INVESTIGATION OF THE BIOPHYSICS OF LIPID DROPLETS

A dissertation submitted to
Kent State University in partial
fulfillment of the requirements for the
degree of Doctor of Philosophy

by
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June 2017
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Acknowledgements

I would like to express my sincere gratitude to Dr. Elizabeth K. Mann and Dr. Edgar E. Kooijman, for their continuous help and support during my PhD study. I would not be able to finish this work without their advice. Dear Dr. Mann, I am grateful of being part of your research group during my first year and having your advice while I was trying to tackle all issues with my experiments. Dr. Kooijman, I am thankful of having the opportunity to work with you during past 5 years and for all the crash courses of biochemistry and biology. I came into your lab as a solid physics student without an experimental biology background. I appreciate all the training I have received.

The tensiometer instrument would not have been working without help of Dr. J. Adin Mann and Dr. Hossein Tavana. Special thanks to Wade Aldhizer and Larry Maurer for their help in making customized pieces.

I also want to say thank you to my committee members Dr. Jakli, Dr. Balci, Dr. Portman, Dr. Novak and Dr. Huang.

I would like to thank everyone who helped me while troubleshooting and analyzing my data: Dr. Weers, Dr. Robin Selinger, Dr. Petratos, Dr. Model, Dr. Katramatou, Dr. Khitrin, Dr. Baldwin, Dr. Secaur, and Dr. Newmann.

Dr. Luchette, Dr. Taheri, and Dr. Kosa who trained me during my internship at Alphamicron Inc.

As a physics student, I appreciate all the time and energy from the office staffs; Loretta, Cindy, Kelly, Connie, Deborah, Ida and Kim. Thank you for all the motivations and
supporting me when I was going through hard time. I also want to thank all the faculty in physics for their support and smiling faces when they are around.

I would like to thank all the previous lab member Dr. Zachary Greber, Dr. Anne Froyd Rankenberg, Dr. Piotr Popov and Dr. Pritam Mandal. I would also thank other student fellow in our lab, who always motivate me: Jeo Thomas, Emily, Larry, Ghadah, Huda, Amber, Jeo Yarzebinski and Nabin,

Priya, there are million reasons for thanking you. Your unlimited kindness and patience are among my precious moments in lab. I wish you best of luck with your dissertation and future career.

Dr. Morteza Mirhaidari and dear Sharon, we had a lot of celebrations together. Thank you for having me as part of your family, I never felt alone here. Shayda, Zach, Cyrus, Gabriel and Adrian, I have enjoyed every single moment of our hangouts. Thank you for always being source of positivity and energy.

My family who always supported even when they are thousands of miles away. Mom, I love you. Thank you for many years of support and for all the love and care you shared with me while I was away. Dad, you are my role model for leadership and hard work. Thank you for always being there for me. I have the most amazing parents in the whole universe. Amir, Ali and Mohammad, thank you for all the childhood and the time we had together. You are best brothers and partners in crimes, I’m counting days to get together and break another window.
Finally, I want to thank my friends from round world. We met here, and shared a lot of moments Marziyeh, Loubna, Bahare, Mahrokh, Adriana, Carine and Kajal. I always enjoyed our gathering.

June 16, 2017
CHAPTER 1
INTRODUCTION

1.1. Overview

Lipid droplets are energy storage organelles that can be found in various cell types of all organisms such as plants, insects, mammals, and fungi. Lipid droplets are all oil-based storage reservoirs for cell energy [1, 2]. They also play a role in other physiological processes, such as metabolism, lipid transport, new membrane formation, and steroid hormone synthesis [3]. Obesity, diabetes, lipodystrophy, atherosclerosis and liver steatosis are diseases which can appear if lipid droplets dysfunction [4].

Surface tension is utilized to explain problems in macroscale engineering, e. g. the distinct element method (DEM, a numerical calculation used to characterize the mechanical behavior) [5]. However, surface (or interfacial, which is always used between two condensed phases) tension results from molecular interactions at the interface. Thus, variations of interfacial tension can be used to probe the interaction of protein with a phospholipid monolayer on the surface of a lipid droplet. Recently, studies have been conducted on the structure and formation of lipid droplets, but there are many questions remaining on the mechanism of lipid droplet formation and growth [6, 7]. The major goal for my project is to investigate protein insertion on the LD surface by means of surface tensiometry. Tensiometry measures the interfacial tension to characterize the protein interaction with phospholipid monolayers.
The first part of my work uses the Langmuir monolayer method to study protein binding to phospholipid monolayers at the air/buffer interface \[8, 9\]. For my second project, I constructed a droplet tensiometer to model protein-lipid interaction at the oil/buffer interface. I use Axisymmetric Drop Shape Analysis (ADSA) to numerically analyze the droplets to determine the interfacial tension \[2\].

Droplet tensiometry can be used to mimic the physiological condition, where the lipid-protein interaction occurs at the oil-buffer interface. Droplet tensiometry contributes to a more profound investigation of protein interaction with the core of lipid droplets.

### 1.2. Biological membranes

Biological membranes are constructs that define cell boundaries, the outer boundaries (plasma membrane, PM) and the inner components (organelles) \[10\]. Membranes control the permeability of substances into and out of the cells. Biological membranes also regulate the flow of information between cells by chemical or electrical signals or through specific signal molecules. They also participate in energy transfer (capture and release of energy). Therefore, biological membrane are vital to keep the cell alive \[11, 12\]. There exist many different cellular membranes in all complex living organisms.

The “fluid mosaic model” (FMM) proposed by Singer and Nicolson was developed for all biological membranes \[11\]. The structure, function and dynamics of the membrane have been studied since 1970s \[13, 14\].

Figure 1.1 shows a cartoon of the PM of an eukaryotic cell. It shows the general structure of a biological membrane, namely the lipid bilayer, and embedded proteins and
sugars (attached to proteins and lipids). The PM is a single bilayer. There are other cell organelles which are also surrounded by a single lipid membrane e.g. Golgi apparatus, endoplasmic reticulum, and lysosomes, while mitochondria and nuclei are enveloped by double membranes [15]. The phospholipids which exist in the membrane and participate in the formation of the bilayer are amphipathic molecules; they have hydrophobic tails and hydrophilic heads [16]. The amphipathic characteristics supports the formation of 2D plane of phospholipid molecules, with the hydrophobic tails facing each other, and thus isolated from water [17].

The hydrophobic effect is the driving force for the self-assembly of lipid bilayers which form the matrix of biomembranes [18, 19]. The long aliphatic chains of lipids contribute the hydrophobic effect on the biological membranes. In addition, the polar headgroups of diacyl phospholipids play an important role in the stability of self-assembled lipid bilayers [18]. Zwitterionic lipids, i.e. lipids with both a negative and positive charge, are present in

Figure 1. 1. One representation of a biological membrane. Figure taken from [https://classconnection.s3.amazonaws.com]
the structure of the mammalian bilayer membrane. The presence of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in the structure contribute to the opposing forces, which are necessary for the stable equilibrium of the biomembrane. These lipids are the major building blocks of most biological membranes [18]. It is known that biological membranes also include significant amount of negatively charged lipids, which are critical for certain functions of the membrane.

Proteins are partially or completely imbedded into the lipid bilayer of biological membranes. These proteins either exist at the surface of the bilayer (extrinsic protein) or are integral (intrinsic protein) to the hydrophobic tail portion of the bilayer [20]. Proteins are attached to lipids by non-covalent bonds e.g. Van-der-Waals bonds (which connect hydrophobic tails) and hydrogen bonds (which lie between the charged head group and water) [21]. Additionally, some proteins are covalently linked to a lipid anchor [22]. Sugars are attached by covalent bonds to some lipids and proteins.

1.3. Membrane lipids

Lipids are the major structural component of biological membranes. Membrane lipids are biological configurations with two parts: a hydrophobic (nonpolar) tail and a hydrophilic (polar) head-group. Most lipids are generally soluble in organic solvents. Variations in the hydrophobic and hydrophilic parts of lipid molecules result in new molecules with different biochemical characteristics. These differences in lipid headgroup and acyl chains result in variations in charge, shape, and curvature of lipids in the structure of the membrane, which regulate the roles of lipids in the cell membrane. Note that the term “lipid” includes many different compounds: fats, oils, some vitamins, hormones, plant
waxes, and a host of other structurally distinct compounds. Here, I focused specifically on membrane lipids that are denoted as “lipids” throughout the rest of my dissertation. The biomembrane is composed of various classes of lipid, which each play important roles in defining the membrane biochemistry and biophysics. The three main classes of lipids are as follows: glycerol-(phospho-)lipids, sterols and sphingolipids.

Plasma membranes of different cells differ in their lipid and protein compositions. In addition, membranes are not homogenous and uniform within each leaflet. Lipids including PC and sphingomelytin (SM) (see below, in section 1.3.5 for lipid definitions) are mainly found in the outer leaflet of the membrane whereas lipids containing phosphoserine (PS), PE and phosphoinositol (PI) headgroups appear in the inner leaflet [23-25]. The negatively charged lipids, e.g. phosphatidic acid (PA) and PS, function as binding sites to interact with positively charged amino acid residues of the membrane proteins. The mammalian sterol, cholesterol, is distributed between both the inner and outer leaflet of the PM.

Membrane phospholipids can move from one leaflet to the other introducing an asymmetrical distribution of phospholipids across the membrane as defined above [26, 27]. There are several enzymes, such as flippases and floppases, associated with the membrane to provide the energy required for the phospholipids to flip from one leaflet to another [28, 29]. Maintaining the asymmetry of the lipid across the plasma membrane is vital to preserve cell function, whereas disruption in the membrane asymmetry leads to pathological conditions [28]. Some membrane lipids like glycolipids are localized on a particular side of the membrane. Additionally, lipids which are attached to the intracellular structures or extracellular matrix experience a limitation in movement [11].
The endoplasmic reticulum is the place where the majority of plasma membrane lipids are produced. Lipid droplets, mitochondria and peroxisomes are other sources which contribute to synthesis of phospholipids [30]. Phospholipids like PA and lysophosphatidic acid (LPA) that play roles as building blocks for the de novo biosynthesis of all other membrane glycerophospholipids and are present in the biomembrane at lower concentrations (1-4 %) [31, 32].

1.3.1. Fatty acids

Fatty acids are carboxylic acids with a long aliphatic chain, which is either saturated (all the carbons in the structure are protonated) or unsaturated (where there exists at least one double bond in the structure) and can be found free or as part of complex lipids (e.g. membrane glycerophospholipids). Fatty acids are rarely free in nature; however, their esterified form is a major component of lipids. The combination of the carboxylic acid group with an alcohol (−OH) results in fatty acid esters, Fig 1. 2. Fatty acylglycerols form a major building block of mammalian biomembranes. Fatty acids have three major roles: As a part of the membrane lipids they are essential for membrane structure. In addition, as part of the triacylglycerides, they represent energy storage [33]. Finally, they are part of some signaling lipids. Eicosanoids are signaling lipids that are derived from polyunsaturated fatty acids, such as arachidonic acid (20:4, n-6). Eicosanoids regulate many different types of processes such as the inflammatory response and blood clotting [34].
The physical properties of fatty acids vary with the number of unsaturated bonds. Saturated fatty acids are flexible molecules as they can rotate freely around each of their $C - C$ bonds [35]. The stretched configuration of a fatty acid is at its minimum internal energy, as this structure would have the least steric interaction between neighboring methylene groups [36]. However, the stretched configuration, with all trans carbon bonds, also has the lowest entropy since it has only one configuration ($S = k_B T \log \Omega$). As temperature increases, the entropy increases by adding occasional cis-bonds to minimize the free energy at constant temperature. Above the chain melting point $T_C$, there are enough cis-defects to prohibit the acyl chains from ordered packing.

Fatty acids with longer chains and larger molecular masses have higher melting points. In addition, fatty acids with saturated bonds have higher melting points [37]. Further, a decrease in the degree of saturation increases the fluidity of lipids, caused by the disordered packing introduced by one or more double bonds. Stearic acid (18:0) vs. oleic acid (18:1 Δ9) gives an example for differences in the melting point and fluidity. Both of these fatty acids have the same number of carbons (18), but they are very different in fluidity at room temperature. The melting point of stearic acid (candle wax) is much higher than oleic acid (olive oil); since stearic acid is saturated and can pack in an orderly manner.

Figure 1. 2 Schematic view of unsaturated fatty acid taken from
This is due to the Van der Waals interactions between the molecules. This phenomenon has an important impact on membrane properties [38].

1.3.2. Triacylglycerols

Fats and oils in plants and animals consist of triacylglycerol mixtures; triacylglycerols are nonpolar and water insoluble substances. Obesity occurs when these fats and oils store excessively in cells. Considerable attention is now directed at finding medical treatments for this condition. Figure 1.3, shows the schematic structure for a) fatty acid and b) Glycerol. R represents the hydrocarbon chain in the structure of the molecules in number of carbons and unsaturated double bonds.

![Schematic structure for fatty acid, glycerol, and triacylglycerol](image)

*Figure 1.3 Schematic structure of a) fatty acid b) Glycerol c) Triacylglycerol.*

Triacylglycerol, Figure 1.3. (c), molecules vary according to the identity and placement of their three fatty acid residues [39]. Fats and oil are complex mixtures of simple and mixed triacylglycerols whose fatty acid compositions vary with the organism which produces them [40]. Triacylglycerols provide energy storage in animals and are a rich source of different lipids, although they are not present in the membrane structure [41]. In plants, lipid droplets are stored in tissues or they are preserved as oily seeds, in which
triacylglycerol supports the plants’ growth. These seeds constitute the majority of commercial crops [42]. Triacylglycerols have fatty acids in highly reduced states, which characterizes them as an ideal option for energy storage [43]. Because triacylglycerols are insoluble in water [44], complexes must be formed to store or transport them.

In animals, most of this triacylglycerol is maintained in adipose tissue [41, 45]. When the metabolic condition is under stress, the supply of fatty acids and its derivatives exceeds what is needed in adipose tissue or is beyond the capacity of cell to oxidize, triacylglycerols are also located in liver, heart, and muscles [46]. Triacylglycerol formation inhibits the toxic effect of excess fatty acids, which could damage the membrane [47-49]. When needed, intracellular lipases oxidize the fatty acids through hydrolysis processes.

1.3.3. Glycerophospholipids

Glycerophospholipids are lipids that consists of a glycerol backbone, two fatty acid tails, and a phosphate containing headgroup; they are major components of cell membranes [50]. In mammals, the fatty acid chains are usually 14 to 24 carbon atoms long. One chain is typically unsaturated, containing from one to four cis double bonds. Each double bond bends the fatty acid chain.
Figure 1.4. Molecular structure of glycerophospholipids. a) General structure of diacylglycerol (substitute R for H to get DAG) which carries mixed acyl chains. b) Different head groups of phospholipids shown are PC, PE, PA, PI and PS. Substitute R in a) with these groups to get the corresponding diacylglycerophospholipid.

Most of the glycerophospholipids include two long fatty acyl chains esterified to the hydroxyl groups on the glycerol backbone in sn-1 and -2 positions, Figure 1.4 (a). In mammalian cells, saturated acyl chains generally occur in the sn-1 position whereas unsaturated acyl chains occur in the sn-2 position. At physiological pH (pH ≈ 7.2), PC and PE are zwitterionic lipids whereas PS, PIs and PA are anionic and DAGs are neutral. The head group charge and shape of the phospholipids are important parameters which determine their interactions with membrane. In this dissertation, I have used phospholipids to denote glycerophospholipids [31].
1.3.4. Cholesterol

Cholesterol is part of another class of lipids called sterols, also known as steroid alcohols. Sterols are a subgroup of the steroids which is an important class of organic molecules. Sterols are present in animals, plants, and fungi [51]. This type of molecule has a four-ring steroid structure (see Figure 1.5), followed by a short carbon hydrocarbon side chain and a hydroxyl group; these make this molecule distinct from phospholipids and triacylglycerols [52]. Approximately 30 to 40 mol % of cholesterol is present in animal plasma membrane. A lower concentration of cholesterol exists in the membrane of other subcellular organelles [35]. The OH group in the cholesterol structure provides the molecule with some low amphiphilic character, while the fused rings provide greater rigidity in this molecule compared to other lipid molecules [53]. This characteristic makes cholesterol an important molecule in determining the membrane properties. Cholesterol can be incorporated in the lipid bilayer structure; however, it cannot form a bilayer on its own.

Figure 1.5 Cholesterol structure
In addition, cholesterol is the starting pathway for biosynthesis of steroid hormones and bile acid [54]. Steroid hormones regulate different physiological tasks, such as carbohydrate metabolism [55]. Plants also contain a small amount of sterol in the form of stigmasterol and β-sitosterol, whereas in yeast and fungi sterols would be found in an ergosterol-like form.

1.3.5. Sphingolipid

![Sphingomyelin structure](image)

**Figure 1.6. Sphingomyelin structure.**

Sphingolipid is the other class of lipid that is present in the structure of plasma (and other) membranes. They have a backbone of sphingosine, which is indicated in Figure 1.6. Sphingolipids were first discovered in brain extract and they participate in signal transmission. When sphingolipid metabolism does not function properly, neural tissue is affected [56]. This is because myelin sheet, which protects and function in nerve pulse propagation, are composed primarily of sphingolipids.

Sphingolipids have the same type of polar headgroup as glycerophospholipids and the hydrophobic component, ceramide, is the sphingolipid counterpart of diacylglycerol (see Figure 1.4) in the glycerophospholipids, but with its own unique chemical structure and thus physiological function. Sphingomyelin is the type of sphingolipid that is found
widely in the animal cell plasma membrane. Sphingolipids are rarely found in plants and bacteria [57].

1.3.6. Glycolipids

Glycolipids are another class of membrane lipids that plays roles in maintaining proper membrane function [35]. Glycolipids belong to both the phospholipids as well as the sphingolipids but are often grouped in their own class. They facilitate cell-cell interaction and are amphiphilic molecules that have a sugar headgroup and a hydrophobic part (e.g. diacylglycerol or ceramide). The head group can vary from small saccharide units to large polysaccharides. According to the sugar part present in the structure of these molecules, glycolipids play roles that are important for many processes, e.g. cell signaling, lipid rafts (domain formation) and membrane fluidity [58].

1.4. Membrane proteins

To produce a full understanding of biological membranes, it is necessary to investigate the types and effects of lipid-protein interactions. These interactions can be classified in two groups. In the first group, a hydrophobic segment exists which can penetrate through the lipid bilayer (intrinsic/integral proteins). The second group includes the proteins that are water soluble. They can interact electrostatically with negatively charged groups at the lipid headgroup interface (extrinsic/peripheral proteins) [59].

1.4.1. Intrinsic protein

This type of protein needs detergent to solubilize in water and contains one or more hydrophobic regions in the sequence that completely spans the lipid bilayer. Multiple
hydrophobic regions, α-helices, are connected by flexible loops and create so-called multi-spanning membrane proteins [60]. Additionally, proteins with beta-sheet secondary structure can form transmembrane complexes called beta-barrels. Much work has examined protein-lipid interactions for integral membrane proteins [61, 62].

These studies have shown that lipids involved in interactions at lipid-(integral)protein interfaces behave completely differently than the lipids that are present in the bulk bilayer. These lipids are called annular lipids and they form a shell around the protein [63]. Previous ESR (electron spin resonance) studies have shown that in the vicinity of the integral proteins, incorporated lipids experience a higher order parameter (rigidity in acyl chain.) This rigidity affects the motion of the phospholipid [64].

Intrinsic proteins include a nonpolar sequence that binds the protein hydrophobically to the membrane. It is speculated that lipids present on the surface of intrinsic proteins experience a different interface compared to lipids in the bulk bilayer. It is hypothesized that these lipids are specific to the unique hydrophobic region of proteins, and that the lipids provide appropriate environment for the protein function. [65-67]. Recent investigations have determined that the probability of non-bilayer structures play a role in protein insertion into or across the membrane [66].

1.4.2. Extrinsic proteins

Peripheral/extrinsic proteins are a class of membrane protein that is present at/attached to the inner or outer phospholipid bilayer with which they are associated. These extrinsic proteins are connected to the bilayer through such molecular interactions as ionic,
Van der Waals, hydrophobic or hydrogen bonding. Extrinsic protein can also attach to the membrane through intrinsic proteins. Various proteins (Cytochrome, Polyllysine, Perilipin family proteins, apolipoprotein family proteins) are used to study the interaction of extrinsic proteins with phospholipids [68-71]. To increase the probability of lipid interaction with these basic proteins (positively charged), the presence of acidic (negatively charged) lipids is vital.

Generally, extrinsic proteins can be extracted from the membrane by changing the pH or ionic conditions of the medium. Some of the extrinsic protein that are found on the cell surface have lipids attached to them. These lipids anchor the proteins to the membrane covalently [66].

Studying the protein-lipid interaction has the potential to answer major questions, including on the protein structure. Does the protein undergo conformational changes while interacting with the lipid membrane? Is there a specific domain of the protein that is more favorable to bind? On the other hand, protein-lipid interaction can answer questions regarding the role of lipids present in the membrane.

Lipid droplets and lipoproteins are lipid particles that share similar structure. Protein-lipid interaction plays important roles in the dynamics and metabolism of these lipid particles. These characteristics make LDs and lipoproteins unique structures to be used to study protein-lipid interactions.
1.5. Lipid droplets

A constant flux of metabolic energy and fat (lipids) is needed to maintain the activity of a living system [72]. Lipid droplets (LDs) are dynamic cell organelles that in the past were thought to just store energy. However, recent studies demonstrate that they also participate in many other physiological processes. Specifically, lipid droplets play an important role in maintaining cellular energy homeostasis as well as in the regulation of lipid metabolism [73, 74]. LDs are found in all tissues and cells; however the size and number of LDs is different and depends on cell type [75]. The size of LDs varies in the range of 20 nm to 100 μm [76]. For example, the LDs in white adipocytes are larger as they occupy the majority of the cell [76]. In contrast, there are multiple smaller size LDs in brown adipocytes, which increases the LD’s surface area and lipid flux.

1.5.1. LDs vs. Lipoprotein particles

Despite their presence in different cellular components (inside cells or in the bloodstream), vertebrate lipoproteins and LDs share a similar structure [77], and see Figure 1.7. It is beneficial to compare and contrast them. Lipoprotein structure consists of a core of neutral lipids, such as triacylglycerol, surrounded by a phospholipid monolayer. Some proteins are embedded in the coating phospholipid monolayer, Figure 1.7(a). [72, 78]. Apolipoproteins are associated with the surface of lipoprotein particles, where the exchangeable apolipoproteins can commute between lipoprotein particles, or between lipoprotein and blood [79]. ApoA, apoC and apoE are exchangeable proteins coupled to the surface of mammalian lipoproteins, whereas apoB-100 is an unexchangeable protein present on the surface and also embedded in the interior of lipoprotein particle [80, 81].
Figure 1. Schematic structure of lipoprotein particles and lipid droplets. The structure consists of a hydrophobic core [triacylglycerol(tag) + cholesterol esters], surrounded by a phospholipid monolayer. a) The surface of the lipoprotein particle is coated with apolipoproteins. Except apoB proteins, the other proteins associated with the surface of lipoprotein particles are exchangeable. b) Proteins from perilipin family coat the surface of the LDs.

The exchangeable apolipoproteins associated with the surface of lipoproteins have many similarities with the proteins (perilipin family proteins) coupled to LDs [82]. The N-terminal domain of the apoE is one example. The N-terminus includes 4 amphipathic α-helices organized in a bundle in solution. The C-terminus of the exchangeable LD binding protein, perilipin 3, has a similar structure despite minimal amino acid sequence overlap. Some studies have shown that these helical bundles play major roles in protein binding to the surface of neutral lipid particles [83-86].

In this dissertation, I have used two apolipoproteins and one LD binding protein to study the mechanism by which exchangeable LD binding proteins interact with neutral lipid particles.
1.5.1. LD structure

With their roughly spherical shape, LDs are dispersed as an oil-in-water emulsion in the aqueous cell cytosol. Lipid droplets consist of a hydrophobic neutral lipid core of triacylglycerol (some diacylglycerols) and some cholesterol esters, Figure 1.7. (b), and are thus very similar to the lipoproteins. LDs are surrounded by a phospholipid monolayer composed of phospholipids and free cholesterols, with proteins coating the surface of the phospholipid monolayer [87-89]. In addition, these particles and LDs have the same origin, which is the ER (endoplasmic reticulum) [90].

The monolayer surrounding the lipid droplet core has a unique phospholipid composition, quite different from cellular membranes [88]. Some studies have mentioned that this phospholipid monolayer has a rigid structure and the hydrocarbon molecules are saturated. Other work indicates that most of the phospholipid acyl chains in this monolayer are highly unsaturated. Thus, even the composition of this monolayer is controversial [91].

PC is the most abundant phospholipid present in the lipid monolayer. PE, PI, and lyso-phosphatidylcholine (LPC) are the other phospholipids residing in the phospholipid monolayer. Studies have shown that there is a low concentration of sphingomyelin, PA, and PS in the lipid monolayer [88].

Other research shows that the proteins which are associated with the surface of lipid droplets belong to different classes with various functional characteristics [92]. Perilipin family proteins (1,2,3,4 and 5) are the major proteins, which are shared by all mammalian
LDs [74]. In addition to perilipin proteins, there are enzymes, hormones, and signaling molecules involved in lipid synthesis and hydrolysis [93].

The similarity between low density lipoprotein particles and vertebrate LDs means that they provide interesting complementary systems for studying the mechanisms associated with lipid-protein interaction.

1.5.2. **LD formation**

Researchers have suggested different hypotheses for the formation and growth of LDs. In all cases, the ER (endoplasmic reticulum) is considered to be the location where LD formation starts, since most of the enzymes associated with the triacylglycerol or sterol ester synthesis are present in the ER (prior to LDs formation) [1, 94, 95]. Moreover, proteins with hydrophobic domains (e.g. hairpin domains as found in caveolin 1) are also found to have dual localization between ER and lipid droplets. A more recent study on LD formation in yeast supports the models suggesting that an important function of the ER is to form lipid droplets [96]

The initial step in the formation of the LD is still unknown. Most models suggest that lipid esters start accumulating in between the two leaflets of the ER membrane and gradually form a lens shape. When growth finishes, the LD finally detaches from the ER and forms an independent lipid droplet [97, 98], see Figure 1.8. Physics models suggest that this process is a complete dewetting of LD from the membrane. When a liquid is deposited on a surface, it starts spreading over the surface (as when water forms a film on a glass surface) or it partially wets the surface (oil droplet on the water interface). If the
droplet partially wets the surface, it forms a contact angle $\theta$ with the interface, leading to an almost spherical droplet, Fig 1.8, (a). The dewetting process is well-understood and can serve as an explanation for the budding of a lipid droplet.

Figure 1.8. Models for LD formation. (a) Presentation of the dewetting of the droplet on different subphases (b) Interfacial tension controls the dewetting process. Different lipids can affect the dewetting process of the LD (c). Studies suggest that two different population of basic LDs can be present. The first one refers to smaller LDs which bud from ER and DGAT1 enzyme leads this process. The second one refers to cytosolic LDs, which are called expanding LDs. DGAT2 is present in the synthesis pathway (right panel) [1].
from the bilayer membrane [99-101]. When the lens shape converts to a lipid droplet, the contact angle decreases until the LD detaches from the bilayer.

The dewetting of the oil is also affected by the bilayer lipid composition, since those lipids will form a monolayer on the LD. Thermodynamically, the budding of the LD occurs when the interfacial tension of the monolayer and bilayer decreases, Fig 1.8 (b) [101-104]. This model suggests that during LD formation, a huge source of diacylglycerol and fatty acid must be present to act as a surfactant in lowering the interfacial tension and inducing curvature on the monolayer to facilitate the budding process [105-107].

It is still unknown if lipid droplets with different neutral cores form in the same way. The most recent model [1] suggests that there exists a subpopulation with a different process present among lipid droplets containing triacylglycerol after their initiation. The enzymes responsible for this process through the synthesis of triacylglycerol are glycerol-3-phosphate acyltransferase 4 (GPAT 4), 1-acylglycerol-3-phosphate O-acyltransferase 3 (AGPAT 3) and diacylglycerol O-acyltransferase (DGAT 2), see Fig 1.8. (C), that mediate this process through synthesis of the triacylglycerol.

1.5.3. LD function

LDs play a significant role in many metabolic processes that are vital for continuing life. Their fundamental role is to preserve hydrocarbon chains (in the form of fatty acids) for metabolic energy and for lipid membrane synthesis [108, 109]. LDs use their stored lipid for building new membranes, cell signaling and vesicular trafficking. In addition, they also provide the lipid molecules necessary for synthesizing steroid hormones [110].
Hydrolyzation of the cholesterol esters stored in the LDs produces steroid hormones and new membrane [51, 111]. LDs prevent lipotoxicity which occurs due to presence of free fatty acids [112]. LDs also act as the temporary storage site for certain proteins. The hydrophobic environment inside the LDs is favored for the storage of hydrophobic molecules such as lipophilic vitamin and xenobiotic compounds; these molecules are abundant in the digested food present in the lumens of the gut [93, 113, 114]. Moreover, proteins bound to the surface of LDs also regulate the LDs` synthesis and degradation of neutral lipids [115, 116], see Figure 1.8.

Several diseases are associated with the malfunctioning of LDs. It is beneficial to have a better understanding of the function of LDs. The major role of LDs is to regulate the mechanism of influx and efflux of the lipid to maintain cellular homeostasis. So, mis-regulation in LDs metabolism leads to diseases such as obesity, diabetes, atherosclerosis and hypertension [109, 117].

1.6. Structure and function of different classes of lipoprotein

The relation between structure and function is an important focus of investigation for lipids and lipid-protein structures. Different classes of lipoprotein particles exhibit differences in lipid composition and types of apolipoprotein associated with the surface. These distinct variations in structure are associated with distinct functions.

1.6.1. Lipoprotein structure

There are a variety of different kinds of lipoproteins, including HDL, LDL, VLDL and chylomicron. Figure 1.7. (a), shows a schematic representation of lipoprotein particle.
A lipoprotein consists of a hydrophobic core, rich in triacylglycerol and a small amount of cholesterol ester, surrounded by a phospholipid monolayer embedded with free cholesterol. Proteins from the apolipoprotein family are associated with the phospholipid monolayer surface [118, 119].

The unique monolayer coating the lipoprotein plays an important role in aiding the metabolism of the triacylglycerol while maintaining the lipoprotein particle integrity [120, 121]. Although the hydrophobicity of the phospholipid monolayer surrounding the hydrophobic core is limited, it is sufficient to allow access to the aqueous compartment when hydrolytic enzymes such as lipases (enzymes that break down lipids, e.g. remove acylchains from the glycerol backbone) are present. This allows the triacylglycerol core to release its content in the form of fatty acids and glycerol.

The lipoprotein, rich in triacylglycerol, experiences a fast metabolism which begins immediately upon entering the blood plasma [122]. Soon these lipoprotein particles become metabolized by lipases. The major lipase activity in plasma occurs through lipoprotein lipase, which is bound to the endothelial surface of the cell, and connects to the phospholipid monolayer surface of the lipoprotein particle [123]. Triacylglycerol enters the enzyme through the phospholipid monolayer surface which is associated with the active site of the lipase. Apolipoprotein C-II facilitates this interaction [124].

VLDL and chylomicron both include several different types of apolipoproteins. ApoB-100 is the major apolipoprotein found on VLDL, whereas chylomicrons have apoB-49 [125, 126]. Other apolipoproteins (apoE, apoAs and apoCs), which are associated with
the surface of lipoprotein particles, participate in direct metabolism and tissue targeting [127-129].

1.6.2. Chylomicron and very low density of lipoprotein (VLDL)

If a blood plasma of a fasting person is left in a test tube overnight, no changes appear on the surface of the sample. However, if the sample is taken from a person immediately after a meal, a creamy layer of foam forms on the surface. Chylomicrons are the major components of the foamy layer. These lipid particles are the major secretory lipids of intestine. Triacylglycerols and phospholipids are the major dietary lipids, which are hydrolyzed (undergo chemical breakdown by lipases) in the intestinal lumen [130, 131]. Later, lipids are adsorbed by the intestinal enterocyte, re-esterified to triacylglycerol and form chylomicrons rich in triacylglycerol [132]. Since these lipoprotein particles are >99% lipid, their density is less than blood plasma, and they float on the top of the test tube. Since chylomicrons are not present in samples from fasting people, chylomicrons are only present for a limited time. The lipid composition of chylomicrons depends on the dietary composition. For example, a meal high in triacylglycerols results in large chylomicron particles rich in triacylglycerol, while a diet rich in cholesterol leads to relatively small chylomicrons high in cholesterol [130].

VLDL is the major type of lipoprotein produced by the liver. This type of lipoprotein particle oversees delivering the newly synthesized fat (in the form of triacylglycerol) to extrahepatic (outside the liver) cells [133]. VLDL particles are less buoyant than chylomicrons, but they share many similarities; they also transfer lipid to
other tissues. The size of VLDL particles varies from 30 to 100 nm [134]. The liver reacts to changes in lipogenic state (i.e. the balance of lipids presents in the blood) by varying VLDL secretion in two ways: (1) changing the particle size, and (2) altering the number of VLDL particles [135, 136].

1.6.3. Low density Lipoprotein (LDL)

Low density lipoprotein (LDL) is derived from VLDL by lipoprotein lipase. Lipolysis of VLDL results in a pool of intermediate particles that contain different concentrations of triacylglycerols. This group is called intermediate density lipoproteins (IDL) [118, 137]. IDL particles are of great importance in lipoprotein balance. IDLs may be cleared up from the circulation by the liver or they may be further processed to become LDL. Since both processes are available simultaneously, the efficiency of IDL affects the rate of LDL production. For example, rats have very low levels of LDL, which is due to efficient clearance pathway of IDL [138-140].

The concentration of LDL in plasma is associated with the risk of atherosclerosis, an important finding that prompts interest in examining the structure, metabolism, and origin of LDL [141, 142]. If there is an increase in LDL production or a slow rate removal of LDL from blood plasma, the concentration of LDL increases. Sluggish removal of LDL from the blood stream prolongs the residence time of LDLs, results in structural modifications that may boost the interaction of LDL particles with cells in arterial walls [143, 144].
1.6.4. High density lipoprotein (HDL)

HDL particles are the smallest lipoproteins. Among all classes of lipoproteins, HDL is the most condensed, which reflects the highest proportion of protein to lipids in the structure. These lipoprotein particles are synthesized in the liver as complexes of apolipoproteins and phospholipids [83, 145, 146]. Lecithin-cholesterol acyltransferase (LCAT) is a plasma enzyme that modifies the free cholesterol to cholesterol esters [147, 148]. This type cholesterol, which is more hydrophobic, is then sequestered into the lipoprotein core, which cause the newly synthesized HDL to have a spherical shape [149]. During circulation through the blood stream, HDL particles enlarge in size, as they obtain more cholesterol and phospholipids molecules from cells or other lipoprotein particles [150, 151]. HDL mostly carries cholesterol to the liver and steroidogenic organs, e.g. adrenals, ovary and testes, through direct or indirect pathway [152].

HDL receptors such as the scavenger receptor BI (SR-BI) remove HDL from blood plasma. This process assists in mediating the uptake of cholesterol from HDL [153, 154]. In humans, this pathway is mostly indirect through the cholesteryl ester transfer protein (CETP). This protein swaps the triacylglycerols of VLDL against HDL cholesterol. Then, VLDLs are processed to LDL and are removed from the circulation by the LDL receptor pathway [155, 156].

There are several different lipoprotein particles within the HDL density class that are different in apolipoprotein composition, phospholipids, sizes and functions [134, 157].
1.7. Apolipoproteins vs. lipid droplet proteins

Apolipoproteins refers to a group of proteins that are associated with the surface of lipoprotein (from “lipid” + “protein”) particles. They are of considerable physiological importance and are involved with different diseases and disorders e.g., atherosclerosis, hypolipidemia, and Alzheimer’s disease [71, 158, 159].

1.7.1. Apolipoproteins

Apolipoproteins help to solubilize the lipid in the blood by binding to them. They also play a pivotal role as a structural component in lipoprotein particles as discussed above. Lipoprotein particles participate in carrying lipids to provide an energy source and for synthesizing hormones, vitamins and bile acids [160]. ApoB is essential for assembly and secretion of triacylglycerol. In addition to apoB, apoE is also responsible for transporting dietary and endogenous lipids to target tissues [127, 161-163]. Whereas apoA1 (structural component of HDL) is vitally responsible for returning excess cholesterol from the tissue to the liver [127]. Apolipoproteins are also involved in neurological processes, e.g., apoE and apoJ participate in transporting lipids to the brain [164].

1.7.2. Perilipin/PAT family proteins

Proteins belonging to the perilipin family are significantly associated with the surface of lipid droplets; the ratio of these proteins is different depending on the cell type. This protein family has 5 members: Perilipin 1, Perilipin 2/ADPR (Adipose Differentiation Related Protein), Perilipin 3/TIP47 (Tail-interacting 47 KDa), Perilipin 4 (S3-12), and Perilipin 5 (OXPAT), see Figure 1.9 [73].
This family’s members share a PAT domain which includes the first 100 amino acids present in the N-terminal region (Figure 1.9) [165]. The 11-mer repeat domain is also shared among all perilipins. 11-mer repeat is a protein domain where an 11 amino acid stretch is repeated. It is found not only in perilipins but also other proteins such as α-synuclein. Most perilipins have imperfect 11-mer repeats where not all the amino acids in the repeat are perfectly replicated in each occurrence of the 11 amino acid stretch. Perilipin 4 forms the best repetitive pattern for the 11-mer-repeat. The 11-mer repeat is also capable of forming amphipathic α-helices [9, 166].

Perilipin 1 and perilipin 2 are almost always associated with LDs, whereas perilipin 3, 4 and 5 can exchange between the LDs’ surface and cytoplasm; the presence of each of these proteins depends on the state of the cellular metabolism [82]. Recent studies have
shown that perilipin proteins may also be found at the core of LDs [167]. The role of these internal proteins is not known yet and this finding is highly controversial [168].

1.7.2. Perilipin 3 /TIP47

A significant focus of this research is on LD protein perilipin 3. We investigated the role of several protein motifs in interaction to the lipid droplet interface. There is approximately 43% similarity in structure and 63% of homology between perilipin 3 and perilipin 2 [73, 169]. Perilipin 3 was first discovered in yeast hybrid as a protein that bound to the C-terminus of mannose-6-phosphate receptor (M6RP). Additionally, this work observed that perilipin 3 protein associated with Rab 9 protein, when M6RP moved from endosomes to the trans-Golgi network [170, 171]. More recent work showed that perilipin 3 does not affect the localization or formation of M6RP. Moreover, the N-terminus of perilipin 3 does not participate in mannose transport [169, 172].

As perilipin 3 is associated with the surface of the lipid droplet and found in cytosol, it is considered to be an exchangeable protein. When the level of fatty acid increases, perilipin 3 is transferred from the cytosol to the lipid droplet surface, which leads to the hypothesis that perilipin 3 participates in the formation of LD [172, 173].

The only structure known for perilipin 3 is the x-ray crystal structure of the C-terminus of the mouse protein; 1SZI.pdb. The structure of the C-terminus of the perilipin 3 includes 4 amphipathic α-helices that form a helix bundle, Figure 1. 10 (b), which are linked to an α/β domain through a hydrophobic cleft [169]. This structure of the C-terminus of perilipin 3 is close to the N-terminus of apolipoprotein E, which is associated with
In vivo studies have suggested that binding of perilipin 3 increases when the concentration of diacylglycerols accumulated in the ER (during biogenesis or LD expansion) increases [173]. It is also suggested that diacylglycerols would be the major partner in perilipin 3 interactions where the hydrophobic cleft present in the C-terminus plays a role in the targeting of this protein to the LD surface [87, 169]. Studies have also shown that the PAT domain is not involved in the binding of perilipin 3 to LD while the 11-mer repeats are vital for the protein interaction [166, 172, 175]. Further studies on other exchangeable proteins such as α-synuclein and apolipoprotein support the hypothesis that 11-mer repeats are involved in perilipin 3 targeting to LD surface [175].

Experiments suggest that perilipin 3 is involved in several pathological conditions e.g. it binds to proteins from pathogens of the Dengue and the Hepatitis virus and to
envelope proteins of HIV [176-178]. Interaction of perilipin 3 with the coating proteins of these pathogens facilitates the infectious life cycle. However, recent work has suggested that while perilipin 3 interacts with the matrix protein of HIV, this binding is not necessary for the completion of the viral life cycle [179]. Apolipoproteins can solubilize phospholipid vesicles, made up of short saturated acyl chains (such as DMPC and DMPG), into small disc like structures that are called nanodiscs [180-182]. Perilipin 3 also has similar ability to form nanodiscs. Bulankina et al. show that full-length perilipin 3 and the C-terminal domain can restructure lipid vesicles of DMPC into disc structures at the gel-liquid-crystalline phase transition of DMPC \textit{in vitro} [175, 180, 183].

In order to study the lipid protein interaction associated with the lipid droplets and lipoproteins, in this study we have used two techniques. Langmuir monolayer tensiometry measures the variation in surface pressure at the air/water interface. However, the structure of the LD and lipoprotein is more complicated. We built a droplet tensiometer to characterize the lipid-protein interactions through the surface tension at the oil-water interface.

1.8. Methods

Here, I briefly discuss the main techniques used in my research. Further details of these experiments can be found in chapter 2 of this dissertation.

1.8.1. Langmuir monolayer tensiometry

“Monolayer” refers to a thin film of molecules that are located and packed at an interface. This single-layer film can also be formed from atoms or cells [184]. In chemical
physics, the study of monolayers is significant because of their applications in biology, chemistry and material sciences.

A Langmuir monolayer is a single layered film formed by insoluble amphiphilic molecules on an aqueous subphase. In biology, this is a well-defined system mimicking half of the biomembrane for studying the interaction of amphiphilic molecules [185, 186]. This representation of membranes helps in investigating the membrane processes that mostly occur on the surface of the membrane. The advantage of this technique is its ability to control the concentration and density of lipids on the interface. Surface tension is the parameter used to characterize the energy variation in the system [184]. Imaging techniques such as Brewster angle microscopy (BAM), fluorescence microscopy and light scattering are used to characterize the formation and structure of the surface domains [187, 188].

1.8.2. Droplet tensiometry

For many years, the study of interfacial tension has received attention. Interfacial tension is a phenomenon at the interface between liquids caused by intermolecular (cohesive) forces. It is defined as the excess energy per unit area associated with the interface between two phases. Interfacial tension can be used to study the contact in between two fluids or a solid and fluid. Interfacial tension has been used for many years to solve problems in microscale engineering (for example, to design a virtual check valve using surface tension of vapor bubbles in microchannels) Advances in material sciences at the micro and nano-scale have led to the manufacture of new instruments. To control adhesion and wetting in those instruments, surface parameters like surface tension are significant.
Today, many instruments use interfacial tension to perform crucial tasks, e.g. pumped devices for micro and nano-fluids [189, 190], microfluid circuits for Micro Total Analysis System where drops are transferred from one area of circuit to another [191], electrowetting (e.g. a fluidic actuation method used as a reversible switch for high speed air-fluid two phase flow) [192], microelectromechanical systems (MEMS) [193], nanoelectromechanical systems (e.g. oscillators made from carbon nanotubes to oscillate droplets back and forth with electrical current) [194], microfluid based flow cytometry [195], orthopedic technology [196], and crude oil recovery [197].

These examples indicate the significance of the interfacial tension. To have a well-designed device for any of the applications mentioned above, good knowledge about the interfacial tension of the solid and/or fluid phases is necessary.

To measure the interfacial tension directly, any of the following methods can be used: the Willhemy plate method [198, 199], the drop weight method [200], the oscillating jet, the capillary wave method, the spinning drop method, and shape analysis techniques [201, 202].

We used the Wilhelmy plate method to measure the surface tension associated with Langmuir monolayers and a droplet shape analysis (ADSA) technique to measure the interfacial tension associated with oil droplets in buffer. This method allows us to use very small amounts of oil compared to a similar Langmuir technique. In this method the interfacial tension is measured by analyzing the images taken successively of an oil droplet
We used this technique to explore the protein interaction at the surface of phospholipid-buffer interface.

The shape of the droplet is determined by the balance between gravity force and surface tension; since gravity is known, shape analysis allows us to characterize the oil-buffer interfacial tension. This method was first employed by Small [204], using several proteins to study the lipid protein interaction for several apolipoproteins. ADSA (axisymmetric drop shape analysis) is the method developed by Neumann et al. [2] to implement the shape analysis. They have shown that it provides an accurate method for image analysis and droplet shape fitting, and discuss the conditions under which it is reliable. Despite the availability of commercial instruments, the home built tensiometer provides higher accuracy in the tension measurement and is easy to calibrate.

1.9. Outline

Lipid droplets are physiologically important cell organelles. However, studies are needed to better understand the physical mechanisms of their formation, growth, and stability. The first goal of my work is to use Langmuir monolayer method to study the lipid-protein interaction at the air/buffer interface. Our long-term goal is to study the interaction of perilipins at the oil/buffer interface.

In chapter 2, I discuss the Langmuir monolayer method, in addition to TLC, sample preparation and materials used for experiments. I also explain the instrumentation and the process followed to build our droplet tensiometer. Methods used to troubleshoot the system and check the accuracy of the set-up are also explained in detail.
Chapter 3 further discusses the interaction of apoLp-III at the air/ buffer interface. ApoLp-III has an amphipathic α-helix bundle domain that is similar to the C-terminus of the perilipins. This study sheds light on the interaction of the α-helix bundle domain with the phospholipid monolayer at the air/water interface. We also investigated the effect of lipid head group size and charges in this interaction.

In Chapter 4, I use perilipin 3 and different mutants of this protein to characterize the protein binding to the air/buffer interface. The major question is to identify the domain which majorly participates in the lipid-protein interaction on the LD surface. This lipid-protein interaction is characterized by changing the headgroup size and charges for all protein constructs.

In Chapter 5, I discuss how I used apoLp-III as a model protein to mimic protein interaction at the oil-phospholipid/ buffer interface. This study is supported by investigating the effect of charges on the surface of the oil droplet.

In Chapter 6, first we looked at the dynamics of the oil droplet through successive compression and decompression. This study helps to understand the behavior of the phospholipid monolayer formed on the LD surface and the protein associated with the surface when the oil drop undergoes variation in surface area (i.e. growth and shrinkage of the LD). The second part of the study focuses on the effect of lipid packing on the protein insertion to the phospholipid monolayer. Here, I used two apolipoproteins apoLp-III and apoE 3 (N-terminus), which have amphipathic α-helix bundle domains. This work
demonstrates the importance of the α-helix bundle domain in lipid protein interaction on the surface of lipid particles.

Chapter 7 suggests future studies and names potential projects for using this technique. Conclusions to and discussions of this project will be considered.

References:


CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Materials were chosen to mimic interactions between lipids and proteins at lipid droplet and lipoprotein surfaces. Lipid droplets and lipoproteins share a common structure (Figure 1.7.) The hydrophobic core is loaded with neutral lipids e.g. triglyceride and some cholesterol esters. A phospholipid monolayer surrounds the hydrophobic core and there are proteins associated with the surface. The material choice was based on 1) the information available in the literature, 2) physiological relevance and 3) to systematically vary physical/chemical properties.

2.1.1. Lipids, oils, and proteins

Here, we discuss lipids that belong to diverse chemical classes, and are present in the membrane. Other lipids like triglycerols (oil) are excluded from the bilayer, but are found in the structure of lipid droplets. In addition, it is important to consider the different parts of LD and lipoprotein binding proteins in order to study the role of unique protein domains in targeting to neutral lipid particles.
2.1.1.1. Phospholipids

One of the major roles of lipid droplets is to provide a reservoir of lipids such as fatty acids, sterols, and phospholipids for membrane synthesis [1]. PC, PE, PI and PS are the phospholipids known to be present on the surface of lipid droplets rich in perilipin [2, 3]. Such perilipin-enriched lipid droplets include longer saturated fatty acids [4]. Meanwhile, studies have also shown that several fatty acids are present in the structure of lipid droplets, including C16:1, C18:1 and C20:2 [5, 6].

The lipids used in our experiments, and their physical properties, are given in Table 2.1. In order to stay consistent with the literature, for our model system we have used POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine), POG (1-palmitoyl-2-oleoyl-sn-glycerol), POPA (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (sodium salt)) and POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol) [7-9]. Note that all of these have one saturated and one unsaturated chain of the same length. We have also used DO- lipids to investigate the effect of unsaturated chains on lipid-protein interaction [10].
PC is a cylindrically shaped zwitterionic lipid that readily forms bilayers. PC is thus an important glycerol-phospholipid that is used in biophysical experiments to mimic the lipid bilayer and to study lipid rafts. Lipid bilayers are negatively charged under physiological conditions; they are mixtures of anionic/zwitterionic lipids.

PE is the other major class of phospholipids present in the membrane. PE is a zwitterionic lipid which induces negative curvature stress in the bilayer; it mostly appears in the inner leaflet of the plasma membrane in mammalian cells, and is found in numerous other intracellular membranes [11]. An intriguing observation is that the *E. coli* membrane consists of ~75% PE. It plays a role in membrane fusion and regulates the membrane curvature [12]. PA is a precursor for the synthesis of all glycerophospholipids [13]. The physical chemistry of PA affects the surface curvature of the membrane [14]. Table 2.1

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Abbreviation</th>
<th>Charge</th>
<th>Chains</th>
<th>“Curvature”</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycerol</td>
<td>POG</td>
<td>Neutral</td>
<td>Mixed</td>
<td>Negative, Induces HII phase</td>
</tr>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
<td>POPC</td>
<td>Zwitterionic</td>
<td>Mixed</td>
<td>Infinite, forms Bilayer</td>
</tr>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine</td>
<td>POPE</td>
<td>Zwitterionic</td>
<td>Mixed</td>
<td>Negative, forms HII phase</td>
</tr>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol)</td>
<td>POPG</td>
<td>Anionic</td>
<td>Mixed</td>
<td>Infinite, forms Bilayer</td>
</tr>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (sodium salt)</td>
<td>POPA</td>
<td>Anionic</td>
<td>Mixed</td>
<td>Negative, Induces HII phase</td>
</tr>
<tr>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine</td>
<td>DOPC</td>
<td>Zwitterionic</td>
<td>Unsaturated</td>
<td>Infinite, forms Bilayer</td>
</tr>
<tr>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine</td>
<td>DOPE</td>
<td>Zwitterionic</td>
<td>Unsaturated</td>
<td>Negative, forms HII phase</td>
</tr>
<tr>
<td>1,2-dioleoyl-sn-glycero-3-phosphate (sodium salt)</td>
<td>DOPA</td>
<td>Anionic</td>
<td>Unsaturated</td>
<td>Negative, Induces HII phase</td>
</tr>
</tbody>
</table>

Table 2.1. The list of lipids used. Table includes the physicochemical properties of the lipids.
includes a list of lipids used for our study with their physical-chemical properties. More details on the lipid shape and behavior are given in the chapter where they are used. Lipids were purchased from Avanti Polar Lipids. Thin layer chromatography (TLC) was used to check the purity of the lipids each time before use.

2.1.1.2. Triolein

Triolein is a triglyceride derived from glycerol. It has three unsaturated fatty acid chains made up of oleic acid. Olive oil is made up of approximately 30% triolein. Triolein is one of the two components of Lorenzo oil [15, 16].

The lipid droplet core is composed of neutral lipid (triacylglycerol) which forms a liquid oil at room temperature, and some cholesterol esters. Since triolein is a symmetrical triglyceride, we selected that to form our oil droplet. Triolein was first purchased from Avanti. Figure 2.1 (a) shows variation in interfacial tension of a triolein droplet maintained in water for 1 hr. The interfacial tension of the oil droplet decreases approximately 18 mN/m. This decrease in tension reflects the presence of a surface active compound on the oil interface. In order to check the purity of water, I ran the same experiment. This time the oil droplet was formed in salt solution from the beginning. Figure 2.1 (b) shows the variation in interfacial tension for the oil droplet formed in salt solution following the Hofmeister solution series. Our data demonstrate that the decrease in interfacial tension is independent of salt type.
Figure 2. 1. The surface activity of a triolein droplet in contact with water and salt solutions following the Hoftmeister series. a) A triolein droplet formed and kept in water for 1 hour. The interfacial tension of the oil droplet decreases approximately 18 mN/m. b) Variation in interfacial tension for a triolein droplet formed in Hoftmeister series solutions. The data shows that the variation in tension is nearly salt independent.

Since the variation in tension was high, we ran other tests to control the purity of the oil. After running several purity tests, e.g. surface tension measurements, TLC and mass spectroscopy (done in Dr. Shelly’s lab, Chemistry Department, Kent State University), it was determined that there was some amount of impurity in the triolein purchased from Avanti.

Mass spectroscopy is an analytical chemistry technique that ionizes the chemical species and categorizes the ions by their mass to charge ratio. Mass spectrometry measures the ions which are sorted and displays them as charts (using a detector) [17]. The mass spectroscopy data taken from triolein samples provided from Avanti and Nu preptech (the company where triolein was subsequently purchased) are given in Figure 2.2. Figure 2.2 a) shows the spectroscopy data for 1 μL triolein from Avanti dissolved in a 9:1 Methanol: water mixture. The spectrum shows several high peaks close to the actual peak for triolein. Mass spectroscopy from a triolein sample purchased from NU-preptech is shown in Figure
2.2. (b). Here, the spectrum is dominated by a single high peak corresponding to the triolein molecule. If the Nu-preptech sample was kept for 3 days in the Mass spectroscopy solution, it also began to show several major peaks in mass spectroscopy. One likely contaminant of the Avanti sample is diacylglycerol, a metabolite of triacylglycerol, which can come to the oil surface due to molecular rearrangement and form a monolayer. The literature has also mentioned using triolein purified by Nu-preptech MN [18, 19].

Surface tensiometry experiments are exceptionally sensitive to the presence of any surface-active impurities (see Figure 2.1). Mass spectroscopy was performed to thoroughly investigate the composition of the samples purchased from Nu-preptech. Our results from TLC along with mass spectroscopy determined that the triolein purchased from Nu-preptech is of >99% purity.
In addition to triolein, there are other triglyceride molecules that are of great importance to study. Those molecules differ in the length of the acyl chain and the number of double bonds present. Due to limitations in time we focused here on triolein.

2.1.1.3. Proteins

Since there is a similarity in the structure of lipoprotein particles and intracellular lipid droplets, I used two apolipoproteins (proteins associated with the surface of lipoprotein particles) in addition to the LD protein perilipin 3 to study the biophysics of lipid-protein interactions at neutral lipid-water interfaces. All the proteins of our choice are exchangeable (they can commute from one particle to the other) and they have amphipathic α-helical bundle domains.

Figure 2. 3D structures of the proteins used in our experiment. The structures were drawn by the pdb file provide by protein data bank source, and plugging into VMD software. a) NMR structure of Locusta Migratoria apoLp-III, which is a fully amphipathic α-helical bundle protein. apoLp-III is from apolipophorin family associated with the lipophorin particles in insects, 1ls4.pdb. b) The crystal structure of the c-terminal structure of mouse perilipin 3, 1szl.pdb. Perilipin protein is present on the surface of lipid droplets. c) Crystal structure of the N-terminus of apoE 3, 1nfn.pdb.
Figure 2.3 shows the 3D structure of the protein constructs we used in our experiment. ApoLp-III, Figure 2.3. (a), is a fully amphipathic α-helix bundle protein while perilipin 3 and apoE 3 each have such a bundle and an additional amphipathic domain. We used the mouse model reported by Hickenbottom to draw the crystal structure of the c-terminus of perilipin 3, Figure 2.3. (b) [20]. However, studies have shown similarities in structure for perilipin 3 present in human LD and the mouse model. Faustino et al. also showed that the C-terminus of perilipin 3 shares similarity in structure with the N-terminus of apoE 3 [21, 22]. ApoE 3 (NT) is the other protein construct we used to study the lipid-protein interaction. Since the N-terminus of apoE 3 has a 4 helix bundle which is similar to the C-terminus of perilipin 3, we become interested to use it as a model protein. Other parts of the protein also have the capacity to form amphipathic α-helices, but here our focus is on the N-terminus of the apoE 3 protein. The crystal structure of apoE 3 (NT) is shown in Figure 2.3. (c).

ApoLp-III was a kind gift from Dr. Paul Weers from the University of California Long Beach. The protein was provided in powder form. The protein was dissolved in HPLC grade water and the concentration was set to 1 mg/mL. Perilipin 3 and its mutants were purified in Dr. Kooijman’s lab. Further information on the purification and the procedures is provided in reference [23]. ApoE 3 NT was a precious gift from Dr. Narayanaswami also from the University of California Long Beach. The protein was in powdered form and I followed the protocol provided by their lab to dialyze the protein (as discussed in Chapter 6).
2.1.2. Buffer preparation

A Tris buffer was prepared according to the protocols available in the lab [10, 23]. Tris is a common biological buffer used in labs. Tris helps to maintain the pH of the system, when it is critical to have a stabilized pH for biological experiments. It keeps the pH between 7 to 9 (since its pKa is around 8.1). However, tris buffer is temperature sensitive; it should be preserved at the temperature where its pH is first set to avoid any inaccuracy. All salts were HPLC grade obtained from VWR. Surface tensiometry experiments are sensitive to any contamination present in the buffer. In some cases, it is necessary to anneal salts for further purification (see Chapter 5). Buffer was made using Fischer HPLC purity water (Fischer W5SK). The pH was set to 7.22 ±0.05 to mimic the physiological environment. All glassware was cleaned using KOH solution (24 g KOH, 25 g water, and 164 g of Ethanol/Reagent alcohol).

2.1.3. Thin layer chromatography (TLC): testing for purity

TLC analyzes mixtures by separating the compounds in the sample mixture. It is also used to identify the compounds and testify their purity. TLC also can be used as a scale to monitor the progress of a chemical reaction. Like all other chromatography system, TLC is based on two phases, a mobile phase and stationary phase, which affect the rate at which the compound migrates. The mobile phase runs through the stationary phase and carries the compound of the mixture with it. The goal of TLC is to produce well-defined and well-separated spots.
Thin layer chromatography is performed, as the name suggests, using a thin and uniform layer of gel silica coated on a piece of glass or rigid plastic. Here the silica gel is the stationary phase for the TLC, and the mobile phase is the proper liquid solvent or mixture of solvents.

TLC consists of three steps: spotting, development, and visualization. It is preferable to treat the TLC plate before spotting the sample. The plate should be preheated in a vacuum oven for 1 Hr at 100 °C to remove any organic contamination on the plate. While the plate is heating in the oven, the solvent should be made and left for approximately 30 min. This helps the solvent become homogeneous and uniform. After cooling under nitrogen, the plate is placed about 5 mm into the solvent. This step lets the solvent migrate along the TLC plate, which facilitates the development step. When the whole plate is soaked with the solvent, it is removed from the pool of solvent and dried under nitrogen.

**Spotting**: Using a micro-pipet, a small amount of the sample is spotted on one end of the plate. The spotting solvent is then evaporated by flowing nitrogen over the plate, leaving of the material on the plate.

**Development**: The plate is placed in the shallow pool of the solvent, which travels upward by capillary action. While the solvent moves up in the TLC plate, it carries the sample as well. If the solvent is polar enough, then the sample will move from the original place for some distance. Different compounds with different polarities and acyl chains will show different behavior travelling along the TLC plate. When the solvent has traveled
enough, about 1 cm away from the other end of the TLC plate, the plate should be removed from the solvent and dried under nitrogen.

![TLC plate image](image)

**Figure 2.4.** An image from TLC done for Triolein stock provided by Avanti. In order to do a control on the purity of the Triolein, a diacylglycerol spot was also spotted on the TLC plate. After development and visualization of the TLC plate, one can see that for 1μL of sample, there is no trace of DOG left on the line.

**Visualization:** To track the motion of the sample (i.e. detect the compounds), the plate has to be dyed. In our lab, we primarily use iodine to mark the sample spot, since iodine has the highest affinity for both unsaturated and aromatic compounds. However, there are other ways to mark the spot e.g. spraying sulfuric acid.

The TLC method was used to check the purity of our samples. For polar lipids, the solvent was a mixture of chloroform (65 ml), methanol (25 ml), and water (4 ml). There are two protocols for testing the neutral lipids. In the first one, which has been applied for years in our lab, the solvent consists of acetic acid (3 ml), hexane (40 ml), diethyl ether (20 ml), and methanol (2 ml).
The new protocol was obtained from Nu-preptech Company, which is our major provider for Triolein. The protocol suggests a 9:1 ratio of hexane and diethyl ether as the solvent to run the plate spotted with triolein. Figure 2.4 shows thin layer chromatography (TLC) of different concentration of triolein samples. In order to check for the purity of the triolein, I also included a sample of diacylglycerol, since it is a metabolite of triolein. My TLC plate demonstrates that DOG does not run in this solvent, so that if there is any DOG impurity present in the triolein sample, we expect that it does not travel along the TLC plate. For higher concentrations of the triolein, the sample has moved along the plate, leaving a visible trace exactly where we expect the spot for DOG. We thus believe our sample from Avanti contained diacylglycerol, which indicates that purifying the triolein at higher degree is necessary.

2.1.4. Vesicle preparations:

In biology, vesicles are spherical particles that have at least one layer of bilayer which encloses fluid (Figure 2.5). In physiological conditions, vesicles are formed inside cytoplasm. If the vesicles are prepared artificially, they are called liposomes. If the vesicles involve only one phospholipid bilayer, they are called unilamellar vesicles (Figure. 2.5); otherwise they are multilamellar [24].
The composition of the lipid bilayer (anionic, cationic, and neutral lipid species) can vary depending on the experimental goal. Variation of vesicle lipid composition may affect the vesicle curvature.

When the lipid film is hydrated, and stacked into liquid crystalline bilayers, large multilamellar vesicles (MLVs) are formed. MLVs inhibit water molecules from interacting with the hydrocarbon core of the bilayer. In order to break the MLVs and form SUVs, the size of the particle should be reduced by applying energy, through sonication (sonic energy) or extrusion (mechanical energy).

2.1.4.1. Water bath sonicator

In this technique, the MLVs sample is hung in an Avanti lipid cylindrical sonicator for ~40 min. During this process, the sonic energy helps break the MLVs and form the SUVs. The water bath should always be refilled with fresh water, as the water becomes degassed during the sonication process and starts heating up. It is important to keep the

Figure 2.5. Cartoon of a lipid vesicle. The structure has a bilayer, where the polar headgroups are facing the water or buffer used to form the vesicles and the hydrocarbon chains face towards each other.
temperature of the water bath below the lipid melting temperature (transition temperature).

When the temperature increases, the lipids might break down [25]. When the sonication is over, the lipid suspension changes from a milky color to clear. This is due to the breakdown of larger vesicles and MLVs to SUVs. The size of the vesicles made by this method ranges from 30-50 nm. The size was checked via dynamic light scattering using the Horiba DLS 7100 instrument.

2.1.4.2. Using extruder

![Extruder diagram]

*Figure 2.6. Shows different part of the extruder. Image taken from https://avantilipids.com/divisions/equipment.*

Extrusion is the other technique commonly used for making vesicles [26]. Here, the lipid suspensions are pressed through a polycarbonate membrane with a defined pore size by applying force.

The Avanti syringe-based membrane extruder is shown in Figure 2.6 and is easy to use. When the instrument is assembled, 1 ml of the sample is running through the membrane using a syringe. Press the syringe loaded with lipid mixture to pass the whole lipid mixture through filters to the other syringe opening on the other side. This process
needs to be repeated 11 passes. Always stick with odd number since you do not want to contaminate the final product with the multilamellar vesicles left on the original side. The Horiba DLS 7100 was again used to measure the particles sizes.

2.1.4.3. Tip sonicator

Tip sonication is the other method that uses sonic energy to break MLVs. This technique is mostly used to make SUVs. The probe tip sonicator puts high energy into the lipid suspension; this can make the lipid sample overheat and degrade. To limit overheating, we apply sonication in 10 s periods, with 10 s intervals in between each step, 12 times. Lipid suspension should be kept on a bucket of ice to avoid overheating. The probe is made of titanium. During sonication, a very small amount of titanium detaches from the end of the probe and floats in the sample. After the sonication, the sample should be centrifuged for 30 min to pellet down the nanoparticles.

2.1.4.4. DLS

Differential light scattering, by means of a Horiba SZ-100 instrument, is the technique used for measuring the size of SUVs. In this method, the size of the particles suspended in the solution can be measured by using monochromatic laser light. In order to have an exact measurement, it is preferable to run the sample through a filter first, to remove any dust and large particles [27].

When the laser light passes through the sample, it hits the particles and is scattered in all directions (Rayleigh scattering), as long as the size of the particle is smaller than the
light wavelength [28]. Scattered light from all the particles can form constructive or destructive patterns. The scattered intensity fluctuates over time, which is due to Brownian motion of the particles. The particles’ dynamic information is derived by an autocorrelation function of the intensity trace:

\[ g^2(q; \tau) = \frac{\langle I(t)I(t+\tau) \rangle}{\langle I(t) \rangle^2} \]  

(2.9)

Here, \( g^2(q; \tau) \) is the autocorrelation function for the specific \( q \) wave vector, \( \tau \) is the time delay and \( I \) is the light intensity. Delay time refers to the time delay over which the correlation is found. For short delay times, the particles do not have the chance to move far from the initial state, which yields high correlation: the autocorrelation function is nearly unity. An increase in delay time reduces the correlation exponentially, until there is no correlation between the scattered intensity of the initial and final state. The fusion of particles also affects the exponential decay. The decay is later fit by using numerical methods based on the assumed distribution [27].

If the sample is monodispersed (all the particles in the sample are in a same size range), then the decay is a simple exponential:

\[ g^1(q; \tau) = \exp(-\Gamma \tau) \]  

(2.10)

\( \Gamma \) denotes the decay rate in the equation above; \( \Gamma = q^2D \) at a specific wave vector. Here, \( D \) is the translational diffusion coefficient (defined by the equation \( \langle X^2 \rangle = 2Dt \)) and \( q = \frac{4\pi n_0}{\lambda} \sin(\frac{\theta}{2}) \). \( n_0 \) is the refractive index of the sample, \( \theta \) is the angle of the detector, and \( \lambda \) is the laser wavelength. Finally, a plot of \( (\Gamma/q^2) \) vs. \( q^2 \) is made [29]. If the particles are
small spheres, then the plot is not angularly dependent, meaning there is no anisotropy. If the particles have shapes other than spheres, then the plot depends on the angle of detection. The intercept of the plot is $D$. For a high-quality analysis, it is preferable to do DLS at different angles, especially when size measurements are performed on polydispersed samples. DLS gives the particle size or more accurately the hydrodynamic radius (where the diameter of the particle $d$ is given by translational diffusion coefficient $D$, $d = \frac{K T}{3 \pi \eta D}$ where $\eta$ is the fluid viscosity) [27].

Lipid-protein interactions address how the lipid influences the membrane protein and vice versa. There are 4 major questions that researchers have been trying to answer about the dynamics of the protein-lipid interaction: 1) How strongly do the proteins bind to lipids [30, 31]? 2) What is the influence of the protein on the dynamics of the lipid membrane and does it cause defects in the membrane [32]? 3) Can the lipids change the protein configuration [33-35]? 4) What are the major types of interaction between the membrane proteins and lipids [36].

Lipid-protein interaction also occurs on the surface of LDs and lipoproteins. Here, we are interested to understand the dynamics of lipid-protein interaction and investigate the parameters that affect this interaction. Surface tension is a parameter that has been used for years to study the interaction between molecules at the interface. We are using this physical property to investigate the interaction between lipid and protein molecules.
### 2.2. Surface tension

The cohesive forces existing between like molecules on a fluid surface lead to surface tension. Surface tension is the surface energy per unit area. It is the excess energy associated with the surface. Figure 2.7 sketches the cohesive forces present between the molecules. The molecules inside a fluid, experience cohesive forces from all neighboring molecules. Molecules at the surface do not have neighboring molecules from the same type on one side (molecule 1 in Figure 2.7), which exposes those molecules to a stronger intermolecular cohesive force compared to their neighbors further from the surface (while molecule 2 experiences forces in all directions). Interfacial tension is sometimes called surface tension when one of the phases is a gas.

Higher values of surface tension correspond to states with stronger interactive forces among molecules, while lower values reflect that the molecules do not strongly interact. When two bulk phases are brought together, the surface molecules diffuse into the other fluid until the system reaches an equilibrium state. In this equilibrium state, the mean atomic separation decreases and the intermolecular force increases relatively. This phenomenon is similar to the force present on the surface of the membrane where there are various types of molecules present.
Interfacial tension varies with temperature; it decreases when temperature increases. Interfacial tension is measured as mN/m, which is the force applied to stretch a surface in one direction by a length of 1 m. Due to hydrogen bonding between the water molecules, water has one of the highest fluid surface tensions. Pure hydrocarbon molecules like hexane have lower surface energy. Fluorinated molecules have very small surface tension, as fluorine does not share electrons well. One can conclude that the contamination of water alters the interfacial tension.

2.3. Langmuir Monolayers

Here I will introduce the techniques used to measure interfacial tension at the air-water interface and at the interface of two immiscible liquids. I will start with an introduction to the concept of interfacial tension.

2.3.1. Introduction

Humans have long made use of the effect of oil on the water surface. Babylonians were the first group who recorded this effect while practicing divination, in 18th century BC [37]. A Japanese painting technique (black ink jet flow) was one of the early applications of a floating monolayer [38]. The first known scientific manuscript on surface chemistry was written by Benjamin Franklin; among other things, he studied the calming effect of one teaspoon of oil on the surface of water. He showed that right after dropping the oil on the surface of water, it spread out and formed a thin layer [39].
Rayleigh, Pockels, Devaux and Hardy further defined the nature of these monomolecular films [40, 41]. Langmuir showed that the area occupied by molecules such as acids, alcohols, and esters is independent of the hydrocarbon chain length, by studying the variation of surface pressure vs. molecular area [42, 43].

2.3.2. Experimental set-up

In order to determine the interfacial tension of a lipid film at the air/buffer interface, we used the Langmuir monolayer method. The set-up includes a surface pressure sensor (Nima PS4; Figure 2.8 b) with a Wilhelmy plate attached to it. The surface pressure sensor is a force sensor, from which the Wilhelmy plate can be suspended. The pressure sensor is connected to the computer through an interface.

Through the Wilhelmy plate we can measure the interfacial tension. However, we are more interested in how this tension changes in the presence of lipids and proteins, through the surface pressure. At the beginning of each experiment, we set the baseline and calibrate the sensor to measure 0 interfacial tension when the plate is in contact with buffer. Then, in the presence of surface-active reagents at the interface, the instrument measures it as the surface pressure ($\pi = \gamma_{\text{buffer}} - \gamma_{\text{reagent}}$).

There are two different types of Wilhelmy plates we can use in this set-up: paper plates and platinum plates. Here, I used a platinum plate (Figure 2.8 a) as it can be completely cleaned after each experiment, and it is more stable on the surface since it is heavier.
Usually, the troughs used for Langmuir monolayer experiments are fabricated from polytetrafluoroethylene (PTFE), known as Teflon. This polymer is the most known hydrophobic material and has the least coefficient of friction, approximately 0.04 (zero friction coefficient is only possible theoretically). It is less likely to leach contaminants into the solvent in the trough, and there are few materials that stick to it; most compounds can be easily removed from the surface. Teflon has high resistance towards many chemical agents and solvents. It is also stable over a wide range of temperatures (-73 °C to 200 °C). Figure 2.8 (b) shows the Nima pressure sensor. The Wilhelmy plate is connected to the sensor through a hanging hinge.

For our study of lipid-protein interaction, we have designed our own trough based on that in Demel et al., Figure 2. 9 [44]. In part (a), an arrow marks the shallow depression in the bottom of the trough where the stirring magnet is placed. The magnet helps to make the subphase homogeneous during the experiment by gently mixing the aqueous solvent.
Figure 2.9(b) indicates the hole on the side of the trough that helps protein insertion without disturbing the interface.

2.3.3. Surface pressure measurements

In the Nima system, the surface pressure is measured with a Wilhelmy plate. When the plate is suspended at the air-water interface, it is pulled down to the sub-phase by the water surface tension. The force acting on the plate is the sum of three forces: the gravity and surface tension both acting downward, and the buoyancy force on the plate due to displaced water, which acts upward. This can be shown by drawing the force diagram, Figure 2.10. a. For a Wilhemy plate with $l$, $w$, and $t$ (length, width, and thickness) dimensions and $\rho$ density, immersed in the water at depth of $h$, the net force is expressed as the following equation:

$$F_{\text{net}} = \left(\frac{\rho_l w t \cdot g}{\text{weight}}\right) - \left(\frac{\rho_l h w t \cdot g}{\text{buoyancy}}\right) + \frac{2(w + t) \cdot (\gamma) \cos \theta}{\text{surface tension}}$$  (2.1)
where $\gamma$ is the surface tension, $\rho_l$ the water density, $\rho_p$ the plate density, and $\theta$ is the contact angle in between liquid and plate. At the beginning of each experiment, the pressure reading is set to zero, which cancels out the first two terms, as long as the plate is always kept at the same height during the experiment, Figure 2.10 b. If the plate is thoroughly wetted with the water, then $\theta = 0$, and $\cos \theta = 1$, which simplifies the equation above to

$$F_{net} = 2(w + t)(\gamma)$$  \hspace{1cm} (2.2)

2.3.4. Sample preparation

Prior to each experiment the lipid sample is made according to the protocol available in lab. However, we always make an initial stock that has higher concentration. To dissolve the lipid: the goal for this procedure is to suspend lipid in a solvent for further analysis. To dissolve neutral lipids, non-polar solvents, such as diethyl ether or chloroform, must be used. However, for membrane-associated lipids, which are polar, polar solvents...
like ethanol and methanol are sometimes (depending on the chemistry of the membrane lipid) better because they disrupt the formation of hydrogen bonding and electrostatic forces between the lipids. All membrane lipids that I used in my work dissolve well in chloroform and chloroform/methanol mixtures. Because experiments on surface variations are sensitive to any contamination in the samples, it is important to use the purest possible solvent: we consistently used HPLC-grade solvents for dissolving lipids.

First, a higher concentration stock (mother stock) is made by dissolving lipid in chloroform. To inhibit any lipid oxidization, we used nitrogen to cover the lipid solution. For the daughter solution to form the lipid monolayer, we diluted the mother stock with a 2:1 mixture of chloroform and methanol. The final concentration for the lipid solution was 0.1 mM.

2.4. Droplet tensiometer

While the Langmuir monolayer set-up is in principle capable of measuring the interfacial tension of two immiscible fluids, in practice, this is not straightforward. Hence, an attractive method, liquid droplet tensiometry, is used instead. This method uses either pendant or sessile drops and one of several data analysis procedures.

Droplet tensiometry is a technique that measures the interfacial tension of a pendent or inverted pendent fluid droplet. In this method, the interfacial tension is measured by analyzing the shape of the droplet. Small fluid droplets are spherical. Large droplets would be deformed due to the action of gravity while their interfacial tension tends to make the
droplets more spherical. The equilibrium droplet shape is determined by the balance between the gravitational force and the interfacial tension.

Here, we built our own tensiometer per the design suggested by Dr. Tavana at the University of Akron [45] and calibrated the system for precise measurement. We used the software developed by Neumann [46] group to measure the interfacial tension by using drop shape analysis. We had help with the calibration from Dr. J. Mann, a collaborator of all of our groups (Dr. Elizabeth K. Mann, Dr. Edgar E. Kooijman, Dr. Hossein Tavana, and Dr. Wilhelm Neumann).

2.4.1. Instrumentation

Figure 2. 11. Schematic view of the set-up. It includes a light source, diffuser, J-shaped needle and CCD camera with mounted lenses.

Figure 2. 11 shows a schematic setup for droplet tensiometry. To have uniform background light, a combination of light source (Thorlabs OSL2) and frosted diffuser are used. The droplet is formed on the tip of a J-shaped needle connected to Hamilton syringe (250 μl Model. 725 RN SYR). The needle is shaped in the machine shop of the physics
department. A combination of high-resolution CCD camera and microscope lenses is connected through a USB port to a computer to transfer images. Labview version 14 is used to control the camera and store captured images.

The whole set-up is placed on a TMC vibration isolation table (AMETEC 78-23761-01) which isolates the system from environmental vibrations in the building. An enclosure surrounding the set-up helps to prevent dust from entering the sample cells, as the interfacial tension measurements are so sensitive to any contamination. (The enclosure is made from polycarbonate sheets in the physics department machine shop). All experimental instruments are regularly (prior to each experiment) cleaned using 100% HPLC methanol.

2.4.2. Interfacial tension measurements

Figure 2. 12. Schematic view of the droplet where radii $R_1$ and $R_2$ are marked in the image. $R_1$, turns in the plan of paper and $R_2$, rotates in the plane perpendicular to the plane of paper. $R_0$ is the curvature at the origin (curvature at apex). $\varphi$ is the angle that $R_1$ makes with the Z axis. $S$ is the arc length measured from the origin.
Axisymmetric drop shape analysis (ADSA) is a code developed by Neumann group (at the University of Toronto) to analyze images and calculate interfacial tension, droplet volume, surface area, and curvature at the droplet apex (see Figure 2.12). Assuming that the experimental profile of the droplet is axisymmetric and Laplacian, ADSA calculates the best matching profile numerically calculated from the real droplet image. Rotenberg first developed ADSA [47] and Cheng later optimized the software [48].

The balance between the gravitational force and surface tension determines the shape of the droplet. Interfacial tension minimizes the surface area of the droplet and makes it more spherical. The surface energy of the droplet is proportional to the droplet area ($\propto R^2$, where R is the droplet radius.) The gravitational energy of the droplet is proportional to the volume of the droplet ($\propto R^3$) and the difference in densities between the two fluids ($\Delta \rho = \rho_{int} - \rho_{ext}$). If $\Delta \rho > 0$, a pendant droplet is elongated, while with $\Delta \rho < 0$ an inverted pendant droplet is elongated. I used inverted pendant droplets for running the lipid droplet tensiometry experiments since oil is less dense than the water/buffer (used as subphase). The Laplace equation of capillarity governs the local mechanical equilibrium between two homogenous fluids which are separated by interfacial tension [49]. This equation relates the pressure difference $\Delta P$ across the two fluids` interface with the interfacial tension $\gamma$ and the interface curvature:

$$P_2 - P_1 = \Delta P = \gamma \left( \frac{1}{R_1} + \frac{1}{R_2} \right) \tag{2.3}$$

where $R_1$ and $R_2$ are the radii of the curvature. If the gravity is the only force applied on the droplet, $\Delta P$ can be written as:
\[ \Delta P = \Delta P_0 + (\Delta \rho) g Z \]  \hspace{1cm} (2.4)

where \( \Delta P_0 \) is the pressure difference at arbitrary reference plane. \( \Delta \rho \) is the density difference between the two fluid phase, \( g \) is the local gravitation acceleration and \( Z \) is the height on the interface above the reference plane. If the center of the coordinate system is relocated to the apex point as shown in Figure 2.12, equations 2.3 and 2.4 become

\[ \gamma \left( \frac{1}{R_1} + \frac{\sin \varphi}{x} \right) = 2 \frac{\gamma}{R_0} + (\Delta \rho) g z \]  \hspace{1cm} (2.5)

\( R_1 \) is the radius which turns in the plane of the paper and \( R_2 = \frac{x}{\sin \varphi} \) rotates in the plane perpendicular to the paper (it spins about the axis of symmetry). \( R_0 \) is the radius of curvature at the apex where \( R_0 = R_1 = R_2 \). \( \varphi \) is the turning angle measured in between the arbitrary point \((x_i, z_i)\) on the horizontal plane and the tangent to the interface, which represents the contact angle at the contact point between the droplet profile and the solid surface.

Since the droplet is axisymmetric, a meridian section is used to describe the interface. If the interface is given by

\[ u = u(x, z) \]  \hspace{1cm} (2.6)

the meridian curve would be expressed by the functions below:

\[ x = x(s) \quad \text{and} \quad z = z(s) \]  \hspace{1cm} (2.7)
where $s$ is the parameter representing the arch length from the center of coordinate. After plugging (2.7) in (2.5), the Laplace equation is parameterized and provides three equations with three boundary conditions:

$$\frac{d\varphi}{ds} = \frac{2}{R_0} + \frac{(\Delta \rho)g}{\gamma} Z - \frac{\sin \varphi}{x} \quad (2.8a)$$

Notice that equation 2.8a depends on two parameters, $R_0$ and $\frac{(\Delta \rho)g}{\gamma}$, while $x, z$ and $\varphi$ are the geometrical variables. The boundary conditions are:

$$\frac{dx}{ds} = \cos \varphi \quad (2.8b)$$

$$\frac{dz}{ds} = \sin \varphi \quad (2.8c)$$

$$x(0) = z(0) = \varphi(0) \quad (2.8d)$$

In order to start the image analysis, the operator should provide the software with the vertical coordinates of the droplet edges (the two points connecting the droplet to the needle) in pixels. Canny, SUSAN, and LoG are the choices of edge detectors one can use to find the droplet shape profile. I used Canny, since it traces the edge of the droplet well. These are pre-defined functions in MATLAB. Once the droplet profile is identified, ADSA randomly chooses 20 points along the edge of the droplet profile. A series of equations is solved and $\frac{(\Delta \rho)g}{\gamma}$ is calculated, which gives the interfacial tension, since values for $\Delta \rho$ and $g$ are provided to the program. This process is repeated 10 times, and the output for the interfacial tension is calculated as an average over all values. This process is well-explained in the references by Cheng et al. [50, 51].
A Ronchi ruling, Figure 2.13 (a), or other reference calibrates and provides the software with the actual size of each pixel. The software uses the pixel size to calculate the area and volume of the droplet. To check the optical distortion caused by the microscope, an image of the ruler was taken prior to the experiment. That image is also used to find the size of each pixel in centimeters. In order to calculate the pixel size, a certain number of lines are selected from the Ranchi ruler image. The plot profile function within ImageJ helps counting the number of lines, Figure 2.13 (b) [52].

2.4.3. Technical troubleshooting

**Camera:** Since imaging is the major source of all data for these experiments, it is critical to have a sharp image from the droplet. In the first step of the image analysis, the software tries to fit the droplet image with a line representing the droplet edge using Canny or other defined procedures from MATLAB as discussed above. Since this profile is used later to pair the theoretical fit with the experimental profile, it is important to have a sharp image from the droplet. The choice of camera is important as it is required to detect the
border of the droplet clearly. Initially, we used camera purchased from JenOptics to capture images for further analysis of the oil droplet. However, images taken through JenOptics camera were noisy and the ADSA code could not recognize the profile of the oil droplet properly. Since the profile resolution is very important for image analysis, we purchased another camera from PixeLink which is more light sensitive. The graph below demonstrates the differences between the two cameras in defining the droplet border. Images were taken from the same drops. Figure 2.14 (a) is an image taken by a PixeLink (PL-B776U) camera. Here the image is sharp and the droplet border are marked exactly at the profile of the droplet. Figure 2.14 (b) is an image captured by the JenOptics (D-07739 Jena) camera. The software can fit the droplet profile. However, since the droplet edge is not perfectly distinguishable for the software, it averages over the neighboring pixels on the droplet profile, resulting in the noisy profile shown in Figure 2.14 (c).

Using ImageJ, we looked at the grey values captured by the two cameras across the droplet and the background, Figure 2. 14 (c). The profile of the PixeLink camera had a larger width (~ 14 microns, is the size for 3 pixels); The PixeLink camera is more light sensitive and works better with reduced aperture. The smaller aperture leads to an increase depth of field and less background light. Note that the grey-level vs. position curve is much less noisy than with the JenOptics camera. Finally, we decided to use PixeLink camera, since it is more light-sensitive and the images are sharper.
**Platform:** The Neumann group always uses a platform to form the droplet [53, 54], Figure 2.15 (a). The platform is then loaded on the tip of the J-shaped needle. The platform helps with the formation of sessile droplet and the calibration. However, I had trouble with formation of the droplet on the platform, since the oil droplet did not spread all over the platform surface. In principle, we can treat the platform surface, so it becomes hydrophobic and oleophilic. I was unable to perform this optimization, due to time limitation. However, I was able to use the J-shaped needle in most cases for further experiments.

Another problem I encountered with the platform was the appearance of air droplets during droplet formation. This would reduce the accuracy for the volume measurement of the droplet. Also, the interfacial tension calculated from the drop is not a valid number, due to the inhomogeneity of the oil mixed with air bubbles, Figure 2. 15 b. Also, it was difficult
to make the platform horizontal, which is critical for the software to calculate the interfacial tension. More than 10 pixels difference between the two edges makes the droplet insufficiently symmetric as assumed by the theoretical fit.

2.5. Effect of surface ions on interfacial tension: testing the reproducibility of droplet tensiometer

In order to check the accuracy of our set-up, I ran series of experiments to study the effect of surface ions on the interfacial tension of triolein. Following the study by Ghimire et al., we consider different ions following the Hofmeister series of anions, to study the effect of water structure on the fusion of two oil droplets. Different anions in the Hofmeister series influence water structure differently and Ghimire et al. described a dependence of fusion on the type of anions. This study is important as it shed light on the formation and maturation of LDs.

LDs mature in cell through processes like fusion, but little is known about this process. We are interested to deal with the physiological media of LDs, where there are a lot of ions, which are conveniently ordered according to the Hofmeister series:
$SO_4^{2-} > HPO_4^{2-} > Cl^- > NO_3^- > I^-$

The Hofmeister effect was first studied by Franz Hofmeister, who looked at the effect of cations and anions on protein solubility [55]. Research has shown that anions have a larger effect compared to cations [56].

Moving from the right side of the series to the left, the solubility of non-polar molecules decreases (salt-out). Two mechanisms guide the Hofmeister ions interaction. The first one is the interaction of the ions and protein, and the second one is the interactions of ions with the water molecules associated with the proteins.

Ghimire et al. examined the collision of lipid droplets and showed that the docking rate (the step at which the droplets are close to each other, but not fused yet) is affected by the type of anion according to the Hofmeister series given above. These results revealed the importance of the interfacial water shedding during this process [57].

*Figure 2. 16. a) Interfacial tension measurements for the triolein droplet in Hofmeister series solution. b) measurement for interfacial tension of triolein droplet in Hofmeister solutions series made by preheated salts. The final concentration of these salts solutions is set to 100 mM.*
Here, we studied the effect of ions on the interfacial tension. These experiments also provided a first test for our interfacial tension droplet technique and checked the reproducibility of the interfacial tension measurement. All sodium salts were bought from VWR (NJ). Fisher water (HPLC grade) was used for making the solutions. Salt solutions were made according to a previously published protocol and had a concentration of 100 mM [57]. The first trial measured the interfacial tension of the triolein droplet in a buffer composed of Na$_2$HPO$_4$ + NaH$_2$PO$_4$ at a concentration of 100 mM.

These data points are the average values over 3 sets of independent experiments (two measurements were done for each set), Figure 2.16. Error bars represent the standard deviation over all measured values for interfacial tension, typically 6 totals. Our data showed that the presence of the ion increases the interfacial tension ~ 2 mN/m compared to pure water. Other solutions were mixtures of phosphate buffer (to hold the pH constant) and Hofmeister series sodium ions with a ratio of 9:1. Measurements were done to find the interfacial tension of triolein droplets in the Hofmeister series solutions. Our data showed that salt type does not affect the interfacial tension significantly. The same set of experiments were run by preheating the salt in the oven at 100 °C for 12 Hr. Annealing the salts reduces the error bars, where it become more reproducible on different days. The data show that it is necessary to anneal the salt, since surface-related experiments are sensitive to any contamination or impurity.

We can use colligative properties to further discuss the data from Figure 2.16. The colligative theory suggests that the property of the solution depends on the number of particles of solute and solvent and not the nature of the chemical species. Na$_2$SO$_4$, when it
is in solution releases 2 cations (2Na\(^+\)) and one anion (SO\(_4^{2-}\)). In the case of Na\(_2\)HPO\(_4\) it releases one H\(^+\), two Na\(^+\) and one PO\(_4^{3-}\). So, theoretically, the solution that releases higher number of ions should have a higher interfacial tension. However, the rest of the salt solutions such as NaCl, NaI and NaNO\(_3\) release the same number of anions and cations. So, for these solutions we expect the same interfacial tension but somewhat higher than pure water. We indeed observe the highest interfacial tension for the sodium phosphate solution but the differences in interfacial tension for the other solutions is not that significantly different from each other.

References:


[47] Y. Rotenberg, Determination of the Shape of Non-Axisymmetric Drops and the Calculation of the Surface Tension, Contact Angle, Surface Area and Volume of Axisymmetric Drops, University of Toronto, Toronto, 1983.


CHAPTER 3

INSERTION OF APOLP-III INTO A LIPID MONOLAYER IS MORE FAVORABLE FOR SATURATED, MORE ORDERED, ACYL-CHAINS

3.1. Abstract

Neutral lipid transport in mammals is a complicated procedure, which involves different types of apolipoproteins. The exchangeable apolipoproteins mediate the transfer of hydrophobic lipids between tissues and particles, and bind to cell surface receptors. Amphipathic α-helices form a common structural motif that facilitates their lipid binding and exchangeability. ApoLp-III is the only exchangeable protein found in insects which can be used as a model amphipathic α-helix bundle protein. The three-dimensional structure and function of apoLp-III protein mimics that of the mammalian proteins apoE, and apoAI present on the surface of lipoprotein particles. Even the intracellular exchangeable lipid droplet protein TIP47/perilipin 3 associated with the surface of LDs contains an α-helix bundle domain with high structural similarity to that of apoE and apoLp-III. Here, we investigated the interaction of apoLp-III from Locusta migratoria with lipid monolayers. ApoLp-III present in lipophorin particles, which supplies the flight muscles with energy during flight.

1 This chapter is submitted as an article in BBA
Consistent with earlier work we find that insertion of apoLp-III into fluid lipid monolayers is the highest for diacylglycerol. We observe a preference for saturated and more highly ordered lipids suggesting a new mode of interaction for amphipathic α-helix bundles. These results thus shed important new insight into the protein-lipid interactions of a model exchangeable apolipoprotein with significant implications for its mammalian counterparts.

3.2. Introduction

Neutral lipids (e.g. triacylglycerols and cholesterolesters) need to be packaged in order to exist in stable form in solution. In the blood stream as well as inside cells this packaging is mediated by a phospholipid-protein monolayer. The protein component consists of both intrinsically associated and exchangeable proteins. Protein-lipid interactions for exchangeable proteins are critical for their reversible interaction with the neutral lipid particle (lipoprotein in the case of transport) [2]. Lipoprotein particles in both mammals and insects have similar structures and functions, including intrinsically associated proteins (so-called non-exchangeable apolipoproteins) that provide structural integrity. Mammalian lipid transport is complex and involves distinct types of particles and many different kinds of apolipoproteins. Compared with mammalian lipid transport, insect hemolymph carries neutral lipids in a single type of lipoprotein called lipophorin (HDLp). The non-exchangeable apolipoproteins, apolipophorin-I (apoLp-I) and apolipophorin-II (apoLp-II), (homologues of mammalian apoB100) form an integral part of the structure of lipophorin. Aside from apoLp-I and apoLp-II some insects also have a single exchangeable
apolipoprotein, apolipophorin-III (apoLp-III). This low molecular weight (18-20 kDa) exchangeable apolipoprotein associates with lipophorins in the hemolymph facilitating the delivery of neutral lipids into the insect flight muscle during flight (for recent reviews: [3-5]). The structure and function of apoLp-III is similar to that of mammalian proteins apoE, and TIP47/perilipin 3 [6-8]. ApoLp-III can reversibly associate with the lipophorin particle existing in both lipid-bound and lipid-free forms depending on the status of lipid metabolism in the insect body [9, 10]. The incorporation of ApoLp-III molecules to growing lipophorin particles (called LDLp after acquiring additional lipid and apoLp-III) is thought to stabilize them [10-12].

In contrast, mammals contain many different types of exchangeable apolipoproteins, such as apoE, apoA-I, apoA-II, apoC-I, apoC-II and apoC-III [13]. Apolp-III from insects shares many of the characteristics of the mammalian exchangeable apolipoproteins, as all possess amphipathic α-helices that are involved in lipid binding [14]. The monomeric lipid-free state of apoLp-III circulates in the hemolymph of a resting insect until it associates with the expanding lipophorins during insect flight to supply fuel to the flight muscle tissue by delivering neutral lipids from the fat body [12].

ApoLp-III from the migratory locust Locusta migratoria and the tobacco hornworm Manduca sexta (two evolutionarily divergent species) is commonly used as model apolipoprotein (see e.g. [3, 4, 15]). Highest concentration of apoLp-III is present in both species of hemolymph adults[16-18]. Despite the low sequence similarity between these two apolipoproteins, they share a great degree of structural and functional similarity [3, 4]. A similar degree of structural and functional similarity exists between apoLp-III and the
N-terminus of the mammalian apolipoprotein apoE and the C-terminus of the mammalian lipid droplet binding protein TIP47/perilipin 3 [7, 8]. We use apoLp-III from *Locusta migratoria* as model apolipoprotein to investigate the interaction of an amphipathic α-helix bundle protein with (phospho-) lipid monolayers.

ApoLp-III interacts preferentially with diacylglycerols (DAGs) and to a lesser degree with phospholipids [19, 20]. Since most insect lipophorin is rich in DAG and other hydrocarbons and phospholipids [21, 22], this preference for DAG appears to make sense. On the other hand, mammalian lipoproteins contain a core of non-polar lipids including triacylglycerols (TAGs) and cholesterol esters, and are covered by a monolayer of phospholipids and cholesterol interspersed with apolipoproteins [13]. However, it is not well studied whether the interaction of apoLp-III to lipophorin is facilitated by actual chemical recognition (as in the case of an enzyme and substrate) of DAG or is due to physical properties of the lipid monolayer is unclear and has not been investigated in great detail. The objective of this work is to delineate the specific interactions required for apoLp-III phospholipid interaction. Lipids chosen for these experiments vary in a range found in insects lipophorin. In order to most closely mimic the *in-vivo* conditions we used native apoLp-III isolated from *Locusta migratoria*.

### 3.3. Experimentation

**3.3.1. Material and Method**

POG (1-palmitoyl-2-oleoyl-*sn*-glycerol), POPC (1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphocholine) POPE (1,2-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphoethanolamine)
POPG (1,2-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol), DOG (1,2-dioleoyl-sn-glycerol), DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), and DOPG (1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol) were purchased from Avanti Polar Lipids (Albaster, Alabama). All the lipids were purchased in powdered form except for POG and DOG which were dissolved in chloroform. lipids were used without further purification. Powdered lipids were dissolved in a 2:1 (v/v) mixture of chloroform and methanol. Thin layer chromatography combined with iodine staining was used regularly to check the purity and integrity of all lipids used (at least >99% on HPTLC plates) at various times after solubilization; no breakdown was observed during the course of our experiments (after solubilization and storage at -20°C). Apolp-III from Locusta migratoria was a kind gift from Drs. Koert N. J. Burger and Kees Rodenburg (Division of Endocrinology and Metabolism)[23, 24].

All other chemicals used were at least 99.8% pure (analytical grade for buffer components and HPLC grade for solvents) and purchased from Sigma Aldrich Co. All glass-wares were cleaned using concentrated KOH (24g H2O, 25g KOH, and 164g C2H5OH) cleaning solution to avoid contamination of stocks, water and buffer. The Teflon trough and stirrer were cleaned thoroughly before each monolayer experiment by several washes of detergent, distilled water, and KOH cleaning solution. The Wilhelmy plate was rinsed with chloroform, distilled water and KOH cleaning solution.
3.3.2. Insertion isotherms

LDs have a similar structure to lipoprotein, and like them they are surrounded by a single phospholipid monolayer (Figure 1.7). Here, Langmuir monolayer experiment is used to characterized the protein interaction with the phospholipid monolayer at the air/water interface. Surface tension is a parameter which is used to investigate the protein binding.

Insertion isotherms were carried out at room temperature according to [19]. The protein insertion experiments were performed within a Plexiglas enclosure/ chamber. The circular Teflon trough was filled with 6.5 mLs of the Tris buffer used as the subphase in this experiment. Temperature of the subphase was monitored within the Plexiglas enclosure and generally kept at 21 ± 2 °C, except as noted. Monolayer experiments were formed at the air/buffer interface by the drop wise addition of the lipids dissolved in chloroform or chloroform/ methanol on to the surface of the buffer using a Hamilton microsyringe until the desired initial surface pressure was reached. The solvent was allowed to evaporate for about 10 minutes before the introduction of apoLp-III into the subphase (0.1 µM final concentration) via injection with a micropipette through a hole at the side of the trough. Continuous mixing of the sub phase was ensured by a magnetic bar. The change in surface pressure with time was monitored with a platinum Wilhelmy plate and a conventional PS4 Nima surface pressure sensor. All experiments were carried out on a vibration-isolated table.
For all the lipid samples, the Maximum Insertion Pressure (MIP) and $\Delta \pi_{\text{max}}$ were determined by plotting the initial pressure vs. variation in surface pressure after protein insertion to the phospholipid monolayer. The uncertainty of these values is determined by calculating the 95% confidence interval for both $\Delta \pi_{\text{max}}$ and MIP. For $\Delta \pi_{\text{max}}$ the 95% confidence interval is found by multiplying the SE, given by the SigmaPlot 11 software used to generate a linear fit, by 1.96. The 95% confidence interval for MIP is found as described by Calvez et al. [25]. The lipids for these experiments were chosen in a way that they are different in charge properties, head group and effective molecular shape (curvature). All the lipids used were not present in the native biological membrane (liophorin monolayer), however, they could mimic the physiochemical properties of the

![Figure 3. 1. a) A typical insertion experiment for apoLp-III interaction with a DOG monolayer. Surface pressure of the lipid monolayer at the point of protein injection is considered as the initial pressure, $\pi_{\text{int}}$. $\Delta \pi$ is the change in surface pressure recorded 30 minutes after protein injection at which point a stable surface pressure is reached. This time is kept constant for all the subsequent measurements. Subphase buffer: 10 mM Tris, 150 mM NaCl, and 0.2 mM EDTA at pH 7.2. b) A cartoon representing the lipid monolayer after stabilization. The protein is inserted, when the surface is in equilibrium and the rest...](image)
lipid monolayer in lipid-protein interaction. Table 1, presents the list of lipids used in this works with their biophysical characteristics.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Abbreviation</th>
<th>Charge</th>
<th>Chains</th>
<th>“Curvature”</th>
</tr>
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<tbody>
<tr>
<td>1-2-dioleoyl-sn-glycerol</td>
<td>DOG</td>
<td>Neutral</td>
<td>Unsaturated</td>
<td>HII phase</td>
</tr>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycerol</td>
<td>POG</td>
<td>Neutral</td>
<td>Mixed</td>
<td>HII phase</td>
</tr>
<tr>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine</td>
<td>DOPC</td>
<td>Zwitterionic</td>
<td>Unsaturated</td>
<td>Bilayer</td>
</tr>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
<td>POPC</td>
<td>Zwitterionic</td>
<td>Mixed</td>
<td>Bilayer</td>
</tr>
<tr>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine</td>
<td>DOPE</td>
<td>Zwitterionic</td>
<td>Unsaturated</td>
<td>HII phase</td>
</tr>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine</td>
<td>POPE</td>
<td>Zwitterionic</td>
<td>Mixed</td>
<td>HII phase</td>
</tr>
<tr>
<td>1,2-dioleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)*</td>
<td>DOPG</td>
<td>Anionic</td>
<td>Unsaturated</td>
<td>Bilayer</td>
</tr>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol)</td>
<td>POPG</td>
<td>Anionic</td>
<td>Mixed</td>
<td>Bilayer</td>
</tr>
</tbody>
</table>

Table 3. 1. *List of glycero-(phospho)-lipids used in this study*

3.4. Results and discussion

In this work, we used apoLp-III native protein to investigate the effect of biophysical properties of glycerol (phosphor)-lipids in the interaction with the protein at the air/buffer
interface. Lipoprotein-particles and LDs surface are the biological systems where this lipid-protein binding mainly occurs.

3.4.1 Structure of apoLp-III at the air-buffer interface

The first step in performing the monolayer experiments was to determine the protein concentration which maximally varies the surface pressure. The concentration of apoLp-III used for our experiments corresponds to the minimal amount of protein required to reach the maximum surface pressure of the apoLp-III monolayer (see Figure. 3.2). In order to determine the optimum amount of protein for protein insertion isotherms and to investigate to what degree apoLp-III is surface active by itself, we investigated the self-assembly of apoLp-III after injection into a buffer (10mM Tris, 150mM NaCl, pH 7.2) as a function of apoLp-III subphase concentration. Figure 3.2, clearly shows that increased concentrations of apoLp-III lead to increased surface pressure of the self-assembled monolayer up to a plateau (saturation) pressure of ~14 mN/m. From these results, we determined the optimum concentration of apoLp-III for our insertion experiments to be 0.11µM. Here, we assume that for each of the lipid monolayers investigated this concentration also saturates the protein-lipid interaction. This fact is verified by the observation that additional insertion of apoLp-III underneath the lipid monolayer does not result in a further increase in surface pressure. In our typical insertion experiments this
translates into 12.5 µg of 1 mg/ml protein (for a subphase volume of 6.5ml, and a MW of apoLp-III of 20kD).

**Characterization of diacylglycerols used:** we characterized the lipid packing properties of three diacylglycerols, namely, dipalmitoylglycerol (1,2-DPG), dioleoylglycerol (1,2-DOG) and 1-palmitoyl-2-oleoyl-sn-glycerol (1,2-POG) using Langmuir monolayers. These diacylglycerols were characterized because they are likely to occur in the glycerophospholipid monolayer of lipoproteins and LDs. For *L. migratoria* lipophorin, this is relevant not only because DAG is a direct metabolite of TAG, but also because DAG is the main neutral lipid transported in insect blood. Representative

![Figure 3.2](image_url)
Isotherms at 20°C for the DAGs used for the study are shown in Figure 3.3. The area per molecule occupied by 1,2-DPG (data shown in blue) at low surface pressures is significantly lower than those of 1,2-DOG (data shown in red) and 1,2-POG (data shown in black). In fact, the surface pressure of the 1,2-DPG monolayer increases sharply and asymptotically around 45 Å²/molecule. Data also show that 1,2-DPG forms a solid monolayer at any appreciable surface pressure. However, 1,2-DOG and 1,2-POG form monolayers that exist in a liquid disordered state, i.e. the pressure increases gradually at much larger molecular areas.

It was experimentally difficult to form monolayers of 1,2-DPG with defined initial surface pressure by sequentially applying small volumes of lipid stock solution to reach the desired initial lipid pressure (see, e.g. [19]). The insertion isotherm data for 1,2-DPG did not yield consistent results (data not shown), therefore, was not further investigated. The packing properties of diacylglycerols were also investigated via x-ray reflectivity and diffraction techniques sensitive to a monomolecular layer at the air-buffer interface. These data are discussed in detail in Rathnayake et al. 2014, and confirm the Langmuir monolayer isotherms in that no diffraction was observed for 1,2-DOG at all surface pressures and for 1,2-POG below 25 mN/m[26]. Interestingly, 1,2-POG, did show diffraction at surface pressures above 25mN/m.
3.4.2. Insertion of apoLp-III into a diacylglycerol monolayer

Most insect lipophorin transports diacylglycerols not triacylglycerols, and apoLp-III interacts strongly with diacylglycerol exposed at the phospholipid-protein monolayer surrounding the lipophorin particle. Soulages and coworkers showed that concentrations as low as 2 mol % trigger apoLp-III–lipid binding [20]. What is not clear is whether or not this lipid binding is chemically specific or simply a function of the differential packing (i.e. physical) properties of diacylglycerol. More generally the question can be stated as; “What is the driving force behind lipid binding of amphipathic α-helix bundle proteins such as apoLp-III?” The generally accepted model is that hydrophobic defects on the surface of the lipoprotein particle trigger protein binding [9, 20]. However, this does not satisfactorily answer the question as it does not address the specific type of hydrophobic defect.
distinct modes of hydrophobic interaction can be considered that might drive the interaction, namely:

i. Hydrophobic interactions arising from packing defects between the lipids making up the monolayer covering the droplet, which thus depend on packing density.

ii. Hydrophobic “defects” arising from a mismatch in effective molecular area between lipid headgroup and acyl chain area, i.e. the spontaneous curvature of the lipid making up the lipid monolayer. Increases in negative curvature stress in the monolayer, leaving more room in the headgroup region, would facilitate apolar protein contacts, whereas additional positive curvature stress would impede monolayer insertion. Note that these defects, unlike the ones in (i) are delocalized over the entire monolayer. The definition of negative and positive lipid (monolayer) curvature was defined by Helfrich in his seminal work from 1973 and is now common convention [27].

iii. Hydrophobic “defects” arising from line boundaries between different lipid and lipid/protein domains on the surface of the particle [28].

The first two of these hydrophobic interactions are addressed by our Langmuir monolayer insertion studies below. The third one, domain boundaries, will be touched upon in the discussion. The first (i) hydrophobic defect is addressed by measuring the insertion, a requirement for stable incorporation into the surface monolayer, as a function of lipid molecular area (as expressed by the surface pressure). The second (ii) is addressed by choosing lipids that differ in their spontaneous curvature.
Diacylglycerol, due to its high negative spontaneous curvature (i.e., it is cone-shaped, see Figure 3.9), creates so-called hydrophobic defects in a phospholipid monolayer as shown recently by Vamparys et al. and further investigated by Vanni et al. in an accompanying paper [29, 30]. Vamparys compared the packing defects created by diacylglycerol to those created by high positive curvature of a lipid bilayer leaflet and concluded that both types act in the same way to recruit hydrophobic domains/residues to the membrane.

![Figure 3.4](image)

**Figure 3.4.** Change in surface pressure versus initial lipid monolayer pressure after interaction with apoLp-III for 1,2-DOG and 1,2-POG. Subphase buffer: 10 mM Tris, 150mM NaCl, 0.2 mM EDTA at pH 7.2.

The effect of apoLp-III insertion into monolayers of 1,2-DOG and 1,2-POG is shown in Figure 3.4. The insertion of apoLp-III at low lipid monolayer pressure (large
molecular area as seen from the isotherms in Figure. S2) leads to a large increase in the initial lipid monolayer pressure. This increase (at low lipid surface pressure) is larger for 1,2-POG than for 1,2-DOG as shown by the maximum insertion pressure, $\Delta \pi_{\text{max}}$, which is the increase in surface pressure upon protein insertion extrapolated to zero initial lipid monolayer pressure.

The $\Delta \pi_{\text{max}}$ for 1,2-POG is $3 \text{ mN/m}$ (95% confidence intervals do not overlap) higher than for 1,2-DOG. The increase is not due to a displacement of 1,2-DOG or 1,2-POG as the observed pressure is significantly higher than that of the self-assembled apoLp-III monolayer (Figure. 3, 2, ~$14 \text{mN/m}$). Instead the increase in surface pressure is due to interaction of apoLp-III with 1,2-DOG and 1,2-POG.

At increasingly higher lipid monolayer pressures the increase in surface pressure due to protein insertion decreases. Extrapolating this increase in lipid monolayer pressure upon protein insertion to zero leads to a lipid monolayer pressure called the Maximum Insertion Pressure (MIP) [25], the lipid monolayer surface pressure above which apoLp-III is no longer able to insert itself into the lipid layer. Further insertion of apoLp-III no longer results in a decrease of the surface free energy beyond this lipid monolayer pressure. For DOG we find a MIP, $35.0 \pm 1.0 \text{ mN/m}$, identical to that found previously by Demel et al. [19]. The MIP for POG (MIP = $34.4 \pm 0.7$) is the same as that of DOG.
3.4.3. Insertion of apoLp-III into phospholipid monolayers; effect of headgroup and acylchain species.

Next, we systematically varied the headgroup and acyl-chain composition of the lipid monolayer to alternate both the spontaneous curvature and chemical nature of the lipids (see Table 1). We determined the Δπ_{max} and MIP of apoLp-III for each of these lipids (see Table 2).

In order to study the effect of chemical species (chemical specificity and charge) and hydrophobic defects of type i and ii (localized packing defects vs. effective lipid molecular shape), we changed the lipid headgroup, charge, and acyl-chain composition. The lipid headgroups were: 1) phosphocholine(PC), 2) phosphoethanolamine (PE) and 3) phosphoglycerol.(PG). The acyl-chains chosen were: 1,2-dioleoyl (DO), and 1-palmiltoyl, 2-oleoyl (PO). These lipids are summarized in Table 1 together with their relevant physical and chemical properties.
For diacylglycerol (1,2-DOG and 1,2-POG), we observed a MIP around 35mN/m. Since diacylglycerol is a neutral lipid with strong negative spontaneous curvature [31, 32], and to further investigate the effect of spontaneous curvature on apoLp-III insertion we used DOPE, a lipid with strong negative curvature, if not as strong as that of 1,2-DOG and 1,2-POG ($C_{DOPE} \approx -1/30 \text{ Å}^{-1}$[31, 33], whereas $C_{DOG} \approx -1/10.1 \text{ Å}^{-1}$ [31, 32]). If spontaneous curvature were a strong determinant for insertion of apoLp-III, then one would expect DOPE to show more insertion than DOPC, a lipid with very small (-1/143 to -1/200 Å$^{-1}$, i.e. essentially zero) spontaneous curvature [32]. Data shown in Figure 5 and summarized in Table 2 suggests that while DOPE allows slightly more insertion of apoLp-III into the lipid monolayer, the increase in MIP of DOPE over that for DOPC is very minor (95%
confidence intervals overlap). This increase is much less than that for 1,2-DOG and 1,2-POG.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>$\Delta \pi_{\text{max}}$ (mN/m)</th>
<th>MIP (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOG</td>
<td>21.8 ± 1.2</td>
<td>35.0 ± 1.0</td>
</tr>
<tr>
<td>DOPE</td>
<td>17.3 ± 1.6</td>
<td>28.6 ± 1.4</td>
</tr>
<tr>
<td>DOPC</td>
<td>17.3 ± 2.0</td>
<td>26.3 ± 1.0</td>
</tr>
<tr>
<td>DOPG</td>
<td>19.9 ± 1.8</td>
<td>26.4 ± 1.2</td>
</tr>
</tbody>
</table>

Table 3. 2.: The maximal increase in the surface pressure ($\Delta \pi_{\text{max}}$) and the maximum insertion pressure (MIP) after insertion of apoLp-III for the different lipids is shown.

Next, we investigated the effect of negative charge on the interaction of apoLp-III with a lipid monolayer. We chose DOPG because its spontaneous curvature has been characterized (identical to that of DOPC; $C_{\text{DOPG}}= -1/150 \, \text{Å}^{-1}$ [34]). Also, PG is an important bacterial lipid and apoLp-III from *Galleria mellonella* has been implicated in the immune response [35-37]. The immune protective function of apoLp-III is not unique as it has also been found for apoA-I, apoE, and apoB [38-41]. Interestingly we observe that the $\Delta \pi_{\text{max}}$ pressure for DOPG is larger (by 2.5 mN/m) than for DOPC and DOPE suggesting that the negative charge aids in the recruitment of apoLp-III to the lipid monolayer. This is consistent with the finding that apoLp-III of *Locusta migratoria* contains several basic amino acid residues on the hydrophilic face of the amphipathic $\alpha$-helix bundle, similar to what is found on apoA-I [38]. On the other hand, the MIP of apoLp-III for a DOPG monolayer is identical to that for DOPC, consistent with their spontaneous curvature.

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Next, we changed the acyl-chain composition to investigate the effect of chain saturation while maintaining an essentially fluid lipid monolayer (i.e. at 20°C these lipids form a liquid disordered monolayer at low pressures and a liquid ordered monolayer at high pressure). The MIP curves for the 1-palmitoyl, 2-oleoyl containing phospholipids are shown in Figure 3.6. Compared with the data for the dioleoyl lipids we find that the MIP for POPE, POG and POPC are very similar and significantly different from that of 1,2-POG. Interestingly we find that the MIP follows a slightly different trend namely that the MIP for POPC is the smallest followed by those of POPE and POG (which are essentially

Figure 3.6. A comparison of insertion properties of apoLp-III to all the 1-palmitoyl, 2-oleoyl (PO) lipids used in the study. Highest affinity as well as insertion is observed for POG. Subphase buffer: 10 mM Tris, 150 mM NaCl, and 0.2 mM EDTA pH at pH7.2
identical) with the highest value observed for 1,2-POG. This is opposite from the trends in the dioleoyl data where we found that the MIP for DOPC and DOPG were identical. This observation is further discussed below.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>$\Delta \pi_{\text{max}}$ (mN/m)</th>
<th>MIP (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POG</td>
<td>24.9 ± 1.2</td>
<td>34.4 ± 0.7</td>
</tr>
<tr>
<td>POPE</td>
<td>18.7 ± 1.4</td>
<td>30.1 ± 1.5</td>
</tr>
<tr>
<td>POPC</td>
<td>18.1 ± 1.2</td>
<td>26.9 ± 1.0</td>
</tr>
<tr>
<td>POPG</td>
<td>20.6 ± 1.2</td>
<td>29.2 ± 1.8</td>
</tr>
</tbody>
</table>

Table 3. The maximal increase in the surface pressure ($\Delta \pi_{\text{max}}$) and the maximum insertion pressure (MIP) after insertion of apoLp-III for the different lipids is shown.

3.4.4. Effect of chain saturation and increased acyl-chain order

Figure 3. 7. Comparison of the insertion of apoLp-III into a di-unsaturated lipid monolayer and a phospholipid monolayer containing a sn 1-saturated, and 2-unsaturated lipid. A, insertion of apoLp-III into DOPG and POPG, B, insertion of apoLp-III into DOPC and POPC, and C, insertion of apoLp-III into DOPE and POPE. Subphase buffer: 10mM Tris, 150mM NaCl, 0.2 mM EDTA at pH 7.2.
The insertion of apoLp-III into monolayers containing saturated acyl-chains is more favorable than those containing two unsaturated chains. This is particularly striking for the 1,2-POG and 1,2-DOG monolayers as shown in Figure 3. 4, but also clear from the data for DOPG and POPG shown together in Figure 3.7 a. In fact, if we compare the best linear fit to our insertion data for all lipid headgroup species independently, the data for the more saturated lipid monolayer lies above that of the di-unsaturated lipid monolayer (See Figure 3.7 b and c).

Figure 3. 8. Effect of increased temperature (increased fluidity) on the insertion of apoLp-III. Insertion of apoLp-III into a monolayer of POPG at 21 ± 2 °C with insertion of apoLp-III into a monolayer of POPG at 26 ± 1 °C. Subphase buffer: 10mM Tris, 150mM NaCl, 0.2 mM EDTA at pH 7.2.

The data thus suggests that apoLp-III prefers a more ordered lipid monolayer for insertion. This observation was further tested by increasing the temperature of the POPG
monolayer. Figure 3.8 indeed shows that an increased temperature, and hence a decreased order in the POPG monolayer, leads to a decreased insertion of apoLp-III into the lipid monolayer.

Interaction of apoLp-III with lipid monolayers is more favorable for saturated acyl-chain containing lipids. This observation is consistent with a recent paper by Storey et al. that showed that the lipid monolayer of perilipin enriched LDs is more saturated than other bio membranes found in the cells [42]. Both apoLp-III and perilipin 3 contain structurally homologous amphipathic α-helix bundles, and our results for apoLp-III suggest that this forms a lipid sensor for more ordered lipid domains. Unfortunately, there is limited data on the acyl-chain composition of the phospholipid monolayer covering low density lipophorin (LDLp) while more extensive data is available on the fatty acid composition of the core of these particles. The core of LDLp isolated from L. migratoria is enriched in C16:0 and C18:1 lipids, whereas that of the phospholipid monolayer is enriched in C18:0 and C18:2 and C18:3 fatty acids [43, 44].

The finding by Wan et al. that apoLp-III is able to transform vesicles made of DMPC, or shorter saturated lipids into lipid nanodiscs [45] may be related to our observations. In the gel phase the DMPC vesicles consist of many connected gel phase domains and apoLp-III might be able to interact with these domain boundaries. However, apoLp-III is able to transform the lipid bilayer into lipid (membrane) nanodiscs only very near the phase transition. At the phase transition the compressibility of the bilayer is increased and hence deformability is likely enhanced. In light of the results from the disc assay it is timely to investigate the interaction of apoLp-III with domain boundaries in lipid
monolayers. One possible method by which to explore this further is light scattering microscopy [46, 47]. It is intriguing that the monolayers of perilipin-enriched LDs contain more saturated phospholipids [42].

The basic structure of apolipoproteins closely resemble that of a LD in that they both consist of a neutral lipid core surrounded by a phospholipid-protein monolayer. Some of the proteins found at the LD surface, for example perilipins contain amphipathic $\alpha$-helix bundles similar to apoLp-III. Our results now show that a model amphipathic $\alpha$-helix bundle protein interacts favorably with more ordered lipid monolayers. This favorable interaction might differentiate the LD monolayer from most other intracellular and infinitely more abundant, lipid membranes. It can thus be one mechanism by which perilipin 3 may be sorted to the surface of growing LDs. This hypothesis is consistent with the finding that proteins such as caveolins also reside on LDs (their native membrane environment is more highly ordered than the rest of the plasma membrane). The high concentration of cholesterol in caveolae serves to increase the order of the lipid bilayer in these structures. So why then does, e.g. perilipin 3, not bind to caveolae? One reason might be that caveolae are formed by the simultaneous recruitment of caveolin and cholesterol and hence there is no additional space for perilipin 3, however, this suggestion clearly requires experimental verification. The compression – decompression (or vice versa) experiments with diacylglycerol – apoLp-III monolayers show that apoLp-III is able to assemble from solution and unfold its helix bundle at the interface (data shown in Rathnayake et al., 2014) [26]. Subsequent compression does not result in a folded helix-bundle attached to the lipid monolayer as far as x-ray reflectivity experiments are able to
observe [26]. The results instead suggest that apoLp-III is able to quickly dissociate from the lipid monolayer as soon as the bundle has folded, consistent with its function *in vivo*. These compression-decompression isotherms for apoLp-III lipid monolayers show significant hysteresis. This suggests that lipidated apoLp-III may not refold in the α-helix bundle revealed by x-ray crystallography. This observation clearly deserves more attention in the future and is being explored at an oil-water interface in our lab.


The results indicate that though more apoLp-III is incorporated in the lipid monolayer after expansion and compression, or right after deposition of lipid on top of a protein monolayer, the density of apoLp-III at the interface of a tightly packed lipid layer is insufficient to observe the protein. The protein α-helix is thus entirely embedded in the headgroup region (consistent with previous observations/suggestions, see e.g. [48]). Excess apoLp-III that moved from the subphase to the lipid-protein monolayer at high lipid molecular area is most likely expelled again after compression, however, folded apoLp-III is never observed underneath the lipid layer (within the time frame of the reflectivity experiment). This suggests that once apoLp-III is folded it diffuses away into the subphase. Several compression-decompression (or expansion-compression) cycles lead to significant hysteresis suggesting a loss of diacylglycerol from the interface, similar to that observed for the C-terminus of apo-A1 [49].
3.5. Conclusions

We did perform limited insertion experiments with 1,2-DPG and found insertion is not very reproducible and occurs even at high initial lipid monolayer pressures. A similar observation was made for POPE monolayers (data not shown). At high surface pressures POPE undergoes a phase transition to a more ordered phase. In this regime (π<35 mN/m) we routinely observe a limited amount of insertion in the range of 1 to 2 mN/m apparently independent of monolayer pressure. This observation is again consistent with the observation that apoLp-III interacts more favorably with saturated/ordered acyl chains.

An alternative explanation may be that apoLp-III interacts with line boundaries in the POPE and 1,2-DPG monolayer. Solid monolayers (as in 1,2-DPG) are only quasi 2-D crystals, as there is no real long-range order in individual molecule packing. Instead, these layers are built of many crystalline domains separated by domain boundaries. The structure of POPE monolayers at high pressure has been investigated via BAM microscopy and AFM spectroscopy [50-52]. In BAM, at low POPE surface pressures, tiny, brightly scattering specks are observed indicating some type of three dimensional structures ([50], and our own observations ). The exact nature of these spots has not been studied to date. At the phase transition, clear domains become visible in both BAM and AFM with some of the bright spots still present. Domain formation at the phase transition appears to be reproducible. It is possible that apoLp-III – POPE interaction at high pressures is driven by this domain formation if apoLp-III has a high affinity for either one of the domains or for the domain boundaries. In the case of apoLp-III – 1,2-DPG interaction it is possible that
apoLp-III interacts with the boundaries surrounding each semi-crystalline domain. One possible method by which to explore this further is light scattering microscopy [46, 47].

It is intriguing that the monolayers of perilipin-enriched LDs contain more saturated phospholipid [42]. Our results now show that a model amphipathic α-helix bundle protein interacts more favorably with more ordered lipid monolayers. This favorable interaction with more ordered lipid monolayers differentiates the LD monolayer from most other intracellular, and infinitely more abundant, lipid membranes. It is thus one mechanism by which TIP47/perilipin 3 may be sorted to growing LD surfaces.

This hypothesis is consistent with the finding that, e.g. caveolins, also reside on LDs as their native membrane environment is more highly ordered than the rest of the plasma membrane. The high concentration of cholesterol in caveolae serves to order the lipid bilayer in these structures. So why then does, e.g. perilipin 3, not bind to caveolae? One reason might be that caveolae are formed by the simultaneous recruitment of caveolin and cholesterol and hence there is no additional space for perilipin 3, however, this suggestion clearly requires experimental verification.

The compression – decompression (or vice versa) experiments with diacylglycerol – apoLp-III monolayers show that apoLp-III is able to assemble from solution and unfold its helix bundle at the interface. Subsequent compression does not result in a folded helix-bundle attached to the lipid monolayer as far as our x-ray reflectivity experiments are able to observe. The results instead suggest that apoLp-III is able to quickly dissociate from the lipid monolayer as soon as the bundle has folded, consistent with its function in vivo.
3.5.1. Model of apoLp-III – preferential DAG interaction.

Our results clearly show that insertion of apoLp-III into a monolayer of diacylgllycerol is more favorable than into a monolayer of the six phospholipids that we tested here. One measure of this interaction is given by the $\Delta \pi_{\text{max}}$ and MIP data shown in Table 2. The maximum increase in the surface pressure gives an indication of the affinity of the protein to the lipid monolayer whereas the MIP is an indication of the degree of insertion. For example, the higher $\Delta \pi_{\text{max}}$ for PG indicates some affinity of the protein for negative charge consistent with the basic amino acid residues however the degree of insertion is not increased as indicated by the MIP values.

We suggest that preferential interaction (as measured via the MIP) of apoLp-III with diacylgllycerol is mediated by differences in headgroup size (steric interaction, length of the headgroup) of the lipid, and to a much smaller degree the spontaneous curvature (effective lipid molecular shape) of the lipids in the lipid monolayer. The cartoon in Figure 9 shows this interaction model to scale.

Our data support this model in the following manner:

I. Insertion into a lipid monolayer is more favorable for DAG than PE and PC which both have a considerably larger headgroup. The depth at which the amphipathic $\alpha$-helix has to insert in order to reach the hydrophobic interior of the lipid monolayer is considerably larger for PE and especially for PC than for DAG (see Figure 9).
II. Insertion into a monolayer containing saturated acyl-chain containing lipids was shown to be more favorable compared to unsaturated chains (with less order). This is especially evident when we compare 1,2-DOG and 1,2-POG at large molecular areas. At low initial lipid surface pressure the 1,2-DOG and 1,2-POG monolayer isotherms overlap (see Figure S2). Insertion into a monolayer of these lipids in these conditions is considerably more favorable for 1,2-POG than for 1,2-DOG. 1,2-POG has a saturated acyl-chain at the sn-1 position and 1,2-DOG does not. Note that 1,2-POG is more ordered than 1,2-DOG, as also shown by the diffraction data (Figure S3).

III. The effect of a more ordered lipid monolayer, was reiterated by the experiment where the POPG monolayer was heated. In this case, we found a significantly reduced, over all molecular areas, insertion of apoLp-III into the POPG monolayer. Increased temperature means increased disorder for the acyl-chains.
of POPG. This thus follows the model where increased disorder in the hydrophobic interior of the lipid monolayer significantly perturbs the interaction.

Interaction of hydrophobic side residues of apoLp-III with the hydrophobic interior of the lipid monolayer is thus crucial for apoLp-III – lipid interaction.

3.5.2. Future directions

These monolayer experiments on the model amphipathic α-helix bundle protein apoLp-III set the stage to investigate in exquisite detail the interaction of intracellular LD proteins containing this domain. Additionally, the interaction of human apolipoproteins such as apoE have not yet been examined in as much detail as we do here, i.e. different headgroups and acyl chain species that systematically vary lipid biophysical properties [25]. In addition to pure lipid monolayers, as in the present study, we will expand our work to include lipid mixtures with specific focus on the potential role of cholesterol. The interaction between LPS and apoLp-III of Galleria m. should be investigated as this will shed light on the immune protective function of apoLp-III.

References


Kooijman, Insertion of apoLp-III into a lipid monolayer is more favorable for saturated, more ordered, acyl-chains, *Biochim Biophys Acta* 1838(1 Pt B) (2014) 482-92.


CHAPTER 4

INSERTION OF PERILIPIN 3 INTO A GLYCERO(PHOSPHO-)LIPID MONOLAYER DEPENDS ON LIPID HEADGROUP AND ACYLCHAIN SPECIES

4.1. Abstract

Lipid droplets are organelles that contribute to various cellular functions which are vital for life. Aside from acting as a neutral lipid storage depot, they are also involved in building new membranes, synthesis of steroid hormones and cell signaling. Many aspects of LD structure and function are not yet well understood. Here we investigate the interaction of perilipin 3, a member of the perilipin family of LD binding proteins, and three N-terminal truncation mutants with lipid monolayers. The interaction is studied as a function of surface pressure for a series of systematically chosen lipids. We find that the C-terminus of perilipin 3 has different insertion behavior from that of the longer truncation mutants and the full length protein. Inclusion of N-terminal sequences with the C-terminus decreases the ability of the protein construct to insert in lipid monolayers. Anionic lipids, coupled to negative spontaneous curvature, facilitates protein interaction and insertion.

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2 This chapter is submitted as an article in Journal of Lipid Research.
The C-terminus shows strong preference for lipids with more saturated fatty acids. This work sheds light on the LD binding properties and function of the different domains of perilipin 3.

4.2. Introduction

Lipid droplets (LDs) are dynamic cell organelles that carry out a multitude of cellular functions vital for life, and protein-lipid interactions are crucial to the structure and function of LDs. While much work has focused on peripheral and integral membrane proteins, the mechanism by which lipid droplet binding proteins recognize and target to LDs is still poorly understood [1]. This is especially true for dedicated LD binding proteins of the perilipin family, i.e. perilipin 1 through 5, that function in the biogenesis and metabolism (lipolysis) of LDs. No work has directly investigated the interaction of a perilipin family member with a phospholipid monolayer interface. In order to address how this family of proteins interacts with lipid interfaces we investigated the interaction of perilipin 3 with phospholipid monolayers at the air-buffer interface. We chose perilipin 3 because it is found both in the cytosol as well as on the LD surface, and because previous work has characterized the structure and LD association of the protein [1-3].

In addition to providing cellular energy, LDs take part in many other cellular functions including signal transduction, formation of new cellular membranes, hormone synthesis, and lipid trafficking [4-8]. Under certain physiological conditions, LDs have been found to act as store houses for several different types of enzymes and proteins including histones
[9, 10], and they also facilitate virus replication [11-13]. An understanding of how proteins associate with the LD surface is crucial for our understanding of LD biology.

Although the composition of the lipid droplet interior might vary from cell to cell, the basic structure consists of a neutral lipid core of triacylglycerols (TAG) and cholesterol esters (CE) surrounded by a phospholipid-protein monolayer [14, 15]. The phospholipid composition of the monolayer surrounding the LDs has not been clearly defined but appears to consist mostly of phosphatidylcholine (PC) with a series of other phospholipids, diacylglycerol (DAG), and cholesterol [16, 17].

A major fraction of the proteins associated with the lipid monolayer belong to the perilipin family. The perilipins are synthesized on cytosolic ribosomes [18-20] and are thus sorted to the LD interface in a different manner than those proteins that originate from the ER, such as, for example caveolin [21]. Sorting of caveolin is cooperatively mediated by a dual motif consisting of a hydrophobic sequence located close to a positively charged sequence [21]. Targeting has been investigated for perilipins 1 through 5 in cellular studies and different regions of the proteins have been identified [1, 22-27]. Based on currently available data it appears likely that amphiphatic sequences, especially the 11-mer repeat region of the proteins, target perilipins to LDs [1, 23, 28-31]. The 11-mer repeat is a protein region with a repeating 11 amino acid pattern [32]. These 11-mer repeats are found in the N-terminus of all perilipins (see Figure 1), with the largest such domain on perilipin 4 [33]. The 11-mer repeats occur in other lipid binding proteins such as α-synuclein and apoAI [34, 35]. They form amphipathic α-helices and have been ascribed a function in the
reversible binding to lipid interfaces [34]. However, the function of this domain in perilipin 3-lipid binding has not been explored in detail in model systems.

The mammalian perilipin family includes five distinct members that share sequence homology especially in their N-terminal regions [20]. The five mammalian proteins are perilipin1, perilipin 2 (previously named adipophilin or ADRP), perilipin 3 (previously named TIP 47), perilipin 4 (previously named S3-12) and perilipin 5 (previously named OXPAT) [29, 36]. Perilipins 1 and 2 are found exclusively on LDs and are classified as constitutively LD associated proteins [18, 37-41] whereas perilipins 3, 4 and 5 circulate between LDs and the cytoplasm, and are therefore called exchangeable perilipin proteins [40, 42, 43]. Some research suggests that perilipin 2 might also be found free in the cytosol [44, 45]. The expression of perilipins 1 and 4 is confined to adipocytes and steroidogenic cells [18, 37-39, 41, 46] while perilipin 2 and perilipin 3 are ubiquitously expressed [19, 42, 43].

The structure of perilipin 3 has been investigated by two different groups. Hickenbottom et al. resolved the crystal structure of the C-terminal region of murine perilipin 3 and revealed that it consists of an α/β domain and a bundle of 4 amphipathic α-helices [3]. The helix bundle structure of the C-terminus of perilipin 3 closely resembles the LDL receptor binding domain in the N-terminal region of ApoE [3], and even shares sequence homology [47]. Similar amphipathic α-helix bundle structures are found in other apolipoproteins such as insect apolipophorin III [48]. The function of this amphipathic α-
helix bundle domain of perilipin 3 is unknown, and the detailed protein-lipid interactions have not been investigated [1].

Hynson et al. recently studied the structure of full length human perilipin 3, and showed that, as predicted by the crystal structure work, the protein has a highly helical C-terminus in solution [2]. This work additionally revealed a disordered N-terminal region and showed that the full length protein assumes an extended confirmation in solution suggesting that the N and C termini might perform separate functions [2].

To shed light on the function of perilipin 3 and the mechanisms of lipid interaction, we studied the interaction of the C-terminal domain of perilipin 3 with model phospholipid monolayers at the air-buffer interface. Results are compared with those for two constructs that contain additional segments of the N-terminal portion of the protein, and those for the full-length protein (see Figure 1). Model lipid monolayers formed at the air-buffer interface are especially useful model systems for the study of LD proteins. In vivo, LD proteins interact with a (phospho-) lipid monolayer and the composition and physical properties of this monolayer can be made to closely resemble that surrounding the lipid droplet.

The Langmuir monolayer technique is a biophysical method frequently used to explore the lipid interaction of proteins and peptides, specifically protein insertion [49, 50]. It must be stressed here that the lipids in this study are not necessarily those that occur naturally on the LD surface. Only limited data on the phospholipid composition of the LD monolayer is available, for a very limited set of cell types [16, 17]. Instead we choose those lipids that vary properties that might, based on our previous work,[50] influence the interaction of
perilipin 3 with the phospholipid monolayer in vivo: spontaneous curvature (i.e. curvature stress of the monolayer, and access to hydrophobic interior of the LD), and charge.

We find that all constructs of perilipin 3 are surface active. The more of the protein N-terminus is included, the more surface active the construct is, as assessed from changes in the surface pressure of the self-assembled protein monolayer. The C-terminal α-helix bundle domain of perilipin 3 has markedly different insertion properties as compared to the full-length protein. Inclusion of more N-terminal sequences from the full-length protein reduces the maximum lipid monolayer pressure at which the protein construct remains able to insert into the lipid monolayer. At the same time, the affinity for the lipid monolayer is increased. The C-terminus of perilipin 3 shows a high affinity for saturated fatty acids as evidenced by a reduced insertion into a lipid monolayer containing unsaturated fatty acids. Taken together our results suggest that the C-terminus of perilipin 3 carries out distinct functions compared to the N-terminus, which is dominated by the highly amphipathic 11-mer repeat region.

4.3. Materials and Methods

4.3.1. Materials

POG (1-palmitoyl-2-oleoyl-sn-glycerol), POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine), POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine), DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine), PO PG (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1’-rac-glycerol), POPA (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (sodium salt)), and DOPA
(1,2-dioleoyl-sn-glycero-3-phosphate (sodium salt)) were purchased from Avanti Polar Lipids (Alabaster, Alabama). NaCl, EDTA and Tris were purchased from Sigma Aldrich Co. All of the chemicals were > 99% pure. Chemicals for protein purification were purchased from Amresco. HPLC grade water was purchased from Fisher Scientific. Proteins used for these experiments were purified in Kooijman lab. Further information on protein purification can be found in the published paper [51].

4.3.2. Monolayer insertion experiments

The monolayer insertion experiments followed the procedure by Demel et al., 1992 [52]. Briefly, the set-up consisted of a circular Teflon trough enclosed within a Plexiglas enclosure placed on a vibration isolation table. The trough has an inner diameter of 3 cm and 5 cm outer diameter, and is 0.5 cm deep with, in the middle, space for a small magnetic stirrer bar (1 cm diameter X 0.5 cm deep). In the upper rim (1 cm) is a port through which protein is injected below the monolayer without disturbing the lipid monolayer. The trough was filled with 6.5 mLs of Tris buffer, pH7.2 (10 mM Tris, 150 mM NaCl and 0.1 mM EDTA): the subphase for the experiment. Note that other common biological buffers (e.g. HEPES) and reducing agents (e.g. DTT and beta mercaptoethanol) affect the surface pressure of lipid monolayers and should thus be avoided. A lipid monolayer is formed by drop wise addition of lipids (0.1 mM) dissolved in chloroform/methanol (2:1 volume ratio) until the desired surface pressure is reached. The solvent is allowed to evaporate for about 10 minutes until a stable pressure is reached, and protein is injected into the subphase through a port in the side of the trough. The monolayer experiments are carried out in a temperature controlled room at 22.0 ± 1.0 °C, and the subphase is continuously stirred with
a magnetic bar to ensure proper mixing of the proteins in the subphase. All glassware, the Teflon trough and magnetic stirrer were cleaned thoroughly before each monolayer experiment by a wash with KOH cleaning solution (164 g of ethanol, 24 g of water, and 25 g of KOH), several rinses with deionized water before final rinsing with ultrapure water (HPLC grade water, Catalog No: W5-4, Fisher Scientific). The platinum Wilhelmy plate was rinsed with distilled water, KOH cleaning solution, and finally ultra-pure water. Proteins used in these experiments were purified in Kooijman lab. Further information about protein purification protocols are available through paper published in 2016 [53].

4.4. Results

4.4.1. Surface activity of perilipin 3

We first determined the surface activity of the amphipathic α-helix bundle of perilipin 3 (named: perilipin 3D), followed by two additional N-terminal truncation mutants and finally the full length ecombinant protein (see Figure 1).

This surface-activity determination is an important control. If the change in lipid monolayer pressure, after insertion of the protein into the interface, is not larger than the surface pressure supported by the self-assembled protein alone, then it is possible that the protein does not insert into (i.e. in between) the lipid monolayer at all. It may instead simply form patches of protein at the interface, in equilibrium with a more-compressed lipid monolayer.
Figure 2 shows the increase in surface pressure of a bare buffer interface as function of the bulk protein concentration. As expected, the amphipathic α-helix bundle of perilipin 3 (perilipin 3D) is highly surface active with a final surface pressure of ~12mN/m for the self-assembled (Gibbs[54]) monolayer. This is comparable to the maximum surface pressure reached by the self-assembled monolayer of the insect protein apoLp-III, ~14mN/m, which consists entirely of an α-helix bundle that in structure resembles that of the C-terminus of perilipin 3 [50]. Addition of further domains from the N-terminus (see Figure 1) to the α-helix bundle of perilipin 3 increases the surface activity of the protein, such that the full-length protein (perilipin 3A) has the highest surface pressure of ~
19mN/m. It is interesting to note here that addition of 35 amino acids to the sequence of the α-helix bundle essentially maximizes the surface activity.

Figure 4.2. Surface activity of perilipin 3 and its truncation mutants. Increase in surface pressure of self-assembled monolayers of perilipin 3A-D as a function of subphase concentration. Proteins were inserted beneath an air-buffer interface and surface pressure was allowed to equilibrate. Values shown are averages of three independent experiments and error bars represent the standard deviation. Buffer subphase: 10mM Tris-HCl, 150 mM NaCl, 0.2 mM EDTA at pH7.2.

These data also show that a subphase concentration of 0.04 µM is required to maximize the surface pressure of the monolayer, in our particular set-up. In order to assure that we used sufficient protein to maximize interaction with the lipid monolayers
investigated we used a subphase concentration of at least 0.07 µM in all subsequent experiments.

4.4.2. Perilipin 3D behaves similarly to apoLp-III, a model α-helix bundle protein

Next, we determined the interaction of the α-helix bundle of perilipin 3, perilipin 3D, with model lipid monolayers. The lipids we used in this study are listed in Table 4. 1 together with the relevant biophysical properties.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Abbreviation</th>
<th>Charge</th>
<th>Chains</th>
<th>“Curvature”</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol</td>
<td>PGO</td>
<td>Neutral</td>
<td>Mixed</td>
<td>Negative, induces H₃ phase</td>
</tr>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine</td>
<td>POPC</td>
<td>Zwitterionic</td>
<td>Mixed</td>
<td>Infinite, forms Bilayer</td>
</tr>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine</td>
<td>POPE</td>
<td>Zwitterionic</td>
<td>Mixed</td>
<td>Negative, forms H₃ phase</td>
</tr>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1-rac-glycerol)</td>
<td>POGG</td>
<td>Anionic</td>
<td>Mixed</td>
<td>Infinite, forms Bilayer</td>
</tr>
<tr>
<td>1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (sodium salt)</td>
<td>POPEA</td>
<td>Anionic</td>
<td>Mixed</td>
<td>Negative, induces H₃ phase</td>
</tr>
<tr>
<td>1,2-dioleoyl-sn-glycero-3-phosphocholine</td>
<td>DOPC</td>
<td>Zwitterionic</td>
<td>Unsaturated</td>
<td>Infinite, forms Bilayer</td>
</tr>
<tr>
<td>1,2-dioleoyl-sn-glycero-3-phosphoethanolamine</td>
<td>DOPE</td>
<td>Zwitterionic</td>
<td>Unsaturated</td>
<td>Negative, forms H₃ phase</td>
</tr>
<tr>
<td>1,2-dioleoyl-sn-glycero-3-phosphate (sodium salt)</td>
<td>DOPA</td>
<td>Anionic</td>
<td>Unsaturated</td>
<td>Negative, induces H₃ phase</td>
</tr>
</tbody>
</table>

Table 4. 1. Physical chemical properties of lipids used in this study

Specifically, these lipids were chosen such that they had the same acyl-chain composition but differed in the headgroup (i.e. size, hydration, and charge), resulting in lipids that spanned a range of relevant physicochemical properties. The sn-1-palmitoyl, and
sn-2-oleoyl acylchains were chosen as it is representative of mammalian glycerophospholipid fatty acid composition [55].

Figure 3 shows the cumulative data for the interaction of perilipin 3D with monolayers of POG, POPC, POPE, POPA, and POPG. Representative insertion kinetics are shown in Supplementary Figure S2. The resulting change in surface pressure of the lipid monolayer is plotted as a function of initial lipid monolayer pressure. Note that generally the Δπ values are larger than the surface pressure of the protein at the air-buffer interface alone (compare Figure 2 and 3). A possible exception is POPC where the pressure of the resulting protein-lipid monolayer is comparable with that of the protein alone (see discussion of acyl chain saturation below).

![Graph showing insertion isotherms for perilipin 3D for a series of (phospho-)lipids.](image)

**Figure 4.** Insertion isotherms for perilipin 3D for a series of (phospho-)lipids. Comparison of the insertion of perillipin 3-D into monolayers of POG (black circle), POPC (yellow triangle), POPE (green square), POPA (red diamond), and POPG (blue cross). MIP and Δπmax values are shown in Table 2. Buffer subphase: 10mM Tris-HCl, 150 mM NaCl, 0.2 mM EDTA at pH7.2.
We extract two key quantities from these insertion isotherms, namely the MIP (maximum insertion pressure) and the $\Delta \pi_{\text{max}}$ (maximal change in monolayer pressure). The MIP is the lipid monolayer pressure at which the protein is no longer able to insert in between the lipids in the monolayer (see Figure 2, and reference [49]). This pressure has also been referred to as the exclusion pressure. The MIP value, therefore, is an indication of the propensity of insertion of a given protein into a given lipid monolayer. MIP values above 30-35 mN/m are used to indicate proteins with propensity to insert into a lipid bilayer [56, 57]. $\Delta \pi_{\text{max}}$ is the maximal change in lipid monolayer pressure after protein insertion and is taken as the intercept of the change in lipid monolayer pressure axis at 0 lipid monolayer pressure (see Figure 2). This value is also used as an indication of affinity of the protein for the lipids in the monolayer.

<table>
<thead>
<tr>
<th>Perilipin 3D</th>
<th>$\Delta \pi_{\text{max}}$ (mN/m)</th>
<th>MIP (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POG</td>
<td>19.8 ± 0.6</td>
<td>38.1 ± 1.4</td>
</tr>
<tr>
<td>POPC</td>
<td>14.2 ± 1.3</td>
<td>26.0 ± 0.5</td>
</tr>
<tr>
<td>POPE</td>
<td>13.6 ± 1.4</td>
<td>39.6 ± 0.4</td>
</tr>
<tr>
<td>POPA</td>
<td>23.3 ± 0.8</td>
<td>34.0 ± 1.2</td>
</tr>
<tr>
<td>POPG</td>
<td>15.3 ± 0.8</td>
<td>23.7 ± 1.2</td>
</tr>
</tbody>
</table>

Table 4. 2. Maximum change in monolayer pressure $\Delta \pi_{\text{max}}$ and maximum insertion pressure MIP data derived from data shown in Figure 3 for the amphipathic alpha helix bundle of perilipin 3 (perilipin 3D).

MIP and $\Delta \pi_{\text{max}}$ values for perilipin 3D are shown in table 2. The 95% confidence interval for these values are calculated according to reference [49]. We note that the insertion of the $\alpha$-helix bundle of perilipin 3 is highest for POG and POPE, both of which have negative spontaneous curvature, and for POPA which has both negative curvature, and a negative...
charge [58-60]. Note that the $\Delta \pi_{\text{max}}$ for POPE is much reduced compared to POG and POPA, suggesting that the affinity of the helix bundle domain for POPE is limited. Interestingly, addition of a negative charge (POPA) significantly enhances the interaction with phospholipid monolayers, but negative charge alone is not sufficient (compare POPG, also negatively charged, to POPC and POPA). Only the MIPs for POG and POPE are sufficiently above the 30-35 mN/m typical of glycero(phospho-)lipid bilayers [56] to suggest that this domain can interact with such hypothetical bilayers. Note that POG or POPE alone do not form fluid lipid bilayers under physiological conditions.

### 4.4.3. Addition of further N-terminus results in a reduced MIP and an increase in $\Delta \pi_{\text{max}}$

<table>
<thead>
<tr>
<th>Perilipin 3C</th>
<th>$\Delta \pi_{\text{max}}$ (mN/m)</th>
<th>MIP (mN/m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>POG</td>
<td>21.4 ± 1.1</td>
<td>27.4 ± 0.6</td>
</tr>
<tr>
<td>POPC</td>
<td>20.2 ± 1.1</td>
<td>29.4 ± 0.7</td>
</tr>
<tr>
<td>POPE</td>
<td>20.7 ± 1.2</td>
<td>26.5 ± 1.0</td>
</tr>
<tr>
<td>POPA</td>
<td>26.6 ± 1.5</td>
<td>32.3 ± 0.7</td>
</tr>
<tr>
<td>POPG</td>
<td>25.4 ± 2.0</td>
<td>27.3 ± 0.8</td>
</tr>
</tbody>
</table>

Figure 4. Insertion isotherms for perilipin 3C for a series of (phospho-) lipids. Comparison of the insertion of perilipin 3C into monolayers of POG (black circle), POPC (yellow triangle), POPE (green square), POPA (red diamond), and POPG (blue cross). MIP and $\Delta \pi_{\text{max}}$ values are shown in Table 4.3. Buffer subphase: 10mM Tris-HCl, 150 mM NaCl, 0.2 mM EDTA at pH7.2. Table 4.3. Maximum change in monolayer pressure $\Delta \pi_{\text{max}}$, and maximum insertion pressure MIP data derived from data shown in Figure 4 for construct perilipin 3C.
Next, we investigated the effect of addition of a small (35 amino acids, 152 through 187) portion of the N-terminus to perilipin 3D (perilipin 3C). Figure 4 shows the resulting insertion isotherms and indicates a significant increase in the affinity of the protein for the lipid monolayer (higher $\Delta \pi_{\text{max}}$), consistent with the increase in surface activity of the protein (see Figure 2). Interestingly, the addition of negative charge to the lipid monolayer significantly increases the affinity of the protein for the lipids (POPA and POPG), but the MIP is only increased (with respect to PC) for POPA which has negative spontaneous curvature. Representative insertion kinetics are shown in Supplementary Figure S3.

Addition of the 11-mer repeat region to the protein, to yield perilipin 3B, does not significantly alter its monolayer interaction except that it appears POG has the highest MIP, compared to POPA for perilipin 3C (see Figure 5). It should be noted that the spread in the...
POG data at higher initial pressure is significant resulting in a larger confidence interval. Representative insertion kinetics are shown in Supplementary Figure S4.

Like perilipin 3B, the full length protein, perilipin 3A, does not show major differences in lipid insertion compared to perilipin 3C and 3B (see Figure 6). Representative insertion kinetics are shown in Supplementary Figure S5.

![Perilipin 3A](image)

**Figure 4.6**: Insertion isotherms for perilipin 3A (full length protein) for a series of (phospho-)lipids. Comparison of the insertion of full length perillipin 3 into monolayers of POG (black circle), POPC (yellow triangle), POPE (green square), POPA (red values are shown in Table 5) and POPE (blue cross). MIP and Δπ\textsubscript{max}. **Table 4.5**: Maximum change in monolayer pressure Δπ\textsubscript{max}, and maximum insertion pressure MIP data derived from data shown in Figure 6 for construct perilipin 3A.
4.4.4. Perilipin 3 prefers saturated acylchains

Our previous work on apoLp-III, a model amphipathic α-helix bundle protein, suggested that this protein prefers phospholipids with saturated acyl chains [50]. We thus investigated the interaction of perilipin 3D with the unsaturated lipids DOPC, DOPE, and DOPA. Fig 4.7(a) compares the results for DOPC and POPC (POPC data replicated from Figure 3). Here it should be noted that it took a considerable time for perilipin 3D to start inserting into the DOPC monolayer (see Supplementary Figure S6 for insertion kinetics). This was previously observed for POPC (Supplementary Figure S2), but for DOPC this observation was especially noticeable. The data for DOPC is thus also taken at 40 min after protein insertion compared to 30 min for all other lipids. The MIP for perilipin 3D is significantly (>10mN/m) lower for DOPC compared to POPC.

![A)](image1)  ![B)](image2)  ![C)](image3)

Figure 4. 7 Insertion isotherms for perilipin 3D in saturated and unsaturated phospholipids. Comparison of the insertion isotherms of perilipin 3D into a lipid monolayer of A, POPC and DOPC, B, POPE and DOPE, and C, POPA and DOPA. The change in lipid monolayer pressure was recorded after 30 minutes, except for DOPC where data was recorded after 40 minutes due to the slow insertion kinetics of the protein. Note the large difference in MIP for the more saturated (PO) and unsaturated (DO) lipids. Buffer subphase: 10mM Tris-HCl, 150 mM NaCl, 0.2 mM EDTA at pH7.2

Since our previous work showed that negative spontaneous curvature affects protein insertion (see Figures 3 through 6) we next compared the insertion of perilipin 3D
into monolayers of POPE and DOPE (Fig 4. 7(b); Data for POPE taken from Figure 3). In this case as well, the PO lipid shows significantly more insertion of the amphipathic α-helix bundle, with the MIP for POPE ~22mN/m higher than for DOPE. Fig 4. 7(c) compares the interaction of perilipin 3D with monolayers of POPA and DOPA. Again, we observe significantly more (MIP >15mN/m) insertion for monolayers of the more saturated lipid.

4.5. Discussion

For the lipid monolayers, we utilized single lipids that systematically varied in physical chemical properties (charge, shape, hydration, acyl-chain saturation, etc.) as shown in Table 1. The systematic variation of these properties allows us to identify which physical and chemical properties of the lipid interface are important for perilipin 3 interaction. The use of our different constructs also allows us to make conclusions on the role of the different perilipin 3 domains in lipid interaction.

4.5.1. *The amphipathic α-helix bundle of perilipin 3 inserts more strongly into a phospholipid monolayer than the full-length protein.*

Our results show that the C-terminal domain (consisting of an α/β domain and 4-helix α-helix bundle) shows the largest insertion, i.e. highest MIP, into monolayers composed of lipids with negative spontaneous curvature, namely POG, POPE, and POPA (see Figure 3). Lipids with negative spontaneous curvature are known to facilitate the interaction of peripheral membrane proteins as the asymmetry in the headgroup area (small) and acyl chain area (larger; see table 1) facilitates insertion of hydrophobic domains
into the hydrophobic interior of the membrane bilayer [61, 62]. A similar mechanism appears to be at play for the C-terminus of perilipin 3 (perilipin 3D), where the negative curvature stress in the monolayer of POG, POPE and POPA allows for further insertion of amino acids into the hydrophobic interior of the lipid monolayer. In the case of LDs this may result in the interaction of the protein with the neutral lipids inside the droplet. Addition of further segments of the full-length protein to the C-terminus (resulting in perilipin 3B, perilipin 3C and finally the full length protein) results in a dramatic decrease in MIP (>10mN/m) for both POG and POPE, suggesting that the additional segments of the protein (i.e. regions of the N-terminus, including the 11-mer repeats (see below)) results in less insertion of the protein into a phospholipid monolayer of neutral or zwitterionic lipids.

This is a puzzling finding since energetically the higher MIP value for the C-terminus suggests that its interaction is more favorable than that of the full-length protein even though the full-length protein still contains the C-terminus. What could explain such perplexing behavior? One possibility is the following: The addition of N-terminal regions of the protein to the C-terminus might sterically hinder the interaction of the C-terminus with the phospholipid monolayer. The region between the α/β domain and 4-helix α-helix bundle is designated as the hydrophobic cleft [3]. This region was suggested to be involved in lipid binding and it is possible that addition of N-terminal sequences close to this region affects the folding and rearrangement of the amphipathic α-helix bundle. Yet, in vivo, the hydrophobic cleft was shown to be dispensable for LD targeting [1]. Another possibility is that, instead of steric hindrance, the addition of amino acids from the N-terminus (35 amino
acids are sufficient to essentially maximize the effect) increases the affinity of the C-terminus for the aqueous phase. Clearly this finding needs to be further explored.

The exception to the decrease in MIP observed for POG and POPE is the lipid POPA, which in addition to having negative spontaneous curvature carries a negative charge in its headgroup. The charge of PA is usually considered to be -1 in membranes but can easily reach -2 under favorable environmental conditions (pH, presence of hydrogen bond donors, e.g. the lipid PE [58, 63]). The charge of PA will also be stabilized at -2 when it interacts with basic amino acid residues via the electrostatic hydrogen bond switch model [63]. The preference for insertion of perilipin 3 into a PA monolayer does not diminish (surface pressure stays around 34mN/m) with the addition of 35 amino acid residues to perilipin 3D (perilipin 3C, see Figure 4). The MIP for perilipin 3B and the full-length protein (perilipin 3A) does reduce to 29 and 26 mN/m respectively for the POPA monolayer (see Figures 5 and 6). The effect of negative charge is also reflected in the $\Delta \pi_{\text{max}}$ values, qualitative measures of lipid affinity that are highest for POPA for all four constructs investigated.

4.5.2. Negative charge influences the recruitment of perilipin 3 to the LD surface

The effect of charge is not novel in LD-associated proteins. For caveolins it was shown that a cationic domain close to a hydrophobic region is required to sort the protein to LDs in the ER [21]. Attaching these domains to non-LD binding proteins conferred to them LD localization [21].
How can the effect of negative charge be explained for perilipin 3? Since all constructs show sensitivity to anionic lipids we hypothesized that cationic residues in one or more of the amphipathic α-helices of the C-terminal domain of perilipin 3 facilitated the electrostatic interaction of the protein with the monolayer. Figure 8 shows the amino acid sequence and helical wheel representation [64] of all four helices (alignment taken from Hickenbottom et al 2004 [3]) in the α-helix bundle of human perilipin 3, together with the calculated value for the hydrophobicity and hydrophobic moment of each of these helices [64]. The first α-helix of the bundle is highly charged and is in fact not amphipathic (see Figure 4.8: Helix wheel representation of the 4 α-helices in the amphipathic helix bundle (C-terminus) of perilipin 3. To test for electrostatic and hydrophobic interactions, each helix is graphed separately. Note that only the first helix has a net (positive) charge, while the other three are neutral. In helices 2, 3, and 4, positively charged amino acids (R and K; R and R; and R and R respectively) bound a hydrophobic lower face.
Figure 4. 8). It contains 9 cationic and 4 anionic amino acid residues whereas the additional helices show an equal or lower number of cationic and anionic residues. In solution at neutral pH, helix 1 is likely to have a net charge of +3 as the two Histidine residues are likely to be neutral (depending on local electrostatic environment in the protein structure and solution pH).

We propose that this substantial charge facilitates the initial electrostatic interaction with the lipid monolayer. This charge can potentially increase to +9 when we consider the following: An anionic lipid interface has a local surface pH that is considerably (potentially by 2 pH values or more) lower than that found in the bulk solution [65]. At low pH the Histidine residues in helix 1 will be positively charged and the anionic residues, Aspartate and Glutamate, might protonate and become neutral. The fact that Histidine residues can act as a switch in local protein structure upon interaction of lipid binding domains to an anionic lipid interface is well known [66, 67], but the likelihood of Asp and Glu protonation is often overlooked (Ben de Kruijff, personal communication). While helix 1 of the amphipathic α-helix bundle carries the largest concentration of charge in the C-terminus (again it is barely amphipathic), the additional helices are considerably more amphipathic as evidenced by larger positive values of the hydrophobic moment (Figure 4.8). However, protonation of Asp and Glu residues in the α-helices of the helix-bundle near anionic lipid interfaces can potentially contribute to the effect of negatively charged lipids on perilipin 3 binding to a lipid interface.
4.5.3. **Negative charge and negative spontaneous curvature both contribute to lipid binding for the C-terminus of perilipin 3.**

For the C-terminal domain, negative charge is not the only driving factor for monolayer insertion as the MIP and $\Delta \pi_{\text{max}}$ for POPG (anionic) are identical to those for POPC (zwitterionic), and both lipids have very small spontaneous curvatures (i.e. induce essentially no negative curvature stress in a lipid monolayer). Coupled with the results for POPA, POPE, and POG this thus suggests that indeed negative spontaneous curvature is important as well. This is also exemplified by the MIP and $\Delta \pi_{\text{max}}$ values for the full-length protein (perilipin 3A, see Fig 4. 6) where the $\Delta \pi_{\text{max}}$ is highest for the anionic POPA (which combines both negative charge and negative spontaneous curvature), but the MIP is significantly higher for neutral POG, although both induce negative curvature stress.

While the effect of positive charge (highest $\Delta \pi_{\text{max}}$ value) in the protein is largest for POPA (constructs perilipin 3B and 3C also show higher $\Delta \pi_{\text{max}}$ for POPG), hydrophobic interactions, as exemplified by the role of negative spontaneous curvature, is thus important as well. While LDs are covered by mainly a phosphatidylcholine monolayer, it is known that PC interfaces have a slight negative charge (PC vesicles show a negative zeta potential [68]). Furthermore the structure of the lipid monolayer surrounding LDs is not known and it is likely that patches of neutral lipids become exposed at least part of the time to the aqueous environment [69]. Oil (hydrophobic) interfaces in water are known to be negatively charged due to the accumulation of hydroxyl ions from solution [70]. We demonstrated this previously in a study on oil droplet fusion. Pure triolein droplets in water
do not want to fuse (supplementary data to reference [71]) and screening by salt dramatically reduced the docking time (time before fusion takes place) of two triolein droplets. The role of these observations for the interaction of perilipin 3 with LDs is unknown.

4.5.4. Recruitment and Targeting of Perilipin 3

Multiple studies have addressed the recruitment and targeting of perilips to the LD surface. Cells expressing perilipin 1, 2, and 3 show perilipin 3 localized on small droplets at the cell periphery. Increasingly larger LDs become coated with perilipin 2, and eventually the largest LDs at the center of these cells contain predominantly perilipin 1 [40]. The reason for this distribution of perilips is unknown but may be due to maturation of LDs and movement to the cell center. LDs are dynamic organelles and much is still unclear concerning their lipid composition and how this changes during the lifecycle of a single droplet.

There is no universal agreement on the exact region (amino acid sequence) on perilips required for recruitment and targeting to the LD surface. However, an overview of the literature suggests that amphipathic $\alpha$-helices in the N-terminus of the protein are necessary and sufficient [1, 25, 28]. This region overlaps with the so-called 11-mer repeat region. The 11-mer repeats found in the N-terminus of perilipin 3 are imperfect, in that the sequence homology within the repeating sequences is not as conserved as in the repeats found in perilipin 4 [33].

Different authors have indicated different stretches of amino acids as belonging to these so-called 11-mer repeats [3, 28]. Careful examination of the perilipin 3 sequence
suggests that the imperfect 11-mer repeats span the entire region from amino acid 73 to amino acid 204. Supplementary Figure S7 shows the helical wheel representations (assuming that this part of the protein indeed folds into α-helices) of this region, both represented as a perfect α-helix as well as a 3/11 α-helix (3 full turns per 11 amino acids). We show both these conformations since the amphipathic α-helices of the 11-mer repeats in apolipoproteins and α-synuclein were proposed to adopt this 3/11 conformation [34]. Comparison of the hydrophobic moment of these helices with those of the α-helix bundle shows that they are comparable. What is striking is the presence of 4 proline residues in the first part of the 11-mer repeat like region. The role of these residues is unknown but is likely to affect the secondary structure of this segment of the protein. If folded in an amphipathic helix or helices, most of the N-terminus could potentially interact with a (phospho)-lipid monolayer via hydrophobic interactions. Indeed, we now show that the ∆π_max (a qualitative measure for the affinity of the protein for the lipid monolayer) increases upon addition of N-terminal sequences (perilipin 3C, B, and the full-length protein). This agrees with the cell based studies that show that the N-terminus is necessary and sufficient for LD targeting and binding [1, 25, 28]. For example, compare the insertion isotherms (∆π_max) for perilipin 3D with that of perilipin 3C and 3B. Interestingly, inclusion of the entire N-terminus, i.e. the so-called PAT domain (perilipin 3B and C compared to the full length protein) decreases ∆π_max again, at least for most of the lipids investigated (the only exception to this decrease in ∆π_max is the lipid POPA which combines negative charge with negative spontaneous curvature, i.e. access to the hydrophobic interior of the droplet.) The PAT domain was named after the founding members of the perilipin family, perilipin 1
through 3, and is based on the old names for these proteins. The name PAT-domain has since been used for the N-terminus of these proteins and constitutes a region of ~100 amino acids that is conserved among the perilipin family members, except for perilipin 4 [40, 72]. In human perilipin 3 the PAT domain is located between amino acid 21 and amino acid 120, and thus includes a significant part of the 11-mer repeat region (see Figure 1 and discussion above) [72, 73].

The function of the C- and N-terminal domain of perilipin 3 is mostly unknown. Our data clearly suggest that each of these domains contributes separately and independently to lipid binding. The N-terminus with net positive charge (see above and Fig 4.8) is likely responsible for initial recruitment to negatively-charged lipid interfaces. Once the initial interaction commences, it is the N-terminus that takes over lipid binding and firmly anchors the protein to the phospholipid-oil interface of the LD. Since the MIP of the full-length protein is so much lower than the MIP of perilipin 3D for POG, POPA, and POPE, one possible scenario is that as soon as the N-terminus of the protein interacts, the C-terminal α-helix bundle detaches from the LD surface. Another possibility is that the α-helix bundle is easily displaced from this surface by additional perilipin 3 molecules that bind. Our data suggests also that the full-length protein, while electrostatically attracted to anionic lipid interfaces, does not bind to other membranes inside the cell as the MIP is lower than that expected for a lipid bilayer [56, 57].

4.5.5. Role of phospholipid saturation on perilipin 3 LD interaction.

Previously, we showed that the insertion of the amphipathic α-helix bundle protein apoLp-III from Locusta migratoria is sensitive to the saturation of lipid acyl chains.
Replacing the palmitic acid from the sn-1 position with an oleic acid decreased the insertion of apoLp-III for each of the lipids investigated [50]. We now show that the helix bundle domain of perilipin 3 (perilipin 3D) is also acutely sensitive to the saturation of the phospholipid acyl chain. The di-oleoyl lipids DOPC, DOPE, and DOPA showed a dramatic decrease of MIP by more than 10 mN/m compared to the PO species. This decrease is much more significant than what we observed for apoLp-III.

What are the biological implications of these findings? Very little is known about the phospholipid composition of the lipid monolayer surrounding lipid droplets [16, 17]. What has been published shows great differences in lipid composition. Tauchi-Sato et al. show that lipid droplets contain a significant fraction of lyso-lipids, something not observed for biomembranes [74]. On the other hand, Storey et al. show that LDs containing mainly perilipin 1 have phospholipids with more saturated fatty acids while LDs containing mainly perilipin 2/perilipin 3 have phospholipids with more unsaturated fatty acids [16]. The differences between these two studies are perhaps not surprising as the phospholipid composition may depend sensitively on cell type and maturation state (i.e. function) of the LD. Thus, the phospholipid composition is likely to vary spatially and temporally in the cell. Previous work by the Atshaves group seems to suggest that lipid droplets coated with perilipin 3 contain a lipid monolayer with more unsaturated chains than the pool of LDs that are primarily coated with perilipin 1 [16]. If this is indeed the case, then it is unlikely that the amphipathic α-helix bundle of perilipin 3 is bound to the phospholipid monolayer. Future work will investigate the role of lipid acyl chain saturation for the full-length protein.
4.6. Conclusion

The results, discussed above, raise a number of substantial questions that need to be addressed before the interaction of perilipin 3 with LDs can be fully understood. Our data appear to be at odds with those from cell biological studies. For example, we find that the C-terminus of perilipin 3 has a higher maximum insertion pressure, MIP, than any of the other constructs tested, including the full-length protein. On the other hand, cell biological work shows that the C-terminus is not required for targeting and binding to LDs [1, 28]. Additionally, the C-terminus interacts less favorably with unsaturated lipids but Atshaves et al. appear to show that perilipin 3 is enriched on LDs with a more unsaturated phospholipid monolayer [16].

Can these findings be reconciled? One possibility is that indeed the C-terminus does not bind to the phospholipid monolayer surrounding the lipid droplet [28]. However, in light of our data we find this possibility unlikely. Bulankina et al. show that both the C-terminus as well as the full length protein are able to transform lipid vesicles (of DMPC and DMPG at or very near their main phase transition temperature) into lipid discs [1]. This clearly indicates that the full length protein as well as the C-terminus interacts with lipid interfaces. Also, the amphipathic α-helix bundle domain is the only domain present in the insect protein apoL-III, whose function in lipophorin stability and thus lipid interaction is extensively studied and well-established [48, 75-78]. When perilipin 3 first interacts with the surface of a LD, it is likely that the amphipathic α-helix bundle domain opens up so that the hydrophobic side of the 4 helices is able to insert into the phospholipid interface, identically to what has been described for apoLp-III.
What is not clear is the exact role this binding and opening up of the helix bundle has for the function of the full-length protein. One possibility is that the amphipathic α-helix bundle domain further stabilizes the interaction of the protein with the phospholipid-neutral lipid (i.e. triacylglycerol and cholestereoler) interface and further mediates the exchangeability of the protein, as has been proposed by Narayanaswami and Ryan [79]. The ability to exchange between the cytosol and LD surface might be mediated by the saturation of LD (phospho)lipids and could thus be related to maturation of the LD. We show that the interaction of the amphipathic α-helix bundle domain is less favorable for unsaturated lipids than it is for saturated lipids. In fact, the amphipathic α-helix bundle is not likely to insert at all into a fully formed PC monolayer with unsaturated acyl chains as the MIP for this interaction is well below 30 mN/m.

It is also possible that the interaction partner for the amphipathic α-helix bundle domain is not the phospholipid monolayer at all but the neutral lipids underlying this monolayer. Further work on the full-length protein and more sophisticated model systems (a buffer-phospholipid-oil interface) are needed to address these questions and are currently underway.

References


CHAPTER 5

INTERACTION OF A MODEL APOLIPOPROTEIN, APOLP-III, WITH AN
OIL-PHOSPHOLIPID INTERFACE

5.1. Abstract

Lipid droplets are “small” organelles that play an important role in de novo synthesis of new membrane, and steroid hormones, as well as in energy storage. The way proteins interact specifically with the oil-(phospho-)lipid monolayer interface of lipid droplets is a relatively unexplored but crucial question. Here, we use our home built liquid droplet tensiometer to mimic intracellular lipid droplets and study protein-lipid interactions at this interface. As model neutral lipid binding protein, we use apoLp-III, an amphipathic α-helix bundle protein. This domain is also found in proteins from the perilipin family and in apoE. Protein binding to the monolayer is studied by the decrease in the oil/water surface tension. Previous work used POPC (one of the major lipids found on lipid droplets) to form the phospholipid monolayer on the triolein surface. Here we expand this work by incorporating other lipids with different physico-chemical properties to study the effect of charge and lipid head-group size. This study sheds light on the affinity of this important protein domain to interact with lipids.

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3 This chapter is submitted as an article to BBA.
5.2. Introduction

Vertebrate lipoprotein (VLDL, LDL, and HDLs) and insect lipophorin share structural features with intracellular lipid droplets (LDs), namely a neutral lipid core covered with a phospholipid-protein monolayer [1-4]. To date there is little understanding of how the structure and interactions of protein and lipids at this oil/aqueous interface relates to lipid droplet function. Here we consider one of the simplest such proteins and how the presence of mixtures of lipids with different physicochemical properties affects the attraction of the protein to the oil/water interface and its insertion there.

Proteins associated with the surface of vertebrate lipoproteins (apolipoproteins) and LDs (e.g. proteins from the perilipin family) have many similarities with the proteins (apolipophorins) coupled to the lipophorin surface [5-7]. One recurring structure in these neutral lipid particle-binding proteins is the amphipathic α-helix, and more specifically an amphipathic α-helix bundle. For example, apoLp-III, apoE, and perilipin 2, 3, and 5 all have such a helix bundle [8].

In the case of insect lipophorin the protein matrix on the surface of the particle consists of apolipophorin-I and II (apoLp-I and II), which are two nonexchangeable proteins, and apoLp-III, an exchangeable apolipoprotein [9, 10]. In the same way, vertebrate lipoproteins have both non-exchangeable and exchangeable apolipoproteins but the diversity of proteins is considerably more complex [1, 11, 12]. (V)LDL has as matrix (non-exchangeable) protein apoB-100, while a good example of an exchangeable apolipoprotein is apoE [13]. Lipid droplets appear to have a similar complement of non-exchangeable and exchangeable LD binding proteins, e.g. the proteins of the perilipin family. Of the perilipins, perilipin 1,
which is found mainly in adipocytes, and perilipin 2, which is ubiquitously expressed, are considered to be non-exchangeable, and perilipin 3, 4, and 5 exchangeable [14-17]. It is believed that the amphipathic α-helical bundles in exchangeable neutral lipid particle-binding proteins play an important role in the reversible binding of the particle surface [7, 18, 19].

Here we use apoLp-III, the insect exchangeable apolipoprotein, from Locusta migratoria, as a model apolipoprotein to study how amphipathic α-helix bundle proteins interact with a neutral lipid interface. We use apoLp-III for several reasons: (a) The structure of apoLp-III consists of five amphipathic α-helices that form the helix bundle and thus the entire protein is an α-helix bundle. The helix bundle of apoLp-III is similar to the structure of the N-terminus of apoE and C-terminus of perilipin 3 [7, 20-23]. The fact that the entire apoLp-III protein forms an α-helix bundle makes data interpretation simpler than if we used full-length apoE or perilipin 3. (b) ApoLp-III is an exchangeable protein, like apoE and perilipin 3, and is found on the surface of lipophorin particles in insect hemolymph. (c) Investigations of apoLp-III and apoE have shown that these proteins undergo a conformational change upon binding to a lipid interface, which results in unfolding of the amphipathic α-helix bundle of the protein [18]. This opening of the helix bundle exposes the hydrophobic side of the α-helices and facilitates protein interaction [24-26]. (d) Expression and purification of apoLp-III is straightforward and large quantities of the protein are available, which is a prerequisite for our biophysical experiments. (e) And lastly, to the best of our knowledge, the interaction of a full length protein with an oil-
phospholipid-water interface as a function of phospholipid composition has not yet been studied.

The group of Donald Small has studied apolipoproteins at the neutral lipid interface, although not the interaction of a model amphipathic α-helix bundle protein like apoLp-III. For example, Mitsche, Small and coworkers investigated the interaction of several peptides derived from apoB-100 and concluded that the amphipathic β strands of the protein anchor it to the oil interface, whereas the amphipathic α-helix domains of the protein are able to desorb and resorb upon compression and expansion of the surface respectively [27, 28]. Additionally, the Small group extensively studied peptides derived from apoA-I as well as the full length protein. Mitsche and Small concluded that the C-terminal peptide of apoA-1 is able to remove phospholipid from the water/phospholipid/oil interface while the N-terminal peptide is not [29]. Also, Meyers et al. investigated amphipathic α-helix peptides of apoC-1 as well as the full length protein and concluded that the full length protein has a stronger interaction with the phospholipid-coated oil interface than with a neat oil surface [30, 31].

Additionally, all of these previous studies were done with a phospholipid monolayer consisting of a single phospholipid, namely phosphatidylcholine (PC). The phospholipid content of the lipid monolayer that coats neutral lipid particles is still poorly explored, but while PC is the most common phospholipid found, it is certainly not the only one[32-34]. Additionally, it is well known that specific lipids facilitate protein binding to membranes, but for LD binding proteins this is entirely unexplored in systems utilizing an oil-aqueous (physiologically relevant) interface. Also, in order to understand how lipid composition
effects protein interaction, the possible range of lipid properties must be taken into account [35, 36]. Important parameters to vary are lipid (spontaneous) curvature, and charge-hydration of the lipid headgroup. Lipids with negative curvature, i.e. lipids with a small headgroup area compared to its acylchain are (so called Type-II lipids), are known to facilitate membrane protein interaction [37]. Specifically, proteins with amphipathic α-helices are better able to insert the hydrophobic side of an amphipathic α-helix into the lipid headgroup-acylchain interface.

Here we use our home-built liquid droplet tensiometer to study the interaction of our model protein, apoLp-III, with phospholipid monolayers at aqueous-phospholipid-oil interfaces. The aqueous phase is either very pure water or a buffer, which allows us to test the importance of long-range electrostatic interactions to both adsorption and insertion. Oil (triolein) droplets were coated with a phospholipid monolayer formed by 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC). PC is a major component of the phospholipid monolayer covering the neutral lipid core of lipid droplets and has been used by others to mimic the phospholipid monolayer covering neutral lipid particles [35, 38, 39]. Identical experiments were then performed with lipid mixtures containing POPC and increasing concentrations of POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine), POG (1-palmitoyl-2-oleoyl-sn-glycerol), and POPA (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (sodium salt)). We choose PE, DAG, and PA based on the currently available mass spectrometry data of (phospho)lipid species present in the LD lipid monolayer, and to systematically change lipid curvature and charge. Our results are
discussed in terms of lipid adsorption and protein insertion as a function of charge screening, i.e. electrostatic shielding.

5.3. Materials

5.3.1. Protein:

Our model protein, recombinant apoLp-III, was a kind gift from Prof. Paul Weers from CSU-Long Beach, and was prepared as described previously [40]. ApoLp-III was expressed and purified as published [41] and provided to us as a powder. A stock solution of the protein was made by adding 1 ml of HPLC grade water to 1 mg of protein, to set the final concentration at 1 mg/mL. The protein concentration was also checked by Nanodrop 1-position spectrophotometer (ND-2000) instrument.

5.3.2. Lipids:

POG (1-palmitoyl-2-oleoyl-sn-glycerol), POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine), POPA (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (sodium salt)) were purchased from Avanti Polar Lipids (Alabaster, Alabama). Triolein glyceride was purchased from NU-Check Prep (MN). Thin layer chromatography was used to regularly test the purity of lipids. For polar lipids, we use a solvent consisting of CHCl₃/ CH₃OH/H₂O (64 ml /25 ml /4 ml) and for neutral lipid we used C₆H₁₂/ (C₂H₅)₂O (10:1).
5.3.3. Chemicals:

KCl, EDTA and Tris were purchased from Sigma Aldrich Co. All the chemicals were > 99% pure. HPLC grade water was purchased from Fisher Scientific (product # WSK5)

5.4. Methods:

5.4.1. Buffer:

Tris buffer was made using 150 mM KCl, 10 mM Tris and 0.2 mM EDTA, carefully mixed in HPLC grade water. The buffer for all experiments had a pH set (with ultra-pure HCl) to 7.20 ± 0.05. All salts were annealed under vacuum for 18 hours at 250 °C.

Through the process of developing our set-up, we looked at the dependence of interfacial tension of an oil droplet on the ion type and concentration. Initially we observed poor reproducibility and subsequently found several reports on contamination of inorganic salts with organics [42, 43]. These studies suggest treating salts to remove these contaminants. We repeated our experiments with annealed salts, and our results indeed showed that heating up salts increased the reproducibility (supplementary data Figure. 1).

After the buffer was prepared, it was kept in the experiment room (21.4 ± 0.2 °C) to minimize temperature fluctuations and the accompanying density variations (see liquid droplet tensiometer section). All glassware was cleaned using KOH cleaning solution (164 gr ethanol, 25 gr water and 24 gr KOH).
5.4.2. Vesicle formation:

Vesicles were made by a standard procedure [44]. First lipid films were made in a borosilicate glass test tube by drying specific volumes of lipid in organic solution (chloroform or chloroform/methanol) under a stream of Nitrogen. The film was kept under vacuum for 12 hours to remove residual traces of organic solvent and was stored at -20°C before use. Prior to vesicle formation, the lipid film was moved to room temperature for about 20 minutes. In order to form small unilamellar vesicles (SUV), 4 ml of ultra-pure water was used to solubilize the film. The mixture was sonicated for ~45 minutes in a cylindrical sonicator (Avanti, Alabaster). DLS (differential light scattering, Horiba DLS 7100, SZ -100 series) was used to measure vesicles size. Normally the vesicles prepared this way were around 70 nm in size. For some of the experiments, large unilamellar vesicles (LUVs) of ~100 nm were used. These were prepared with the Avanti liposome extruder (Liposomes used for making monolayer in buffer were produced by using extruder)(Avanti; Alabaster, AL). Lipid absorption to the oil surface showed no significant difference between the 70 and 100 nm liposomes.

5.4.3. Liquid Droplet Tensiometer:

Our homebuilt tensiometer (see Figure 1) is a combination of CCD camera (Pixelink PL-B776F), Light source (Thorlab), diffuser (Thorlab), syringe (Hamilton) with a J-shaped needle, and cuvette (made from borosilicate glass, supplied by Wale Apparatus PA., at the machine shop located in the Department of Chemistry, Kent State University).
The set-up is cleaned regularly using pure (HPLC grade) methanol to remove any organic residues. Syringe and needle are rinsed a minimum of 3 times with chloroform for cleaning.

The cuvette is cleaned using KOH solvent (164gr MeOH, 24 gr Water and 25 gr KOH), and rinsed with DI-water and ultra-pure water a minimum of three times each. Two pumps are involved, one to vacuum water/buffer from cuvette and the other one to fill the cuvette with fresh buffer/water.

A typical experiment is shown in Figure 2. The cuvette is filled with 10 ml of water or buffer and a triolein droplet is formed at the end of the J-shaped needle (point 1 in the Figure). The droplet is allowed to stabilize in the aqueous solution for at least 5 min prior further experimentation. At point 2, liposomes of either POPC, or POPC mixed with 10, 20, or 40 mol% of POPE, POG, or POPA, are added. This is done by the removal of 4 ml of water/buffer from the top of the cuvette after which 4 ml of liposome solution, approximately 0.115 mM, is added cautiously. Deposition of the self-assembled phospholipid monolayer at the aqueous-oil interface is then followed by the automatic
capture of images (discussed further below) from time point 2 until 3 (where the spontaneous formation of a lipid monolayer has reached equilibrium). At point 3, the remaining vesicles are washed out by carefully flushing fresh water/buffer (50 mL in 40 min) through the cuvette. Buffer/water enters the bottom of the cuvette through a needle connected to a flushing pump. To keep the volume of the solution in the cuvette constant, a vacuum pump removes excess water/buffer from the top of the cuvette (see Figure 1).

Figure 5. 2. Typical experiment: a triolein droplet was formed at the end of the J-shaped needle. After droplet stabilization with the media (1), 4 ml of vesicles was added to the cuvette cautiously (2). For 30 min the lipid vesicles were incubated to allow the vesicles to form a phospholipid monolayer around the triolein droplet. $\pi_L$ is the difference in tension between clean and lipid coated triolein surface. The rest of vesicles were removed from the cuvette by carefully flushing with fresh water/buffer (50 mL in 40 min) (3). Fresh water/buffer entered the bottom of cuvette through a needle connected to flushing pump. To keep the volume of the solution in the cuvette constant, there is a vacuum pump that removes excess water/buffer from the top of the cuvette. 0.1 nmol/L of protein was added
to the cuvette (4). $\Delta \pi_p$ is the difference in tension between the surface coated with lipid/clean surface and surface with bound protein.

Note that the interfacial tension does not change during the removal of remaining liposomes (between 3 and 4). At point 4, a total of 0.1 nmol/L of protein (determination of this concentration is further discussed with the results) is added to the cuvette and left to interact with the interface until the interfacial tension again reached equilibrium. The following values for lipid, and protein interaction (absorption) are now derived for each of these experiments, as shown in Figure 2, and are collected in Figures 4 through 6. The first value of importance is the steady state decrease in interfacial tension upon the formation of a phospholipid monolayer. In other words, this decrease, called the lipid surface pressure, $\pi_L$, indicates the amount of phospholipid absorbed to the interface. The decrease in interfacial tension resulting from the interaction of the protein is then denoted as $\Delta \pi_p$ i.e. the pressure change due to insertion of the protein into the oil or phospholipid-oil interface.

The surface tension, volume, droplet area and radius at apex are determined using the ADSA code package developed by the Neumann group at the University of Toronto [45]. For a typical experiment, 1500 images are captured and analyzed simultaneously. To initiate the image analysis process, we have to provide the software with some physical properties of the oil droplet. The vertical coordinate of the points (in pixels) where the drop is attached to the needle is marked. Gravity elongates the droplet whereas interfacial tension forces the droplet towards a circular shape. The density difference between the droplet and the environment and gravity are thus parameters that are provided to the ADSA code. The code uses the 4th order Runge-Kutta method to solve and fit the droplet profile with the Young-Laplace equation and calculate the interfacial tension [46]. The interfacial
tension is calculated for random points on the droplet profile. The ADSA code averages over all the values calculated for the interfacial tension to generate the final value for interfacial tension. The image analyzing procedure is repeated for all acquired images. The interfacial tension between water and triolein (oil) measured with our apparatus was 32.6 ± 0.5 at 22°C, which agrees well with values found in the literature [47].

All experiments were run first in water and later in physiologically relevant buffer, i.e. 150 mM KCl, 10 mM Tris, and 0.1 mM EDTA set to pH 7.20 ± 0.05.

5.5. Results
Figure 5.3. Looking at the surface activity of different concentrations of the protein in water. It shows that, all the concentrations are highly surface active and interact with the oil interface; however, the rate of protein interaction is higher for larger concentrations.

We began our study by investigating the surface activity of apoLp-III at the oil/water interface, Figure 3. Different concentrations (0.06, 0.08, 0.09, and 0.11 nM) of the protein were added to the ultra-pure water subphase and the interfacial tension was measured as a function of time. The blue (dashed) curve refers to the lowest protein concentration investigated, 0.06 nM.

Here the surface tension decreases at a very slow rate. With an increase in protein concentration, we notice a much faster reduction in surface tension.

For these higher concentrations (0.08, 0.09, 0.11 nmol/L) of apoLp-III, the interfacial tension suddenly falls after protein addition, followed by gradual stabilization over time. The fast reduction in the interfacial tension indicates rapid protein binding and incorporation into the oil/water interface. The steady state tension after this rapid drop reveals that no additional protein is binding to the oil interface so that it has reached its maximum insertion to the interface. For our further experiments, we use 0.1 nmol/L of protein apoLp-III. Note that the change in interfacial tension in this case is ~20 mN/m (also see Figure 3(b)) which represents a ~60% reduction in the interfacial tension of the oil-water interface. This is a significantly higher reduction in interfacial tension than found for the self-assembled Gibbs monolayer for the protein at the air/water interface, ~14 mN/m or ~19% decrease in the interfacial tension of a neat water interface [40].
5.5.1. **PE facilitates protein insertion in the phospholipid-oil interface at the oil-buffer interface**

Next, we continued by forming a fresh oil drop in pure water and injecting lipid vesicles (SUVs) to form a lipid monolayer on the surface of the oil droplet. All lipid vesicles contain PC. Figure 4 (a) shows the decrease in tension after POPC vesicle adsorption to the oil/water interface and formation of a self-assembled phospholipid monolayer on the water/oil (triolein) interface. The decrease in tension, i.e. $\pi_L$, is $\sim 7.5$ mN/m, or a reduction in oil droplet interfacial tension of $\sim 23\%$, much less than that achieved by the protein ($\sim 60\%$; see Figure 3). The addition of PE to the lipid monolayer mixture reduces values of $\pi_L$, suggesting that less lipid is absorbed to the interface in the pure water subphase. Table 1 (a) shows these data for water. $\pi_L$ decreases from 7.49 to 2.93 mN/m, with increases in PE concentration from 0 to 40 mole %.
Figure 5. Interaction of apoLp-III with aqueous-(phospho-)lipid-oil interfaces, effect of phosphatidylethanolamine (PE). $\pi_L$ is the difference in tension between clean and lipid coated triolein surface. The data recorded in water are represented by open circles. a) Decrease in surface tension due to adsorption of lipid onto the triolein surface in ultra-pure water. b) Decrease in surface tension due to adsorption (insertion) of apoLp-III into the lipid-oil interface. The same experiments were performed in buffer. Closed circles represents the measurements in buffer. c) and d) are same measurements but now conducted in a buffer solution of 150 mM NaCl, 10 mM Tris, and 0.1 mM EDTA at pH 7.2. In both C and D the total change in tension due to lipid and protein is indicated as well. POPE in POPC, in red (open diamonds) in Figure 4 (b). Green data points (i.e. open squares) gives the total decrease in interfacial tension, $\pi_L + \Delta \pi_p$, on addition of lipid vesicles and protein interaction with the lipid coated interface.

These data points (last column Table 1a) show that the total reduction of the interfacial tension is close to the same whether the protein interacts directly with the triolein
interface or after a POPE-containing monolayer is deposited. Interestingly POPC by itself appears to result in a reduction of protein interaction with the oil interface in the pure water subphase.

Table 5. Maximum change in monolayer pressure $\pi_L$, and maximum insertion pressure $\Delta \pi_p$ derived from data shown in Figure 4 for PC:PE mixture interaction with apoLp-III. a) in water, b) in buffer

Next, we consider electrostatic screening by incorporating physiological pH and salt concentration, namely a pH 7.2 buffer containing 150 mM KCl. Note that for these experiments we had to be extremely careful with the preparation of our buffer. Most commercial buffer components and salts contain significant (for the purposes of our experiments) amounts of organic and surface active contaminants that need to be removed before buffer preparation. We hence baked our salts as discussed in the methods section.

Figure 4 (c) and Table 1b show that a physiological salt concentration significantly increases lipid adsorption to the interface. In the case of pure POPC the difference from the pure water case is 3.5 ± 0.2 mN/m and increases to 15 ± 4 mN/m for the 40 mol% POPE-containing monolayer. In buffer, the increase of PE concentration in the lipid mixture thus leads to significantly more lipid adsorption to the interface, in contrast to $\pi_L$ values measured in pure water.
Unlike for the pure water subphase, we do not find a PE concentration-dependent increase in $\Delta \pi_p$, i.e. for PC:PE mixtures $\Delta \pi_p$ values are close, Figure 4 (d). This implies that a similar amount of protein interacts with the oil-phospholipid/buffer interface for all PC:PE mixtures, Table 1 (b). $\Delta \pi_p$ values are still significantly less than $\Delta \pi_p$ found with no lipid on the triolein interface (21.2 ± 1.2 mN/m), identical to what we observed for the water subphase. In contrast to $\pi_L + \Delta \pi_p$ values in pure water, $\pi_L + \Delta \pi_p$ values in buffer systematically increase as a function of PE concentration.

5.5.2. Diacylglycerol increases protein insertion in the phospholipid-oil interface in water and buffer subphase

Diacylglycerol, like PE, has negative spontaneous curvature, but carries no charge compared to zwitterionic PE [48, 49]. In Figure 5 (a) and (c), we looked at the effect of POG (1-palmitoyl-2-oleoyl-sn-glycerol) on the formation of a self-assembled phospholipid monolayer on the triolein interface in water (open black circles) and buffer (closed black circles) respectively. POPC data is taken from Figure 4. In the first set, we use 10 mol% POG to investigate its effect on liposome binding to the oil droplet in water, Figure 5 (a). $\pi_L$ for POPC:POG (9:1) changes dramatically compared to $\pi_L$ for pure POPC liposomes. Interestingly, $\pi_L$ decreases for higher concentrations of POG to approximately the same value observed for pure POPC liposomes (Table 2 (a), indicates that $\pi_L$ decreases from ~18 to 7 mN/m). We then tested lower concentrations of POG (see supplementary data Figure. 2) and did not see a significant change in liposome absorption for lower POG concentrations compared to ones above 10 mol%. We observed essentially the same
behavior for POPC:POG liposome fusion in the buffer subphase, i.e. we observe significantly more absorption of the 10 mol% POG containing liposomes than the pure PC ones, or the 20 and 40 mol% POG ones.

Figure 5. $\pi_L$ is the difference in tension between clean and lipid coated triolein surface. Open squares are used to marked the measured data in water. a) Decrease in surface tension due to adsorption of lipid onto the triolein surface in ultra-pure water. b) Decrease in surface tension due to adsorption (insertion) of apoLp-III into the lipid-oil interface. Followed by measurement in buffer, here, we used closed squares to marked the data recorded for buffer. c) and d) are the same measurements as in A and C but now conducted in a buffer solution of 150 mM NaCl, 10 mM Tris, and 0.1 mM EDTA at pH 7.2. In both C and D the total change in tension due to lipid and protein is indicated as well.

Followed our experiments by protein insertion; see Figure 5 (b). For the 10% POG mixture there is less protein binding (insertion) to the interface compared to the uncoated
triolein droplet directly interacting with the protein in water. Protein insertion is higher in this case than that observed for the POPC-covered oil interface, and we find a POG concentration-dependent increase in $\Delta \pi_p$ for all POG concentrations, starting at 10 mol% POG (5.5, 10.5, 16.1, and 19.1 mN/m for 0, 10, 20, and 40 mole% POG respectively). Values for $\pi_L + \Delta \pi_p$ are shown in green (open and closed squares) and demonstrates that the largest interaction of the self-assembled lipid and protein monolayer occurs for 10 mol% POG in the water subphase.

<table>
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<th>a)</th>
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Table 5.2. Maximum change in monolayer pressure $\pi_L$, and maximum insertion pressure $\Delta \pi_p$ derived from data shown in Figure 5 for PC: POG mixture interaction with apoLp-III. a) water, b) buffer.

Here the interfacial tension of the oil droplet is reduced to only a few (~4) mN/m. In buffer, see Figure 5 (d), a similar trend is observed in that increasing concentrations of POG in the liposomes result in increases in $\Delta \pi_p$ that depend on concentration. Again, for 10 mol% POG we observe an anomalously higher deposition of lipid on the oil interface, compared to pure POPC liposomes.
5.5.3. Phosphatidic acid reduces lipid absorption to the oil interface and increases protein insertion in the water but not the buffer subphase.

Previously, we showed that negative charge facilitates apoLp-III binding to phospholipid monolayers. Here we use PA to model negative charge and negative spontaneous curvature and investigate the effect of charge on lipid absorption and protein binding to the oil interface (see Figure. 6). Again, POPC data is identical to that shown in Figure 4. Compared to POPC liposome absorption, addition of POPA to the liposomes systematically decreases $\pi_L$ values from $7.40 \pm 0.12$ to $2.3 \pm 0.7$ mN/m in water and from $11.1 \pm 0.1$ to $5.1 \pm 0.9$ mN/m in buffer. This illustrates that the addition of negative charge to the liposomes reduces lipid binding to the oil/water or buffer interface, Figure 6 (a and b). Interestingly, lipid absorption, $\pi_L$, in the buffer subphase is considerably higher (almost double) when compared to the pure water subphase (compare Table 3 a and b).
Figure 6 shows that $\Delta \pi_p$ increases upon the addition of POPA to the liposomes (from $5.5 \pm 1.7$ to $15.1 \pm 1.2$ mN/m) but then quickly stabilizes upon an increase in POPA concentration in the water subphase (see Table 3a). No such PA-dependent increase in protein insertion is observed for buffer subphase (see Figure 6 (d) and Table 3b). $\Delta \pi_p$ in buffer is $\sim 11$ mN/m for pure POPC, and does not significantly increase for higher POPA concentrations. Since lipid accumulation is reduced for increasing concentrations of POPA
and protein insertion is more or less independent of POPA, \( \pi_L + \Delta \pi_p \) values for POPC:POPA mixtures are reduced compared to pure POPC.

<table>
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</table>

Table 5.3. Maximum change in monolayer pressure \( \pi_L \), and maximum insertion pressure \( \Delta \pi_p \) derived from data derived from Figure 7 for PC: PA mixture interaction with apoLp-III

a) in water and b) in buffer

5.6. Discussion

The interaction of neutral lipid particle binding proteins, i.e. apolipoproteins and lipid droplet binding proteins, with neutral lipid particles is an active and important area of research [38, 50]. Here we focus on a prevalent protein domain that is found in cytosolic, exchangeable neutral lipid particle-binding proteins; the amphipathic \( \alpha \)-helix bundle. As model protein, we use apoLp-III, which is entirely composed of such a helix bundle. We study the interaction of apoLp-III with a neat oil surface as well as with an oil surface coated with a self-assembled phospholipid monolayer of different lipid compositions. We, and others, have previously shown that lipid composition influences apoLp-III – lipid interaction [40, 51, 52]. It is well known that lipid composition influences membrane protein – lipid interactions. Hence it is crucial to change lipid composition of the (phospho-)lipid monolayer coating the oil droplet. The (phospho-)lipsids we use here were chosen based on previous lipidomics studies [32, 33, 53], and to vary the physical properties of the
lipids coating the oil surface. Phosphatidylcholine, PC, is the main phospholipid found in the monolayer surrounding intracellular lipid droplets; it is zwitterionic, and has zero spontaneous curvature. Phosphatidylethanolamine, PE, is the second most abundant phospholipid found [53]; it is zwitterionic like PC, but has negative spontaneous curvature. Diacylglycerol, DAG, is an intermediate in the synthesis and breakdown of triglycerides and present in the lipid monolayer. Diacylglycerol is neutral and has strong negative spontaneous curvature. Lastly, phosphatidic acid, which is a precursor and product of DAG metabolism, is anionic, and also has negative spontaneous curvature [54, 55].

_ApoLp-III has a stronger interaction with an oil-aqueous than an air-aqueous interface._

Interaction of apoLp-III with a neat triolein interface leads to a ~21 mN/m reduction in the interfacial tension of the oil/aqueous subphase interface (~32.5 mN/m). This represents a reduction of the interfacial tension of the oil droplet by nearly 60%. Compared to a self-assembled apoLp-III monolayer at the air-aqueous interface (a surface pressure of 14 mN/m or a 19% reduction in the interfacial tension), the much larger reduction in the oil surface tension suggests that apoLp-III has a more favorable interaction with the latter. This can be understood based on the proposed mechanism of interaction of amphipathic α-helix bundle proteins with phospholipids vesicles, i.e. the so-called disc assay [56]. In solution, the hydrophobic interior of the helix bundle is shielded from the aqueous environment. However, upon binding to lipids, apoLp-III is known to undergo a conformational change and unfold its helix bundle (but not the α-helical secondary structure) and expose the hydrophobic residues of the interior to the hydrophobic acyl chains of lipids [57, 58]. Figure 7 shows the helical wheel representations of the five
amphipathic α-helices of apoLp-III (assuming a normal α-helix, three and half amino acid residues pert turn) [59]. Helix 2, 3, 4, and 5 have at least one large hydrophobic amino acid (such as phenylalanine or tryptophan) residue on the hydrophobic side of the amphipathic α-helix. We propose that the interaction of the hydrophobic side of the amphipathic α-helices, and especially those of the large hydrophobic residues, with neutral lipids is more favorable than that with the air-aqueous interface. For example, van der Waals interactions with neutral lipids would be more favorable than simply exposing hydrophobic residues to air. We speculate that the same holds for the interaction of α-helix bundle proteins with phospholipid monolayers at the oil-aqueous interface.
Figure 5. 7. Helical wheel structure for apoLp-III [59]. Each helix is drawn separately. Hydrophobic surfaces is marked with yellow and gray circles, while, polar amino acids are displayed with red and blue colors. Table shows the value for hydrophobicity, hydrophobic moment and net charge for each helix. The protein sequence is taken from Protein Data Bank 1ls4.pdb.

Phosphatidylcholine prevents interaction of apoLp-III with the oil interface in the pure water but not the buffer subphase
Formation of a self-assembled lipid monolayer of POPC in the pure water subphase leads to a reduction in the interfacial tension of the oil droplet of ~7.5 mN/m, significantly less than that induced by apoLp-III alone. Addition of apolp-III to the subphase (after removal of excess lipid) decreases the interfacial tension another ~5.5 mN/m, leading to a final surface pressure of the POPC-apoLp-III monolayer of ~13 mN/m, significantly less than that of a self-assembled apoLp-III monolayer in the absence of POPC (~22mN/m). In pure water, the formation of a POPC monolayer at a surface pressure of 7.5 mN/m results in a reduction of apoLp-III binding and insertion into the lipid coated oil-water interface.

In buffer, however the situation is completely different. First of all the formation of a self-assembled POPC monolayer results in a significantly larger interfacial pressure for the lipid monolayer, namely 11 mN/m compared to 7.5 mN/m for water. It should be noted here that the interfacial tension of the triolein interface is not significantly affected by the physiologically relevant buffer. We will come back to this effect later in this discussion. What is striking is that the interaction of apoLp-III with this more tightly packed POPC monolayer leads to a change in the interfacial pressure induced by the protein that is twice that observed for the pure water subphase (i.e. a $\Delta \pi_p$ of 11 compared to 5.5 mN/m). Compared to the pure water subphase this appears to show that the POPC monolayer facilitates the binding and insertion of apoLp-III to the lipid monolayer coated oil interface. However, the total surface pressure of this apoLp-III-POPC monolayer is identical to that formed by just the protein at the oil interface. So, while we can clearly conclude that in pure water POPC prevents binding and insertion of apoLp-III to the lipid-coated oil interface, we cannot conclude that in buffer POPC facilitates apoLp-III binding and
insertion. An alternative explanation is that in buffer apoLp-III interacts with the partially POPC-coated oil interface more neutrally. That is, apoLp-III interacts with the oil interface and pushed the POPC away till the natural interfacial pressure of apoLp-III has been reached. This would thus suggest islands of POPC and apoLp-III at the oil-buffer interface. A model is shown in Figure 8a.

Figure 5.8. Shows the two possible models for the lipid protein interaction on the oil interface. Model a) indicates that the protein can push away the lipid molecules on the surface until it reaches the maximum insertion pressure of the apoLp-III. b) the model shows the insertion of the apoLp-III to PC:PE monolayer.

The difference in interaction of lipids and protein with the oil interface between water and buffer subphases may be understood based on simple electrostatic considerations. In buffer (pH 7.20, 150 mM KCl) the Debye length is considerably smaller than that in pure water (1 nm vs. 10 nm). In water, the electrostatic interactions are screened less than in buffer. For both lipids and proteins this should lead to a reduced packing in the self-assembled monolayer in the water subphase compared to the buffer subphase. Remember that POPC is zwitterionic in both subphases, i.e. carries both positive and negative charges, and apoLp-III has numerous charged residues on the hydrophilic side of the amphipathic α-helices.

PC is not the only (phospho-)lipid found in the monolayer surrounding LDs. Hence, we also studied lipid mixtures containing PC, and different concentrations of lipids that differed in their spontaneous curvature and charge properties.
**Phosphatidylethanolamine facilitates the interaction of apoLp-III with the lipid coated oil surface.**

Phosphatidylethanolamine is the second most abundant phospholipid on lipid droplets [33, 60] and is known to influence protein–lipid interaction. The negative spontaneous curvature (small headgroup area vs. acyl chain area) of PE facilitates insertion of proteins containing amphipathic α-helices, or those containing other, hydrophobic domains, that facilitate membrane interaction [37, 61]. Since lipids with negative spontaneous curvature facilitate protein insertion into lipid membranes, we hypothesized that a similar mechanism might function for the interaction of neutral lipid binding proteins (such as apoLp-III) with the neutral lipid particle.

In the pure water subphase, the absorption of liposomes containing increasing concentrations of PE is significantly less compared to that of pure POPC liposomes as judged by the decrease in \( \pi_L \). The opposite is observed in the buffer subphase where absorption increases as the PE concentration is increased. At 40 mol% PE the decrease in the interfacial tension of the oil interface is almost twice that compared to the pure POPC liposomes (~18 compared to 11 mN/m). The difference in Debye screening length between buffer and pure water is likely responsible for this difference. Electrostatic interactions are discussed further below.

For the pure water subphase we observe increased binding and insertion of apoLp-III as the PE concentration in the liposomes used to coat the oil surfaces is increased. This increase is not surprising as the lipid monolayer pressure on the oil interface is significantly reduced (\( \pi_L \) decreases from 7.5 to 3.8 mN/m as PE concentration increases from 0 to 10
mol%). The lower the lipid monolayer pressure, the higher the absorption (insertion) of apoLp-III into the phospholipid monolayer [40]. I.e. the energetics of protein insertion at significantly lower lipid monolayer pressures is considerably more favorable than at high lipid packing. This increased insertion may thus be explained by the reduced lipid packing, rather than specificity for PE, at least at these low concentrations of PE (up to 10 mol%).

When we compare apoLp-III insertion for 10 and 40 mol% PE, we observe an increase in \( \Delta \pi_p \) of ~5 mN/m while the decrease in \( \pi_L \) is only 0.9 mN/m, i.e. \( \pi_L \) is comparable. This seems to suggest that high concentrations of PE result in increased apoLp-III insertion. However, \( \pi_L + \Delta \pi_p \) is comparable to that of the decrease in interfacial tension induced by the protein alone (\( \Delta \pi_p \)). Similar to the POPC monolayer in buffer, POPC-POPE monolayers in pure water result in lipid-protein monolayers best explained by the model shown in Figure 8a.

We observe a similar trend for apoLp-III insertion for the buffer subphase. First there is no significant difference in protein insertion for monolayers containing 0 and 10 mol% POPE, \( \Delta \pi_p \)'s of 11 and 11.7 mN/m respectively. At 20 mol% PE we observe a higher insertion compared to that at 0 and 10 mol%, while the lipid monolayer pressure is essentially identical. This suggests that PE facilitates insertion of apoLp-III at higher concentrations. Additionally, the sum of the lipid and protein monolayer pressure, \( \pi_L + \Delta \pi_p \), is significantly higher than the \( \Delta \pi_p \) for the neat oil interface, 27 compared to 21 mN/m. This shows that apoLp-III insertion into the POPC/POPE monolayer at the oil-buffer interface is a cooperative process, see Figure 8b for a cartoon of this model. This conclusion is further strengthened by the results for 40 mol% PE. Here the protein insertion
is not significantly different from that observed at 20 mol% PE, but the lipid monolayer pressure is significantly higher. At higher lipid pressures we generally observe reduced protein insertion [40, 62]. In conclusion, at higher monolayer concentrations POPE facilitates insertion of apoLp-III into a POPC monolayer at the oil-buffer interface.

**Diacylglycerol shows anomalous absorption behavior at the oil-buffer interface.**

Absorption of liposomes containing 1-palmitoyl-2-oleoyl-sn-glycerol shows unexpected behavior. In the pure water subphase the absorption of liposomes containing 0, 20, and 40 mol% POG is essentially identical. However, at 10 mol% the lipid absorption more than doubles (from 7.5 to 17 mN/m). In the buffer subphase we observe similar behavior, i.e. highest lipid monolayer pressure for 10 mol% POG, except that the increase in lipid monolayer pressure is not nearly as high (from 11 to 15.4 mN/m) as for the pure water subphase.

Previously we have shown that high concentrations of DAG in lipid films used for liposome preparation results in liposomes with strange, multi-layer morphology (rosette-like structure) [37]. This is not surprising as DAG is a neutral phospholipid and creates hydrophobic regions in lipid bilayers that increases membrane fusion [63]. The reason we observe less lipid absorption for liposomes with 20 and 40 mol% POG is thus likely related to this phenomena as a significant portion of the lipid may be on the inside of these liposomes and thus not available to readily spread on the oil interface.

We do observe an increase in the insertion of apoLp-III, $\Delta \pi_p$, as the POG concentration increases for both the pure water as well as the buffer subphase. Additionally, for 10 and 40 mol% POG, the pressure of the lipid-protein monolayer is higher than that of the apoLp-
III monolayer on the bare interface, i.e. insertion of apoLp-III into the lipid monolayer is a cooperative process described by the model shown in Figure 8b. The increased values of $\Delta \pi_p$ suggest that while liposome fusion with the oil interface is reduced (for 20 and 40 mol% POG), the lipid monolayer does contain increasing concentrations of POG, which ultimately leads to increased apoLp-III binding and insertion. This is not unexpected, as previous work has shown that DAG is an important interaction partner for apoLp-III [51, 52, 64, 65], and we previously showed the highest insertion for native apoLp-III into monolayers of POG and DOG [40]. We now show that at the physiologically more relevant oil-phospholipid-buffer interface as well, DAG increases apoLp-III insertion.

*Electrostatics controls the absorption of liposomes from aqueous solution, and facilitates the interaction of apoLp-III with the oil interface.*

Several observations suggest that electrostatics is an important parameter that drives lipid and protein interaction with the oil interface. First, we observe considerably less absorption of POPC liposomes in the pure water subphase compared to the buffer subphase. Second, we observe decreased absorption for liposomes with increasing concentration of the cone-like POPE in the pure water subphase, but not the buffer subphase. Lastly, absorption of liposomes containing increasing concentrations of phosphatidic acid, an anionic phospholipid, is reduced in both the pure water as well as the buffer subphase. In buffer, more PA containing liposomes absorb, similar to our initial observation for POPC liposomes.

This last observation clearly suggests that negative charges diminish liposome absorption. Intuitively this may be difficult to understand as the neat oil surface might be
expected to be electrostatically neutral. However, previous work has shown that the oil surface is instead negatively charged due to the adsorption of hydroxyl ions from solution [66]. We also showed that oil droplets of ~1 µm in diameter do not (easily) fuse in pure water, but readily fuse as the salt concentration in the solution is increased from 0 to 100 mM NaCl (see supplementary information of Ghimire et al. [67]). Thus, electrostatic repulsion between liposomes containing anionic PA and a negatively charged oil-water interface is the most plausible explanation for the reduced absorption of PA containing liposomes at increasing concentrations of PA. The increased absorption of PA-containing liposomes observed for the buffer subphase (compared to water) is due to the decrease in the Debye length in buffer (more electrostatic screening) which allows tighter packing of the PC-PA monolayer, identically to what we proposed for the absorption of POPC liposomes.

The absorption of PE containing liposomes in pure water is more surprising as the increase in negative curvature stress in bilayer membranes (by the addition of Type II lipids) results in an increased propensity for membrane fusion [37, 68]. One would thus expect that an increase in PE concentration results in increased fusion of liposomes with the oil interface, opposite to what we observe in the pure water subphase. We do observe increased lipid absorption in the buffer subphase. In the water subphase we propose that the absorption of PE-containing liposomes to the oil interface is thus less dependent on curvature effects (i.e. the spontaneous curvature of the liposomal membrane) but instead depends more strongly on electrostatic considerations. PE apparently changes the electrostatics in the absorbed phospholipid monolayer in such a way that this results in
more electrostatic repulsion of lipids and thus less dense packing. It is well known that the orientation of the PE headgroup in lipid monolayers is very different from the orientation of the PC headgroup, and it is likely these differences translate to lipid monolayers at the oil-aqueous interface. However, for the buffer subphase the charges in both the PC and PE headgroups are screened and the increase in PE does indeed lead to increased liposome absorption onto the oil surface.

In conclusion, the interaction of apoLp-III with an oil-(phospho-)lipid interface depends on the packing density and type of lipids present in the lipid monolayer. For pure PC (in a water subphase) an interfacial pressure of 7.5mN/m is sufficient to perturb protein insertion into the oil-phospholipid interface. In buffer, under the same conditions, we propose that protein interaction leads to protein and PC rich domains on the surface of the oil droplet. For PE and DAG, under the right conditions, we observe cooperative interaction between the self-assembled lipid monolayer and apoLp-III.

Also, our lipid absorption results show that when small LDs grow, i.e. gain additional neutral lipids, hydrophobic patches may not be easily filled via membrane (from, e.g., the ER) fusion. Instead, it is more likely that proteins are required to prevent growing LDs from becoming unstable in the aqueous cytosol.

**References**


[64] V. Narayanaswami, P.M. Weers, J. Bogerd, F.P. Kooiman, C.M. Kay, D.G. Scraba, D.J. Van der Horst, R.O. Ryan, Spectroscopic and lipid binding studies on the amino and


6.1 Introduction

Lipoproteins are lipid particles that share a similar structure with lipid droplets. They have a hydrophobic core rich in triacylglycerol and some cholesterol esters. A phospholipid monolayer surrounds the hydrophobic core, and apolipoproteins are attached to the lipoprotein interface.

These lipid particles transport nutrients that are insoluble in water through the bloodstream to peripheral tissue. Depending on the type of these particle, the apolipoproteins present in the structure of lipoprotein particles will be different. All the apolipoproteins share amphipathic α-helices as a common feature (sharing similar amino acid residues). The presence of the α-helix bundle in the protein structure facilitates the protein binding to the phospholipid monolayer [1].

Recently, the study on the dynamic of lipoproteins has become of a great importance. Donald Small is the pioneer in studying the interaction of apolipoproteins using the tensiometry system. His major focus is studying different peptides of apoB, which is present in larger lipoprotein particles such as VLDL and LDL [2-4].
Apolipoprotein A-I is the other apolipoprotein that has an amphipathic α-helical domain and has been studied extensively [5-8]. Except apoB, all apolipoproteins are exchangeable proteins; they can move from one lipoprotein particle to another, or to the surrounding cytoplasm.

ApoLp-III is an apolipoprotein that is almost fully amphipathic α-helix. Additionally, apoE3 (NT) has an amphipathic α-helical domain similar to the structure of perilipin 3 C-terminus. Here, for the first time, we study the interaction of these proteins with the phospholipid monolayer at the oil interface. All studies on the dynamics of lipoproteins have used POPC to form a phospholipid monolayer on the oil interface. Here, we investigate the lipid-protein interaction at oil surfaces covered with monolayers of different mixtures.

We report two distinct types of experiments: First, dynamics measurements in which we first allow the droplet to come into equilibrium with a solution of lipid vesicles and then remove those vesicles. The droplet volume is then oscillated to change its area, allowing us to deduce a surface pressure vs. area isotherm for the lipid mixture. Finally, the lipid pressure is returned to its value after deposition, and the process is repeated with the addition of a protein instead of lipid solution. These dynamic measurements allow us to test the stability of the interface and study reorganization of the protein.

Second, we do insertion measurements very much like those in Chapter 4, but with three different types of protein associated with lipid droplet-like structures and composed almost entirely of α-helices (apoLp-III). First, we consider native apolipophorin-III, an insect
protein, along with a mutant whose helix bundle cannot unfold into separate α-helices, which tests the role of such unfolding. Finally, we compare these two cases with that of a segment of apolipoprotein E, a mammalian protein, consisting entirely of α-helices.

6.2. Associated proteins (apolipoproteins)

Apolipoproteins are a group of proteins associated with the surface of lipoprotein particles. They are of considerable physiological importance and are involved with different diseases and disorders e.g. atherosclerosis, hypolipidemia and Alzheimer [9-11].

Apolipoproteins help to solubilize the lipid in the blood by binding to them. They also play a pivotal role as a structural, active component in lipoprotein particles, acting as ligand receptors and as co-factors of enzymes. Lipoprotein particles carry lipids to provide an energy source and for synthesizing hormones, vitamins and bile acids [12]. ApoB is essential for the assembly and secretion of triacylglycerol. In addition to apoB, apoE is also responsible for transporting dietary and endogenous lipids to target tissues [13-16]. ApoA1 is vitally responsible for returning the excess cholesterol from the tissue to the liver [15]. Apolipoproteins are also involved in the neurological processes, e.g. apoE and apoJ participate in transporting lipids into the brain [17].

6.2.1. Apolipoprotein E

Apolipoprotein E is a member of apolipoprotein family that is mostly found in chylomicron and intermediate density lipoprotein. These lipid particles play a role in the catabolism of the triacylglycerol-rich lipoprotein constituents [18]. The full-length protein has 299 amino
acids (a.a.); the lipid binding domain is located at the residues 130 to 150 and from 200 to 280 [19]. Investigation of the crystal structure of the protein (pdb: 2L7B) shows that the N-terminal and C-terminal are connected through a hinge. The N-terminal domain includes the a.a.1-167, which form four anti-parallel amphipathic α-helical bundle. The C-terminal, a.a. 206-299, also contains 3 large helices with hydrophobic surfaces. The hydrophobic surfaces from the N-terminal and C-terminal interact through hydrogen bond and salt bridges. The receptor for low density lipoprotein (LDL) is also located at the C-terminus [20]. A considerable amount of apoE is produced in the lung, liver, brain, spleen, kidney, muscles, ovaries and adrenal glands [21]. ApoE supports the homeostasis of the lipid by mediating the lipid transport from one cell type to the other. In the target tissue, apoE is produced by the liver and macrophages, and handles the cholesterol metabolism in an isoform-dependent manner [22].

ApoE was first recognized due to its importance in lipoprotein metabolism and cardiovascular disease. Deficiency in apoE causes hyperlipidemia type III, where the amount plasma cholesterol and triacylglycerol increases due to accumulation of chylomicron, VLDL and LDL particles. Such individuals show a risk for premature atherosclerosis type 1 [23].

There exist three isoforms of apoE; apoE 2, apoE 3 and apoE 4. These alleles differ by an amino acids pair; apoE 2(Cys 112, Cys 158), apoE 3(cys 112, Arg 158) and apoE 4 (Arg 112, Arg 158) [24]. This difference in amino acid residues is important to the binding ability of the protein to lipid or cell receptors. Compared to apolipoprotein E3, apoE 2 has
lower activity for binding to receptors (the two cysteine on the structure of the protein can form disulfide bond and prohibit the protein from unfolding). Approximately 90% of patients with dysbetalipoproteinemia type 2 have a homozygote of apoE 2. The rest is due to a rare toxic mutation of apoE 3[25].

ApoE 3 is the most common type of apoE, present in half of the population, where apoE 2 is the rarest. Presence of an allele of apoE 2 decreases the risk of Alzheimer`s disease emerging. On the other hand, the presence of apoE 4 increases the probability of developing Alzheimer`s disease.

6.2. 2. Apolipophorin-III (apoLp-III)

Insects possess an efficient transport system of lipids to support sources of energy for flight activity [26]. Insect lipophorin particles (lipoprotein), complexes of multifunctional molecules, are well studied. There are three different apolipoproteins associated with the surface of lipophorin: apolipophorin-I, apolipophorin-II and apolipophorin-III. ApoLp-I and apoLp-II form non-exchangeable lipid-binding core of the lipophorin particle [27]. When high-density lipophorin (HDLp) takes up diacylglycerol (DAG), it transforms into low-density lipophorin (LDLp). This process results in an increase in the lipid content of the particles [28]. ApoLp-III is an exchangeable lipid-free protein; it changes the conformation to prevent lipids in lipophorin particles from coming into contact with the aqueous environment [27].

ApoLp-III is a fully amphipathic α-helical bundle protein which forms five anti-parallel amphipathic helices arranged in an up-and-down topology (anti-parallel), as shown in
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Figure. 2.3a. These helices are connected through hinges [29]. The arrangement of the bundle motif stabilizes the protein in the hemolymph. Hydrophobic residues are mainly located in the inner surface of the protein while the hydrophilic residues are mainly revealed to the aqueous environment [30]. The protein can be found in the lipid-free or lipid-bound state in physiological conditions. In the lipid-free state, the intrinsic stability of the helix bundle is low, which facilitates interaction with lipid surfaces. When complexes of apoLp-III and lipid form, the protein undergoes considerable changes in conformation, unfolding with a possibility of helix-helix interaction [31, 32].

In this study, we look at the interaction at the surface of the oil droplet of two proteins, apoLp-III and apoE 3 NT. These proteins include an amphipathic α-helical bundle domain, which is similar to the C-terminus of the protein perilipin 3. We use our homebuilt droplet tensiometer to change the lipid packing on the surface of the oil droplet by varying the droplet volume and thus surface area. This variation decreases the oil surface available for protein interaction. Investigation of these model proteins sheds light on the role of the hydrophobic surface in lipid-protein interaction more generally.

6.2.3. Lipids

We use the same lipid mixtures as in Chapter 5. Lipids are chosen based on studies [33] of the lipid composition of the monolayer surrounding the lipid droplet. All of the lipids have the same PO acyl chains; only the head group varies. The lipids have different shapes and charges, to test the effect of lipid physical chemistry on lipid-protein interactions on the oil droplet:
• POPC is the dominant lipid on lipid droplets. It is a cylindrical zwitterionic lipid, which has a positive and a negative charge available in the lipid structure. Because it is cylindrical, it forms vesicles well, and will be at least 90% of all of the lipid mixtures studied.

• POPE is a zwitterionic lipid in the 2-10 pH range and is anionic over the range 10-12. Addition of PE lipid to the lipid mixture induces negative curvature stress to the vesicles [34].

• POG, (palmitoyl-oleoyl glycerol) is a neutral hydrophobic molecule with a small headgroup compared to POPC, leading to negative spontaneous curvature.

• POPA has both negative charge and negative spontaneous curvature.

A cone-shaped lipid, with a smaller head group than tail, may induce defects in the packing and allow the protein to come more easily in contact with the acyl chains and with the triolein at the oil droplet surface.

6.3 Materials and method

6.3.1 Materials

1. Protein: Here we used two model proteins. ApoLp-III was a kind gift from Dr. Paul Weers from California State University Long Beach. The protein was provided in powder form. We used HPLC grade water (W5SK) to suspend the protein. 1mg of protein was dissolved in 1 mL of water, setting the final concentration for protein stock to 1 mg/mL.
ApoE 3 NT was a generous gift from Dr. Narayanaswami from the California State University Long Beach. We used the protocol provided to dissolve the protein. Protein was mixed with 2M DDT (to forbid disulfide bond formation from C112 a.a.) and 6M guanidine-HCl (gn-HCl), suspended in PBS buffer. The mixture was incubated for 1 hour at 37 °C, and then dialyzed to remove excess of gn-HCl for 48 hours (3 buffer changes).

2. Lipids: POG (1-palmitoyl-2-oleoyl-sn-glycerol), POPC (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine), POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine), POPA (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphate (sodium salt)) were purchased from Avanti Polar Lipids (Alabaster, Alabama). Triolein glyceride was purchased from NU-Check Prep (MN). Thin layer chromatography was used regularly to test the purity of lipids, as discussed in Chapter 2. For polar lipids, we use a solvent consisting of CHCl₃/ CH₃OH/H₂O (64 ml /25 ml /4 ml) and for neutral lipid we used C₆H₁₂/ (C₂H₅)₂O (10:1).

3. Chemicals: KCl, EDTA and Tris were purchased from Sigma Aldrich Co. All the chemicals were > 99% pure. HPLC-grade water was purchased from Fisher Scientific (WSK5). Salts were heated in an oven at 200 °C for 24 hours, to eliminate any organics.

6.3.2. Methods

1. Buffer: Tris buffer is made using 150 mM KCl, 10 mM Tris and 0.2 mM EDTA, carefully mixed in HPLC-grade water.

After buffer preparation, the buffer is kept in the experiment room (21.4 ± 0.2 °C) to assure the absence of temperature fluctuations and thus density variations (see liquid
droplet tensiometer section). All glassware are cleaned using KOH cleaning solution (164 g ethanol, 25 g water and 24 g KOH).

2. Vesicle formation: Lipid films are made by drying 460 nmol of lipid in organic solution (chloroform or chloroform/methanol) under a stream of Nitrogen. The film is kept under vacuum for 24 hours to remove residual traces of organic solvent and was stored at -20 °C before use. Prior to vesicle formation, the lipid film is moved to room temperature for about 20 minutes. In order to form small unilamellar vesicles (SUV), 4 ml of water is used to suspend the lipid film. The mixture is sonicated for 2 minutes every 10 secs using a probe sonicator (Avanti, Alabaster). The sample is kept on ice through the sonication process, to avoid heating the lipid mixture. Later the sample is centrifuged for 20 minutes to pellet the nano particles produced from the end of titanium probe (Qsonica Q55 sonicator). DLS (differential light scattering; Horiba 7100) is used to measure vesicles size. Normally the vesicles prepared this way are around 50 nm in size.
3. Liquid Droplet Tensiometer: Our homebuilt tensiometer (see Figure. 1) is a combination of CCD camera (PixeLINK PL-B776F), light source (Thorlab), diffuser (Thorlab), syringe (Hamilton) with a J-shaped needle, and cuvette (made from borosilicate glass, supplied by Wale Apparatus PA., at a machine shop located in the Department of Chemistry, Kent State University). The set-up is cleaned regularly using pure methanol to remove any organic residues. Syringe and needle are rinsed 3 times with chloroform for cleaning. The cuvette is cleaned using KOH solvent (164gr MeOH, 24 gr Water and 25 gr KOH), and rinsed with DI-water, and ultra-pure water a minimum of three times each. Two pumps are involved, one to vacuum water/buffer from the cuvette and the other one to fill the cuvette with fresh buffer/water. The syringe is attached a micrometer screw (designed and fabricated by Wade Aldhizer in the machine shop in the Department of Physics at Kent State University) to control the volume of the droplet.

Figure 6. 1. Schematic view of the set-up. It includes a light source, diffuser, J-shaped needle and CCD camera with mounted lenses. Not to scale
The surface tension, volume, droplet area and radius at apex are measured using the ADSA code package developed by the Neumann group at the University of Toronto [35]. For a typical experiment, 3000 images are captured and analyzed simultaneously. Details on image analysis are given in Chapters 2 and 5.

6.4. Investigation on the dynamics of a triolein droplet covered by a phospholipid monolayer in the presence of apoLp-III protein

Here we investigate the dynamics of the lipid and protein adsorptions to the oil surface. We perform compression/decompression cycles to study the stability of the adsorption. A major parameter will be the hysteresis during those cycles. Depending on the lipid mixture, protein (absent or inserted), and environment (pure water vs. buffer), we observe both reversible hysteresis, in which compression and decompression of the surface follow different paths but successive cycles retrace the same paths, and irreversible hysteresis, in which successive cycles trace very different paths.

6.4.1. A typical experiment

A typical experiment is shown in Figure 6.2. The cuvette is filled with 10 ml of buffer and a triolein droplet is formed at the end of the J-shaped needle (point 1 in the figure). The droplet is allowed to stabilize in the aqueous solution for at least 5 min prior to further experimentation. At point 2, liposomes of either POPC or POPC mixed with 10 mol% of POPE, POG, or POPA are added. This is done by the removal of 4 ml of water/buffer from the top of the cuvette after which 4 ml of liposome solution was added cautiously. Deposition of the self-assembled phospholipid monolayer at the aqueous-oil
interface is then followed from time point 2 until 3 (where the spontaneous formation of a lipid monolayer has reached a steady state). At this point (3), remaining vesicles are washed out by carefully flushing fresh water/buffer (50 mL in 10 min) through the cuvette. Buffer/water enters the bottom of cuvette through a needle connected to a flushing pump. To keep the volume of the solution in the cuvette constant, a vacuum pump removes excess water/buffer from the top of the cuvette (see Figure 6. 1).

![Triolein droplet coated with POPC and apoLp-III](image)

**Figure 6. 2. Typical experiment: a triolein droplet was formed at the end of the J-shaped needle in water.**

Note that the interfacial tension does not change significantly during the removal of any remaining liposomes (between 3 and 4). At point 4, we start doing successive
compression and decompression cycles of the lipid droplet surface, by changing the droplet volume through the syringe.

During compression, the volume of the oil droplet, and thus its surface area, decreases, the lipid monolayer on the oil surface becomes more compact, and surface tension decreases (surface pressure increases.) During decompression, the oil droplet expands and with a larger surface area available for the lipid molecule to relax, the tension increases (pressure decreases). In order to characterize the stability of the lipid monolayer, several compression/decompression cycles are done between specific droplet volumes. At the end of these cycles, the droplet volume is brought to its initial value, and the droplet is left at rest to stabilize.

At point 5, a total of 0.1 nmol/L of protein is added to the cuvette and left to interact with the oil/lipid interface until the interfacial tension again reaches equilibrium. After 1 hr, any protein that does not bind to the oil interface is removed from the cuvette, step 6. 100 ml of buffer/water is flushed through the cell for approximately 20 min (between 6 and 7). While rinsing, any proteins bound loosely to the lipid monolayer also detach from the surface. This protein detachment is indicated on the graph by the variation in tension after rinsing. In step 7, we study the stability of the protein interaction with the oil- phospholipid monolayer interface. This process was run for POPC, PC: POG, PC:PE and PC:PA mixture in water and buffer. I also aim to observe the effect of variations in lipid physical chemistry on the dynamics of the oil droplet.

Lipids are chosen based on studies [33] of the lipid composition of the monolayer surrounding the lipid droplet. All experiments are run first in water and then in
physiologically relevant buffer, i.e. 150 mM KCl, 10 mM Tris, and 0.1 mM EDTA set to pH 7.2 ± 0.5.

6.4.2. Interaction of the apoLp-III protein with a bare triolein droplet:

The first set of experiments was performed to study the compression and decompression of protein layer on a bare triolein droplet formed in water. When the interfacial tension of the droplet stabilized after droplet formation, 0.1 nmol/L of protein was added to the cuvette. It took approximately 60 min for the protein to bind to the oil interface and the interfacial tension to reach its maximum decrease. The unbound protein was washed out of the cuvette with a fresh water rinse. Figure 6.3. (a) shows successive compression and decompression cycles induced on the protein-coated triolein droplet in water. During compression, the interfacial tension decreases (surface pressure increases) significantly below the original steady state level as the protein is compressed on the surface. This may mean that the layer is more concentrated than in equilibrium. Part of the protein might leave the interface. Decompression of the triolein droplet increases the surface area available for the protein to spread out and if necessary to rebind to the oil interface. From Figure 6.3. (b) we see that the droplet surface tension shows nearly reversible hysteresis: the tension follows different
paths during compression and decompression, whereas successive cycles follow nearly the same set of paths.

6.4.3. Dynamics of a triolein droplet coated with POPC interacting with apoLp-III.

The cylindrical, zwitterionic lipid POPC has both a positive and a negative charge available. Figure 6. 4 (a) and (c) show compression/decompression isotherms for the oil droplet coated with a POPC lipid monolayer in water and buffer respectively. Figure 6. 4 (b) and (d) repeats Figure 6. 4 (a) and (b) respectively with the addition of apoLp-III protein to the interface.

Figure 6. 3. a) A typical isotherm experiment for a triolein droplet covered with apoLp-III in water. The oil droplet was first formed and stabilized in water. Deposition of the protein leads to a decrease in interfacial tension. When the interfacial tension reaches a steady state, successive compression and decompression cycles are applied on the droplet. b) Variation in surface pressure versus droplet area during successive compression and decompression cycles.
Decreasing the droplet volume decreases the droplet area, putting the lipid/(protein) monolayer under compression. During each compression the surface pressure increases, as the lipid/(protein) packing at the oil interface is increased. Increasing the volume of the oil droplet increases the surface area; there are more sites available for the lipid/(protein) molecule to return to their initial state (initial configuration of the lipid molecules on the oil surface).

Figure 6. 4. Comparison of successive compression and decompression isotherms for an oil droplet first after coating with POPC lipid (a,c), and then after apoLp III protein insertion (b,d). The same experiments are performed with the droplet in pure water (a,b) and in a physiologically relevant buffer, pH ~7.2 (c,d).
For the lipid alone on the droplet surface in pure water, each successive cycle follows the same path, indicating that the compression/decompression of the lipid monolayer is reversible (Figure 6.4 (a)). Due to a higher level of hysteresis, the PC coated oil droplet in buffer could not undergo more than one oscillation cycle, Figure 6.4 (c); further attempted changes in the droplet volume did not affect the surface area of the oil droplet. However, the decompression pathway is systematically below the compression one; the difference is bigger when the droplet sits in buffer (Figure 6.4 c) than in pure water (Figure 6.4a). This area between the compression and decompression paths can be interpreted as the work required for the lipid molecules to reorganize.

For the protein/POPC-coated oil droplet covered with a POPC lipid monolayer, in water, the decompression/compression paths lie near each other, but at a significant distance from the next decompression path (Figure. 6.4. (b,d).) In buffer, the decompression paths lie further from each other, but closer to the next decompression path, until the surface area was reduced by a factor >1.5.

The maximum pressure attained with the lipid alone was similar in both water and buffer, ~15 mN/m. With the protein, the maximum pressure in pure water was much higher, ~27 mN/m, compared to ~20 mN/m in buffer. After several compression/decompression cycles, the minimum pressure reduced from 22 mN/m to 12 mN/m in pure water, while remaining at ~7 mN/m in buffer.
6.4.4. Dynamics of a triolein droplet coated with PC:PE (9:1) interacting with apoLp-III.

We repeated the experiments above with a PC:PE (9:1) mixture. PE has a small head and is a zwitterionic lipid. The results were qualitatively very similar, with only subtle changes. For the lipid alone on the droplet surface in pure water, each successive cycle follows the same path, indicating that the compression/decompression of the lipid monolayer is reversible (Figure 6.5 (a).) However, the decompression pathway is systematically below the compression one; the difference is bigger when the droplet sits in buffer (Figure 6.5 c) than in pure water (Figure 6.5 a). This area between the compression and decompression paths can be interpreted as the work
required for the lipid molecules to reorganize.

For the protein/PO:PE-coated oil droplet in pure water, the decompression/compression paths lie near each other, but at a significant distance from the next decompression path (Figure 6.5. (b)). In buffer, the distances between paths is much reduced.

The maximum changes in surface tension we attained was much less in pure water than in the buffer: 12mN/m compared to 25 mN/m, although if the droplet in pure water could
have been compressed more, it might have reached higher pressures. With the addition of protein, the pressure saturates at 25 mN/m in pure water, while it can rise to at least 30 mN/m in buffer. In pure water after several compression/decompression cycles, the minimum pressure reduced from 24 mN/m to 7 mN/m at similar surface areas. The minimum remained about ~15mN/m in buffer again at similar surface areas.

6.4.5. Dynamics of a triolein droplet coated with PC:POG (9:1) interacting with apoLp-III.

The set of compression/decompression experiments were then repeated with PC:POG mixtures. POG is a neutral hydrophobic molecule with a small headgroup compared to POPC. The results were qualitatively very similar to those for pure POPC. For the lipid alone on the droplet surface in both pure water and buffer, successive cycles follow the same path, indicating that the compression/decompression of the lipid monolayer is reversible (Figure 6.6 (a,b).) However, these cycles showed significant hysteresis in both cases.

For the protein/PC:POG-coated oil droplet with inserted protein in water, the decompression/compression paths again lie near each other, but at a significant distance from the next decompression path (Figure. 6. 6 (b)) In buffer, the paths are again much closer to each other, without consistent hysteresis.

Again, this pressure much less in pure water than in the buffer: 15 mN/m compared to 25 mN/m, although if the droplet in pure water could have been compressed more, it might have reached higher pressures. With the addition of protein, the pressure saturates at 23 mN/m in pure water, while it can rise to at least 35 mN/m in buffer. After several
compression/decompression cycles, the minimum pressure reduced from 20 mN/m to 11 mN/m in pure water, while remaining at ~15 mN/m in buffer.

Figure 6.6. Comparison of successive compression and decompression isotherms for an oil droplet first after coating with PC:POG (9:1) lipid mixture (a,c), and then after apoLp III protein insertion (b,d). The same experiments are performed with the droplet in pure water (a,b) and in a physiologically relevant buffer, pH ~7.2 (c,d).

6.4.6. Dynamics of a triolein droplet coated with PC:PA (9:1) interacting with apoLp-III.

The set of compression/decompression experiments were then repeated with PC:PA mixtures (Figure 6.7); remember that PA has both negative charge and negative
spontaneous curvature. These results were significantly different from both pure POPC and the other lipid mixtures.

Figure 6. 7. Comparison of successive compression and decompression isotherms for an oil droplet first after coating with PC:PA (9:1) lipid mixture (a,c), and then after apoLp III protein insertion (b,d). The same experiments are performed with the droplet in pure water (a,b) and in a physiologically relevant buffer, pH ~7.2 (c,d).

For the lipid alone on the droplet surface in pure water, each successive cycle follows the same path, indicating that the compression/decompression of the lipid monolayer is reversible (Figure 6. 7 (a,b).) However, these cycles showed significant hysteresis in both cases, although much smaller in the buffer solution.
The maximum surface tension we attained was similar in pure water and in the buffer: 20 mN/m. With the addition of protein, the pressure saturates at 28 mN/m in pure water, while it can be pushed to 30 mN/m in buffer.

6.4.7. Discussion of interfacial tension dynamics during compression/decompression cycles.

Here in Section 6.4, we present the dynamics of a triolein droplet covered by different lipid mixtures, with or without an inserted protein. The triolein droplet undergoes a series of compression and decompression cycles, which mimics the stress applied on the lipid particles.

Table 6.1 compares the observed hysteresis for compression/decompression of the different protein/phospholipid interfaces presented above. We see that the hysteresis is much more complex with protein than with just lipid at the interface. We will begin by comparing the behavior of the different lipids.

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<th>Lipid</th>
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<td>Pure water success</td>
<td>Decompression</td>
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<tr>
<td></td>
<td>Pure water success</td>
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<tr>
<td>Pure Triolein</td>
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<td>POPC</td>
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<td>PC:PE</td>
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<td>PC:POG</td>
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<td>PC:PA</td>
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<td>2nd &lt;0.01</td>
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Table 6.1. Hysteresis in the compression/decompression surface pressure isotherms on different triolein droplets coated with different phospholipid mixtures, with or without protein, in pure water or buffer. Columns compare the difference in the area to arrive at
the same intermediate pressure between successive compression or decompression cycles, as well as the difference in area to reach that pressure between decompression and the next compression. In one set of case, that of protein in buffer, these area differences depended on how much the droplet surface was compressed, so that another column, giving the ratio of the final to the initial area is added in that case. Finally, in the PC:PA case in water, the surface pressure behaved quite differently in the first compression, so both cases are given.

6.4.7.1. Hysteresis in the absence of protein.

As the area of a phospholipid-coated triolein droplet is compressed, the lipid molecules on the triolein surface pack more tightly and increase the surface pressure. Lipid packing changes with the space available for the lipid molecules to locate. In principle, phospholipids on the surface and the triolein core may exchange particles. During decompression, the packed lipid molecules on the surface of the triolein droplet start to relax and can go back to their previous configuration, or occupy a closer empty spot. Either process decreases the surface pressure. In stiff monolayers, cracks can occur and the pressure decreases rapidly until the lipids rearrange to fill space.

In most cases with only lipids on the droplet, successive compression and decompression cycles (each about 20 minutes total in length) follow the same paths but the decompression curves lie systematically under the compression ones. During compression, the lipids pack more tightly, to increase the surface pressure. The work done by lipid molecules to relax during droplet decompression is given by the area between the compression and decompression isotherm. However, our curves are not yet sufficiently smooth and over sufficiently well-defined droplet areas for this work to be a basis of comparison between the different cases. We will focus on other quantities, such as hysteresis.
Hysteresis implies a change in the layer configuration upon compression that cannot relax immediately on decompression, because of an energy barrier or because the process is complex and takes a longer time than available. There are several different possibilities for such hysteresis.

As just discussed, the molecules may remain on the surface, but compression forces the system to explore new, more tightly packed configurations. There may be an energy barrier to relaxing back to the old configuration, or the system may find a new, lower energy configuration. In the latter case, the hysteresis will be irreversible. If the lipids desorbed from the surface, they would need to diffuse back to the surface, reabsorb and rearrange. In this case, we would expect to see irreversible hysteresis over short time scales, while the hysteresis could be reversible over sufficiently long time scales. We see essentially reversible hysteresis in all cases, so that it is most likely that the phospholipids remain on the surface.

We note that the hysteresis is much larger for the mixture with PC:PE than with either the pure PC layer or the other mixtures. Possible reconfigurations of this case include the bending and binding of the PE head groups to the interface (Figure 6.8). This binding is affected during compression and decompression. In addition, there can be repulsion between negative charge of the PE head group and the negative charges present on the oil interface when the oil droplet is in contact with water [36]. With PC:PE, the hysteresis is notably smaller in buffer than in pure water. Charge screening of the oil surface may
enhance lipid adsorption to the interface and lower barriers to reconfiguration at the surface.

Figure 6. 8 Cartoon of a lipid monolayer at an aqueous(above)/oil (below, yellow) interface. Phospholipids with green headgroups represent the cylindrical, zwitterionic lipid POPC. The minority lipid with a blue headgroup represents POPE, which has a relatively slender head group. This headgroup can bend and bind to the interface as shown, depending on the packing. The presence of buffer partially shields the repulsion between the negative charge of PE headgroup and the negatively charged oil interface, over a characteristic length scale given by the Debye length.

6.4.7.2. Hysteresis in the presence of protein.

The insertion of a large molecule such as the protein into the droplet brings more possibilities for configurations and thus for hysteresis. The protein may not be confined to the surface, but may rather dangle into either the triolein interior or the aqueous exterior of the drop. Compression may squeeze the protein entirely or partially out of the interface, perhaps taking triolein and/or phospholipids with it, as suggested by Mitsche et al. [37], whose experiments suggest that at higher surface pressure, the apolipoprotein A-I does leave the surface. In the case of a protein bundle, perhaps one or more helices within the bundle desorb. The one(s) with the strongest hydrophobic surface might remain. Alternatively, some other part of the protein may tether the protein to the surface.
Weers et al. demonstrate that apoLp-III undergoes a major conformational change when it interacts with a lipid bilayer [38]. During interaction, apoLp-III unfolds completely, with helix 1, 2 and 5 separated from helix 3 and 4, Figure 6. 9 (a). Figure 6. 9 (b) shows the NMR structure of the intact protein bundle for comparison. Weers et al. also suggests that the protein remains connected to the oil surface through the hinge between the 3rd and 5th helix. We would expect the protein to behave similarly on the phospholipid monolayer.

![Image](image.png)

*Figure 6. 9. Configuration changes of apoLp-III in contact with phospholipid layers. a) The helix bundle unfolded inside the membrane. it is assumed that the amphipathic helix bridging the 3rd and 4th helix play major role in binding to the membrane. b) The folded native protein. The NMR structure drawn using 1ls4.pdb."

On a bare droplet, the protein undergoes reversible hysteresis. These experiments were done only in pure water, because the presence of buffer had no influence on the initial protein adsorption in this case. We expect the same to hold for the dynamics, although it might be useful to verify this. Reversible hysteresis implies that the protein does return to its original configuration. It is thus likely that the protein remains firmly attached to the surface, although some part of the protein must undergo conformational changes that may force part of it to leave that surface.
With most of the lipid coating, the protein layers undergoes a kind of irreversible hysteresis. The compression of the triolein droplet is likely to force the protein to come off (partially/completely) from the surface, with or without phospholipids and triolein. Decompression increases the triolein surface area, which facilitates the protein rebinding to the triolein interface. In pure water, the pressure increases during compression until reaching a quasi-plateau. We continued to compress along this plateau. During decompression, the pressure descended immediately, parallel to the compression curve but at significantly lower droplet areas. During recompression, the pressure followed the path of the last decompression until reaching another plateau. It appears that almost all of the irreversible behavior occurs during this plateau (at about 25 mN/m for POPC and PC:PE; 22 mN/m for PC:POG.) With the droplet in the buffer solution, the droplet can be compressed to a much lower area before reaching a pressure plateau, and any hysteresis is much less consistent. Table 6.1 compares the observed hysteresis for compression/decompression of the different protein/phospholipid interfaces presented above. We see that the hysteresis is much more complex with protein than with just lipid at the interface. We will begin by comparing the behavior of the different lipids.

**Hysteresis in the pure lipid cases.**

The behavior of the surface pressure with the protein inserted into the PC:PA coated surface was distinctly different from all of the other cases. In pure water, some conformation change during the first compression is irreversible. However, after that the layer undergoes entirely reversible hysteresis, to higher pressures and with a very different
shape than with the lipid alone. In buffer, the behavior was much more like that of the other protein/phospholipid systems.

6.4.8. **Conclusions from the interfacial tension dynamics during compression/decompression cycles.**

While these results are certainly preliminary, they suggest several things about protein/lipid interactions at the surface.

*Charge screening binds the protein more tightly to the droplet interface.*

The isotherms in the presence of buffer, which decreases the Debye length (the characteristic length over which charge is screened by the redistribution of ions in solution), are significantly more reversible. Note that the pH in this case is also higher.

The irreversible behavior in water occurs at high pressures, ~25 mN/m. It suggests that at these pressures, the protein partially or completely leaves the interface, perhaps taking some of the phospholipid and/or triolein with it. The removal of protein at a single high pressure is not apparent in the presence of buffer, and the isotherms are nearly reversible for modest compression.

As discussed below, the PC:PA system behaved distinctly differently.

*The effect of head size on protein binding.*

POG and, to a lesser extent, POPA, both have smaller head groups. In both cases, the surface pressure vs. droplet area curves for the lipid mixtures alone showed much more hysteresis. Here, lipid molecules apparently do more work to relax and occupy empty sites
on the droplet surface during decompression. The hydrophobic interaction between the monolayer and triolein molecules also affects the work needed for these molecules to relax over time.

However, the hysteresis observed with the protein was not obviously different from the POPC and PC:PE cases.

*Phosphatidic acid facilitates protein rebinding to the interface.*

The PC:PA case showed very different dynamics than the other three lipid mixtures. First, the lipid layer itself showed much greater hysteresis. During decompression, the pressure dropped very quickly to low values: ~4mN/m in water and ~2mN/m in buffer. However, the pressure quickly increases during recompression and follows the original compression isotherm: the hysteresis is reversible.

Because of this reversibility, it seems unlikely that the lipids actually leave the surface. It would be interested to see the actual structure of this layer during compression and decompression, but this is difficult at the oil/water interface. At the air/water interface, we most often see such behavior when the film is quite stiff on compression, and cracks rather than respreads on decompression. On the other hand, such layers usually show hysteresis on at least the first compression/decompression cycle.

With the protein on the interface, in water, the first compression leads to irreversible rearrangement of the lipid layer: the minimum pressure decreases from 25 to 5 mN/m. However, succeeding compression/decompression cycles are reversible, so that after the
first compressions the lipids and proteins are almost certainly staying partially bound to the interface. The presence of PA thus seems to facilitate rebinding of the protein to the interface. We speculate that the low surface pressures, and perhaps any associated defects in the layer, facilitate the rebinding.

It would be particular interesting to compare the structure of the lipid and protein layers in the PC:PA and the POPC cases.

6.5. Insertion of alpha-helices into a lipid monolayer at the oil/buffer interface

Now we turn to the influence of lipids to the affinity and the cooperativity of protein binding to the triolein/aqueous interface, with insertion measurements very much like those in Chapter 5.

The monolayer insertion experiments followed the procedure by Donald Small [2]. They are run very much like the dynamics experiments just discussed, except that after depositing the phospholipid mixture on the droplet surface (point 2 on Figure 6.10), the droplet area is not oscillated, but rather simply changed to reach one of a series of fixed pressures. Then we deposit 0.1 nmol/L of the protein into the cuvette through the pipette, point 5 on Figure 6.10.

6.5.1 Characterization of protein insertion into a lipid monolayer.

\( \pi_0 \) and \( \Delta \pi \) are the two variables I use to characterize the insertion data. \( \pi_0 \) is the decrease in interfacial tension after the droplet volume is decreased to its desired value, while \( \Delta \pi \) is the additional decrease in surface tension after protein addition. These
parameters are shown in the typical insertion experiments shown in Figure 6.10. We characterize protein insertion from the variation of $\Delta \pi$ as a function of the initial lipid pressure $\pi_0$.

![Figure 6.10](image)

Figure 6.10. A typical insertion experiment. 1) A droplet of triolein droplet is formed. 2) After 5 min 4 ml of the buffer in the cuvette is replaced by 4 ml of freshly made PC:PA vesicles. 3) the droplet volume is changed to the desired value. 4) after stabilization of the lipid monolayer the rest of the vesicles were rinsed off from the cuvette. 5) 0.1 nmol/L of the protein was deposited into the cuvette. The droplet was incubated with the protein for 1 hr to let the protein to interact with the oil interface.

6.5.2 Insertion of apoLp-III protein into monolayers of lipid mixtures coating a triolein droplet

POPC, PC:PE (9:1), PC: POG (9:1) and PC:PA (9:1) are the lipid mixtures used to form the lipid monolayer on the triolein interface. The lipids were chosen to all have the same acyl chains with differences in headgroup size and charge. These differences lead to
a range of lipids with relevant physicochemical properties. The PO (sn-1-palmitoyl, and sn-2-oleoyl) acyl chains were chosen because they are common in the mammalian glycerophospholipid fatty acid compositions [39].

Maximum insertion pressure refers to a monolayer pressure at which the protein does not insert anymore. The results show that apoLp-III has the highest maximum insertion pressure into the monolayer formed from POPC and from PC:POG lipid mixtures, Figure 6.11. The protein has the lowest MIP for the lipid monolayer formed from PC:PA and PC:PE mixtures. $\Delta \pi_{\text{Max}}$ is the maximal changes in the pressure of lipid monolayer after protein insertion. The values for $\Delta \pi_{\text{Max}}$ reflect the affinity of the protein to the lipid monolayer. Here, apoLp-III shows the highest affinity toward the PC:PA monolayer. The

![Figure 6.11. Comparison of the insertion of apoLp-III into monolayers of POPC (black circle), PC:PE (red triangle), PC:POG (blue square), PC:PA (green diamond). Tris buffer: 10mM Tris-HCl, 150 mM KCl, 0.2 mM EDTA at pH7.2.](image)
affinity of the protein is significantly lower for the apoLp-III protein interaction with other lipid mixtures.

**Discussion**

POG is from a class of lipid with negative spontaneous curvature: they can be visualized as cone shaped, with a smaller head than tail. This class of lipid is known to facilitate the interaction of some membrane protein since defects around the lipid helps the insertion of the hydrophobic domain of the protein into the hydrophobic interior of the membrane bilayer [40, 41]. In addition, when the droplet volume decreases, the lipid packing on the surface increases. POPC has a cylindrical shape, so that the addition of POG is likely to lead to more gaps between the lipid headgroup to facilitate the insertion of the hydrophobic domain through the lipid monolayer. The core of our oil droplet consists of a neutral lipid, triolein. Our results suggest that indeed, at the highest MIP the hydrophobic domain of the protein can insert and interact with the neutral core (triolein). It would however be interesting to visualize the PC:PG layer to test its uniformity and any gaps.

ApoLp-III shows the highest propensity for the PC:PA-coated triolein droplet. The $\Delta\pi$ is ~ 18 mN/m, close to the variation in surface pressure when the apoLp-III protein directly interacts with the triolein. The apoLp-III protein shows very similar affinities for other lipid mixtures ~ 15 mN/m.
6.5.3. *Comparison on the insertion of locked apoLp-III with native apoLp-III.*

Studies have showed that apoLp-III unfolds its amphipathic α-helix bundle prior to insertion into a phospholipid monolayer [42]. In order to investigate the importance of helix bundle unfolding, I used a helix-locked protein, in which the protein is mutated by two cysteine amino acid residues. The presence of cysteine residues causes the formation of a disulfide bond, which inhibits the protein from unfolding.

*Figure 6.12. a) representation of the locked apoLp-III through mutation of T26 and A136. b) the structure of the native protein*

The protein was a kind gift from Dr. Paul Weers from California State University (Long Beach). The mutation of the protein was done on amino acids Threonine (T) 26 and (A)136, as seen in Figure 6.12. Our control experiment was to deposit L(locked)-apoLp-III on a triolein droplet coated with POPC monolayer to study the protein interaction with the interface. The presence of the disulfide bond locks all the hydrophobic surface of the protein in the interior surface of the protein [42]. The insertion experiments for this protein were performed just as before.

Both $\Delta \pi$ and $\pi_0$ decreased significantly with the locked protein. Maximum insertion pressure, above which the protein cannot insert into the monolayer decreases by
~10 mN/m. $\Delta \pi$, reflecting the protein affinity also decreases ~ 10 mN/m for the locked as compared to the unlocked protein.

**Discussion**

![Comparison of the two protein](image)

*Figure 6. 13. Comparison of the insertion of apoLp-III into monolayers of POPC (black circle), PC:PE (red triangle), PC:POG (blue square), PC:PA (green diamond). Tris buffer: 10mM Tris-HCl, 150 mM KCl, 0.2 mM EDTA at pH7.2.*

Our data suggest that the helix bundle unfolding plays major role in lipid-protein interaction. Most of the hydrophobic residues are located in the inner surface of the helical bundle. Protein unfolding accommodates the access of the amino acid residues located on the inner surface of the helix to the hydrophobic surface of the oil droplet. In addition to electrostatic interactions, one should also consider the hydrophobic interaction as a source of protein interactions with the oil interface.
6.5.4. Insertion of apoE 3 (NT) into different monolayers at the oil/buffer interface.

ApoE 3 is an apolipoprotein that is present on the surface of lipoprotein particles (in humans). The whole proteins include two amphipathic α-helix bundles, one is located at the N-terminal and the other one is located at the c-terminus. The N-terminus includes a domain with 4 helix bundle which is similar to the C-terminus of the perilipin 3. Since there is a similarity in structure between apoE 3 (NT) and perilipin 3 (CT), I become interested to study the insertion of this protein into different monolayer.

Later, the protein was incubated for 1 h at 37°C. Three dialysis cycles were run over 48 hr to rinse out excess Gdn-HCl. The protocol was provided by Dr. Narayanaswami. I used Nanodrop 1-position spectrophotometer model ND-2000 to measure the concentration of the protein constructs, ~ 0.48 mg/mL. Since I wanted to be consistent with the amount of protein used for the insertion experiment, I first tested different protein concentrations to measure the surface activity. Here, a droplet of triolein is formed in water. When the droplet is stabilized with the media, the desired concentration of the protein is added to the cuvette. The protein is incubated with the triolein droplet for 1 hr, to let the protein fully interact with the oil interface. In order to keep the protein in contact with the triolein droplet, I started using a stirring magnet. At low speed, it inhibits the protein from staying at the bottom of the cuvette. Since the magnet causes drift to the buffer surrounding the oil droplet which result in the leakage of the oil droplet, it is important to set the speed as low as possible.
In Figure 6.14, we see that at 0.048 nmol/L of apoE 3(NT) (black line), the protein is able to interact with the oil interface and change the interfacial tension, but the change in tension is small. The interfacial tension decreases more and faster for higher protein concentrations. Data showed that the interfacial tension saturates for concentrations higher that 0.07 nmol/L the variation in interfacial tension is stabilized. In order to be consistent with my data from apoLp-III and have the same protein concentration in the media, I used 0.1 noml/L of protein to deposit into cuvette.

![Graph showing interfacial tension over time for different protein concentrations.]

*Figure 6. 14. Looking at the surface activity of different concentrations of the protein in water. It shows that, Lower concentrations are less surface active with the oil interface; however, the rate of protein interaction follows the same trend.*

Since I wanted to compare the behavior of apoE 3 (NT) protein with apoLp-III, I studied the protein insertion into the same series of lipid monolayers at the triolein-buffer interface. Before depositing the protein to the cuvette, I started the stirring magnet to help the protein float in the media and interact with the triolein surface.
ApoE 3 (NT) has the highest maximum insertion pressure (MIP) for the monolayer formed by PC: POG and PC: PE mixtures, while it shows the lowest MIP for PC:PA and POPC lipid mixture. We expect the MIP to be a maximum for the lipid monolayer that lets the protein insert and interact most with the oil interface. POG and PE are lipids with negative spontaneous curvature.

\[ \Delta \pi_{\text{max}} \], which is \( \Delta \pi \) in the limit where \( \pi_0 \) is zero, gives the maximal changes in surface pressure when protein interacts with the monolayer interface. Here, apoE 3 (NT) has the highest propensity to interact with PC:PA mixture, with a \( \Delta \pi_{\text{max}} \) of \(~20\) mN/m. As is seen in Figure 6.15, the other lipid mixtures show \( \Delta \pi_{\text{max}} \) close to each other at \(~15\) mN/m, which is close the variation of the surface pressure when the protein is directly

![Figure 6.15. Comparison of the insertion of apoE 3(NT) into monolayers of POPC (black circle), PC: PE (red triangle), PC: POG (blue square), PC: PA (green diamond). Tris buffer: 10mM Tris-HCl, 150 mM KCl, 0.2 mM EDTA at pH7.2.](image-url)
interacting with the triolein droplet and also similar to what I observed for apoLp-III protein in Figure 6.11.

**Discussion.**

POG and PE are lipids with negative spontaneous curvature. This class of lipid are known to facilitate the interaction of some membrane protein since the structure of these lipids helps the insertion of the hydrophobic domain of the protein into the hydrophobic interior of the membrane bilayer [40, 41]. In addition, when the droplet volume decreases, the lipid packing on the surface increases. POPC has a cylindrical shape; introduction of lipids such as POG that have a cone shape with a smaller head group should lead to more gaps between the lipid head groups to facilitate the insertion of the hydrophobic domain through the lipid monolayer. The core of our oil droplet consists of a neutral lipid, triolein. A higher MIP means that the hydrophobic domain of the protein can insert and interact with the neutral core (triolein). We see that the PC:POG mixture indeed has the highest MIP.

Similarly to apoLp-III, apoE 3(NT) shows the highest affinity for the triolein droplet coated with PC:PA mixture. PA is a negatively charged lipid, where the electrostatic interaction is the dominant interaction for this lipid. However, the hydrophobic acyl chains of the lipid can interact with the hydrophobic residues of present in the structure of the protein. The question is still open: do these proteins interact with the triolein core or with the phospholipid monolayer surrounding the core?
A helical wheel presentation, showing how the amino acid residues are placed around the helical axis, can relate the preference of any type of interaction between the protein and lipid to the physical and chemical properties of the different α-helices in the proteins. Figure 6.16 shows the helical wheel structure for apoE 3 (NT) and Figure 6.17 shows the helical wheel structure for apoLp-III. The helical wheel representation shows that apoE 3 (NT) is highly positively charged, which increases the affinity of the molecule with the negatively charged lipid. In addition, the oil surface is also negatively charged, which will also increase the affinity of the protein with the surface. Further, presence of large aromatic amino acid residues such as tryptophan, tyrosine and phenylalanine may help the insertion
of the protein into the monolayer interface, as suggested by Small 2017.

The helical wheel structure indicates that the 4th helix has a charge +5, which should play a major role in electrostatic interaction of the protein with the oil interface, although...
there are charged amino acid residues present in other helical bundles. The helical wheel structure also make clear that all the helices have hydrophobic surfaces, which is likely to be important for the hydrophobic interaction between the hydrophobic residues and the hydrophobic surface of the oil. However, the relative stability of the bundle may make it less likely to present these hydrophobic surfaces.

In comparison, the helical wheel representation of the apoLp-III protein shows that the hydrophobic surfaces have residues like alanine, threonine, and serine present in each bundle, which make the bundle less stable.

Although the two proteins are different in charge, the affinity of the proteins towards PC:PA monolayer is slightly different. Further studies are necessary to identify other factors important in the interaction of the protein with the oil droplet.
Figure 6. 17. Helical wheel structure for apoLpIII). Each helix is drawn separately. Hydrophobic surfaces are marked with yellow and gray circles, while, polar amino acids are displayed with red and blue colors. Table shows the value for hydrophobicity, hydrophobic moment and net charge for each helix. The figure was prepared form protein data bank 1ls4.pdb
References:


CHAPTER 7

PERSPECTIVE AND FUTURE DIRECTIONS

The major goal of this research was to explore the biophysics of lipid-protein interactions on the surface of lipid particles. Lipid droplets and lipoproteins are lipid particles that share similar structures, but play completely different roles. Lipid droplets are dynamic cell organelles that store energy as neutral lipid in their structure. Excessive and deficient storage of lipid droplets is associated with human diseases. On the other hand, lipoprotein particles are considered to be lipid shuttles floating in the blood stream. Fatty liver, atherosclerosis and Alzheimer disease are examples of diseases that result from misregulation in the metabolism of lipoproteins.

In my study, I focused on three different proteins; two are associated with the surface of lipoprotein particles and one is found on the lipid droplet surface. All three proteins are exchangeable proteins and have an amphipathic α-helix bundle domain, while apoLp-III has almost nothing else. Investigations have shown a high degree of similarity in the structure of these three proteins [1]. Detailed studies have investigated different types of lipoprotein and their associated apolipoproteins, but the structural and functional role of lipid droplets needs to be elucidated in further detail.
Perilipins are the major class of LD binding proteins in mammals. Except for perilipin 1, the function of the perilipins (2, 3, 4, and 5) is not yet fully resolved. Especially the role of perilipin 3 remains unclear. It is thus important to determine how perilipins are associated with the surface of the LD and what their major role in lipid metabolism is. Study of lipid-protein interactions on the surface of lipoprotein and comparing this interaction with LDs sheds light on the ways structural similarities of apolipoproteins and perilipins affect their interactions with lipids.

In different studies, I showed that the amphipathic α-helix bundle domain plays major roles in binding to the lipid monolayer interface. However, it is not yet clear if it is necessary in vivo for lipid-protein interactions. Studies by Donald Small on a series of human apolipoproteins (and peptides derived from these proteins) suggest that during lipoprotein shrinkage, apolipoproteins can come off from the surface and fold, although it is not yet clear if the same thing occurs for all neutral lipid particle binding proteins [2, 3]. However, the same process is assumed to occur for lipid droplets. During lipolysis (the enzymatic breakdown of lipids for energy production or other processes), the lipid droplet shrinks, which leads to an increase of surface pressure in the lipid-protein monolayer covering the droplet. Shrinkage of the lipoprotein particle also causes an increase in the of lipid droplet particle density (the protein/lipid ratio increases and the particle thus becomes heavier) and crowding of the protein on the surface of lipid droplets [4]. The shrinkage of lipid droplets causes some proteins, or parts of proteins, to come off from the LD surface. We would then expect competition for the proteins to rebind to the LD surface if the LD
increases in size again. Indeed, the shrinkage causes changes in the protein composition on the surface of LD [5].

Proteins interacting with the LD surface can be categorized in two classes. Class one proteins enter the phospholipid monolayer surrounding the LD via the synthesis of LDs at the ER. That is, they are targeted from the ER membrane (i.e. they are also ER membrane resident proteins) directly to the LD surface. These proteins often have proline residues that cause kinks in α-helix bundles and thus form hairpins that are suggested to be important in the interaction with the phospholipid monolayer covering the LD [6-8]. Class two proteins are synthesized on free ribosomes and thus need to be targeted to the phospholipid monolayer covering the LD surface from the cytosol. This process is thought to involve binding of amphipathic α-helices or some sort of other hydrophobic domain [5, 9].

Perilipin family proteins contain amphipathic domains that may directly interact with the oil surface. Perilipin 2, 3, and 5, have an amphipathic α-helix bundle, and all perilipins have an 11-mer repeat domain. This 11-mer repeat domain is also able to theoretically form amphipathic α-helices but the structure of this domain when in interaction with lipids is currently unknown. It is established that the 11-mer repeats are crucial to target perilipins to the LD surface [10-12]

Protein binding to the triacylglycerol-phospholipid monolayer interface is the major process that facilitates access to the neutral lipids present in the core of lipid droplets. Localization of proteins to the LD surface is thus crucial in understanding lipid metabolism and cell signaling. There have been studies that have investigated the lipid metabolism
process, but questions remain as to the factors that affect this process (e.g. activators, deactivators, etc.).

The main goal of my study was to investigate the lipid-protein interaction of perilipin 3 through a model system, *in vitro*, and associate my findings with the *in vivo* system. In the first part of my work, I studied an apolipoprotein, apoLp-III, which mimics the helix bundle domain of perilipin 3. I followed up on this work with an investigation of different mutants of perilipin 3 in order to define the possible roles of different domains of the protein. Beside full length protein, I also used three N-terminal truncation mutants. These two studies were performed at the air-buffer interface. In order to shed further light on the physiological system I built an instrument to study lipid-protein interactions at the oil-phospholipid-buffer interface. The liquid droplet tensiometer was subsequently used to study apoLp-III at the oil-buffer and oil-phospholipid-buffer interface.

The main goal of this work is thus to define the major lipid-protein interactions that are necessary for class II LD binding proteins to interact with the hydrophobic core of the LD. The work is focused on studying the mechanism through which exchangeable perilipin proteins interact with the phospholipid-triacylglycerol interface. This is crucial for a general understanding of LD biology.

The four major questions that drove this work are:

1) What are the mechanisms through which the exchangeable proteins from the perilipin family interact with the LD surface?
2) How do individual domains of exchangeable proteins contribute to this binding?

3) What are the biophysical and physical chemistry properties involved in this binding?

4) How do cells regulate the protein composition of the LD surface?

Using Langmuir monolayer system to study the interaction of apoLp-III and perilipin 3 at air-buffer interface.

The amphipathic α-helix bundle is a protein domain found in both apoLp-III and perilipin 3. Each of these proteins is an exchangeable lipid binding protein, i.e. they can desorb or readsorb to the surface of neutral lipid particles (lipoproteins and LDs) [13-16]. Both apoLp-III and perilipin 3 have similar structure and function to another mammalian apolipoprotein, namely apoE [17, 18]. The structure and function of apoLp-III in insects has been widely studied, however, the function of perilipin 3 in the biology of lipid droplet is still unknown [19-24].

It is not yet clear how these proteins interact with lipid particles, whether the protein interaction occurs through chemical recognition (e.g. for a specific lipid headgroup) or via physical properties of the lipid monolayer surrounding the lipid particles (lipoproteins and LDs). Additionally, how class II LD binding proteins, especially the perilipins, recognize and target LD monolayer is not yet understood. It is worthwhile to compare the lipid interaction of perilipin 3 with the model insect protein apoLp-III to provide further information on the lipid binding as well as biological functions.
One of the major goal of this study was to identify the biophysical parameters of the phospholipid which affect the protein insertion and binding. Through Langmuir monolayer, I could characterize the lipid composition of the lipid monolayer in order to study the given biophysical parameter. It should be mentioned that not all the lipids I used for the lipid monolayer formation are per se physiologically relevant. However, our results shed light on the interaction of apoLp-III and perilipin 3, which is comparable to the mammalian apolipoprotein apoE.

ApoLp-III is a surface active protein by itself. Our data shows that apoLp-III favorably inserts into the lipid monolayer composed of more ordered lipids (saturated) compared to lipids with unsaturated acylchains. Our data is consistent with previous data, which show that apoLp-III has maximum insertion for diacylglycerol [24, 25]. A wide range of lipids was chosen to study, where it determines that the molecular shape of the lipid affect apoLp-III lipid-binding. The negatively charged lipids have the highest “affinity” in binding to the apoLp-III. As the lipid-bound structure of t apoLp-III is unknown, it is challenging to relate some of our observations with the lipid particle system. The insect lipoprotein is high in DAG. Our data also indicates that apoLp-III shows the highest affinity for DAG, which makes sense. It is speculated that islands of DAG grow on the surface of lipoprotein to act as binding sites for apoLp-III.

Langmuir monolayer system was also used to study lipid interaction with full-length perilipin 3 and its N-terminal mutants. No work had yet studied the association of perilipin
3 with the lipid monolayer directly. Our goal was to determine the role of different protein domains in lipid – perilipin 3 interaction.

In the first step of our experiments, we showed that all the perilipin 3 constructs are surface active, where the full length protein has the highest surface activity and the C-terminus has the least surface activity. This data shows that the N-terminus of the protein helps increase the surface activity. Our data also shows that the C-terminal of the protein shows different insertion behavior compared to the full length protein and other N-terminal truncation mutants. The addition of the N-terminus to α-helix bundle domain reduces the insertion to the lipid monolayer (i.e. the maximum insertion pressure decreases).

Previous work by others suggested that perilipin 3 is located on LDs where there is more unsaturated phospholipid in the lipid monolayer [26], which is contrary to our finding. [27] Our study shows that similarly to apoLp-III, perilipin 3 has the highest insertion for saturated lipid. In fact, this observation is much stronger for the helix bundle domain of perilipin 3 than for apoLp-III. Our data is also consistent with another study indicating that the N-terminal and C-terminal of perilipin 3 have distinct functions [28]. Since the C-terminus of the protein shows the highest insertion for the lipid monolayer, it can be said that the C-terminus is responsible for the protein insertion to the LD surface, where the N-terminus contributes to the targeting of the perilipin 3 protein to the LD surface.
Using droplet tensiometry to study the interaction of apoLp-III and apoE 3(NT) at air-water interface.

Here, I developed our home built tensiometer to measure the interfacial tension. Through this system, one can mimic the intracellular lipid droplets and study the lipid-protein interaction at the oil interface. I used apoLp-III as a lipid binding model protein, since apoLp-III is an amphipathic α-helix bundle protein. Here the protein binding is demonstrated by a decrease in interfacial tension for the oil droplet. Previous work has used POPC (one major lipid found on the LD surface) to form the phospholipid monolayer on the oil interface. However, here I studied the lipid-protein interaction by incorporating other lipids with different physical-chemical properties. This change in lipid composition contributes in studying the influence of the head group charge and the molecular shape in lipid-protein interaction.

To the best of our knowledge, the interaction of the full-length protein has never been studied on the oil-phospholipid-water interface as a function of lipid composition, as in this work. The aqueous phase is either pure water or buffer, which allows us to test the importance of electrostatic interactions on both lipid adsorption and protein insertion. POPC is chosen as the major lipid present in the phospholipid monolayer. PE, DAG and PA are selected according to the mass spectrometry results available on the lipid species present on the LD monolayer. Data shows that the lipid packing density and the type of lipid present in the lipid monolayer affects the interaction of the apoLp-III protein with the oil-phospholipid interface.
The adsorption data also suggests that during the formation of small LDs, while LDs is gaining additional neutral lipid, the hydrophobic patches on the oil surface are not filled via membrane fusion, i.e. the additional supply of more phospholipid (further explanation is provided in Chapter 5). This idea suggests that the protein on the LD surface are necessary to make LD stable in aqueous cytosol.

Aside from studying the self-assembly of a phospholipid-protein monolayer, I also considered the dynamics on changing the oil droplet size. Here, I was able to change the size of the oil droplet, which allowed me to: 1) study the lipid packing on the oil interface and 2) investigate protein behavior when the droplet is under stress.

Compression and decompression of the oil droplet causes variation of the surface pressure of the oil droplet vs. droplet area. In order to better interpret our finding, we introduce two mechanisms, reversible and irreversible hysteresis. Reversible hysteresis refers to the mechanism through which successive compression/decompression cycles for the oil droplets follows the same path. When successive compression/ decompression cycles follow totally different trends, the mechanism is irreversible hysteresis. When the protein is just interacting with the oil droplet, the isotherm for the variation in surface pressure vs. the droplet surface area has reversible hysteresis.

In order to study the effect of lipid packing on the oil interface, the surface of the oil droplet was covered with a monolayer of different lipid mixture: POPC, PC:PE, PC:POG, and PC:PA. The oil droplet follows reversible hysteresis for all the lipid monolayer formed.
by all lipid mixtures in water. The lipid packing follows the same hysteresis (reversible) in buffer as well.

While applying the compression and decompression on the oil droplet covered with different lipid monolayer, the apoLp-III protein shows different behavior. Except for the lipid monolayer formed by PC:PA mixture, the surface pressure isotherm with inserted protein follows irreversible hysteresis in water. However, charge screening the oil surface affects the protein behavior during compression and decompression (in buffer).

Our results reflect the importance of headgroup size and charge for the hysteresis that the oil droplet follows in the presence of the protein on the interface. We speculated that the protein can undergo two different mechanism during compression. The first one would be that during compression, the oil surface becomes smaller, and that with fewer binding sites for the protein, the protein leaves the surface completely or partially. The second possibility is that the protein removes some of the oil or the lipid monolayer from the interface during compression. We do not know which of these mechanisms are more favorable for the protein. The measurement of surface tension has its limits.

We follow this study by investigating protein insertion into to a lipid monolayer formed on the surface of an oil droplet as a function of the initial pressure of the lipids alone. In addition to apoLp-III, I also studied apoE 3, which is a mammalian apolipoprotein. ApoE3 shares structural and functional factors with perilipin 3. The N-terminus of the apoE 3 has a 4 helix amphipathic α-helix bundle, similar to the C-terminus of perilipin 3. Our data shows that both apoLp-III and apoE 3 (NT) has the highest affinity for negatively charged
lipids, which is similar to our data on full length perilipin 3 and its N-truncation mutants [27]. In correlation with our Langmuir monolayer data, the highest insertion of the two proteins occurs for the lipid monolayer composed of PC:POG [29]. Yet it is challenging to relate our data to the physiological system and more studies need to be done in this regard.

Protein unfolding plays important roles in protein insertion into lipid monolayers [30]. To test this, I used a mutant of apoLp-III where two amino acid residues from helix 1 and 5 were replaced by cysteine. A disulfide bond forms, when two cysteine residues are directly adjacent to each other, so that this mutant protein bundle does not unfold. Our data shows that for this mutant of apoLp-III, both insertion and affinity are significantly less than for native apoLp-III protein. Since the hydrophobic residues are located in the inner surface of the helix bundle, it is necessary for the amphipathic helix-bundle to unfold when the protein binds to a lipid interface.

Suggested future experiments

Identifying the parameters that affect the lipid-protein interaction at the oil interface leads to better understanding of the biophysics of LDs. Through my studies in chapter 4 and 6, I emphasized the role of the amphipathic $\alpha$-helix bundle in lipid-protein interaction. However, many proteins are not fully $\alpha$-helical, and can form other hydrophobic surfaces that can facilitate protein binding. I thus suggest a set of further studied, classified here by their specific experimental method, such as tensiometry and laser tweezers.
Droplet tensiometry and related studies

The first step in this study is to characterize different domains present in the structure of the proteins associated with the surface of LD. Our study with Langmuir monolayer showed that the amphipathic α-helix bundle domain has the highest insertion into the lipid monolayer. Our study also showed that the insertion of the protein to the lipid monolayer decreases for the full-length perilipin 3 protein. We should test the surface activity of different protein domains on the oil interface, since it is a more physiologically relevant system. Additionally, we can understand if these domains interact with the oil interface.

The structure of proteins from the perilipin family share both an amphipathic α-helix bundle and 11-mer domains. The 11-mer repeat is a protein domain with a repeating 11 amino acid pattern. In addition to the α-helix bundle, the 11-mer domain also forms hydrophobic surfaces as we showed in chapter 4 [27]. The pattern from the 11-mer domain is similar for all perilipin family proteins, although only perilipin 4 contains a complete 11-mer repeat domain. Through the helical wheel representation, we observe that the 11-mer repeat domain of the perilipin 3 protein can form hydrophobic surfaces. It is critical to understand if these hydrophobic surfaces affect the protein interaction with the hydrophobic surface of the oil. Through mutation we are trying to answer two questions.

1) Does stitching the helix bundle together, like the stitching apoLp-III, affects the affinity and insertion of the protein? This teaches us something about the role of this domain in lipid interaction. And we can also introduce this mutant into cells to study the physiological role of the protein (if there are specific phenotypes).

2) How replacing the proline residues
in 11-mer repeat of perilipin 3 would affect the lipid-protein interaction? Since proline residues are highly conserved in the structure of 11-mer repeat. Protein mutation can help in identifying the amino acid residues necessary in the protein structure, to facilitate the protein interaction with the lipid membrane. This process contributes to characterizing the binding sites for each protein in contact to the phospholipid monolayer.

*Protein engineering*

Since the crystal structures of many human proteins are not yet available, the protein can be modeled by a protein of the same type with known crystal structure. Through this method, one can better choose the amino acid residues to replace. Tensiometry can then characterize the changes in insertion and dynamics due to those changes, as I did using the mutant alpha helix bundle which could not unfold.

*Surface fluorescence screening*

Surface fluorescence screening can address questions on monolayer reconfiguration during LD shrinkage, at least for the same model triolein droplet we use in the tensiometer studies. One can tag the lipid monolayer with fluorescence dye, or use fluorescence lipid to form the lipid monolayer surrounding the neutral core. Successive compression and expansion of oil droplet leads to surface tension hysteresis that can, with further automation of the tensiometer, be characterized by the variation in work done on each path. The work would be correlated to the changes in the monolayer configuration on the surface of oil droplet. Fluorescence screening can further address whether the reconfiguration is confined
to the phospholipid monolayer, or if it is accompanied by variation in the oil droplet structure.

This work can further explain lipid-protein interaction on the LD interface, which will shed light on the structure and function of LD. In order to reveal the role of the amphipathic \(\alpha\)-helix domain of the protein associated with the surface of LD, we need to understand the fundamental mechanism through which the protein interacts with the LD phospholipid monolayer surrounding the hydrophobic core.

*Laser tweezers (Optical trapping)*

Force spectroscopy is a method to measure the interaction and binding forces between molecules. Through force spectroscopy one can study the behavior of the molecule under some mechanical force or stretching. Optical tweezers (optical trapping) is an instrument which uses a focused laser beam to investigate the behavior of the molecule undergoing any attractive or repulsive forces. Optical traps are very sensitive instrument that detect any displacements in the nanometer range.

Through tensiometry we observe the changes in interfacial tension when the protein interacts with the oil interface. We had to guess if the whole protein or some part of it dominates the interaction. However, applying mechanical force on the oil droplet to detach each protein helix from the surface would give insight into the role of each helix when the whole protein interacts with the oil interface. As I have mentioned in chapter 5 and 6, each helix has different physical chemistry in interacting with the oil interface. Yet, we remain
uncertain if there exist protein-protein interactions when the protein comes off from the surface.

Through helical wheel representations, we can gain a better perspective about the arrangement of amino acid residues in one helix, if the 3D structure of the protein is not available. The helical wheel presentation of apoLp-III shows that the presence of polar amino acid residues in the hydrophobic face of the protein destabilizes the helix bundle. These residues are present in helix 1, 2 and 5. However, helix 3 and 4 would have a more stable hydrophobic surface since they have no polar head-group to perturb the hydrophobic surface. I speculate that applying mechanical force to detach the helices separately would further test the interactions of the helix with the oil interface. It would be possible to address the interaction (electrostatic or hydrophobic) which has the highest impact on the helix binding.

**In Conclusion:**

Here, we focused on studying the lipid-protein interaction on the LD surface. Our study of the amphipathic α-helical bundle of the C-terminus of perilipin 3, full-length apoLp-III and the N-terminus of apoE shows that the amphipathic α-helix bundle is necessary for the protein insertion into the lipid monolayer surrounding the neutral core of the lipid droplet. All proteins mentioned above show the highest affinity for the negatively charged lipid, which indicate an effect of electrostatic interactions in lipid-protein binding at the LD surface.
Using our homebuilt droplet tensiometer, we also showed that protein inserts and it interacts with the oil interface. Our data also indicates that the protein unfolding facilitates the protein insertion and interaction to the LD surface.

References:


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