [13], [107], [132]. Compared to purely organic polymers, siloxane polymers display high flexibility, which is due to large bond angles and bond lengths compared to those found
in more basic carbon polymers such as polyethylene. A C-C backbone unit has a typical bond length of 1.54 Å and a bond angle of 109.5°, whereas the siloxane backbone unit Si-O has a bond length of 1.63 Å and a bond angle that can reach 145°[133]-[135].

The methyl groups on silicon in siloxane polymers, provide a hydrophobic character to the siloxane chain allowing the polymer chain to reorient itself into a more stable orientation when at interfaces [64]. To more efficiently identify silicones, a shorthand nomenclature system known as the “MDTQ” nomenclature has been developed. This system describes silicones according to the presence of various siloxane monomer units that make up the silicone. The symbol M denotes the mono-functional unit (CH\textsubscript{3})\textsubscript{3}SiO\textsubscript{0.5}, D denotes the di-functional unit (CH\textsubscript{3})\textsubscript{2}SiO, T denotes the tri-functional unit (CH\textsubscript{3})SiO\textsubscript{1.5}, and Q denotes the tetra-functional unit SiO\textsubscript{2}[136].

Silicones are generally prepared via the hydrolysis of alkylchlorosilane and polymerization of the resulting silanol (Figure 11) [137].

![Figure 11. Preparation of Silicone. Adapted from Ref. 11. Copyright © 2009 by Scientific Information Database (SID).](image-url)
The key to the versatility of silicone materials is the result of their chemical properties. Silicones are semi-inorganic polymers, composed of repeating Si-O-Si units, with organic substituents typically attached to the open valences of the silicon atom. Silicones can exist in many different forms such as fluids, gels, elastomers, and resins which are all obtained by appropriately functionalizing the silicone polymer or adding fillers such that the silicone physical state changes [138].

Silicone fluids can be used in lubrication and dampening applications. They are made up of non-reactive silicone polymers formulated with methyl, phenyl, diphenyl, dimethyl or trifluoropropylmethyl moieties. The viscosity of these silicone fluids materials is highly dependent on the molecular weight of the polymer and the steric hindrance of the functional group that is part of the polymer chain [138], [139].

Silicone gels, because of their designed very soft and compliant feel when cured, find their application in tissue-mimicking [140]. These silicone gels are comprised of reactive silicone polymers and reactive silicone cross-linkers.

Silicone elastomers can be broken down into two very general categories: mouldable elastomers and adhesives. Adhesives, used to make things stick firmly together, are often of low-viscosity and differ from mouldable elastomers in that they require a humidity of 5% to 95% to cure, while mouldable elastomers often require addition of catalysts heat and pressure to cure effectively and mold high precision parts such as valves, O-rings, and seals [141]–[143]. These silicone elastomers also contain reactive polymers like gels and cross-linkers but differ in that they contain a reinforcing filler in order to achieve the desired physical property. High levels of reinforcing fillers and longer polymer chains, for example, result in higher viscosities of elastomers than gels and fluids [144].
Dimethylsilicones are found to be the most widely used silicone polymers industrially, due to the fact that they are cost effective and yield good physical properties in silicone elastomers and gels [107]. Figure 12 shows the structure of polydimethylsiloxane (PDMS) commonly referred to as silicone oil and widely used in drug delivery [11].

![Figure 12. Structure of polydimethylsiloxane.](image)

The flexibility of the Si-O bond in the inorganic chain of PDMS causes the glass transition ($T_g$) of PDMS to be amongst the lowest of all polymers (-125°C). This very low $T_g$ due to low intermolecular interactions in silicones causes PDMS based devices to remain flexible even in extremely cold environments [64], [145]

2.5.1 Biomedical silicones

The beginning of the use of silicone in medical applications can be traced down to the 1950s after its extensive use in the aerospace industry in the 1940s [146]. An experiment conducted by McDougall in 1954, which turned out to be a control experiment, demonstrated that silicone oils and cross-linked siloxane systems were biologically and toxicologically inert [11].

In general, the design of drug delivery systems based on silicones are typically either matrices or reservoirs containing the API of interest (Figure 13) [11], [145]. The decision as to which configuration should be chosen depends on the profile of the API and the type of release pattern needed to achieve therapy. In a matrix design, the API is mixed with the silicone and then moulded to the required geometry. This route implies that an API should be soluble in the
silicone for it to be moulded into a matrix configuration [145], [147]. Most silicones are known to be hydrophobic in nature. Knowing the extent of hydrophilicity of an API can be a good lead in the configuration choice. If the API is hydrophilic for example, the matrix design is no longer a favourable option. The release pattern of a matrix configuration, in general, is such that the API experiences a burst release and then the release rate gradually becomes smaller, while in the reservoir configuration an initial spike in the release is observed, which then normalizes into a lower but consistent release rate. In every reservoir design, the API is concentrated in a void at the center of a moulded silicone elastomer. This configuration is preferable for APIs that are insoluble in silicone as well as sensitive to the heat from the moulding process. After appropriate incorporation of the API has been done, the device is then capable of eluting or releasing the API [144].

2.5.2 Siloxane liposomes in drug delivery

To date, no studies have been done to explore the drug delivery capacity of liposomes possessing siloxanes units as part of the phospholipid acyl chain. The only related work saw silicones used to impart stability in cationic liposomes from egg yolk phosphatidylcholine within a pH range of 8.5 and 10.2, by coating a thin layer of silicone on the liposomes [148].
2.6 Phospholipid synthesis

The biogenesis of phospholipid *in vivo* occurs at the endoplasmic reticulum (ER), which not only provides the required hydrophobic conditions suitable for distinct reactions but also organizes and carries out complex processes essential for the production of PLs [149]. These natural phospholipids can then be isolated and purified from living species (vegetable oils or animal tissues) and used for targeted purposes. Even though the isolation cost of natural PLs is low, the fact that they typically have a low level of purity (increasing the purity increases the cost) and are relatively unstable due to the presence of polyunsaturated fatty acids, leads many researchers to invest in the chemical synthesis of phospholipids for production [150]–[152]. The purity in the case of laboratory-synthesized lipids is higher as is the stability relative to natural PLs since single component PLs with defined structures and configurations is obtainable [6]. The
synthesis of lipids can either be via a semi-synthesis or total synthesis. In a semi-synthesis, fewer synthetic steps are required as the process typically involves the modification of natural PL at the level of the tail group, phosphate head group, or both (e.g., hydrogenation of an unsaturated natural PL to yield a saturated PL) [150]. In the total synthesis, more steps are required as the process involves the esterification or etherification of apolar moieties to a glycerol backbone, which is in turn linked to a polar head group [153], [154].

Catalytic steps can be performed by enzymes that either act at the sn-1 position, at the sn-2 position, or at the sn-3 position of the glycerol backbone. This trend of using enzymes has gained popularity in organic synthesis as the enzymes provide mild alternatives to harsh metals and toxic catalysts and exclude the need to perform chemical transformations at extreme temperatures or pressures. Also, using enzymes permits chemistries to be performed in solvent-free conditions and permits the use of convenient and easy purification methods between the reaction mixture and enzymes [155].

2.6.1 Lipases

Throughout this thesis, the use of lipases (Enzyme Commission number; EC 3.1.1.3), a serine family of hydrolases is favoured for the synthesis of various lysophospholipids and phospholipids [156]. This class of enzymes is one of the most commonly used in biocatalysis and shows a broad range of substrate specificity [157]–[159]. Lipases are able to catalyze the hydrolysis of triacylglycerols to diacylglycerol, monoacylglycerol, glycerol, and free fatty acids (Figure 14), and in reverse conditions (i.e., under anhydrous conditions) the enzyme is able to perform esterification, alcoholysis, and transesterification reactions [160]. Lipases perform various chemistries providing high regio- and enantioselectivity under mild conditions [161].
X-Ray crystallography analysis revealed that lipases generally share a serine catalytic triad signature (i.e., Ser$_{105}$ His$_{224}$ and Asp$_{187}$) as part of their active site with the serine moiety acting as the active nucleophile [162]. *Candida antarctica* lipase B (CalB), whose crystal structure was elucidated in 1994, has a molecular weight of 33,273 Da and is composed of 317 amino acids. This lipase, unlike other lipases, is not thought to require interfacial activation for catalysis, as the active site is not surrounded by a hydrophobic lid (which provides a closed conformation when blocking the active site and active conformation when opened) but is instead solvent exposed [163].

2.6.2 Chemoenzymatic synthesis of siloxane phospholipids

In 2016, Frampton and Zelisko introduced the first trisiloxane-containing phospholipids; 1,2-SiPC and 1,3-SiPC (Figure 15) [75]. These lipids contained siloxane moieties as part of the acyl unit or fatty acid tail of a PC glycerol backbone at the $sn$-1 and $sn$-2 position for 1,2-SiPC
and the sn-1 and sn-3 position for 1,3-SiPC. The cornerstone of the success of this synthesis was biocatalytic, using heptamethyltrisiloxane-modified decenoic acid ester as a starting material.

![Chemical structures and reactions](image)

Figure 15. General scheme of first-generation synthesis of 1,2-SiPC and 1,3-SiPC. (A) 10wt% Lipozyme RM, 40°C, 72h; (B) 2-chloro-1,3,2-dioxophospholane-2-oxide, NEt₃, PhMe; (C) NMe₃, AcCN, -40°C to RT M. B. Adapted with permission from Ref. 75. Copyright © 2017 John Wiley & Sons, Inc.

As shown in Figure 15 above, the synthesis started with glycerol and subsequently attaching FA tails and a phosphate head group. Among the prospective applications of this research was the exploration of the potential of these lipids as nano-carriers for delivery vehicles [75]. One of the most fascinating discoveries made from these two lipids after small angle x-ray scattering (SAXS) analysis in parallel with dynamic light scattering (DLS) measurements was the capacity of these new lipids to spontaneously self-assemble into ULVs (~150 nm diameter) when in an aqueous environment. This presented a potential methodology for liposome
formation that eliminated time-consuming steps such as extrusion and sonication typically employed to obtain ULVs with specific diameters [164].

2.6.3 Nomenclature of silicon-containing phospholipids

After the introduction of 1,2-SiPC and 1,3-SiPC as the first siloxane-containing phospholipids in the lipid library found in the literature, several other siloxane-containing phospholipids followed [75]. These emerging siloxane-PLs created a necessity for a naming convention. In order to understand the naming system used across all those lipids, let us consider the illustration in Figure 16 below:

Figure 16. Unsymmetrical silicon-containing lipid.
The pattern used in naming the lipids is A-BC-D;

where “A” represents the carboxylic acid moiety at the *sn*-1 position, “B” represents the carboxylic acid moiety at the *sn*-2 position, “C” represents the silicon-containing portion at the *sn*-2 position and D represent the head group at the *sn*-3 position.

In regard to the phosphate head group portion, the conventional two-letter abbreviation (i.e., PC, PE, PG, etc.) is used, which corresponds to the biologically relevant phospholipid family [6]. As for the silicon-containing portion, the lipid name is made up of an abbreviated “XS”, where “X” represents the first letter of the quantifier (i.e., di, tri, etc.) indicating the length of the siloxane unit “S”.

The carboxylic acid moieties’ names were abbreviated according to Table 3 below:
<table>
<thead>
<tr>
<th>Total carbon number in fatty acid chain</th>
<th>Trivial name</th>
<th>Three-letter abbreviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁</td>
<td>formic acid</td>
<td>For</td>
</tr>
<tr>
<td>C₂</td>
<td>acetic acid</td>
<td>Ace</td>
</tr>
<tr>
<td>C₃</td>
<td>propionic acid</td>
<td>Pro</td>
</tr>
<tr>
<td>C₄</td>
<td>butyric acid</td>
<td>But</td>
</tr>
<tr>
<td>C₅</td>
<td>valeric acid</td>
<td>Val</td>
</tr>
<tr>
<td>C₆</td>
<td>caproic acid</td>
<td>Cpr</td>
</tr>
<tr>
<td>C₇</td>
<td>enanthic acid</td>
<td>Ena</td>
</tr>
<tr>
<td>C₈</td>
<td>caprylic acid</td>
<td>Cpy</td>
</tr>
<tr>
<td>C₉</td>
<td>pelargonic acid</td>
<td>Pel</td>
</tr>
<tr>
<td>C₁₀</td>
<td>capric acid</td>
<td>Cpc</td>
</tr>
<tr>
<td>C₁₁</td>
<td>hendecanoic acid</td>
<td>Hen</td>
</tr>
<tr>
<td>C₁₂</td>
<td>lauric acid</td>
<td>Try</td>
</tr>
<tr>
<td>C₁₃</td>
<td>tridecylic acid</td>
<td>Try</td>
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<tr>
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<td>myristic acid</td>
<td>Myr</td>
</tr>
<tr>
<td>C₁₅</td>
<td>pentadecylic acid</td>
<td>Pen</td>
</tr>
<tr>
<td>C₁₆</td>
<td>palmic acid</td>
<td>Pal</td>
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<tr>
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<td>margaric acid</td>
<td>Mar</td>
</tr>
<tr>
<td>C₁₈</td>
<td>stearic acid</td>
<td>Ste</td>
</tr>
</tbody>
</table>
Based on this system, the names of the following silicon-containing lipids in Figure 17 below would be as follows:

![Chemical structures of silicon-containing lipids]

Figure 17. Some named silicon-containing lipids.

2.7 Fluorescence spectroscopy

This section will focus on fluorescence spectroscopy briefly explaining the central idea behind the technique and how it contributes to the analysis of drug delivery.

The onset of fluorescence spectroscopy can be traced back to 1565, when a Spanish scientist, Nicolás Monardes observed a blue colour coming out of a Mexican tree soaking in water. This Mexican tree later called *Lignum nephriticum* (kidney-wood) was used in the treatment of kidney ailment. Matlaline, which is the oxidation product of one of the flavonoids found in the wood, was the fluorescent compound responsible for the appearance of the beautiful bluish colour tone [165], [166]. The discovery of fluorescent molecules such as fluorescein then
followed (1871) but it was not until the Second World War that scientists striving to treat malaria created a proper instrument that could help in identifying how much of the antimalarial drug was present in a patient’s plasma sample for proper treatment [167].

Fluorescence is defined as the emission of light by the singlet excited state of molecules following absorption of photons from an external source. The two basic components needed for fluorescence to occur is the presence of a fluorophore or a fluorescent dye, which is usually an aromatic hydrocarbon or heterocycle. The second requirement is excitation of the dye through the absorption of a photon [168]. Fluorescence competes for other non-radiative processes occurring after excitation such as energy transfer and heat loss as well as eligible radiative processes such as phosphorescence. The process of fluorescence can be depicted using a Jablonski diagram as illustrated in Figure 18 below [168], [169].

![Figure 18. Illustration of the Jablonski Diagram. Adapted from Ref. 168. Copyright © 2006 by Springer Science+Business Media, LLC.](image-url)
From the Jablonski diagram above, one can see that light energy excites an electron from the ground state to a higher energy state. From the excited state, internal conversion affected by vibrational relaxation and thermal energy loss to the environment can occur. A photon is then emitted from the lower excited state in the form of fluorescence.

The emission wavelengths are conventionally of lower energy (hence higher wavelength) than the ones that are absorbed and can be from the UV through to the near infrared (NIR) region [168]. This emission can be affected by factors such as pH, temperature, energy transfer to and from other molecules, and quenching. Fluorescence has a wide range of applications in the biochemical, medical and chemical research fields for analyzing organic compound. In regard to drug delivery, fluorescence is beneficial for spatial and temporal interactions of DDSs with cells producing live cell imaging of dynamic events occurring in vivo or in vitro [98], [170].

2.7.1 Calcein

Calcein (Figure 19) is a negatively charged hydrophilic fluorescent molecule with a logP value of -4 and a pKₐ of 6.4, exhibiting its maximum absorbance at 495 nm and maximum emission at 515 nm[125], [171], [172]. Calcein is known to self-quench at concentrations above 70mM [173] and is used in liposomal work as a fluorophore to characterize IVR due to its self-quenching property. Incorporating calcein at a quenched concentration in a liposome enables a periodic increase in the fluorescence of calcein when it is diluted upon release from the liposome to the buffer system surrounding it. The encapsulation of quenched calcein in liposome is hence a strategy used to investigate release profiles of liposomes derived from synthetic and naturally occurring phospholipids.
2.8 Aim of the Thesis

The aim of this thesis is to investigate the capacity of siloxane-containing phospholipids to entrap and release a model drug, after forming liposomes of an ideal size for drug delivery (70-200 nm), and exploring siloxane-containing phospholipids as nano-vesicular drug delivery systems in different pH media. It is hypothesized that liposomes containing siloxanes will possess increased flexibility and rotation displayed by the siloxane moiety which will in turn cause an increase in the membrane fluidity and a faster release of the encapsulated model compound calcein (Figure 19).

![Chemical structure of Calcein](image.png)

Figure 19. Chemical structure of Calcein.
3 Results and Discussion

3.1 Hydrolysis of siloxane ester to siloxane acid

3.1.1 Chemoenzymatic synthesis of trisiloxane acid (3)

Compound 3, the trisiloxane acid to be used in the construction of the FA tails of siloxane-containing phospholipids (Figure 20) and the precursor of the newly synthesized lysophospholipid LysoTSPC (4) was synthesized using two different enzymes: Candida antartica lipase A (CalA) and Candida antartica lipase B (CalB). These two enzymes investigated as potential biocatalysts for this synthesis are both lipases from Candida antarctica shown to catalyze esterification and transesterification reactions [174]. The use of CalB specifically for this reaction was based on a literature report, which clearly showed that CalB had the capacity to accommodate bulky substrates in its active site and to convert siloxane-containing esters to corresponding acids [175]. CalA was also explored as a potential biocatalyst because it has been shown to exhibit activity towards various straight chain primary alcohols and carboxylic acids [176].

![Compound 2, 3, and 4 structures](image)

Figure 20. Structure of trisiloxane ester (2), trisiloxane acid (3) and LysoTSPC (4).

In contrast to CalB, CalA exhibits interfacial activation, such that the probability of its lid to remain closed in an aqueous environment is high, hence lowering its activity [177]. This
interfacial activation was reflected in the yield of both enzymatic reactions; the yield achieved using CalB was 31% higher than that from CalA suggesting that CalB is more suitable and efficient for the hydrolysis of the trisiloxane ester (2) (Figure 20) to the trisiloxane acid (3).

Figure 21 illustrates the scheme for this reaction.

The maximum yield using CalB was 34% with the yield for the majority of reactions ranging from 20-24%. A minimum of two columns was usually required to purify the product (i.e., one column to separate the acid from a majority of the ester starting material, and another column to separate the acid (already 95% pure) from trace amount of ester that were not visible on the TLC plates from the first column but revealed in $^1$H-NMR). It was hypothesized that as the carboxylic acid was being formed during hydrolysis and liberated from the enzymes’ binding pocket, the acid formed micelles entrapping some starting materials ester and coordinated with the ester such that they were really hard to separate.

CalA and CalB are not the only types of serine lipases that exist. Lipozyme RM an immobilized lipase from *Rhizomucor miehei* and Lipase D from *Rhizopus delemar* have also been used as biocatalyst [75] and it was of interest to see if these enzymes could out-perform CalB to optimise the yield of this reaction.
3.1.2 Chemoenzymatic synthesis of disiloxane acid (8)

This reaction, as the one previously discussed, was crucial in that it produced another required siloxane acid moiety necessary for the synthesis of various siloxane-phospholipids used in this thesis. For the hydrolysis reaction resulting in compound 8, only CalB (from N435) was
used as a biocatalyst. The yield from this reaction was only 19% but enough product to be used as a starting material for the next step (i.e. syntheses of PLs).

Even though this present thesis was not focusing in optimising reactions but instead in obtaining enough PL material (≥ 10 mg) in order to perform drug delivery experiments, a study exploring the efficiency of other lipases for this enzymatic reaction would be useful.

The scheme for the enzymatic reaction discussed above follows the same pattern as previously presented in Figure 21.

3.2 Hydrosilylation of mono-unsaturated esters and mono-unsaturated acids

Hydrosilylation is extensively used in silicon chemistry and industry to obtain a silicon-carbon (Si-C) bond. This generally occurs by reacting a silicon-hydrogen (Si-H) bond with an unsaturated carbon-carbon (C=C) bond using an appropriate catalyst and solvent system if necessary [178]. A proposed reaction scheme is illustrated in Figure 22 below.

In order to obtain siloxane acid moieties that would serve as FA tails (i.e., compounds 3 and 8), two different platinum catalysts, Karstedt’s catalyst (Figure 23) and platinum oxide (Pt₂O), were used for the hydrosilylation of a hydrosiloxane with an unsaturated ester and an unsaturated acid respectively. Platinum oxide was first favoured over Karstedt’s catalyst as it is efficient in reactions involving unsaturated acids directly [179], implying that the siloxane acid moiety could be obtained in a single step. Karstedt’s catalyst, on the other hand, involves more than one step to obtain the siloxane acid moiety. The unsaturated acid of interest needed first to be converted to its unsaturated ester (step 1) before hydrosilylation could be performed (step 2) [178], [180]. The hydrosilylated ester that was produced could then be subjected to hydrolysis (in this case using lipases) to transform the latter to its corresponding acid (step 3, time-consuming
step due to purification as mention earlier in Section 3.1.1 for compound 3). Unfortunately, with every hydrosilylation reaction that was attempted, platinum oxide was unable to fully hydrosilylate any of the unsaturated acid such that this unsaturated starting material was still present as an impurity after the hydrosilylation reaction and could not be removed completely. Being unable to obtain pure siloxane acid moieties after using platinum oxide, Karstedt’s catalyst was favoured, which fully hydrosilylated every unsaturated ester. The drawback of this method was the purification method in the subsequent hydrolysis step, but this was the only accessible alternative to obtain pure siloxane acids.

![Reaction Scheme](image)

Figure 22. Proposed reaction scheme of platinum catalyzed hydrosilylation of an unsaturated moiety.
3.3 *sn*-2 Substitution of lysophospholipids to synthesize phospholipids

Total synthesis was the approach used for the synthesis of various phospholipids throughout this thesis, starting with GPC and subsequently attaching a FA tail to its *sn*-1 and *sn*-2 position. When only one FA tail is present on the glycerol backbone, the structure is referred to as a lysophospholipid [181]. CalB, being *sn*-1 selective was used to couple a fatty acid tail to the GPC to yield either 16:0 lysoPC (50% yield) or LysoTSPC (23% yield) depending on the acid used [182]. These lysophospholipids were important in that they served as precursors for the synthesis of various siloxane-phospholipids (Figure 24). After obtaining the lysophospholipid, 16:0 LPC, for example, dicyclohexylcarbodiimide (DCC) / 4-dimethylaminopyridine (DMAP) coupling was favoured for the substitution of compound 8 at its *sn*-2 position to produce PalValDSPC (10) in 59% yield. Using compound 3 produced PalCpcTSPC (11) in 41% yield. On the other hand, the lysophospholipid LysoTSPC, DCC-coupled with compound 3 could produce 1,2-SiPC (5) at 42% yield. All these yields produced enough of the PL product for release study experiments to be performed (60.0 mg of PalValDSPC, 50.0 mg of PalCpcTSPC and 20.0 mg of 1,2-SiPC).
General schemes of the synthesis of compounds 5, 10 and 11 are illustrated in Figure 25-27 respectively.

CalB, even though sn-1 selective, was also used to perform an sn-2 substitution of the disiloxane acid 8 onto 16:0 LPC in order to obtain PalValDSPC (10). The idea came from a report in the literature showing that lipozyme RM, which is sn-1,3 selective, could also yield sn-1,2 substituted products via acyl migration [75], [84], [183]. Unfortunately, CalB was unable to perform an sn-2 substitution on the lysophospholipid, and it was hypothesized that the phosphate group in 16:0 LPC was too big to fit into its active site. DCC/DMAP esterification was concluded to be the best alternative for sn-2 substitution for every system in this thesis to obtain unsymmetrical and symmetrical lipids.
Figure 25 General reaction scheme for the second-generation total synthesis of 1,2-SiPC.
Figure 26 General reaction scheme for the total synthesis of PalValDSPC.
3.4 Size analysis of liposomes before extrusion

Dynamic light scattering (DLS) measurements, as presented in detail in Section 5.2, were carried out at various steps of the release experiment to monitor vesicles’ sizes. Before extrusion of each phospholipid, vesicle formation was initiated by vortexing the rehydrated thin lipid film with calcein in Tris buffer (100 mM calcein in 50mM Tris 100mM NaCl, pH 7.5). POPC,
PalValDSPC, 16:0 LPC, LysoTSPC and 1,2-SiPC were all subjected to DLS before extrusion (i.e., after vortexing).

LysoTSPC was observed to self-assemble to form ~100 nm diameter vesicles after vortexing (Figure 28). Being a lysophospholipid, LysoTSPC was expected to form micelles which can be up to about 20 nm in diameter [184]. 16:0 LPC another lysophospholipid used for syntheses of PLs, formed vesicles ~40 nm in diameter (Figure 28). The fact that LysoTSPC vesicles exhibited a larger diameter than its aliphatic counterpart 16:0 LPC suggested that the siloxane tail in LysoTSPC conferred a different surfactant parameter in terms of the shape of the acyl chain than the tail that did not contain a siloxane unit [84]. Instead of having an overall cone configuration as in molecules forming micelles, LysoTSPC likely has a cylindrical shape usually seen in molecules forming bigger vesicles like liposomes (Figure 8). Lipid membrane analysis experiments using small-angle X-ray scattering (SAXS) were solicited as DLS only provided size measurements. Results are still pending to confirm or reject the hypothesis made about LysoTSPC. However, we know from previous studies [164] that siloxane-containing lipids tend to have larger lipid membrane volume than their purely aliphatic counterparts due to the presence of the siloxane-containing acyl chains. A study comparing 1,2-SiPC with POPC in terms of volume illustrated that 1,2-SiPC exhibited a larger lipid membrane volume than POPC and was more similar to diphytanoylphosphatidylcholine (DPhPc) in that regard [164].

The phospholipids POPC and PalValDSPC were observed to self-assemble to large vesicles (>2000 nm) and were subjected to extrusion in order to obtain smaller vesicles ideal for release studies (Figure 28). 1,2-SiPC was also expected to form larger vesicles like its biologically-relevant phospholipid counterparts as phospholipids usually self-assemble to form large MLVs [185]. The fact that 1,2-SiPC self-assembles to ~100 nm ULVs provides an
opportunity to bypass the time-consuming and strenuous extrusion step [75]. However, fractions of the self-assembled siloxane-containing liposomes were also extruded using a 100 nm polycarbonate membrane in order to compare release from extruded and non-extruded 1,2-SiPC vesicles of the same size.

3.5 Size analysis of liposomes after extrusion

All extrusions, as presented in detail in Section 5.3.2.1, were carried out at room temperature using a 100 nm pore diameter polycarbonate membrane mounted in a mini-extruder fitted with Hamilton syringes. Liposome suspensions were then subjected to DLS measurements to ensure successful formations of small vesicles ideal for release studies in the following step. Analyses, done after extrusion (or after vortexing if extrusion was not performed) were hence considered as pre-release data.

The phospholipid POPC and the siloxane-phospholipid PalValDSPC formed 100 nm vesicles after extrusion (Figure 28). 16:0 LPC was not extruded using the 100 nm pore membrane as it formed vesicles of ~40 nm (less than the membrane pore size). The corresponding siloxane-lysospholipid LysoTSPC, formed 100 nm vesicles after vortexing and after extrusion became slightly smaller (~70 nm). Like 1,2-SiPC, an interest in doing a horizontal comparison between extruded and non-extruded LysoTSPC vesicles arose as both were of ideal size for release [186].

Due to mechanical failure of the DLS instrument partway through the study, siloxane-phospholipids PalCpcTSPC, OleCpcTSPC, and 1,2-SiPC were hypothesized to also form ~100 nm vesicles following extrusion based on results obtained from the siloxane-phospholipid PalValDSPC after extrusion, in parallel to the fact that it is shown that extrusion of phospholipids using 100 nm pore membranes results in ~100 nm vesicles [187], [188].
Figure 28 DLS measurements of vesicles before release experiments. Lysophospholipid 16:0 LPC after vortexing exhibiting 40nm in size, siloxane lysophospholipid LysoTSPC exhibiting ~100nm in size after vortexing and ~ 90 after extrusion, phospholipids PalValDSPC and POPC exhibiting ~100nm in size. Experiments were done in triplicates.

3.6 Entrapment efficiency of liposomes

Entrapment efficiency (ee) is based on how much of a drug can be sequestered in a liposome [189]. Several factors, such as the type of phospholipid family, charge of lipids, membrane thickness and fluidity, lipid concentration, buffer medium, lipid to rehydration volume ratio, liposome preparation technique, etc. can affect the encapsulation of hydrophilic drugs in liposomes [190]. All of the lipids listed below correspond to the same PL family (i.e., phosphocholine) and these PLs were all calibrated to the same concentration (i.e., 20 nM of lipid) and were all investigated in the same buffer medium. Also, every liposome was prepared using the same method; lipid film rehydration, and the fact that each formulated liposome was
neutral (due to PC) implied there was no electrostatic effect contribution to the ee [191]. This means that the main factor affecting or differentiating the ee between all of these lipids is narrowed down to what differentiates them, namely the lipid membrane.

The entrapment efficiency of lysoTSPC, 34%, was observed to be greater than that of 16:0 LysoPC, 13% (Table 4). This observation can be correlated to the DLS result obtained for these two lipids after vortexing (16:0 LPC 40 nm and LysoTSPC 100 nm), with LysoTSPC having more room than 16:0 LPC as it has a relatively larger diameter than 16:0 LPC and hence more potential to entrap calcein during vortexing. Also, 1,2-SiPC was seen to have a greater entrapment efficiency before extrusion than after extrusion (Table 4). The non-extruded or vortexed liposome had a 50% ee while the extruded liposome, even though of the same size as its non-extruded counterpart, only had a 24% ee.

Before vortexing, when a thin lipid film is rehydrated in an aqueous environment, the lipid lamellae only swell and do not detach from the support. An external energy input such as vortexing (usually for 10 min) is usually employed to mechanically agitate and break the stack for vesicle formation to occur as hydrophobic edges reseal [192]. During the extrusion process (that followed for this thesis), the external pressure applied is associated with the lysis tension required to rupture the phospholipid bilayer membrane with the rate at which the lipid membrane separates ultimately responsible for the observation of either MLVs (if slow) or ULVs (if fast) [187], [193]. It is believed that the reduction in the ee of 1,2-SiPC is in part due to the loss of material that occurs during the extrusion process, and also due to the fact that extrusion causes vesicles to be formed at a higher rate than during vortexing and hence offering less potential for encapsulating calcein [194]. Like the case of 1,2-SiPC, these factors are believed to be responsible for the reduction of ee of LysoTSPC vesicles after extrusion (Table 4).
Based on the overall results, siloxane-containing lipids were shown to have a potential for greater entrapment capacity than the corresponding aliphatic analogue. The siloxane group most likely confers a different surfactant parameter in terms of the shape of the acyl chain such that each layer has an increase lipid volume and thickness compare to POPC. SAXS analyses from previous studies support these findings [164]. Extruded 1,2-SiPC and PalValDSPC, however, were shown to have a lower entrapment efficiency than POPC. From Table 4 we can see that extruded 1,2-SiPC had a 24% ee, extruded PalValDSPC had a 25% ee, while extruded POPC had a 31% ee. This suggests that extrusion of the siloxane lipids PalValDSPC and 1,2-SiPC in this case, most likely caused bifurcation of the lipid membrane and vesicle formation rate to be faster than that of POPC reducing the time for calcein to be entrapped before lipid membrane closure [187]. This is likely why, even though all these different PLs end up having the approximately same diameter after extrusion, they do not have the same ee.

Even though the pressure was always applied manually, further studies investigating extrusion with an automated extruder at a fixed pressure will eliminate potential human error and also confirm the variability of ee in terms of the lipid membrane. It should be noted that these studies have used homogenous monodisperse vesicles with a dispersity (Đ) of less than 0.5 (range 0.0 to 0.2) in order to avoid any uncertainties [195], [196]. The dispersity, as a measure of the heterogeneity of sizes of particles in a mixture, was determined using the equation below [197]:

\[ Đ = \frac{\sigma^2}{M_n^2} \]  
(Eq. 8)

where \( \sigma \) is the standard deviation and \( M_n \) the mean diameter of liposome vesicles.
Table 4. Entrapment efficiency obtained for various liposomes. Extrusion was done using a 100 nm membrane (Mean ± SEM, n=3).

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Liposome diameter (nm)</th>
<th>Entrapment efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0 LPC non-extruded</td>
<td>40±3.8</td>
<td>13±0.4</td>
</tr>
<tr>
<td>LysoTSPC non-extruded</td>
<td>100±8.0</td>
<td>34±1.1</td>
</tr>
<tr>
<td>LysoTSPC extruded</td>
<td>70±6.6</td>
<td>15±1.4</td>
</tr>
<tr>
<td>POPC extruded</td>
<td>100±5.4</td>
<td>31±0.6</td>
</tr>
<tr>
<td>1,2-SiPC non-extruded</td>
<td>100±31.9</td>
<td>50±0.0</td>
</tr>
<tr>
<td>1,2-SiPC extruded</td>
<td>100</td>
<td>24±1.8</td>
</tr>
<tr>
<td>PalCpcTSPC extruded</td>
<td>100</td>
<td>35±1.3</td>
</tr>
<tr>
<td>OleCpcTSPC extruded</td>
<td>100</td>
<td>38±1.1</td>
</tr>
<tr>
<td>PalValDSPC extruded</td>
<td>100±8.4</td>
<td>25±0.0</td>
</tr>
</tbody>
</table>

The fact that silicon-containing liposomes confer potential for higher entrapment capacity implies that a higher drug-to-lipid ratio can be obtained with these systems, which means that the bioavailability of desired molecules can be increased with a reduced frequency of drug administration and reduced risk of lipid-induced toxicity after administration [198].

3.7 Calcein release profiles

The release profile of different formulated liposomes was obtained using the formula:
\[
RF(\%) = 100 \frac{(I_t - I_0)}{(I_{max} - I_0)} \quad (Eq. 9)
\]

where RF is the fraction of calcein released, \(I_0\), \(I_t\), and \(I_{max}\) are the fluorescence intensities measured at the beginning of the experiment, at time \(t\), and after the addition of 3\% Triton X-100, respectively [172].

Release profiles of liposomes were obtained at room temperature at three different pH values: 4.5, 7.5, and 10 reflecting the different pH environments encountered in the human body [199]. The attempt of this thesis to model *in vivo* release calcein profiles of siloxane liposomes at pHs 4.5, 7.5, and 10, as well as POPC at pHs 4.5 and 10, is a new contribution to the existing body of knowledge: current reports in the literature only report release profiles of POPC liposomes at pH 7.4. The main reason for this is that liposomes formulations from those studies are to be used for IV administration. Using liposomes for oral administration usually present challenges in the variability of pHs encountered by the liposome before reaching the systemic circulation as well as the presence of bile salts which tend to degrade the liposomes [200], [201]. The pH in the stomach which can go up to 3 is largely different from the neutral pH in the intestine for example, which is enough to initiate pH sensitive behaviours from liposomes [202]. This means that for ideal oral delivery, pH-independent release from liposomes could be of great value.

The major purpose of the release profile study was to explore release profiles from synthesized siloxane-PCs and to compare them with those from POPC (a biologically relevant phospholipid). Interest rose along the road to also compare extruded with non-extruded vesicles of 1,2-SiPC and LysoTSPC of the same and or ideal size for drug delivery. This study is the first investigating release studies of not only these siloxane-based liposomes but also release in different pH media.
POPC, as a commercially available, biologically relevant PC-lipid, was used as a reference to be compared with various synthesized siloxane-PC lipids. It was observed that vesicles from POPC had unique release profiles at each pH, presenting an overall first-order release of calcein (Figure 29).

![Figure 29 Release profiles of POPC at different pH values All points were measured in triplicate, and error bars represent the standard deviation in the measurements.](image)

At this point, we know that 1,2-SiPC self-assembles directly into ULVs avoiding the need for extrusion to achieve an ideal vesicle size. It was previously seen that extruded and non-extruded samples of this siloxane-PC had the same size. In order to compare the effect of extrusion the release from liposomes based upon this particular lipid, release profiles from both extruded and non-extruded samples were performed. Figure 30 illustrates the release profile of extruded liposomes from 1,2-SiPC. Interestingly extruded liposomes based upon 1,2-SiPC each exhibited a first-order release profile as well as a pH independent behaviour.
16:0 LPC or 16:0 LysoPC is a lysophospholipid that was used as a precursor for the synthesis of siloxane-phospholipids such as PalValDSPC. The same way this lipid was used as a precursor in phospholipid synthesis, LysoTSPC, its siloxane-containing lipid counterpart, was also used as a precursor for siloxane-lipid synthesis. LysoTSPC being a new molecule and presenting certain features like self-assembling into vesicles larger than micelles, raised interest in its potential release profile. For a direct comparison, release from 16:0 LPC needed to be performed. Figure 31 illustrates the release profile of non-extruded vesicles from 16:0 LPC. Vesicles from this lipid were not extruded simply because they spontaneously self-assembled into ~40 nm vesicles. These were probably micellar structures as lysophospholipids are known to self-assemble into this particle vesicle type. Three different release profiles were observed in various pH media, all exhibiting a first-order release profile.
LysoTSPC, a newly synthesized molecule used as a precursor for siloxane-lipid synthesis was investigated for its release profile as well in three different pH media in Tris buffer. Its non-silicon-containing counterpart was 16:0 LPC. LysoTSPC self-assembled into vesicles with a diameter of ~ 100 nm as compared to 16:0 LPC, which presented vesicles with a smaller diameter of ~ 40 nm. Again, three different release patterns were observed in the various pH media but as opposed to 16:0 LPC, LysoTSPC presented a first-order release in every pH media (Figure 32).
Figure 32 Release profiles of LysoTSPC at different pH values. All points were measured in triplicate, and the error bars represent the standard deviation in the measurements.
Even though LysoTSPC self-assembled into a liposome with an ideal diameter for release studies, samples were also extruded and analysed for release studies to see if any difference would be observed for extruded and non-extruded samples. For extruded LysoTSPC pH 7, 4.5, and 10.5 each presented a first-order release (Figure 33).

![Figure 33](image)

**Figure 33** Release profiles of extruded LysoTSPC at different pH values. All points were measured in triplicate, and the error bars represent the standard deviation in the measurements.

In **Figure 30** the release profile of extruded 1,2-SiPC is illustrated. **Figure 34** below illustrates the release profile of non-extruded liposomes from 1,2-SiPC (i.e., of self-assembled 1,2-SiPC). Even though extruded and non-extruded 1,2-SiPC have the same size, pH behaviours from the self-assembled 1,2-SiPC present unique characteristics that still need further investigations to be fully understood.
Figure 34 Release profiles of 1,2-SiPC at different pH measurements. All points were measured in triplicate, and the error bars represent the standard deviations in the measurements.
PalValDSPC is another siloxane-PC lipid that was studied in this thesis. This lipid did not self-assemble directly into ULV liposomes like 1,2-SiPC, so extrusion was necessary to obtain vesicles with the desired diameter. Figure 35 illustrates the release profile of extruded liposomes from PalValDSPC, all pHs presenting a first-order release profile signature.

![Figure 35 Release profile of PalValDSPC at different pH values. All points were measured in triplicate, and the error bars represent the standard deviation in the measurements.](image)

The self-assembling characteristic characteristics of PalCpcTSPC could not be determined due to mechanical failure of the DLS machine and was hence directly treated via extrusion after the initiation of vesicles during vortexing. Figure 36 illustrates the release profile of extruded liposomes from PalCpcTSPC and the release kinetics were seen to be overall first-order in the three different pH media.
Figure 36 Release profile of PalCpcTSPC at different pH values. All points were measured in triplicate, and the error bars represent the standard deviation in the measurements.
The synthesis of OleCpcTSPC was achieved after the mechanical failure of the DLS machine and hence no self-assembly size was determined for this lipid. Direct treatment of vesicles via extrusion was performed after vortexing. Figure 37 illustrates the release profile of extruded liposomes from OleCpcTSPC. Interestingly the release kinetics were seen to be zero-order in the three different pH media as well as exhibiting a pH-independent behaviour.

Figure 37 Release profile of OleCpcTSPC at different pH values. All points were measured in triplicate, and the error bars represent the standard deviation in the measurements.
Table 5 illustrates the different rate constants obtained from each lipid per medium.

The rate of each first order release was obtained using the equation:

\[ RF(t) = RF_{(\text{max})}(1 - e^{-kt}) \quad (Eq. 10) \]

This equation [172] was rearranged as follows to isolate \( k \) and obtain the rate constant for each first order release profile.

\[ RF(t) = RF_{(\text{max})} - RF_{(\text{max})}e^{-kt} \]

\[ e^{-kt} = \frac{RF_{(\text{max})} - RF(t)}{RF_{(\text{max})}} \]

\[ e^{-kt} = \frac{-RF(t)}{RF_{(\text{max})}} + 1 \]

\[ -kt = -\ln RF(t) + \ln RF_{(\text{max})} \]

\[ -k = \frac{\ln RF_{(\text{max})} - \ln RF(t)}{t} \]

The rate constant for zero-order release profiles was obtained using the line of best fit \((R^2 > 0.8)\) displayed in Appendix 2.

After each \( k \) was deduced, the corresponding permeability coefficient \( P_m \) was obtainable through Eq. 11 below;

\[ P_m = \left( \frac{r}{3} \right)k \quad (Eq. 11) \]

where \( r \) is the radius of liposomes derived as half the diameter size after extrusion (or after vortexing if extrusion was not performed).
Table 5. Illustrating the different rate constants of lipids as well as their corresponding permeability coefficient. Pm is the permeability coefficient of calcein, which is calculated in terms of k, k here being an average of each individual release profile.

<table>
<thead>
<tr>
<th>Lipids</th>
<th>k at pH 7.5</th>
<th>Pm</th>
<th>k at pH 10.5</th>
<th>Pm</th>
<th>k at pH 4.5</th>
<th>Pm</th>
</tr>
</thead>
<tbody>
<tr>
<td>POPC</td>
<td>5.3 ± 0.2</td>
<td>88.7±3.3</td>
<td>6.3 ± 0.1</td>
<td>105.7±1.7</td>
<td>-0.0 ± 0.0</td>
<td>-0.5±0.0</td>
</tr>
<tr>
<td>PalcpcTSPC</td>
<td>12.5 ± 0.8</td>
<td>208.5±13.3</td>
<td>10.8 ± 0.5</td>
<td>180.9±8.3</td>
<td>10.9 ± 0.3</td>
<td>181.2±5.0</td>
</tr>
<tr>
<td>OleCpcTSPC</td>
<td>13.3 ± 1.4</td>
<td>221.1±23.3</td>
<td>12.6 ± 0.7</td>
<td>210.2±11.7</td>
<td>13.9 ± 0.4</td>
<td>232.1±6.7</td>
</tr>
<tr>
<td>1,2-SiPC</td>
<td>9.8 ± 1.2</td>
<td>163.0±20.0</td>
<td>9.5 ± 1.2</td>
<td>158.2±20.0</td>
<td>10.8 ± 1.3</td>
<td>180.9±21.8</td>
</tr>
<tr>
<td>1,2-SiPC*</td>
<td>8.6 ± 0.3</td>
<td>143.0±5.0</td>
<td>-0.0 ± 0.0</td>
<td>-0.0±0.0</td>
<td>-0.2 ± 0.0</td>
<td>-4.2±0.0</td>
</tr>
<tr>
<td>PalValDSPC</td>
<td>8.6 ± 2.1</td>
<td>143.4±35.0</td>
<td>5.4 ± 0.3</td>
<td>90.9±5.1</td>
<td>-0.0 ± 0.0</td>
<td>-0.5±0.0</td>
</tr>
<tr>
<td>16:0 LPC*</td>
<td>4.3 ± 0.4</td>
<td>71.9±6.7</td>
<td>3.3 ± 0.2</td>
<td>55.9±3.4</td>
<td>-0.0 ± 0.0</td>
<td>-0.1±0.0</td>
</tr>
<tr>
<td>LysoTSPC</td>
<td>9.8 ± 1.2</td>
<td>164.0±20.1</td>
<td>-0.9 ± 0.0</td>
<td>-14.8±0.0</td>
<td>-0.3 ± 0.5</td>
<td>-4.8±8.0</td>
</tr>
<tr>
<td>LysoTSPC*</td>
<td>-0.0 ± 0.0</td>
<td>-0.1±0.0</td>
<td>-0.0 ± 0.0</td>
<td>-0.1±0.0</td>
<td>-0.0 ± 0.0</td>
<td>-0.2±0.0</td>
</tr>
</tbody>
</table>

Note * represents non-extruded vesicles.

From Table 5 (in-depth data in Appendix 2) and Eq. 11 [172] we can conclude that the greater the rate constant k, the greater the permeability coefficient (Pm) of the liposome system. In other words, if a lipid membrane is more permeable to a molecule than another lipid, its Pm will be reflected in its rate of release being higher and vice versa. At pH 7.5, 10 and 4.5 OleCpcTSPC was observed to have the greatest Pm (221.1, 210.2 and 231.1 respectively) and hence the fastest rate among the various lipids. Excluding LysoTSPC and 16:0 LPC because they are lysophospholipids, non-extruded 1,2-SiPC was observed to have the lowest permeability (−4.2) coefficient at pH 4.5 followed by POPC (−0.5). POPC was observed to have the lowest Pm
at pH 7.5 (88.8) and at pH 10.5 vortexed 1,2-SiPC was observed to have a lowest Pm of -0.0. All these data combined with the order of release kinetics can help a researcher to decide on suitable lipids for specific release applications. For example, 1,2-SiPC has a first-order release in every pH media according to its release profile and a higher to lower Pm moving from pH 7.5 to pH 10.5. This implies that we can have a controlled release from 1,2-SiPC at a fast or slower rate depending on which media we are targeting. OleCpcTSPC, on the other hand, can provide a fast release in every pH medium, which can be used for drugs such as captopril to quickly alleviate heart attack conditions [203].

3.7.1 Proposed release mechanism(s)

The use of liposomes as drug delivery systems was first investigated by Gregoriadis where he suggested that the release was operating through rapid diffusion of drugs through the liposomal membranes [204]. Release from liposomes occurs because the stability of the membranes is altered in different media such that they expel some or all of their encapsulated content to the external environment [191].

Irrespective of the type of order of release, we can see that SiPCs release calcein at a faster rate than POPC, that non-extruded 1,2-SiPCs releases calcein at a lower rate than its extruded counterpart, and that maximum release always occurred at pH 4 for both pH dependent and independent profiles. The mechanism of release for each system is believed to be a combination of factors such as pH environment, lipid membrane, and extrusion in some cases.

It was observed that extrusion caused an increase in the Pm of vesicles (Table 5). This increase in permeability is believed to be the reason why calcein was released to a greater extent in extruded 1,2-SiPC as compared to its non-extruded counterpart of the same diameter [205].
From the DLS data, it was not possible to determine the lamellarity of the liposomes but diffusion across a liposome membrane is also dependent on the number of lipid bilayers present in the system (i.e., one bilayer releases material faster than a multi-layer system). It was also seen that the extruded LysoTSPC released calcein to a greater extent than its non-extruded counterpart in every media. Even though vortexed and extruded vesicles from LysoTSPC were of relatively different sizes, their vesicles were both of sizes ideal for drug delivery studies and applications (i.e., 70 nm and 100 nm). However, it should be noted that lysophospholipids are not widely used in drug delivery applications as they are known to instead lyse cells (the prefix lyso from lysis) [206]. Release data from lysophospholipids are hence mainly considered for characterisation and comparison purposes. From Figures 32 and 33 we can see that at pH 4.5 for example, non-extruded LysoTSPC released up to 60% release of entrapped calcein while in the same condition extruded LysoTSPC released up to 80%. This feature illustrates that even though non-extruded vesicles had a greater ee than their corresponding extruded vesicles, the Pm of the membrane was altered by extrusion and caused a higher rate of release from extruded vesicles as well as their percentage of calcein release[205].

Another trend that was evident from the various release profiles is that maximum release always occurred at pH 4 followed by pH 7.5, and finally pH 10 (Figures 29-37)

As mentioned in Section 2.7.1, calcein has a pKa of 6.4. This implies that calcein is always negatively charged inside each vesicle as every liposome was formulated with calcein in Tris buffer at a pH of 7.5. Figure 38 depicts the different protonation states of calcein at different pH values.
Figure 38 Different protonation states of calcein during release. Calcein is always at pH 7.5 inside each vesicle.

At pH 4, excess H$_3$O$^+$ in the outer environment causes the creation of an electrochemical gradient, or proton gradient, across the lipid membrane, which constitutes a driving force for passive H$^+$ influx across the membrane. This influx causes initial deprotonated calcein inside the liposome to become protonated and ultimately neutral, contributing to a higher permeation of calcein out of the membrane [207].

At an external pH 7.5 there is an equal amount of H$_3$O$^+$ outside and inside the liposome, as these were formulated with calcein in Tris buffer pH 7.5. This means that no electrochemical
gradient is present such that there is no gradient-based driving force across the membrane [208]. Calcein, being deprotonated at pH 7.5, is released via the semi-permeable membrane via simple diffusion.

At pH 10.5, the electrochemical gradient built across the membrane causes a net influx of OH\(^-\) in parallel with a net efflux of H\(^+\) from an internal pH 7.5 such that the pH inside the liposome is further reduced with a higher population of calcein being deprotonated than it was at pH 7.5 making the release slower as it has to operate against the H\(^+\) gradient [209].

The mechanism of release was previously discussed in terms of pH and extrusion but not in terms of lipid membrane composition. It is believed that the lipid membrane is the major contributory factor to the mechanism of release from SiPC vesicles. As mentioned earlier SiPCs are seen to release calcein at a faster rate than POPC.

It is known that the unsaturated fatty acid that constitutes part of the phospholipid tail of lipids tends to increase the fluidity of the membrane by decreasing the compactness of the fatty acid chains [210]. An increase in unsaturation also causes an increase in the internal mobility of the membrane as rotation occurs around the unsaturated bond [211]. As unsaturation increases, the permeability of the membrane to water and small hydrophilic molecules will also increase [212]. As a result, POPC is expected to experience a greater level of internal mobility than SiPCs and hence release. Ironically, the reverse was observed. This observation can be explained by a combination of two factors. Firstly the Si atom next to the alpha carbon in the siloxane moiety is shown from the literature to present restriction in rotation [213]. Secondly, the Si-O-Si backbone presents more flexibility than C-C backbones as Si-O-Si bond angle can go from the usual \(sp^3\) hybridization state of 109.5\(^\circ\) up to 145\(^\circ\)[213], [214]. This higher flexibility in the siloxane backbone than its aliphatic counterparts is believed to cause more fluidity and hence less rigidity
in the siloxane lipid membrane as interactions between the lipid membrane will be lower than the relatively tighter packed POPC. This feature displayed by the siloxane backbone causes the internal mobility of siloxane containing lipid to be greater than POPC and hence the rate of calcein release from SiPCs release profiles is also greater.

POPC displayed a zero-order release profile for calcein at pH 7.5, which is in agreement with the literature [172]. Another interesting factor seen with release from the SiPC liposomes is the pH independence; a zero-order release profile was observed at all pH values tested. This implies that SiPCs can be used to achieve zero-order release kinetics of an entrapped molecule under various pH conditions. SiPCs can hence be the next generation of liposomes facing different pH conditions in the human body without showing pH sensitive behaviours offering an opportunity to provide various formulations with different modes of administration of the API.

The desired outcome from SiPCs may not always be for fast release purposes, in which case cholesterol may be a solution to decrease the release rate if needed. Cholesterol, as part of a membrane, decreases the compactness of the acyl chain by inducing conformational ordering [190]. A decrease in compactness of the lipid membrane will decrease its fluidity and hence the release of molecules. However, no studies from the literature report incorporation of cholesterol in siloxane lipid systems and until done, one cannot fully be sure of the ordering capacity of cholesterol in SiPCs.

From the rate calculations in Table 5, one can observe that POPC has the smallest release rate at pH 7.5 (5.3 h⁻¹) and that SiPCs releases calcein at a faster rate (PalCpcTSPC, 12.5 h⁻¹). As mention earlier, with this information, and depending on the profile of the API and the type of release pattern needed to achieve therapy, formulation choices for delivery of drugs at rates
sufficient to provide enough bioavailable drug to address a biological issue can be accurately made [215].

3.8 Size analysis of liposomes after release studies

Size analysis of liposomes after each release experiment, where possible, was performed using DLS to ensure the presence of liposomes after release measurements. As shown in Figure 39 DLS indicated the presence of liposomes after completion of the release experiments. After this confirmation, Triton X-100 was used to obtain a maximum release of calcein ($I_{\text{max}}$). In cases where DLS could not be used, an increase in intensity after introducing Triton-X was correlated to the presence of liposome before Triton X and hence after release studies.

![Figure 39 DLS measurements of vesicles before release experiments.](image)

3.9 Size analysis of liposomes after application of Triton X-100

Triton X-100 was the surfactant used at the end of release studies to burst the liposomes and obtain $I_{\text{max}}$ necessary for deriving the RF data (Eq. 9). DLS measurements were done after
the application of Triton X to confirm a shift in the DLS spectrum from 100 nm, the average initial size of the liposomes, to <10 nm after release. DLS measurements of each liposome sample performed were ~5 nm after the application of Triton X confirming destruction of the liposomal colloidal systems (Figure 40). This shift in the size of vesicles due to Triton X was always accompanied by an increase in calcein intensity (I_{max}). This increase in intensity was observed in every single system such that in scenarios where DLS was not used to observe the shift, the increase in intensity was an indicator of burst liposomes.

![Figure 40 DLS measurements of liposomes after Triton X-100.](image)

3.10 Specific rotation of lipids

After the release profiles and ee determined, the specific rotation of synthesized lipids listed below in Table 6 was also determined (Section 5.2 ) in order to see if they preserved the chirality passed from GPC, used as a starting material for the total synthesis of these lipids.
The specific rotation was obtained using the formula:

\[ \alpha_B = \frac{\alpha_{\text{obs}}}{cl} \]  

(Eq. 12)

where \( \alpha_{\text{obs}} \) is the observed optical rotation, \( c \) is the concentration of the solution in g/ml and \( l \) is the length of the cell in dm.

Table 6. Specific rotation of GPC and some synthesized siloxane lipids.

<table>
<thead>
<tr>
<th>Lipid in methanol</th>
<th>Specific rotation/°</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPC (in water)</td>
<td>-2.8</td>
</tr>
<tr>
<td>GPC</td>
<td>-0.8</td>
</tr>
<tr>
<td>PalValDSPC</td>
<td>+6.9</td>
</tr>
<tr>
<td>OleCpcTSPC</td>
<td>-0.3</td>
</tr>
<tr>
<td>PalCpcTSPC</td>
<td>-3.3</td>
</tr>
<tr>
<td>LysoTSPC</td>
<td>-1.3</td>
</tr>
<tr>
<td>1,2 Si-PC</td>
<td>-2.4</td>
</tr>
</tbody>
</table>

From the above table, except for PalValDSPC, which showed a dextrorotary characteristic, the rest of the lipids were levorotary. Lipids are known to be small rotors and every synthesized lipid in this thesis, PalValDSPC, PalCpcTSPC, OleCpcTSPC, and 1,2-SiPC, was obtained using alpha-GPC as a starting material. Lipids synthesized by chiral enzymes \textit{in vivo} are known to be levorotary [216], [217] and most of the synthesize lipids mentioned above retained that stereochemistry. This adds an additional feature to these lipids for use as drug delivery systems \textit{in vivo}, as pharmaceutical companies tend to use chirality for better drug delivery system across membranes [218]. It should be noted that chirality is a feature that helps in the good functioning of lipid membranes surrounding various living cell as other compatible chiral biomolecules, such as proteins, can then be embedded in the membranes or other biomolecules that pass through them [217].

The feature observed in PalValDSPC is believed to be due to stereoconversion during DCC coupling, caused by the nucleophile having the same stereochemical priority as the leaving
group resulting to an inversion in the overall stereochemistry of the lipid molecule. However, drug delivery does not always involve chirality as a feature and hence PalValDSPC can still be considered for that application [219].

3.11 Stability of liposomes with time

DLS was also used to determine the stability of vesicles. This was done (as stated in Section 5.2) as an extension experiment using cuvettes containing vesicles that were used to derive pre-released data.

Liposomes made up of unsaturated lipid are far less stable than liposomes composed of saturated phospholipids [191]. Over time small vesicles were seen to become large due to aggregation but larger vesicles were seen to form smaller vesicles at a certain point. This switch from large to small vesicle is most likely due to the budding of liposomes to form smaller vesicles such that the population of these smaller vesicles eventually exceeded that of larger ones, which was reflected in the DLS [220].
Table 7. Stability experiment of PalValDSPC and POPC. Sizes were obtained at room temperature using DLS.

<table>
<thead>
<tr>
<th>Time/ days</th>
<th>Diameter of non-extruded PalValDSPC liposomes (nm)</th>
<th>Diameter of extruded PalValDSPC liposomes (nm)</th>
<th>Diameter of non-extruded POPC liposomes (nm)</th>
<th>Diameter of extruded POPC liposomes (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2000</td>
<td>105</td>
<td>3000</td>
<td>109</td>
</tr>
<tr>
<td>5</td>
<td>3000</td>
<td>112</td>
<td>4000</td>
<td>115</td>
</tr>
<tr>
<td>23</td>
<td>124</td>
<td>141.8</td>
<td>5000</td>
<td>126</td>
</tr>
<tr>
<td>30</td>
<td>147</td>
<td>154.8</td>
<td>5000</td>
<td>124</td>
</tr>
<tr>
<td>37</td>
<td>149</td>
<td>177.1</td>
<td>87 (59.6%)</td>
<td>34135 (40.5%)</td>
</tr>
<tr>
<td>44</td>
<td>199</td>
<td>60 (13%)</td>
<td>0.4 (2.3%)</td>
<td>3492 (49.7%)</td>
</tr>
</tbody>
</table>

Note: Values with no displayed brackets were obtained at ≥ 95% (homogenous monodisperse population) and values with brackets display fractions of the 100% population size.
4 Conclusions

The aim of this thesis was to investigate the capacity of siloxane containing phospholipids to entrap and release a model drug after forming liposomes of desired vesicle sizes for drug delivery, using POPC as a conventional lipid for comparison and reference.

In order to perform release studies for the purpose of this thesis one fundamental requirement was to synthesize various siloxane-containing lipids. Even though the objective of the synthesis was to obtain enough pure lipid product for the release experiments, and even though enough product was always obtained after synthesis to be used for the release experiments, there is still room for improvement in terms of the yield of various intermediates and products. One of the factors or steps contributing to the lowest amount of yield was the use of the serine lipase enzyme CalB. The benefit of this enzyme among others was its required selectivity and ease of purification post reaction but further studies exploring the potential of other lipases could be an opportunity to optimise the yield of various siloxane containing phospholipids while preserving the advantages enzyme catalysis.

After each synthesis was accomplished entrapment capacity and release experiments were performed. From various analyses, it can be concluded that liposomes from siloxanes phospholipids present favourable properties to be considered as novel drug delivery systems. These polymers as drug carrier matrices did not degrade the drug due to their chemical inertness. Some of them (1,2-SiPC) are known to self-assemble to ULVs when in an aqueous medium, avoiding the need for extrusion or sonication in the preparation of liposomal formulations of desired smaller sizes.
The loading capacity of some SiPCs showed a higher entrapment capacity than POPC and some SiPCs (PalValDSPC), which could be investigated for stability, showed a greater stability than POPC liposomes over time. One implication of these features is that a higher drug-to-lipid ratio could be obtained with SiPCs and an increase in bioavailability required to address a biological issue can hence be achieved, which could then decrease the frequency of drug administration thereby improving patient compliance. It is believed that the rate at which vesicles were formed during extrusion contributed to the entrapment capacity of various extruded liposomes and that the rate was lipid membrane-dependent. In order to further confirm this fact, it was proposed that further studies could move from manual extrusion to automated extrusion at a fixed pressure just to eliminate the possibility of potential human error that could have caused a slight variability in pressure during manual extrusion of every single system.

Release experiments were performed on eligible vesicles in terms of size or between lipid membrane analogues for comparison. Horizontal comparisons were performed between non-extruded and extruded vesicles of same or desired vesicles size and vertical comparisons were made between every extruded vesicle. Release data from lysophospholipids were mainly considered as characterisation data, as lysophospholipids have not been greatly used for drug delivery application but instead for cell lysis. Siloxane phospholipids, SiPCs, presented pH independent behaviours and showed a zero-order release profile at various pH values. Zero-order kinetics is generally desirable in drug delivery systems, where drug levels released would remain constant throughout the delivery periods. This is generally considered to be important in improving therapeutic outcomes and patient compliance and it also reduces the frequency of drug administration. The pH-independent behaviour, on the other hand, can be exploited in oral delivery of liposomes which always presents a challenge, as administered liposomes have to
surmount the effect of different biological media together with the exposure to bile salts and digestive enzymes. To confirm the capacity of oral delivery of pH-independent identified siloxane liposomes, experiments should hence be conducted in a simulated state of the GI tract reflecting enzymes and bile salts conditions *in-vitro* and if successful experiments could be pursued *in vivo*.

The release rates from SiPCs were relatively faster than the non-siloxane lipids. Cholesterol was suggested to decrease the release rate of siloxane liposomes if ever needed due to its capacity to reduce membrane fluidity. At the time of this thesis, no studies identifying the effect of cholesterol on siloxane-PC liposomes have been performed. It will be good to consider future directions in regard to that, verifying if cholesterol could really help in fluidity reduction in these systems. Assays such as Laurdan assay where Laurdan acts as a fluorescent dye can be used to monitor membrane permeability and fluidity to assess the impact of cholesterol.

After using calcein as a hydrophilic drug model and fluorescent dye marker, a hydrophobic drug model should also be considered for a broad-spectrum analysis of these liposomes. With *in vitro* data from a hydrophilic drug model the next step could be to switch from a dye to an actual drug before moving to *in vivo* testing.

This study opens the door to a broad range of prospective and results from these findings could contribute to bring the design of liposomes for drug delivery to the next level.
5 Experimental

5.1 Materials

Calcein, *Candida antarctica* lipase A immobilized on Immobead 150 (599 U/g), Novozym-435 lipase B on an acrylic resin from *Candida antarctica* (≥5,000 U/g), palmitic acid (≥95% purity), 9-decenoic acid (≥95% purity), 4-pentenoic acid (≥98% purity), sepharose 4B, \(N,N'\)-dicyclohexylcarbodiimide (99% purity), 4-dimethylaminopyridine (99% purity), platinum(0)-1,3-divinyl-1,1,3,3-tetramethyldisiloxane complex solution in xylenes, activated carbon decolourising, chloroform, toluene (≥ 99.5% purity), ethyl acetate (≥ 99.5%), polycarbonate membrane (pore size 100 nm), filter agent Celite\textsuperscript{®} 545, hydrochloric acid were acquired from Sigma-Aldrich (Oakville, Ontario, Canada).

1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-hydroxy-\(sn\)-glycero-3-phosphocholine (16:0 LPC), polyester filter supports of 100 µm thickness, and Avanti mini extruder were acquired from Avanti Polar Lipids Inc. (Alabaster, Alabama, USA).

\(sn\)-3-glycerophosphocholine (GPC) was acquired from Toronto Research Chemicals.

Heptamethyltrisiloxane (99% purity) and pentamethyldisiloxane were acquired from Gelest, Inc. (Morristown, Pennsylvania, USA).

Chloroform-\(d\) (99.8% deuterated) was obtained from Cambridge Isotope Laboratories, Inc. (Landover, Maryland, USA).
Triton®X-100 and p-toluensulfonic acid (98% purity) were obtained from Alfa Aesar (Ward Hill, Massachusetts, USA)

Sodium hydroxide, sodium chloride, benzene, and methanol were acquired from ACP Chemical Inc. (Montreal, Quebec, Canada)

Petroleum ether, tris(hydroxymethylaminomethane), vortex mixer (Variable speed: 300 to 3200rpm), and disposable empty polypropylene columns (5 mL), were acquired from Thermo Fisher Scientific (Fair Lawn, New Jersey, USA)

UV/vis quartz cells (volume 3500 µL, height 45 mm, width 12.5 mm, depth 12.5 mm path length 10 mm) were acquired from Hellma Analytics (Plainview, New York, USA)

Disposable polystyrene fluorescence cuvettes (volume 4000 µL, height 46 mm, width 13 mm, depth 13 mm path length 10 mm) were acquired from Ocean Optics Spectrometers (Largo, Florida, USA)

Distilled water was used for all preparations. All reagents were used as received without further modification
5.2 General Experimental Procedures

*Nuclear Magnetic Resonance Spectroscopy (NMR).* NMR spectra were acquired using a Bruker Avance 300 MHz equipped with a broadband probe. Samples were typically dissolved in CDCl₃, unless otherwise stated. ¹H NMR spectra were recorded at 300 MHz and the residual protons of CHCl₃ were used as the internal standard at 7.26 ppm. ¹³C NMR spectra were recorded at 75 MHz and the CDCl₃ was used as the internal standard, and set to 77.0 ppm. ²⁹Si NMR spectra were recorded at 59.6 MHz, ³¹P NMR spectra were recorded at 121.5 MHz. The resonance frequency for each nucleus was ¹H - 300 MHz, ¹³C - 75 MHz, ²⁹Si – 59.6 MHz and ³¹P – 121.5 MHz [221]. Spectra were analyzed using the Bruker TopSpin v3.5 pl 7 software platform.

*Fourier-Transform Infrared Spectroscopy (FTIR).* FTIR spectra were acquired using a Bruker Alpha scanning infrared spectrometer. Samples were prepared as neat films on an ATR diamond window. Each spectrum was the average of 24 scans at 2 cm⁻¹ resolution. Spectra were analysed using the OPUS v7.2 software platform.

*Dynamic Light Scattering (DLS).* DLS spectra were acquired using a Dynapro Dynamic light scattering instrument. Liposome suspensions formulated with quenched calcein (dark brown in colour) after vortexing and/or extrusion were diluted in the same exact rehydration buffer (50 mM Tris buffer, 100 mM NaCl) used in their preparation. The dilution (2 µl: 998 µl) was done in a 1 mL cuvette in order to obtain a clear solution (light green in colour) suitable for
measurement. Liposome suspensions formulated with calcein after release experiments were not further diluted. Those liposomes were directly transferred in the appropriate cuvettes for DLS measurement. Each spectrum was the average of 20 acquisitions and the final reported measurement an average of three samples minimum. Spectra were analyzed at 37°C and processed using the DYNAMICS V6 v6.3.40 software platform.

*Ultra Violet/Visible Spectroscopy (UV/Vis).* UV-Vis spectra were recorded using a SpectroVis Plus spectrometer/fluorimeter. Proper wavelength selection depended on the nature of the sample. Absorption (using UV/vis quartz cells) and emission (using disposable polystyrene fluorescence cuvettes) spectra were analyzed using the Logger Pro v3.10.2 software platform.

*Optical Spectroscopy.* The optical rotation of lipids was acquired using a Rudolph Autopol IV Polarimeter. Pure lipids samples of known mass were each dissolved into 7 mL of methanol to fill up the cell without any air bubble. The optical rotation $\alpha$, was the average of 5 acquisitions at 23.1°C, and was used to calculate the specific rotation of each lipid using Biot’s law [221].

5.3 Detailed Experimental Methods

5.3.1 Synthetic Experimental Procedures

*Methyl-9-decanoate (1)*

\[
\begin{align*}
&\text{CH}_3-\text{CH}
\end{align*}
\]
A round bottom flask was charged with 3.31 g (19.4 mmol) of 9-decenoic acid, 0.34 g of pTsOH (1.97 mmol) and dissolved into 33.0 mL of methanol and refluxed for 4 h. The progress of the reaction was monitored by FTIR by following the decrease in the carbonyl resonance associated with the carboxylic acid at ~1720 cm\(^{-1}\). When this resonance was no longer visible the reaction was refluxed for an additional hour. The solvent was removed \textit{in vacuo} and the remaining crude residue was extracted into 15 mL ethyl acetate and washed with 15 mL of water, 15 mL of saturated NaHCO\(_3\), and 15 mL of saturated NaCl. The combined organic fractions were dried over Na\(_2\)SO\(_4\), and then decanted in a weighed round bottom flask. Removal of the solvent \textit{in vacuo} gave 2.74 g (14.9 mmol, 77\%) of a clear colourless liquid, directly used for the synthesis of compound 2. \(^1\)HNMR (300 MHz, CDCl\(_3\)): \(\delta\) 1.317 (s, 8H), \(\delta\) 1.61 (m, 2H, \(J= 7\)Hz), \(\delta\) 2.053 (q, 2H, \(J= 6\)Hz), \(\delta\) 2.31 (t, 2H, \(J= 9\)Hz), \(\delta\) 3.67 (s, 3H), \(\delta\) 4.95 (m, 2H, \(J= 8\) Hz) \(\delta\) 5.81 (m, 1H, \(J= 7\)Hz). The spectra were in agreement with the literature [222] and compound 1 was used for the synthesis of compound 2 below.

**Heptamethyltrisiloxanemethyldecanoate (2)**

![Chemical Structure](image)

An oven-dried round bottom flask was charged with methyl-9-decenoate (2.74 g, 14.9 mmol) and dissolved into 14 mL of toluene. To this mixture Karstedt’s platinum complex (2 % in xylenes, 17 \(\mu\)L) was added and the reaction mixture stirred at room temperature for 10 min. 1,1,1,3,3,5,5-Heptamethyltrisiloxane (3.68 g, 16.5 mmol) was then added drop-wise at room temperature. The reaction mixture was allowed to reflux for 3 h. The completion of the reaction was assured on \(^1\)H NMR by the disappearance of the alkene peak characteristic of the ester
starting material at 4.95 ppm and 5.81 ppm. When this resonance was no longer present, activated carbon was added to remove colloidal platinum by stirring overnight. The carbon was separated by filtering through Celite 545 and ethyl acetate, and the solvent was removed in vacuo to yield 5.62 g (13.8 mmol, 93%) of a clear and colourless liquid. $^1$HNMR (300 MHz, CDCl$_3$): δ 0.01 (s, 9H), δ 0.05 (s, 6H), δ 0.08 (s, 6H), δ 0.51 (t, 2H, J= 8Hz), δ 1.27 (s, 12H), δ 1.61 (m, 2H, J= 7Hz), δ 2.29 (t, 2H, J= 7Hz), δ 3.66 (s, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 0.18 ppm, 1.25, 1.79, 18.27, 23.19, 24.96, 29.14, 29.29, 29.37, 33.39, 34.10, 51.39, 174.32; $^{29}$Si NMR (59 MHz, CDCl$_3$): δ -20.92, 6.98, 7.49; EI-MS (m/z): M$^+$ 406.24; FTIR (cm$^{-1}$): 793, 839, 1043, 1255, 1436, 1743, 2923.

**Heptamethyltrisiloxanedecanoic acid synthesis 1 (trisiloxane acid, 3)**

![Chemical Structure](image)

Compound 2 (3.05 g, 7.50 mmol), obtained by hydrosilylation, was used as a starting material together with 5 mole equivalents of water for the synthesis of the trisiloxane acid. The acid was prepared in a round bottom flask using 10 wt% of N435 (CalB) with respect to the mass of the ester at 50°C with gentle stirring for 48 h. Upon completion, the enzyme beads were separated by filtration using ethyl acetate. The solvent was removed in vacuo and TLC of the crude mixture in a 4:1 ratio of petroleum ether and ethyl acetate as the elution solvent was done to confirm the presence of the product by the visualisation of two spots; one for the ester and the other for the acid. Chromatography was then performed using 60 g of silica gel where the loaded crude sample was eluted with 250 mL of 99:1, 95:5 and 4:1 ratio of petroleum ether:ethyl acetate and 150 mL ethyl acetate to wash the column. Identical fractions based on $R_f$-values (0.76 cm
and 0.36 cm) were combined and the solvent removed in vacuo to yield 1.01 g (2.57 mmol, 34%) of a clear and colourless liquid acid identified through $^1$H NMR spectroscopy by the disappearance of the ester resonance at 3.66 ppm. $^1$HNMR (300 MHz, CDCl$_3$): δ 0.14 (s, 9H), δ 0.05 (s, 6H), δ 0.08 (s, 6H), δ 0.51 (t, 2H, J= 7Hz), δ 1.28 (s, 12H), δ 1.63 (m, 2H, J=7Hz), δ 2.34 (t, 2H, J=7Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 0.17 ppm, 1.24, 1.78, 18.26, 18.67, 23.19, 24.67, 29.06, 29.27, 29.30, 29.36, 33.39, 34.12, 180.495; $^{29}$Si NMR (59 MHz, CDCl$_3$): δ -21.09, 6.98, 7.42; ESI+/MS: (M-Na)$^+$ 415; EI-MS (m/z): (M-CH$_3$)$^+$ 377.19 FTIR (cm$^{-1}$): 791, 837, 1039, 1254, 1412, 1710, 2923. Analytical calculation for C$_{17}$H$_{40}$O$_4$Si$_3$ C 51.99 %; H 10.57%. Found C 51.79 %; H 10.54%.

Heptamethyltrisiloxanedecanoic acid synthesis 2 (trisiloxane acid, 3')

Compound 2 (3.17 g, 7.79 mmol), obtained by hydrosilylation, was used as a starting material together with 5 moles equivalent of water for the synthesis of the trisiloxane acid. The acid was prepared in a round bottom flask using 10 wt% CalA (with respect to the mass of the ester) at 50$^0$C with gentle stirring for 48 hours. Upon completion, the enzyme beads were separated by filtration using ethyl acetate. The solvent was removed in vacuo and TLC of the crude mixture in 4:1 ratio of pet-ether and ethyl acetate was done to confirm the presence of the product by the visualisation of two spots; one for the ester and the other for the acid. Chromatography was then performed through silica gel where the loaded crude sample was eluted with 250 mL of 99:1, 95:5 and 4:1 ratio of pet-ether: ethyl acetate and 150 mL ethyl acetate to wash the column. Identical fractions based on Rf-values (0.71 cm and 0.30 cm) were
combined and the solvent removed in vacuo to yield 80 mg (0.20 mmol, 3%) of clear and
colourless liquid acid identified through $^1$H NMR spectroscopy by a significant disappearance of
the ester resonance at 3.66 ppm. $^1$HNMR new spectra (300 MHz, CDCl$_3$; $\delta$ 0.14 (s, 9H), $\delta$ 0.05 (s,
6H), $\delta$ 0.08 (s, 6H), $\delta$ 0.51 (t, 2H, $J$= 7Hz), $\delta$ 1.28 (s, 12H), $\delta$ 1.63 (m, 2H, $J$=7Hz), $\delta$ 2.34 (t,
2H, $J$=7Hz); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 0.17 ppm, 1.24, 1.78, 18.26, 18.67, 23.19, 24.67,
29.06, 29.27, 29.30, 29.36, 33.39, 34.12, 180.495; $^{29}$Si NMR (59 MHz, CDCl$_3$): $\delta$ -21.09, 6.98,
7.42; FTIR (cm$^{-1}$): 791, 837, 1039, 1254, 1412, 1710, 2923.

The two preceding methodologies display the synthesis of the same compound,
heptamethyltrisiloxanedecanoic acid, with the first one using Novozym-435; a CALB lipase
immobilized on an acrylic resin, and the second one using CalA, every other variable was kept
constant. Based on the yield from CalB; 30%, and that from CalA; 3%, it is evident that CalB is
more efficient than CalA in regard to this hydrolysis reaction. A discussion for this observed
difference is provided in Section 3.1.1.

1-(Heptamethyltrisiloxane)decanoyl-sn-glycero-3-phosphocholine (lysoTSPC, 4)

![Chemical Structure]

Compound 3 (1.15 g, 2.93 mmol) was used as a starting material at 10 mole equivalents
of GPC (90.0 mg, 0.14 mmol), for the preparation of lysoTSPC. The lysoTSPC was prepared in
a Teflon round bottom flask using 10 wt% N435 (with respect to the mass of the acid and GPC)
under vacuum at 80°C without stirring for 48 h. Upon 2 days of reaction, the enzyme beads were
separated by filtration using chloroform. The solvent was removed in vacuo and TLC of the
crude, viscous, clear, liquid was performed using a 65:25:4 ratio of chloroform:methanol:water to confirm the presence of the product through the visualisation of two separate spots one corresponding to the starting material compound 3 (confirmed by spotting the starting material as well) and the other to the product. Chromatography was then performed using preconditioned silica gel with 9:1 CHCl₃: methanol. The loaded crude sample was then eluted with 600 mL of 65:25:5 CHCl₃: methanol: water. Identical fractions based on Rf-values (0.9 cm and 0.25 cm) were combined and the solvent removed in vacuo. Fractions containing the product were foamy due to interaction with the remaining water. Benzene (2 mL) was used to remove any remaining water by freeze-drying to yield 50.0 mg (79.12 μmol, 23%) of a clear and colourless gel.

$^1$HNMR (300 MHz, CDCl₃): $\delta$ 0.01 (s, 9H), $\delta$ 0.05 (s, 6H), $\delta$ 0.08 (s, 6H), $\delta$ 0.52 (t, 2H, $J$ = 7Hz), $\delta$ 1.27 (s, 12H), $\delta$ 1.57 (m, 2H, $J$ = 7Hz), $\delta$ 2.30 (t, 2H, $J$ = 8Hz) $\delta$ 3.30 (s, 9H) $\delta$ 3.76 (s, 2H), $\delta$ 3.76, $\delta$ 3.95 (s, 2H), $\delta$ 4.07 (s, 2H), $\delta$ 4.32 (s, 2H); $^{13}$C NMR (75 MHz, CDCl₃): $\delta$ 0.20 ppm, 1.27, 1.81, 18.32, 23.28, 24.94, 29.35, 29.49, 29.52, 29.62, 33.55, 54.29, 173.91; $^{29}$Si NMR (59 MHz, CDCl₃): $\delta$ -20.98, 6.98, 7.39; $^{31}$P NMR (121 MHz, CDCl₃): $\delta$ -0.56; ESI+/MS: (M-Na)$^+$ 654.3; EI-MS (m/z): M+ 632.32; FTIR (cm$^{-1}$): 792, 838, 1043, 1254, 1466, 1728, 2853, 2921, 2957, 3367. Analytical calculation for C$_{25}$H$_{58}$NO$_6$PSi$_3$C 47.51%; H 9.25%. Found C 42.15% H 8.70%. Compound 4 was then used as a precursor for the synthesis of compound 5 below in association with recycled compound 3 from this reaction.

1,2-SiPC (5)
An oven-dried round bottom flask cooled under N₂ gas was charged with compound 3 (70 mg, 0.18 mmol) dissolved in 4 mL CHCl₃, DCC (40 mg, 0.19 mmol), and a catalytic amount of 4-dimethylaminopyridine (2.0 mg, 16.37 μmol). The mixture was stirred at room temperature for 10 min. Upon completion, lysoTSPC; compound 4 (30.0 mg, 47.47 μmol), dissolved in 4 mL of CHCl₃ was added to the flask and left stirring for 24 h. The white precipitate (N-N dicyclohexylurea) was filtered off using a medium porosity fritted Büchner funnel and chloroform, and the solvent was removed under reduced pressure. The crude sample was then purified via chromatography using a pre-conditioned column of 25 g of silica gel with CHCl₃: MeOH (9:1 v/v) using 400 mL of CHCl₃: MeOH: H₂O (65:25:4 v/v) as the eluent. Identical fractions based on Rf-values (0.62 cm and 0.20 cm) were combined and the solvent removed in vacuo. The synthesized compound with a yield of 20.0 mg (19.87 μmol, 42%) was obtained as a clear and viscous gel. ¹H NMR (300 MHz, CDCl₃): δ 0.14 (s, 18H), δ 0.05 (s, 12H), δ 0.08 (s, 12H), δ 0.51 (t, 4H, J= 6Hz), δ 1.26 (s, 27H), δ 1.56 (m, 4H), δ 2.28 (q, 4H, J= 8Hz) δ 3.34 (s, 9H) δ 3.79 (s, 2H), δ 3.93 (s, 2H), δ 4.11 (s, 2H), δ 4.30 (s, 2H), δ 5.18 (s, 1H); ¹³C NMR (75 MHz, CDCl₃): δ 0.19 ppm, 1.18, 1.27, 1.81, 18.31, 23.25, 24.89, 24.98, 29.21, 29.42, 29.53, 33.49, 34.13, 34.32, 54.44, 173.222, 173.571; ²⁹Si NMR (59 MHz, CDCl₃): δ -21.09, 6.98, 7.39; ³¹P NMR (121 MHz, CDCl₃): δ -0.56; ESI+/MS: (M-Na)+ 1028.5; FTIR (cm⁻¹): 792, 838, 1043, 1254,1466, 1728, 2853, 2921.

**Methyl-4-pentenoate (6)**
A round bottom flask was charged with 1.95 g (19.4 mmol) of 4-pentenoic acid, 0.17 g of pTsOH (0.99 mmol) and dissolved into 20.0 mL of methanol and refluxed for 4 h. The progress of the reaction was monitored by FTIR by following the decrease in the carbonyl resonance associated with the carboxylic acid at ~1720 cm\(^{-1}\). When this peak was no longer visible the reaction was refluxed for an additional hour. The solvent was removed \textit{in vacuo} and the remaining crude residue was extracted into 15 mL ethyl acetate and washed with 15 mL of water, 15 mL of saturated NaHCO\(_3\), 15 mL of saturated NaCl. The combined organic fractions were dried over Na\(_2\)SO\(_4\), and then decanted in a weighed round bottom flask. Removal of the solvent \textit{in vacuo} gave 1.78 g (15.6 mmol, 80\%) of a clear colourless liquid, directly used for synthesis of compound 7.

\underline{Pentamethyldisiloxanemethylpentanoate (7)}

\begin{center}
\includegraphics[width=0.5\textwidth]{pentamethyldisiloxanemethylpentanoate.png}
\end{center}

An oven dried round bottom flask was charged with methyl-4-pentenoate (1.31 g, 11.48 mmol) and dissolved into 7 mL of toluene. To this mixture Karstedt’s platinum complex (2\% in xylenes, 9 \(\mu\)l) was added and the reaction mixture stirred at room temperature for 10 min. 1,1,1,3,3-Pentamethyltrisiloxane (1.89 g, 12.7 mmol) was added drop-wise at room temperature. The reaction mixture was refluxed for 3 h. The completion of the reaction was assured by the disappearance of the alkene resonance at 4.95 ppm and 5.81 ppm characteristic of the ester starting material in \(^1\)H NMR. When this resonance was no longer present, activated carbon was added to remove colloidal platinum by stirring overnight. The carbon was separated by filtering through Celite 545 and ethyl acetate and the solvent was removed \textit{in vacuo} to yield 2.89 g (11.01
mmol, 96%) of a clear and colourless liquid. $^1$HNMR (300 MHz, CDCl$_3$): $\delta$ 0.02 (s, 6H), 0.03 (s, 9H), $\delta$ 0.49 (t, 2H, $J$= 5Hz), $\delta$ 1.33 (m, 2H, $J$= 7Hz), $\delta$ 1.63 (m, 2H, $J$= 8Hz), $\delta$ 2.29 (t, 2H, $J$= 8Hz), $\delta$ 3.64 (s, 3H); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 0.26 ppm, 1.91, 17.98, 22.91, 28.48, 33.84, 51.39, 174.29; $^{29}$Si NMR (59 MHz, CDCl$_3$): $\delta$ 7.28; ESI+/MS: (M$-$Na)$^+$ 285.1; EI-MS (m/z): (M$-$CH$_3$)$^+$ 247.11; FTIR (cm$^{-1}$): 753, 804, 838, 1045, 1196, 1251, 1362, 1742, 2955.

**Pentamethyldisiloxanepentanoic acid synthesis 1 (Disiloxane acid, 8)**

\[
\begin{array}{c}
\text{O} \\
\text{Si} \\
\text{Si}
\end{array}
\]

Compound 7 (3.26 g, 12.42 mmol) was used as a starting material together with 5 mole equivalents of water for the synthesis of the disiloxane acid. The acid was prepared in a round bottom flask using 10 wt% N435 (with respect to the mass of the ester) at 50°C with gentle stirring for 48 h. Upon completion, the enzyme beads were separated by filtration using ethyl acetate. The solvent was removed in vacuo and TLC of the crude mixture in 4:1 ratio of petroleum ether and ethyl acetate was done to confirm the presence of the product by the visualisation of two spots; one for the ester and the other for the acid. Chromatography was then performed through 40 g of silica gel where the loaded crude sample was eluted with 250 mL of 99:1, 95:5 and 4:1 ratio of petroleum ether: ethyl acetate and 130 mL ethyl acetate to wash the column. Identical fractions based on $Rf$-values (0.75 cm and 0.43 cm) were combined and the solvent removed in vacuo to yield 0.57 g (2.29 mmol, 19%) of a clear, viscous, colourless liquid. $^1$HNMR (300 MHz, CDCl$_3$): $\delta$ 0.02 (s, 6H), 0.04 (s, 9H), $\delta$ 0.52 (t, 2H), $\delta$ 1.40 (m, 2H), $\delta$ 1.66 (m, 2H), $\delta$ 2.36 (t, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$): $\delta$ 0.26 ppm, 1.90, 17.95, 22.83, 28.18,
33.65, 179.42; $^{29}$Si NMR (59 MHz, CDCl$_3$): $\delta$ 7.25, 7.34; ESI-/MS: M$^+$ 247.0; EI-MS (m/z): (M-CH$_3$)$^+$ 233.10 FTIR (cm$^{-1}$): 752, 804, 837, 1045, 1251, 1412, 1708, 2956.

**Pentamethyldisiloxanepentanoic acid synthesis 2 (Disiloxane acid, 8')**

![Pentamethyldisiloxanepentanoic acid structure](image)

An oven dried round bottom flask cooled under N$_2$ gas was charged with 4-pentenoic acid (511 $\mu$L, 0.05 mol) and a catalytic amount of PtO$_2$ (1.20 mg, 5.0 $\mu$mol). To this mixture pentamethyldisiloxane (945 $\mu$L, 0.05 mol) was added dropwise via an addition funnel and the reaction mixture stirred at room temperature for 24 hours. $^1$H NMR revealed the presence of the alkene peak characteristic of the ester starting material. Activated carbon was then added to remove colloidal platinum by stirring overnight. The carbon was separated by filtering through Celite 545 and ethyl acetate and the solvent was removed in vacuo. Vacuum distillation was then performed to remove the bi-product (pentanoic acid) plus unreacted 4-pentenoic acid (Boiling point: 83-84°C). After three rounds of vacuum distillations, $^1$H NMR of the initial flask still revealed the presence of the alkene peak. Product was unable to be purified.

The two latest methodologies above present the synthesis of the pentamethyldisiloxanepentanoic acid required for the synthesis of compound 10 below. The enzymatic reaction, (i.e., using CalB (from N435)) was successful with a yield of 19%. The scheme for this reaction is the same as that provided in Section 3.1.1. This enzymatic reaction hydrolyzed compound 7 (obtained by the hydrosilylation of compound 6) to compound 8, making the whole synthesis of compound 8 a continuous three-step process. The second
proposed methodology (using PtO₂) pentamethyldisiloxanepentanoic acid in only one step (i.e.,
no need to synthesize compounds 6 and 7 before obtaining compound 8). However, compound 8
synthesized using PtO₂ was unable to be purified, and hence not useful for the sake of the
synthesis of compound 10, PalValDSPC. Karsted’s catalyst used in the hydrosilylation of
compound 6 to 7 was hence favoured in this present document over PtO₂ as discussed in Section
3.2, for every hydrosilylation reaction.

1-Palmitoyl-sn-glycero-3-phosphocholine (16:0 lysoPC, 9)

![1-Palmitoyl-sn-glycero-3-phosphocholine](image)

Palmitic acid (1.14 g, 4.43 mmol) was used at 10 moles equivalent of GPC (0.11 g,
0.443 mmol) as starting material for the preparation of lysoPC. The lysoPC was prepared in a
Teflon round bottom flask using 10 wt% N435 (with respect to the mass of the acid and GPC)
under vacuum at 80 °C without stirring for 48 h. Upon completion, the enzyme beads were
separated by filtration using chloroform. The solvent was removed in vacuo and TLC of the
white crude solid mixture in 65:25:4 ratio of chloroform, methanol and water was done to
confirm the presence of the product through the visualisation of two separate spots.
Chromatography was then performed through a preconditioned silica gel with CHCl₃: methanol
(9:1, v/v). The loaded crude sample was then eluted with 600 mL of CHCl₃: methanol: water
(65:25:5 v/v). Identical fractions based on Rf-values (0.80 cm and 0.29 cm) were combined and
the solvent removed in vacuo. Fractions containing the product were foamy due to interaction
with the remaining water. Benzene was used to remove any remaining water to yield 0.11 g (0.22
mmol, 50 %) 16:0 lysoPC as a white powder. ¹H NMR (300 MHz, CDCl₃): δ 0.86 (t, 3H, J=
7Hz), δ 1.24 (s, 27H), δ 1.55 (m, 2H), δ 2.29 (t, 2H, J= 8Hz), δ 3.31 (s, 9H) δ 3.78 (s, 2H), δ 3.92 (s, 2H), δ 4.05 (s, 2H), δ 4.31(s, 2H); $^{13}$C NMR (75 MHz, CDCl$_3$): δ 14.11 ppm, 22.69, 24.95, 29.33, 29.39, 29.49, 29.69, 29.78, 31.93, 34.15, 54.29, 173.93; $^{31}$P NMR (121 MHz, CDCl$_3$): δ -0.54; ESI+/MS: (M-Na)$^+$ 518.3; FAB-MS (m/z): M$^+$ 496.34 FTIR (cm$^{-1}$): 764, 874, 1051, 1085, 1197, 1285,1467, 1733, 2298, 2849, 2916, 3264. This lysophospholipid was used as a precursor for the synthesis of compounds 10 and 11 presented below.

**PalValDSPC (10)**

![PalValDSPC (10)](image)

An oven-dried round bottom flask cooled under N$_2$ gas was charged with compound 8, disiloxane acid (0.11 g, 0.35 mmol) dissolved in 5 mL CHCl$_3$, DCC (0.07 g, 0.35 mmol), and a catalytic amount of 4-dimethylaminopyridine (6.0 mg, 49.11 µmol). The mixture was stirred at room temperature for 10 min. Upon completion, 16:0 lysoPC; compound 9 (70.0 mg, 0.14 mmol), dissolved in 2 mL of CHCl$_3$ was added to the flask and left stirring for 24 h. The white precipitate (N-N dicyclohexylurea) was filtered off using excess chloroform and the solvent removed under reduced pressure. The crude sample was then purified via chromatography over a pre-conditioned column of 30 g silica gel with CHCl$_3$: MeOH (9:1 v/v) using 400 mL of CHCl$_3$: MeOH: H$_2$O (65:25:4 v/v) as eluent. Identical fractions based on Rf-values (0.65 cm and 0.27 cm) were combined and the solvent removed in vacuo. The synthesized compound with a yield of 60 mg (82.63 µmol, 59%) was obtained as a clear and colourless gel. $^1$H NMR (300 MHz,
CDCl₃: δ 0.02 (s, 6H), 0.04 (s, 9H), 0.49 (t, 2H, J= 8Hz) 0.86 (t, 3H, J= 7Hz), δ 1.23 (s, 27H), δ 1.56 (m, 4H, J= 6Hz ), δ 2.26 (q, 4H, J= 8Hz), δ 3.35 (s, 9H) δ 3.81 (s, 2H), δ 3.88 (s, 2H), δ 4.09 (s, 2H), δ 4.36 (s, 2H), 5.16 (s, 1H); ¹³C NMR (75 MHz, CDCl₃): δ 0.31 ppm, 1.973, 14.09, 18.07, 22.67, 22.95, 24.88, 28.51, 29.21, 29.35, 29.57, 29.65, 29.70, 31.90, 34.06, 34.11, 54.35, 59.35, 63.00, 63.45, 66.24, 70.51, 173.20, 173.57; ³¹P NMR (121 MHz, CDCl₃): δ -0.50; ²⁹Si NMR (59 MHz, CDCl₃): δ 7.22, 7.32; ESI+/MS: (M-Na)+ 748.4; EI-MS (m/z): M+ 726.46 FTIR (cm⁻¹): 795, 968, 1051, 1188, 1249,1466, 1734, 2852, 2922, 3387.

**PalValDSPC (10')**

Compound 8, Disiloxane acid (0.57 g, 2.29 mmol) was used at 10 moles equivalent of 16:0 lysoPC (0.10 g, 0.20 mmol) as starting material for the preparation of PalValDSPC. The PalValDSPC was prepared in a Teflon round bottom flask using 10 wt% N435 (with respect to the mass of the acid and GPC) under vacuo at 80°C without stirring for 48 hours. Upon completion, the enzyme beads were separated by filtration using chloroform. The solvent was removed in vacuo and TLC of the white crude solid mixture in a 65:25:4 ratio of chloroform, methanol, and water was done to confirm the presence of the product. Two spots were instead seen out of three and purification via chromatography using a preconditioned silica gel with CHCl₃: methanol (9:1, v/v), loading the crude sample and eluting with 600 mL of CHCl₃: methanol: water (65:25:5 v/v), recovered only the starting materials. No product was observed.
Looking at the two preceding methodologies, it can be observed that only one reaction was successful for the synthesis of the unsymmetrical lipid PalValDSPC. This successful reaction went through a DCC coupling with a yield of 59% as compared to no yield from the enzymatic reaction. CalB (i.e., from N435) was hypothesized to be unable to accommodate 16:0 LPC in its active site and hence unable to catalyze this reaction.

**PalCpcTSPC (11)**

An oven-dried round bottom flask cooled under N₂ gas was charged with compound 3, trisiloxane acid (140 mg, 0.36 mmol) dissolved in 4 mL CHCl₃, DCC (60 mg, 0.29 mmol), and a catalytic amount of 4-dimethylaminopyridine (6.9 mg, 56.47 µmol). The mixture was stirred at room temperature for 10 min. Upon completion, LPC; compound 9 (70.0 mg, 0.14 mmol), dissolved in 4 mL of CHCl₃ was added to the flask and left stirring for 24 h. The white precipitate (N-N dicyclohexylurea) was filtered off using a medium porosity fritted Büchner filter using chloroform and the solvent removed under reduced pressure. The crude sample was then purified via chromatography over a pre-conditioned column of 25 g of silica gel with CHCl₃: MeOH (9:1 v/v) using 400 mL of CHCl₃: MeOH: H₂O (65:25:4 v/v) as eluent. Identical fractions based on Rf-values (0.60 cm and 0.36 cm) were combined and the solvent removed in vacuo. Compound 11 was obtained as a clear and viscous gel (50.0 mg, 57.45 µmol, 41%).

¹HNMR (300 MHz, CDCl₃): δ 0.11 (s, 9H), δ 0.05 (s, 6H), δ 0.08 (s, 6H), δ 0.49 (t, 2H, J= 6Hz), δ 0.87 (t, 3H, J= 6Hz), δ 1.25 (s, 34H), δ 1.56 (m, 4H), δ 2.27 (q, 4H, J= 8Hz) δ 3.38 (s, 9H) δ 3.85 (s, 2H), δ 4.07 (s, 2H), δ 4.14 (s, 2H), δ 4.32 (s, 2H), δ 5.19 (s, 1H); ¹³C NMR (75
107 MHz, CDCl$_3$): $\delta$ 0.18 ppm, 1.18, 1.26, 1.01, 18.31, 22.27, 24.89, 25.01, 29.21, 29.36, 29.46, 29.56, 29.71, 31.92, 33.51, 34.13, 34.34, 54.42, 173.24, 173.57; $^{29}$Si NMR (59 MHz, CDCl$_3$): $\delta$ -21.06, 7.02, 7.46; $^{31}$P NMR (121 MHz, CDCl$_3$): $\delta$ -0.54; EI-MS (m/z): M$^+$ 870.55; FTIR (cm$^{-1}$): 792, 839, 1045, 1253, 1466, 1735, 2853, 2921. Analytical calculation for C$_{41}$H$_{88}$NO$_{10}$PSi$_3$ C 56.58%; H 10.19%. Found C 55.00% H 9.98%.

**OleCpcTSPC (12)**

![OleCpcTSPC](image)

An oven-dried round bottom flask cooled under N$_2$ gas was charged with compound 3 trisiloxane acid (114 mg, 0.29 mmol) dissolved in 4 mL CHCL$_3$, DCC (60 mg, 0.29 mmol), and a catalytic amount of 4-dimethylaminopyridine (6.1 mg, 49.93 $\mu$mol). The mixture was stirred at room temperature for 10 mins. Upon completion, 18:1 OlePC (61.1 mg, 0.12 mmol) dissolved in 4 mL of CHCL$_3$ was added to the flask and left stirring for 24 hours. The white precipitate formed ($N$-$N$ dicyclohexylurea) was filtered off via a Buchner filter using chloroform and the solvent removed under reduced pressure. The crude sample was then purified via chromatography over a pre-conditioned column of 25 g of silica gel with CHCL$_3$: MeOH (9:1 v/v) using 400 mL of CHCL$_3$: MeOH: H$_2$O (65:25:4 v/v) as eluent. Identical fractions based on $R_f$-values (0.80 cm and 0.33 cm) were combined and the solvent removed in vacuo. The synthesized compound with a yield of 70.0 mg (78.08 $\mu$mol, 67%) was obtained as a clear and viscous gel and characterized by IR, $^1$H-NMR $^{13}$C-NMR $^{29}$Si NMR and mass spectral data. $^1$HNMR (300 MHz, CDCl$_3$): $\delta$ 0.11 (s, 9H), $\delta$ 0.05 (s, 6H), $\delta$ 0.08 (s, 6H), $\delta$ 0.51 (t, 2H, J=
6Hz), δ 0.87 (t, 3H, J= 6Hz), δ 1.26 (s, 32H), δ 1.56 (m, 4H), δ 2.00 (m, 4H), δ 2.26 (q, 4H, J= 8Hz) δ 3.35 (s, 9H) δ 3.79 (s, 2H), δ 3.91 (s, 2H), δ 4.09 (s, 2H), δ 4.35 (s, 2H), δ 5.17 (s, 1H) δ 5.32 (m, 2H); 13C NMR (75 MHz, CDCl3): δ 0.18 ppm, 1.26, 1.79, 1.96, 14.01, 18.29, 22.66, 23.26, 24.87, 24.99, 27.20, 29.16, 29.19, 29.26, 29.30, 29.45, 29.51, 29.56, 29.74, 31.89, 33.51, 34.10, 34.32, 54.37, 59.31, 62.99, 66.26, 70.53, 129.65, 129.98, 173.20, 173.51; 29Si NMR (59 MHz, CDCl3): δ -21.09, 6.98, 7.39; 31P NMR (121 MHz, CDCl3): δ -0.62; EI-MS (m/z): M⁺ 896.57; FTIR (cm⁻¹): 793, 840, 1047, 1254,1458, 1736, 2853, 2922. Analytical calculation for C₄₉H₉₀NO₁₀PSi₃C 57.62 %; H 10.12 %. Found C 56.57 % H 10.23 %.

Note: The 18:1 OlePC was kindly provided by Dr. Mark Frampton in the Zelisko’s group.

5.3.2 Drug Delivery Experimental Methods

Release experiments were a modified protocol from Shimanouchi et al.(2009) [172].

5.3.2.1 Liposome preparation and formulation with Calcein

5.3.2.1.1 POPC

In a methanol-cleaned round bottom flask, 10 mg of POPC were dissolved with 100 μL of chloroform. The organic solvent was then removed in vacuo to prepare a thin lipid film. This lipid film was then rehydrated with 0.7 ml of calcein in Tris buffer pH 7.5 (100 mM calcein in 50 mM Tris 100 mM NaCl) to make up 20 mM of POPC. The flask was then capped with a septum and vortexed at 1500 rpm for 10 min at room temperature to initiate liposome formation. DLS was then used to determinate the size of these liposomes which were all assumed to be MLVs based on their size (>2000 nm). Extrusion via a 100 nm was then performed up to 11 passages using 1 mL syringes in order to have ULVs ideal for release studies. DLS was again used after extrusion to confirm the presence of ULVs (0-100 nm)
using 2 µL of liposome suspension diluted into 998 µL Tris buffer pH 7.5 (50 mM Tris 100 mM NaCl).

5.3.2.1.2 PalValDSPC (10)

A freeze-dried thin lipid film of PalValDSPC (5 mg) was rehydrated with 314 µL of calcein in Tris buffer pH 7.5 (100 mM Calcein in 50 mM Tris 100 mM NaCl) to make up 20 mM of PalValDSPC. The flask was then capped with a septum and vortexed at 1500 rpm for 10 min to initiate liposome formation. DLS was then used to determinate the size of these liposomes which were all assumed to be MLVs based on their size (>2000 nm). Extrusion via a 100 nm was then performed up to 11 passages using 1 mL syringes in other to have ULVs ideal for release studies.

5.3.2.1.3 16:0 LPC

10 mg of freeze-dried thin lipid film of LPC was rehydrated with 1.0 ml of Calcein in Tris buffer pH 7.5 (100 mM Calcein in 50 mM Tris 100 mM NaCl) to make up 20 mM of 16:0 LPC. The flask was then capped with a septum and vortexed at 1500 rpm for 10 min to initiate vesicle formation.

5.3.2.1.4 LysoTSPC (4)

10 mg of freeze-dried thin lipid film of lysoTSPC was rehydrated with 0.8 ml of Calcein in Tris buffer pH 7.5 (100 mM Calcein in 50 mM Tris 100 mM NaCl) to make up 20 mM of LysoTSPC. The flask was then capped with a septum and vortexed at 1500 rpm for 10 min to initiate vesicles formation. DLS was then used to determinate the size of these liposomes which were 100 nm). Extrusion via a 100 nm was then performed up to 11 passages using 1 mL syringes in other to have ULVs (ideal for release studies). DLS was again used after
extrusion to confirm the presence of ULVs (0-100 nm) using 5 μL of liposome suspension into 995 μL Tris buffer pH 7.5 (50 mM Tris 100 mM NaCl).

5.3.2.1.5 1,2-SiPC (5)

10 mg of freeze-dried thin lipid film of (5) was rehydrated with 0.5 ml of Calcein in Tris buffer pH 7.5 (100 mM Calcein in 50 mM Tris 100 mM NaCl) to make up 20 mM of 1,2-SiPC. The flask was then capped with a septum and vortexed at 1500 rpm for 10 min to initiate liposomes formation. Half of the flask volume was then subjected to extrusion via a 100 nm (11 passages) using 1 mL syringes.

5.3.2.1.6 PalCpcTSPC (11)

10 mg of freeze-dried thin lipid film of PalCpcTSPC was rehydrated with 300 μl of Calcein in Tris buffer pH 7.5 (100 mM Calcein in 50 mM Tris 100 mM NaCl) to make up 20 mM of PalCpcTSPC. The flask was then capped with a septum and vortexed at 1500 rpm for 10 min to initiate liposomes formation. Extrusion via a 100 nm was then performed up to 11 passages using 1 mL syringes in other to have ULVs (ideal for release studies).

5.3.2.1.7 OleCpcTSPC (12)

10 mg of freeze-dried thin lipid film of OleCpcTSPC was rehydrated with 300 μl of Calcein in Tris buffer pH 7.5 (100 mM Calcein in 50 mM Tris 100 mM NaCl) to make up 20 mM of OleCpcTSPC. The flask was then capped with a septum and vortexed at 1500 rpm for 10 min to initiate liposomes formation. Extrusion via a 100 nm was then performed up to 11 passages using 1 mL syringes in other to have ULVs (ideal for release studies).
5.3.2.2 Liposome purification

Liposome purification was performed to separate non-encapsulated calcein from calcein-containing liposomes. This purification was necessary because for the sake of release profiles we only need calcein entrapped within liposomes. The purification was achieved using size exclusion chromatography (SEC), where Sepharose 4B was packed in a 5.0 mL disposable column and thoroughly pre-equilibrated with Tris buffer solution pH 7.5. Each liposome suspension obtained after extrusion was then loaded and eluted with Tris buffer (50 mM Tris 100 mM NaCl) pH 7.5. Fractions were analysed using a fluorimeter at a 405 nm excitation wavelength with low to zero emission of calcein reflecting the required quenched state of calcein in liposomes (brown in colour). Calcein in liposomes, being larger in size, always eluted first followed by free calcein or unentrapped calcein (green in colour), which was also confirmed by the high emission intensity of calcein at 515.4 nm. Free calcein was then used to calculate the entrapment capacity of each liposome prepared above. The absorbance at 495.1nm was recorded and using the calibration curve of calcein (Figure 41) together with the Beer-Lambert law [223], the concentration of free calcein was obtained. Using the total volume of free calcein and its derived concentration, the mass of free calcein was then obtained. The entrapment capacity (ee) could then be calculated using the formula:

\[ ee = \frac{\text{free calcein}}{\text{total calcein}} \times 100\% \]  
(Eq. 13)

5.3.2.3 Liposome release

The liposome formulated with calcein was then subjected to a release kinetic experiment directly after SEC (~20 seconds). 10 µL of each liposome suspension was immersed in 2 mL of Tris buffer (0.1 mM phospholipid, 50 mM Tris 100 mM NaCl) at pH values of 7.5, 10.5, and 4.5. Release studies were done up to 10 h maximum, at room temperature. Calcein’s fluorescence
was used to monitor the release of calcein from each liposome capsule at 405 nm excitation wavelength and 515.4 nm emission wavelength. Release profile were then calculated using the formula:

\[ RF(\%) = 100 \frac{(I_t - I_0)}{(I_{\text{max}} - I_0)} \]  

(Eq. 9)

where \( I_0 \) was the intensity of calcein at time 0, \( I_t \) the intensity of calcein at time \( t \) and \( I_{\text{max}} \) the intensity of calcein after the application of 3% Triton X-100 (1 mL per cuvette). The actual \( I_{\text{max}} \) intensity was corrected using the dilution factor of each cuvette after triton X-100 application.

DLS measurement were done before the addition of Triton X-100, to check whether liposomes were still present and also after the application of Triton X-100 to confirm the bursting destruction and hence disappearance of ULVs. The release profile obtained for each sample was then used to derive the kinetic constant \( k \), and the corresponding permeability coefficient \( P_m \).

The line of best fit applied in the excel book was used to infer whether the profile was zero order (\( R^2 > 0.8 \)) or first order release. In case the release was zero order, the gradient of the equation of the line was derived as \( k \). In the case of first order release, \( k \) was derived using the first order equation

\[ RF(t) = RF_{(\text{max})} \left(1 - e^{-kt}\right) \]  

(Eq. 10)

\( P_m \) could then be obtained for every system as a factor of \( k \) using the equation

\[ Pm = \left(\frac{r}{3}\right)k \]  

(Eq. 11)
Figure 41 Calcein absorbance calibration curve. Absorbance was measured at 495 nm. All points were measured in triplicate, and error bars represent the standard deviation.

5.3.3 Liposome Stability experiment

DLS cuvettes prepared after extrusion of liposomes or directly after vortexing of lipid film were sealed, kept under the dark at room temperature, and were periodically subjected to DLS analysis to infer how long the ULVs or MLVs maintain their characteristic size. This experiment was performed over 4 weeks.

5.3.4 Buffers preparation

The eluent buffer used during size exclusion chromatography (SEC) and as the release buffer medium pH 7.5 was prepared as such: Tris buffer (3.02 g), together with 2.93 g of NaCl, was dissolved in 300 mL of distilled water. The pH was adjusted to 7.5 using 1 M HCl and the volume was topped up to 500 mL to achieve a 50 mM Tris 100 mM NaCl. This was used as required.
The release buffer media pH 4.5 and 10.5 were prepared as follows; Tris buffer (0.12 g), together with 0.12 g of NaCl were both dissolved in two separate vials containing 10 mL distilled water. The pH was adjusted to 4.5 in one and 10.5 in another using 1 M HCl and 1 M NaOH. The volume was then topped up to 20 mL to achieve a 50 mM Tris 100 mM NaCl. These were used as required.

The lipid film rehydration buffer was prepared as follows; Calcein (0.62 g), was immersed in 5 mL of Tris buffer (50 mM Tris 100 mM NaCl). The pH was adjusted to 7.5 and the volume topped up to 10 mL both using 1 M HCl and 1 M NaOH to obtain a 100 mM Calcein in Tris buffer. This was used as required.
Appendix Figure 1 $^1$H NMR Spectrum of heptamethyltrisiloxanemethyldecanoate (2) in CDCl$_3$
Appendix Figure 2 $^{13}$C NMR Spectrum of heptamethyltrisiloxanemethyldecanoate (2) in CDCl$_3$
Appendix Figure 3 $^{29}$Si NMR Spectrum of heptamethyltrisiloxanemethyldecanoate (2) in CDCl$_3$
Appendix Figure 4 FTIR spectrum of heptamethyltrisiloxanemethyldecanoate (2)
Appendix Figure 5 EI-MS spectrum of heptamethyltrisiloxanemethyldecanoate (2)
Appendix Figure 6 $^1$H NMR Spectrum of heptamethyltrisiloxanedeconoic acid (3) in CDCl$_3$
Appendix Figure 7 $^{13}$C NMR Spectrum of heptamethyltrisiloxanedecanoic acid in CDCl$_3$
Appendix Figure 8 $^{29}$Si NMR Spectrum of heptamethyltrisiloxanedecanoic acid (3) in CDCl$_3$
Appendix Figure 9 FTIR spectrum of heptamethyltrisiloxanedecanoic acid (3)
Appendix Figure 10 EI-MS spectrum of heptamethyltrisiloxanedecanoic acid (3)
Appendix Figure 11 ESI+/MS spectrum of heptamethyltrisiloxanedecanoic acid (3)

Appendix Figure 12 $^1$H NMR Spectrum of LysoTSPC (4) in CDCl$_3$
Appendix Figure 13 13C NMR Spectrum of LysoTSPC (4) in CDCl3
Appendix Figure 14 31P NMR Spectrum of LysoTSPC (4) in CDCl3
Appendix Figure 15 29Si NMR Spectrum of LysoTSPC (4) in CDCl3
Appendix Figure 16 FTIR spectrum of LysoTSPC (4)
Appendix Figure 17 EI-MS spectrum of LysoTSPC (4)

Appendix Figure 18 ESI+/MS spectrum of LysoTSPC (4)
Appendix Figure 19 $^1$H NMR Spectrum of 1,2 Si-PC (5) in CDCl$_3$
Appendix Figure 20 $^{13}$C NMR Spectrum of 1,2 Si-PC (5) in CDCl$_3$
Appendix Figure 21 $^{31}$P NMR Spectrum of 1,2 Si-PC (5) in CDCl$_3$
Appendix Figure 22 \(^{29}\text{Si NMR Spectrum of 1,2 Si-PC (5) in CDCl}_3\)

Appendix Figure 23 ESI+/MS Spectrum of 1,2 Si-PC (5)
Appendix Figure 24 $^1$H-NMR spectrum of Pentamethyldisiloxanemethylpentanoate (7) in CDCl$_3$
Appendix Figure 25 $^{13}$C NMR Spectrum of Pentamethyldisiloxanemethylpentanoate (7) in CDCl$_3$
Appendix Figure 26 $^{29}$Si NMR Spectrum of Pentamethyldisiloxanemethylpentanoate (7) in CDCl$_3$
Appendix Figure 27 FTIR spectrum of Pentamethyldisiloxane methylpentanoate (7)
Appendix Figure 28 EI-MS spectrum of Pentamethyldisiloxanemethylpentanoate (7)
Appendix Figure 29 ESI+/MS spectrum of Pentamethyldisiloxanemethylpentanoate (7)

Appendix Figure 30 $^1H$ NMR Spectrum of Pentamethyldisiloxanepentanoic acid (8) in CDCl$_3$
Appendix Figure 31 $^{13}$C NMR Spectrum of Pentamethyldisiloxanepentanoic acid (8) in CDCl$_3$
Appendix Figure 32 $^{29}$Si NMR Spectrum of Pentamethyldisiloxanepentanoic acid (8) in CDCl$_3$
Appendix Figure 33 FTIR spectrum of pentamethyldisiloxanepentanoic acid (8)
Appendix Figure 34 El-MS spectrum of pentamethyldisiloxanepentanoic acid (8)
Appendix Figure 35 ESI-MS spectrum of pentamethyldisiloxanepentanoic acid (8)

Appendix Figure 36 $^1$H NMR Spectrum of 16:0 LPC (9) in CDCl$_3$
Appendix Figure 37 $^{13}$C NMR Spectrum of 16:0 LPC (9) in CDCl$_3$
Appendix Figure 38 $^{31}$P NMR Spectrum of 16:0 LPC (9) in CDCl$_3$
Appendix Figure 39 FTIR spectrum of 16:0 LPC (9)
Appendix Figure 40 FAB-MS spectrum of 16:0 LPC (9)
Appendix Figure 41 ESI+/MS spectrum of 16:0 LPC (9)
Appendix Figure 42 $^1$H NMR Spectrum of PalValDSPC (10) in CDCl$_3$
Appendix Figure 43 $^{13}$C NMR Spectrum of PalValDSPC (10) in CDCl$_3$
Appendix Figure 44 $^{31}$P NMR Spectrum of PalValDSPC (10) in CDCl$_3$
Appendix Figure 45 $^{29}$Si NMR Spectrum of PalValDSPC (10) in CDCl$_3$
Appendix Figure 46 FTIR Spectrum of PalValDSPC (10)
Appendix Figure 47 El-MS Spectrum of PalValDSPC (10)
Appendix Figure 48 ESI+/MS Spectrum of PalValDSPC (10)
Appendix Figure 49 $^1$H NMR Spectrum of OleCpcTSPC (12) in CDCl$_3$
Appendix Figure 50 $^{13}$C NMR Spectrum of OleCpcTSPC (12) in CDCl$_3$
Appendix Figure 51 $^{31}$P NMR Spectrum of OleCpcTSPC (12) in CDCl$_3$
Appendix Figure 52 $^{29}$Si NMR Spectrum of OleCpcTSPC (12) in CDCl$_3$
Appendix Figure 53 FTIR spectrum of OleCpcTSPC (12)
Appendix Figure 54 FAB-MS spectrum of OleCpcTSPC (12)
Appendix Figure 55 $^1$H NMR Spectrum of PalCpcTSPC (11) in CDCl$_3$
Appendix Figure 56 $^{13}$C NMR Spectrum of PalCpTSPC (11) in CDCl$_3$
Appendix Figure 57 $^{29}$Si NMR Spectrum of PalCpcTSPC (11) in CDCl$_3$
Appendix Figure 58 $^{31}$P NMR Spectrum of PalCpcTSPC (11) in CDCl$_3$
Appendix Figure 59 FTIR spectrum of PalCpcTSPC (11)
Appendix Figure 60 FAB-MS spectrum of PalCpcTSPC (11)
Appendix 2 - Release studies' selected data

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Appendix Figure 61 Different rate constants of lipids as well as their corresponding permeability coefficient

Appendix Figure 62 Line of best fit for zero-order release profiles of OleCptTSPC
7 Vita

Anne Patricia Tongkoua Nkamou was born in Douala, Cameroon on January 1, 1995. She was raised with her four siblings by their parents Florette and Jean Tongkoua. Her high school education was completed at Horizon Bilingual Educational Complex in 2009 at which point she attended University of Buea in Cameroon. In 2011, she obtained her BSc in Biochemistry and Molecular Biology and began her Masters studies at Brock University in St. Catharines, Ontario, Canada under the supervision of Dr. Paul M. Zelisko. She is presently completing her MSc. in Biotechnology.
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