FORMATION OF CALCIUM PHOSPHATE CRYSTALS UNDER LIPID MONOLAYERS

By

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To all my family members and friends for loving and supporting me
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TABLE OF CONTENTS

ACKNOWLEDGMENTS.................................................................................................. 4

LIST OF TABLES............................................................................................................ 7

LIST OF FIGURES.......................................................................................................... 8

ABSTRACT................................................................................................................... 11

CHAPTER

1 INTRODUCTION.................................................................................................... 13

1.1 Scope of Thesis ................................................................................................ 13
1.2 Background....................................................................................................... 13
1.3 Kinetics of Crystallization .................................................................................. 15
  1.3.1 Calculation of Relative Supersaturation Ratio (σ)..................................... 15
  1.3.2 Factors Affecting the Relative Supersaturation ....................................... 16
1.4 Lipids and Membranes...................................................................................... 16
1.5 Langmuir Monolayers and Langmuir-Blodgett Films......................................... 17
1.6 Brewster Angle Microscopy and its Applications............................................. 18

2 LITERATURE REVIEW .......................................................................................... 23

2.1 Close Look at Domain Formation in DPPC Monolayer ..................................... 23
2.2 Study of Hydrolysis of DPPC by Phospholipase A2 Using Langmuir
  Monolayer and BAM ............................................................................................ 24
2.3 Condensing Effect of Palmitic Acid on DPPC Monolayer .................................. 25
2.4 Studies of Lysophosphocholine/DPPC Mixtures ............................................... 26
2.5 Study of Calcium Phosphate Formation under Three Different Langmuir
  Monolayers .......................................................................................................... 28

3 FORMATION OF CALCIUM PHOSPHATE CRYSTALS UNDER LIPID
  MONOLAYERS ...................................................................................................... 37

3.1 Introduction ....................................................................................................... 37
3.2 Experimental Section ........................................................................................ 39
  3.2.1 Materials .................................................................................................. 39
  3.2.2 Preparation of Supersaturated Calcium Phosphate Solution ................... 40
  3.2.3 Preparation of Surfactant Solution ........................................................... 40
  3.2.4 Glass Slides ............................................................................................ 41
3.3 Brewster Angle Microscopy .............................................................................. 41
3.4 Crystal Counting Procedure .............................................................................. 42
3.5 Procedure ......................................................................................................... 42
3.6 Study of Hydrolysis of Phospholipid DPPC....................................................... 43
3.7 Crystallization of Calcium Phosphate under the DPPC Monolayer ...................... 44
3.8 Crystallization of Calcium Phosphate under the Ternary Mixture Monolayer
with Palmitic Acid .................................................................................................... 46
3.9 Behavioral Study of the Binary Mixture (DPPC: PA, DPPC: Lyso PC) ............... 47
3.10 Crystallization of Calcium Phosphate under the Binary Mixtures ................. 48

4 IMPACT OF CHAIN LENGTH OF FATTY ACIDS ON CALCIUM PHOSPHATE
FORMATION ............................................................................................................. 57

4.1 Experimental Section .......................................................................................... 57
4.2 Crystallization of Calcium Phosphate under the Ternary Mixture Monolayer
with Arachidic Acid ............................................................................................... 58
4.3 BAM Images of Ternary Mixture at Different Pressures .................................... 59
4.4 Isotherm and BAM Images of Pure Fatty Acids .............................................. 60

5 CONCLUSIONS AND RECOMMENDATIONS ...................................................... 66

LIST OF REFERENCES .............................................................................................. 67

BIOGRAPHICAL SKETCH ......................................................................................... 72
LIST OF TABLES

Table | page
------|------
3-1   | The average number of crystals\textsuperscript{a} per BAM image under different monolayers at low and high pressure. ................................................................................... 50
4-1   | Average number of crystals\textsuperscript{a} per BAM image under different monolayers at low and high pressure. ..................................................................................... 61
<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-1</td>
<td>Structural features of lipids, using a glycerophospholipid (phosphatidylcholine) as an example.</td>
<td>20</td>
</tr>
<tr>
<td>1-2</td>
<td>Schematic representation of a Langmuir monolayer experiment.</td>
<td>20</td>
</tr>
<tr>
<td>1-3</td>
<td>Schematic illustration of the principle behind Brewster Angle Microscopy.</td>
<td>21</td>
</tr>
<tr>
<td>1-4</td>
<td>Experimental setup utilizing Brewster angle microscopy to monitor crystal growth at phospholipid Langmuir monolayers.</td>
<td>21</td>
</tr>
<tr>
<td>1-5</td>
<td>Schematic representation of a pressure-area isotherm showing the possible phases present during the compression of an amphiphile.</td>
<td>22</td>
</tr>
<tr>
<td>2-1</td>
<td>DPPC domain growth with a compression rate of 0.86 Å² molecule-1 min-1.</td>
<td>30</td>
</tr>
<tr>
<td>2-2</td>
<td>Compression isotherm of 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) on a water subphase at 25 °C.</td>
<td>31</td>
</tr>
<tr>
<td>2-3</td>
<td>Structures of DPPC (1), lysolipid 22:0 Lyso PC (2), and palmitic acid (PA) (3).</td>
<td>31</td>
</tr>
<tr>
<td>2-4</td>
<td>Pressure vs mean chain area isotherms of DPPC and of a palmitic acid taken over a 0.35 mM CaCl₂ subphase at 25 °C.</td>
<td>32</td>
</tr>
<tr>
<td>2-5</td>
<td>Pressure vs mean chain area isotherms of the binary monolayers of DPPC with PA and DPPC with the 22:0 Lyso PC.</td>
<td>32</td>
</tr>
<tr>
<td>2-6</td>
<td>Brewster angle microscopy images of a DPPC (A) and ternary mixture (DPPC: PA: Lyso PC) (B) and pure palmitic acid (C) monolayer.</td>
<td>33</td>
</tr>
<tr>
<td>2-7</td>
<td>BAM images of 70:30DPPC/Lyso PC (A) and 70:30 DPPC/PA (B) binary monolayers over a 0.35 mM calcium oxalate subphase.</td>
<td>33</td>
</tr>
<tr>
<td>2-8</td>
<td>Surface pressure (mN/m)-area (Å²) isotherms at 24 °C on a pure water subphase: (A) neat DPPC-d62 (gray curve) and neat PA (black curve), (B)</td>
<td>34</td>
</tr>
<tr>
<td>2-9</td>
<td>DPPC/C&lt;sub&gt;22&lt;/sub&gt;PC isotherms at 20 °C.</td>
<td>35</td>
</tr>
<tr>
<td>2-10</td>
<td>DPPC/C&lt;sub&gt;18&lt;/sub&gt;PC domains formed by compression at 0.86 Å² molecule-1 min-1.</td>
<td>35</td>
</tr>
<tr>
<td>2-11</td>
<td>Isotherms of monolayers on pure water and HAp supersaturated solution for different times: (A) DPPC, (B) AA, and (C) ODA.</td>
<td>36</td>
</tr>
<tr>
<td>2-12</td>
<td>EPM spectra of calcium phosphate formed under the DPPC, ODA and AA monolayer.</td>
<td>36</td>
</tr>
</tbody>
</table>
3-1 Control experiment showing BAM images of calcium phosphate subphase in the absence of a DPPC Langmuir monolayer. ................................................................. 50

3-2 BAM images of CaCl₂ subphase (A) and Na₃PO₄ subphase (B) prepared in the NaCl, Tris HCL buffer solution. Images taken after 24 hours at 20°C. ............. 50

3-3 Structures of DPPC (1), lysolipid 22:0 Lyso PC (2), and palmitic acid (PA) (3). . 51

3-4 Pressure vs mean molecular area isotherms of a pure DPPC taken over a 0.43 mM CaCl₂ subphase at 20°C. ........................................................................... 51

3-5 Brewster angle microscopy images of a DPPC monolayer at 20°C after 24 h over a calcium phosphate subphase at (A) 35 and (B) 15 mN/m. ...................... 52

3-6 Pressure vs mean molecular area isotherms of a ternary 2:1:1 mixture of DPPC, 22:0 Lyso PC, and palmitic acid taken over a 0.43 mM CaCl₂ subphase ........................................................................................................ 52

3-7 Brewster angle microscopy images of a ternary monolayer, a 2:1:1 mixture of DPPC, 22:0 Lyso PC, and palmitic acid monolayer ........................................ 53

3-8 Pressure vs mean molecular area isotherms of the binary monolayers of DPPC with PA and DPPC with the 22:0 Lyso PC.......................................................... 54

3-9 Shape of domains formed in LC/LE coexistence region (A) pure DPPC, (B) Ternary mixture with DPPC:PA:Lyso PC at 20°C. .............................................. 54

3-10 BAM images of a 70:30 DPPC/palmitic acid binary monolayer over a calcium phosphate subphase. ..................................................................................... 55

3-11 BAM images of a 70:30 DPPC/lyso PC binary monolayer over a calcium phosphate subphase. ......................................................................................... 55

3-12 BAM images of a palmitic acid monolayer at 15mN/m over a calcium phosphate subphase. ................................................................................................. 55

3-13 BAM images of a palmitic acid monolayer at 35mN/m over a calcium phosphate subphase containing NaCl and tris HCl at 20°C after 24 hr 20°C. .... 56

4-1 Structures of DPPC (1), lysolipid 22:0 Lyso PC (2), and arachidic acid (AA) (3). ..................................................................................................................... 61

4-2 Pressure vs mean molecular area isotherms of ternary mixture of DPPC: lyso PC: AA (50:25:25) taken over a 0.43 mM CaCl₂ subphase at 20°C. ............... 62

4-3 BAM images of the ternary monolayer, a 2:1:1 mixture of DPPC, 22:0LysoPC, and Arachidic acid, over a calcium phosphate subphase................. 62
4-4  BAM images of a arachidic acid monolayer over a calcium phosphate subphase............................................................................................................ 63

4-5  Pressure vs area isotherm of a ternary 2:1:1 mixture of DPPC, 22:0 Lyso PC, and palmitic acid......................................................................................................................... 63

4-6  Pressure vs area isotherm of a ternary 2:1:1 mixture of DPPC, 22:0 Lyso PC, and arachidic acid......................................................................................................................... 64

4-7  Pressure vs area isotherm of a pure palmitic acid over a CaCl₂, NaCl, tris HCl buffer at 20°C. .............................................................................................................................. 64

4-8  Pressure vs area isotherm of a pure arachidic acid over a CaCl₂, NaCl, tris HCl buffer at 20°C.......................................................................................................................... 65

4-9  SEM image of the calcium phosphate crystals formed under the fatty acid monolayer................................................................................................................................. 65
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This study looks at the precipitation of calcium phosphate crystals at phospholipid Langmuir monolayers. Urinary stones are commonly composed of an inorganic component, calcium phosphate, or calcium oxalate and an organic matrix of lipids, carbohydrates, and proteinaceous matter. Hyperoxaluria, elevated oxalate concentration in the kidney, is a condition frequently associated with individuals suffering from kidney stones. This condition causes the breakdown of membranes and creates free radicals at the cellular surface. Once free radicals are generated, lipid peroxidation begins to occur which further leads to the hydrolysis of phospholipids. Hydrolysis can also be caused by an enzyme called phospholipase A2, which hydrolyzes the sn-2 position of a phospholipid. Two of the products at the cell surface when hydrolysis occurs are a single chain lysolipid and also a fatty acid. In this study, products of lipid hydrolysis are examined for their effect on calcium phosphate precipitation using Langmuir monolayers as model lipid membrane assemblies. Brewster angle microscopy is employed to monitor the calcium phosphate crystals which appear as a bright spots at the air/water interface. Crystal precipitation was monitored at monolayers of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC),
palmitic acid (PA), the binary mixture of DPPC:PA, and DPPC:22-carbon chain lysophospholipid (22:0 Lyso PC) and the ternary mixture of DPPC: PA: lyso PC in liquid condensed (LC) and liquid expanded (LE) phases. It is found that a ternary mixture of DPPC and its hydrolysis products, a lysolipid and a fatty acid, cause a significant increase in heterogeneous calcium phosphate precipitation when compared to DPPC alone. It is demonstrated that the fatty acid PA generated during lipid hydrolysis causes a significant increase in the extent of heterogeneous nucleation of calcium phosphate from supersaturated solutions. The results imply a possible link between break down of phospholipid into its hydrolysis products and calcium phosphate precipitation. We also studied a ternary mixture using Dipalmitoylphosphatidylcholine (DPPC), arachidic acid (AA), and a 22-carbon chain lysophospholipid (22:0:Lyso PC) to see the effect of chain length of fatty acid on calcium phosphate precipitation. It was observed that only the short chain fatty acid generated during lipid hydrolysis causes a significant increase in the extent of heterogeneous nucleation of calcium phosphate.
CHAPTER 1
INTRODUCTION

1.1 Scope of Thesis

Chapter 2 describes the literature review which includes the study of domains formed by pure DPPC, the effect of palmitic acid and lysolipid on DPPC and the formation of calcium phosphate under different monolayers. Chapter 3 of this dissertation describes the formation of calcium phosphate crystals under lipid monolayers. At the end, in Chapter 4, the effects of chain length of fatty acids on calcium phosphate precipitation at lipid interfaces are discussed.

The motivation for this project was initiated by the success of Sharbaugh’s experiment in our group in observing the effect of phospholipase A2 hydrolysis products on calcium oxalate precipitation at lipid interfaces.  

1.2 Background

Biomineralization is an important phenomenon in nature. Studies on the biomineralization mechanism have been aimed at developing a detailed understanding of interfacial interactions associated with biomineralization and template-directed crystallization. Formation of biomineral like calcium oxalate monohydrate has been studied previously by Whipps and Backov in our group. For a long time, crystallizations of calcium phosphates and of calcium carbonates on the surfaces of thin films have constituted model systems for understanding the role of the surface chemistry in the nucleation and crystal growth processes. Langmuir-Blodgett films of surfactant molecules bearing different kinds of hydrophilic moieties, and self-assembled monolayers of surfactant molecules on noble metal surfaces have been used to study the formation of minerals. Calcium phosphates and calcium oxalates are
investigated extensively under the Langmuir films because they are the main inorganic components of kidney stones and hard tissues.\textsuperscript{15-23}

Kidney stones afflict patients worldwide. In the United States, alone, kidney stones account for over one million hospital admissions per year.\textsuperscript{24} It has been found that stones can be deposits of calcium phosphates, uric acid, struvite, or even calcium carbonate, but by far the most common are those that contain calcium phosphate as the principal inorganic component. This crystalline inorganic material is always mixed with an organic matrix which is comprised of proteins, carbohydrates, lipids, and other cellular components.

Phosphate and oxalate are concentrated in the kidney resulting in supersaturation with respect to their calcium salts, but the solution is metastable, meaning that heterogeneous nucleation is the predominant precipitation process. Calculations show that urinary concentrations and the rate of fluid flow in the kidney provide insufficient transit time for crystals to grow large enough to be occluded and retained.\textsuperscript{25} Indeed, crystals formed in the urinary tract of most humans are harmlessly excreted with the aid of protein and small molecule inhibitors. Specific urinary substances such as citrate, glycosaminoglycans, and the proteins osteopontin, bikunin, and CAI (crystal adhesion inhibitor) are thought to aid the process.\textsuperscript{26-28} However, in some cases, the crystals remain inside the kidneys and initiate the process of stone formation. Crystal attachment to the kidney’s tubular cell surface is therefore a critical step in pathological calcification, and it is thought that cell injury can provide sites for crystal nucleation, aggregation, and retention within the kidneys.\textsuperscript{29-38} In addition, cell death and
degradation result in the production of membrane vesicles,\textsuperscript{29, 30} which can further facilitate heterogeneous crystal nucleation and aggregation.

1.3 Kinetics of Crystallization

It is now well established that a number of calcium phosphate phases such as dicalcium phosphate dihydrate ($\text{Ca}_2\text{H}_2\text{(PO}_4\text{-2H}_2\text{O, DCPD})$ and octacalcium phosphate ($\text{Ca}_8\text{H}_2\text{(PO}_4\text{)6. 5H}_2\text{O, OCP})$ participate as precursor phases in the precipitation of the thermodynamically most stable hydroxylapatite ($\text{Ca}_5\text{(PO}_4\text{)3OH, HAP})$. During the reactions, kinetic factors may determine the nature of the initially formed solids and it is important to evaluate the supersaturations with respect to each of the phases.\textsuperscript{39} Two other phases, anhydrous dicalcium phosphate (DCPA) and tricalcium phosphate ($\text{Ca}_3\text{(PO}_4\text{)2, TCP})$ have also been invoked as possible precursor phases although there is little evidence for their formation at ambient temperatures.

1.3.1 Calculation of Relative Supersaturation Ratio ($\sigma$)

In studying the precipitation of calcium phosphate it is important to control the concentrations so that the degree of supersaturation of the solution with respect to the various calcium phosphate phases is defined. Robertson and co workers have emphasized the importance of considering the concentrations of calcium and phosphate ions in studies of heterogeneous nucleation by crystals of HAP. The relative supersaturation of the solution with respect to each of the calcium phosphate phases can be expressed in term of the appropriate ion activity products and is calculated by

$$\sigma = (\text{IAP}^{1/v} - K_{sp}^{1/v})/K_{sp}^{1/v} = S - 1$$

where $K_{sp}$ and IAP are the solubility product and the ionic activity product, respectively, and $v$ is the total number of ions in a formula unit. $S$ is the supersaturation ratio.\textsuperscript{43}
1.3.2 Factors Affecting the Relative Supersaturation

Although the precipitation of calcium phosphates and in particular hydroxyapatite has been extensively studied, there remains considerable uncertainty as to the nature of the phases formed during the early stages of the precipitation reaction. \(^{39-42}\) It is first necessary to express the degree of supersaturation in the stable supersaturated solutions with respect to the various possible calcium phosphate phases, DCPD, octacalcium phosphate, tricalcium phosphate, and HAP for studying the crystallization. In addition to the concentration of calcium and phosphate ions in the solution, the supersaturation depends upon the pH, temperature, and the concentration of other "neutral" electrolytes. For the precise evaluation of supersaturation level, it is important that the balance among all the ionic and complexed species can be quantitatively assessed. However, since large number of different phases of calcium phosphate is formed as a precursor, it is difficult to find the ion activity product considering a particular phase.\(^{43}\)

1.4 Lipids and Membranes

Lipids are molecules of biological origin, which are soluble in organic solvents such as ether and chloroform and insoluble in water. \(^{23}\) To better understand the mechanism of the crystal growth it is important to study the cell membrane. Most lipids are composed of three essential features: a polar head group which is hydrophilic and can consist of any number of charged or uncharged moieties, one or more hydrophobic tail regions, which can contain aromatic groups, saturated aliphatic chains, or unsaturated aliphatic chains and a backbone structure that connects the head and tails as shown in Figure 1-1. Owing to the large variability in head group and tail chemistries,
Lipids are often categorized according to backbone structure: isoprenoids, phospholipids, sphingolipids, ceramides, fatty acids, triacylglycerols, eicosanoids, waxes, and glycerophospholipids. Within each of these categories, numerous variants exist that are the result of head group or tail modifications. For example, glycerophospholipids, the primary constituents of biomembranes, are composed of two fatty acid chains esterified at C-1 and C-2 of glycerol-3-phosphate, Figure 1-1. The acyl chains are typically 8 to 24 carbons long, and they can be saturated, unsaturated, or even coupled to form a macrocycle. Typically, an ester linkage couples the glycerophosphate to one of several head group compounds such as choline, ethanolamine, or serine to form phosphatidylcholine, phosphatidylethanolamine, or phosphatidylserine, respectively. Such head group modifications may render the lipids either charged, uncharged, or zwitterionic at physiological pH.

1.5 Langmuir Monolayers and Langmuir-Blodgett Films

Langmuir monolayers are monomolecular films prepared at an air-water interface. They are generally comprised of molecules that contain both hydrophobic and hydrophilic regions. The films are formed by amphiphilic molecules where the polar head group is immersed in the aqueous subphase and the alkyl tail remains in the air. These films can be compressed by the barriers, Figure 1-2 altering their phase behavior and interaction with other molecules in the subphase. A Wilhelmy balance is used to monitor the state of the monolayer. Langmuir monolayers of phospholipids are often used as membrane mimics. The monolayers can be transferred to a solid support in order to extend their applications as thin films, and perform additional characterization not accessible at the air-water interface and to form multilayer. The transferred monolayer is called a Langmuir-Blodgett or LB film. The power of a monolayer,
particularly when compared to a bilayer, lies in its controllability. A monolayer’s properties can be carefully tuned, allowing the ability to define molecular density by varying the area per molecule on a Langmuir film balance. In addition, the planar geometry of this experiment makes it accessible to several optical techniques. An extensive review on the formation of these films can be found in the original papers by Irving Langmuir and Katherine Blodgett on the subject.

1.6 Brewster Angle Microscopy and its Applications

Brewster angle microscopy (BAM) has emerged as a widely used technique to image Langmuir monolayers in situ over the past 10 years. The first report of a home-built Brewster angle microscope appeared in 1991 by Hénon and Meunier. BAM is based on the physical fact that plane-polarized light is not reflected from an interface between two materials with different refractive coefficients if the incident angle is at the Brewster angle. The existence of domains in a Langmuir monolayer changes the local refractive index such that at the Brewster angle for the pure air–water interface, light is now reflected with varying intensity from the different phases. The reflected light can then be used for imaging purposes, Figure 1-3. BAM can thus be used to visualize details of the inner structure of condensed phase domains at the air/water interface and to follow morphological changes of the reactive process occurring in monolayers under constant surface pressures.

The principle of operation is based on the Brewster angle, $\theta_B$, at which linearly polarized light directed towards a boundary of two media having different refractive indices will transmit completely from one medium to the other. The Brewster angle is given by
\[ \theta_B = \tan^{-1}(n_1/n_2) \]

where \( n_1 \) and \( n_2 \) are indices of refraction of the media involved. The Brewster angle for the air-water interface is 53°.

In this thesis, BAM is used to observe the monolayer and the crystals formed underneath the monolayers, Figure 1-4. The crystals appear as a bright spots. Although most BAM experiments are performed at the air-water interface, an air-solid interface can also be interrogated by use of the appropriate Brewster angle.

A very useful application of BAM is that it enables visual characterization of the phase behavior of monolayers at the air-water interface. As a Langmuir monolayer is compressed, it will exhibit distinct phases analogous to the gas, liquid and solid states in three-dimensional materials. At high Mean Molecular Area a disorganized Gas (G) phase is present, although practically a pure G phase is not observed as it requires very large trough areas. Compression of the monolayer will yield a liquid expanded (LE) phase where the molecules are still disorganized but in closer proximity and finally a liquid condensed phase (LC) where the amphiphiles are closed packed and uniformly oriented. These phases can be identified in an isotherm by sharp slope changes as shown in Figure 1-5. There can also be coexistence region of LE/LC phases which appear as plateaus in the isotherm. When the monolayer is further compressed it reaches the collapse pressure, Figure 1-5. The shape of the isotherm pictured in Figure 1-5 is not necessarily observed for all surfactants, as coexistence regions may not occur, or a LC phase may not form due to the inability of the molecules to close-pack. The presence or lack of these phases can be easily observed by BAM for any Langmuir monolayer at the air-water interface.
Figure 1-1. Structural features of lipids, using a glycerophospholipid (phosphatidylcholine) as an example.

Figure 1-2. Schematic representation of a Langmuir monolayer experiment.
Figure 1-3. Schematic illustration of the principle behind Brewster Angle Microscopy.

Figure 1-4. Experimental setup utilizing Brewster angle microscopy to monitor crystal growth at phospholipid Langmuir monolayers.
Figure 1-5. Schematic representation of a pressure-area isotherm showing the possible phases present during the compression of an amphiphile.
CHAPTER 2
LITERATURE REVIEW

2.1 Close Look at Domain Formation in DPPC Monolayer

Insoluble monolayers of phospholipids at the air-water interface have long been of interest, both because of their intriguing behavior and because of their biomimetic applications. Monolayers of phospholipids have thus been applied as a simple model for the cellular lipid bilayer. Dipalmitoylphosphatidylcholine (DPPC) has frequently been the phospholipid of choice for many monolayer studies. Phosphatidylcholines are the primary phospholipids in the mammalian cell membrane and a significant component of alveolar fluid. Thus, DPPC is a natural focus for study. In addition, DPPC exhibits a phase transition at room temperature from what is called a liquid-expanded (LE) phase to a liquid-condensed (LC) phase.

Cary W. McConlogue studied the domain formation in pure DPPC monolayer and found that the basic domain shape is an asymmetric “bean” with a flattened lobe and a distinct cavity, Figure 2-1. It is seen that on further compression of the monolayer, the domain fills the unused space and appears as polygons since domains are repulsive. And finally the domains are dispersed uniformly about their boundaries, resulting in domains that appear to be solubilized.

McConlogue and coworker performed the detailed examination of the shapes of DPPC domains throughout the coexistence region. Multilobed shapes can also be observed, but these originate as beans and over time transform back to beans. In this paper the important and interesting features are pointed out and it also demonstrate methods that cause divergence from the fundamental shape are demonstrated. The DPPC isotherm is characterized by a kink and a subsequent plateau, indicating a liquid-
expanded/liquid-condensed (LE/LC) coexistence region. A DPPC pressure-mean molecular area isotherm is shown in Figure 2-2. Domain nucleation occurs at the kink in the isotherm (typically at 3.6-3.8 mN/m). Initially, domains appear completely round; whether this is the case in reality or due to limits in the resolution of the microscope is unclear. The domains are asymmetric: if the bean is oriented with its cavity facing upward, the left lobe has a flattened edge. This was noted by Florscheimer and Mohwald, who also proposed a model for the orientations of the molecular tilt within the domain. 59 they also observed that, this flattened edge plays a role in the domain’s growth process at higher pressures.

2.2 Study of Hydrolysis of DPPC by Phospholipase A2 Using Langmuir Monolayer and BAM

Ternary mixed monolayers of phospholipid/lysolipid/fatty acid have been studied both for their intrinsic behavior 60 and for effect on phospholipase A2 activity. 61 In this literature, Sharbaugh studied the effect of phospholipase A2 hydrolysis products on calcium oxalate formation 1. Phospholipase A2 (PLA2) is a water-soluble enzyme which catalyzes the region-specific hydrolysis of the sn-2 acyl ester linkage of sn-glycero-3-phospholipids. The products are fatty acids (FA) and 1-acyl-lysophospholipids (lysoPC) Figure 2-3.

First pressure vs mean chain area isotherms of DPPC and of palmitic acid taken over a 0.35 mM CaCl2 subphase were obtained, Figure 2-4. Then BAM images of pure DPPC monolayer on calcium oxalate subphase were obtained and compared with the ternary mixture. A significant growth in number of crystals is observed in case of ternary mixture as compared to DPPC alone, Figure 2-6A-C.
With the help of the isotherms and Brewster Angle Microscope images of DPPC and its hydrolysis products, the LE, LC phase, the condensing effect and collapse pressure of the monolayer were studied. To study the cause of enhanced crystallization Sharbaugh examined the isotherm, Figure 2-5 and BAM images, Figure 2-7 of two binary monolayers i.e. DPPC/palmitic acid and DPPC/lysolipid for their effect on calcium oxalate generation under various pressure versus area conditions. In both cases, there is very little change in the extent of crystal formation relative to DPPC alone. However, when PA is examined alone as a pure monolayer, crystal nucleation is again very high, similar to that observed with the ternary mixture, Figure 2-6C. It was concluded that among the hydrolysis products, palmitic acid is the one which is a highly active component and causes the enhanced growth of calcium oxalate crystals at the lipid interface.

2.3 Condensing Effect of Palmitic Acid on DPPC Monolayer

The condensing effect between lipids has long been reported in the literature. Adam and Jessop used an intuitive way to explain the condensing effect.\textsuperscript{62, 63} They suggested that bulky or rigid molecules could mechanically impede the motions of more flexible molecules and hence reduce their tendency to expand. The all-trans configuration of the chain makes PA a rigid molecule in the monolayer. PA is in the condensed phase at 12mN/m as indicated by the phase diagram in Figure 2-8A. The collapse pressure drop has been demonstrated by the isotherm measurements in Figure 2-8B.

Ma and Allen (2006) studied the condensing effect of palmitic acid on monolayers of DPPC using vibrational sum frequency generation (VSFG) spectroscopy. They showed that palmitic acid condenses DPPC upon spreading. They postulated that the
rigid PA molecule can hinder rotational isomerism of the DPPC chains in the expanded phase and hence reduce or eliminate the number of gauche defects, forcing the neighboring DPPC molecules into a condensed phase. They explained that PA also reduces the DPPC head-tail mismatch by hydrogen bonding interaction between the PA’s COOH hydroxyl group and oxygen of the PO2– group of DPPC. The isotherm clearly shows the condensing effect of PA on DPPC monolayer, Figure 2-8B.

According to a previous phospholipid X-ray study, the surface area occupied by the bulky headgroup of a PC is about 50 Å². On the other hand, the minimum cross-sectional area of the two hydrocarbon chains of a PC is about 38 Å². In a closely packed environment as in the liquid condensed (LC) phase, the chain must be tilted to some extent to compensate for the head-tail mismatch to form a stable monolayer at the air-water interface. So they discovered that insertion of a relatively small molecule like PA into the DPPC condensed phase can compensate for the head-tail mismatch and reduce the chain tilt. The orientational ordering effect caused by PA can also be considered as a condensing effect since it decreases the mean area per DPPC hydrocarbon chain.

It is observed that on one hand, PA increases the chain ordering of DPPC and promotes the phase separation between DPPC and other unsaturated lipids. On the other hand, due to the miscibility between DPPC and PA in the condensed phase, PA decreases the collapse pressure of the DPPC monolayer.

### 2.4 Studies of Lysophosphocholine/DPPC Mixtures

Mixtures of phospholipid and a single-chain lysophospholipid provide an intriguing multicomponent monolayer, both from physicochemical and biological perspectives. By choosing a double-chained phospholipid and a single chained
lysophospholipid with the same headgroup, the effect of hydrophobicity can be isolated. More specifically, by using different chain lengths of lysolipid, one can tune the solubility of the lysolipid while maintaining a constant electrostatic environment at the interface. In addition, the single-chain lysolipid forms a very different volumetric profile in a monolayer when compared to that of the phospholipid; this has implications in packing at the interface. Lysolipids comprise one product and they serve as an activator of phospholipid hydrolysis by phospholipase A2, \(^{65-67}\). They carry implications in membrane structure and fluidity, including permeability, \(^{68-72}\) and fusion. \(^{73-75}\)

McConlogue C.W., (1998) used three different groups of mixtures of DPPC and Lyso lipids\(^5\). From the isotherm Figure 2-9 and the BAM images figure 2-10, the effects of lysolipid on DPPC surface pressure/mean molecular area isotherm behavior, as well as on the shapes of domains that form in the DPPC liquid-expanded/liquid-condensed coexistence region, were examined. A key result of this study is that the behavior of the lysolipids enables their assignment by chain length to one of three well-defined groups each with distinct effects on DPPC monolayers.

McConlogue and coworkers also observed that the lysolipids 22:0 are hydrophobic enough to remain present at the interface despite stress caused by the compression. This is supported by the isotherm data where the increase in the mean molecular area indicates that the lysolipid is still present at the interface interaction with the DPPC. All kinks and the plateau are shifted to higher surface pressure and the plateau slope is increased. The author concluded that, apart from the solubility of the lyso PC, the intermolecular forces also influence monolayer properties. Group I lysolipids (C\(_8\) through C\(_{12}\)) are most soluble and have minimal effect on monolayer properties. Group II
lysolipids (C\textsubscript{14} and C\textsubscript{16}) can be kinetically trapped at the interface and show hints of line activity. Group III lysolipids (C\textsubscript{18} through C\textsubscript{22}) remain at the interface and exhibit significant line activity.

2.5 Study of Calcium Phosphate Formation under Three Different Langmuir Monolayers

L. J. Zhang et al (2003) studied the formation of calcium phosphate under three different monolayers. They employed Langmuir films that formed from amphiphilic molecules with different headgroups at the air/water interface as templates to mimic calcium phosphate formation\textsuperscript{76}. The template inducing and controlling, molecular recognition, and, especially, electrostatic attraction and lattice matching were investigated in this paper. A phase transformation process from an initially deposited amorphous phase to a crystalline phase during the initial stage of calcium phosphate formation was observed in this study of different monolayers, Dipalmitoylphosphatidylcholine (DPPC), arachidic acid (AA), and octadecylamine (ODA) were the compounds used to form the monolayer template. The different charged head group and its effect on calcium phosphate initial stage of nucleation and crystallization was studied.

Zhang and coworkers also observed that calcium phosphates were formed through a multistage assembly process. First, a thermodynamically unstable calcium phosphate dehydrate (DPCD) in amorphous phase was formed. Then it transformed into a crystalline phase and at the end the most stable hydroxyapatite (HAp) was formed. To show the phase change, pressure–molecular area isotherms of the monolayers were analyzed, Figure 2-11. From these curves, it can be seen that the mean molecular areas of DPPC, AA, and ODA on a HAp supersaturated aqueous
solution are larger than those on pure water, indicating that there is a very strong interaction between the monolayers and calcium ions (or phosphate), and calcium ions (or phosphate) could bind into the monolayers. On the other hand, it was found that the collapsed pressures of the last curves were lower than those of the former curves in each group of isotherms. This also suggested that more and more calcium ions (or phosphate) bound to the monolayer/subphase interface, which led to brittle films.

Zhang and coworkers also acquired the TEM images and SAED patterns of calcium phosphate to confirm the phase transformation. The compositions of calcium phosphates formed under DPPC, AA, and ODA monolayers at different times were examined with an EPM, respectively. From Figures 2-12, it can be seen that the atomic ratios of Ca/P of calcium phosphate formed under a DPPC monolayer are about 0.85, 0.98, 1.33, and 1.67 for 1, 2, 5, and 7 days; under an AA monolayer they are about 0.90, 1.01, and 1.49 for 1, 2, and 7 days; and under an ODA monolayer they are about 0.91, 0.95, and 1.6 for 1, 2, and 7 days, respectively. It could be considered that the particles with the ratios of Ca/P close to the theoretical value of calcium phosphate dihydrate (DPCD) (1.0) were DPCD. In addition, the ratios of Ca/P of calcium phosphate formed under three kinds of monolayers on the 7th day all were close to the theoretical value of Hap (1.67). From these results, they could conclude that, during the crystallization of calcium phosphate, its unstable precursor (DPCD) was formed first and finally transferred to the stable crystalline phase HAp.
Figure 2-1. DPPC domain growth with a compression rate of 0.86 Å² molecule-1 min-1: a) 3.8 mN/m; b) 3.9 mN/m; c) 4.2 mN/m; d) 4.3 mN/m; e) 5.0 mN/m; f) 7.5 mN/m., [Cary W. McConlogue, 1997, reproduced with permission]
Figure 2-2. Compression isotherm of 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) on a water subphase at 25 °C., [Benitez I., 2004, reproduced with permission]

Figure 2-3. Structures of 1) DPPC, 2) Lysolipid 22:0 Lyso PC, and 3) Palmitic acid (PA). The 22:0 lysolipid is used in this study instead of the complementary 16-carbon tail lysolipid because it is more stable in Langmuir monolayers, [Sharbaugh D., 2009, reproduced with permission]
Figure 2-4. Pressure vs mean chain area isotherms of DPPC and of a palmitic acid taken over a 0.35 mM CaCl₂ subphase at 25 °C.

Figure 2-5. Pressure vs mean chain area isotherms of the binary monolayers of DPPC with PA and DPPC with the 22:0 Lyso PC, taken over a 0.35 mM CaCl₂ subphase at 25°C.
Figure 2-6. Brewster angle microscopy images at 24 °C after 24 h over a 0.35 mM calcium oxalate subphase A) DPPC, B) ternary mixture (DPPC: PA: Lyso PC) and C) pure palmitic acid. [Sharbaugh D., 2009, reproduced with permission]

Figure 2-7. BAM images of binary monolayers over a 0.35 mM calcium oxalate subphase containing NaCl and Tris HCl after 24 h at 24°C A) 70:30 DPPC/Lyso PC and B) 70:30 DPPC/PA. [Sharbaugh D., 2009, reproduced with permission]
Figure 2-8. Surface pressure (mN/m)-area (Å²) isotherms at 24 °C on a pure water subphase: A) neat DPPC-d62 (gray curve) and neat PA (black curve), B) mixtures of DPPC-d62 and PA with three molar ratios (black curve, 3:1; gray curve, 1:1; dashed curve, 1:3). G, gas phase; LE, liquid-expanded phase; TC, tilted-condensed phase; Untilted, untilted-condensed phase; G-LE, coexistence of G and LE; G-TC, coexistence of G and TC; LE-TC, coexistence of LE and TC. All isotherms are the average of three measurements., [Ma and Allen, 2006, reproduced with permission]
Figure 2-9. DPPC/C<sub>22</sub>PC isotherms at 20 °C., [Cary W. McConlogue, 1998, reproduced with permission]

Figure 2-10. DPPC/C<sub>18</sub>PC domains formed by compression at 0.86 Å<sup>2</sup> molecule<sup>-1</sup> min<sup>-1</sup> at 20 °C: a) 7.5 mN/m; b) 9.3 mN/m; c) 11.6 mN/m; d) 12.0 mN/m; e) 10.7 mN/m; f) 11.7 mN/m; g) 12.6 mN/m; h) 13.1 mN/m; i) 13.5 mN/m; j) 13.9 mN/m., [Cary W. McConlogue, 1998, reproduced with permission]
Figure 2-11. Isotherms of monolayer on pure water and HAp supersaturated solution for different times: A) DPPC, B) AA, and C) ODA at 25 °C., [Zhang L. J., 2003, reproduced with permission]

Figure 2-12. EPM spectra of calcium phosphate formed under the DPPC, ODA and AA monolayer at different times: a, 1 day; b, 2 days; c, 5 days; d, 7 days., [Zhang L. J., 2003, reproduced with permission]
CHAPTER 3
FORMATION OF CALCIUM PHOSPHATE CRYSTALS UNDER LIPID MONOLAYERS

This chapter provides the details about formation of calcium phosphate crystals under the DPPC and its hydrolysis products. The isotherms and the BAM images of every component used here are thoroughly studied. The results are rigorously analyzed and reasons for the variation in number of crystals seen over the different experiments are discussed.

3.1 Introduction

Biomineralization is characterized by the close association of inorganic and organic substances. In biological systems, an organism creates the proper organic matrix on which inorganic crystals can precipitate and the interfacial interactions between them provide the control over the resultant composite structures. The molecular interactions at the inorganic-organic interfaces are an important aspect of biomineralization since the nucleation, growth and organization of biominerals are mediated by the organic supramolecular system.

Kidney stones, which generally describe any crystalline deposit in the urinary tract, are most commonly calcific deposits within an organic matrix. Calcium phosphate is one of the predominant inorganic components of most stones. Although the bulk of the mass in a stone is from the inorganic matter, the organic matrix, including lipids, carbohydrates, and proteins, constitutes a significant volume, making the relationship between inorganic and organic components of the urinary stones important to understand. Urine is metastable with respect to calcium phosphates and calcium oxalates, meaning that salt formation is not spontaneous in the absence of an interface. Furthermore, the transient time of urine in the kidney is quite rapid, about
3 to 4 min, which makes it unlikely for any inorganic component to grow large enough during transit to block the tubular lumen. Stone formations, therefore, requires either the heterogeneous nucleation and growth of the inorganic crystals or crystal attachment. The study of lipid assemblies and their influence on the nucleation of calcium salts, in particular calcium phosphate, is of interest because of the potential role that such a process plays in urinary stone formation.

Hypercalciuria is the most common metabolic abnormality observed in patients with nephrolithiasis. Hypercalciuria raises urine supersaturation with respect to the solid phases of calcium phosphate and calcium oxalate, leading to an enhanced probability for nucleation and growth of crystals into clinically significant stones.

Phospholipase hydrolyses lipids, liberating a fatty acid and a one-tailed lipid, a lysolipid. Lysolipids are associated with changes in the function of the mitochondria, gene expression, and bioactive molecules that can trigger cell apoptosis. Lipid hydrolysis products are also found at elevated levels in stone formers.

In this study, we use Langmuir monolayers as membrane mimics and monitor calcium phosphate growth at lipid interfaces having high concentrations of lipid hydrolysis products. Phospholipid Langmuir monolayers are frequently used as membrane mimics and our group has previously used phospholipid monolayers to study heterogeneous calcium oxalate precipitation. Previous studies showed that the lipid interface promotes the crystallization of calcium oxalate monohydrate (COM), the form most often found in urinary stones and that the identity of the lipid influences crystallization, with anionic lipids generating more crystallization than neutral, zwitterionic lipids. Langmuir monolayer studies also demonstrated that membrane
heterogeneities such as phase boundaries provide preferred sites for crystal formation. In a recent publication by our group, a systematic study of the nucleation of calcium oxalate monohydrate crystals beneath the hydrolysis products of a DPPC monolayer was carried out.\textsuperscript{1}

In the present work, formation of calcium phosphate beneath the hydrolysis products of DPPC monolayer is studied. A significant increase in crystal formation is observed at the lipid interface in the presence of a short chain fatty acid. Also to understand better how the enzyme affects crystallization, lipid monolayers containing the different lipid hydrolysis products, such as the binary mixture of DPPC:PA and DPPC:Lyso PC, are studied. The results indicate that small chain fatty acid, liberated upon hydrolysis of the lipids lead to rapid nucleation from the metastable calcium phosphate subphase, providing a possible mechanism linking the hydrolysis of DPPC and urinary calcific deposits.

3.2 Experimental Section

3.2.1 Materials

All reagents were purchased from commercially available sources and used without further purification. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 22:0 Lyso PC (purity >99\%) were purchased from Avanti Polar Lipids (Alabaster, AL). Palmitic acid was purchased from Acros Organic. Sodium phosphate and tris (hydroxymethyl) aminomethane hydrochloride (Tris·HCl) were purchase from Aldrich Chemical Co. (Milwaukee, WI). Calcium chloride dihydrate and sodium chloride were obtained from Fisher Scientific (Pittsburgh, PA).
3.2.2 Preparation of Supersaturated Calcium Phosphate Solution

This was prepared by dissolving NaCl and tris HCl in 4 L of deionized water with a resistivity of 17.0-17.5 MΩ.cm to create a solution 150 mM NaCl and 5mM tris HCl. Once all solid was thoroughly dissolved, the pH of the solution was adjusted to 7.0 using a 30%w/v KOH solution. The pH-adjusted solution was split evenly into two flasks. To one, 0.43 mM of calcium chloride was added, and 0.28mM of sodium phosphate to the other, so that the final concentrations of CaCl₂ and Na₃PO₄ solution was 0.86 mM and 0.56 mM, respectively. The Ca/P ratio is 1.53, which is close to the Ca/P ratio in human body fluids. Using a large Erlenmeyer flask and an addition funnel, the calcium chloride solution was added into an equal amount of constantly stirred sodium phosphate solution. This final solution contains 150 mM NaCl, 5mM tris HCl, and 0.43 mM calcium and 0.28 mM of phosphate, for this solution the corresponding relative supersaturation was 5.95 for tricalcium phosphate. To remove any Ca₃(PO₄)₂ crystals that may be formed in the addition, the solution was vacuum filtered through a Millipore 0.45 μm nylon filter and was immediately poured onto a Langmuir trough. The supersaturated solution prepared was kept undisturbed for 24 hr and did not yield any visible precipitate for at least 24 hours.

3.2.3 Preparation of Surfactant Solution

To spread the surfactant, lipids was dissolved in 10 mL of a 90:10 chloroform/methanol mixture to make a 0.15mg/mL solution and then sonicated for 5 min. For the fatty acid a solution of concentration 0.20 mg/mL was used. Solutions used in this study consisted of pure 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), pure palmitic acid (PA) and arachidic acid (AA), 70:30 DPPC/1-behenoyl- 2-
hydroxy-sn-glycero-3-phosphocholine (22:0 Lyso PC), 70:30 DPPC/PA, or a 2:1:1 mixture of DPPC, 22:0 Lyso PC, and PA.

### 3.2.4 Glass Slides

Glass microscope coverslips were used as substrates for the SEM analyses. The slides were cleaned using a piranha etch (3:1 H₂SO₄: H₂O₂) solution. The slides were immersed in freshly prepared piranha etch solution for one hour and were cleaned with distilled water.

### 3.3 Brewster Angle Microscopy

All BAM images were taken using a Nanofilm Technologie GmbH BAM2plus system with the above-described KSV 2000 Langmuir-Blodgett system. The instrument is equipped with a polarized Nd:YAG laser (532 nm, 50 mW) and a CCD camera (572 X 758 pixels). The operating software also controls a scanner that moves the objective along the optical axis to generate a focused image and to correct for distortion from the objective motion.

For all experiments a 10X objective was used with a laser power of 50% or less as required. The incident beam is set to the Brewster angle in order to obtain minimal reflected light from the subphase. To minimize stray light from the Teflon, a piece of highly polished black glass was set on the bottom of the trough to absorb refracted light. When necessary, the shutter speeds and gain could be adjusted to generate images with reasonable contrast; however, unless noted, the same shutter speed of 1/50 s and a maximum gain were used to enable the comparison of different images. The polarizer and analyzer are normally set at 0°, and these are the settings for all images presented here. To verify that the bright spots under the monolayer are crystals, the polarizer can be set to 0° and the analyzer can be set to 90°, blocking all but the scattered light from...
crystals under the monolayer. Finally, to sample a large area of the trough and track the monolayer if it drifts, the laser and camera are mounted on an x-y stage that is user controlled by video monitoring. Images were taken 24 hours after compression

3.4 Crystal Counting Procedure

The extent of crystal formation for a given surface pressure and time interval was estimated by counting and averaging the number of visible crystals in a series of images taken at random over the surface of the monolayer. From 20 to 40 images of each experiment, around 15 clearest images were selected and the crystals were hand counted. The average of these 15 images was then combined with at least 2 other sets of 15 images from separate experiments under the same conditions to obtain the data that are reported.

3.5 Procedure

To determine the extent to which homogeneous precipitation of calcium phosphate occurs in the Langmuir-Blodgett trough, control experiments was performed, in which a calcium phosphate subphase was allowed to sit in the LB trough in the absence of a Langmuir monolayer. No crystals of calcium phosphate were seen in the BAM images after periods of up to 24 h, Figure 3-1, indicating that homogeneous precipitation in the LB trough is not a significant source of calcium phosphate crystals within the timescale of the experiments.

Also CaCl₂ and Na₃PO₄ subphases were prepared in the NaCl/Tris HCL buffer with the same concentration which was used in the experiment. DPPC monolayer was spread on either of these subphases and BAM images were taken after 24 h, Figure 3-2. No crystals were seen for either of the subphase. This step was needed to prove that
the crystals seen under the monolayer on calcium phosphate supersaturated solution is calcium phosphate only.

Lipid monolayers were prepared by spreading the lipids at the air/water interface from a chloroform/methanol solution and compressing the films using opposing moveable barriers. The stability of the lipid monolayers on the supersaturated calcium phosphate subphases was checked by monitoring the area of the compressed monolayers as a function of time while holding a constant surface pressure of 20 mN/m. All lipid monolayers were very stable with no loss of film after several hours. For the crystal growth experiments, the monolayers were compressed and held at a surface pressure of 35 mN/m and 15 mN/m to observe the film at high and low pressures respectively. Calcium phosphate crystals appeared at the air-water interface due to the electrostatic attraction. BAM images of monolayers were taken after 1 h, 15 h and 24h. These BAM images obtained after different intervals of time will be discussed below. Number of crystals and their size will be the issue of concern.

To verify that the features in the BAM images are calcium phosphate crystals, the monolayer surface from underneath the subphase was collected on a slide to transfer the film and crystals for SEM imaging. The SEM image of crystals grown under the monolayer confirmed the crystal habit of the calcium phosphate crystal formed. In this manner calcium phosphate formation at the air-water interface using different molecules was studied.

3.6 Study of Hydrolysis of Phospholipid DPPC

Hyperoxaluria, elevated oxalate concentration in the kidney, is a condition frequently associated with individuals suffering from kidney stones. This condition causes the breakdown of membranes and creates free radicals at the cellular surface.
Once free radicals are generated, lipid peroxidation begins to occur and as a repair mechanism after peroxidation, the hydrolysis of phospholipids takes place. Two of the products at the cell surface when hydrolysis occur, are a single chain lysolipid and a fatty acid, Figure 3.3. Also due to the high concentration of oxalate in the urine, an enzyme called phospholipase A$_2$ (PLA$_2$) is generated. It is a water-soluble enzyme which catalyzes the regio-specific hydrolysis of the $sn$-2 acyl ester linkage of $sn$-glycero-3-phospholipids. PLA$_2$ is an interfacially activated enzyme and its activity as well as the hydrolysis kinetics depends on the morphology and the physico-chemical state of the substrate.

Langmuir monolayers have been exploited by other researchers as models for studying lipid hydrolysis. For example, the latency period of phospholipase PLA$_2$ was studied at Langmuir monolayers, showing that once hydrolysis begins, enzyme activity accelerates, leading to composition and phase heterogeneities in the monolayer. Product-enriched domains have disproportionate levels of hydrolysis products compared to the rest of the membrane, and these hydrolysis-rich domains have also been modeled using Langmuir monolayers. In this study, products of lipid hydrolysis are examined for their effect on calcium phosphate precipitation using Langmuir monolayers as model lipid membrane assemblies.

**3.7 Crystallization of Calcium Phosphate under the DPPC Monolayer**

Before starting the crystal formation under the DPPC monolayer experiment, pure DPPC isotherm was studied, Figure 3-4. Once the surfactant solution of DPPC was prepared 500 uL of it was used to spread on the calcium phosphate subphase and, as the monolayer was stabilized, it is compressed using the barriers at the rate of 1mm/min at high and low pressure and images were taken after 24 hours. BAM images of crystals
formed at the DPPC monolayer held at 15 and 35 mN/m over a calcium phosphate subphase are shown in Figure 3-5. Bright spots in the BAM image correspond to calcium phosphate crystals, which have a similar size and number density to those observed previously for this system. 23, 24, 92, 93 Figure 3-5 also includes an SEM image of crystals grown under the monolayer, confirming the crystal morphology of the tricalcium phosphate.

DPPC is a zwitterionic phospholipid with a phosphate and amino group in its polar head. The crystals formed here under the DPPC monolayer are due to the electrostatic force of attraction between the negatively charged phosphate group of the choline and the positive calcium ions in the subphase. The polar head of DPPC has a methylene bridge separating the phosphate and \( N^+ (CH_3)_3 \) group. Due to the steric hindrance, the phosphate group is less assessable to interact with the calcium ions and thus high number of crystals was formed.

The activation energy required for the heterogeneous nucleation is much less than that for the homogenous nucleation. Any foreign materials, lipids here, which can provide an interface substantially, reduce the free energy barriers for nucleation. The lipid interface catalyzes heterogeneous nucleation by lowering the surface energy of the nascent crystal. The wetting effect of the polar head of lipids provides a contact angle to the ions which lowers the surface energy and facilitates nucleation. Thus polar heads groups of the lipids can stabilize the surface ions by forming bonds with them. The high surface to- volume ratio of the crystal nuclei accounts for the activation energy associated with their formation, thus at the interface this activation energy is minimized by polar headgroups of the lipids. The relative effectiveness of the different polar groups
can be estimated by considering the strength of interaction with \( \text{Ca}^{2+} \) ions. The binding constant of \( \text{Ca}^{2+} \) to phosphatidylcholine headgroups is considered to be rather weak. 97 For example; using deuterium NMR, Altenbach and Seelig reported a binding constant of 13.8 M\(^{-1}\) for \( \text{Ca}^{2+} \) and POPC bilayers. 98 Thus few crystals are formed under DPPC monolayer.

3.8 Crystallization of Calcium Phosphate under the Ternary Mixture Monolayer with Palmitic Acid

After studying the crystal formation under the DPPC monolayer, the ternary mixture containing DPPC:PA:Lyso PC (50:25:25) was studied. The pressure versus area isotherm of the ternary monolayer is shown in Figure 3-6. The 2:1:1 ratio of DPPC/PA/lysolipid was previously used to model local concentrations of the hydrolysis products in membrane domains 5, and we used the model systems here to explore the influence of these domains on calcium phosphate precipitation. As with pure DPPC, the ternary mixture is characterized by a long LE/LC coexistence region although at slightly lower pressure and on a liquid like background of gradually increasing pressure.

Once the ternary mixture solution was spread on subphase and compressed, BAM images were taken at high and low pressure. From the BAM images, Figure 3-7 it can be seen that more crystals are formed under the ternary mixture. Also from the isotherm it seems that the condensing effect of PA is not present in the ternary monolayer. Here the lysolipid reduces the interaction between DPPC and PA, freeing the carboxylic acid to interact with the subphase. Under similar conditions the binding constant of \( \text{Ca}^{2+} \) to carboxylate headgroups is strong. It is reported that approximately 0.4 mol of \( \text{Ca}^{2+} \) is bound to every mole of palmitic acid. 99 The significantly stronger binding interaction
between Ca\(^{2+}\) and carboxylate on the surface of crystal nuclei most likely accounts for
the enhanced heterogeneous precipitation at the PA and ternary monolayers.

3.9 Behavioral Study of the Binary Mixture (DPPC: PA, DPPC: Lyso PC)

To understand better which component of the ternary mixture is predominantly
responsible for the increase in heterogeneous nucleation, binary mixtures of DPPC with
PA and DPPC with lysolipid are studied and explained further. The isotherm of the
binary mixtures containing DPPC:PA or DPPC:Lyso PC are first obtained and were
studied, Figure 3-8.

From the isotherm of DPPC:PA, the condensing effect of the palmitic acid on
DPPC monolayer was clearly seen. The condensing effect between lipids has long been
reported in the literature which is already discussed in chapter 2. It is suggested that
bulky or rigid molecules could mechanically impede the motions of more flexible
molecules and hence reduce their tendency to expand. Also from the DPPC/Lysolipid
isotherm it is seen that lysolipids have a dramatic impact on DPPC isotherms which
changed its surface pressure/ mean molecular area, as shown in Figure 3-8. It is
observed that the lysolipid 22:0 used here is hydrophobic enough to remain present at
the interface despite stresses caused by the compression. This is supported by the
isotherm data where the increase in the mean molecular area indicates that the lysolipid
is still present at the interface interaction with the DPPC. As a result, all kinks and
plateaus are shifted to higher surface pressure, and the plateau slope is increased as
compared to pure DPPC isotherm. It can be seen that the shape of DPPC domains
formed in the LE/LC coexistence region in presence of lysolipid is different from those
formed in pure DPPC, Figure 3-9. With compression, the domain of the DPPC/Lyso PC
mixture tends to expand its perimeter at the expense of its area and thus it spreads
DPPC giving rises to the surface pressure, while the DPPC domains appear as a kidney shape bean with a distinct cavity.

### 3.10 Crystallization of Calcium Phosphate under the Binary Mixtures

After the isotherm data analysis the crystal formation under the binary mixtures was studied, a solution of DPPC:PA and DPPC: lyso PC in 70:30 ratio was prepared as mentioned earlier. Once the monolayer was stabilized it was compressed by moving the barriers at the rate of 1mm/min. Once the target pressure was reached monolayers were held for 24 hr and images were taken.

In both of these mixtures, it was observed that very few crystals were formed, similar to the behavior of pure DPPC monolayer. Though, for the binary mixture of DPPC/PA it was surprising to see few crystals, it is relatively inactive despite the high percentage of palmitic acid, Figure 3-10. This is because of the condensing effect of the palmitic acid on the monolayer of DPPC which makes the carboxylate group to rise up away from the aqueous subphase, which thus reduces its ability to interact with subphase ions. The condensing effect is already discussed in chapter 2. The observation that crystal growth under the DPPC/PA mixture is essentially unchanged relative to DPPC alone supports this description.

In case of the binary mixture of DPPC:Lyso PC, Figure 3-11 few crystals were formed because the head groups are still the same as of pure DPPC. Lyso PC and DPPC bears the same head group i.e. the phosphatidylcholine head group with relatively weak binding constant with the Ca^{2+} ions in the subphase, so very few crystals were expected.

Our group has previously demonstrated that for a given lipid system the phase behavior can influence heterogeneous nucleation. For example, fewer crystals form at
DPPC in the condensed phase than if the monolayer is expanded.\textsuperscript{94, 95} Phase boundaries at which lipids can exchange between phases provide high-energy sites along with an avenue for the lipids to reorganize to accommodate the surface of the insipient crystal, increasing the incidence of crystal formation. These trends are seen again in the experiments reported here for DPPC and the binary DPPC/lysolipid and DPPC/PA monolayers for which the extent of crystal formation increases as the monolayers are expanded.

However when PA was examined alone as a pure monolayer, the larger number of big crystals consistent with a rapid growth was seen. The monolayer was compressed at low pressure and images were taken after 1, 15 and 24 hours, Figure 13-12. At high pressure, there was rapid crystallization with plenty of crystals formed. It was seen that the crystal formed at the different pressure differ in their sizes. When the monolayer was compressed at low pressure, crystals were formed within less time and grow in size with time until the equilibrium was reached and because of the free space available. However, at high pressure, no significant change in crystal size with time was seen, Figure 13-13 because the monolayer is highly compressed and is in liquid condensed phase which makes difficult for the incipient crystals to accommodate at the air/water interface. The reason for high number of crystals is in this case, the activity of the fatty acid is so high that lipid fluidity and the presence of phase boundaries are minor effects and the extent of crystal formation increases as the surface density of fatty acid sites increases and it was difficult to quantify them.
Table 3-1. The average number of crystals\textsuperscript{a} per BAM image under different monolayers at low and high pressure.

<table>
<thead>
<tr>
<th></th>
<th>DPPC</th>
<th>ternary\textsuperscript{b}</th>
<th>DPPC/PA\textsuperscript{c}</th>
<th>DPPC/lysolipid\textsuperscript{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mN/m</td>
<td>4±1</td>
<td>48±5</td>
<td>4±2</td>
<td>2±1</td>
</tr>
<tr>
<td>35 mN/m</td>
<td>2±1</td>
<td>60±4</td>
<td>3±1</td>
<td>2±1</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Reported as the number of crystals in a 536 μm x 430 μm frame after 24 h. Each value is the average of 3 different experiments using at least 20 frames from each experiment.

\textsuperscript{b} 2:1:1 ternary mixture of DPPC, 22:0 Lyso PC, and palmitic acid. \textsuperscript{c} 70:30 mixtures of DPPC/PA and DPPC/lysolipid.

Figure 3-1. Control experiment showing BAM images of calcium phosphate subphase in the absence of a DPPC Langmuir monolayer. Images taken after A) 1hr and B) 24 hr at 20°C.

Figure 3-2. BAM images of different phases prepared in the NaCl, Tris HCL buffer solution after 24 hr. A) CaCl\textsubscript{2} subphase B) Na\textsubscript{3}PO\textsubscript{4} subphase.
Figure 3-3. Structures of 1) DPPC, 2) Lysolipid 22:0 Lyso PC, and 3) Palmitic acid (PA). The 22:0 lysolipid is used in this study instead of the complementary 16-carbon tail lysolipid because it is more stable in Langmuir monolayers.

Figure 3-4. Pressure vs mean molecular area isotherms of a pure DPPC taken over a 0.43 mM CaCl$_2$ subphase at 20°C.
Figure 3-5. Brewster angle microscopy images of a DPPC monolayer at 20°C after 24 h over a calcium phosphate subphase at A) 35 and B) 15 mN/m. Crystals formed at the lipid interface appear as bright spots in the images. C) Scanning electron microscopy image of crystals grown under a DPPC monolayer.

Figure 3-6. Pressure vs mean molecular area isotherms of a ternary 2:1:1 mixture of DPPC, 22:0 Lyso PC, and palmitic acid taken over a 0.43 mM CaCl$_2$ subphase at 20°C.
Figure 3-7. Brewster angle microscopy images of a ternary monolayer, a 2:1:1 mixture of DPPC, 22:0 Lyso PC, and palmitic acid monolayer at 20°C after 24 h over a calcium phosphate subphase at 35 mN/m A) and 15 mN/m B). Crystals formed at the lipid interface appear as bright spots in the images. C) Scanning electron microscopy image of crystals grown under ternary mixture monolayer.
Figure 3-8. Pressure vs mean molecular area isotherms of the binary monolayers of DPPC with PA and DPPC with the 22:0 Lyso PC, taken over a 0.35 mM CaCl$_2$ subphase at 20°C.

Figure 3-9. Shape of domains formed in LC/LE coexistence region A) pure DPPC, B) Ternary mixture with DPPC:PA:Lyso PC at 20°C.
Figure 3-10. BAM images of a 70:30 DPPC/palmitic acid binary monolayer over a calcium phosphate subphase containing NaCl and tris HCl after 24 h at 20°C. A) 35mN/m and B) 15mN/m. Crystals appear as bright spots in the images.

Figure 3-11. BAM images of a 70:30 DPPC/lyso PC binary monolayer over a calcium phosphate subphase containing NaCl and tris HCl after 24 h at 20°C. A) 35mN/m and B) 15mN/m.

Figure 3-12. BAM images of a palmitic acid monolayer at 15mN/m over a calcium phosphate subphase containing NaCl and tris HCl at 20°C after A) 1 hr, B) 15 hr, and C) 24 hr 20°C.
Figure 3-1. BAM images of a palmitic acid monolayer at 35mN/m over a calcium phosphate subphase containing NaCl and tris HCl at 20°C after 24 hr 20°C.
CHAPTER 4  
IMPACT OF CHAIN LENGTH OF FATTY ACIDS ON CALCIUM PHOSPHATE FORMATION

In this chapter, the effects of chain length of fatty acids on calcium phosphate precipitation at lipid interfaces are discussed. As mentioned earlier, hydrolysis of phospholipid DPPC leads to a formation of a fatty acid and a lyso PC. Here arachidic acid is used as a fatty acid liberated upon hydrolysis, Figure 4-1. Previously, in chapter 3 the formation of calcium phosphate under the hydrolysis product of DPPC using a shorter tail palmitic acid (PA) with 16-carbons was discussed. In this chapter, a longer chain arachidic acid (AA) with 20-carbons is used to see its impact on calcium phosphate formation.

4.1 Experimental Section

All reagents were purchased from commercially available sources and used without further purification. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), and 22:0 Lyso PC (purity >99%) were purchased from Avanti Polar Lipids (Alabaster, AL). Arachidic acid and palmitic acid were purchased from Acros organic. Sodium phosphate and tris (hydroxymethyl) aminomethane hydrochloride (Tris HCl) were purchase from Aldrich Chemical Co. (Milwaukee, WI). Calcium chloride dihydrate and sodium chloride were obtained from Fisher Scientific (Pittsburgh, PA). The supersaturated calcium phosphate solution was prepared in the same way as described in chapter 3. To prepare all the surfactant solution, the same procedure was adopted as prescribed in chapter 3. Brewster Angle Microscope (Nanofilm Technologie GmbH BAM2plus system) was used to monitor the monolayers.
4.2 Crystallization of Calcium Phosphate under the Ternary Mixture Monolayer with Arachidic Acid

First a ternary mixture solution containing DPPC: AA: Lyso PC in the ratio 50:25:25 was prepared. The pressure versus area isotherm of the ternary monolayer with AA is shown in Figure 4-2. Once it was spread on the calcium phosphate subphase and compressed, BAM images were taken at high and low pressure after 24 hours to see the crystal formation. From the BAM images, Figure 4-3, it was seen that very few crystals were formed. These results were quite surprising. In the ternary mixture, the lysolipid is adsorbed to the exterior of the DPPC domain, lowering the line tension and allowing it to “extend” to less compact shapes and thus it reduces the interaction between DPPC and fatty acid, freeing the carboxylic acid to interact with the subphase ion. So it is expected to see calcium phosphate crystals. But here, in the ternary mixture very few crystals, similar to DPPC, were seen indicating that the carboxylic group is not interacting with the subphase ions.

There could be many reasons for this. From the BAM image at low pressure it can be postulated that the DPPC monolayer is still in the condensed phase while some DPPC is in expanded phase. This means that the lyso PC was not able to expand the monolayer and thus DPPC is still in condensed phase. Longer chain of the arachidic acid reduces the polarization of carboxylic group by pulling it above the subphase making it more hydrophobic. This causes the reduction in the interaction of COO⁻ ion with the Ca²⁺ ions in the calcium phosphate subphase solution.

When the monolayer is compressed at high pressure, molecules are packed at an interface and a cohesive force develops between their hydrocarbons chains due to van der Waals forces of attraction. The cohesive force grows as the chains increase in
length and as they are brought closer together. This makes difficult for the lyso PC to extend the domain freeing the carboxylic acid from the condensed phase to expanded phase and thus fewer crystals are seen similar to DPPC. However, when AA is examined alone as a pure monolayer, Figure 4-4, crystal nucleation is very high due to the interaction of carboxyl group with the calcium ions which was hindered in case of the ternary mixture.

4.3 BAM Images of Ternary Mixture at Different Pressures

To confirm the above hypothesis, isotherms with BAM images at different phases of compression were obtained. We first tried to obtain the BAM images of ternary mixture with palmitic acid, Figure 4-5 and then compared them with the ternary mixture with arachidic acid, Figure 4-6. From the BAM images it was seen that ternary mixture with PA is liquid as seen in the BAM image at zero pressure where no pressure is acting on the monolayer and it is in the liquid expanded phase. On compression, the domains were growing in size to fill the space available. Whereas the ternary mixture with AA looks solid and on compression there was no change in size of domain rather the domains were moving closer.

In the ternary mixture containing PA, Figure 4-5 it is seen that the domains appear expanded and circular in shape and grows as the pressure increases and slowly fills the whole image with high pressure is reached. This means that the domain is expanding (increasing in size) with pressure and is in the LE phase. Whereas in the case of ternary mixture with AA, Figure 4-6 most of the DPPC is still in the condensed phase (LC) and little amount of DPPC is in expanded phase. With the increase in pressure, the domains come closer with no change in size and thus it seems that the phase separation effect is not present in case of ternary mixture with arachidic acid. Due to the longer chain length
(C\textsubscript{20}) of AA, the DPPC remains in the condensed phase, keeping the carboxylate group raised up from the subphase.

4.4 Isotherm and BAM Images of Pure Fatty Acids

Since, as studied in chapter 3, the fatty acid of the ternary monolayer was the active component of crystallization, we tried to compare the isotherm and BAM images of pure fatty acids at different pressure. In case of palmitic acid, Figure 4-7 it is observed that the monolayer is dissolved and liquefied. Palmitic acid has 16 carbon atoms which make it a short chain fatty acid and its tails is not hydrophobic enough to hold itself away from the subphase. In case of arachidic acid, the monolayer appears to be more solid, Figure 4-8 The 20 carbon atoms make arachidic acid more hydrophobic so that it pulls away it chain from the subphase.

Thus it can be concluded that in the presence of arachidic acid, the phase separation of the ternary mixture is not seen because of the comparatively strong Van der Waals forces of attraction between the DPPC and AA chains and thus less crystals, similar to DPPC, are seen. The surface topography of the crystal grown on the Langmuir monolayer film was investigated using the scanning electron microscopy. This figure 4-9 shows the SEM image of the calcium phosphate surface formed under the fatty acid monolayer. The surface of the substrate is partly covered with hemispherical aggregates.
Table 4-1. Average number of crystals per BAM image under different monolayers at low and high pressure.

<table>
<thead>
<tr>
<th></th>
<th>DPPC</th>
<th>ternary\textsuperscript{b}</th>
<th>AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 mN/m</td>
<td>4±1</td>
<td>2±1</td>
<td>60±7</td>
</tr>
<tr>
<td>35 mN/m</td>
<td>2±1</td>
<td>2±1</td>
<td>200±25</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Reported as the number of crystals in a 536 μm x 430 μm frame after 24 h. Each value is the average of 2 different experiments using at least 15 frames from each experiment. 
\textsuperscript{b} 2:1:1 ternary mixture of DPPC, 22:0 Lyso PC, and arachidic acid.

Figure 4-1. Structures of 1) DPPC, 2) Lysolipid 22:0 Lyso PC, and 3) Arachidic acid (AA). The 22:0 lysolipid is used in this study instead of the complementary 16-carbon tail lysolipid because it is more stable in Langmuir monolayers.
Figure 4-2. Pressure vs mean molecular area isotherms of ternary mixture of DPPC: lyso PC: AA (50:25:25) taken over a 0.43 mM CaCl$_2$ subphase at 20°C.

Figure 4-3. BAM images of the ternary monolayer, a 2:1:1 mixture of DPPC, 22:0LysoPC, and Arachidic acid, over a calcium phosphate subphase after 24 h at 20°C. The monolayer in A was maintained at 35mN/m, and that in B was maintained at 15 mN/m.
Figure 4-4. BAM images of a arachidic acid monolayer over a calcium phosphate subphase containing NaCl and tris HCl after 24 h at 20°C. Monolayers were held at a two pressures: A) 35 and B) 15 mN/m.

Figure 4-5. Pressure vs area isotherm of a ternary 2:1:1 mixture of DPPC, 22:0 Lyso PC, and palmitic acid over a CaCl₂, NaCl, tris HCl buffer at 20°C. BAM images at various stages of compression are included.
Figure 4-6. Pressure vs area isotherm of a ternary 2:1:1 mixture of DPPC, 22:0 Lyso PC, and arachidic acid over a CaCl₂, NaCl, tris HCl buffer at 20°C. BAM images at various stages of compression are included.

Figure 4-7. Pressure vs area isotherm of a pure palmitic acid over a CaCl₂, NaCl, tris HCl buffer at 20°C. BAM images at various stages of compression are included.
Figure 4-8. Pressure vs area isotherm of a pure arachidic acid over a CaCl₂, NaCl, tris HCl buffer at 20°C. BAM images at various stages of compression are included.

Figure 4-9. SEM image of the calcium phosphate crystals formed under the fatty acid monolayer.
CHAPTER 5  
CONCLUSIONS AND RECOMMENDATIONS

This study demonstrates that the hydrolysis products of DPPC monolayer over a supersaturated calcium phosphate subphase leads to a greatly enhanced incidence of heterogeneous calcium phosphate precipitation. It is the lipid hydrolysis products, in particular, the locally high concentration of fatty acid, that cause the rapid precipitation. The results suggest a possible link between hydrolysis of phospholipid and formation of calcium phosphate. Calcium phosphate precipitation can occur at lipid interfaces, but the process is slow and precipitation is controlled. Our results show that lipid hydrolysis and the generation of locally high concentrations of small chain (C16) fatty acids i.e. palmitic acid can provide highly active sites for rapid calcium phosphate precipitation. Also it is found that in the ternary mixture with a long chain fatty acid like arachidic acid (C20) few crystals are formed. This is because of the arachidic acid, which does not allow the phase separation to occur because of the cohesive forces between the lipids tails and thus leads to very few crystals. Also it is seen that the size of the crystal increase with time at low pressure since the monolayer can arrange itself in order to accommodate its growth.
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BIOGRAPHICAL SKETCH

Hrishikesh P Bhase was born in Nashik, India in 1984. He went to HPT Arts and RYK College of Science in June 2002 to pursue his bachelor’s degree. He earned his bachelor’s degree in chemistry in April 2005. He joined Pune University in 2005 where he got his master’s degree in 2007. He was admitted to the University of Florida in fall 2008 and started working on his research program in biomineralization. He graduated from the University of Florida in December 2010 and continued for his PhD program in analytical chemistry at University of Florida.