Electrochemically Controlled Interaction of Liposomes with a Solid-Supported Octadecanol Bilayer

by

Amanda Musgrove

B.Sc., University of Alberta, 2003
M.Sc., The University of British Columbia, 2006

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

in

The Faculty of Graduate and Postdoctoral Studies

(Chemistry)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

October 2013

© Amanda Musgrove 2013
Abstract

Transmembrane proteins and ion channels are a major target for new drug development. Incorporating them into sensors requires a method to produce stable, easily modifiable solid-supported phospholipid bilayers. This thesis demonstrates a method for using potential control on the electrode to mediate liposome adsorption, allowing them to interact with a previously deposited octadecanol layer through potential-created defects.

Compression isotherms and electrochemical measurements were used to establish the effect of the incorporation of a small amount of fluorescent dye on the octadecanol layers. Using these fluorescently-labelled octadecanol layers, electrochemical measurements both independently and coupled with in-situ fluorescence measurements were used to characterize the interaction of liposomes with these layers under potential control. It was found that application of moderate potentials - more negative than the onset of defect formation but less than that required for desorption of the layer - facilitated the effective incorporation of liposome material into the octadecanol bilayer. The length of time spent at the poration potential had little effect on the degree of liposome interaction with the adsorbed layer. The incorporation was seen as a change in the double-layer capacitance and the creation of small fluorescent structures in the layer after exposure to liposomes at the poration potential. A shift in the characteristic desorption potential was also seen with liposome incorporation.

Atomic force microscopy coupled in-situ with electrochemical control was also used to investigate the interaction of liposomes with the adsorbed octadecanol layer. The structure of the adsorbed layer was observed and with liposomes present in solution, the creation of three-dimensional structures similar in nature to those seen by fluorescence was noted. The incorporation of liposomes into the octadecanol was shown to be easily controlled by application of an electrical potential, opening a path for a new method of producing supported lipid bilayers in-situ for biosensing applications.
Preface

All of the work presented henceforth was conducted in the Bizzotto group lab in the Advanced Materials and Process Engineering Laboratory (AMPEL) at the University of British Columbia, Point Grey campus.

A previously published journal article (Potential dependent interaction of DOPC liposomes with an octadecanol covered Au(111) surface investigated using electrochemical methods coupled with in-situ fluorescence microscopy, Langmuir) [1] has been published that encompasses the material presented here in Chapter 5 and portions of the material in Chapter 4. This article may be accessed free of charge at http://pubs.acs.org/articlesonrequest/AOR-wsP5riyY8kpIhQztXDzq. I was the lead investigator for this work, responsible for the major areas of concept formation, experimental design, data collection and analysis, and manuscript composition, with the exception of the work performed by the following authors. Colin R. Bridges was an undergraduate researcher in the laboratories of Drs. Bizzotto and Sammis, and performed the synthesis of the BODIPY-C19-OH fluorophore used in this investigation and composed the portion of the manuscript submitted as Supplementary Information (not included in this thesis). Glenn M. Sammis was the research supervisor of Colin R. Bridges, and contributed edits to the Supplementary Information portion of the manuscript. Dan Bizzotto was the supervisory author on this project and was involved throughout the project in concept formation, data analysis, and manuscript composition.

Colin R. Bridges also performed the data collection of the Langmuir isotherms presented in Section 3.1 under the supervision of myself and Dan Bizzotto. I was responsible for the remainder of the work presented in this thesis, including data collection and analysis, experiment design, and manuscript composition. Dan Bizzotto was the supervisory author on this project and was involved throughout the project in concept formation, data analysis, and manuscript composition.
# Table of Contents

Abstract ................................................................. ii

Preface ............................................................... iii

Table of Contents ..................................................... iv

List of Tables .......................................................... ix

List of Figures ........................................................ x

Nomenclature ........................................................... xviii

Acknowledgements ...................................................... xix

Dedication ............................................................... xx

1 Introduction ......................................................... 1
   1.1 Biosensors ....................................................... 1
   1.2 Phospholipids ................................................... 2
   1.3 Thin Films ....................................................... 4
       1.3.1 Surface Pressure and Langmuir Trough Measurements  4
       1.3.2 Isotherms and Phase Diagrams for Thin Films .......... 5
       1.3.3 Multi-component Monolayers ............................. 8
   1.4 Solid-Supported Bilayers and Biosensors .................... 8
       1.4.1 Formation of Solid-Supported Layers .................... 11
   1.5 Electrochemical Background .................................. 18
       1.5.1 Theory ...................................................... 18
       1.5.2 Octadecanol Electrochemistry ............................ 25
       1.5.3 Electrochemistry of DOPC and Vesicles ................ 33
   1.6 Fluorescence Background ....................................... 42
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.6.1</td>
<td>Theory</td>
<td>Theory</td>
<td>42</td>
</tr>
<tr>
<td>1.6.2</td>
<td>In-situ Fluorescence Imaging of Adsorbed layers at Electrode Surfaces</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>1.7</td>
<td>Atomic Force Microscopy Background</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>1.7.1</td>
<td>Theory</td>
<td>Theory</td>
<td>56</td>
</tr>
<tr>
<td>1.7.2</td>
<td>Atomic Force Microscopy of Vesicles and Adsorbed Layers</td>
<td>61</td>
<td></td>
</tr>
<tr>
<td>1.7.3</td>
<td>In-situ Atomic Force Microscopy with Electrochemistry</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>1.8</td>
<td>Scope of the Project</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Experimental Methods</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>2.1</td>
<td>Materials</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>2.1.1</td>
<td>Electrodes</td>
<td>71</td>
<td></td>
</tr>
<tr>
<td>2.2</td>
<td>Liposome Formation</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>2.2.1</td>
<td>Chemical Stability of Liposomes</td>
<td>72</td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>Surface Pressure Measurements</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>2.3.1</td>
<td>Compression Isotherms and ESP</td>
<td>73</td>
<td></td>
</tr>
<tr>
<td>2.3.2</td>
<td>In-situ Fluorescence Measurements</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>2.4</td>
<td>Preparation of Modified Electrodes</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>2.4.1</td>
<td>Liposome-coated Electrodes</td>
<td>75</td>
<td></td>
</tr>
<tr>
<td>2.4.2</td>
<td>Octadecanol-coated Electrodes: Single-touch (monolayer)</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>2.4.3</td>
<td>Octadecanol-coated Electrodes: Double-touch (bilayer)</td>
<td>78</td>
<td></td>
</tr>
<tr>
<td>2.4.4</td>
<td>Multiple Depositions From Octadecanol Monolayers</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>Electrochemical Methods</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>2.5.1</td>
<td>Cyclic Voltammetry</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>2.5.2</td>
<td>Differential Capacitance</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>2.5.3</td>
<td>Incorporation of Liposomes</td>
<td>81</td>
<td></td>
</tr>
<tr>
<td>2.5.4</td>
<td>In-situ Fluorescence Methods</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>2.6</td>
<td>Fluorescence Image Analysis</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>2.7</td>
<td>Atomic Force Microscopy Methods</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>2.7.1</td>
<td>Instrumentation</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>2.7.2</td>
<td>Substrates</td>
<td>84</td>
<td></td>
</tr>
<tr>
<td>2.7.3</td>
<td>Surface Modification</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>2.7.4</td>
<td>Imaging Conditions</td>
<td>86</td>
<td></td>
</tr>
</tbody>
</table>
3 Effects of Fluorescent Dye on Octadecanol Monolayers
   3.1 Compression Isotherms
      3.1.1 Experimental Methods
      3.1.2 Results
   3.2 Electrochemical Characterization
      3.2.1 Experimental Methods
      3.2.2 Results
   3.3 Fluorescence - Floating Layers and Compressions
      3.3.1 Experimental Methods
      3.3.2 Uncompressed Layers
      3.3.3 Compressions of Layers
   3.4 In-situ Fluorescence with Electrochemistry
      3.4.1 Experimental Methods
      3.4.2 Results
   3.5 Conclusions

4 Electrochemical Studies of Liposome Interaction with Solid-Supported Octadecanol Bilayers
   4.1 Electrochemistry of DOPC on Au(111)
   4.2 Interaction of DOPC with Floating Octadecanol Monolayers
   4.3 Interaction of DOPC Liposomes with Octadecanol on Au(111) at 0 V
   4.4 Effect of Poration Potential on Liposome - Octadecanol Interaction
      4.4.1 Experimental Design
      4.4.2 Capacitance During Potential Steps
      4.4.3 Desorption of the Modified Bilayers
   4.5 Effect of Poration Time on Liposome - Octadecanol Interaction
      4.5.1 Experimental Design
      4.5.2 Capacitance During Application of Poration Potentials
      4.5.3 Desorption of the Modified Layers
   4.6 Conclusions

5 In-situ Fluorescence Studies of Liposome Interaction with Solid-Supported Octadecanol Bilayers
   5.1 Experimental Methods
   5.2 Fluorescence at 0 V
List of Tables

3.1 Average red:green (R/G) ratios for octadecanol monolayers containing 1 mol% and 3 mol% BODIPY-C19-OH.......... 102

4.1 Equilibrium surface pressure (ESP) of octadecanol and mixed octadecanol/DOPC monolayers. ......................... 112

A.1 Mean particle sizes for liposome solutions as measured using dynamic light scattering. ......................... 195

B.1 Conversion factors to kcts/sec used for fluorescence images. . 196
# List of Figures

1.1 dioleoyl phosphatidylcholine (DOPC) ........................................... 3
1.2 Differences in aggregation shapes of a) single-tailed lipids and b) double-tailed lipids (e.g. phospholipids) ........................................ 3
1.3 Schematic diagram of a Langmuir trough ..................................... 5
1.4 Langmuir isotherm for octadecanol at 20°C ................................. 7
1.5 Phase diagram for octadecanol .................................................. 7
1.6 Brewster angle microscopy images of floating octadecanol monolayers .......................................................... 9
1.7 Schematic of miscibility of mixed monolayers ................................ 10
1.8 Collapse pressures of immiscible and miscible layers ..................... 10
1.9 Deposition techniques for forming solid-supported bilayers from floating monolayers ............................................................ 12
1.10 Bilayer arrangements formed from Langmuir-Blodgett depositions... 12
1.11 Proposed mechanisms of vesicle bursting and fusion to form supported lipid bilayers ......................................................... 15
1.12 Depiction of PEG-PE containing vesicle and bilayer ..................... 17
1.13 Examples of different methods of forming bilayers on templated surfaces ................................................................. 19
1.14 Schematic model of the electrode-solution interface ...................... 20
1.15 RC Circuit .............................................................................. 21
1.16 Effect of the increase of bulk activity of the surfactant (a: lowest, d: highest) on the interfacial tension ........................................... 23
1.17 Differential capacitance-potential curve of a HMDE in: (1) a 0.5 M Na$_2$SO$_4$, (2) 0.5 M Na$_2$SO$_4$ solution saturated with camphor.... 24
1.18 Differential capacity of a Hg electrode in 1 M KNO$_3$ ................. 26
1.19 Capacitance behaviour of octadecanol on a HMDE ................. 27
List of Figures

1.20 Cyclic voltammogram (a) and differential capacitance (b - dashed line: negative scan, solid line: positive scan) of an octadecanol monolayer on Au(111) in 0.05 M KClO$_4$ .................................. 29
1.21 Effect of varying initial surface pressure on differential capacitance scans ................................................................. 29
1.22 Elastically scattered light on octadecanol monolayer Au(111) ........................................ 30
1.23 Changes in octadecanol chain tilt angle as determined by PM-IRRAS .................. 31
1.24 Proposed mechanism of desorption and readsoption of a bilayer of octadecanol on Au(111) ................................................................. 32
1.25 Differential capacitance of Langmuir-Schaefer, X-type, and Y-type bilayers of octadecanol on Au(111) ................................................................. 33
1.26 Differential capacitance of L-α-lecithin (open circles) and DL-α-lecithin (closed circles) monolayers ................................................................. 34
1.27 Differential capacitance of a DOPC monolayer adsorbed from the gas|solution interface on a mercury drop electrode .......................... 35
1.28 Differential capacitance desorption scans of DOPC (dotted line) and DMPC (solid line) bilayers formed from vesicles in solution .......................... 36
1.29 Cartoon representation of the behaviour of DMPC on an Au electrode ................................................................. 37
1.30 Adhesion signal for multilamellar DOPC vesicles adhering to a mercury electrode ................................................................. 39
1.31 Differential capacitance of DOPC on a mercury electrode ................................................................. 40
1.32 Proposed mechanism for liposome interaction with an existing monolayer of DOPC ................................................................. 41
1.33 Partial energy diagram for a photoluminescent system ................................................................. 43
1.34 Application of FRET to RNA sensing ................................................................. 45
1.35 Proposed structures of the BODIPY dimers ................................................................. 45
1.36 Excited state lifetime of Eu$^{3+}$ in air near a silver mirror surface ................................................................. 46
1.37 Oscillating field of dipole interacting by interference with a plane mirror ................................................................. 46
1.38 Fluorescence sensor for DNA binding ................................................................. 48
1.39 Components of fluorescence microscopy ................................................................. 49
1.40 Light cone of a microscope objective ................................................................. 50
1.41 Rayleigh criterion for spatial resolution ................................................................. 51
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.42</td>
<td>Fluorescence imaging of an octadecanol monolayer with 3 mol % DilC&lt;sub&gt;18&lt;/sub&gt;(5) dye on a Au(111) electrode.</td>
</tr>
<tr>
<td>1.43</td>
<td>Fluorescence images of an octadecanol monolayer before adsorption (a) and the same region after adsorption.</td>
</tr>
<tr>
<td>1.44</td>
<td>Tip-sample interactions at specific points in a force-curve cycle for AFM.</td>
</tr>
<tr>
<td>1.45</td>
<td>Frequency and phase response of a cantilever modeled as a damped harmonic oscillator.</td>
</tr>
<tr>
<td>1.46</td>
<td>AFM images of a polycrystalline gold sample.</td>
</tr>
<tr>
<td>1.47</td>
<td>AFM height and phase images of a polyvinyl alcohol thin film.</td>
</tr>
<tr>
<td>1.48</td>
<td>AFM image (taken in contact mode) of a mica surface modified with a partial-double-bilayer of phosphatidylethanolamine, with the centre of the imaging area additionally modified by a high-force imaging scan.</td>
</tr>
<tr>
<td>1.49</td>
<td>Force vs distance curve of an AFM tip placed directly onto an egg phosphatidylethanolamine liposome.</td>
</tr>
<tr>
<td>1.50</td>
<td>Cartoon schematic of a lipid raft.</td>
</tr>
<tr>
<td>1.51</td>
<td>AFM images of mixed sphingomyelin/DOPC monolayers (1:1 mol/mol) containing various amounts of cholesterol.</td>
</tr>
<tr>
<td>1.52</td>
<td>Atomic force microscopy images of N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate.</td>
</tr>
<tr>
<td>1.53</td>
<td>Atomic force microscopy image (MAC mode, in 0.1 M NaF) of a dimyristyl phosphatidylethanolamine layer under potential control.</td>
</tr>
<tr>
<td>1.54</td>
<td>Illustration of the proposed hybrid bilayer formation process.</td>
</tr>
<tr>
<td>1.55</td>
<td>Differential capacitance scans during desorption of a DOPC bilayer (top frame) and octadecanol bilayer (bottom).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Epi-fluorescence microscope configurations used for in-situ fluorescence imaging.</td>
</tr>
<tr>
<td>2.2</td>
<td>Transmission characteristics of filter cubes used for fluorescence measurements.</td>
</tr>
<tr>
<td>2.3</td>
<td>Schematics of electrochemical cells used, shown with Au(111) electrode in hanging meniscus configuration.</td>
</tr>
<tr>
<td>2.4</td>
<td>Potential step profiles used in testing liposome incorporation.</td>
</tr>
<tr>
<td>2.5</td>
<td>Assembly of AFM electrodes used for imaging.</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>3.1</td>
<td>Compression isotherms for monolayers of octadecanol containing 0, 1, 3, 5, and 8 mol% BODIPY-C19-OH. Film pressure range plotted is limited to the region below the minimum collapse pressure for all layers.</td>
</tr>
<tr>
<td>3.2</td>
<td>Equilibrium spreading pressure (ESP) and minimum capacitance values for octadecanol monolayers containing 0, 0.5, 1, 3, 5, and 8 mol% BODIPY-C19-OH fluorophore.</td>
</tr>
<tr>
<td>3.3</td>
<td>Cyclic voltammograms (top) and differential capacitance scans (bottom) of octadecanol monolayers containing various concentrations of BODIPY-C19-OH on Au(111).</td>
</tr>
<tr>
<td>3.4</td>
<td>Cyclic voltammograms (top) and differential capacitance scans (bottom) of octadecanol bilayers containing various concentrations of BODIPY-C19-OH on Au(111).</td>
</tr>
<tr>
<td>3.5</td>
<td>Fluorescence images of a floating octadecanol monolayer containing 1 mol% BODIPY-C19-OH fluorophore in a Langmuir trough.</td>
</tr>
<tr>
<td>3.6</td>
<td>Fluorescence images of a floating octadecanol monolayer containing 3 mol% BODIPY-C19-OH fluorophore in a Langmuir trough.</td>
</tr>
<tr>
<td>3.7</td>
<td>Fluorescence images of a floating octadecanol monolayer containing 1 mol% BODIPY-C19-OH fluorophore in a Langmuir trough. Images taken with barriers partially closed.</td>
</tr>
<tr>
<td>3.8</td>
<td>Fluorescence images of a floating octadecanol monolayer containing 3 mol% BODIPY-C19-OH fluorophore in a Langmuir trough. Images taken with barriers partially closed.</td>
</tr>
<tr>
<td>3.9</td>
<td>In-situ fluorescence and differential capacitance measurements of a bilayer of octadecanol containing BODIPY-C19-OH fluorophore.</td>
</tr>
<tr>
<td>4.1</td>
<td>Differential capacitance scans during desorption of a DOPC bilayer (top frame) and octadecanol bilayer (bottom).</td>
</tr>
<tr>
<td>4.2</td>
<td>Differential capacitance measurements of DOPC on Au(111).</td>
</tr>
<tr>
<td>4.3</td>
<td>Surface pressure (Π) during exposure to liposomes.</td>
</tr>
<tr>
<td>4.4</td>
<td>Differential capacitance of octadecanol bilayers at 0 V/SCE during exposure to DOPC liposomes for 60 min.</td>
</tr>
<tr>
<td>4.5</td>
<td>Potential step profiles used in testing liposome incorporation.</td>
</tr>
</tbody>
</table>
List of Figures

4.6 Differential capacitance of octadecanol bilayers on Au(111) with and without liposomes in solution during application of potential steps ................................................. 118

4.7 Replicate measurements of octadecanol capacitance with and without liposomes in solution during application of the -0.6 V and -0.4 V/SCE poration potential ........................................ 119

4.8 Capacitance during a potential sweep to desorption (+0.15 to -0.8 V/SCE) of octadecanol layers subjected to the potential profiles in Figure 4.6 .................................................. 122

4.9 Differential capacitance of octadecanol bilayers on Au(111) with and without liposomes in solution during application of -0.4 V/SCE for various times ........................................... 124

4.10 Capacitance during a potential sweep to desorption (+0.15 to -0.8 V/SCE) of octadecanol layers subjected to the potential profiles in Figure 4.9 .................................................. 125

5.1 Cartoon illustration of a possible mechanism of increasing fluorescence during liposome incorporation .......................................................... 128

5.2 Average fluorescence intensity and differential capacitance measurements for layers held at 0 V/SCE ............................................................... 130

5.3 Spectroelectrochemical behaviour of adsorbed octadecanol bilayers with and without liposomes in solution during application of a potential profile with a 1 min poration step ............. 136

5.4 Representative fluorescence images taken during application of a potential profile with a 1 min poration step ....................................................... 137

5.5 Spectroelectrochemical behaviour of adsorbed octadecanol bilayers with and without liposomes in solution during desorption of the layer after modification by a 1 min poration potential profile ... 140

5.6 Representative fluorescence images taken during application of a potential profile with a 1 min poration step ....................................................... 141

5.7 Fluorescence images of Figure 5.6 after background subtraction by a 50 pixel radius rolling ball filter ............................................................... 142

5.8 Spectroelectrochemical behaviour of adsorbed octadecanol bilayers with and without liposomes in solution during application of a potential profile with a 15 min poration step ............. 144
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.9</td>
<td>Representative fluorescence images taken during application of a potential profile with a 15 min poration step.</td>
</tr>
<tr>
<td>5.10</td>
<td>Spectroelectrochemical behaviour of adsorbed octadecanol bilayers with and without liposomes in solution during desorption of the layer after modification by a 15 min poration potential profile.</td>
</tr>
<tr>
<td>5.11</td>
<td>Representative fluorescence images taken during application of a potential profile with a 15 min poration step.</td>
</tr>
<tr>
<td>5.12</td>
<td>Fluorescence images of Figure 5.6 after background subtraction by a 50 pixel radius rolling ball filter.</td>
</tr>
<tr>
<td>6.1</td>
<td>AFM image of an octadecanol monolayer containing 3 mol% BODIPY-C19-OH on the Au(111) facet of a bead electrode. Images were acquired in ACAFM mode in air.</td>
</tr>
<tr>
<td>6.2</td>
<td>AFM image of an octadecanol monolayer containing 3 mol% BODIPY-C19-OH on a Au/mica substrate. Image acquired in MAC mode in air.</td>
</tr>
<tr>
<td>6.3</td>
<td>AFM topography image (left) of the Au(111) facet with octadecanol monolayer. (Right) Mask of major topographical features from the AFM image.</td>
</tr>
<tr>
<td>6.4</td>
<td>Fluorescence image of the region surrounding the area imaged in Figure 6.3.</td>
</tr>
<tr>
<td>6.5</td>
<td>In-situ AFM images of an octadecanol bilayer containing 3 mol% BODIPY-C19-OH: topography channel. Potentials are measured vs. an Au bead reference.</td>
</tr>
<tr>
<td>6.6</td>
<td>In-situ AFM images of an octadecanol bilayer containing 3 mol% BODIPY-C19-OH: phase channel. Potentials are measured vs. an Au bead reference.</td>
</tr>
<tr>
<td>6.7</td>
<td>Example features of each category from the AFM topography images of octadecanol bilayers. Images are cropped from those in Figure 6.5.</td>
</tr>
<tr>
<td>6.8</td>
<td>In-situ AFM images of an octadecanol bilayer containing 3 mol% BODIPY-C19-OH and liposomes in solution: topography channel. Potentials are measured vs. an Au bead reference.</td>
</tr>
</tbody>
</table>
List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.9</td>
<td>In-situ AFM images of an octadecanol bilayer containing 3 mol% BODIPY-C19-OH and liposomes in solution: phase channel. Potentials are measured vs. an Au bead reference.</td>
</tr>
<tr>
<td>6.10</td>
<td>Example features that were present in the initially deposited layer of each category from the AFM topography images.</td>
</tr>
<tr>
<td>6.11</td>
<td>Example features that appeared during liposome adsorption from the AFM topography images.</td>
</tr>
<tr>
<td>6.12</td>
<td>Fluorescence images taken during the application of potential steps after background subtraction by application of a 50 pixel rolling ball filter.</td>
</tr>
<tr>
<td>7.1</td>
<td>Cartoon schematic of protein incorporation into a raised liposomal bilayer structure.</td>
</tr>
<tr>
<td>7.2</td>
<td>A possible procedure for creating multifunctional sensors in-situ in a microfluidic cell.</td>
</tr>
<tr>
<td>A.1</td>
<td>TLC plates taken at various points during the lifetime of a liposome solution.</td>
</tr>
<tr>
<td>C.1</td>
<td>False-color fluorescence images processed with a rolling-ball filter of various ball sizes. False-color scale is the same for all images, and all images are 150 x 150 μm.</td>
</tr>
</tbody>
</table>
Nomenclature

$\varepsilon$  dielectric constant

$\varepsilon_0$  permittivity of free space

$\Gamma$  Surface excess (Gibbs excess)

$\gamma$  Surface (or interfacial) tension

$\mu$  chemical potential

$\Pi$  Surface pressure

$\sigma$  Charge density

12-AS  12-(9-anthroyloxy) stearic acid

AC  Alternating current

AC  Intermittent contact AFM imaging mode

AFM  Atomic force microscope/microscopy

BAM  Brewster angle microscopy

BODIPY-C19-OH  4,4-difluoro-1,3,5,7-tetramethyl-8-(18-octadecanol)-4-bora-3a,4a-diaza-s-indacene

CV  Cyclic voltammetry/voltammogram

DilC$_{18}(5)$  1,1´-dioctadecyl-3,3,3´,3´-tetramethylindodicarbocyanine perchlorate

DMPC  Dioleoyl myristoyl phosphatidylcholine

DNA  Deoxyribonucleic acid

DOPC  dioleoyl phosphatidylcholine
Nomenclature

EQCM  Electrochemical quartz crystal microbalance
ESP   Equilibrium surface pressure
FRET  Förster Resonance Energy Transfer
HMDE  Hanging mercury drop electrode
IHP   Inner Helmholtz Plane
kcts/sec  kilocounts per second
MAC   Magnetic AC (AFM vibrational imaging mode)
NA    Numerical aperture
OHP   Outer Helmholtz Plane
PM-IRRAS  Polarization modulation infrared reflection absorption spectroscopy
pzc    Potential of zero charge
QCM   Quartz crystal microbalance
RMS   Root mean square
SAM   Self-assembled monolayer
SCE   Saturated caolmel electrode
STM   Scanning tunneling microscopy
TLC   Thin layer chromatography
Acknowledgements

Many people have helped me along the way - but some special thank yous are warranted.

First, to my supervisor, Dr. Dan Bizzotto. Your guidance and advising has been invaluable, and makes a lab worth coming back to.

To the members of the Bizzotto lab group, past and present - Dr. Jeff Shepherd, Dr. Robin Stoodley, Dr. Aya Sode, Jeffrey Murphy, Jannu Casanova Moreno, Landis Yu, Santa Maria Gorbunova, and Isaac Martens. You are a great group to work with, and your discussions, friendship, and teatimes have been immensely helpful, both in the lab and out.

All of the glass electrochemical cells used in this work were created by the department’s glassblower, Brian Ditchburn. Thanks Brian - this work, especially with the fluorescence cells, literally could not have happened without your skill.

Thank you to Dr. Guillaume Bussiere for the use of your Langmuir trough to acquire the data shown in Chapter 3, and for assisting Colin Bridges in his measurements on the same.

A special thank you to Lyndsey Earl - your friendship has brightened my time here, and your scientific discussions (notably on optimizing the TLC analysis) have gotten me unstuck many a time.

Thank you to my family - my husband, parents, and sisters - your love and support brought me here and kept me going.

Financial support from the Agnes and Gilbert Hooley Scholarship in Chemistry is gratefully acknowledged.
Dedication

This thesis is dedicated to my husband Laurent - Thank you. You have supported me through more schooling than is really right, and your love, support, and understanding have made me a better person through it all.
Chapter 1

Introduction

The use of solid-supported lipid bilayers as a platform for developing biosensors is a growing field of study, especially as up to 60% of new drug targets are membrane proteins [2]. The ability to support these proteins in their natural environment is essential to create devices that rely on monitoring their functionality. This thesis will describe a novel process for producing a solid-supported bilayer assembly for development as a biosensor platform. The rationale and perceived challenges for this project [3] will be outlined below, along with a brief introduction to the necessary theory and a review of the state of the art regarding the techniques used in this study.

1.1 Biosensors

From the now-ubiquitous electrochemical glucose sensor [4] to detection of new drug targets, sensors for biological molecules are an actively developing area. In its broadest definition, a biosensor is a device that is able to detect chemical compounds based on biochemical reactions and transduces the reaction or binding event into a signal that is readily measured. [5] The reaction used for detection in the sensor could involve the analyte interacting with a specific ligand, such as an antibody or DNA aptamer designed to interact with the target compound. Enzymes and peptides as detection targets present a special challenge as they often have a complex three-dimensional structure that requires a carefully controlled environment to maintain – including the availability of a phospholipid bilayer similar to a cell membrane for some proteins. Happily, with careful design these sensors can be incredibly effective, even detecting concentrations in the attomole range [6], and even down to the single molecule level. [7]

Biosensors based on lipid bilayers supported on a planar substrate are more robust than those based on other bilayer models, such as a black lipid membrane. The stability of the supported layers may be further enhanced by forming tethered
1.2. Phospholipids

bilayers wherein a molecule, such as polyethylene glycol or a thiolipid, acts as a mediator between the bilayer and the substrate and both anchors the bilayer to the surface and provides a cushioning space to ensure room for water and ion flow between the electrode and the bottom leaflet of the bilayer. Maintaining a water gap between the supported bilayer and the electrode surface is also important for incorporating proteins with large extra-membranal domains, as they may denature if they come into direct contact with the electrode surface.

Embedding a protein or ion channel in a supported lipid membrane provides sensor functionality. Since ions cannot pass through the hydrophobic core of the bilayer without a carrier molecule, biomolecules that transport ions across the membrane will generate an easily detectable perturbation in the electrochemical characteristics of the system. Incorporation of tetrachloro-o-benzoquinone or tetrachloro-p-benzoquinone in electrode-supported bilayers creates a pH sensitivity, while sensitivity to ions can be created by use of ion carriers such as valinomycin or crown ethers. Ions may also pass through a lipid bilayer via an ion channel. These are either voltage-gated or ligand-gated, and can be based on natural or synthetic ion channels. Gramicidin was among the first ion channels used to produce an electrochemical sensor by measuring the change in conductance across the membrane. This design has been developed to produce a sensor for influenza virus designed for point-of-care analysis. Other peptides have been incorporated into solid supported membranes as well as ion channels. Although solid supported bilayers as a platform are less developed than other models, they are the only current option for sensing with membrane proteins that is sufficiently stable and suited for making robust, commercially viable sensors.

1.2 Phospholipids

The membrane that surrounds biological cells is composed primarily of a mixed-phospholipid bilayer, embedded with proteins and carbohydrates, especially cholesterol. Liposomes resemble the cell membrane, though proteins and carbohydrates are not usually added and the phospholipid composition is controlled, usually consisting of only one or two types of molecule.

There are two main classifications of phospholipids: glycerophospholipids and sphingomyelins. The focus of this study is on liposomes formed from glycerophospholipids. Sphingomyelins, though an important membrane component, will not be
1.2. Phospholipids

Glycerophospholipids consist of a glycerol backbone to which are attached two esterified fatty acids at the C1 and C2 positions, and a phosphate group on C3. Another group may be linked to the phosphate group as well. One common phospholipid, dioleoyl phosphatidylcholine (DOPC), is depicted in Figure 1.1. Note that this particular phospholipid, though uncharged overall, is zwitterionic at neutral pH, enhancing the hydrophilicity of the phosphate head group, while the hydrocarbon “tails” are hydrophobic. This amphiphilic behavior causes phospholipids to aggregate when placed in aqueous solution. However, unlike some other surfactants, which tend to form spherical micelles, the more cylindrical cross section of a phospholipid molecule favors a planar structure - the bilayer - as shown in Figure 1.2. Liposomes may be unilamellar, having only one bilayer, or multilamellar, being composed of several nested spherical bilayers. They may also be classified by size, “small” vesicles being on the order of 20 - 200 nm, “large” vesicles 200 - 1000 nm, and “giant” liposomes larger than 1 μm, often on the same scale as a typical cell. [27]

For most phospholipids, the bilayer is a liquid crystalline structure at biologically relevant temperatures [28], though it does have at least one phase transition, depending on the type of phospholipid involved. All such bilayers undergo a “melt-
1.3. Thin Films

The behaviour of thin films at the gas|solution interface is important for the deposition methods used in this work, as well as for characterizing the structure and behaviour of insoluble surfactants. Thin films, especially monomolecular films and bilayers, are used extensively in forming the biosensors discussed above, and an understanding of their behaviour is essential for designing and creating such tools.

1.3.1 Surface Pressure and Langmuir Trough Measurements

The method of using a trough with movable barriers to examine the effects of thin films on surface pressure were developed first by Pockels [30] and further refined by others, notably Langmuir and Blodgett who developed a technique for depositing monolayers and multilayers from the gas|solution interface onto a solid substrate. [31–33] Typically, the behaviour of a thin film of insoluble surfactant (such as octadecanol, a fatty alcohol) is measured by the use of a Langmuir Trough (Figure 1.3). Briefly, the trough consists of a reservoir filled with solvent and bound by one or two movable barriers. A microbalance with Wilhelmy plate is arranged so that the plate is in contact with the solvent between the barriers, and a small amount of the surfactant molecule is introduced to the gas|solution interface. Once the surfactant film has formed, the film pressure Π can be determined from the net force on the Wilhelmy plate. The net downward force for a plate of density ρ_p, known width w, length l and thickness t, immersed in a solvent of density ρ_s to a
1.3. Thin Films

Figure 1.3: Schematic diagram of a Langmuir trough.

depth $h$, is the balance of gravity, surface tension, and buoyancy:

$$F = \rho_p g l w t + 2\gamma(t + w)\cos\theta - \rho_s g t w h \quad (1.1)$$

where $\theta$ is the contact angle of the solvent with the plate, $g$ the acceleration due to gravity, and $\gamma$ the surface tension of the liquid. By taking the difference between the clean subphase and the surfactant-modified surface, the film pressure of the surfactant can be found:

$$\Pi = \gamma_{water} - \gamma_{film} = -\frac{\Delta F}{2(t + w)} \approx -\frac{\Delta F}{2w} \quad (1.2)$$

where the final form of the equation holds when the plate thickness is negligible compared to the width, as is generally true for plates made from paper or similar thin materials. By monitoring the film pressure as the barriers are brought closer together, reducing the area available for the surfactant, insight is gained into the structure and interaction of the surfactant molecules as their surface concentration increases.

1.3.2 Isotherms and Phase Diagrams for Thin Films

The plot of $\Pi$ vs. molecular area for compression of a surfactant at a constant temperature is called a Langmuir isotherm. A typical Langmuir isotherm for octadecanol is shown in Figure 1.4. In this isotherm, several regimes of response to changes in area can be clearly seen, based on changes in linearity and slope of the isotherm. These follow the generally predicted trends for compression of a long-chain alcohol. Described using the phase descriptions introduced in [34] and
summarized in [33, 35], up to six distinct phases are distinguishable in long-chain alcohols. According to the phase diagrams predicted for long-chain molecules collected in [36] and further investigated in [35] and [37], octadecanol does not have access to this lowest density phase at the film pressures and temperatures measured. Instead the lowest density phases observed in the long-chain alcohols is the $L_2$ liquid-condensed phase. This phase is liquid-crystal like with some translational order, although the layer remains compressible. At room temperature, higher film pressure will result in the LS phase, the so-called ‘superliquid’ phase. This phase is also a liquid-crystal like mesophase, but with higher order - a hexagonal unit cell, and the aliphatic chains oriented perpendicularly to the gas|solution interface. At lower temperatures, the solid (S) and compact solid (CS) states are accessible. Despite the term 'solid' used in these phases, x-ray diffraction studies [38] suggest that these phases are also more liquid-crystal like than true solid phases. The empirical phase diagram for a monolayer of octadecanol is shown in Figure 1.5. In the isotherm of Figure 1.4, the transitions between these phases are visible as changes in the slope, or 'kinks' in the isotherm plot. Eventually, as compression increases, the layer begins to buckle and form multilayers, collapsing, and the film pressure remains at a maximum value as the area decreases. Typically, this collapse pressure is at or above the equilibrium spreading pressure (ESP) of the monolayer - the film pressure it will naturally obtain when a film is formed with an excess of surfactant in a fixed area. For an uncollapsed monolayer at its ESP, any excess surfactant material will be located in small crystallites or lenses of multilayer interspersed with the monolayer. Octadecanol has a well-characterized ESP typically at 32-35 mN/m [33, 35], which suggests that it will be in the LS phase - a high-density liquid crystal - when formed at equilibrium conditions at room temperature.

As the terminology used for the phases suggests, the monolayers of octadecanol and similar surfactants formed at the gas|solution interface are not perfectly crystalline or defect free. As a demonstration of the defects present in a compressed octadecanol layer, Slevin et al. [39] used an ultramicroelectrode placed facing upwards beneath an octadecanol layer in a Langmuir trough. The interfacial resistance of the oxygen transfer across the air|solution interface was measured. From a pressure of 5 mN/m to approximately 50 mN/m (the point of collapse), oxygen transfer across the monolayer was detected. The rate of the transfer depended linearly with the molecular area, and interfacial resistance increased as the surface pressure increased, fitting a model of the monolayer containing defects that permit oxygen
1.3. Thin Films

Figure 1.4: Langmuir isotherm for octadecanol at 20°C. Phase symbols are as defined in Figure 1.5. Reprinted from [35] with permission from Elsevier.

Figure 1.5: Phase diagram for octadecanol. Phase abbreviations: S - solid, CS - compact solid, LS - superliquid, $L_2$ - liquid condensed. Reprinted from [35] with permission from Elsevier.
1.4 Solid-Supported Bilayers and Biosensors

transfer into the subphase that are gradually removed as the layer is compressed.

Brewster angle microscopy (BAM) has shown that even pure octadecanol monolayers have regions of coexisting phases at a range of film pressures. [40] Higher film pressures (corresponding to lower molecular area) have larger regions of condensed-phase octadecanol, but even near the molecular area at collapse (~19 Å²/molecule), regions of uncompressed layer exist, as seen in Figure 1.6e. The presence of defects in octadecanol monolayers formed in a Langmuir trough may also be inferred from capacitance measurements, as discussed in Section 1.5.1

1.3.3 Multi-component Monolayers

Mixed monolayers may be formed that contain more than one type of surfactant molecule. If the compounds in the mixture are completely miscible, the composition across the floating monolayer will be even (Figure 1.7), and the collapse pressure of the film will vary depending on the film composition. For compounds that are completely or partially immiscible, pure regions of each component will exist (Figure 1.7), and the film will collapse at the lowest collapse pressure of the individual components, as regions of this component collapse. [33] An example of this is shown in Figure 1.8 for mixtures of C₁₆ and C₂₄ fatty acids. The immiscible combination of C₁₆ and C₂₄ shows a constant collapse pressure near the lower of the two compounds’ ESP for all mixtures containing both compounds. A plateau also appears in the isotherm at an intermediate pressure, believed to be due to the ‘squeezing out’ of the lower-pressure component. [41] For miscible compounds, such as C₁₆ and C₂₀ fatty acids in Figure 1.8, the collapse pressure increases with the increasing amount of C₂₀ to a maximum at 100% C₂₀. The similar segregation of long chain (16 to 22-carbon) alkyl alcohols has been observed by AFM, [42] confirming that alcohol combinations where the chain lengths are less than 6 carbons different tend to be miscible, with no segregated domains visible by AFM. Greater differences in chain length lead to domain formation visible by Π/area isotherm measurements and AFM.

1.4 Solid-Supported Bilayers and Biosensors

Thin monomolecular films of the type described in Section 1.3 can be studied directly, and phospholipid bilayers as “black lipid membranes” can be formed across
Figure 1.6: Brewster angle microscopy images of floating octadecanol monolayers at various film pressures. Light areas correspond to condensed phase regions, and dark regions are less-condensed 'gaseous' phase. a) 120 Å²/molecule b) 100 Å²/molecule c) 60 Å²/molecule d) 30 Å²/molecule e) 20 Å²/molecule. Reprinted with permission from [40]. Copyright 1996 American Chemical Society.
1.4. Solid-Supported Bilayers and Biosensors

Figure 1.7: Schematic of miscibility of mixed monolayers. (a) Components completely miscible (b) Components partially miscible (small domains). (c) Components completely immiscible (complete phase segregation).

Figure 1.8: Collapse pressures of immiscible and miscible layers. Reproduced from [43] with kind permission from Springer Science and Business Media.
a small hole and used to investigate transport processes, as summarized in [44]. However these membranes are delicate, and have a short lifetime. By associating the bilayer onto a solid support, greater stability of the layer and more facile methods of formation can be accessed, making this platform a popular topic of recent study.

1.4.1 Formation of Solid-Supported Layers

Solid-supported layers can be broadly categorized into two types: chemisorbed and physisorbed layers. Chemisorbed species are more strongly attached to the solid support, generally involving formation of a chemical bond between the adsorbate and surface. A thiol self-assembled monolayer (SAM) on gold, attached by a gold-sulfur bond, is a typical example of a chemisorbed species. Physisorbed species are held at the solid support only by van der Walls attraction between the adsorbate and surface, and are therefore generally less robust than chemisorbed layers, as evidenced by the typical enthalpy of adsorption for physisorption being on the order of 10 times less than for chemisorption. [45] Although solid-supported layers are easily formed by deposition from a Langmuir trough or similar, it is also simple to use self-assembly from a solution of the adsorbate to form chemisorbed layers, and in some cases (such as self-assembly from vesicles) to self-assemble physisorbed layers.

Langmuir-Blodgett deposition

Solid-supported mono-, bi-, and multi-layers may be conveniently formed from a floating surfactant monolayer of the type described in Section 1.3 by the process of Langmuir-Blodgett deposition. [31, 33] In this technique, a floating monolayer is formed and compressed to the desired film pressure in a Langmuir trough, while the solid support is suspended vertically above the air|solution interface. The solid support is then slowly dipped through the interface and the monolayer supported thereon, depositing the monolayer onto the solid support. Figure 1.9a depicts this process, as well as a variant where the solid support is initially in the subphase below the interface, and is pulled up to deposit the monolayer.

The surface tension of the interface and direction of motion of the solid support during the monolayer transfer onto the solid support will influence the final orientation of the surfactant molecules; for a monolayer of surfactant on an aque-
1.4. Solid-Supported Bilayers and Biosensors

(a) Langmuir-Blodgett deposition using the “pull” method (left) and “push” method (right).

(b) Bilayer formation by Langmuir-Schaefer deposition. In this case, the first leaflet of the bilayer has previously been deposited, and the outer leaflet is being added.

Figure 1.9: Deposition techniques for forming solid-supported bilayers from floating monolayers. Adapted with permission from [46]. Copyright 2004 American Chemical Society.

Figure 1.10: Bilayer arrangements formed from Langmuir-Blodgett depositions. Left: X type. Center: Y type. Right: Z type. Adapted with permission from [46]. Copyright 2004 American Chemical Society.

ous subphase, the hydrophobic tail groups will be facing the upper surface of the monolayer, so a solid support pushed downwards through the interface will form a layer with the tail groups closest to the support. This compression and dipping procedure can be repeated multiple times to form multi-layers of surfactant on the solid support. By varying the direction of the dipping, different combinations of molecular orientations can be achieved [41], as summarized in Figure 1.10. The most common type of multi-layer deposition, where the leaflets are deposited with alternating “push” and “pull” directions, is the Y-type, and results in layers that have leaflets facing head-to-head and tail-to-tail. Layers of X and Z type have a head-to-tail leaflet orientation, and result from all-push and all-pull layer formation, respectively.
A related method for depositing monolayers onto solid supports is the Langmuir-Schaefer deposition (Figure 1.9b). This method uses a floating monolayer as does Langmuir-Blodgett deposition, however rather than moving the substrate through the interface, it is brought in parallel to the interface and gently touched to the surface, then lifted away. The process may be repeated to form multi-layers, but because there is only one possible orientation for the surfactant molecules in each deposition, all multilayers formed in this way will necessarily be X-type.

A special case of mixed layers are those of alkyl alcohols mixed with alkylthiols. Although forming these mixed monolayers on the liquid|gas interface is less favourable because the thiol group is less polar than the alcohol group, when deposited onto a suitable substrate (such as gold), the added attraction from the chemisorption of the thiol fraction causes the deposited layer to be more stable. With the addition of octadecanethiol to Langmuir monolayers of octadecanol, it has been shown that with increasing fraction of octadecanethiol, the monolayer had a decreasing collapse pressure, but when deposited onto a gold electrode, higher octadecanethiol content resulted in a lower capacitance and lower signal from a redox probe, suggesting fewer defects in the layer. See Section 1.5.2 for further discussion on this topic.

Self-assembly of chemisorbed monolayers

A simple and accessible method for forming monolayers on solid substrates is self-assembly from a solution of the monomer. This approach has been taken with many classes of molecules and suitable substrates, such as silanes on glass [48] and on oxidized metal surfaces such as aluminum and titanium, [49, 50] diazonium on silicon, [51] and perhaps the most studied system, thiols and related sulfur compounds on metals, especially gold. Thiol SAMs are typically formed by immersing the substrate in a solution of the thiol compound for a given time (from a few hours to several days [52]) and allowing the layer to self-assemble onto the surface. The process can be aided by application of an electrical potential [53], encouraging the oxidation of the thiol into the metal-thiolate complex.

Thiol SAMs are generally robust, and the method has been used for attaching important biological molecules, both by incorporating a thiol group onto the molecule, as for DNA-cyclodextrin, peptides, and other compounds [52], or making use of naturally accessible sulphur in cysteine for attaching suitable proteins to a surface.[54] As in the Langmuir monolayers, it is possible for chemisorbed thiol
SAMs with more than one component to phase segregate into regions rich in one or the other component, as shown by observing height differences in regions of thiols with differing chain lengths by AFM in [55], for example.

**Self-assembly of physisorbed phospholipid bilayers**

Though Langmuir-Blodgett and Langmuir-Schaefer depositions provide a convenient means for depositing physisorbed layers onto a solid support, it is also possible to self-assemble layers of certain molecules, especially phospholipid bilayers. There are several techniques available for self-assembling solid-supported bilayers. In one method, a freshly exposed metal surface is immersed in a solution of the lipid, then removed and immersed in an aqueous solution. [56] A more flexible method involves immersing the substrate into a suspension of liposomes composed of the desired lipid and allowing them to burst onto the surface, forming a bilayer. [57] The exact mechanism for the formation of these bilayers from vesicles remains under investigation, but a number of theories have been proposed, as summarized in Figure 1.11. Though both hydrophobic and hydrophilic substrates can be used, the mechanisms of formation differ greatly. On hydrophilic surfaces, the final bilayer formed is cushioned by a 10-20 Å water layer near the support surface, that hydrophobic substrates necessarily lack. Adsorption of vesicles onto hydrophilic substrates begins with intact vesicles on the surface, with fusing and then rupturing to form a bilayer [59, 60]. Fusion of the liposomes is dependent on many factors, including surface chemistry, temperature, and osmotic pressure. [61] Simulations support this theory, indicating that the vesicle-surface interaction and membrane tension are driving forces in vesicle bursting and bilayer formation. [62] The membrane tension of a vesicle is directly proportional to its radius, following Laplace’s Law [63], and so vesicles of different sizes may be expected to have correspondingly different fusion behaviour. On some substrates, adsorbed intact vesicles will not naturally form a bilayer even at high vesicle concentrations and other stimuli, such as freezing, must be employed in order to induce vesicle bursting. [64]

Observations of this process for unilamellar DMPC (1,2-dimyristoyl-sn-glycero-3-phosphatidylcholine) vesicles by STM [65] showed that on a slightly hydrophobic Au(111) substrate, the process of vesicle deposition and fusion proceeds by vesicle rupture to first form a monolayer that transforms in to a hemimicellar state, then to a complete bilayer as more vesicles burst onto the surface. Studies of cholesterol-containing DMPC vesicles showed a similar behaviour. [66] On more
Figure 1.11: Proposed mechanisms of vesicle bursting and fusion to form supported lipid bilayers (SLB). Reprinted from [58] with permission from Elsevier.
1.4. *Solid-Supported Bilayers and Biosensors*

hydrophobic surfaces, such as thiol SAM-modified substrates and uncharged mercury electrodes, liposomes will burst to form a monolayer at the interface. [67–69] This process appears to follow a similar two-step process to the deposition onto gold - fluorescence microscopy studies of giant unilamellar vesicles bursting onto a surface modified with a hydrophobic SAM showed that the vesicles first underwent 'hemifusion', creating an island of material from the outer leaflet, then bursting to form a monolayer across the surface. [70] Why multiple modes of adsorption exist for these substrates is not entirely clear, however there is some suggestion that the mechanism is similar to a nucleation event, and can be described by the same models. [71] In this proposed model, the liposomes are substantially not attracted to a hydrophobic surface, but may be 'activated' and interact with the surface. A possible mode of activation previously proposed [72] was that some of the hydrophobic 'tails' of the phospholipid molecules randomly become exposed on the aqueous side of the membrane, providing a point of interaction with the hydrophobic surface. The interaction of vesicles with metal surfaces such as these is highly dependent on the surface potential and charge density. These relationships will be discussed in detail in Section 1.5.3.

An advantage of forming solid supported bilayers by vesicle deposition is that transmembrane proteins can be easily incorporated into the vesicles at formation, and will create a solid supported bilayer containing these proteins. [57, 73] Other methods, such as the Langmuir-Blodgett deposition, are much less suited to protein incorporation, as at some point the protein will be exposed to air and denature. A further improvement to the solid supported bilayers can come from modifying the initial liposomes to contain polyethylene oxide oligomers conjugated to phosphatidylethanolamine (PEG-PE) lipids [74]. The long polymer tails on these lipids extend into the aqueous region just outside the membrane (as shown in Figure 1.12) and help improve the stability of the solid supported bilayer when removed from the aqueous environment into air. However, these PEG layers do not provide a substantial distance between the solid support and the bilayer, so incorporation of membrane proteins can be difficult if the protein extends into the space between the bilayer and the solid support as it can interact unfavorably with the surface, adhering or denaturing. [75]
1.4. Solid-Supported Bilayers and Biosensors

Figure 1.12: Depiction of PEG-PE containing vesicle and bilayer. Adapted with permission from [74]. Copyright (2005) American Chemical Society.

Interaction with an already present layer

Vesicles in solution are not limited to interacting with bare surfaces - as has been described above, they are capable of bursting onto thiol SAM-modified substrates [60, 70] - if these SAMs are carefully chosen to contain a percentage of constituents with an attached moiety (such as cholesterol [76] or a transmembrane protein [77]) that can intercalate into a lipid bilayer, then vesicles from solution can burst onto the interface, forming a bilayer that is attached to the underlying SAM through the linker moieties. These 'tethered' bilayers have an advantage over bilayers deposited directly in that they are more stable than the physisorbed bilayers, and the length of the molecule forming the tethering SAM provides a space between the bilayer and the solid support into which transmembrane proteins can project, reducing denaturing of even bulky proteins. [78, 79]

It is also possible for liposomes in solution to incorporate into a solid supported bilayer previously formed on a solid substrate. In the presence of proteins that mediate membrane fusion, liposomes of egg phosphatidylcholine in solution were observed by fluorescence to incorporate into a solid supported bilayer of the same lipid. [80] The ability of liposomes to incorporate into such layers may not be a complete surprise, as incorporation of similar vesicles into cell membranes by endocytosis is common, however the observation of the process in vitro is worthy of note.
Creation of bilayers with templated patterns

Creation of solid supported bilayers that encompass the entire substrate area is easily achieved by the methods described above. However, it is not always efficient to have an entire surface modified with one type of bilayer; individually addressable regions of bilayer with different lipid composition or a variety of incorporated proteins may be desired. In these cases, there are several methods in development for creating surfaces that have spatially separated regions of lipid bilayer distributed across the substrate. Diffusion barriers (Figure 1.13a) formed by standard photolithographic techniques can be used to create individual islands of bilayer on the surface that will not mix [81, 82], as confirmed by fluorescence microscopy of the surface. Similarly, microwells may be created on a surface, and different materials introduced into each well by micropipette. [83, 84] Microcontact printing [85] using a stamp formed of a compatible material such as polydimethylsilane (PDMS) can be used to remove bilayer material in a pattern (“blotting”) or deposit bilayer domains onto a clean substrate (“stamping”, Figure 1.13b). Other techniques, such as nanolithography [86], oxidative removal of regions by UV illumination with a photomask [87], and polymer stripping [88] have been demonstrated.

Although many parameters of surface chemistry and geometry may be manipulated to influence the formation of solid supported bilayers, when using a conductive substrate one of the easiest to manipulate is the electrical potential (and thus the charge density at the interface). The electrode potential may be manipulated to encourage or discourage adsorption, or alter the structure of the adsorbed bilayer. These phenomena will be discussed in more detail in Section 1.5.1, but first an introduction to the electrochemical background is warranted.

1.5 Electrochemical Background

1.5.1 Theory

The behaviour of solvent and electrolyte molecules and ions near a charged surface is important for understanding electrochemical processes. At a molecular level, the solution side of the metal|solution interface may be imagined as consisting of a mixture of solvent, anions, and cations, which are able to approach and interact with the electrode surface in response to changes in electrical potential. According to the Gouy-Chapman-Stern model of the interface [89], there are two distinct
1.5. Electrochemical Background

(a) Lithographically formed diffusion barriers. From [81]. Reprinted with permission from AAAS.

(b) Patterning by microcontact stamping.

(c) Dip-pen nanolithography of lipid bilayers.

Figure 1.13: Examples of different methods of forming bilayers on templated surfaces.
1.5. Electrochemical Background

Figure 1.14: Schematic model of the electrode-solution interface. Inner Helmholtz Plane (IHP) and Outer Helmholtz Plane (OHP) are shown along with potential ($\phi$) and charge density ($\sigma$). The hydration energy for anions is smaller than for cations and therefore a solvation shell is not shown.

regions near the electrode surface. Closest to the electrode is a monolayer of solvent molecules and any specifically adsorbed species (ionic or molecular), shown in Figure 1.14. The electrical centre of this layer is the Inner Helmholtz Plane (IHP), at a distance $x_1$ from the electrode surface. Solvated ions cannot approach as close to the electrode surface; their closest approach is at distance $x_2$, named the Outer Helmholtz Plane (OHP). Because of thermal motion, the entirety of the surface charge on the electrode will not be compensated by ions within the OHP, but rather the charge compensation by electrolyte ions extends into the solution as the diffuse layer for a distance (typically less than 100 Å). Since the interface is described in this way as having two layers of charge distribution, the solution side of the interface is often referred to as the “double layer”.

This model of the metal|solution interface suggests a behaviour similar to a simple capacitor conceptualized as two parallel plates, one the electrode surface, and the other the compensating charges in solution, beginning at the OHP and encompassing the diffuse layer. The capacitance of a parallel-plate capacitor may
1.5. Electrochemical Background

be generally described by the equation

\[ C = \frac{\varepsilon \varepsilon_0}{d} \]  

(1.3)

where \( \varepsilon \) represents the relative dielectric constant of the material between the plates, \( \varepsilon_0 \) the permittivity of free space, and \( d \) the distance between the two plates. In the absence of specifically adsorbed ions, the solvent and uncharged adsorbates at the IHP appear similar to the dielectric material. In fact, this model explains very well electrode behaviour in solution, and the interface is typically modeled as an RC circuit - a resistor and capacitor in series, as highlighted in Figure 1.15, representing the so-called double-layer capacitance and the solution resistance. The double-layer capacitance differs from a simple electrical capacitance in that it is often potential-dependent due to changes occurring at the metal|solution interface such as changes in double-layer structure for low electrolyte concentrations, surface reconstruction of crystal faces [90–92], or adsorption/desorption events (see below for description). The presence of an electric field at the interface creates an electrostatic pressure across the double-layer, compressing the solvent molecules present at the IHP and altering their effective dielectric constant. [93] The double-layer capacitance is thus an important tool for monitoring the state of the metal|solution interface, and is often calculated experimentally by applying a small AC perturbation to the electrode and measuring the differential capacitance:

\[ C = \left( \frac{\delta \sigma_M}{\delta E} \right) \mu \]  

(1.4)

where \( \sigma_M \) represents the charge density of the electrode, \( E \) the electrode potential, and \( \mu \) chemical potential.

Adsorption of substances onto an electrode surface can alter the capacitance by displacing the solvent (taken here to be water) at the IHP with material with different dielectric properties. An electrode surface with adsorbed material at the surface
may successfully be modeled as two parallel capacitors [94], one representing the capacitance of the unmodified surface \((C_{\theta=0})\) and the other the adsorbate-modified area \((C_{\theta=1})\), where \(\theta\) is the fraction of surface coverage. The total capacitance is thus an area weighted average of the two capacitances. Equation 1.5 illustrates this concept, using \(\theta\) as the fraction of electrode area covered by the adsorbate.

\[
C_{\text{total}} = (\theta)C_{\theta=1} + (1 - \theta)C_{\theta=0}
\]  

(1.5)

This model may also be used to estimate the fraction of surface covered by the adsorbate for systems where the adsorbate capacitance and bare-electrode capacitance are known.

Adsorbates may be desorbed or encouraged to adsorb onto an electrode surface by application of an electrical potential, because of changes in the interfacial tension at the electrode surface. For the adsorption of a neutral adsorbate \(L\) onto an electrode surface in a generalized electrolyte \(M^+X^-\) at constant temperature and pressure, the relation of the surface tension to electrode potential is given by the electrocapillary equation:

\[
-d\gamma = \sigma Me^{+} + \Gamma_-d\mu_{MX} + \Gamma_Ld\mu_{L}
\]  

(1.6)

In this equation, \(\gamma\) is the interfacial tension, \(\Gamma_-\) and \(\Gamma_L\) are the Gibbs surface excess (relative to water) of the \(X^-\) ion and the adsorbate \(L\), respectively, and \(\mu_{MX}\) and \(\mu_{L}\) the chemical potentials of the electrolyte and the adsorbate, respectively. A typical electrocapillary curve will be an inverted parabola when plotted versus electrode potential, as shown in Figure 1.16 for two hypothetical surface states. As the electrode potential is changed the system will react to minimize the surface energy, so for a given potential the stable state will be the one with the lowest interfacial tension. In the example system of Figure 1.16, this would imply that the \(\beta\)-state will be the most stable only in a narrow potential window, and outside that region, the \(\alpha\)-state will be most stable. This behaviour is demonstrated for the adsorption of camphor onto a mercury electrode [96] in Figure 1.17. Here, the camphor-modified electrode (analogous to phase \(\beta\) in Figure 1.16) is the more stable state between approximately 0.4 and 1.8 V, and the unmodified electrolyte-electrode interface (phase \(\alpha\)) is otherwise more stable. The presence of camphor on the electrode surface is indicated by the sharp decrease in capacitance in this potential range, caused by the displacement of electrolyte at the metal|solution interface with cam-
1.5. Electrochemical Background

Figure 1.16: Effect of the increase of bulk activity of the surfactant (a: lowest, d: highest) on the interfacial tension $\gamma$, charge density $\sigma$ and differential capacity $C$ for two defined states $\alpha$ and $\beta$. The lowering of the interfacial tension (arrows in a) is larger for the most compact film. $\beta$. Reprinted from [95] with permission from Elsevier.

phor, which has a lower relative dielectric constant. Similar observations have been made for a range of adsorbates, among them isoquinoline [97], tert-pentanol [98], n-butanol [99], octyl alcohol [100], and lipids [101].

Also notable in Figure 1.17 are the large, sharp peaks associated with the transition between desorbed and adsorbed states. These so-called pseudo-capacitance peaks are characteristic of moving between states on the metal|solution interface, due to kinetic limitations of the phase transition. In systems where there is a change in coverage with potential, current will be required to flow to the interface in order to accommodate the changing dielectric as coverage changes. The change in charge density with potential depends on the surface excess and the charge transferred as the adsorbate is transferred on or off the surface. Although this response is complex, Equation 1.4 can be modified to account for the charge transfer (and thus change in charge density) to become:

$$C = \left( \frac{\partial \sigma_M}{\partial E} \right)_\mu + \left( \frac{\partial \sigma_M}{\partial \Gamma} \right)_E \left( \frac{\partial \Gamma}{\partial E} \right)_\mu$$

The second term in this new equation will be dependent on the AC frequency used,
1.5. Electrochemical Background

Figure 1.17: Differential capacitance-potential curve of a HMDE in: (1) a 0.5 M Na$_2$SO$_4$, (2) 0.5 M Na$_2$SO$_4$ solution saturated with camphor. Frequency: 45 Hz. Reprinted from [96] with permission from Elsevier.
as the change in coverage with potential \( \left( \frac{\partial \Gamma}{\partial E} \right)_T \) will vary depending on the kinetics of the phase transition. In other words, as the rate of the phase change decreases, the ability of the system to respond to the higher frequency AC perturbations decreases. This effect is shown for the adsorption of octyl alcohol in Figure 1.18. Note that as the AC frequency used to measure the differential capacitance increases, the height of the pseudo-capacitance peaks decreases. At the limit of infinite frequency, the peaks disappear entirely and the transition between \( C_\alpha \) and \( C_\beta \) is smooth. At the limit of zero frequency, the expression reduces to Equation 1.4.

The frequency of any applied AC perturbation will have an additional effect on solid electrodes, due to the special nature of their interfaces. Due to microscopic or atomic-scale roughness \([103]\), they may exhibit a distribution of relaxation times, rather than the single relaxation time implied by the simple RC circuit model generally used in capacitance calculations. In this case, a constant-phase element \([104]\) resembling an infinite series of parallel RC circuits may be used rather than a capacitor when modeling the cell. Despite this, it has been shown \([105]\) for polished Au(111) electrodes that the simpler RC circuit is a good approximation of the interface behaviour, as little frequency dispersion in the range 1 kHz to 0.1 kHz was found in the absence of specifically adsorbed material.

### 1.5.2 Octadecanol Electrochemistry

Octadecanol (\( C_{18}H_{37}OH \)) as a thin film has been studied extensively. Its characteristics as a monolayer are well known, as summarized in Section 1.3. As such, it has lent itself well to electrochemical studies of its properties also. As a floating monolayer, it has already been noted that at pressures close to the ESP there are defects in the layer such that oxygen is able to diffuse from the air into the subphase below. \([39]\) As will be described below, the presence and characteristics of these defects will dominate the electrochemical behaviour of octadecanol adsorbed onto an electrode surface.

**Monolayers of octadecanol on gold and mercury electrodes**

Although essentially similar to the soluble surfactants described in Section 1.5.1, the electrochemical behaviour of electrodes coated with a monolayer of octadecanol has some distinguishing properties, mostly linked to its insolubility in aque-
1.5. Electrochemical Background

Figure 1.18: Differential capacity of a Hg electrode in 1 M KNO₃. Solid lines show the capacitance of the electrode in a saturated octyl alcohol solution at 0.24 and 10 kHz. Heights of the pseudocapacitance peaks at intermediate frequencies are marked with arrows. The dashed line shows the capacitance of the electrode in an octyl alcohol-free solution. Reprinted from [102], Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission.
1.5. Electrochemical Background

Figure 1.19: Capacitance behaviour of octadecanol on a HMDE. Potential is given versus the pzc of the interface. Dotted line: 0.05 M KClO₄. Solid line: 0.05 M KClO₄ with a monolayer of octadecanol deposited from the gas|solution interface. Reproduced from [107], Copyright Elsevier 1997.

ous electrolyte. For a monolayer deposited onto a mercury electrode by gently pushing the electrode through an octadecanol-coated gas|solution interface [106], the capacitance behaviour has similar general characteristics to that of the previously described for camphor. (Camphor: Figure 1.17, octadecanol: Figure 1.19.) In the potential range at which the adsorption of the octadecanol is favoured, from approximately 300 mV measured against the potential of zero charge (pzc) to approximately -350 mV/pzc, the capacitance is significantly lowered as compared to measurements in the absence of octadecanol, much as for the camphor previously described. In contrast with the camphor example, however, the pseudocapacitance peaks characteristic of adsorption/desorption events are muted in the octadecanol system. This difference is believed to be due to the slow kinetics of adsorption/desorption of octadecanol, which is evident also in the shift in potential of readsoption vs. desorption. In mercury, the minimum capacitance is found near the pzc, and was measured to be approximately 10 μF/cm².

On a single-crystal Au(111) electrode, the positive desorption potentials are not accessible due to an irreversible oxidization of the adsorbate, [106] but the negative desorption limit is accessible. A typical differential capacitance measurement of the desorption and readsoption of an octadecanol monolayer is shown in Figure 1.20. The minimum capacitance (again near the pzc, which is 0.330 V/SCE for Au(111) in 0.1 M NaF [108]) is similar to that on Hg, indicating that the mono-
layer is not disturbed by the deposition onto a solid surface, which will necessarily have some roughness, rather than the smooth mercury surface. Owing to the more complex phase transition involved in the desorption from Au(111), the number and height of the pseudocapacitance peaks is different, but the hysteresis between desorption and readsoption potential remains. The position of these peaks (and thus the associated desorption/readsoption events) was found to depend on the electrolyte concentration, [109] shifting the peaks approximately -100 mV as the concentration was varied from 100 mM to 5 mM. Therefore, the transition is driven by changes in interfacial tension.

The quality of the layer, as measured by the number and height of pseudocapacitance peaks, also strongly depends on the surface pressure of the octadecanol monolayer before it is transferred to the electrode surface. [110] As shown in Figure 1.21, at the lowest film pressures the minimum capacitance is greater, which is expected given the lower molecular density of the layer and higher number of defects in the less condensed phases (as also measured in [39]). Studies of mixed monolayers of octadecanol and oleyl alcohol, miscible with each other, showed a decrease in the minimum capacitance as the mole fraction of oleyl alcohol in the monolayer was increased. [111] Taken with the findings on film pressure, this supports the conclusion that octadecanol monolayers, especially those deposited at moderate film pressures (i.e. at the ESP) contain defects at deposition that do transfer to the electrode surface. Comparing theoretical and actual differential capacitance measurements of octadecanol monolayers deposited on Au(111) by both Langmuir-Schaefer and Langmuir-Blodgett methods suggest that the best surface coverage is about 0.9. [112] As the initial film pressure increases, the pseudocapacitance peaks at desorption become gradually smaller and broader, and shift toward more negative potentials, exaggerating the hysteresis in potential at desorption/readsoption (not shown in Figure 1.21). These investigations, supported by studies of mixed monolayers of the immiscible pyrenenonanol/octadecanol mixture [111] highlight that the state of the monolayer at the gas|solution interface before transfer to the electrode is a major factor in the state of the adsorbed monolayer.

Although the pseudocapacitance peaks inform us that there is a phase change occurring, as a transition from one state’s electrocapillary curve to another, they do not provide any information about the nature of this change. Investigations on the film pressure changes of Au(111) modified with an octadecanol monolayer [114] showed a fit to three electrocapillary curves with transitions occurring at -
1.5. Electrochemical Background

Figure 1.20: Cyclic voltammogram (a) and differential capacitance (b - dashed line: negative scan, solid line: positive scan) of an octadecanol monolayer on Au(111) in 0.05 M KClO$_4$. Dotted lines show CV and capacitance of electrode in the absence of octadecanol. Reproduced from [113]. Copyright Elsevier 2004.

Figure 1.21: Effect of varying initial surface pressure on differential capacitance scans. Scans are negative-going desorption scans of an octadecanol monolayer on Au(111). Reprinted from [110] with permission from Elsevier.
1.5. Electrochemical Background

250 mV/SCE and -600 mV/SCE, implying that three states are necessary to describe the desorption process. Adding another form of measurement in-situ, such as fluorescence (discussed later in Chapter 5), infrared spectroscopy, or elastically scattered light measurements, can help determine the processes involved in the transition. Light scattering studies done on octadecanol monolayers adsorbed on Au(111) [106, 113] (Figure 1.22) show constant scattering at potentials more positive than -600 mV/SCE, and a sharp increase in scattering negative of -600 mV/SCE as the potential is scanned negatively. This behaviour suggests that the octadecanol layer remains very near the electrode surface at the more positive potentials, then moves away from the surface as some form of small aggregate causing scattering at -600 mV/SCE. These particles or aggregates must remain relatively close to the electrode surface to remain within the focal volume of the measurement. On the positive readsorption scan, the scattering stays high until approximately -300 mV/SCE, matching the onset of the first change in capacitance, indicating that the octadecanol does remain desorbed from the surface. The hysteresis in the light scattering measurements matches with those in the electrochemical measurements, supporting the hypothesis that the octadecanol desorbs from the electrode surface, but remains near, and that a difference in mechanism of desorption vs. readsorption must contribute to the potential shift between these two processes. Polarization modulation infrared reflection absorption spectroscopy (PM-IRRAS) of the octadecanol monolayer on Au(111) [112] also show an increase in the octadecanol tilt angle that loosely correlates with the changes in capacitance (Figure 1.23).

Figure 1.22: Elastically scattered light on octadecanol monolayer Au(111). Reproduced from [113]. Copyright Elsevier 2004.
1.5. Electrochemical Background

Figure 1.23: Changes in octadecanol chain tilt angle as determined by PM-IRRAS. Squares represent the first negative scan; circles the first positive scan. Solid line is the differential capacitance trace of the negative scan. Reproduced from [112] with permission from Elsevier.

Bilayers of octadecanol on gold electrodes

Fewer studies of octadecanol bilayers have been undertaken than of the monolayers. However, the electrochemistry is well-characterized. The capacitance behaviour (Figure 1.24) shows a pattern of behaviour related to the monolayer capacitance. The capacitance is initially lower than a monolayer, as expected since not only is the dielectric octadecanol layer thicker, but most defects present in a monolayer are covered over by the second layer, better isolating the electrode surface. At approximately -200 mV/SCE, a small pseudocapacitance peak is associated with an increase in capacitance of the layer, indicating a phase change in the layer. This has been hypothesized to be a formation of defects in the adsorbed layer, as depicted in the cartoon (e) of Figure 1.24. At approximately -600 mV/SCE, a large increase in capacitance corresponds with the desorption of the bilayer from the electrode surface (Figure 1.24a). Elastically scattered light measurements [106] indicate that, as for the monolayers, desorption results in small aggregates that remain near the electrode surface. The mechanism of readsorption of these presumed aggregates must be different from the desorption, as indicated by the large hysteresis in the capacitance scan. One possible mechanism (Figure 1.24b-c) is that the aggregates first form a defective monolayer, then undergo a phase transition (indicated by the
1.5. Electrochemical Background

Figure 1.24: Proposed mechanism of desorption and readsorption of a bilayer of octadecanol on Au(111). Reprinted with permission from [115]. Copyright 1999 American Chemical Society.

The structure of the adsorbed octadecanol bilayer on Au(111) has been further investigated by PM-IRRAS [46]. Layers deposited by two sequential Langmuir-Schaefer touches, as well as X- and Y-type Langmuir-Blodgett depositions, were compared electrochemically and spectroscopically. Although all three types of depositions formed layers with some basic similarities in their electrochemical behaviour (e.g. low initial capacitance, desorption at negative potentials, hysteresis between desorption and readsorption potentials), both the Langmuir-Schaefer and X-type layers showed an irreversible change in behaviour after the first desorption scan, becoming more like the Y-type layers after repeated scans, while the Y-type layers remained stable, as seen in Figure 1.25. Based on this behaviour and the changes in carbon-chain angle measured by PM-IRRAS, it was determined that the X-type and Langmuir-Schaefer bilayers, which initially have a head-to-tail configuration (see Figure 1.10) rearrange upon desorption to form a more kinetically stable Y-type configuration in the readsorbed layer. This transformation begins after a single desorption, although it takes several cycles to become complete. Although the...
1.5. Electrochemical Background

(a) Langmuir-Schaefer bilayer

(b) X-type bilayer

(c) Y-type bilayer

Figure 1.25: Differential capacitance of Langmuir-Schaefer, X-type, and Y-type bilayers of octadecanol on Au(111). Trace 0: clean Au(111). Trace 1: Initial desorption (negative-going) scan. Trace 2: Initial readsoption (positive-going) scan. Trace 3: Equilibrium desorption & adsorption scans. Reprinted with permission from [46]. Copyright 2004 American Chemical Society.

X-type layers are stable while adsorbed, the conversion to Y-type is irreversible. This state change has important implications for layers formed by the Langmuir-Schaefer touch method, as the as-deposited layer will not be directly comparable to a layer that has experienced a desorption-readsorption cycle.

1.5.3 Electrochemistry of DOPC and Vesicles

Phospholipid bilayers and monolayers may be deposited directly onto an electrode surface, either by deposition from a floating layer or from vesicles in solution. Understanding the electrochemical behaviour of such layers is essential to designing functional electrochemical biosensors.

Monolayers of phospholipid on mercury electrodes

The electrochemical study of monolayers was quickly identified as an important pathway for characterizing bilayer behaviour at a surface. Monolayers of phospholipids deposited onto a HMDE were first studied by Miller et. al. [116–119], including studies on the incorporation of lipoproteins into the monolayer and oxygen transport across the interface. Monolayers of synthetic lecithin (a mixture of phosphatidylcholines) show a similar differential capacitance behaviour to other adsorbed surfactants on mercury electrodes, viz. a “capacitive pit” at potentials where it is readily adsorbed onto the electrode, with pseudocapacitive peaks marking the departure from the adsorbed state into desorbed or alternate phases. For pure
1.5. Electrochemical Background

phospholipids, the capacitive behaviour is well-defined, as in the case of DOPC, where the peaks visible at approximately -1 V/SCE become sharper (Figure 1.27), and are well-characterized enough that they are used to characterize the monolayer behaviour in the presence of membrane-altering additives, such as antibiotics [120] and hydrophobic hydrocarbons [121, 122]. The first peak is believed to be due to a phase change creating defects in the layer [123] and the second, smaller, peak to a nucleation and growth process of these defects. [124] At more negative potentials, the layer is desorbed, although it remains in close proximity to the electrode surface.

Impedance spectroscopy studies of the mercury-supported DOPC monolayer suggested that the simple model of an RC circuit for the electrode-electrolyte interface, as described in Section 1.5.1, may not be adequate for describing the modified Hg interface. [67] In this study, the solution resistance was found to increase dramatically when the DOPC monolayer was present on the mercury surface.
1.5. Electrochemical Background

Figure 1.27: Differential capacitance of a DOPC monolayer adsorbed from the gas|solution interface on a mercury drop electrode. Reprinted from [125].

**Potential-dependent behaviour of phospholipid bilayers on electrode surfaces**

Phospholipid bilayers are only stable on Au(111) in a limited range of potentials. Studies of DOPC and DMPC bilayers formed from vesicles in solution show that the layer is stably adsorbed from ~0.2 V/SCE to ~-0.35 V/SCE, negative of which there is some sort of phase transition, followed by the desorption of the layer from the surface at ~-1.1 V/SCE. Both DOPC and DMPC have similar capacitance behaviour, although the transitions for DOPC are less well defined than for DMPC (Figure 1.28). For supported bilayers at room temperature (20 ºC), DMPC is in the ripple state, and DOPC is in the liquid crystalline state, as determined by PM-IRRAS. [126] Liposomes of DOPC, when adsorbing from a suspension, adsorb without bursting for electrode charge densities less than 8 \( \mu \text{C/cm}^2 \), but at greater charge densities forms a bilayer (or multilayer), as determined by EQCM measurements. [127]

Impedance and chronocoulometric measurements of the DOPC monolayer on mercury drop electrodes have shown [123, 128] that at potentials more negative than -0.65 V/SCE, the monolayer is stable and is defect-free, until the first phase transition at approximately -0.925 V/SCE, and the monolayers are displaced with application of potentials below -1.8 V/SCE.

DOPC, when adsorbed onto a gold surface, is less mobile at positive charges than at negatively charged gold. This is likely due to Coloumbic interactions between the charged headgroup and the electrode surface, likely an increased localization of the negative charge on the phosphate group. [127]
In-situ neutron reflectivity studies of a DMPC bilayer formed by vesicle deposition onto an Au surface show that the layer is thinner at more positive potentials than the bilayer thickness given by X-ray diffraction, suggesting that the tilt angle of the DMPC tails is larger. As well, at these more positive potentials, the bilayer incorporates more of the water solvent into the bilayer structure (as depicted in the cartoon schematic of Figure 1.29). As the electrode potential is moved to more negative values, the layer becomes thicker and eventually desorbs from the electrode surface, although it stays quite nearby, even at -950 mV/SCE, being separated only by a thin layer of the water solvent that is up to approximately 10 Å thick. This “desorbed” membrane closely resembles the structure of bilayers supported on quartz substrates. Here the bilayer is known to be separated from the substrate by a thin layer of solvent. Charge density measurements show that the bilayer is stable on the electrode surface at charge densities of an absolute value less than 8 µC/cm². Outside this range, the charge density curve matches with that of the layer-free electrode surface. This behaviour matches with that observed by neutron reflectivity. [129, 130]

Characterization of DMPE vesicle spreading under potential control suggests that the spreading onto the vesicles is slow, based on hysteresis between positive and negative differential capacitance scan directions. Spreading of vesicles takes place between -600 and -400 mV (positive scan direction), and forms a condensed bilayer phase above -400 mV. However, this layer contains many defects and is not as condensed as layers that form on Hg, as characterized by capacitance and film
1.5. Electrochemical Background

Figure 1.29: Cartoon representation of the behaviour of DMPC on an Au electrode under potential control. Blue dots represent water, red dots the hydrophilic head of the DMPC lipid. Reprinted from [129] with permission from Elsevier.
pressure. Based on capacitance, the coverage for films formed in this way is approximately 80%. PM-IRRAS spectroscopy confirms that the tilt angle of the acyl chains of DMPC is large when adsorbed onto the electrode surface (approximately 55 degrees with respect to the surface normal). [131] The orientation of the polar heads in the two leaflets may differ due to their exposure to different environments. [132]

Further studies of bilayers of DMPC on Au(111) provide an estimate of surface coverage to be ~75% based on differential capacity measurements, and confirm the large tilt angle and increased head group spacing due to hydration in the adsorbed state. [133] Addition of cholesterol or other molecules that increase layer stiffness may be needed to achieve the lowest capacitances in a solid supported lipid bilayer. [134] Fatty alcohols such as octadecanol are soluble in the bilayer and may also be used to similar effect. [135]

**Vesicle behaviour in an electric field**

Adsorption of vesicles of DOPC on polycrystalline gold (sputtered on quartz QCM substrate) depends on the charge density (and thus potential) of the electrode surface. At potentials near the pzc of the interface, data from EQCM suggest that 20 nm vesicles of DOPC adsorb whole, rather than bursting onto the surface. At potentials positive or negative of the pzc, however, the data suggests that a bilayer-like system forms as vesicles interact with the electrode surface. [127]

Adhesion of phospholipid vesicles can be observed by chronocoulometric measurements, pinpointing individual adhesion events. [136] Using multilamellar DOPC vesicles, the current spikes (shown in Figure 1.30) are unidirectional, with the direction of current flow determined by the electrode charge density. The bidirectional signals are produced when the charge density on the electrode surface is less than that of the choline groups of the phospholipid at the vesicle-electrode interface, and were observed as well in unilamellar DOPC vesicles and vesicles of DMPC and DPPC. The bidirectional shape results from the initial current flow due to liposome contact with the electrode, displacing ions (negative portion) followed by the interaction of the positively charged choline groups with the negatively charged electrode (positive portion). The bidirectional peak is only visible for potentials where the charge density is less than the charge density created by the choline groups.

This same technique has previously been used to measure the adhesion of cells.
1.5. Electrochemical Background

(a) Unidirectional signals

![Unidirectional signals](image1)

(b) Bidirectional signals.

![Bidirectional signals](image2)

Figure 1.30: Adhesion signal for multilamellar DOPC vesicles adhering to a mercury electrode. Reprinted from [136] with kind permission from Springer Science and Business Media.

and oil droplets onto mercury electrodes. [137–141]

Liposomes of DOPC have been shown to interact in a potential-dependent manner with a monolayer of DOPC existing on a mercury electrode. [124] The monolayer formed by either deposition from the gas-solution interface or by liposomal deposition were initially similar (Figure 1.31, light traces), however upon excursion to potentials more negative than the second capacitance peak, liposomes in solution are able to interact with the electrode through the newly formed defects. The resulting layer has properties substantially different than the initially formed monolayer, most noticeably the two capacitance peaks are reduced in size and shifted towards more positive potentials. This change in behaviour remains even when the potential is scanned past the desorption potential of the monolayer. The cartoon in Figure 1.32 proposes a mechanism for the interaction of liposomes with the adsorbed monolayer during these potential excursions.

Free vesicles in solution may also be affected by the electric field induced by the electrode potential; in studies of giant unilamellar vesicles (larger than 1 µm diameter), exposure to alternating electrical fields caused an elongation of the vesicle into an oval shape. [142] Compared to the electrochemical behaviour previously discussed for vesicles, these fields are large (ca. 20 V RMS) and higher frequency (ca. 1-15 kHz). [143] Exposure to lower-frequency or DC fields can induce electroporation and rupture of vesicles in solution, and has been explored as a method of drug or gene delivery. The potential and frequency requirements for electroporation are dependent on several factors, such as vesicle size and distance from the
1.5. Electrochemical Background

Figure 1.31: Differential capacitance of DOPC on a mercury electrode. (a) Capacitance-time profile of liposome adsorption. (b) DOPC adsorbed solely from the gas-solution interface. (c) DOPC adsorbed from liposomes in solution. (d) DOPC adsorbed from the gas-solution interface with liposomes in solution. Light lines: initial (negative) scan. Heavy lines: positive scan. Reprinted from [124]. Copyright Elsevier 2001.
Figure 1.32: Proposed mechanism for liposome interaction with an existing monolayer of DOPC. Inset: Formation of a monolayer from liposome solution. Reproduced from [124]. Copyright Elsevier 2001
1.6 Fluorescence Background

1.6.1 Theory

Molecular fluorescence

Photons of light in the UV or visible range may interact with molecules by absorbance, creating an electronic excited state in the molecule. Obeying the quantum mechanical selection rule $\Delta S = 0$, these transitions will result in an excited state of the same electronic multiplicity (usually singlet) as the initial electronic state. Transitions directly between states of differing multiplicities are forbidden, and thus rare, for an absorption event. Once the molecule is in an electronic excited state, however, it is possible to reach a state of a different multiplicity through intersystem crossing. Relaxation of the excited state can occur by any of several pathways, as summarized in Figure 1.33. Most commonly, the molecule can relax non-radiatively, by vibrational transfer of energy to other nearby molecules. This may be a partial relaxation within the excited state, or may release enough energy to return the molecule to the ground state entirely. From the excited state, the molecule may also relax by release of a photon (fluorescence). As some vibrational relaxation generally occurs before release of a fluorescent photon, it is typically of a slightly lower energy than the absorbed photon. This energy loss is typical of molecular luminescence phenomena, and is called the Stokes shift. Phosphorescence may also occur if the molecule has a triplet state accessible from the excited state.

There are many other possible fates for a molecule after achieving an electronic excited state. The excited molecule may react with another molecule in the ground state, forming an excimer. The molecule may also undergo photo-bleaching, a permanent loss of fluorescence due to a breakdown of the fluorescent molecule.
1.6. Fluorescence Background

Figure 1.33: Partial energy diagram for a photoluminescent system.

itself, or be quenched by energy donation to other species (a non-permanent loss of fluorescence). Quenching has many causes, from collisional quenching by oxygen or heavy atoms to energy donation to other molecules or even a metal surface, discussed in more detail below.

**Förster resonance energy transfer (FRET)**

One decay pathway of an excited fluorophore is to lose its energy through non-radiative transfer to another molecule. In the case of resonance energy transfer, the energy is transferred by long range dipole-dipole interactions between the energy donor and acceptor molecules. The acceptor molecule may relax radiatively or non-radiatively after the transfer. The rate of this transfer, $k_T$, is expressed as

$$k_T(r) = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6$$

where $\tau_D$ is the decay time of the donor molecule in the absence of the acceptor, $r$ is the separation between the donor and acceptor, and $R_0$ is the Förster distance - the
1.6. Fluorescence Background

donor-acceptor separation at which the energy transfer is 50% efficient. The rate also depends on factors such as the relative orientation of the dipoles, the quantum yield of the donor, and the spectral overlap between the donor and acceptor molecules, that are incorporated in to the Förster distance value:

\[ R_0 = 0.211(\kappa^2 n^{-4} Q_D J_D(\lambda))^{1/6} \]  

Here, the Förster distance (in Å) is shown to depend on \( \kappa \), describing the relative orientations of the donor and acceptor’s transition dipoles, \( n \), the refractive index of the medium between the donor and acceptor, \( J_D \), the overlap integral describing the degree of spectral overlap between the emission of the donor and the absorption of the detector, and \( Q_D \), the quantum yield of the donor in the absence of the detector. Typically, the Förster distance is between 20 to 60 Å. [146]

From Equation 1.8, it can be seen that the rate is strongly distance dependent, falling off with \( r^{-6} \). This sensitivity to donor-acceptor separation is exploited in several techniques, such as determination of protein folding structure [147–149], DNA and RNA binding (Figure 1.34) [150–153], and lipid distribution in cell membranes [154–157], among others.

The BODIPY fluorophore (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) is known to form two dimers, termed D_I and D_II (Figure 1.35). Mikhalyov et al. [158], Tleggubulova et al. [159], Bergström et al. [160] Both can accept energy through FRET from nearby monomers. The D_I dimer is non-fluorescent and exhibits an absorption band blue-shifted from the monomer, while the D_II dimer has a red-shifted absorbance, and has an emission maximum at approximately 577 nm, red-shifted compared to the monomer emission maximum at approximately 505 nm. The Förster distance for the BODIPY dimers is relatively long, at 57 Å for the monomer - D_I transfer, and 42 Å for the monomer - D_II transfer. [158] This self-dimerization has been harnessed to monitor relative concentration of fluorophore in surfactant layers, Musgrove et al. [1], Casanova-Moreno and Bizzotto [161, 162], Musgrove et al. [163], Shepherd et al. [164], protein folding, [160] membrane organization, [165] and light-harvesting antennae for artificial photosynthesis.[166]

**Fluorescence near a metal surface**

When the oscillating electric field of a fluorophore is near a metal surface, the near-field radiation from the fluorophore can interact with the metal surface in several
1.6. Fluorescence Background

Figure 1.34: Application of FRET to RNA sensing. (A) Two labeled oligonucleotides hybridize to the target sequence, bringing the fluorophores close enough together that RET is observed. (B) Changes in fluorescence spectrum observed with hybridization. (Donor emission maximum: 503 nm, acceptor emission maximum: 670 nm.) (a) Fluorescence spectrum of the unbound probes. (b) Spectrum in the presence of the target DNA sequence. (c) Spectrum in the presence of a nontarget DNA sequence. Reprinted from [150] with permission from Elsevier.

Figure 1.35: Proposed structures of the BODIPY dimers. Left: the $D_I$ dimer. The distance between the ring planes is 4.9 Å. Right: The $D_{II}$ dimer. The angle between the transition dipoles is $55^\circ$, and the distance between centers-of-mass is 3.8 Å.
1.6. Fluorescence Background

Figure 1.36: Excited state lifetime of Eu\textsuperscript{3+} in air near a silver mirror surface. Dots: Experimental data. Solid line: best fit to data. Reprinted with permission from [168]. Copyright 1975, American Institute of Physics.

Figure 1.37: Oscillating field of dipole interacting by interference with a plane mirror constructively (left) and destructively (right). Reprinted from [167] with permission from Elsevier.

ways. At relatively long distances (greater than about 100 nm), the field can reflect off the surface and interfere with the field radiating from the fluorophore. This results in an oscillating pattern of enhanced and reduced fluorescence with separation of the fluorophore from the surface, as established by early measurements of europium fluorescence near a silver mirror, shown in Figure 1.37. [167]

At shorter separations, the behaviour becomes less easily explained. In some cases, fluorescence near a metal surface can be enhanced [169]. Raman scattering may be similarly enhanced, as seen in the surface-enhanced Raman scattering (SERS) technique. [170] Quenching also commonly occurs at very small separations between fluorophore and metal surface, under similar conditions. The quench-
Fluorescence Background

1.6. Fluorescence Background

ing follows a cubic relation with separation, as expected for a Förster-type energy transfer between the fluorophore and surface. [171] The process of quenching is usually described as energy transfer into so-called “lossy surface waves” in the metal.

It was determined by Lakowicz [172, 173] that both enhancement and quenching processes are caused by coupling of the radiative field from the fluorophore into plasmons in the nearby metal. In enhancement phenomena, the plasmons, located at the surface, are able to radiate the energy. However, in quenching the plasmons are restricted by optical properties of the metal surface, are unable to radiate, and must dissipate the energy as heat. The mode of energy transfer to a nearby metal surface depends on the separation between the fluorophore and metal; at distances greater than 100 nm radiative decay of the excited state dominates, from 10 to 400 nm, coupling with plasmons occurs, and below 10 nm, coupling into the lossy surface waves - plasmons that dissipate as heat - is dominant. The effect depends on the nature of the surface (including thickness of the metal) and on the geometry, with fluorescence enhancement being improved by surface roughness or corrugations on the order of the light wavelength. [174] This principle has been applied extensively in the study of adsorbed organic films, discussed below, as well as in the field of Total Internal Reflection Fluorescence (TIRF), where it allows enhanced imaging of structures just above a thin (less than 100 nm) metal-coated interface (eg. cytoplasmic and cellular regions) while quenching fluorescence from the portion of the membrane in direct contact with the interface.[175]

The quenching phenomenon has also been exploited in making DNA-based sensors (Figure 1.38). In these sensors, a SAM of fluorophore-tagged DNA is formed on an electrode surface. By application of an alternating potential, the conformation of the DNA strands can be altered. As DNA has an inherent negative charge at most pH values, it will be attracted towards the electrode surface at positive potentials, bringing the fluorophore closer to the surface and quenching it. At negative potentials, the DNA is repelled from the surface, lifting the fluorophore away from the metal and reducing its quenching. Single-stranded DNA is much more flexible than double-stranded DNA, so changes in the frequency response of this system to potential perturbations can give information as to the binding state of the DNA at the surface. This has been successfully employed to develop DNA sensors able to detect analyte concentrations of less than 10 pM (a surface density of approximately $3 \times 10^8$ interactions per square centimeter). [176, 177]
1.6. Fluorescence Background

Figure 1.38: Fluorescence sensor for DNA binding. At negative potentials (top), the negatively charged DNA backbone is repelled from the surface and stands erect, dequenching the fluorophore. At positive potentials, the DNA is attracted to the electrode surface and lies flat, quenching the fluorophore. Reprinted with permission from [176]. Copyright 2004 American Chemical Society.

Optics and microscopy

Fluorescence is conveniently measured by optical microscopy, providing not only a general intensity measurement, but a spatial distribution of the fluorescence as an image. In order to facilitate in-situ imaging of thin films, either conventional or episcopic fluorescence (epi-fluorescence) microscopy may be used. In an epi-fluorescence microscope, both the excitation light and the emitted light pass through the microscope objective. In order to restrict the light to the appropriate wavelengths, a series of filters is used, as outlined in Figure 1.39a. Typically, holographic filters are used with the sharpest cutoff possible in the wavelength range to minimize spectral overlap between the filters. Transmission profiles for a typical filter set are shown in Figure 1.39b. Inverted microscope geometries, where the objective looks up on a sample from below, may also be used to accommodate a single-crystal electrode in a hanging meniscus configuration for in-situ imaging through liquid subphases.

The choice of objective for epi-fluorescence microscopy is especially important, as it provides both the incident light and gathers the fluorescent light for analysis. The function of the objective is limited by the design and composition of its component lenses. Individual lenses will have some degree of spherical aberration and spectral aberration, causing light of different wavelengths have slightly differ-
1.6. Fluorescence Background

(a) Schematic of the light path for an inverted epi-fluorescence microscope. The excitation filter selects the desired band from the white light source, which is then reflected off of a dichroic mirror. Emitted light from the illuminated object is captured by the objective, and passes through the dichroic mirror to the camera detector. Reflected incident light is removed by the emission filter.

(b) Typical filter profiles for (1) emission (2) excitation and (3) dichroic mirror filter cube components.

Figure 1.39: Components of fluorescence microscopy.

By combining several lenses of different shapes and refractive indices, this aberration can be minimized. Several objective designs are commercially available that correct spectral aberrations to a greater or lesser degree, but currently the best correction comes from “apochromatic” lenses. Minimizing the chromatic aberration in fluorescence microscopy is not only important to obtain crisp focus in images, but since the excitation and emission wavelengths being used are distinct, ensuring that both share a focal plane is important for obtaining the best signal in the fluorescence measurements.

The resolving power and sensitivity of an objective is a function of it’s numerical aperture (NA). The NA of a lens is defined as

\[ NA = n \times \sin(\alpha) \] (1.10)

where \( n \) is the refractive index of the medium (typically air or water), and \( \alpha \) is the half-angle of the light cone (Figure 1.40). The numerical aperture thus affects the focal length of an objective, and also its resolving power. A point source of light, upon passing through the series of optics in a microscope, will produce a diffraction pattern called an “Airy disc”. The Rayleigh criterion for defining the resolu-
1.6. Fluorescence Background

![Light cone of a microscope objective.](image)

Figure 1.40: Light cone of a microscope objective.

Theoretical limit of a microscope assembly is the separation between two point sources at which the central diffraction spot of one point coincides with the first diffraction minimum of the other point. This is depicted graphically in Figure 1.41. The theoretical limit of spatial resolution for an objective is determined by the wavelength used and the numerical aperture using the Abbe formula:

\[
d = \frac{0.61\lambda}{NA}
\]  

(1.11)

where \(\lambda\) is the wavelength of light used, NA the numerical aperture as defined in Equation 1.10. The quantity \(d\) corresponds to the minimum radius of a particle that can be accurately imaged under these conditions. Features with a radius smaller than \(d\) may be visible, but their size will be convolved to the size of the diffraction disk and thus will have an apparent radius of \(d\), regardless of their true size. [178]

For an objective with NA=0.5 and a wavelength of 500 nm, the diffraction limit would be 610 nm, however this resolution is rarely reached in practice. Several techniques may be applied in an effort to extend this resolution limit, such as confocal microscopy, photoactivated localization microscopy, and scanning near field microscopy. [179]
1.6. Fluorescence Background

Figure 1.41: Rayleigh criterion for spatial resolution. Top: Airy discs for point sources. Bottom: Intensity profiles of the discs. (a) Profile of a single point source. (b) Profile of two discs separated at the Rayleigh diffraction limit. (c) Profile of two discs where the maxima align with the second minima of the other point, providing clearer resolution. Reprinted from [178].
1.6. Fluorescence Background

1.6.2 In-situ Fluorescence Imaging of Adsorbed layers at Electrode Surfaces

Fluorescence microscopy is a powerful tool for illustrating the behaviour of surfactants at the electrode surface. Typical electrochemical measurements such as capacitance provide some information on the state of the interface, but are limited to reporting an average across the entire electrode area and are unable to inform on the fate of any surfactants that are not very near the electrode surface. The use of in-situ fluorescence provides an opportunity to gain information on not only differences in behaviour across the electrode area, but to monitor molecules as they move near the electrode surface, through desorption or other responses to potential change.

As outlined in Section 1.6.1, if a surfactant layer containing a fluorophore is adsorbed onto an electrode surface, fluorescence will be quenched through resonance energy transfer into the metal. If the fluorophore is separated from the surface (as by the “flipping” of DNA molecules [176, 177] or desorption of the layer), the fluorophore will be less quenched and fluorescence will return. This phenomenon was harnessed in early studies of the electrochemical behaviour of 12-(9-anthroyloxy) stearic acid (12-AS). [180] This work showed, by fluorescence measurements taken in a similar geometry to the electro-reflectance experiments of Section 1.5.2, that fluorescence of adsorbed 12-AS (one to three monolayers thick, on Au(111)) was quenched by proximity to the electrode surface. For potentials at which the layer was desorbed based on electrochemical measurements, fluorescence signal was observed, both confirming that the layer had been desorbed from the electrode surface, and that it remained within the focal volume of the setup, thus did not move far from the electrode once desorbed. Similar results were found by Li et. al. in studies of monolayers of cystamine tagged with Alexa 488 fluorophore [181, 182].

True fluorescence microscopy of an adsorbed layer began with studies of octadecanol mono- and bi-layers. Confocal microscopy studies [115] of octadecanol mono- and bilayers, mixed with a small amount of a fluorescent dye, showed a similar behaviour to the previously observed 12-AS layers, with a low fluorescence at potentials where the octadecanol was adsorbed and a sharp increase in fluorescence as the potential was moved to the range where the octadecanol was desorbed from the surface. These studies were extended using traditional fluorescence microscopy
1.6. Fluorescence Background

Monolayers of octadecanol, mixed with a small amount of the fluorescent dye $1,1'$-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DilC$_{18}(5)$), showed fluorescence behaviour correlating with the established changes in capacitance. Depicted in Figure 1.42a, images of the electrode surface clearly show a low fluorescence at potentials where the octadecanol layer is adsorbed, and a higher fluorescence at potentials of desorption. The onset of the increase in fluorescence, more easily visible in Figure 1.42b, corresponds to the higher capacitance desorbed state of the layer. The images clearly show a non-homogenous surface with many presumed aggregates composing the layer at desorption. These structures are remarkably stable through several cycles of adsorption and desorption and are influenced by the structure of the octadecanol layer before adsorption rather than the underlying electrode structure.

Casanova-Moreno and Bizzotto, Shepherd et al. The fluorescence images of Figure 1.43 show that although there is distortion in the layer structure upon deposition, the overall fluorescence properties of the octadecanol monolayer are largely determined by the structure of the floating layer. This study also provides an example of using the FRET properties of the BODIPY fluorophore to probe layer structure. The octadecanol layer in these images contains 1 mol % of the fluorophore BODIPY-HPC (2-(4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-dodecanoyl)-1-hexadecanoyl-sn-glycero-3-phosphocholine), a phospholipid modified to include the BODIPY moiety. The octadecanol layers containing this fluorophore were imaged at the wavelength of monomer emission as well as at the D$_{11}$ dimer emission wavelength. As can be seen in Figure 1.43 (a), there are monomer-rich and dimer-rich regions of the layer even at formation. Dimer-rich regions are expected to be either enriched in fluorophore, allowing more dimer to form, or more fluid in nature, allowing the monomers to aggregate into dimers more easily.

These same techniques have been applied to other systems of adsorbed surfactants. Studies of mixed monolayers of octadecanol and oleyl alcohol show similar behaviour to the “pure” octadecanol layers.
1.6. Fluorescence Background

(a) Differential capacitance (top) and fluorescence images (bottom). The dashed capacitance trace (fluorescence images A-H) corresponds to the initial desorption scan, and the solid trace (images I-P) the return scan. The dotted trace is the capacitance of the electrode in the absence of octadecanol. Reprinted from [185]. Copyright Elsevier 2002.

(b) Average grayscale values (a), mean particle area (b) and number of particles (c). Solid squares: images taken with a sweeping potential perturbation; open circles: images taken with a stepping potential perturbation. Reprinted from [185]. Copyright Elsevier 2002.

Figure 1.42: Fluorescence imaging of an octadecanol monolayer with 3 mol % DilC$_{18}$(5) dye on a Au(111) electrode.
1.6. Fluorescence Background

Figure 1.43: Fluorescence images of an octadecanol monolayer before adsorption (a) and the same region after adsorption onto a Au(111) electrode and the application of a potential of (b) -600 mV/SCE and (c) -800 mV/SCE. The monolayer contains 1 mol% of BODIPY-HPC. Scale bars are 50 µm in all images; top (red) images are of the dimer fluorescence, bottom (green) images of the monomer emission. Reprinted from [162]. Copyright Elsevier 2010.
1.7 Atomic Force Microscopy Background

1.7.1 Theory

Atomic Force Microscopy (AFM) generates images of the sample surface by scanning a probe (a pointed tip mounted on the bottom of a flat cantilever) across the sample surface. The cantilever is mounted on the bottom of a piezoelectric tube which controls the position of the tip and cantilever in the $x$, $y$, and $z$ dimensions to nanometer or better precision. The deflection of the cantilever (up/down as well as left/right) is monitored via a laser spot reflected off of the back side of the cantilever and onto a photodetector.

As the sharp tip is brought near the sample surface, a number of forces act upon it that may affect the imaging qualities. As the tip approaches the surface, it begins to experience attractive (mostly Van der Walls) forces. At a certain point during the approach (point 2 in Figure 1.44), the attractive forces are strong enough to overcome the stiffness of the cantilever, and the tip will “snap to contact” with the sample surface. Once in contact with the surface, the tip presses into the surface, experiencing a repulsion as the sample compresses. If the tip is once again withdrawn from the surface, it will remain in contact with the surface past the point at which the initial snap-to-contact occurred. This is partially due to the attractive forces previously mentioned, but can be enhanced by surface tension of a liquid-coated sample or by spontaneous formation of a water meniscus around the point of contact when imaging in humid air which must be broken in order to remove the tip from the surface. Because of this, a more rigid cantilever is often used for imaging in air to aid in overcoming the attractive forces on withdrawal. When imaging in liquid, these forces are less severe and a more flexible cantilever may be used.

The simplest imaging mode in AFM is “Contact Mode”, where the probe tip is held in gentle contact with the surface and swept in a raster-scan pattern across an area of the surface. The tip is maintained at a constant force on the surface by setting a constant cantilever deflection value based on the position of the reflected laser spot. In order to maintain this position relative to the surface while accommodating surface features, the tip is raised and lowered via the piezoelectric tube. This motion provides a height profile as the tip is scanned across the surface, producing a topographical image of the surface. This imaging mode is effective, however since the tip is in constant physical contact with the surface, it is possible to cause distortion or damage to the sample surface during imaging. This property
1.7. Atomic Force Microscopy Background

Figure 1.44: Tip-sample interactions at specific points in a force-curve cycle for AFM. Point (1): Large tip-sample separation - no interaction. (2): Tip approaching sample and experiencing attractive forces; “snap-to-contact”. (3) Maximum approach point; tip presses into sample and experiences repulsive forces. (4) Retraction; tip no longer indents surface and only attractive forces are felt. (5) Final point of contact, where the tip-sample adhesion force is equal to the restoring force from the bent cantilever. From [186].
1.7. Atomic Force Microscopy Background

has been taken advantage of in nanolithography, etching patterns into substrates at a precision difficult to match with other techniques. [187, 188]

An alternate method for AFM imaging is intermittent-contact mode, also variously called tapping mode, or AC mode. In this technique, the cantilever is oscillated at or near its flexural resonant frequency, and held just above the sample surface, such that the cantilever only contacts the surface at the bottom of each oscillation. The amplitude of the cantilever oscillation is monitored, and the piezoelectric tube is used to maintain the cantilever at a position above the sample where the resonant amplitude is constant. Again, in this method, the movement of the z-scanner needed to maintain the constant amplitude provides the topographical profile while scanning the surface. This method is typically more gentle to the substrate than contact mode, although at high enough oscillation amplitudes it is possible for the tip to move loosely adhered particles on the sample surface. [189]

Typically AC mode imaging uses a piezoelectric oscillator in the cantilever housing to drive the oscillation of the cantilever and tip. This can create some problems, especially when imaging in liquid, as the oscillation of the cantilever chip can couple to resonances in the cantilever holder assembly. In liquid, an increase in the driving amplitude is required to overcome the solution resistance to cantilever movement, exacerbating the problem. An alternative method for inducing the oscillations in the cantilever is “Magnetic AC” or MAC mode. It is functionally identical to regular AC mode, however instead of using a piezoelectric actuator to vibrate the cantilever chip, the cantilever is coated with a thin magnetic coating, and an oscillating magnetic field is generated by a solenoid located directly above the cantilever or below the sample. Use of the magnetic field to drive the oscillation avoids many of the issues with noise associated with the typical AC mode, and has been successfully used to image many delicate samples. [190–193]

As the oscillation of the cantilever is being monitored during AC mode imaging, it is possible to measure its phase shift as well as the amplitude. There is some intrinsic phase lag between the oscillation of the tip and the drive signal being fed to the cantilever, which can be easily accounted for by typical imaging software. After accounting for this lag, the phase relation to the oscillation amplitude is shown in Figure 1.45, calculated by modeling the cantilever as a damped driven harmonic oscillator. Near the resonant frequency, the rate of change of the phase with oscillation frequency is quite large, resulting in enhanced phase contrast with small amplitude shifts.
Damping of the oscillation caused by tip-sample interactions will cause a small shift in the oscillation frequency. The accompanying phase shift is easily measured, and used for phase-contrast imaging modes. The tip-sample interactions may include (but are not limited to) Van der Walls interactions, electrostatic attractions, or chemical interactions. Changes in tip-sample adhesion as well as sample elasticity and viscosity will affect the measured phase lag in the image, probing local variations in surface properties.

Although quantitative analysis of the phase shifts is possible, the presence of a number of complicating factors makes such analysis difficult. Although a change in the surface interactions will produce a change in phase, a change in phase is not necessarily due to a change in surface properties. Importantly, the surface topography will be coupled into the phase contrast. Sharp features (especially indentations with a radius similar to the tip radius) will have enhanced Van der Walls type attractions between the tip and surface. These attractions are local in nature but do not reflect a change in surface properties, only geometry. This causes a change in the phase along these features, highlighting topographical features. Figure 1.46 shows images of polycrystalline gold, for which there should be no variance in tip-surface
1.7. Atomic Force Microscopy Background

Figure 1.46: AFM images of a polycrystalline gold sample. Circled regions highlight narrow regions on the sample where a geometry-induced increase in tip-sample interaction causes a change in the phase offset. From [186].

Figure 1.47: AFM height and phase images of a polyvinyl alcohol thin film. Region 1 is a strongly adhered layer covering most of the substrate. Region 2 is a highly crystalline domain approximately 1 nm high, and Region 3 a heterogeneous, thicker layer approximately 3 nm high. From [186].

interaction based on chemical properties. The circled regions highlight valleys in the surface which create a strong tip-surface interaction that is visible in the phase image, despite being chemically identical to the surrounding regions.

Similarly, other properties such as surface potential can affect the tip-sample interaction, causing a change in phase that is not associated with a region of different chemical properties. For these reasons, phase imaging is often used qualitatively, to highlight regions with varying tip-sample interactions, rather than for quantifying the absolute magnitude of these interactions. Figure 1.47 shows a typical topography-phase imaging pair for a thin film of polyvinyl alcohol which has three distinct domains, differentiable by both the height of the layers and by their phase behaviour.
1.7.2 Atomic Force Microscopy of Vesicles and Adsorbed Layers

AFM has been used extensively to probe the properties of thin films and surface layers. Some of these studies have been discussed in the context of bilayer self-assembly in Section 1.4.1 previously, and will be only briefly mentioned here.

Topographical imaging of vesicle-modified substrates has been an invaluable tool in probing the three-dimensional structure of the adsorbed vesicles and self-assembled phospholipid bilayers. As the vesicles and layers are delicate and only loosely adhered to the substrate, AC or MAC mode is typically used. Many studies have used AFM to determine the size of liposomes adsorbed on a solid substrate (reviewed in [194]), although the sizes measured by AFM may be slightly larger than sizes found in solution due to vesicle deformation when resting on the surface. [195] For small unilamellar vesicles, the measured ratio of width to height of adsorbed vesicles was found to increase to approximately 5, suggesting that some significant deformation occurs for vesicles adsorbed intact on glass surfaces. [196]

Applications beyond simple size measurements are also possible; the use of a high-speed, high force image in contact mode was used to characterize the adhesion of phosphatidylethanolamine bilayers on mica substrates. After modification of the mica surface with a single bilayer, this phospholipid will deposit a second bilayer on top of the first. Figure 1.48 shows the AFM image taken of such a surface after a high-force image of a smaller area was made. Based on the height differences between the area imaged under high-force and the unmodified area, it was shown that even with repeated imaging, only the upper bilayer would be removed by contact with the AFM tip. [197]

Rigidity of the vesicles adsorbed on a substrate is also measurable by AFM - both by height correlation in traditional imaging methods and by force-distance analysis. In the rigidity determination by imaging [198], the adsorbed liposomes are compared to rigid polystyrene spheres of known radius and their geometry compared. More rigid liposomes are assumed to hold a more spherical shape, and were characterized by the ratio of their height measured by AFM over their radius as determined by dynamic light scattering particle size analysis. Using this method, it was found that the type of phospholipid used to form vesicles and the amount of cholesterol incorporated into the membrane had an effect on the overall rigidity of the adsorbed liposomes.

Using force-distance spectroscopy to measure liposome rigidity [199, 200] in-
1.7. Atomic Force Microscopy Background

Figure 1.48: AFM image (taken in contact mode) of a mica surface modified with a partial-double-bilayer of phosphatidylethanolamine, with the centre of the imaging area additionally modified by a high-force imaging scan. Comparison of the heights reveals that the cleared area inside the high-force scan region retains the bottom bilayer and only the second bilayer has been moved by the imaging. Reprinted with permission from [197]. Copyright 1999 American Chemical Society.

volves pushing an AFM tip through the vesicle and recording the force required to burst completely through to the underlying substrate. Figure 1.49 shows a sample data set for the approach and retraction parts of a force-curve cycle, with the two “jump-in” points where the tip pushes through each bilayer clearly visible. The stiffness of these vesicles can then be calculated from the relationship of force applied to tip position. Measurements performed using this technique showed that addition of cholesterol to egg phosphatidylcholine vesicles increased the bending modulus dramatically.

Although in the force curve method, it is possible to easily punch the probe through an adsorbed vesicle, AFM imaging can be delicate enough to resolve fine detail of adsorbed structures. One example of such an achievement is imaging of so-called lipid rafts in adsorbed mixed-component bilayers. Lipid rafts, or microdomains, are cholesterol and sphingolipid rich regions in a bilayer membrane. These regions are believed to exist in a liquid-ordered phase distinct from the liquid-disordered phase of the surrounding bilayer (Figure 1.50), causing a slight difference in bilayer thickness between the two regions. Many studies, for example [201–205], have used atomic force microscopy as a method to investigate the formation and characteristics of these domains. Figure 1.51 shows some AFM topography images of a supported sphingomyelin/DOPC mixed monolayer containing various amounts of cholesterol. The slightly taller microdomains are clearly visible, although the height difference between the raft and membrane bulk is only 0.8 nm or less. Combination of AFM with other techniques, such as fluorescence
1.7. Atomic Force Microscopy Background

Figure 1.49: Force vs distance curve of an AFM tip placed directly onto an egg phosphatidylcholine liposome. Point 1 corresponds to the initial contact of the tip to the top surface of the liposome. The transition from Point 2 to Point 3 is the bursting of the tip through the topmost bilayer of the liposome into the vesicle cavity. The transition at Point 4 is the bursting of the tip through the bottommost bilayer and into direct contact with the substrate. The interactions on the retraction portion of the curve are more complex. Note, however, that the final jump-out point is at a distance greater than the liposome radius as it is elongated due to adhesive forces with the tip during retraction. Reprinted from [199] with permission from Elsevier.
1.7. Atomic Force Microscopy Background

Figure 1.50: Cartoon schematic of a lipid raft (gray molecules with cholesterol) in a liquid-ordered state surrounded by other membrane phospholipids (green molecules) in a liquid-disordered state. Adapted with permission from [202]. Copyright 2007 American Chemical Society.

Atomic Force Microscopy, can provide further information on the bilayer behaviour. In one such study [206] it was demonstrated that a selection of fluorescent dyes will partition into different domains that were also identifiable by AFM imaging.

1.7.3 In-situ Atomic Force Microscopy with Electrochemistry

Atomic Force Microscopy is easily adapted for imaging under liquid, and in fact often produces superior images due to the lack of the tip adhesion caused by spontaneous meniscus formation in air. It is then a logical extension to conduct AFM imaging in an electrochemical cell, and in fact a great deal of work has been done combining potential control with in-situ atomic force microscopy. Of especial interest in the context of this work are studies done on solid-supported organic layers.

Imaging of the adsorption N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate from a low-concentration solution onto an Au(111) substrate under potential control [207] revealed that at larger negative charge densities ($\sigma_M < -5 \mu \text{C/cm}^2$) a “spongy”-textured monolayer forms, while at higher charge densities ($\sigma_M > +5 \mu \text{C/cm}^2$) a blistered film is favored. At intermediate charge densities, a film containing long stripes of hemimicellar aggregates forms. These structures are shown in Figure 1.52, along with the structure of the surfactant. Formation of similar long aggregates at positive electrode potentials was previously observed in AFM and STM imaging of bipyridine monolayers on Au(111). [208] In other experiments, potential-induced orientation changes of DNA helices on a solid support was observed using AFM[209], paving the way for the DNA-based fluorescence sensors under current development. [176, 177]
1.7. Atomic Force Microscopy Background

Figure 1.51: AFM images of mixed sphingomyelin/DOPC monolayers (1:1 mol/mol) containing various amounts of cholesterol. (A) no cholesterol, (B) 10 mol% cholesterol, (C) 20 mol% cholesterol, (D) 33 mol% cholesterol, (E) 50 mol% cholesterol, and (F) 50 mol% cholesterol monolayer deposited from a subphase containing methyl-β-cyclodextrin. All layers are formed on mica by Langmuir-Blodgett deposition. Reprinted from [201] with permission from Elsevier.

Figure 1.52: Atomic force microscopy images of N-dodecyl-N,N-dimethyl-3-ammonio-1-propanesulfonate (structure at left) on a Au(111) electrode at large negative charge density ($\sigma_M < -5 \mu C/cm^2$, leftmost image), intermediate charge density (centre image), and large positive charge density ($\sigma_M > +5 \mu C/cm^2$, rightmost image). At each potential, a characteristic molecular orientation and film structure is observed. Reprinted with permission from [207]. Copyright 2007 American Chemical Society.
1.7. Atomic Force Microscopy Background

Figure 1.53: Atomic force microscopy image (MAC mode, in 0.1 M NaF) of a dimyristyl phosphatidylcholine layer under potential control. The fast scan axis is vertical, and the slow scan axis proceeds from left to right across the image. During imaging, potential was changed from 0.2 V/AgAgCl to 0.45 V/Ag/AgCl (in the centre of the image) and back. The potential profile applied is illustrated above the image, with the applied potential aligned to the portion of the image recorded at that potential. Below the image is a height profile along the line marked on the image. Reproduced from [210] with permission from Annual Reviews.
Two potential-linked states were also found for bilayers of dimyristyl phosphatidylcholine adsorbed on Au(111). [65, 210] Figure 1.53 shows an AFM image taken as the electrode (substrate) potential was scanned from 0.2 to 0.5 V/Ag/AgCl and back. The fast scan axis is oriented vertically in this image, so that each column of image data is acquired at a slightly different potential, progressing along the applied profile as the image progresses from left to right. In the image, it is clear that the lower potentials have a distinct corrugated layer structure that is altered at the more positive potentials, adopting a flatter ‘melted’ structure. The corrugated structure reappears as the potential is returned to lower potentials at the right hand side of the image.

1.8 Scope of the Project

Solid-supported bilayers of surfactants and phospholipids form a major motif in biosensor design. Especially for phospholipid bilayers, the ability to incorporate and study transmembrane proteins in an environment where both the chemical and electrical properties can be controlled is an advantage. Such bilayers are easily formed by self-assembly from a solution of liposomes with the desired characteristics, however phospholipid bilayers deposited directly on a solid surface have some notable disadvantages. They do not contain a large hydrated ‘cushion’ into which transmembrane proteins can extend, reducing their usability. As well, they delaminate from the substrate if removed from the aqueous environment. Some strategies, such as tethered bilayers [78, 79] and use of spacer polymers [74] have been attempted in the past to address these issues.

By first modifying the electrode with a layer of insoluble surfactant (Figure 1.54a), interaction of liposomes directly with the electrode surface is prevented. Creating pores or defects in this passivating layer (Figure 1.54b) would then allow liposomes to interact with the electrode through these pores (Figure 1.54c), and to become incorporated into the adsorbed layer (Figure 1.54d) to produce a final hybrid bilayer. Depending on the characteristics of the initially adsorbed layer, it may be possible to produce a hybrid layer where the phospholipid bilayer regions are buckled or lifted from the surface (as with the hemiliposomal structure illustrated in Figure 1.54d) which would facilitate the use of transmembrane proteins by providing a pocket of electrolyte into which they can extend. The initially adsorbed layer, if it is itself more stable, may also impart some stability to the DOPC bilayer,
1.8. Scope of the Project

Figure 1.54: Illustration of the proposed hybrid bilayer formation process. (a) The electrode is first covered in a physisorbed layer (here, octadecanol). (b) Defects are introduced into the adsorbed layer. (c) Liposomes from solution approach the electrode and interact with the defect sites. (d) The phospholipid bilayer incorporates into the adsorbed bilayer, forming a hybrid layer.

preventing it from delaminating on removal from aqueous solution as bilayers of phospholipids are wont to do.

In order for such a system to be feasible, the surfactant layer must be stable on the electrode surface over a range of conditions, and it must have some mechanism by which to induce defect creation. Octadecanol is well-characterized in electrochemical systems, and offers all of these advantages. Defect creation is easily accomplished by application of a moderate electrical potential (see Section 1.5.2) and is reversible. Using DOPC as a model phospholipid, Figure 1.55 highlights a window of electrical potential in which defects can be created in the octadecanol, but adsorbed DOPC remains stable on the electrode surface. Within this region (-0.2 V to -0.4 V/SCE) liposomes in solution are predicted to have the best opportunity to incorporate into an existing octadecanol bilayer, as depicted in the cartoon of Figure 1.54.

The focus of the work presented in this thesis is the development and characterization of a hybrid octadecanol - DOPC bilayer using the scheme described above. Electrochemical methods, primarily differential capacitance, along with in-
1.8. Scope of the Project

Figure 1.55: Differential capacitance scans during desorption of a DOPC bilayer (top frame) and octadecanol bilayer (bottom). The region highlighted in blue extends from the onset of defect formation in octadecanol (-0.2 V) to the beginning of the first phase change in the DOPC bilayer (-0.4 V/SCE), representing the potential range in which liposomes are expected to be most effective at incorporating into a solid-supported octadecanol bilayer. Capacitance measurements were performed with a 5 mV/s scan rate, 5 mV RMS potential perturbation, and a 25 Hz perturbation frequency.
1.8. Scope of the Project

In situ fluorescence and atomic force microscopy are used to observe the system and determine its behaviour.
Chapter 2

Experimental Methods

This chapter contains the general procedures and instrumental information for experiments presented in the following chapters. In all cases, further experimental detail is included alongside the relevant results.

2.1 Materials

All glassware used was cleaned by heating for two hours in a 1:1 H₂SO₄:HNO₃ bath, rinsing in MilliQ (18.2 MΩ) water, and stored filled with MilliQ water overnight. Electrochemical measurements were performed in a solution of 0.05 M KClO₄ (Fluka puriss, triply recrystallized) in MilliQ water or in a 0.1 M NaF solution (Sigma SigmaUltra) in MilliQ water. A 3 mg/mL solution of octadecanol (Fluka SelectoPhore) was prepared in chloroform (Fisher, HPLC grade). Octadecanol solutions containing various concentrations (0.5 to 8 mol%) of 4,4-difluoro-1,3,5,7-tetramethyl-8-(18-octadecanol)-4-bora-3a,4a-diaza-s-indacene (BODIPY-C19-OH), synthesized in-house[1], were prepared by addition of stock BODIPY-C19-OH solution (7 mg/mL in chloroform) to a clean vial, evaporating the solvent under argon, and filling with 3 mg/mL octadecanol solution. The volumes transferred were monitored by weighing the vial at each transfer step, and the final concentration of the BODIPY-C19-OH in the solution was verified using absorbance. The BODIPY-C19-OH concentrations reported in the text are the nominal values. Actual concentrations were within 0.5% of the nominal values (within 0.05% for the 0.5 mol% solution), but exact values are not reported in the text for clarity as several solutions were used of each concentration throughout the time frame of the experiments.

2.1.1 Electrodes

Electrochemical measurements and in-situ fluorescence measurements were performed on a polished Au(111) electrode, prepared as described previously [183,
2.2. Liposome Formation

Before and after each experiment, the electrode was cleaned by flame annealing with a butane torch and quenching in MilliQ water, repeating a minimum of three times. A gold coil, used as the counter electrode, was prepared using the same procedure.

2.2 Liposome Formation

Liposomes were formed using the extrusion method [211], using a commercial apparatus (LIPEX Extruder, Northern Lipids Inc.) fitted with a polycarbonate filter (Nucleopore 0.1 µm, Costar Corporation). Dioleoyl phosphatidylcholine (DOPC, in chloroform, Avanti Polar Lipids, Inc.) was dried under argon (Supelco) from the as-provided chloroform solution, then sonicated to form a suspension in 0.1 M NaF electrolyte. This suspension was cycled through the extruder under high-pressure argon ten times to form a monodisperse suspension of liposomes. The resulting solution was stored in a closed glass vial under refrigeration. After formation, the size distribution of liposomes was measured with dynamic light scattering (Coulter N4+ particle size analyzer). This procedure and results of particle sizing are described in Appendix B.

2.2.1 Chemical Stability of Liposomes

After formation and periodically throughout the lifetime of the solution, the solution was tested for lipid degradation by thin-layer chromatography (TLC). The liposome solution was spotted (1 µL) onto an aluminum-backed silica gel TLC plate (EMD) along with a sample of stock DOPC solution in chloroform (0.5 µL) and a known degraded liposome solution (1 µL) in adjacent lanes. The degraded liposome solution was prepared from liposomes extruded in the perchlorate electrolyte, saturated with oxygen after preparation, and aged for several weeks. After applying the solution to the TLC plate, the spots were allowed to dry thoroughly and then eluted using a 2:1 chloroform:methanol mixture (both: Fisher, HPLC grade). After elution, the plate was dried and visualized by rinsing in dilute sulphuric acid (Fisher ACS, approximately 1 M) and charring in an oven at 190 C. Multiple spots was characteristic of degraded phospholipid material, thus only liposome solutions producing a single TLC spot were used for electrochemical analysis. Details of the monitoring by TLC are provided in Appendix A.
2.3. Surface Pressure Measurements

Measurements of the floating monolayers of octadecanol were made during compression and at equilibrium spreading pressure (ESP), both independently and with simultaneous fluorescence imaging.

2.3.1 Compression Isotherms and ESP

Compression isotherms of the octadecanol monolayers were measured on a Langmuir trough (Nima model 602A) in the lab of Dr. Guillaume Bussiere. The trough was prepared by wiping clean with isopropanol and a laboratory tissue, then rinsing with deionized water. The trough was then filled with the 0.05 M KClO$_4$ electrolyte and tested to ensure that the bare water pressure value was accurate and that compression of the barrier did not change the surface pressure in the absence of a floating monolayer. Once the trough was prepared and confirmed clean, approximately 20 µL of octadecanol solution was deposited onto the electrolyte surface and the solvent allowed to evaporate. Pressure was measured while compressing the barrier at 150 cm$^2$/min, slowing to 50 cm$^2$/min and 20 cm$^2$/min as surface area decreased.

Measurements of the ESP of the various concentrations of BODIPY-C19-OH in octadecanol were taken by partially filling a 100 mL beaker with the perchlorate electrolyte and assembling a microbalance (Nima, model P54) with Wilhelmy plate slightly off-centre of the surface to avoid the floating octadecanol crystal. Octadecanol solution was added in 1-5 µL aliquots to the surface and the pressure allowed to equilibrate after the chloroform solvent evaporated. Aliquots of octadecanol were added until the measured surface pressure reached a maximum and a visible crystal of excess octadecanol was present at the interface. In some cases, the layer was then “re-formed” by depositing approximately 50 µL of chloroform onto the electrolyte-air interface with a microliter syringe and allowing it to evaporate, consolidating any small crystallites on the surface into one large structure. Surface pressure was considered an “equilibrium” surface pressure if it remained stable, within 0.2 mN/m, for at least 200 seconds.
2.3. Surface Pressure Measurements

2.3.2 In-situ Fluorescence Measurements

A modified Langmuir trough with an optical window below the subphase (Nima model 102M) was used with an epi-fluorescence microscope to obtain fluorescence images of the floating monolayers of octadecanol/BODIPY-C19-OH mixtures (Figure 2.1a). The trough was prepared in a similar manner to that described above, with special care to keep the optical window clean and streak-free. To accommodate the time required for image acquisition, rather than a continuous compression, the layer was compressed by several mN/m then allowed to rest while images were taken. To collect the fluorescence images, a Photometrics Evolve 512 CCD camera was used (16 µm x 16 µm pixel size), Peltier cooled to -80 C. Exposure time and electron-multiplier gain settings were varied from experiment to experiment; these settings are noted with the images. Illumination was provided by a broad-spectrum source (Exfo X-cite Exacite), passed through a filter cube custom-assembled to suit either the monomer or dimer fluorescence of BODIPY-C19-OH (transmission profiles shown in Figure 2.2).

Monomer and dimer images were taken sequentially; monomer first, followed by dimer, using a 10x objective (Olympus LMPlanFl, NA=0.25). The lamp shutter was closed between images to minimize photobleaching. As the trough was immobile, only a small area was available for imaging. To obtain a sampling of the structures present, monomer-dimer image pairs were taken several minutes apart to allow different areas of the floating layer to drift into view when possible.

Images of the floating layer at ESP were taken using the electrochemical cell on the same microscope assembly (Figure 2.1b). The cell was filled with electrolyte to a depth of approximately 5 mm, and the electrolyte was purged of oxygen by bubbling with argon. During measurements, an argon atmosphere was maintained in the cell by a minimal flow of argon gas so as to avoid disturbing the layer during imaging. A 20 µL aliquot of octadecanol/BODIPY-C19-OH solution was deposited onto the electrolyte surface with a microliter syringe and the solvent allowed to evaporate. Several images of the layer were then taken, following the same procedure as in the Langmuir trough, but as the cell was mounted on a movable stage, it was possible to take images of a sampling of the different structures across the electrolyte surface.
2.4. Preparation of Modified Electrodes

2.4.1 Liposome-coated Electrodes

To modify an electrode with a phospholipid bilayer, an electrochemical cell was prepared with 0.1 M sodium fluoride electrolyte. The freshly flame-annealed and cooled Au(111) electrode was introduced into the cell in a hanging meniscus configuration and held under potential control. Liposomes were introduced directly into the electrolyte through the cell’s injection port (Figure 2.3) using a microliter syringe, to a concentration of 30 µg DOPC/mL electrolyte. The liposomes were allowed to diffuse through solution and assemble on the electrode surface until a minimum capacitance value was reached (typically one hour), while differential capacitance was monitored (see Section 2.5.2 for settings). To simulate the conditions that would be required in the fluorescence-type cell, the electrolyte was not stirred.

In some cases, the electrode was modified a second time from the same liposome-electrolyte preparation by removing the electrode, flame-annealing, and re-introducing it to the electrochemical cell already containing liposome solution. In these cases, substantially less time was required to reach the minimum capacitance value (approximately five minutes), though measurements continued for the same hour-long
2.4. Preparation of Modified Electrodes

(a) Transmission characteristics of filters used for BODIPY-C19-OH monomer fluorescence.

(b) Transmission characteristics of filters used for BODIPY-C19-OH dimer fluorescence.

Figure 2.2: Transmission characteristics of filter cubes used for fluorescence measurements.
2.4. Preparation of Modified Electrodes

2.4.2 Octadecanol-coated Electrodes: Single-touch (monolayer)

Adsorbed monolayers of octadecanol were prepared on the Au(111) electrode by Langmuir-Schaefer deposition. A monolayer of octadecanol or octadecanol/BODIPY-C19-OH mixture at ESP was prepared in the electrochemical cell by depositing several (20-30) µL of the relevant solution onto the electrolyte surface and allowing the solvent to evaporate under a flow of argon, leaving a small crystal of excess octadecanol on the surface. The freshly flame-annealed and dried electrode was introduced into the cell and allowed to cool in the argon atmosphere for approximately two minutes, then touched to the electrolyte surface and lifted to form a hanging meniscus while under potential control. For electrochemical studies of octadecanol/BODIPY-C19-OH mixtures, the 0.05 M KClO₄ electrolyte was typically used. Where relevant, the type of electrolyte used is noted in the Results sections.
2.4.3 Octadecanol-coated Electrodes: Double-touch (bilayer)

Electrodes with a bilayer of octadecanol were prepared using sequential Langmuir-Schaefer depositions. First, a floating monolayer of octadecanol or octadecanol/BODIPY-C19-OH mixture at ESP was prepared in a 50 mL beaker or small crystallization dish containing electrolyte. An excess of octadecanol solution (20-50 µL) was deposited onto the electrolyte surface, and the chloroform allowed to evaporate. The beaker or dish was kept covered with a loose lid when not in use. A second floating monolayer of octadecanol was prepared in the electrochemical cell, as described in Section 2.4.2. The Au(111) electrode was flame-annealed and cooled, then touched to the first octadecanol layer (prepared in the beaker) and slowly withdrawn. Any remaining droplet of electrolyte was carefully wicked away with the edge of a laboratory tissue. The electrode was then placed into the electrochemical cell and allowed to sit in the argon atmosphere for 30 sec, then touched to the electrolyte interface and pulled into a hanging meniscus configuration. This method produces an electrode which is expected to have a bilayer of octadecanol similar to the “X type” of bilayer described in the literature.[46]

Composition of bilayer leaflets

For investigations of BODIPY-C19-OH in Chapter 3, the composition of both the ex-situ (in beaker) and in-situ layers (in the electrochemical cell) were varied from pure octadecanol to a maximum concentration of 8 mol% BODIPY-C19-OH in octadecanol. This created both symmetrical bilayers, where both leaflets had the same composition, and asymmetrical bilayers, where one leaflet was pure octadecanol and the other contained the BODIPY-C19-OH fluorophore.

For the investigations of liposome interaction with octadecanol bilayers detailed in Chapters 4 and 5, a single composition was used. In these studies, the ex-situ layer was composed of a 3 mol% mixture of BODIPY-C19-OH in octadecanol, and the in-situ layer of pure octadecanol. The fluorophore-containing layer was thus deposited directly onto the electrode surface, minimizing the metal-fluorophore separation and ensuring that the fluorescence was maximally quenched while adsorbed.
2.4.4 Multiple Depositions From Octadecanol Monolayers

Due to the solid nature of octadecanol at room temperature, the number of good-quality Langmuir-Schaefer depositions that can be performed from a single floating monolayer is limited by the relative surface area of the substrate and of the floating monolayer. For example, it was found during these investigations that a maximum of two such depositions onto the Au(111) electrode could be reliably made from a monolayer prepared in a 50 mL beaker. After this point, the monolayer must be re-formed or a new monolayer deposited in order to maintain a consistent layer quality.

For monolayers composed of pure octadecanol, the monolayer can be re-formed by careful application of chloroform to the electrolyte surface, dissolving the octadecanol at the interface and allowing it to redistribute into a monolayer with the same properties as a freshly deposited one. This reformation of the layer is not suitable for monolayers containing the BODIPY-C19-OH fluorophore, however. For reasons that were not investigated here, the addition of chloroform causes an uneven distribution of fluorophore in the re-formed layer and large non-fluorescent regions are formed. For this reason, for every two ex-situ depositions of fluorophore-doped octadecanol required for an experiment, a fresh layer was formed in a clean beaker. For experiments where the BODIPY-C19-OH fluorophore was located in the electrochemical cell, it was not possible to re-form the layers, so the experiment length was limited to one or two depositions, depending on the size of cell used. For the investigations of liposome interaction with the octadecanol bilayers, pure octadecanol was used in the electrochemical cell in order to facilitate multiple depositions in one experiment day without conflict caused by fluorophore leaching after layer reformation.

2.5 Electrochemical Methods

Electrochemical measurements were performed in electrolyte purged of oxygen by bubbling with argon, and maintained under positive argon pressure during the experiment. A gold wire coil was used for the counter electrode, and a saturated calomel electrode (SCE) connected via a salt bridge was used as a reference. The salt bridge was filled with electrolyte, allowing electrical connectivity while the stopcock prevented mixing of solutions and chloride contamination of the elec-
2.5. Electrochemical Methods

trolyte by the SCE. Figure 2.3 shows a schematic of the typical cell set-up, including the hanging meniscus configuration used to ensure only the polished (111) face of the electrode was used for electrochemical measurements. Electrochemical measurements were taken with a potentiostat and lock-in amplifier (electrochemical station: HEKA PG590 with PAR 175 scan generator and EG&G 5210 lock-in. Fluorescence station: FHI-ELAB 0599 with EG&G 5208 lock-in) and recorded using custom National Instruments LabView software. Experiments outlined in Chapter 3 concerning the effect of BODIPY-C19-OH on octadecanol layers were performed in the 0.05 M potassium perchlorate electrolyte. For the investigations of liposome interaction with octadecanol (Chapters 4, 5, and 6), experiments were performed in the 0.1 M sodium fluoride electrolyte to prevent oxidation of the phospholipids.

After assembling the electrochemical cell and purging it of oxygen, cyclic voltammogram (CV) and differential capacitance measurements were taken of the “full-scale” and “double-layer” potential windows to ensure the cleanliness of the electrolyte (described below).

2.5.1 Cyclic Voltammetry

Cyclic voltammetry was performed at a scan rate of 20 mV/s. For unmodified electrolytes in potassium perchlorate electrolyte, “full scale” CV limits were +1.25 V to -0.8 V, bounded at the positive limit by bulk gold oxidation and by hydrogen generation at the negative limit. “Double layer” CV limits, used for characterizing the electrolyte cleanliness and for comparison with modified electrode, were +0.65 V to -0.8 V. In the sodium fluoride electrolyte, the full scale and double layer limits were +1.1 V to -0.8 V and +0.55 V to -0.8 V, respectively, due to the different pH in this electrolyte.

After modification, electrodes were first scanned in a range where the adsorbates are stable and free from defect formation or oxidation: +0.15 V to -0.15 V. After modification with DOPC or octadecanol layers, the electrode was scanned in this range until the current was stable and any wetting on the sides of the electrode from the hanging meniscus was minimized. After the CV was stable, further characterization by differential capacitance or CV measurements with an expanded potential range could be performed.
2.5. Electrochemical Methods

2.5.2 Differential Capacitance

Differential capacitance measurements were performed with a perturbation of 5 mV RMS and a scan rate of 5 mV/s where applicable. For standard electrochemical measurements, a frequency of 25 Hz was used. For in-situ fluorescence measurements where a stepping potential profile was used, the frequency was increased to 200 Hz in order to allow the lock-in amplifier to acquire a stable reading in a shorter time after each step. At the potentials under investigation, this change in frequency has a minimal effect on the capacitance measurements.

All modified electrodes were characterized by measuring the differential capacitance in the +0.15 to -0.15 V potential range. For investigations of liposome incorporation into octadecanol layers (described in Chapters 4 and 5), in order to ensure all measurements were made on comparable layers, any layers with a minimum capacitance greater than 1.08 μF/cm² were discarded as defective deposits. For other experiments, all layers were used as-formed. Once the capacitance in the +0.15 to -0.15 V range was known, further modification of the layer and capacitance measurements at extended potential ranges could be performed.

2.5.3 Incorporation of Liposomes

After modification of the electrode with a bilayer of octadecanol, liposomes were introduced directly to the electrolyte using a microliter syringe through the injection port of the electrochemical cell (see Figure 2.3). In the larger electrochemical cell, the liposome concentration was approximately 30 μg DOPC/mL electrolyte, while in the smaller cell used for in-situ fluorescence measurements, the concentration was higher, approximately 45 μg DOPC/mL electrolyte. As when forming liposome layers directly on the electrode surface, the electrode was held for 60 min at 0 V/SCE while the liposomes diffuse through the electrolyte and differential capacitance measurements were made.

After the hour, a series of potential steps was applied to the electrode while continuous capacitance measurements were made. The magnitude and duration of the steps was varied, but generally involved a maximum of four stages, as depicted in Figure 2.4.

After the application of the potential step profile, the liposome-modified layer was characterized by a scan to desorption of the octadecanol layer (from +0.15 to -0.8 V/SCE) while measuring capacitance.
Figure 2.4: Potential step profiles used in testing liposome incorporation. In experiments following 2.4c, the time at each potential varied from 1 to 45 min, and one of the three 'poration potentials' shown (-0.4, -0.6, and -0.8 V/SCE) was chosen.

2.5.4 In-situ Fluorescence Methods

In order to further characterize the potential dependent behaviour of octadecanol and liposome-modified octadecanol layers at the electrode surface, in-situ fluorescence images were also taken. The electrochemical procedures were the same as described in Section 2.5 with a few minor modifications. To allow image acquisition at a fixed potential, scanning experiments were performed using a 'staircase' profile rather than a smooth potential ramp as in the simple electrochemical experiments. To simulate the 5 mV/sec sweep rate used in the electrochemical experiments, a potential step of 25 mV was used with a step width of 5 seconds. At the beginning of each step, the acquisition of a fluorescence image and a capacitance measurement was triggered. Each capacitance measurement was the average of 100 individual samples taken in succession. The potential applied was set by the LabView software, and image and capacitance data acquisition was triggered by the same software at the beginning of each step.

While holding at a fixed potential, such as during the 60 min diffusion time or during the application of the potential step profile for liposome incorporation, image and capacitance measurement were triggered at a fixed interval (5 sec, 1 min, or 5 min, as indicated with each dataset).

The camera and light source used was the same as described in Section 2.3.2, however a 50x objective was used for all measurements (Olympus, LMPlanFL, NA=0.5). For the octadecanol/BODIPY-C19-OH studies, a 2.5 sec exposure time was used with an electron-multiplier gain of 200 and an 80% lamp intensity. For the studies on liposome incorporation, two imaging settings were used: 2.5 sec exposure and a gain of 200, or a 5 sec exposure time with a gain of 400, both with
an 80% lamp intensity. The exposure times are specified in the data presentations. The longer exposure and higher gain was used to increase signal from the mostly quenched, adsorbed layer. The lamp shutter was closed in between image acquisitions in order to minimize photobleaching.

2.6 Fluorescence Image Analysis

As the specific analysis performed varies considerably across the experiments discussed in this work, a summary of general techniques used is given here, and details of the analysis are included in the respective results sections. Image processing was performed using the ImageJ [212] software suite. All images were initially processed by applying the built-in Despeckle routine to remove single pixel noise, followed by a 2-pixel radius Median or Accurate Gaussian Blur filter and a subtraction of the dark signal (500 counts). The average grayscale value of the image was taken and this value is reported on the ‘intensity’ plots.

Image sequences were aligned using the StackReg plugin in ImageJ to account for a slight drift in electrode position with time. As this plugin subtracts the minimum intensity value from the first image, this quantity was measured and re-added to the image after alignment. Where a fluorescent background was to be subtracted, the “Subtract Background” routine, which implements a rolling ball filter [213] of a given radius, in this case, 50 pixels. When required, in order to compare image intensities taken at different exposure times in the liposome incorporation experiments, images taken at the higher gain setting (400) were divided by an empirically determined conversion factor to convert the values to the equivalent of the lowest gain value used (200). The average intensity value for each image is thus reported in “kilocounts per second” (kcts/sec). The derivation of the calibrations factors used is shown in Appendix B.

2.7 Atomic Force Microscopy Methods

2.7.1 Instrumentation

An Agilent 5500 atomic force microscope (AFM) equipped with a large scanner (84 µm × 84 µm maximum imaging area) was used to obtain all measurements, using the manufacturer’s provided PicoView software suite. The AFM is housed in
an acoustic isolation box, supported on a lead plinth suspended from bungee cords from the box ceiling in order to minimize vibrational noise from the laboratory surroundings. The instrument is equipped with a video camera, which was used to aid in alignment of the cantilever tip with the desired region of the substrate and for recording a reference of the imaging area location.

In-situ AFM measurements were done in the electrochemical liquid cell (Figure 2.5b). An environmental chamber was used with a positive-pressure flow of nitrogen gas in order to provide a clean and oxygen-free atmosphere. As the environmental chamber is not airtight, and the nitrogen gas is not bubbled directly into solution in the liquid cell, oxygen may not be completely removed from the electrolyte during imaging. However, this is not expected to affect the experiments presented here, as oxygen is not significantly electrochemically active at the potentials used.

A µ-Autolab potentiostat (Metrohm) was used to provide potential control for in-situ measurements, controlled via the manufacturer’s provided software. A gold bead pseudo-reference electrode was used, with an Au coil counter electrode. Although the Au bead is not a true reference electrode, it provided a stable enough reference to function in the electrolyte and potential ranges used. This reference, based on the position of the oxide peaks of a bare Au CV in the same electrolyte, was approximately -0.1 V/SCE.

### 2.7.2 Substrates

Atomic force microscopy was performed on either a gold bead electrode or a gold-on-mica substrate. Non-electrochemical measurements were also performed using the back of a gold-coated mica substrate, where the imaging surface was freshly cleaved mica, prepared by lifting off the top layer of mica using a fresh piece of Scotch tape (3M) and dusting away stray mica flakes with a stream of dry argon.

The gold bead electrode was prepared by heating a gold wire with a butane torch until it melted to form a bead, then allowing the bead to cool slowly in the flame, forming large flat facets on the bead surface. The bead was then electropolished in 0.1 M perchloric acid (Fisher Ultima) by application of an 100 mA/cm² current for 30 seconds, followed by a rinse in 0.1 M hydrochloric acid (Fisher ACS) and a rinse in MilliQ water. This polishing procedure was repeated three times. Before and after each experiment, the bead was cleaned by flame annealing.
2.7. Atomic Force Microscopy Methods

Figure 2.5: Assembly of AFM electrodes used for imaging. 2.5a shows the Au bead with wire inserted into the Teflon block and mounted into sample plate. 2.5b is a top-view of the AFM electrochemical cell used for in-situ measurements. WE=working electrode, CE=counter electrode, RE=reference electrode. The WE is Au on mica, and forms the base of the cell, held in place by clamps fixed to the cell plate. The CE and RE are gold wires.

with a butane torch, maintaining a red heat for at least five seconds, then quenching in MilliQ water. The bead was annealed at least three times in this manner, then gently dried in the butane flame and allowed to cool in air before use.

Once formed, the bead was cleaned before use by the flame annealing procedure described in Section 2.1.1. After any necessary surface modifications were performed (see below), the bead was aligned to present a horizontal Au(111) facet for imaging. The electrode stem was inserted into a Teflon sample holder (Figure 2.5a) and a laser spot was reflected from the facet. The bead alignment was manually adjusted to within $1^{\circ}$ of horizontal by bringing the reflected laser spot into alignment with the laser source. The substrate was then mounted in the AFM and ready for imaging.

For in-situ AFM measurements, a gold on mica substrate (Agilent Technologies) was prepared by flame annealing with a butane torch. The substrate was placed on a clean quartz plate and the flame passed over it several times over a period of approximately 15 seconds. The substrate was allowed to cool for several seconds in air on the plate before use. It was then mounted in the liquid cell (Figure 2.5b), which was cleaned using the same procedure as for the glassware, using a $\text{H}_2\text{SO}_4-\text{HNO}_3$ bath and storage in MilliQ water, with the exception of the O-ring, which was cleaned by rinsing in methanol and MilliQ water.
2.7.3 Surface Modification

For AFM analysis, octadecanol monolayers were deposited onto Au bead electrodes or Au/mica substrates using a method similar to that described in Section 2.4.2. The substrates were cleaned and prepared as described above. A floating monolayer of octadecanol or a 3 mol% mixture of BODIPY-C19-OH in octadecanol at ESP was formed on a small crystallization dish of electrolyte (either 0.1 M NaF or 0.05 M KClO₄) in a method similar to that described for the electrochemical cell above, excepting that the dish was not in an argon atmosphere but in air, protected with a loose-fitting lid. The electrode was then oriented so that the flat surface was parallel to the electrolyte surface, gently touched to the electrolyte, and slowly removed. Any small droplet of electrolyte remaining on the electrode was wicked away with the corner of a laboratory tissue (Kimwipe Delicate Task Wiper, Kimberley-Clark), if necessary aided by first moving it to the edge of the electrode with a gentle flow of argon.

Bilayers for AFM imaging (Au bead and Au/mica substrates) were prepared in a similar manner. The floating monolayers were prepared in a larger crystallization dish (diameter approximately 10 cm) to ensure enough monolayer surface area was available to perform two sequential depositions. The flat imaging area of the substrate was touched twice to the surface using the method described above. Between touches, the droplet of electrolyte was wicked away, and the second touch was performed on a region of the surface geometrically as far as possible from the area of the first touch in order to obtain the best quality deposition possible. After the second deposition, the droplet of electrolyte was again wicked away from the surface, and the substrate dried under a stream of Ar for several seconds before assembly into the AFM. When imaging in air, the electrode was also allowed to dry further by resting, mounted in the AFM in the isolation box, for approximately 30 min.

2.7.4 Imaging Conditions

All images of modified electrode surfaces were taken using intermittent-contact mode imaging (AC or MAC). Images for fluorescence correlation and basic layer characterization were taken in air, using either the Au bead electrode or the Au/mica electrode. All in-air images were taken in AC mode, using silicon probes with a nominal resonant frequency of 190 kHz (VistaProbes, model T190R), tuned to a 1.5 V amplitude and -0.1 kHz off-peak, using 80% of the free oscillation amplitude.
2.7. Atomic Force Microscopy Methods

as the setpoint. Images in solution were taken in MAC mode, using Type II MAC cantilevers (Agilent, nominal force constant 2.8 N/m, resonant frequency 75 kHz in air) or Type I MAC cantilevers (Agilent, nominal force constant 0.6 N/m, resonant frequency 75 kHz in air). The same tuning parameters were used with these tips as with the VistaProbes tips. For most images, a scan frequency of 1 Hz was used and a resolution of 256 or 512 lines/image. For the large 84 μm images, a scan rate of 0.1 Hz was used and a resolution of 2048 lines/image.
Chapter 3

Effects of Fluorescent Dye on Octadecanol Monolayers

The multicomponent bilayer model under investigation here (see Section 1.8) relies on using an adsorbed layer of octadecanol covering the electrode surface which both prevents liposomes from interacting with the electrode surface in an uncontrolled manner, and provides the means to create defect areas into which they will later incorporate. In order for potential controlled incorporation of liposomes to proceed in this model, it is important to characterize the initially adsorbed octadecanol layer. This will be accomplished with electrochemical measurements and via in-situ fluorescence microscopy. As neither octadecanol nor the DOPC used in the liposomes is natively fluorescent, a small percentage of a fluorescent dye molecule must also be incorporated into the adsorbed layer.

Adding a second component to the monolayer runs the risk of disrupting the layer structure, creating defects and making the depositions less reproducible. Even though the BODIPY-C19-OH fluorophore was chosen for its structural similarity to octadecanol, its presence will interfere with the organization of the octadecanol monolayer to some degree. The concentration should be kept at a minimum in order to maintain a good quality adsorbed monolayer. However, the need to obtain the sufficient signal from the fluorescence imaging demands that the fluorophore concentration in the layer be kept as high as possible. Especially for studies of the adsorbed layers, where most of the fluorescence will be quenched by energy transfer into the nearby metal, maintaining a strong fluorescence signal is important.

These two conflicting demands require that an optimum fluorophore concentration is used, one that provides sufficient fluorophore in the monolayer while retaining the structural and electrochemical properties of the pure octadecanol monolayer. The studies presented in this chapter will characterize the effects of BODIPY-C19-OH on an octadecanol monolayer, and direct the selection of the fluorophore concentration that is most suitable for use in the liposome incorporation studies.
3.1 Compression Isotherms

One of the simplest methods to characterize the properties of thin monomolecular films is by measurement of the compression isotherms relating film pressure to the mean molecular area at the surface. The measurement of these isotherms for octadecanol monolayers with varying concentrations of BODIPY-C19-OH will measure the influence of the fluorophore on the layer structure.

3.1.1 Experimental Methods

A large surface area Langmuir trough was cleaned and prepared as described in Section 2.3.1 using as the subphase 0.05 M KClO$_4$ electrolyte. Several µL of an octadecanol solution containing 0 to 8 mol% of BODIPY-C19-OH were deposited onto the air-solution interface and the solvent allowed to evaporate. The barrier was closed at 150 cm$^2$/min, slowing to 20 cm$^2$/min as surface area decreased, while measuring the film pressure.

The equilibrium spreading pressures (ESP) of the monolayers were measured separately by spreading a small amount of the octadecanol/BODIPY-C19-OH solution onto the air-solution interface of a beaker filled with 0.1 M KClO$_4$ electrolyte and monitoring film pressure as more of the octadecanol mixture was added, until a maximum film pressure was reached. Details of this procedure are also found in Section 2.3.1.

3.1.2 Results

Typical compression isotherms for each fluorophore concentration are presented in Figure 3.1. The isotherm for pure octadecanol (black line) closely resembles literature reports for the octadecanol isotherm (Figure 1.4, [35]). As the concentration of BODIPY-C19-OH increases, a bump appears at approximately 15 mN/m, which develops into a plateau at higher BODIPY-C19-OH concentrations. The appearance of a plateau at intermediate film pressures is typical of layers containing two completely or partially immiscible components, and suggests that the BODIPY-C19-OH is not distributed evenly in the octadecanol monolayer.

Measurements of the ESP of the octadecanol/BODIPY-C19-OH layers (Figure 3.2) shows a minimum in ESP at approximately 3 mol%. This may be due to some change in layer structure between the low and high concentrations. In the
3.2. Electrochemical Characterization

Figure 3.1: Compression isotherms for monolayers of octadecanol containing 0, 1, 3, 5, and 8 mol% BODIPY-C19-OH. Film pressure range plotted is limited to the region below the minimum collapse pressure for all layers.

The plateau at approx. 15 mN/m is much more pronounced in the 5 and 8 mol% than might be expected from the trend in the 1 and 3 mol% layers, supporting the observation that there is a change in behaviour around the 3 mol% concentration. This observation is supported by electrochemical study of these layers, discussed next.

3.2 Electrochemical Characterization

Examination of the electrochemical behaviour of the octadecanol/BODIPY-C19-OH layers will be influenced by the character of the monolayers formed at the air-solution interface. A layer with many defects will, when deposited onto the electrode, show a higher minimum capacitance value, and different desorption characteristics in the cyclic voltammogram and differential capacitance scans. As well, it is important to ensure that the layer chosen to use for the liposome interaction studies can be formed reproducibly from the floating monolayer and has consistent desorption and poration characteristics.
3.2. Electrochemical Characterization

3.2.1 Experimental Methods

Electrode preparation and electrochemical measurements were performed according to the general procedures outlined in Sections 2.4.1 and 2.5. For these studies, various solutions of octadecanol containing different amounts of the BODIPY-C19-OH fluorophore were prepared for use in forming the floating monolayers used for deposition from the floating monolayer onto the electrode. Monolayers of octadecanol were deposited onto the electrode using the Langmuir-Schaefer method described in Section 2.4.1. Bilayers were formed by performing two sequential Langmuir-Schaefer depositions from the floating monolayer in the electrochemical cell. Between the first and second depositions, the electrode was removed from the cell, and the droplet of electrolyte remaining on the electrode surface gently wicked away with the edge of a laboratory tissue. The electrode was then re-introduced into the electrochemical cell, allowed to equilibrate in the Ar atmosphere for approximately 5 seconds, and then touched to the electrolyte surface to form the second Langmuir-Schaefer deposition.

All measurements are performed in the hanging meniscus arrangement, ensuring that the electrode area is constant across measurements, and that only the Au(111) face is exposed to solution. After deposition, each octadecanol mono-
or bi-layer was monitored by cyclic voltammetry between +0.15 V/SCE and -0.15 V/SCE until subsequent scans overlapped, an indication that the edges of the electrode had dried. The potential scan limits were then increased to +0.15 V/SCE to -0.8 V/SCE and the layer cycled until an equilibrium response, where sequential scans overlapped exactly. This equilibrium CV was recorded, then a differential capacitance measurement was made using the same potential range. As mentioned in Section 1.5.2, significant changes in the layer occur, especially between the first and second potential sweeps. Recording the equilibrium scans removes any uncertainty in comparing the sequentially acquired CV and capacitance scans.

3.2.2 Results

The CV and capacitance scans for monolayers of the BODIPY-C19-OH concentrations tested are shown in Figure 3.3. The leftmost (black) scans show an example of pure octadecanol, with no fluorophore added. As the concentration of the BODIPY-C19-OH increases, deviations from this “ideal” behaviour become more pronounced. At 0.5 mol%, a small shoulder appears on the tallest pseudocapacitance peak of the adsorption scan (at approx. -0.2 V/SCE), showing that there is some disruption in the octadecanol layer even at these low concentrations. This disturbance is also visible in the capacitance scan as a distortion of the peak at the same potential. At higher concentrations, a pair of pseudocapacitance peaks on the CV at approximately -0.15 V/SCE appear, along with a spike in the capacitance scans. These capacitance peaks are caused by potential driven changes in the organization of the adsorbed layer. New peaks are an indication that the dye is causing some disruption in the structure of the octadecanol layer.

A comparison of the minimum capacitance values for each layer relates the general quality of the adsorbed layer. The values for the minimum capacitance of each fluorophore concentration are overlaid with the ESP in Figure 3.2. A layer with fewer defects and higher coverage will have a lower capacitance while adsorbed (for monolayers, between approximately -0.15 and +0.15 V/SCE). The pure octadecanol unsurprisingly has the lowest minimum capacitance. The minimum capacitance of the octadecanol monolayer containing 0.5 mol% of the fluorophore is similar to the pure octadecanol value, reflecting the small amount of disruption caused by the low concentration. The 1 mol% and 5 mol% monolayers show a higher minimum capacitance than the octadecanol reference, indicating that the
3.3 Fluorescence - Floating Layers and Compressions

Monolayer structure is more significantly disrupted, containing more defects. The 3 mol% monolayer shows the lowest minimum capacitance after the 0.5 mol%.

Monolayers of the 8 mol% octadecanol mixture were also studied in this manner, however the quality of the monolayers were uneven, ranging from “good quality” layers similar to pure octadecanol to layers that showed more disruption than the 5 mol%, and a representative scan was unable to be selected. This behaviour suggests that at 8 mol%, there is a high degree of segregation in the layer, resulting in well-organized regions of nearly pure octadecanol, and highly disrupted regions in the floating monolayer that are variously sampled on deposition onto the electrode.

The trend in minimum capacitance does not follow the trend in fluorophore concentration. However, the trend does follow the trend in ESP (Figure 3.2) where the 3 mol% corresponds to a minimum in the ESP value.

Thus, based on the studies of the electrochemical behaviour of the adsorbed octadecanol monolayers, the 3 mol% concentration is recommended as the maximum fluorophore for future study.

Since future studies on liposome incorporation would be performed with octadecanol bilayers, measurements of bilayers with various concentrations of the BODIPY-C19-OH fluorophore were performed in the same manner. Figure 3.4 shows typical CV and capacitance scans at concentrations from 0 to 3 mol% of the fluorophore in octadecanol. Again comparing the minimum capacitance values for each layer, the 0.5 mol% solution shows the lowest capacitance value of the fluorophore-containing layers, and thus the least amount of disruption, followed by the 3 mol%. The 5 and 8 mol% solutions, already shown to be less stable in monolayer studies, were not deemed appropriate for continued study of bilayer electrochemistry.

3.3 Fluorescence - Floating Layers and Compressions

Monolayers of octadecanol containing 1 mol% and 3 mol% of the BODIPY-C19-OH fluorophore were examined using a Langmuir trough equipped for in-situ fluorescence measurements. Fluorescence imaging at the ESP of such layers have been reported previously [114, 162] as well as Brewster angle microscopy [40] (reviewed in Sections 1.3 and 1.6.2), showing a variety of structures present in the floating layer, both in pure octadecanol and in fluorophore-doped octadecanol.
Figure 3.3: Cyclic voltammograms (top) and differential capacitance scans (bottom) of octadecanol monolayers containing various concentrations of BODIPY-C19-OH on Au(111). Gray dotted lines are Au(111) with no octadecanol. From left to right: pure octadecanol, 0.5 mol%, 1 mol%, 3 mol%, and 5 mol% BODIPY-C19-OH in octadecanol. CV measurements were acquired using a scan rate of 20 mV/s. Capacitance measurements were made using a 5 mV/s scan rate, 5 mV RMS potential perturbation, and a 25 Hz perturbation frequency. The increased capacitance (above the bare gold value) for 5 and 8 mol% is believed to be linked to increased area caused by wetting of the sides of the electrode.
3.3. Fluorescence - Floating Layers and Compressions

Figure 3.4: Cyclic voltammograms (top) and differential capacitance scans (bottom) of octadecanol bilayers containing various concentrations of BODIPY-C19-OH on Au(111). Gray dotted lines are Au(111) with no octadecanol. From left to right: pure octadecanol, 0.5 mol%, 1 mol%, and 3 mol% BODIPY-C19-OH in octadecanol. CV measurements were acquired using a scan rate of 20 mV/s. Capacitance measurements were made using a 5 mV/s scan rate, 5 mV RMS potential perturbation, and a 25 Hz perturbation frequency. The increased capacitance (above the bare gold value) is believed to be linked to increased area caused by wetting of the sides of the electrode as the meniscus was held lower due to reduced surface tension in the presence of octadecanol.
layers. Study of the BODIPY-C19-OH containing monolayers will elucidate the structure of the octadecanol monolayers used for adsorption onto the electrode surface, as well as the distribution of the fluorophore within the layer. The ability of the BODIPY fluorophore to form a dimer that emits a red-shifted fluorescence (see Section 1.6.1) can be used as an indicator of the local concentration of the fluorophore within the layer. Regions of high fluorophore concentration should have a stronger dimer fluorescence signal. The higher concentration implies that the fluorophores are closer together, enabling both dimer formation and FRET energy transfer from the BODIPY monomers to create the dimer excited state. Thus, the relative monomer and dimer signal can be used as a rough indicator of the local concentration of the BODIPY fluorophore.

3.3.1 Experimental Methods

A Langmuir trough specially equipped for in-situ microscopy (Figure 2.1a) was prepared according to the procedures in Section 2.3.1. An aliquot of the desired octadecanol solution (1 -3 µL, less than the amount required for monolayer formation at ESP) was deposited onto the air-electrolyte interface of the trough with the barriers fully open. The solvent was allowed to evaporate and the layer to equilibrate until the surface pressure provided a stable value. Fluorescence images of the surface were taken, first using the green emission filter (BODIPY monomer), then the red emission filter (BODIPY DII dimer). As the DII dimer is non-fluorescent (see Section 1.6.1), it is not detectable by this method. After images of the uncompressed layer were taken, the barriers were partially closed to achieve a film pressure of between 25 and 35 mN/m, and further images taken with both the red and green fluorescence filters. This range of film pressure was chosen as it is near the ESP of the floating layers, but below the collapse point.

Images were taken with a 10x objective, using an exposure time of 0.1 s and electron multiplier gain of 200. In between images, the shutter was closed in order to reduce photobleaching. The layer was slightly mobile on the surface, so although it was not possible to move the focal area around the interface to image different areas, by waiting approximately 5 min between image pairs it was possible to view new regions of the floating monolayer with each acquisition.

Because the filter cubes must be switched manually, there is a small time delay between green and red images, during which the floating monolayer may drift
3.3. Fluorescence - Floating Layers and Compressions

Figure 3.5: Fluorescence images of a floating octadecanol monolayer containing 1 mol% BODIPY-C19-OH fluorophore in a Langmuir trough. Images taken with barriers open; film pressure is less than 15 mN/m. Each vertical image pair is of approximately the same region of the interface, using the green emission filter (top) and red emission filter (bottom). Acquisition conditions: 10x objective, 0.1 second exposure time, electron multiplier gain 200. Scale bar is 130 µm.

within the viewing area. For image analysis, the two images were first aligned in ImageJ using visible features and any non-overlapping area cropped out. The aligned images were then processed with a Despeckle filter followed by a 2-pixel Median filter. The aligned images were then divided (Red / Green) using the Image Calculator function to provide the red:green fluorescence ratio.

Regions within each image were identified as either “background” - the lower intensity, flat or slightly mottled regions typically found in most of the image; or “hotspots” - small regions of higher fluorescence intensity, and the red:green ratio analyzed separately for each region type. Other structures, such as stripes or large bright regions, were identified but not included in the ratio analysis as they were not present in all images analyzed.

3.3.2 Uncompressed Layers

In the uncompressed, as-deposited floating monolayer, a variety of structures are visible. In the images with 1 mol% of the BODIPY-C19-OH fluorophore in octade-
Figure 3.6: Fluorescence images of a floating octadecanol monolayer containing 3 mol% BODIPY-C19-OH fluorophore in a Langmuir trough. Images taken with barriers open; film pressure is less than 15 mN/m. Each vertical image pair is of approximately the same region of the interface, using the green emission filter (top) and red emission filter (bottom). Acquisition conditions: 10x objective, 0.1X second exposure time, electron multiplier gain 200. Scale bar is 130 µm.
3.3. Fluorescence - Floating Layers and Compressions

canol (Figure 3.5), the observed structures include an overall mottled texture, inclusions of differently-fluorescent regions, such as in the centre column, and bright stripes and hotspots, prominent in the rightmost image pair. In the small hotspot regions, the increased green fluorescence signal from the BODIPY monomers is accompanied by an increase in red dimer fluorescence. This behaviour is as expected for a region enriched with the fluorophore, as the molecules present in higher concentration should therefore be closer together and more likely to both form the dimer and become electronically excited through FRET with a nearby monomer molecule.

A few regions (such as the “blob” in the centre column in Figure 3.5 and the bottom corner of the leftmost column in Figure 3.6) show an increase in the green fluorescence signal with no corresponding increase in the red intensity. It appears that in these regions the concentration of BODIPY-C19-OH is larger than surrounding areas, but the fluorophores are positioned far enough apart that dimer formation is discouraged.

Despite the differences in structure implied in the electrochemical results in Section 3.2, the structures seen in the 3 mol% layer are similar to those in the 1 mol% layer. As monolayers studied electrochemically were formed from monolayers at ESP, a study of more compressed monolayers will speak more directly to the electrochemical results.

3.3.3 Compressions of Layers

After imaging at low surface pressure, the barriers of the Langmuir trough were closed until the surface pressure had increased to between 25 and 35 mN/m, a value similar to the ESP, and below the collapse pressure of the layer. Although for both 1 mol% and 3 mol% layers large areas of the same mottled texture seen in the uncompressed layers are common, in the 1 mol% layer the large bright regions seen in the centre and rightmost columns of Figure 3.7 are more common than in the 3 mol% layer. The presence of these regions agrees with the hypothesis based on the Langmuir isotherms that the dye is not mixing ideally with the octadecanol in the monolayers. As seen in the uncompressed layers, these regions have an increased green (monomer) signal but no increase in red (dimer) signal, so the molecular structure of these regions must be such that the fluorophores are significantly separated despite the increase in concentration.
3.3. Fluorescence - Floating Layers and Compressions

Figure 3.7: Fluorescence images of a floating octadecanol monolayer containing 1 mol% BODIPY-C19-OH fluorophore in a Langmuir trough. Images taken with barriers partially closed; film pressure is approximately 25 mN/m. Each vertical image pair is of approximately the same region of the interface, using the green emission filter (top) and red emission filter (bottom). Acquisition conditions: 10x objective, 0.1 second exposure time, electron multiplier gain 200. Scale bar is 130 µm.
3.3. Fluorescence - Floating Layers and Compressions

Figure 3.8: Fluorescence images of a floating octadecanol monolayer containing 3 mol% BODIPY-C19-OH fluorophore in a Langmuir trough. Images taken with barriers partially closed; film pressure is approximately 25 mN/m. Each vertical image pair is of approximately the same region of the interface, using the green emission filter (top) and red emission filter (bottom). Acquisition conditions: 10x objective, 0.1 second exposure time, electron multiplier gain 200. Scale bar is 130 µm.
3.3. Fluorescence - Floating Layers and Compressions

<table>
<thead>
<tr>
<th>Feature Type</th>
<th>Layer Status</th>
<th>R/G Ratio (1% layers)</th>
<th>R/G Ratio (3% layers)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>Uncompressed</td>
<td>0.392</td>
<td>0.438</td>
</tr>
<tr>
<td></td>
<td>Compressed</td>
<td>0.538</td>
<td>0.550</td>
</tr>
<tr>
<td>Hotspots</td>
<td>Uncompressed</td>
<td>0.320</td>
<td>0.447</td>
</tr>
<tr>
<td></td>
<td>Compressed</td>
<td>0.283</td>
<td>0.903</td>
</tr>
</tbody>
</table>

Table 3.1: Average red:green (R/G) ratios for octadecanol monolayers containing 1 mol% and 3 mol% BODIPY-C19-OH. Measurements are shown for the mottled “background” regions and for the small bright “hotspot” regions in each image type. Images of the uncompressed layers were taken with trough barriers open; film pressure less than 13 mN/m. Images of the compressed layers were taken with barriers partially closed at film pressures 25-35 mN/m. A selection of the images used for measurement are shown in Figures 3.5-3.8, however these ratio values are based on images of 5-7 regions at each pressure and concentration.

Comparing the ratio of the red:green (dimer:monomer) fluorescence intensity can provide some information on the local variations in concentration of the BODIPY-C19-OH fluorophore within the monolayer. As the fluorophore concentration increases, the distance between the molecules should decrease, causing more dimer to be formed and increasing the likelihood of excitation by FRET with nearby monomers. Thus, as the local concentration of the fluorophore increases, an increase in the red:green ratio should follow.

The regions that were present in all images (the mottled “background” and small hotspots) were analyzed and the average red:green ratios calculated for each image set. These ratios are presented in Table 3.1. In both the compressed and uncompressed image sets, the 3 mol% layer consistently has a higher red:green ratio than the 1 mol% layer for each region, as expected for a layer of higher concentration. As the layers were compressed, the red:green ratio also increased for each region, with the exception of the 1 mol% hotspots. As the mean molecular area decreases with compression, the molecules are closer together, and the amount of dimer present therefore increases, increasing the red:green ratio. The decrease in ratio for the 1 mol% hotspots might indicate that some of the areas identified as “hotspots” in the compressed layer might have a different structure than those analyzed in the uncompressed or 3 mol% layers, perhaps similar to the intensely green regions discussed above.

These ratios do not provide a quantitative measure of the relative amounts of
monomer and dimer present in the layer for several reasons. First, the D_{II} dimer does not absorb strongly in the spectral region passed by the excitation filter, and is instead excited by FRET from nearby monomer molecules. Thus, an increase in the dimer fluorescence must come at the cost of some monomer signal as the monomer excited state is fed into the dimer excitation rather than directly fluorescing. As well, there may be dimer present that is unable to fluoresce due to a lack of nearby monomer molecules. In addition to these chemical considerations, the sensitivity of the CCD used for image acquisition varies across the wavelength range, being more sensitive to red than green light. With these complicating factors, the intensities and ratios from the fluorescence images can report only on the relative changes in the fluorophore state.

3.4 In-situ Fluorescence with Electrochemistry

Based on the Langmuir isotherm, ESP, and floating layer fluorescence imaging, the 3 mol% concentration of BODIPY-C19-OH fluorophore appears to be the best choice for future study, providing the maximum fluorophore concentration and therefore maximum fluorescence signal, while retaining the electrochemical characteristics of a well-organized octadecanol layer.

For the studies investigating the interaction of liposomes with adsorbed octadecanol layers, a bilayer of octadecanol will be used. Therefore the fluorescence and electrochemical characteristics of the BODIPY-C19-OH containing octadecanol bilayers must be established.

3.4.1 Experimental Methods

The Au(111) electrode and in-situ fluorescence cell were cleaned and prepared as described in Chapter 2. A bilayer of octadecanol was deposited by two sequential Langmuir-Schaefer touches, the first ex-situ in a beaker containing a monolayer of octadecanol with 3 mol% BODIPY-C19-OH, and the second in the electrochemical cell, of pure octadecanol. This is the same bilayer configuration that is used for all following work studying liposome incorporation.

The modified electrode was then scanned by cyclic voltammetry between +0.15 and -0.15 V/SCE until successive scans overlapped, indicating that the sides of the electrode in the hanging meniscus had dried, and the deposited layer was stable.
3.4. In-situ Fluorescence with Electrochemistry

The initial capacitance was measured by a differential capacitance scan from +0.15 to -0.15 V/SCE.

In-situ fluorescence measurements were taken using the 50x objective and an exposure time of 2.5 sec and electron multiplier gain of 200, with images taken every 5 sec. The potential was stepped by 25 mV with each image to simulate a 5 mV/sec scan rate. The electrode was first stepped from +0.15 V/SCE to -0.5 V/SCE and back to explore the fluorescence behaviour of the electrode during layer poration. This scan was repeated three times, twice with the green emission filter, and a third time with the red emission filter. The octadecanol bilayer was then desorbed and readsorbed by cycling the potential from +0.15 V/SCE to -0.8 V/SCE and back. This scan was also repeated three times, twice with the green emission filter and a final time with the red emission filter.

The triple cycle method was used in order to measure fluorescence scans with both monomer (green) and dimer (red) fluorescence images that are able to be directly compared. As previously established (see Section 1.5.2) the virgin octadecanol layer formed with the Langmuir-Schaefer method has different electrochemical properties than the subsequent desorptions due to octadecanol molecules changing orientation. Thus, the first scan in these experiments was not used for analysis, and only the second (green) and third (red) desorption scans were used for comparison.

3.4.2 Results

Fluorescence and differential capacitance scans for the octadecanol bilayer are shown in Figure 3.9. The scans to -0.5 V/SCE - Figure 3.9a and b - show a small change in capacitance starting at approximately -0.4 V/SCE associated with the formation of defects in the adsorbed layer. However, there is no corresponding change in fluorescence visible at these imaging conditions in either the green or red fluorescence signals. This is not unexpected as the change in layer structure is subtle. Since the octadecanol bilayer remains adsorbed at the electrode surface, the fluorescence remains significantly quenched.

The capacitance scan produced by the second and third desorptions of the layer (Figure 3.9d) are similar to those already seen for the octadecanol and octadecanol-BODIPY-C19-OH bilayers in Figure 3.4. Although the changes from the first scan (not shown) to the second scan are large, the changes between the second
and subsequent scans are much smaller - this can be seen in the small shift in capacitance between the green (second) and red (third) capacitance scans.

The fluorescence during desorption shows very little change in either the monomer or dimer fluorescence until approximately -0.6 V/SCE, correlating with the large increase in capacitance as the octadecanol layer is desorbed from the electrode surface. The fluorescence reaches a maximum at the minimum potential (-0.8 V/SCE), where the layer is fully desorbed. Fluorescence decreases steadily due to photobleaching until the layer begins to readsoorb at approximately -0.3 V/SCE. The onset of readsorption is accompanied by a small bump in fluorescence, then a sharp decrease to a minimum value as the layer becomes fully readsorbed. The fluorescence continues to decrease on the scan from the positive limit back to 0 V/SCE, as does the capacitance, suggesting that the readsorption process is slow, and some change in the layer structure continues even after the layer is nominally readsorbed.

The dimer (red) fluorescence follows the same basic trend as the green fluorescence, although the absolute intensity is much lower, and the change in fluorescence during desorption is also smaller. The fact that there is still some signal from the BODIPY dimers at desorption indicates that the fluorophores are not separating from each other enough during the desorption process to break all of the dimers. However, interpreting the relative fluorescence between monomer and dimer is fraught with complication - in addition to those described in Section 3.3.3, the quenching characteristics for the monomer and dimer will differ based on the emission wavelength. Although there has been some work to suggest that the red fluorophore will be quenched at greater fluorophore-electrode separations than the green [114], the exact characteristics of the quenching remain unknown. For these reasons, the dimer fluorescence is generally not considered, and future results will only present the monomer fluorescence.

3.5 Conclusions

Based on the compression isotherms and ESP measurements, increasing concentration of BODIPY-C19-OH in the octadecanol monolayer causes significant disruption to layer organization, and the floating layer appears to segregate into octadecanol-rich and fluorophore-rich regions. These observations are supported by fluorescence measurements of the floating monolayer, where various structures formed in the layer are clearly visible. The 3 mol% concentration appears to be the maxi-
3.5. Conclusions

Figure 3.9: In-situ fluorescence and differential capacitance measurements of a bilayer of octadecanol containing BODIPY-C19-OH fluorophore. (a) and (b): Fluorescence and capacitance of a preliminary scan to -0.5 V/SCE, where the layer contains defects but is not desorbed. (c) and (d): Fluorescence and capacitance desorption scans of the same layer (to -0.8 V/SCE). All scans are the second and third scans to their respective potential limits. Images acquired using 50x objective, 2.5 sec exposure time and electron multiplier gain 200. The values for the red (dimer) fluorescence have been multiplied by 2 so that they are visible on the same plot as the green. Images on the right are the maximum fluorescence images at desorption (-0.8 V/SCE) for monomer and dimer.
3.5. Conclusions

The minimum BODIPY-C19-OH concentration at which the structural properties (based on Langmuir isotherms) and electrochemical behaviour (in both mono- and bi-layers) remain essentially similar to pure octadecanol. Based on these findings, the octadecanol layers used for the liposome interaction studies will be the 3 mol% BODIPY-C19-OH concentration.
In the Introduction and Section 1.8, a method for forming a hybrid layer of an adsorbed surfactant bilayer and a phospholipid bilayer was proposed. In this method, surfactant layer is initially deposited onto an electrode surface. Defects are created in this layer, into which phospholipid liposomes may insert themselves and incorporate into the adsorbed layer. Figure 1.54 outlines the scheme graphically.

This chapter describes the initial investigations into forming such a hybrid layer. As highlighted in Figure 1.55, reproduced here as Figure 4.1, there is a potential window where an adsorbed octadecanol bilayer is expected to contain potential-induced defects, while a DOPC bilayer, if adsorbed, will form a stable adsorbed state. Based on this observation, octadecanol has been selected as the model for the adsorbed surfactant layer to which liposomes of DOPC are introduced. Differential capacitance is used to monitor bulk changes in the layer properties. Although this is a convenient method and does not require any special treatment of the electrode or liposomes, capacitance alone is unable to determine the nature of any changes that do occur, and can only provide average values representing the entire electrode surface; no spatial information may be inferred.

In this context, “interaction” or “incorporation” of liposomes into the existing octadecanol bilayer is taken as a change in the capacitance behaviour - either a change in the capacitance value when measured at a constant potential, or as a shift in the potential at which characteristic changes in the capacitance, such as desorption, occur. It is possible that some interactions may take place to which capaci-
Figure 4.1: Differential capacitance scans during desorption of a DOPC bilayer (top frame) and octadecanol bilayer (bottom). The region highlighted in blue extends from the onset of defect formation in octadecanol (-0.2 V) to the beginning of the first phase change in the DOPC bilayer (-0.4 V/SCE), representing the potential range in which liposomes are expected to be most effective at incorporating into a solid-supported octadecanol bilayer. Capacitance measurements were performed with a 5 mV/s scan rate, 5 mV RMS potential perturbation, and a 25 Hz perturbation frequency.
4.1 Electrochemistry of DOPC on Au(111)

The behaviour of an Au(111) electrode exposed to liposomes of DOPC has been previously documented in the literature[126, 129, 132] and summarized in Section...
1.5.3. When a Au(111) electrode is placed in contact with a solution containing phospholipid liposomes, the liposomes spread onto the surface to form a bilayer coating the interface. The layer as-formed is not perfect, and is believed to contain water trapped in the bilayer and possible defects.

At potentials more positive than -0.4 V/SCE, the layer is adsorbed onto the electrode surface as a condensed bilayer phase. Potentials more negative than -0.4 V/SCE cause a thickening of the bilayer and a phase change, evidenced by the sharp rise in capacitance at these potentials, followed by an eventual separation of the bilayer from the electrode at -1.1 to -1.2 V/SCE. This behaviour is illustrated by the red trace in Figure 4.2, showing the negative-going potential scan of a DOPC bilayer formed by exposing the Au(111) electrode to a liposome-containing solution while being held at 0 V/SCE, where the water-covered electrode surface is negatively charged, as the pzc of Au(111) in 0.1 M NaF is approximately 0.330 V/SCE [108]. Note that at potentials more negative than -0.4 V/SCE, the capacitance of the DOPC-modified electrode is higher than that of the unmodified (bare) electrode. This is due to a combination of pseudocapacitance peaks increasing the apparent capacitance (see Sections 1.5.1 and 1.5.3) and wetting of the electrode’s sides, increasing the exposed area.

In order to characterize the ability of liposomes to interact with the electrode at other potentials, a freshly flame-annealed Au(111) electrode was exposed to liposome-containing electrolyte for one hour while being held at various potentials (See Section 2.4.1 for procedures). At the end of the hour, an equilibrium value of capacitance was achieved, shown as the blue points in Figure 4.2. Several repetitions of the procedure at 0 V/SCE are presented for reference on the reproducibility of the depositions. Small changes in the effective electrode area caused by variations in argon flow, meniscus height, etc., as well as possible variations in surface coverage, contribute to the variability of the capacitance values reported. Since the variation in electrode area is the largest source of error in these measurements, the relative distribution of capacitance values of the replicates measured at 0 V/SCE should be representative of those measured at other potentials.

Although only one data point is presented, a similar range of responses can be expected for depositions at all potentials, since the external variables affecting the electrode area are similar across experiments.

From Figure 4.2, it is clear that the liposomes are effectively adsorbing onto the electrode surface at all potentials tested (0, -0.35, -0.45, and -0.8 V/SCE), produc-
4.2 Interaction of DOPC with Floating Octadecanol Monolayers

<table>
<thead>
<tr>
<th>Description</th>
<th>Equilibrium Surface Pressure (Π, mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octadecanol with 0.23 mol% DOPC</td>
<td>30±1</td>
</tr>
<tr>
<td>Pure octadecanol</td>
<td>34±1</td>
</tr>
<tr>
<td>Octadecanol with liposomes in solution</td>
<td>35-37</td>
</tr>
<tr>
<td>Octadecanol with liposomes in solution, after layer re-formed with chloroform</td>
<td>32±1</td>
</tr>
</tbody>
</table>

Table 4.1: Equilibrium surface pressure (ESP) of octadecanol and mixed octadecanol/DOPC monolayers.

ing a layer that is similar (within expected experimental error) to the capacitance of the layer during a potential sweep of a previously formed bilayer. Based on these results, it is shown that the liposomes are able to interact with the Au(111) electrode effectively within the entire potential range of octadecanol stability, from a completely adsorbed layer at 0 V/SCE to complete desorption of octadecanol at -0.8 V/SCE. Thus, the liposome-octadecanol combination is suitable for investigation of the potential-controlled interaction of the layers.

4.2 Interaction of DOPC with Floating Octadecanol Monolayers

In order to determine the degree of interaction between octadecanol and DOPC liposomes in the absence of potential control, the equilibrium surface pressure (ESP) of octadecanol monolayers was monitored with exposure to DOPC and liposomes. Table 4.1 summarizes the data from these measurements. Pure octadecanol in the absence of liposomes has a measured ESP of 34 mN/m. This layer, as described in Section 1.3, is imperfect and contains some defects and domain boundaries that might be expected to interact with liposomes in the subphase. This hypothesis can be tested by comparing the interaction of liposomes with the floating monolayer with layers known to contain a mixture of octadecanol and DOPC.

Mixed layers created by deposition of an octadecanol/DOPC solution have a substantially lower surface pressure than pure octadecanol, at 30 mN/m. The 0.23 mol% concentration used approximates a possible DOPC concentration in an octadecanol layer modified according to the scheme proposed in the Introduc-
4.2. Interaction of DOPC with Floating Octadecanol Monolayers

Figure 4.3: Surface pressure (Π) during exposure to liposomes. Pure octadecanol was initially added and allowed to equilibrate. At approximately 28 minutes, liposome suspension was injected into the subphase. After a further 40 minutes of equilibration (t=75 min), chloroform was added to the floating octadecanol monolayer and allowed to dry.
4.3. Interaction of DOPC Liposomes with Octadecanol on Au(111) at 0 V

Although liposomes may not interact strongly with a floating monolayer of octadecanol, additional defects may be formed in the monolayer upon deposition onto an electrode surface that would allow liposomes to interact with the solid-supported layer interface. Deposition of a bilayer rather than a monolayer is expected to cover many of these defects, however lack of interaction is not guaranteed. In order to investigate the liposome interaction with the as-formed octadecanol bilayer, differ-
4.3. Interaction of DOPC Liposomes with Octadecanol on Au(111) at 0 V

Figure 4.4: Differential capacitance of octadecanol bilayers at 0 V/SCE during exposure to DOPC liposomes for 60 min. All layers were pre-selected as described in Section 2.5.2 to have a similar initial capacitance. Capacitance measurements were performed with a 5 mV/s scan rate, 5 mV RMS potential perturbation, and a 25 Hz perturbation frequency.

Differential capacitance was monitored during the 60 minute mixing time after addition of liposomes to the electrolyte. Note that in the DOPC-only experiments described in Section 4.1, 60 minutes was more than sufficient time for the liposomes to form a layer on the electrode surface with a stable capacitance value.

Exposure of an octadecanol-modified Au(111) electrode to a liposome solution generally produced two types of behaviour, as depicted in Figure 4.4. The most common behaviour was simply no change in the capacitance value with time, within the typical variation of an octadecanol layer (traces 1A and 1B). As in the DOPC measurements described previously, variations in the capacitance can be caused by deposition differences, and by wetting and dewetting of the sides of the electrode in the hanging meniscus. Rarely (3 out of over 27 datasets), a deposition would result in the behaviour in trace 2 - namely, a marked increase of capacitance with time. Despite the pre-screening of layers to have an initial capacitance less than 1.08 \( \mu \text{F/cm}^2 \) (Section 2.5.2), some octadecanol bilayers are nevertheless formed as substantially defective, allowing the interaction of liposomes even at 0 V/SCE. These layers were considered to be faulty depositions, and are excluded from further analysis.

At 0 V/SCE, where the adsorbed octadecanol bilayer has few or small defects, liposomes of DOPC in solution therefore do not incorporate into or disrupt the existing layer, as determined by differential capacitance. This does not exclude the
possibility that liposomes interact with the layer in other ways to which capacitance is insensitive, for example adsorbing on top of the octadecanol layer without disrupting it. Further study (described in Chapter 5) will address these questions.

4.4 Effect of Poration Potential on Liposome - Octadecanol Interaction

After establishing that DOPC liposomes in solution do not incorporate into octadecanol bilayers in the absence of substantial defects in the bilayer, electrochemical methods of artificially forming defects in the layer were attempted. Based on the electrochemical behaviour of octadecanol (summarized in Section 1.5.2), application of a potential more negative than -0.4 V/SCE should result in formation of defects in the octadecanol bilayer. By application of a series of potential steps, the ability of liposomes to interact with the octadecanol bilayer was tested. After measurements at 0 V/SCE to characterize the unmodified bilayer, a “poration potential” - the potential at which defects were formed in the bilayer - was applied. A schematic of possible defect structures for an adsorbed bilayer of octadecanol is shown in Figure 1.24e.

4.4.1 Experimental Design

Electrochemical measurements were performed as outlined in Chapter 2, and differential capacitance monitored during all stages. Briefly, after deposition and screening of an octadecanol bilayer (inner leaflet: octadecanol with 3 mol% BODIPY-C19-OH, outer leaflet: 100% octadecanol), liposomes were injected directly into the electrolyte and the potential held at 0 V/SCE for one hour to allow diffusion into solution (liposome injection was omitted for control experiments). Then a series of potential steps was applied to the electrode to explore the facility of liposome interaction with the adsorbed octadecanol layer as it was perturbed by application of the potentials.

In one variation, outlined in Figure 4.5a, after being held at 0 V/SCE for 15 minutes to establish a baseline capacitance, the potential is scanned to -0.2 V/SCE and held for 15 min before returning to 0 V/SCE for a further 15 min. For most experiments, a four-step profile was used (Figure 4.5b). In these profiles, after the initial 15 minutes at 0 V/SCE, the potential was then scanned to a “poration po-
4.4. Effect of Poration Potential on Liposome - Octadecanol Interaction

Figure 4.5: Potential step profiles used in testing liposome incorporation. (4.5a) In experiments following 4.5b, the time at each potential varied from 1 to 45 min, and one of the three "poration potentials" shown (-0.4, -0.6, and -0.8 V/SCE) was chosen.

tential" which ranged from -0.4 V/SCE to -0.8 V/SCE, and held for a time varying between 1 min and 15 min. After this time, a -0.2 V/SCE potential was applied for 15 min, followed by 0 V/SCE for a final 15 min. Exceptions to this pattern were for the -0.8 V/SCE poration potential, where the potential was not held but merely scanned from 0 V to -0.8 V and then immediately to -0.2 V/SCE. For the 45 minute residence time at -0.4 V/SCE, all potential steps were held for 45 min rather than 15 min.

After application of the stepping potential profile, the potential was scanned to desorption (+0.15 V to -0.8 V/SCE) to further characterize the changes to the modified layer.

4.4.2 Capacitance During Potential Steps

The capacitance data from a data series exploring the effect of an increasing poration potential with a constant hold time of 15 minutes are summarized in Figure 4.6. When using only the -0.2 V/SCE potential (Figure 4.6a), where few if any defects should be created, very little change in the bilayer is observed. Both the layer exposed to liposomes in solution and the liposome-free control show a small increase in capacitance during the -0.2 V/SCE potential step, as expected from known capacitance properties of the layer. Upon return to 0 V/SCE, both samples return to very near the initial capacitance value, with little lasting change to the layer caused
4.4. Effect of Poration Potential on Liposome - Octadecanol Interaction

Figure 4.6: Differential capacitance of octadecanol bilayers on Au(111) with and without liposomes in solution during application of potential steps. Inset plots show the potential profile applied in each experiment. (a) Application of -0.2 V/SCE potential step only. (b) Application of -0.4 V for 15 min, followed by -0.2 V/SCE for 15 min. (c) Application of -0.6 V for 15 min, followed by -0.2 V/SCE for 15 min. (d) Application of -0.8 V, followed by -0.2 V/SCE for 15 min. Capacitance measurements were performed with a 5 mV/s scan rate, 5 mV RMS potential perturbation, and a 25 Hz perturbation frequency.
4.4. Effect of Poration Potential on Liposome - Octadecanol Interaction

Figure 4.7: Replicate measurements of octadecanol capacitance with and without liposomes in solution during application of the (a) -0.6 V and (b) -0.4 V/SCE poration potential (as in Figure 4.6c). Solid lines: Electrochemistry in standard cell with 30 µg/mL DOPC, performed with a 5 mV RMS potential perturbation, and a 25 Hz perturbation frequency. Dashed lines: Electrochemistry in microscope cell with 45 µg/mL DOPC, performed with a 5 mV RMS potential perturbation, and a 200 Hz perturbation frequency. A zoomed view of the capacitance after the potential perturbations is shown in figure (c) for -0.6 V and (d) for -0.4 V.

by the excursion to the negative potential. It is likely that any defects formed at -0.2 V/SCE are too small or too few to facilitate substantial liposome-octadecanol interaction.

Addition of a step to -0.4 V/SCE produces more dramatic results (Figure 4.6b). The capacitance at -0.4 and -0.2 V/SCE is increased, as in the previous experiment, due to the presence of defects in the adsorbed layer and increase in the average dielectric value caused by water at the electrode surface. However, the layer in the presence of liposomes experiences a larger relative change in the capacitance at -0.4 V/SCE. The cause of the “jump” in capacitance at approximately 28 minutes is unknown, and did not occur in other measurements. As well, rather than returning to a value similar to the initial capacitance upon return to 0 V/SCE, the liposome-exposed layer maintains a distinctly higher final capacitance value. This increase in capacitance signals a decrease in the organization of the octadecanol layer, presumably caused by incorporation of liposome material. The addition of a step to -0.4 V/SCE results in greater liposome interaction with the adsorbed octadecanol bilayer, likely due to increased defect formation at this potential.

If the poration potential is extended to -0.6 V/SCE, the change in capacitance at
the poration potential is correspondingly larger, however, upon return to 0 V/SCE, little or no change is seen from the initial capacitance values. A repetition of the measurement in the smaller microscope electrochemical cell (using a slightly higher liposome concentration - 45 rather than 30 µg/mL DOPC) is shown along with the original data in Figure 4.7. Both of the liposome-exposed layers in the microscope cell (gray, dashed lines in Figure 4.7) showed a larger change in capacitance than the standard cell experiments (solid lines), which is easily attributable to the increase in liposome concentration in this cell. More importantly, the control layers (black lines) show markedly different responses to the same potential perturbation, especially during the poration step (15 to 30 min), where the second experiment (dashed line), although it started at a similar value to the first (solid line), experienced a much greater increase in capacitance, and higher capacitance values remain thereafter. Contrast this variation to the typical behaviour of the -0.4 V/SCE potential perturbation (Figure 4.7b), where the control layers, despite being measured in two different cells again, exhibit almost identical capacitance behaviour.

The difference is seen even more clearly in the expanded plots of Figure 4.7c and d. With a -0.4 V/SCE poration potential, both control experiments have very similar capacitances (which are again similar to the initial capacitance values seen in Figure 4.7b). With the -0.6 V/SCE poration potential, although both control layers again started with similar capacitances, there is a much greater difference in final capacitance from run to run. Clearly, the -0.6 V/SCE potential profile suffers from a lack of reproducibility.

The source of this variation is most likely small changes in layer formation conditions, resulting in a shift in the desorption potential. The onset of octadecanol desorption is typically near 0.6 V/SCE, so a small shift in the desorption potential will result in widely varying conditions at the electrode surface. The -0.4 V/SCE poration potential is further from such large-scale transitions, and is less affected by small variations in initial octadecanol conditions. Therefore, the -0.4 V/SCE potential profile is recommended over the -0.6 V/SCE profile, as the -0.6 V/SCE profile suffers from reproducibility problems that are not easily detected at the start of experiments.

The final potential tested was -0.8 V/SCE, at which the octadecanol layer is completely desorbed from the electrode surface. Although previous research has shown that the octadecanol layer does not travel far from the interface while des-
orbed [112, 115, 183–185], this state is expected to be less stable with time than the adsorbed states. Rather than holding the potential at -0.8 V/SCE for 15 minutes, it was scanned out and immediately back to -0.2 V/SCE. Although the time at -0.8 V/SCE is relatively short (a few seconds), based on the previously described capacitance behaviour, the layer should not readsorb onto the electrode surface immediately, staying substantially desorbed until the potential reaches approximately -0.4 V/SCE. Thus, this potential profile results in the liposome solution being exposed to the interface with a desorbed octadecanol bilayer for several minutes overall. The capacitance behaviour during this trial (Figure 4.6d) shows the expected dramatic increase in capacitance as the layer is desorbed at the most negative potentials. Upon return to 0 V/SCE, the liposome-exposed layer does show an increase in capacitance over the control layer, indicating that liposomes indeed are able to interact with the octadecanol layer through the process of desorption and read sorption. However, the overall change is smaller than that of the -0.4 V/SCE poration potential, suggesting that less liposome material has been incorporated into the octadecanol bilayer. This may be due in part to the shorter time spent at the poration potential, but increasing the residence time at desorption is inadvisable as the octadecanol layer is only very loosely associated with the electrode, and will eventually diffuse away.

By observing the capacitance behaviour during the poration and healing of the octadecanol layer, it is shown that the application of a poration potential is necessary in order to achieve liposome interaction with the octadecanol layer. As well, the choice of potential is important in order to achieve reproducibility as well as quality of liposome interaction.

4.4.3 Desorption of the Modified Bilayers

Further characterization of the modified octadecanol layers was done by desorption of the modified layer while monitoring changes in capacitance behaviour. Desorption scans of the layers formed during the procedures shown in Figure 4.6 are shown in Figure 4.8. Generally, a shift in the desorption potential to more positive values indicates an increase in layer disorder, allowing the layer to desorb at lower energy. However, this information is convoluted with the fact that it is impossible to prevent liposomes from interacting with the layer as it becomes porated before desorption. Therefore, even layers that have little liposome incorporation prior to the
4.4. Effect of Poration Potential on Liposome - Octadecanol Interaction

Figure 4.8: Capacitance during a potential sweep to desorption (+0.15 to -0.8 V/SCE) of octadecanol layers subjected to the potential profiles in Figure 4.6. Data is from the first potential sweep taken immediately after application of the poration potential profile. (a) Layers after application of a -0.2 V/SCE potential. (b) Layers after application of a -0.4 V followed by a -0.2 V/SCE potential. (c) Layers after application of a -0.6 V followed by a -0.2 V/SCE potential. (d) Layers after application of a -0.8 V followed by a -0.2 V potential. Capacitance measurements were performed with a 5 mV/s scan rate, 5 mV RMS potential perturbation, and a 25 Hz perturbation frequency.
4.5 Effect of Poration Time on Liposome - Octadecanol Interaction

start of the desorption scan would be expected to have some shift in the desorption potential caused by incorporation during the scan.

Both of the potential profiles that showed little change during the application of poration potentials (-0.2 V and -0.8 V, Figure 4.8a and d respectively) show only a small shift in desorption potential as well, confirming that little change occurred in the octadecanol layer during the application of the potential steps. Both (c) and (d) in Figure 4.8 (-0.4 and -0.6 V/SCE poration) show more significant shifts in the desorption behaviour, corresponding with the larger changes observed during the potential steps. In both cases, the onset of desorption, identified by the steep rise in capacitance, begins at more positive potentials for the layers exposed to liposomes. As there is very little variation run-to-run in the octadecanol layers not exposed to liposomes, these changes can be attributed entirely to the effects of liposomes interacting with the octadecanol layer. Both the -0.4 and -0.6 V poration potentials show a similar ability to facilitate liposome interaction with the layer, as judged by the relative shift in desorption potential - for both, beginning at approximately -0.45 V/SCE compared to -0.65 V/SCE for the unmodified octadecanol.

Desorption scans of the liposome-modified and unmodified octadecanol bilayers show that potentials at which the octadecanol layers are adsorbed but experience disruption are effective in allowing liposomes to interact with the layer, while potentials at which the octadecanol is completely desorbed or weakly porated are less effective, confirming observations made during the application of the poration potentials. The -0.4 V/SCE poration potential is judged to be the most effective of those tested, as it allows significant interaction of liposomes with the octadecanol bilayer while avoiding the variability of the -0.6 V/SCE potentials. Thus, the -0.4 V/SCE potential was chosen for further investigation on the length of poration times.

4.5 Effect of Poration Time on Liposome - Octadecanol Interaction

In order to determine the effect of the duration of the poration potential on the degree of liposome interaction with the layer, a poration potential of -0.4 V/SCE (the best-performing potential as determined previously) was used with a variety of residence times.
4.5. Effect of Poration Time on Liposome - Octadecanol Interaction

Figure 4.9: Differential capacitance of octadecanol bilayers on Au(111) with and without liposomes in solution during application of -0.4 V/SCE for various times. Inset plots show the potential profile applied in each experiment. (a) Application of -0.4 V/SCE for 1 min. (b) Application of -0.4 V for 15 min. Capacitance measurements were performed with a 5 mV RMS potential perturbation and a 25 Hz perturbation frequency.

4.5.1 Experimental Design

The same type of potential step pattern as described in Section 4.4 was applied, all with a minimum potential of -0.4 V/SCE. Generally, as in the previous experiments, the potential was held at 0 V/SCE for 15 minutes, then -0.4 V/SCE for the required time (1 or 15 minutes), then -0.2 V and 0 V/SCE for 15 minutes each. The layers were then scanned to desorption to further characterize the changes made to the layers. An experiment was also performed with the same general parameters, except all the potential steps were held for 45 minutes. Since the octadecanol is stably adsorbed at both 0 and -0.2 V/SCE, the additional time at these potentials should not affect the measurements, and these results may be compared directly to the other, shorter durations.

4.5.2 Capacitance During Application of Poration Potentials

As previously, differential capacitance was monitored during the application of the potential steps used to control the poration of the octadecanol bilayer. Figure 4.9 shows the results of these experiments. Both 1 min and 15 min residence times at -0.4 V/SCE were successful in allowing liposomes to interact with the octadecanol layer. Based on the relative increase in capacitance after application of the potential steps, the 1 min residence time appears to be more effective at allowing liposome interaction, but the difference is small compared to the variability of the
4.5. Effect of Poration Time on Liposome - Octadecanol Interaction

Figure 4.10: Capacitance during a potential sweep to desorption (+0.15 to -0.8 V/SCE) of octadecanol layers subjected to the potential profiles in Figure 4.9. Data is from the first potential sweep taken immediately after application of a potential step profile holding -0.4 V/SCE for (a) 1 minute (b) 15 minutes (solid line) and 45 minutes (dashed line). Capacitance measurements were performed with a 5 mV/s scan rate, 5 mV RMS potential perturbation, and a 25 Hz perturbation frequency.

This finding is interesting, but not entirely surprising - the potential steps are only applied after the liposomes in the electrolyte have been allowed to diffuse for an hour, so the density of liposomes near the electrode surface is initially similar for both experiments. It is therefore possible that if the octadecanol layer is not altered during the residence time at -0.4 V/SCE (after the initial equilibration of the layer) then the liposomes may achieve the maximum amount of interaction relatively quickly, and further time at the poration potential does not enhance the interaction. Rather, it is limited by liposome density at the surface. These hypotheses will be investigated further in Chapter 5, when fluorescence imaging is used to monitor the changes during the application of these potentials.

4.5.3 Desorption of the Modified Layers

After application of the potential step profile, the modified octadecanol layers were subjected to a potential scan to desorption (0.15 to -0.8 V/SCE) while measuring capacitance, shown in Figure 4.10. All exposed layers show evidence of modification by incorporation of liposomes, characterized by a shift in the desorption potential to be positive of -0.65 V/SCE.

The 1 minute poration time (Figure 4.10a) showed a large shift in desorption potential. Longer poration times of 15 and 45 minutes (Figure 4.10b) also showed some shift in the desorption potential, but increasing the time spent at -0.4 V/SCE
4.6 Conclusions

The interaction of liposomes with a surface-supported octadecanol bilayer has been demonstrated, using capacitance measurements. Application of a potential sufficient to cause disruption to the octadecanol bilayer is required in order to achieve significant interaction of the liposomes with the octadecanol layer. In the absence of sufficient defects, liposomes do not interact with the octadecanol layer in a measurable way. Complete desorption of the octadecanol layer does not enhance liposome-octadecanol interaction.

The residence time at the poration potential does not appear to have a strong effect on the degree of interaction of the liposomes with the octadecanol layer, although shorter times do appear to have a slightly larger degree of change. Overall, the optimum conditions for facilitating liposome incorporation into the octadecanol bilayer are application of a poration potential of -0.4 V/SCE for 1 or 15 min.

Although the general properties of the interaction can be observed, the capacitance measurements alone cannot provide information on how the liposomes are interacting with the octadecanol layer, or whether the interactions are localized or heterogeneous across the electrode surface. These questions will be investigated first using in-situ fluorescence methods, discussed in the next chapter.
Chapter 5

In-situ Fluorescence Studies of Liposome Interaction with Solid-Supported Octadecanol Bilayers

The capacitance results presented in Chapter 4 provide convincing evidence that liposome interaction with an adsorbed octadecanol bilayer can be initiated by application of an electrical potential, but electrochemical methods alone provide only average information on the state of the interfacce. In order to learn about the nature of the interaction between liposomes and the adsorbed octadecanol layer, or gain any information on spatial characteristics of the interactions across the electrode surface, electrochemical measurements must be combined with other in-situ methods to probe the interface.

Presented in this chapter are the observations of the interaction of liposomes with adsorbed octadecanol using the combination of electrochemistry and in-situ fluorescence microscopy. By the inclusion of a small amount of a fluorophore into the adsorbed octadecanol layer, information on the adsorption/desorption state of the octadecanol layer can be obtained. The potential-dependent fluorescence behaviour of monolayers of octadecanol is already well characterized [113, 114, 183–185], and some observations of octadecanol bilayers [115] and DOPC monolayers [69, 113] using fluorescence have been published, and are summarized in Section 1.6.2.

The liposomes themselves contain no fluorophore, but as they interact with the adsorbed octadecanol layer, some of the fluorophore contained in the adsorbed layer may diffuse into the liposome walls. If these fluorophores are located in areas that are now raised further from the electrode surface than the adsorbed layer (as
in the hemi-liposome structure depicted in Figure 5.1), the quenching will be less and the fluorescence greater in these regions. Since the relation between separation and transfer of energy from the fluorophore excited state is exponential (see Section 1.6.1), even a small difference in height could result in a measurable difference in fluorescence intensity. Desorption of the layer, forcing all material to move farther from the electrode surface, will enhance these differences.

The structure of the fluorescent layer will be heavily influenced by the initial layer structure and the particular area being imaged. As demonstrated in Chapter 3, the octadecanol/BODIPY-C19-OH layers are non-homogeneous at deposition and this will influence the fluorescence intensity and structure of the layers under investigation. Thus, although individual characteristics of the layers formed may not be directly comparable, the trends in layer behaviors should be consistent between experiments. Increases in fluorescence may therefore be interpreted as either layer desorption, which moves octadecanol and fluorophore away from the electrode surface, or liposome incorporation, depending on the context of the experiment.
5.1 Experimental Methods

The electrochemical procedures used were as described in Sections 2.5 and 4.4.1. Octadecanol bilayers were formed by depositing first a monolayer containing 3 mol% of the BODIPY-C19-OH fluorophore, then depositing a layer of pure octadecanol. Deposition of the fluorophore-containing layer first (and thus nearest the electrode surface) offers several advantages. While adsorbed, fluorescence from this layer will be nearly completely quenched, reducing the background fluorescence as much as possible.

After deposition according to the procedures in Section 2.4.3, the capacitance of each layer was measured and confirmed to be less than the maximum acceptable value of 1.08 µF/cm². Potential step profiles were applied to the layer, as described in Section 4.4.1, followed by a potential scan to desorption (-0.8 V/SCE) and back to 0 V/SCE.

Fluorescence images of the electrode surface were taken using the microscope assembly described in Section 2.5.4. In order to maximize the available signal of the mostly quenched fluorescence in the adsorbed layer, images taken during the 0 V/SCE hold time for liposome diffusion and during the application of potential steps were made using the green emission filter exclusively as the green monomer fluorescence is more intense than that of the dimer. Interpretation of dimer fluorescence is complex, as established in Chapter 3, so analysis of the red dimer fluorescence was not performed on these layers. During potential steps and scans, images were continuously acquired every 5 sec. During the initial hour at 0 V/SCE, images were acquired every 1-5 min in order to reduce the influence of photobleaching.

Exposure time for the 60 min hold at 0 mV/SCE was 5 s, with an electron multiplier gain of 400. Images during the potential stepping experiments were acquired with either a 2.5 s exposure and electron multiplier gain of 200, or a 5 s exposure with an electron multiplier gain of 400. For comparison, these images were converted to an equivalent kilocounts per second by use of an empirically determined calibration factor (2.5 for the shorter exposure, 7.5 for the longer exposure). Derivation of the calibration factors is described in Appendix B.
5.1. Experimental Methods

Figure 5.2: Average fluorescence intensity and differential capacitance measurements for layers held at 0 V/SCE. Black lines are control experiments with no liposomes. Blue and red lines have liposomes in the electrolyte. Blue lines show a small change in fluorescence with time; red trace shows a larger fluorescence change despite similar capacitance behaviour.
5.2 Fluorescence at 0 V

After depositing the octadecanol bilayer and adding liposomes to the electrolyte, the system was allowed to equilibrate for an hour to allow the liposomes to diffuse through the solution. This is also an opportunity to observe the interaction of the liposomes with the as-deposited octadecanol layer in the absence of potential induced defects. In the standard electrochemical cell, little change in capacitance was noted during this wait time at 0 V/SCE (Section 4.3). In the microscope cell, although the liposome concentration in the electrolyte was higher (45 µg DOPC/mL electrolyte rather than 30 µg DOPC/mL electrolyte), the capacitance values over the 60 minutes still show little change with time, as seen in Figure 5.2b. Two control experiments are included to show typical octadecanol behaviour in the absence of liposomes; these show a similar stability.

Despite the similar capacitance values, there is large variation in the initial fluorescence intensity for the layers imaged. As outlined in Chapter 3, the floating monolayer of octadecanol with BODIPY-C19-OH used for deposition has a variety of structures and fluorescence intensities that are transferred to the electrode surface on deposition. These differences in intensity are caused by variations in the local concentration of fluorophore in the layer, resulting in regions that are dark - devoid of fluorophore - and intense fluorescence in fluorophore-rich regions. The electrode surface, at 0.26 cm², will encompass several of these regions for any given deposition while the fluorescence images, at 2.2×10⁻⁴ cm², reflect only a small portion of the surface. Thus, while the capacitance measurements reflect the average value across the electrode surface, encompassing all regions, the fluorescence images reflect only the changes in the specific visible area, and the initial fluorescence intensity depends on the type of region visible. It is probable that all layers described here have these regions of fluorescent behaviour, indistinguishable by capacitance, but obvious to fluorescence imaging. Because the fluorophore is mostly quenched when the layer is deposited, little information is available on the layer structure when choosing an imaging area, and the area imaged is essentially left to chance.

Fluorescence measurements during the 60 minute waiting time reveal that despite the lack of measurable capacitance response, there is some change occurring in the layer while holding at 0 V/SCE. Little photobleaching is seen, likely because the layer is adsorbed onto the electrode and fluorescence is quenched, as well as the
5.2. Fluorescence at 0 V

reduced duty cycle used during imaging (one 5 sec image each 1 to 5 min). In the control layers (black lines in Figure 5.2), the fluorescence intensity either remains constant or increases slightly over the wait time. This small increase - less than 10% of the initial fluorescence value - is explained by a possible reorganization of the adsorbed bilayer such that fluorophore from the inner leaflet migrates to the outer leaflet, moving farther from the electrode surface and increasing fluorescence intensity.

When liposomes are added to the solution, they could interact with any defects present by slightly perturbing the octadecanol layer or leaching fluorophore into the phospholipid bilayer, which would result in a change in fluorescence dependent on the structure of the region imaged. Both mechanisms of interaction would result in an increase in fluorescence by moving fluorophore away from the electrode surface, reducing the amount of quenching. The mechanisms should be distinguishable by comparing the change in capacitance - a change in capacitance accompanying an increase in fluorescence would indicate that the liposomes are incorporating into the layer through existing defects, while a steady capacitance favors the leaching mechanism. In fact, in the majority of layers tested (about 60%) a modest increase in fluorescence of 10-15% of the initial fluorescence value is observed, similar in magnitude to that seen in the control layers (blue lines in Figure 5.2). There is no change in capacitance associated with this increase in fluorescence, indicating that the liposomes are not interacting significantly with the adsorbed octadecanol layer under these conditions. In a few cases (about 20% of all experiments, represented by the red line in Figure 5.2), there was a larger increase in fluorescence, again not accompanied by a change in capacitance. Analysis of these images shows either a general increase in fluorescence across the image, or a change in fluorescence confined to the creation of small intensely fluorescent regions. In the latter case, these layers were abandoned as defective, as the fluorescence during subsequent steps was substantially different than in the remainder of the experiments. Analysis was also not performed on the small fraction of experiments where both the fluorescence and capacitance increased during the waiting time, clearly indicating that liposomes were able to interact with existing defects in the octadecanol layer.

Based on the observations of the octadecanol layers with and without liposomes before any potential perturbation was applied, it is clear that in the majority of depositions, the liposomes are unable to incorporate on a large scale into the octadecanol bilayer. There is some reorganization of the octadecanol bilayer that
5.3 In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 1 min

results in a small fluorescence increase. In a small fraction of experiments, sufficient defects were present at deposition to allow liposome interaction, however these layers were excluded from further analysis.

5.3 In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 1 min

In order to investigate the interaction of DOPC liposomes with the adsorbed octadecanol bilayer, in-situ fluorescence images were taken during application of potential profiles similar to those used in Section 4.5. As the electrochemical measurements suggested, the application of a -0.4 V/SCE potential for 1 min or 15 min facilitated the greatest interaction with the octadecanol layer; these experiments were repeated with the addition of in-situ fluorescence measurements.

5.3.1 Fluorescence During Potential Steps

The capacitance and average fluorescence intensity profiles for layers exposed to a poration potential profile at -0.4 V/SCE for 1 min, identical to that used in the electrochemical experiments of Figure 4.9a, are shown here in Figure 5.3. One sample of a control experiment (black lines) and two experiments with liposomes in solution (blue and green lines) are included. The regions identified as hotspots at 0 V/SCE are analyzed separately and shown as dashed lines. The capacitance in the 15 min spent at 0 V/SCE is similar for all three examples. The effects of photo-bleaching are stronger in these data sets compared to the previous measurements at 0 V/SCE as the imaging is done more frequently - here, one image per 5 sec as opposed to every 1 to 5 min during the 60 min diffusion time. The fluorescence of the hotspots decreases more rapidly than the remainder of the image. As these are believed to be multilayer regions that will be less efficiently quenched, this behaviour fits as the less quenched regions would be more subject to photodegradation.

After changing the potential to -0.4 V/SCE, there is a significant increase in capacitance in the adsorbed layers caused by a change in the layer structure, similar to the increase in capacitance seen in the electrochemistry experiments in Section 4.5. With no liposomes in solution, this change is small, and after returning the potential to 0 V/SCE, the final capacitance value is only slightly increased from the initial value at 0 V/SCE. The change in fluorescence during these changes was also
very small, consisting mostly of photobleaching with time. Very little potential dependence of the fluorescence is seen, with only a slight increase marking the change in layer organization at -0.4 V/SCE.

With liposomes in solution, both the capacitance and the fluorescence show an enhanced response to the changes in potential. Again, there is an increase in capacitance associated with the excursion to -0.4 V/SCE, however upon return to 0 V/SCE, both layers exposed to liposomes retain a higher capacitance value than the initial capacitance at 0 V/SCE. The fluorescence response for these two layers differ significantly, illustrating that the fluorescence response observed can depend on the region used for analysis. In both layers, there is a small but significant increase in the overall fluorescence at -0.4 V/SCE, indicating the quick interaction of the liposomes with the adsorbed layer - the scan from 0 to -0.4 V/SCE takes only 20 s, and the increase in fluorescence is visible even before the final potential is reached. After changing the potential to -0.2 V/SCE, there is a sharp decrease in fluorescence although the overall intensity continues to increase slowly in both cases. On returning to 0 V/SCE, there is again an immediate decrease in fluorescence intensity, and a continued increase with time, although the first experiment (green lines) shows a much smaller increase during this time. These changes in fluorescence suggest that the liposomes have disrupted the adsorbed layer in such a way as to increase the separation of fluorophore from the electrode surface. It has been shown [113] that changes of 10-20 nm in this separation can result in a significant increase in the fluorescence due to decreased quenching efficiency.

The changes seen during poration are small and indicate that either there is not a large degree of liposome incorporation overall into the adsorbed layer or that few fluorophores from the adsorbed layer have diffused into the adsorbed lipid regions at the end of the measurement period. More detail on the interaction of the liposomes and the adsorbed layer can be gleaned from an analysis of the structure of the fluorescence images. A selection of fluorescence images acquired for each of the three experiments discussed here is presented in Figure 5.4. The changes in fluorescence are not uniform across the imaging area, but are localized in particular regions, suggesting that the layer may have specific structures that facilitate liposome incorporation and interaction. These can be seen for example in the first liposome exposure experiment (top row of Figure 5.4), where at the end of the -0.4 V/SCE poration (image C at -0.4 V/SCE and D at -0.2 V/SCE) new localized spots of increased fluorescence are visible that were not included in the initially
identified hotspots region. The effect is less visible in the second experiment as a general increase in fluorescence of the layer makes identification of new spots more difficult. The control layer shows little change in fluorescence structure aside from photobleaching, either within the hotspots or in the surrounding regions.

The increase in fluorescence clearly shows that the adsorbed layer is changing in its structure and supports the interaction of liposomes with the adsorbed layer because these changes are not solely due to the potential perturbation of the adsorbed octadecanol layer. The increase in fluorescence is also not located around the regions initially identified as hotspots, which suggests that the defects created through the potential perturbation are not near the regions of high fluorescence outlined even though these regions would be considered to be a nonideally organized region or a possible defect. Although some detail of this interaction is visible during the poration of the images, analysis is hampered by the relatively low fluorescence as the adsorbed layer’s fluorescence is mostly quenched. Desorption of the layer from the electrode surface will highlight changes in the layer and allow greater contrast between regions of the layer as the fluorescence intensity increases.

5.3.2 Fluorescence During Desorption of the Modified Layers

After modification with the potential step profile, the layers were desorbed by application of a potential scan from +0.15 to -0.8 V/SCE while measuring capacitance and fluorescence images. As demonstrated in the electrochemical measurements (Chapter 4), the interaction of liposomes with the adsorbed layer influences the potential-induced desorption, moving the onset of desorption to less-negative values. Without liposomes, the control layer behaves consistently with previous observations; the black lines in Figure 5.5 show that the increase in fluorescence intensity begins at approximately the same potential as the sharp increase in capacitance, signaling desorption of the layer. As the capacitance reaches its maximum value, so does the fluorescence intensity, reaching a value nearly double the initial intensity of the initial adsorbed layer. The regions identified in the initially formed layer as hotspots have a similar behavior to the bulk of the layer. The strong increase in fluorescence occurs as the adsorbed layer is moved away from the electrode surface, reducing the degree of quenching of the fluorophores. The intensity of the fluorescence signal is also influenced by the amount of dye initially present in the adsorbed layer, the distribution of fluorophore normal to the electrode
5.3. In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 1 min

Figure 5.3: Spectroelectrochemical behaviour of adsorbed octadecanol bilayers with and without liposomes in solution during application of a potential profile with a 1 min poration step. (a) Fluorescence intensity as average grayscale values for hotspot regions (dotted lines) and the remainder of the image (solid lines). (b) Capacitance values of the interface during application of the potential step. Black traces: No liposomes in solution. Green and blue traces: with liposomes in solution, separate trials. Letters A-E correspond to time points of images reproduced in Figure 5.4. Blue-shaded background highlights the time period spent at the poration potential (-0.4 V/SCE), orange shaded background highlights the time period at -0.2 V/SCE, and the unshaded background represents time periods spent at 0 V/SCE. Capacitance measurements were performed with a 5 mV RMS potential perturbation and a 200 Hz perturbation frequency. Fluorescence images were taken with either of the exposure settings described in Section 5.1 and converted to equivalent kilocounts per second.
5.3. In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 1 min

Figure 5.4: Representative fluorescence images taken during application of a potential profile with a 1 min poration step. Images are false colored and contrast enhanced for visibility in print - calibration bars on the right indicate minimum and maximum intensity values presented in each series, in kcts/sec. Scale bars are 20 µm long. Letters A-E correspond to time points marked in Figure 5.3: A - initial image at 0 V/SCE, B - end of 0 V/SCE step, C - end of -0.4 V/SCE step, D - end of -0.2 V/SCE step, E - final image (0 V/SCE). Top row: with liposomes in solution (corresponds to green trace in Figure 5.3). Middle row: With liposomes in solution (corresponds to blue trace in Figure 5.3). Bottom row: without liposomes in solution (corresponds to black trace in Figure 5.3). White outlines in column A outline the image areas identified as hotspots for each experiment.
5.3. In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 1 min

surface, and processes such as dimer formation which will reduce the number of molecules fluorescing in the spectral range selected.

After exposure to liposomes and the poration procedure, the desorption of the adsorbed layer shows significant differences in the potential dependence of fluorescence and its increase in intensity. In both examples (blue and green lines in Figure 5.5), the initial fluorescence values (at +0.15 V/SCE) are higher than in the control layer. Partially, this is caused by selection of a naturally more fluorescent region of the electrode, as identified in the initial image intensities. This effect is compounded by the increase in fluorescence due to liposome incorporation such that both liposome-exposed layers had a substantially increased fluorescence value at the beginning of the desorption scan. For both layers, the onset of desorption occurs at -0.45 V/SCE, coincident with the increase in fluorescence. The shift in the onset of the capacitance and fluorescence increase suggests that the modified layer has interacted with the liposomes, changing the layer organization and enabling desorption at less negative potentials than in the unmodified layer. The increased fluorescence intensity at desorption is indicative of a modified layer structure where more of the fluorophore is further from the electrode surface, reducing quenching and increasing the signal. These fluorescence images also show a less uniform structure, with the brighter regions highlighting the regions furthest from the electrode surface.

The layer is desorbed at the negative scan limit, with fluorescence significantly increasing and a maximum at -0.65 V/SCE for one layer (blue lines in Figure 5.5), or a steady increase until -0.8 V/SCE in the other (green lines in Figure 5.5). The appearance of a fluorescence maximum has been observed previously [184], and is believed to result from a change in fluorophore organization such as dimer formation. Both layers have a much greater relative fluorescence increase at desorption than the control layer, which can be explained by variations in the amount of fluorophore present in the layer at deposition, combined with a significant change in the structure of the layer after exposure to the liposomes and poration potential profile. The layer has apparently been modified by inclusion of liposomes to create structures extending normal to the electrode surface (i.e. 3-D structures such as the hemiliposome structure proposed in Figure 5.1).

The hotspots in the liposome-exposed layers do not show a significantly different behaviour than the remainder of the layer, aside from a slightly higher fluorescence value. Thus, although these hotspots might have been expected to be defec-
5.3. In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 1 min

tive regions in the layer that would be easily accessible to liposome incorporation, more incorporation was not observed in these regions. Electrochemically formed defects throughout the non-hotspot regions of the layer are therefore believed to be the main sites for liposome incorporation.

Examination of the structures visible in the fluorescence images (Figure 5.6) provides further details on the mechanism of the liposome interaction with the adsorbed octadecanol layer. Although the hotspots identified from the initially deposited layer remain visible throughout the desorption process, during desorption, the remainder of the visible layer undergoes a significant increase in fluorescence intensity as well. These changes do not appear to be uniform across the image area, however, and appear to consist of small highly fluorescent regions distributed on the surface. A further study of these features is important as the liposomes may not incorporate evenly across the electrode surface, and their influence on the modified layer structure may be localized. The general increase in fluorescence during desorption masks these smaller details and makes it difficult to separate the creation of heterogeneity in the fluorescence images from the overall desorption process. Analysis of these small features can be accomplished by removing the featureless background intensity through a rolling ball background subtraction (with a 50 pixel (14 µm) radius), making the small features easier to distinguish. This background subtraction was performed on the images of Figure 5.6, and the resulting images are presented in Figure 5.7.

For the layer that was not exposed to liposomes, the number of bright features at desorption is essentially the same as at adsorption (features outlined in white). Only a few relatively low intensity features are generated at -0.8 V/SCE, and are attributed to structures present in the layer at adsorption that were initially too dimly fluorescent to see due to quenching in the adsorbed layer. In contrast, many new bright features appear even in the images just at the onset of desorption at -0.45 V/SCE for the layers exposed to liposomes. These structures, not present in the initially adsorbed layer, are a result of interaction with the liposomes. In both of the examples that were exposed to liposomes and the poration procedure, the number of small fluorescent regions also changed with the applied potential. As regions that are further from the electrode will increase more rapidly in fluorescence as a result of the nonlinear relationship between quenching and fluorophore-metal separation (Section 1.6.1), seeing a substantial increase in fluorescence for these regions. These small features (~ 5 pixels in diameter - ~2 µm) are much larger in size.
5.3. **In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 1 min**

Figure 5.5: Spectroelectrochemical behaviour of adsorbed octadecanol bilayers with and without liposomes in solution during desorption of the layer after modification by a 1 min poration potential profile. Potential was scanned from +0.15 to -0.8 V/SCE. (a) Fluorescence intensity as average grayscale values for hotspot regions (dotted lines) and the remainder of the image (solid lines). (b) Capacitance values of the interface during the potential scan. Black traces: No liposomes in solution. Green and blue traces: with liposomes in solution, separate trials. Capacitance measurements were performed with a 5 mV RMS potential perturbation, a 200 Hz perturbation frequency, and a 5 mV/s potential scan rate. Fluorescence images were taken with a 2.5 second exposure time and electron-multiplier gain of 200, and converted to equivalent kilocounts per second.

than an individual liposome, and so cannot be assigned to one specific interaction event. Importantly, most of these features are not present in the initially adsorbed layer and the control layers not exposed to liposomes do not show similar bright regions when treated with the same analysis. Moreover, a distinct difference in the
5.3. In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 1 min

Figure 5.6: Representative fluorescence images taken during desorption of the octadecanol layer after modification by a 1 min poration potential profile. Images are false colored and contrast enhanced for visibility in print - calibration bars on the right indicate minimum and maximum intensity values presented in each series in kcts/sec. Scale bars are 20 µm. Top row: with liposomes in solution (corresponds to green trace in Figure 5.3). Middle row: With liposomes in solution (corresponds to blue trace in Figure 5.3). Bottom row: without liposomes in solution (corresponds to black trace in Figure 5.3). White outlines highlight hotspot regions identified in the initial fluorescence images.
5.3. In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 1 min

Figure 5.7: Fluorescence images of Figure 5.6 after background subtraction by a 50 pixel radius rolling ball filter. White outlines highlight hotspot regions identified in the initial fluorescence images. Top row: with liposomes in solution (corresponds to green trace in Figure 5.3). Middle row: With liposomes in solution (corresponds to blue trace in Figure 5.3). Bottom row: without liposomes in solution (corresponds to black trace in Figure 5.3). Scale bars are 20 µm.
number of these features is evident when comparing the two layers which were exposed to liposomes, indicating that the potential controlled liposome interaction is dependent on the structure of the adsorbed layer, which is not uniform across the electrode surface.

5.4 In-Situ Fluorescence Imaging: Poration at \(-0.4\) V/SCE for 15 min

The changes observed in the adsorbed layer due to the interaction of liposomes should depend on the time spent at the poration potential (-0.4 V/SCE), resulting in an increased possibility of liposome interaction with increasing poration time. From the electrochemical measurements, increasing the time from 1 min to 15 min resulted in adsorbed layers that were still intact, with capacitance values that did not change significantly after the poration process unless liposomes were present in the sub-phase. Also, desorption of the adsorbed layer showed a shift in the potential of the onset of desorption that suggested a change in the adsorbed layer after interaction with liposomes. The difference between the 1 and 15 minute poration times was small, but the increased liposome concentration in the microscopy cell is expected to increase the chance of interaction with the adsorbed layer, enhancing any differences in the poration procedure.

5.4.1 Fluorescence During Potential Steps

A potential profile identical to those used in Section 4.4 was used to expose an adsorbed octadecanol bilayer to a poration potential of -0.4 V/SCE for 15 min, with and without liposomes in solution. The capacitance and average fluorescence intensity values are presented in Figure 5.8. As in the previous data sets, any “hotspots” or intensely fluorescent areas visible in the initially formed layer are outlined and analyzed separately.

The capacitance changes due to poration are larger than observed in the electrochemical measurements, but the trends are similar. In the control experiment with no liposomes present (black lines in Figure 5.8), the adsorbed layer capacitance increases when the potential is changed to -0.4 V/SCE, and a sharp increase in capacitance is also observed at about 7 min after the move to -0.4 V/SCE. This can be explained as a change in the layer, but since no change in the fluorescence intensity
5.4. In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 15 min

Figure 5.8: Spectroelectrochemical behaviour of adsorbed octadecanol bilayers with and without liposomes in solution during application of a potential profile with a 15 min poration step. (a) Fluorescence intensity as average grayscale values for hotspot regions (dotted lines) and the remainder of the image (solid lines). (b) Differential capacitance values of the interface during application of the potential step. Black traces: No liposomes in solution. Green traces: with liposomes in solution. Letters A-E correspond to time points of images reproduced in Figure 5.9. Blue-shaded background highlights the time period spent at the poration potential (-0.4 V/SCE), orange shaded background highlights the time period at -0.2 V/SCE, and the unshaded background represents time periods spent at 0 V/SCE. Capacitance measurements were performed with a 5 mV RMS potential perturbation and a 200 Hz perturbation frequency. Fluorescence images were taken with the exposure settings described in Section 5.1 and converted to equivalent kilocounts per second.

was seen (for the visible region), it is more likely due to a change in the wetting of the sides of the electrode held in a hanging meniscus. The capacitance decreases
5.4. In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 15 min

Figure 5.9: Representative fluorescence images taken during application of a potential profile with a 15 min poration step. Images are false colored and contrast enhanced for visibility in print - calibration bars on the right indicate minimum and maximum intensity values presented in each series in kcts/sec. Letters A-E correspond to time points marked in Figure 5.3: A - initial image at 0 V/SCE, B - end of 0 V/SCE step, C - end of -0.4 V/SCE step, D - end of -0.2 V/SCE step, E - final image (0 V/SCE). Top row: with liposomes in solution (corresponds to green trace in Figure 5.3). Middle row: With liposomes in solution (corresponds to blue trace in Figure 5.3). Bottom row: without liposomes in solution (corresponds to black trace in Figure 5.3). White outlines in column A indicate the image areas identified as hotspots for each experiment. Scale bars are 20 µm.
5.4. **In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 15 min**

during the time at -0.2 V/SCE and when returning to 0 V/SCE, to value that is slightly above its starting value. In the absence of liposomes in the electrolyte, the adsorbed layer did not experience significant changes during this extended poration process.

The fluorescence intensity initially decreases due to photobleaching, with the hotspots bleaching more quickly, as observed previously. As in the 1 min control in Figure 5.3, a slight increase in fluorescence is associated with the change in potential to -0.4 V/SCE, as expected because the adsorbed layer will slightly change its organization at these potentials. The origin of these small changes could be a displacement of fluorophore to the outer surface of the adsorbed layer.

In the presence of liposomes (green lines in Figure 5.8), the adsorbed layer showed more distinctive changes, similar to those exposed to the poration potential for 1 min. On changing the potential to -0.4 V/SCE, the capacitance increases dramatically and significant jumps in capacitance are observed during the 15 min at the poration potential. Similar events were observed for organic droplet adsorption and bursting onto a bare Hg drop[136], but cannot be used to explain these changes since the electrode surface is coated in an organic layer. The capacitance decreases when the potential is changed to -0.2 V/SCE and to 0 V/SCE, resulting in an adsorbed layer that has a capacitance of 5 µF/cm², a larger change than in the electrochemical measurements, although this is most likely due to the increase in liposome concentration when moving to the microscope cell.

When compared to the 1 min poration time described above, the changes in fluorescence for the 15 min poration time are much greater. The experiment shown in Figure 5.8 had very few identifiable hotspots in the initial image, and their behaviour was identical to the overall fluorescence response, so the profile for the hotspots is not shown here. The initial 15 min spent at 0 V/SCE showed typical photobleaching effects. On changing the potential to -0.4 V/SCE, the fluorescence intensity began a steady increase, distributed evenly across the visible image area. The fluorescence continues to increase steadily with time, showing small decreases as the potential is changed to -0.2 V/SCE and again to 0 V/SCE, again mostly evenly across the image area although there is a slightly stronger increase on the right side of the image, visible in columns D and E in Figure 5.9. The steady fluorescence increase may be due to diffusion of fluorophore into liposome structures after interaction with the adsorbed layer. These structures are expected to extend farther from the electrode surface than the adsorbed layer, so as fluorophore dif-
5.4. In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 15 min

fuses into them, the fluorescence quenching will be reduced, making the intensity brighter. A similar increase was seen in the 1 min poration experiments in Section 5.3, although the effect was not as large as that seen here.

5.4.2 Fluorescence During Desorption of the Modified Layers

After exposure to the poration potential profile, the modified octadecanol bilayer was desorbed by applying a potential scan from +0.15 to -0.8 V/SCE while taking capacitance and fluorescence measurements. The average fluorescence and capacitance values are plotted in Figure 5.10. The behaviour of the control layer is similar to that of the 1 min exposure, with an increase in fluorescence intensity associated with the increase in capacitance accompanying desorption of the layer. Similarly, this indicates that the poration process did not significantly perturb the adsorbed layer in the absence of liposomes, even though the capacitance increased slightly in the poration process.

For the example exposed to liposomes (green lines in Figure 5.10), the desorption behaviour was very different. The capacitance shows the onset of desorption at -0.45 V/SCE, with full desorption attained by -0.8 V/SCE, as signified by the capacitance reaching a value similar to that for a water covered electrode. The fluorescence increased continuously prior to desorption, with a rapid increase beginning around -0.5 V/SCE. The change in fluorescence is most sensitive to the features that are furthest from the electrode surface since these features produce the largest fluorescence signals - small changes in the distance from the electrode result in large changes in the fluorescence. This indicates that features of the modified layer are indeed farther from the electrode surface. At potentials more positive than -0.5 V/SCE, although the fluorescence is increasing, the capacitance changes only slightly - capacitance is most sensitive to changes in the dielectric of material on the electrode surface and less sensitive to changes farther from the surface. At potentials more negative than -0.5 V/SCE, both the capacitance and fluorescence increase sharply as the layer becomes desorbed. Although the details of the fluorescence behaviour are specific to the area being imaged, making direct comparisons difficult, the similarity of the general behaviours between the 1 min and 15 min poration times are clear.

As in the 1 min poration examples, for both the layers with and without liposomes in solution the increase in fluorescence is fairly uniform across the imaging
5.4. In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 15 min

Figure 5.10: Spectroelectrochemical behaviour of adsorbed octadecanol bilayers with and without liposomes in solution during desorption of the layer after modification by a 15 min poration potential profile. Potential was scanned from +0.15 V/SCE to -0.8 V/SCE. (a) Fluorescence intensity as average grayscale values for hotspot regions (dotted lines) and the remainder of the image (solid lines). (b) Differential capacitance values of the interface during the potential scan. Black lines: No liposomes in solution. Green lines: with liposomes in solution. Capacitance measurements were performed with a 5 mV RMS potential perturbation at 200 Hz and a 5 mV/s potential scan rate. Fluorescence images were taken with a 2.5 second exposure time and electron-multiplier gain of 200, and converted to equivalent kilocounts per second.

area (Figure 5.11), although the appearance of some regions of more intense fluorescence are visible. Removal of the general fluorescence increase by applying a rolling ball filter as described previously helps highlight the changes in structure in the images as the layer is desorbed. A comparison of the changes in the small
5.4. In-Situ Fluorescence Imaging: Poration at -0.4 V/SCE for 15 min

Figure 5.11: Representative fluorescence images taken during desorption of the octadecanol layer, after modification by a 15 min poration potential profile. Images are false colored and contrast enhanced for visibility in print - calibration bars on the right indicate minimum and maximum intensity values presented in each series in kcts/sec. Top row: with liposomes in solution (corresponds to green trace in Figure 5.10). Bottom row: without liposomes in solution (corresponds to black trace in Figure 5.10). White outlines in leftmost column highlight hotspot regions as identified in the initial fluorescence images. Scale bars are 20 μm.

Figure 5.12: Fluorescence images of Figure 5.11 after background subtraction by a 50 pixel radius rolling ball filter. White outlines highlight hotspot regions as identified in the initial fluorescence images. Top row: with liposomes in solution (corresponds to green trace in Figure 5.10). Bottom row: without liposomes in solution (corresponds to black trace in Figure 5.10). Scale bars are 20 μm. Hotspot regions are highlighted by white outlines in all images.
features observed both with and without liposomes in solution is shown in Figure 5.12. The outlined features are the “hotspots” identified at the start of the in the initially adsorbed layer before poration. The control layer without liposomes shows very few increases in the number or size of these features after poration, annealing and desorption. In contrast, the layer that was exposed to liposomes through the poration procedure displays a significant number of these features, far above that observed before the poration process. Even while adsorbed at 0 V/SCE after poration, but before desorption, (rightmost column in Figure 5.12), the appearance of brightly fluorescent regions is clear. These features are similar to those observed in the 1 min poration studies, with a density that is between the two 1 min poration examples. Even though it was expected that the time allowed for liposome incorporation should increase the number of features observed as well as the fluorescence intensity, the small region chosen for analysis has a large influence on the extent of liposome interaction and incorporation observed, making a comparative analysis difficult.

5.5 Conclusions

Fluorescence microscopy combined in-situ with electrochemical measurement was used to observe the interaction of liposomes with an adsorbed octadecanol bilayer. The fluorescence behaviour confirms the observations of the electrochemical measurements in Chapter 4 that liposome interaction with the adsorbed layer depends on the potential controlled creation of defects in the adsorbed layer.

In the cases where liposomes are present in the electrolyte, the small increases in the adsorbed layer capacitance after the poration procedure coincides with increases in fluorescence due to a redistribution of fluorophore after liposome interaction. The incorporation of liposomes into the adsorbed layer was found to change the desorption potential of the adsorbed layer, desorbing at less negative potentials due to the defects created through liposome interaction. Fluorescence imaging of the desorption process reveals the presence of small structures that may be regions where liposomes are incorporated. These structures are strongly influenced by the initial quality or nature of the layer deposited, which was found to be neither uniform nor homogeneous. Although the structures observed by fluorescence are larger than an individual liposome, investigation using atomic force microscopy (AFM) may reveal further information on the exact nature of
5.5. Conclusions

the liposome-octadecanol interaction.
Chapter 6

Atomic Force Microscopy
Investigations of
Octadecanol-coated Surfaces

As demonstrated in Chapter 5, fluorescence microscopy is a powerful tool for observing the electrode-solution interface. However, it is limited in resolution by diffraction, and without detailed quenching curves cannot provide exact information on fluorophore-electrode separation. Atomic force microscopy (AFM) provides a much higher lateral resolution - much more than needed to resolve 100 nm liposomes. Fluorescence is only able to provide limited information on structures raised above the electrode surface, however by its nature, AFM can provide direct information on the three dimensional size and shape of structures on the surface. When using intermittent contact mode, analysis of the phase of the cantilever oscillation can also provide some information on surface characteristics via tip-surface interaction.

To this end, exploratory measurements were done using AFM in ex-situ (air) and in-situ (in liquid, under potential control) on octadecanol layers adsorbed onto gold substrates. These measurements are complimentary to those done with fluorescence.

6.1 Ex-situ Imaging of Octadecanol Monolayers on Au

Octadecanol monolayers have been shown, both in the literature [40, 162] and in the work presented in this thesis, to be non-homogenous in nature despite their reproducible electrochemical properties. Before proceeding to more complex measurements, the typical characteristics of octadecanol adsorbed on an electrode surface in AFM must be established.
6.2 Correlation with Fluorescence Imaging

6.1.1 Experimental Methods

Monolayers of octadecanol containing 3 mol% of the BODIPY-C19-OH fluorophore were prepared on either a Au bead with Au(111) facet or Au/mica substrate as described in Section 2.7. The prepared substrates were then allowed to dry in air before imaging by AFM. All images were performed in intermittent contact mode, using either ACAFM with VistaProbes cantilevers (model T190R-25, nominal force constant 48 N/m, resonant frequency 190 kHz) or MAC mode with Type II cantilevers (Agilent, nominal force constant 2.8 N/m, resonant frequency 75 kHz). All measurements were performed in air under ambient conditions.

Topography images were processed using Gwyddion [214]. First a median line correction was applied, followed by a polynomial background subtraction (degree of 2 in both x and y). The lowest measurement value was set to 0, and the image contrast adjusted. Phase images were treated only with the median line flatten and contrast adjustment.

6.1.2 Results

The adsorbed monolayers of octadecanol including 3 mol% BODIPY-C19-OH fluorophore showed a small variety of structures upon deposition. Two representative images, one from each substrate, are shown in Figures 6.1 and 6.2. In some regions, fine structural details of the octadecanol layer structure could be observed, such as the small holes or indentations visible in Figure 6.1. A common motif was relatively large “blobs” scattered on the surface, as visible in Figure 6.2, and to a lesser degree in Figure 6.1. These blobs are visible as raised regions in the topography, and also show in the phase imaging as distinct regions, suggesting that they have a different tip-sample interaction compared to the rest of the layer. These blobs are most likely small regions of multilayer octadecanol, which might be softer or more compressible than the surrounding monolayer regions, resulting in the contrast observed in the phase measurement.

6.2 Correlation with Fluorescence Imaging

Although measurements correlating fluorescence and AFM imagery have been previously shown [215, 216], the exact nature of the structures seen in the adsorbed octadecanol layers is unknown. The fluorescent “hotspots” noted in previous chap
6.2. Correlation with Fluorescence Imaging

Figure 6.1: AFM image of an octadecanol monolayer containing 3 mol% BODIPY-C19-OH on the Au(111) facet of a bead electrode. Images were acquired in ACAFM mode in air.

Figure 6.2: AFM image of an octadecanol monolayer containing 3 mol% BODIPY-C19-OH on a Au/mica substrate. Image acquired in MAC mode in air.
6.2. Correlation with Fluorescence Imaging

ters are presumed to be due to 3-D structures separating fluorophore from the electrode surface, but could also be caused by 2-D aggregation of fluorophores within the layer locally increasing the fluorophore concentration. A correlation of the fluorescence images to the AFM should give some idea of the nature of the fluorescent structures seen in an adsorbed octadecanol layer.

6.2.1 Experimental Methods

A monolayer of octadecanol containing 3 mol% BODIPY-C19-OH was deposited onto the Au(111) facet of the bead substrate, as described in Section 2.7. After mounting in the sample holder, the bead was allowed to dry for approximately one hour in air. An area of the facet surface was imaged by AFM, using ACAFM mode (intermittent contact mode) and VistaProbes tips (model T190R-25, nominal force constant 48 N/m, resonant frequency 190 kHz). The approximate cantilever location and orientation on the surface was recorded using the AFM-mounted camera. The sample was then removed from the AFM and transferred to the fluorescence microscope. Fluorescence and brightfield images of the surface were acquired in the region near the AFM imaging location. Both brightfield and fluorescence images were taken using the 50x objective.

Topography images were processed using Gwyddion [214]. First a median line correction was applied, followed by a polynomial background subtraction (degree of 2 in both x and y) and a “remove scars” filter. The lowest measurement point was set to 0, and the image contrast adjusted.

Alignment and analysis of the images was performed in GIMP [217]. The fluorescence, brightfield, AFM camera, and topography AFM images were opened as stacked layers in a single image. The fluorescence and brightfield data, as well as that from the AFM camera, were scaled to have the same pixel scale as the AFM image. The AFM camera image was rotated so that visible surface features aligned with the brightfield image, and the topography channel of the AFM topography image rotated to match. The position and rotation angle of the topography image was manually adjusted to align visible features in the topography to similar features in the fluorescence image. A mask of features visible in the topography image was made by hand-tracing the features onto another image layer using the pencil tool.
6.2. Correlation with Fluorescence Imaging

Figure 6.3: AFM topography image (left) of the Au(111) facet with octadecanol monolayer. (Right) Mask of major topographical features from the AFM image.

6.2.2 Results

Ex-situ fluorescence and AFM measurements were performed on a monolayer of octadecanol containing 3 mol% of the BODIPY-C19-OH fluorophore. The AFM topography image acquired is shown in Figure 6.3. The background texture of the octadecanol layer on the Au-mica terraces, similar to that seen in Figures 6.1 and 6.2, is visible. Some more distinct features are also visible, as outlined in the mask on the left in Figure 6.3. Most of these features were well-defined regions, however a few, such as the diagonal stripe in the top third of the image, and the large blob in the top right, appear to be composed at least in part of loose material that moved with the tip as it scanned the surface. Two notable features, the large stripe originating at the bottom of the image and the v-shape against the right image border, were actually flatter regions that lacked the typical “octadecanol texture” found in the rest of the image, rather than raised regions.

Using the methods described above, these features identified from the AFM image were overlaid with the same region in the fluorescence image. The fluorescence image and overlay of the AFM features onto it are shown in Figure 6.4. Several of the topographically visible features, such as the large hotspot near the centre of the fluorescence image, as well as the dark streaks at the right hand side, correlate across the two images. Features from the AFM image mask that match with fluorescent features are outlined in green in the overlay in Figure 6.4. However, there are a number of features visible to AFM that do not appear to correspond with fluorescent features, and vice versa.
6.3 In-situ Imaging under Potential Control

The results of this correlation show that some fluorescence features are visible in an AFM topography image, as expected for hotspots that are caused by 3-D aggregates of fluorescent material on the surface. Some lower-fluorescence regions are visible as depressed regions in the AFM image, such as the curved stripes, which may be caused by cracks in the octadecanol layer formed at deposition. A number of features are visible to topography but not fluorescence, as would be expected for aggregates of non-fluorescent material on the surface. As well, many fluorescence features were not visible on the topography image. This may indicate that they are not 3-D aggregates as hypothesized, but possibly regions of higher fluorophore concentration within the monolayer. However it is also possible that these regions are raised away from the electrode surface, but the octadecanol layer is soft enough that it was deformed during imaging, making regions with a small height difference difficult to discern. Imaging of the octadecanol under liquid with a cantilever of lower force constant would be required in order to remove this ambiguity.

6.3 In-situ Imaging under Potential Control

With the characteristics of octadecanol layers established, the ground is laid for in-situ imaging under potential control. Roughly replicating the experiments per-
6.3. In-situ Imaging under Potential Control

formed with fluorescence microscopy, these experiments provide a higher-resolution view of the interaction of octadecanol with the electrode surface and with liposomes.

6.3.1 Experimental Methods

All in-situ imaging was done using Au/mica substrates, prepared with a bilayer of octadecanol containing 3 mol% BODIPY-C19-OH as described in Section 2.7. The substrate was then mounted in the liquid cell, which was filled with 0.1 M NaF electrolyte. If required, 1 µL of the liposome solution was then added. The entire sample assembly was placed in the environmental chamber and allowed to rest in a nitrogen atmosphere for one hour to minimize oxygen in solution before imaging. Potential was held constant during imaging. After completing two images, the potential was stepped directly to the next value, and the system allowed to equilibrate for approximately 10 seconds before imaging again. All potentials were measured against an Au bead pseudoreference. Based on the position of Au oxidation peaks (measured independently), this reference electrode is approximately -0.1 V/SCE. Therefore the 0 and -0.5 V/Au potentials used are approximately equal to 0.1 and -0.4 V/SCE, similar to what was used in the electrochemical and fluorescence studies.

Although the substrate was not moved during the measurements, accidental contact between the cantilever holder assembly and the reference or counter electrodes required the tip to be lifted and re-engaged with the surface. During the octadecanol-only experiment, this resulted in slightly different areas being imaged at each potential. This drift was less of a problem during the measurements with liposomes in solution due to a better initial sample placement, so the area imaged at each potential is nearly identical.

All in-situ images were taken in intermittent contact MAC mode. The experiment without liposomes in solution was imaged using Type II MAC cantilevers (Agilent, nominal force constant 2.8 N/m, resonant frequency 75 kHz in air). The experiment with liposomes in solution used a Type I MAC cantilever (Agilent, nominal force constant 0.6 N/m, resonant frequency 75 kHz in air).

AFM images were processed using Gwyddion. The images were subjected to a median line correction followed by a second-degree polynomial background subtraction. The minimum height value was set to zero and contrast adjusted for
6.3. In-situ Imaging under Potential Control

visibility. The images with liposomes in solution were originally taken as 3 \( \mu m \) square images, and cropped to 2 \( \mu m \) squares for a more direct comparison with the liposome-free images. Particle heights were obtained by manually drawing line profiles through the center of the feature in question, and the height taken as the average of the height measured at each edge of the feature.

6.3.2 Results

The topography images acquired for an octadecanol bilayer with no liposomes in solution are presented in Figure 6.5. A sample of representative height profiles and average values for different features seen in the images is shown in Figure 6.7.

Little change in the layer is seen as the potential is stepped from 0 to -0.5 V/Au and back. Initially (Figure 6.5a) the same low flat features as previously seen in the ex-situ octadecanol layers are visible. At -0.5 V/Au, similar features are seen, at approximately the same height, as seen in the profiles of Figure 6.7. Although at this potential defects should be present in the octadecanol layer, none are visible in the images. This may be because the defects are too small in either height or diameter to be detected with the AFM, or because the force of the cantilever contacting the surface is enough to blur out the defect edges and render them invisible.

Upon return to 0 V/Au, where the potential-created defects will be healed, a few of the low flat blobs visible in other images are still present at a reduced height, but the layer is mostly flat and featureless. Generally, all of the visible features are slightly smaller than their counterparts at the initial -0.5 V/Au images (See Figure 6.7). This is not necessarily indicative of a change in the layer itself, as the imaging area is different from the previous two images. The layer may have flattened after application of the potential profile, or it may simply be a more featureless region of the layer being imaged.

Analysis of the phase channel of the images (Figure 6.6) shows that, as demonstrated in the ex-situ measurements, the low blobs exhibit a visibly different phase behaviour than the surrounding layer.

A repeat of the same potential profile with liposomes in solution was also performed. In this case, the imaging area remained the same across all potentials, so features can be tracked more directly. The topographical images are shown in Figure 6.8, and sample cross sectional profiles of the features are shown in Figures 6.10...
6.3. In-situ Imaging under Potential Control

Figure 6.5: In-situ AFM images of an octadecanol bilayer containing 3 mol% BODIPY-C19-OH: topography channel. Potentials are measured vs. an Au bead reference.
Figure 6.6: In-situ AFM images of an octadecanol bilayer containing 3 mol% BODIPY-C19-OH: phase channel. Potentials are measured vs. an Au bead reference.
6.3. In-situ Imaging under Potential Control

Figure 6.7: Example features of each category from the AFM topography images of octadecanol bilayers. Images are cropped from those in Figure 6.5. Profiles shown in plots correspond to the cross-sections drawn on each image. Features in each category were measured on the full images to provide average height values at each potential. Scale bars are 200 nm.
6.3. In-situ Imaging under Potential Control

(a) 0 V/Au (initial).
(b) -0.5 V/Au.
(c) 0 V/Au (final).

Figure 6.8: In-situ AFM images of an octadecanol bilayer containing 3 mol% BODIPY-C19-OH and liposomes in solution: topography channel. Potentials are measured vs. an Au bead reference.
6.3. In-situ Imaging under Potential Control

(a) 0 V/Au (initial)  
(b) -0.5 V/Au  
(c) 0 V/Au (final)

Figure 6.9: In-situ AFM images of an octadecanol bilayer containing 3 mol% BODIPY-C19-OH and liposomes in solution: phase channel. Potentials are measured vs. an Au bead reference.
6.3. In-situ Imaging under Potential Control

Figure 6.10: Example features that were present in the initially deposited layer of each category from the AFM topography images of octadecanol bilayers with liposomes in solution. Images are cropped from those in Figure 6.8. Profiles shown in plots correspond to the cross-sections drawn on each image. The features measured in the 0 V (initial) images are re-measured at the other two potentials. Features in each category were measured on the full images to provide average height values at each potential. The “small features” were not visible except in the initial 0 V image. Scale bars are 200 nm.
6.3 In-situ Imaging under Potential Control

Figure 6.11: Example features that appeared during liposome adsorption from the AFM topography images of octadecanol bilayers. Images are cropped from those in Figure 6.8. Profiles shown in plots correspond to the cross-sections drawn on each image. The features measured in the 0 V (initial) images are re-measured at the other two potentials (image #1 for each). Image #2 in the last two potentials is a feature that was not visible at the initial 0 V image - the same feature in both -0.5 V and 0 V (final). Features in each category were measured on the full images to provide average height values at each potential. The “small features” were not visible except in the initial 0 V image. Scale bars are 200 nm.
6.3. *In-situ Imaging under Potential Control*

and 6.11. Since it was possible to image the same region at all potentials, an analysis of features visible in all images is presented alongside features that appeared only after poration in the profiles of Figure 6.10.

Initially, the layer is relatively flat, with a few larger features and the flat blobs typical of an octadecanol layer. At -0.5 V/Au, a large number of new features appear. Most of these features are significantly taller than the flat blobs previously seen, although some features of a similar height also appear. This can be seen in the profiles in Figures 6.10 and 6.11, where the average heights of both the flat blob features and the tall features is increased over those measured at 0 V/Au. Many of these new features persist upon return to 0 V/Au, and retain some of the increased height.

The appearance of these taller features is absent in the octadecanol-only images, and must be due to the interaction of liposomes with the adsorbed layer. None of the feature heights measured is comparable to the 100 nm size expected of a whole liposome adsorbed onto the surface, even accommodating for some distortion caused by tip effects on a soft structure. Therefore the features must be regions where the liposomes have burst to incorporate into the octadecanol layer, forming 3-D structures above the electrode surface. Based on height measurements, these structures may be hemiliposomes or some partially flattened structure.

Analysis of the phase channel of the AFM images (Figure 6.9) shows another remarkable change with liposome incorporation. Whereas in the absence of liposomes, the structures in the adsorbed layer are distinguishable in the phase images, upon liposome incorporation the contrast between the topographical features and layer background is largely lost. This certainly indicates that the structures seen in Figure 6.8b and c are of a different nature than those seen in the unmodified octadecanol layers. It may also be possible that the phase contrast is erased because liposomes are depositing across the entire electrode surface on top of the octadecanol layer, causing the entire surface to have similar tip-sample interactions. This cannot be determined from the measurements done here, however film thickness measurements, easily performed in-situ, may provide future insight.

Comparison with the fluorescence results from Section 5.3.1 provides further insight. By using the same rolling-ball subtraction performed on the desorption images, it is clear that during the poration phase of the experiment, no new structures are formed in the octadecanol layer without liposomes (Figure 6.12, top). With liposomes in solution, a large number of new features are formed upon application
6.4 Conclusions

Figure 6.12: Fluorescence images taken during the application of potential steps after background subtraction by application of a 50 pixel rolling ball filter. Original fluorescence images are found in Figure 5.4. Top: without liposomes in solution. Bottom: with liposomes in solution. Scale bar is 20 µm.

of the -0.4V/SCE potential, some of which persist as the potential is changed back to 0 V/SCE. This behaviour is exactly analogous to that observed in the AFM measurements. Although the scale of the images is different between the two imaging modes, they share the same potential-dependent changes. If the changes in fluorescence intensity are proportional to changes in height, then the changes seen in the fluorescence images of Figure 6.12 can be interpreted as showing the same behaviour seen in the AFM images.

6.4 Conclusions

Atomic force microscopy, both in-situ and ex-situ, has been performed on solid-supported octadecanol layers. Ex-situ measurements show that the layer is non-uniform, containing structures of approximately 15 nm height that are visible to both topography and phase contrast imaging. Some features visible to AFM imaging can be identified as fluorescent regions in the layer, however a complete correlation is not possible.

In-situ imaging of the octadecanol bilayer under potential control showed little change in the layer in the absence of liposomes. With liposomes in solution, a number of large features appeared across the image area upon creation of defects in the octadecanol layer. Many of these structures persisted upon return to 0 V/Au and
may be assigned to liposomes incorporating into the defects created in the octadecanol layer. Phase contrast analysis shows no difference between these structures and the surrounding layer, indicating that the layer after potential perturbation is different with liposomes in solution than in the absence of liposomes.

The incorporation of liposomes into the adsorbed octadecanol layer while under potential control has been directly observed by in-situ atomic force microscopy. The behaviour seen correlates with the previously observed fluorescence behaviour, providing further evidence for the potential-controlled incorporation of liposomes into the adsorbed octadecanol layer.
Chapter 7

Summary and Future Work

The development of stable, easily modifiable solid-supported layers is critical for the creation of robust biosensor platforms involving transmembrane proteins and ion channels, which are the major targets for modern drug discovery [8]. Including phospholipid bilayers in the design is essential in order to support these proteins, as their structure and function greatly depend on their environment, and the hydrophobic regions of the membrane are an important factor. Building the sensor directly onto an electrode surface builds in the ability to control the transmembrane potential, mimicking the natural cell membrane, however proteins may denature if in direct contact with an electrode surface. Phospholipid bilayers on their own are easily and conveniently created, but are not well adhered to electrode surfaces, requiring the use of a supporting molecule or tether to anchor them to the electrode surface and provide a space for proteins to extend outside the membrane on either side, without contacting the metal directly.

The work presented in this thesis describes a method for producing supported phospholipid layers in-situ by control of the electrode potential. This method produces a three-dimensionally structured layer of modified lipid bilayer with some regions separated from the electrode surface. These structures have the potential to house transmembrane proteins safely away from the electrode surface without the use of special tethering molecules, as illustrated by the cartoon in Figure 7.1. The presence of a small pocket of electrolyte would make sensors based on this platform suitable for direct detection of ion channel activity, and as proteins are separated from the electrode surface, fluorescence-based detection of binding events is also possible.

By using an initially deposited octadecanol bilayer, the electrode surface is protected from liposome deposition until the application of a negative electrode potential. This potential opens defects in the adsorbed octadecanol layer, and allows the liposomes to incorporate into the adsorbed layer, forming regions of incorporated liposomal bilayer across the electrode surface. This process was refined and
Figure 7.1: Cartoon schematic of protein incorporation into a raised liposomal bilayer structure (not to scale). One possible location for a transmembrane protein is shown in green.

characterized as described as follows.

The physical, electrochemical and fluorescence properties of adsorbed octadecanol layers modified with a fluorescent probe were characterized in Chapter 3. Few methods allow for gathering information on the properties of an adsorbed surface layer in-situ and with spatial resolution. Fluorescence microscopy is one such technique, however it requires that the adsorbed layer under study be either inherently fluorescent or doped with a small amount of a fluorescent molecule to make it visible. Since octadecanol is not fluorescent, a fluorophore must be added, in this case the BOCIPY-C19-OH molecule designed specifically for use in octadecanol monolayers. Compression isotherms of octadecanol monolayers containing various concentrations of the fluorophore showed that despite its’ structural similarity to octadecanol, the BODIPY-C19-OH causes some disruption to the monolayer organization even at low concentrations. At concentrations above 3 mol% however, the disruptions became more pronounced and characteristic of a poorly mixed system. Electrochemical characterization of monolayers and bilayers of octadecanol with the fluorophore confirmed this pattern, with 0.5 mol% and 3 mol% showing the least altered electrochemical characteristics of the concentrations tested, relative to pure octadecanol. Fluorescence microscopy of the BODIPY-C19-OH containing octadecanol bilayers showed that 3 mol% of the fluorophore was sufficient to ensure a detectable fluorescence signal, at least at desorption. These results were used to design the octadecanol layers used for continuing study of liposome incorporation.

The explorations of the potential-controlled interaction of DOPC liposomes with the octadecanol bilayer were presented in Chapter 4. Liposomes would not incorporate into the as-deposited octadecanol layer at moderate potentials, even given over an hour of exposure. Application of a potential sufficient to cause sig-
significant disruption to the adsorbed layer structure was required to facilitate interaction with the liposomes - in this case a potential more negative than -0.4 V/SCE. The magnitude of the potential used to create defects in the octadecanol layer had some effect on the characteristics of the liposome-modified layer, but interestingly, complete desorption of the layer did not increase the apparent degree of liposome incorporation into the adsorbed layer. As long as a sufficiently large potential was applied, the time spent at that potential was found to have only a weak effect on the interaction of liposomes with the adsorbed layer. Shorter times, such as 1 min, were both convenient and effective at allowing liposomes to incorporate into the octadecanol layer.

Changes in the adsorbed octadecanol layer were easily observed by a shift in the double-layer capacitance of the electrode as the layer structure was altered due to changes in surface tension with potential. These measurements are simple and easy to implement, but do not provide any detail on the exact form of the changes made to the layer. In-situ fluorescence microscopy, presented in Chapter 5, gives insight into the changes occurring on the electrode surface. As any fluorescent material near the electrode surface is quenched, changes in layer structure that result in a separation of the adsorbed layer from the surface are highlighted by a local increase in fluorescence intensity. Observations of the general fluorescence properties confirmed that liposomes do not alter the octadecanol layer until after application of the poration potential. Analysis of the fine structure visible in the fluorescence images revealed the creation of small raised (and therefore fluorescent) structures on the electrode surface whose appearance coincided with the capacitance changes attributed to liposome incorporation. Although the resolution of optical microscopy is not sufficient to assign these structures to individual liposome incorporation events, the formation of these structures is evidence of the incorporation of liposomes at specific locations on the surface, creating a heterogeneous layer structure.

The improved resolution of atomic force microscopy was leveraged in the experiments documented in Chapter 6 to provide further detail on the nature of the interaction at the electrode surface. The octadecanol layer itself was shown to be quite heterogeneous, as might be expected from the structures visible to fluorescence microscopy. In-situ imaging provided a unique view into the incorporation process, showing the creation of tall structures during exposure to the poration potential that remained visible even after a return to 0 V/SCE. Although these structures can not
Directions for Future Study

Although the mechanism of forming the multi-component layers has been proven sound, many avenues for continuing study of this system remain. As this method is intended to become a biosensor platform, adding sensor functionality to the system is an obvious next step. Sensors based on transmembrane proteins have already been demonstrated using other models of solid-supported bilayers, by incorporating ion channels both natural [13–15, 218] and synthetic, [16, 219] and transport proteins, [12] among others. These transmembrane proteins are relatively easily incorporated into liposomes, and could therefore be applied to the modified octadecanol model. By using liposomes prepared with incorporated gated ion channels, for example, a structure similar to that envisioned in Figure 7.1 could be created as an analog to similar sensors made with tethered bilayers. This would allow a direct comparison of ease of formation and sensitivity of sensors made with the two platforms.

The ability to control deposition could be leveraged to deposit different liposomes onto the various microelectrodes of an interdigitated electrode chip. The ability to create a single chip with several different supported bilayer functionalities would be a great advantage in creating multifunctional sensors, especially if the bilayers can be added in-situ. Figure 7.2 illustrates how this process could be carried out inside a microfluidic chamber. Proof of concept of this process could be easily tested before application to a microfluidic chip by using a series of liposome solutions, with each liposome created with different fluorescent tags. The electrode chip, after modification with the octadecanol bilayer, would be immersed in each
7.1. Directions for Future Study

Figure 7.2: A possible procedure for creating multifunctional sensors in-situ in a microfluidic cell. Liposomes with three different functionalities are deposited, each containing for example a different ion channel or fluorescent probe. Step (1) shows the electrodes and microfluidic chamber in the initial condition before liposome incorporation. In Step (2) the poration potential is applied to one electrode only, while a solution of Liposome A flows through the chamber. Steps (3) and (4) repeat this process using different liposome solutions and poration at sequential electrodes. In Step (5), all three electrodes have been modified with liposomes, and the analyte can interact to produce three simultaneous analytical signals.

solution sequentially with potential control to allow liposome incorporation to one electrode in each solution. The results of the incorporation could then be easily verified with fluorescence microscopy and the model applied towards building a multichannel sensor.

Before the system demonstrated here could be truly called functional as a sensor system, the issues surrounding use of octadecanol as the base layer must be addressed. Although octadecanol has appropriate electrochemical properties, it is very sensitive to small levels of contamination in the electrolyte, and even as a single-component floating monolayer forms phase-segregated structures [40] that reduce the reproducibility of depositions onto the electrode. Use of a more liquid layer, such as oleyl alcohol (cis-9-octadecen-1-ol) which is liquid at room temperature, or a mixed monolayer of a liquid and a solid-phase component, such as octadecanol/oleyl alcohol, may improve reproducibility of the deposited layers by reducing phase segregation.
7.1. Directions for Future Study

Variations in the liposome composition will also have an effect on the incorporation behaviour. Model cell membranes designed to support transmembrane proteins are often made of a mix of phospholipids, and may also include cholesterol. The particular mix of lipids used as well as cholesterol content is known to affect the rigidity of the liposomes formed [198–200], which should also affect the electrochemical incorporation properties of the liposomes. Liposome size may also influence their incorporation characteristics, as smaller vesicles are under greater pressure due to the increased curvature.

Continued study using in-situ AFM would further characterize the incorporation behaviour of the liposomes. Use of force-distance spectroscopy would help establish the thickness of the adsorbed layer at various structures, as well as some details of its nature (for example, bilayer or multilayer). Characterizing both the inhomogeneities of the octadecanol layer and the newly created liposomal structures in this way would further clarify the nature of the liposome-octadecanol interaction, for example determining if a mono- or bilayer is deposited on top of the octadecanol layer, remaining invisible to fluorescence and affecting the phase contrast in the AFM images. Force curve mapping would also determine the rigidity of the adsorbed liposomal structures, and the nature of their 3-D arrangement.

The ability to incorporate liposomes onto an electrode, mediated by potential control, opens a wide variety of options for future sensor design. The primary advantages are the ability to create layers in-situ, and the ease of creating bilayers that contain transmembrane proteins or other membrane components directly on a platform that allows manipulation of the transmembrane potential. After identifying a more robust base layer, this method has the potential to create a wide range of biosensors applicable to drug discovery and health care applications.
References


References


<table>
<thead>
<tr>
<th>Reference</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>[33] Gaines, G. L. In Insoluble Monolayers at Gas-Liquid Interfaces; Prigogine, I., Ed.; Interscience Monographs on Chemistry; John Wiley &amp; Sons Ltd., 1966; p 386.</td>
<td></td>
</tr>
</tbody>
</table>
References


179
References


References


References


References


References


References


185


References


References


Appendix A

Liposome Characterization and Stability

A.1 Characterization by Thin Layer Chromatography

Phospholipids, such as the DOPC used in these studies, are subject to degradation over time. In aqueous solution, the carboxy esters linking the fatty acid residues to the glycerol backbone may be hydrolyzed to release the fatty acids, eventually producing glycerophosphoric acid if both fatty acid chains are hydrolyzed. [220] Thin layer chromatography (TLC) can be used to identify liposomal suspensions in which the phospholipid has degraded and used as a screening method to ensure that solutions used are not decomposed. [221]

A.1.1 Experimental Methods

Liposome solutions were spotted onto TLC plates (1 \( \mu \text{L} \)) onto an aluminum-backed silica gel TLC plate (EMD) along with a sample of stock DOPC solution in chloroform (0.5 \( \mu \text{L} \)) and a known degraded liposome solution (1 \( \mu \text{L} \)) in adjacent lanes. The degraded liposome solution was prepared from liposomes extruded in the perchlorate electrolyte, saturated with oxygen after preparation, and aged for several weeks. After applying the solution to the TLC plate, the spots were allowed to dry thoroughly and then eluted using a 2:1 chloroform:methanol mixture (both: Fisher, HPLC grade). After elution, the plate was dried and visualized by rinsing with dilute sulphuric acid (Fisher ACS, approximately 1 M) and charring in an oven at 190°C for 5-10 minutes until spots appeared. Solutions were monitored in this manner from formation until the end of use of the solution. During the characterization phase, liposomes were initially measured every few days. After it was established that the liposomes in the NaF electrolyte were more stable, confirmation of purity by TLC was performed every few weeks.
A.1.2 Results

Selected TLC plates from the monitoring of Solution 1 of liposomes are shown in Figure A.1. For clarity in viewing, the plates have been electronically scanned, the color desaturated, and the brightness-contrast adjusted to enhance visibility. Spots corresponding to the fatty acid degradation product are marked with a black circle. Two solutions of liposomes prepared on the same day, one in 0.1 M NaF and one in 0.05 M KClO₄, are compared with a known degraded liposome solution and a stock solution of DOPC in chloroform. It is easily seen that in just a few weeks, the liposomes in the KClO₄ electrolyte have begun to degrade, producing a second spot on the TLC plate. After nearly 19 months of storage and use, the final TLC taken of the solution before it was used up shows no evidence of degradation in the NaF electrolyte, while the second spot in the KClO₄ electrolyte solution has grown darker.

The instability of liposomes in the 0.05 M KClO₄ electrolyte is not unexpected, as perchlorate is a known oxidizer and would be expected to enhance the hydrolysis of the ester bonds. A previous study [222] suggested that perchlorate does not contribute to peroxidization of the phospholipids, however other mechanisms of degradation are possible and are clearly occurring in storage. For this reason, studies done with liposomes prepared in perchlorate electrolyte (for example [127]) should be considered carefully and caution taken if work is done on liposome suspensions that were not freshly prepared.

A.2 Characterization by Elastic Light Scattering

In order to confirm that the liposomes produced were of uniform and correct size, an initial characterization of the liposome solution was performed by dynamic light scattering. A Coulter N4+ particle size analyzer was used, and sizes taken as an average of 3 measurements each integrated over 120 s. The sample was 5 μL of the liposome solution diluted to approximately 4 mL using the same 0.1 M NaF electrolyte in which the liposomes were prepared. Temperature was held at 20°C, equilibrated for 5 min, and scattering measured at 90°. For each of the three measurements, a particle size histogram from 1 - 1000 nm was recorded (31 bins) as well as a size histogram from 50 - 250 nm (31 bins). The larger range ensures that there are no particles measured well outside the expected range, while the sec-
A.2. Characterization by Elastic Light Scattering

Figure A.1: TLC plates taken at various points during the lifetime of a liposome solution. From left to right, the plates were measured on: Oct 25 2010 (solution age 0 days), Nov 2 2010 (solution age 8 days), Dec 20 2010 (solution age 8 weeks), and May 25 2011 (solution age 7 months - final use). All plates are spotted with Lane 1 - Liposome solution in NaF, Lane 2 - Liposome solution in KClO₄, Lane 3 - Known degraded liposome solution, Lane 4 - DOPC stock solution in chloroform. Lanes 1-3 have a 1 µL spot, Lane 4 is 0.5 µL. Spots with a lower retention factor (closer to the solvent front) are the fatty acid degradation product and are highlighted with black circles. In Lane 3 and the final measurement of Lane 2, the higher retention factor spot from the lysophospholipid degradation product is also visible. Identity of the markings along the solvent front is unknown but is present in blank runs.

Histogram provides greater detail on the size distribution within the maximum number of bins available. The data from the histogram was fit to a log-normal distribution using QTIPLOT (Ion Vasilief, http://soft.proindependent.com/qtiplot.html) and the mean particle size calculated and averaged for the three measurements. The data for the two liposome preparations used experimentally is shown in Table A.1. As the nominal particle size was 100 nm based on the pore size of the filter used, the size was deemed acceptable if it was roughly similar. Both preparations were considered acceptably close to 100 nm.
### A.2. Characterization by Elastic Light Scattering

Table A.1: Mean particle sizes for liposome solutions as measured using dynamic light scattering.

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Mean Size (nm)</th>
<th>Standard Deviation (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution 1</td>
<td>133</td>
<td>15</td>
</tr>
<tr>
<td>Feb. 2011</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solution 2</td>
<td>122</td>
<td>20</td>
</tr>
<tr>
<td>Aug 2012</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**Appendix B**

**Fluorescence Calibration Factors**

Correction factors for comparing the two exposure time / gain settings used was done empirically. Based on measurements of a calibration sample (A3 on FocalCheck Test Slide #1, Molecular Probes), the average fluorescence intensity was found to be directly proportional to the exposure time with the same electron-multiplier gain settings, and revealed a dark signal of 500. The conversion factor to kcts/sec for matching the images acquired at 2.5 sec and 200 gain to the 5 sec, 400 gain images was determined by dividing the average grayscale values of several images taken at these settings, and testing the conversion factor against further images that were not a part of the analysis. The conversion factor was found to be related to the exposure time and gain ratio, but not perfectly. The values used in the analyses presented in this work are summarized in Table B.1.

<table>
<thead>
<tr>
<th>Image Settings</th>
<th>Conversion Factor to kcts/sec</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exposure: 2.5 sec</td>
<td></td>
</tr>
<tr>
<td>E.M. gain: 200</td>
<td>2500</td>
</tr>
<tr>
<td>Exposure: 5 sec</td>
<td></td>
</tr>
<tr>
<td>E.M. gain: 400</td>
<td>7500</td>
</tr>
</tbody>
</table>

Table B.1: Conversion factors to kcts/sec used for fluorescence images.
Appendix C

Rolling-Ball Image Processing

In Chapters 5 and 6, a rolling-ball filter is used to remove background intensity gradients from fluorescence images while maintaining local changes in image intensity. The rolling-ball filter, also called a “top-hat” filter, can be imagined as rolling a ball of diameter $X$ over the image, where the intensity value is mapped as height. [223] An average intensity value is taken over the radius of the ball, and this background value is subtracted from that point. If the radius is sufficiently large, it will remove low-frequency background fluctuations as well as the average background intensity while leaving unaltered features with a smaller radius. Choosing an appropriate radius is important as a too small radius will result in loss of some image features. For comparison, in Figure C.1 a fluorescence image from Chapter 5 is processed using three different ball sizes.
Figure C.1: False-color fluorescence images processed with a rolling-ball filter of various ball sizes. False-color scale is the same for all images, and all images are 150 x 150 µm.