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Studying the Interaction of Antifreeze Proteins (Type I and Type III) with the Phospholipid Monolayers at Air/Water Interface

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STUDYING THE INTERACTION OF ANTIFREEZE PROTEINS (TYPE I AND TYPE III) WITH THE PHOSPHOLIPID MONOLAYERS AT AIR/WATER INTERFACE

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HEART transplantation is a successful therapeutic procedure for the patients with end stage heart disease. Various preservative techniques have been developed to increase the shelf life of organs, but still there can be tissue damage during cold storage. One mechanism leading to damage is disruption of cell plasma membranes as they pass through temperature induced phase transitions. The addition of antifreeze protein (AFP) or glycoprotein (AFGP) to storage solutions has been hypothesized to reduce the leakiness of lipid membranes. From studies using liposomes it has been shown that AFPS can prevent leakage through lipid membranes, although the mechanism of interaction is not known. We hope to elucidate the mechanism by investigating AFP-lipid interactions at the air-water interface.

We measured surface pressure-area isotherms using a mini Langmuir Trough (Nima 112D) to observe the interactions between lipids and AFPS. We investigated two phospholipids with zwitterionic head groups (DPPC, DMPE) and four proteins - one Type I AFP, two Type III AFPS (RD1, RD3) and BSA control. Our results showed weak or no interactions between all protein-lipid combinations except for the combinations of
RD3 with DPPC and Type I with DMPE. These interactions were observed as an increase in molecular area at high surface pressure compared to pure lipid monolayers. These results suggest that not all antifreeze proteins behave similarly with any particular type of lipid. Our future plan is to compare these isotherms with liposome leakage studies and predict which proteins are good preservation agents, so that we will be able to develop a protocol by which one will be able to determine a priori whether a compound will be a successful membrane protector.
# TABLE OF CONTENTS

ABSTRACT ................................................................................................................. iv
LIST OF TABLES .......................................................................................................... viii
LIST OF FIGURES ....................................................................................................... ix

CHAPTER I
INTRODUCTION ........................................................................................................... 1
  1.1 Organ Preservation Problem ............................................................................... 1
  1.2 Preservation Techniques ..................................................................................... 2
  1.3 Description and Model of Plasma Membrane .................................................... 3
  1.4 Mechanism of Disruption .................................................................................... 3
  1.5 Introduction to Lipids .......................................................................................... 4
  1.6 Antifreeze Proteins Interact with Lipids ............................................................... 6
  1.7 Introduction of Antifreeze Proteins (AFPs) .......................................................... 8
  1.8 Surface Pressure-Area Measurements .................................................................. 9
    1.8.1 Isotherm ......................................................................................................... 10
    1.8.2 Langmuir-Trough .......................................................................................... 11
  1.9 Studies of protein-lipid interactions at air-water interfaces ................................... 13
  1.10 Direction of research .......................................................................................... 14

CHAPTER II
MATERIALS AND METHODS ..................................................................................... 15
  2.1 Summary .............................................................................................................. 15
  2.2 Synthesis of Type III AFP (RD3N) ....................................................................... 15
    2.2.1 Single Domain RD3 (RD3N) Gene ............................................................... 15
    2.2.2 Digestion and Ligation of RD3N and pET 20b ............................................. 16
    2.2.3 Transformation and Plasmid Extraction ...................................................... 17
    2.2.4 Expression of RD3N .................................................................................... 17
    2.2.5 Extraction and purification of RD3N ............................................................ 18
    2.2.6 High performance Liquid Chromatography (HPLC) .................................... 19
    2.2.7 Thermal Hysteresis ...................................................................................... 19
  2.3 Surface Pressure-Area Isotherms ......................................................................... 20

CHAPTER III
RESULTS ....................................................................................................................... 23
  3.1 Protein Expression, Purification, and Characterization ......................................... 23
    3.1.1 DNA Sequencing Result ............................................................................. 23
    3.1.2 Thermal Hysteresis ..................................................................................... 24
    3.1.3 HPLC and Mass Spectrophotometry ............................................................ 25
  3.2 Isotherms of Pure Proteins .................................................................................. 27
    3.2.1 Isotherms of Type III (RD3N, RD3) AFPs ................................................... 27
    3.2.2 Isotherm of Type I AFP .............................................................................. 28
    3.2.3 Isotherm of Pure BSA .................................................................................. 29
  3.3 Isotherms of Pure Lipids ..................................................................................... 30
    3.3.1 Isotherm of DPPC ....................................................................................... 30
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Amino acid sequences of RD3N and RD1</td>
<td>23</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

Figure 1. Structure of DMPE and DPPC lipids ......................................................... 5
Figure 2. AFP Structures .......................................................................................... 9
Figure 3. Schematic of a typical Isotherm ................................................................. 11
Figure 4. Photograph of Langmuir trough ................................................................. 12
Figure 5. Structure of ice crystal in the presence of RD3N .......................................... 25
Figure 6. Plot showing Molecular weight of RD3N .................................................. 26
Figure 7. Isotherms of RD3 and RD3N ..................................................................... 27
Figure 8. Isotherm of Type I AFP ............................................................................ 28
Figure 9. Isotherms of BSA ..................................................................................... 29
Figure 10. Isotherm of pure DPPC ........................................................................... 30
Figure 11. Isotherm of pure DMPE .......................................................................... 31
Figure 12. Isotherm of DPPC with BSA ................................................................... 32
Figure 13. Isotherm of DPPC with RD3N ................................................................. 33
Figure 14. Isotherm of DPPC with RD3 .................................................................. 34
Figure 15. Isotherm of DPPC with Type I ................................................................. 35
Figure 16. Isotherm of DMPE with BSA .................................................................. 36
Figure 17. Isotherm of DMPE with RD3N ................................................................. 37
Figure 18. Isotherm of DMPE with RD3 .................................................................. 38
Figure 19. Isotherm of DMPE with Type I ................................................................. 39
Figure 20. Schematic showing lipid interactions with Type III (RD3) ......................... 45
Figure 21. Schematic showing lipid interactions with Type III (RD3N) ....................... 46
Figure 22. Schematic showing lipid interactions with Type I ..................................... 47
CHAPTER I
INTRODUCTION

1.1 Organ Preservation Problem

For end stage heart disease, heart transplantation has been a successful therapeutic procedure starting in the early 1980s, and since that time there has been constant progress in this procedure. With the increase in world’s population there has been increase in number of patients with heart disease and on the other side there is a scarcity of donor hearts. Maintaining the viability and minimizing damage to the donor heart during its harvest, transport, and implantation into the recipient is an important part of transplant success. Due to this, the preservation of donor hearts has a huge importance. Explanted hearts are stored in hypothermic (4-8°C) ischemia to maximize the time available for getting the heart to the implantation site [1]. The hypothermic conditions slows down the metabolic rate and by the addition of various preservative solutions keeps the tissue in good condition but still the transplantation should occur within six hours [2]. This shortage of time for transplantation can be overcome by increasing the shelf life of explanted hearts, with which there would be a greater flexibility in their distribution and increase in success rate of transplantation. We need some new techniques and storage solutions which will increase the storage life of explanted hearts. That is why there is a
significant interest in the development of new preservative techniques and storage solutions [3].

1.2 Preservation Techniques

Storage at low temperature increases the time that tissues and cells can remain viable, because as the temperature decreases, metabolic processes and physical and chemical degradation are slowed. Freezing can further increase storage time, so cells and embryos are routinely stored at sub-freezing temperatures for long-term storage. Even though important strides have been made in cryogenic storage through new cryopreservation agents and the use of vitrification [4], a significant loss of cells still is expected during cryopreservation [5]. Such loss of a fraction of cells is acceptable in cases when only some of the cells need to remain viable following preservation, for example sperm, oocytes, or cell cultures. This cell loss from the freeze-thaw cycle is not acceptable for whole organ storage, limiting organ storage to temperatures above the freezing point.

Unfortunately, some damage can still occur to cells even at above freezing temperatures through various mechanisms. To help minimize tissue injury, organ preservation solutions already can contain various additives including statins, channel blockers, complement inhibitors, and others, which act to maintain the cell electrolyte concentrations and to prevent inflammation upon repurfusion of the organ [6]. A specific mechanism leading to cellular damage at above freezing hypothermic temperatures is the disruption of the plasma membrane as cells pass through temperature induced phase transitions [7, 8].
1.3 Description and Model of Plasma Membrane

All living cells have plasma membrane, which separates the cytoplasm of cell from extracellular fluid. Plasma membrane is a semipermeable membrane which is made up of lipids and proteins. The function of this membrane is to regulate the transport of material required for living of the cell.

Plasma membranes generally exist in a two-dimensional fluid-like state where all lipids and proteins are free to move with in the membrane as described by the fluid mosaic model [9]. According to this model the proteins are embedded in lipid bilayer where the hydrophobic tails of lipids are pointed towards center and the hydrophilic heads are pointed towards the surface of the membrane. These embedded proteins could be in different positions like projecting one or the other side out, projecting both sides out or lying between the lipid bilayers. These proteins have reduced mobility and can have no mobility if their function is linked to the position on plasma membrane. Since the development of the original model, it has been shown that mobility in the membrane is not as free as originally thought – groups of lipids within the membrane can associate in a gel-like state that diffuses together within the membrane as so-called “rafts” [10]. In any case, sufficient amounts of lipids must be in a fluid state to maintain the fluidity of the membrane.

1.4 Mechanism of Disruption

When the temperature of the membrane is decreased (as for hypothermic storage), its lipids can go through a phase transition from the fluid state to a gel-like state. Such a transition will occur at different temperatures [11], depending primarily on the geometry of the lipid tails and their ability to pack together. Additional compounds incorporated in
the membrane, such as cholesterol, can also affect the lipid phases. As the plasma membrane goes through these transitions, the integrity of the membrane can be breached resulting in leakage of the material into or out of the cell, leading to cell damage. The mechanism has not been clearly determined, but is likely the result of poor packing of domains in the gel and fluid-like states [12, 13].

1.5 Introduction to Lipids

A typical lipid consists of a hydrophilic head and two hydrophobic tails making it amphiphillic in nature. Lipids generally are saturated or unsaturated depending up on the presence of double bonds on the hydrocarbon tails. A kink is observed if the hydrocarbon tail has a double bond and this kink results in a poor packing of the lipids during freezing. The important phospholipids present in the cells of the human heart are phosphatidylcholine (PC), phosphatidylethanolamine (PE), diphosphatidylglycerol (DPG), and sphingomyelin (SPH) [27]. The common lipids used in the liposome and protein-lipid interaction studies are L-\(\alpha\)-distearoyl-phosphatidylcholine (DSPC), dipalmitoylphosphatidylcholine (DPPC), L-\(\alpha\)-dimyristoyl-phosphatidylcholine (DMPC), L-\(\alpha\)-dimyristoyl-phosphatidylethanolamine (DMPE), and L-\(\alpha\)-dimyristoyl-phosphatidic acid (DMPA), which are biologically important surfactants. The structure of DMPE and DPPC lipids are shown in Figure 1 where they have different head groups and different tails. The hydrophilic head groups of these two lipids are zwitterionic and also they are saturated as there are no double bonds on the hydrocarbon tails.
Various lipids make up large portion of the cell membranes and can be approximated as two-dimensional materials, confined to the two-dimensions of the bilayer surfaces. Therefore they can be described as being in a two-dimensional state such as 2-D liquids (fluid phase) or solid/crystals (gel phase). Analogous to three-dimensional materials the state changes of such two-dimensional materials can be altered by changing the temperature, surface pressure (analogous to pressure), or area (analogous to volume). It is important to note that cell membranes are not pure lipid materials, but contain many “additives” such as cholesterol, membrane bound proteins, glycolipids,
etc., which will affect their phase behavior. Additionally, the structure of the lipids, especially the saturation of the hydrocarbon tails, will dictate how they will pack into 2-D solids [28], for example unsaturated fatty acid results in poor packing and lowers the melting point.

As cells are cooled the membranes will go through various transitions based on their composition. Phase separation during these transitions has been suggested as one mechanism by which the integrity of the cell membranes is compromised as they are cooled for low temperature storage [13]. As the membrane goes through a transition, the inability of it to smoothly rearrange may lead to transient “leakiness” of the membrane allowing a loss of intracellular fluid and/or the influx of extracellular components. The interaction of antifreeze proteins with lipids has been demonstrated to affect the membrane during these transitions [7].

1.6 Antifreeze Proteins Interact with Lipids

The addition of antifreeze protein (AFP) or glycoprotein (AFGP) to storage solutions has been shown to reduce the leakiness of lipid membranes [7, 14]. AFP and AFGP were shown to have beneficial effects on cell viability during cryopreservation and for storage at above freezing temperatures [15]. Initially it was proposed that the mechanism of protection at temperatures above freezing was through an interaction with channel proteins to regulate ion flow at low temperature [16], but subsequent experiments provided convincing evidence for a more general non-specific lipid stabilization mechanism [7]. These leakage studies were done using liposomes which were made up from different lipids dielaidoylphosphatidylcholine (DEPC), dielaidoylphosphatidylethanolamine (DEPE), dielaidoylphosphatidylglycerol (DEPG),
and DPPC. While making the liposomes carboxyfluorescein (CF) were incorporated in them in order to detect the leakage. The leakage was detected by measuring the loss of fluorescence from the liposomes. It was found that AFPs and AFGPs significantly reduced such leakage from liposomes. Further experiments using such liposome leakage experiments have revealed that this process is both AFP [17] and lipid composition [18] dependent. The liposome studies using lipids such as DEPC, DEPE, and DEPG which are commonly found in animal plasma membranes showed that only AFGPs were successful in stabilizing these lipids while Types I, II, and III fish AFPs actually lead to destabilization of the lipid membrane at low temperatures [14, 17]. However, Type I AFP was found to stabilize thylakoid membrane lipids from plants [21], which is somewhat consistent with earlier measurements that showed that Type III AFPs lead to damage of thylakoid membranes while Type I AFP did not affect them [22]. These experiments determine that the effectiveness of AFPs in preventing the leakage of lipid membranes depends up on the type of lipid.

Rubinsky et al. used pig oocytes which are sensitive to hypothermic storage (4°C) to see the effect of AFGP during the hypothermic storage. The leakage was detected by measuring membrane potential of the oocytes which were exposed to hypothermic temperatures and compared to fresh oocytes of the same batch. Ion leakage was observed when using low concentrations of AFGPs, but the leakage was reduced with the increase in concentration of AFGPs [19, 20]. So, the AFPs can inhibit/induce leakage depending upon its structure and composition of the lipid.
1.7 Introduction of Antifreeze Proteins (AFPs)

AF(G)Ps were first discovered in artic fish that live in icy waters at temperatures below the freezing point of their bodily fluids, and since have been found in a wide range of organisms that live in environments that experience temperatures below freezing [23-25]. Antifreeze proteins are amphiphillic in nature i.e they both hydrophobic and hydrophilic regions. Antifreeze proteins have several unique characteristic properties that can help organisms avoid or tolerate freezing. For one, AFPs depress the freezing point of solutions below their colligative melting temperature by kinetically inhibiting the growth of ice crystals. The difference in the melting and freezing temperatures (termed thermal hysteresis) is the most common measure of AFP activity, and functionally defines what proteins belong to this group. In addition to non-colligative freezing point depression, AFPs inhibit recrystallization (or the growth of large crystals at the expense of smaller ones) and cause ice to grow with specific crystal habits not typically observed.

Four structurally different types of antifreeze proteins plus antifreeze glycoprotein have been discovered in various Arctic and Antarctic fish. Type I fish AFP is helical in nature while Types II, III, and IV are globular proteins with very different folding motifs. Two known insect AFPs have two different beta-helical structures [15]. Given the diversity in structure a commonality of AFPs seems to be a relatively flat, slightly hydrophobic surface of the protein that is able bind to specific ice crystal lattices [26]. The most commonly used antifreeze proteins in previous liposome leakage studies are Type I and Type III AFPs. Representative structures of these proteins are shown in Figure 2.
Figure 2. A) Type I AFP B) RD3 Type III AFP which has two identical domains attached to a nine residue linker C) Single domain RD3 (RD3N) which is identical to the individual domains of RD3.

1.8 Surface Pressure-Area Measurements

Surface-pressure isotherms have long been used to probe the behavior of insoluble or slightly soluble molecules at air-water interface. These isotherms are characteristic of each material and indicate the type of packing and arrangements the molecules take at an interface. The basic premise of the Langmuir trough is that a known quantity of amphiphilic material is isolated at the air-water interface and the area of this interface can be increased or decreased. The interfacial area divided by the number of molecules at the interface gives the molecular area, i.e. the area available for each
molecule. Surface pressure measurements (the difference between the measured surface tension and the surface tension of pure water) are used to monitor the state of the molecules at the interface.

1.8.1 Isotherm

A typical surface-pressure isotherm is a plot of the surface pressure against the molecular area at constant temperature. The way the molecules interact is observed as different features of the isotherm. When the molecules have ample space that they do not completely cover the surface, the surface pressure is close to zero. When the area is decreased so that the molecules begin to interact, the surface tension will decrease, resulting in a positive surface pressure of the monolayer. During this process various phases of the system can be observed for large areas, the low $\Delta P/\Delta A$ corresponds to a 2-D gas; at smaller areas, a larger $\Delta P/\Delta A$ to a 2-D liquid (fluid phase); and at even smaller areas, the larger $\Delta P/\Delta A$ corresponds to a 2-D crystal or solid (gel phase). If the layer is compressed to smaller areas, the film will collapse by various possible mechanisms. As with bulk materials, there can be a gas to solid transition without forming a liquid. Figure 3 illustrates prototypical surface-pressure isotherms of lipids.
Figure 3. Schematic of 2-D phases of amphiphiles at an air-water interface. Isotherms represent amphiphiles that (A) have a direct “gas” to gel phase transition and (B) have a “gas” to fluid and fluid to gel transition.

1.8.2 Langmuir-Trough

The previous experiments on surface pressure measurements were done using either Langmuir trough or KSV trough. Langmuir troughs are generally computer controlled and consists of two polytetrafluoroethylene (PTFE) barriers which can apply symmetric compression to the surface, while the surface pressure is measured using a Whilhelmy plate which has a resolution of 1µN/m. This computer controller can run with a constant compression rate or hold the film at a constant surface pressure. The temperature of the instrument can be controlled by circulating heated or chilled water through channels in the aluminum support of the trough. This circulating water does not
mix with the protein solution in the trough, but controls the solution temperature by changing the trough temperature. The general figure of the Langmuir trough is shown in Figure 4. It is advantageous to use Langmuir mini trough due to its small volume (50 ml) of sub-phase which minimizes the amount of protein solution needed for each experiment.

Figure 4. A) Photograph of Langmuir trough. The PTFE trough is mounted on an aluminum support. B) Schematic of Tensiometer

Surface tension is measured using a tensiometer, which is a balance with a probe attached to it. This measures the change in weight of the probe when it is in contact with the liquid. The excess weight comes from the weight of the meniscus attached to the probe which is shown in Figure 4. The general equation of surface tension is given by

$$\gamma \cos \theta = \Delta W / P$$

where \( \Delta W \) is the change in weight, \( P \) is the perimeter of the probe, \( \gamma \) is the surface tension, and \( \theta \) is the liquid-solid contact angle [28]. The primary factors affecting surface tension measurement are geometry of the probe and the contact angle. The probe’s size
and shape can be controlled easily. As the setup is small it is difficult to measure the contact angle and there is a chance of getting improper readings which results in a different surface tension. To avoid this, the contact angle is made zero by selecting high energy surface probes. There are different types of probes which can be used to measure the surface tension, such as Wilhelmy plate, DuNouy ring and metal rod. Commonly, a Wilhelmy plate is used with a Langmuir trough.

1.9 Studies of protein-lipid interactions at air-water interfaces

The previous studies used surface pressure measurements to see the adsorption and penetration of protein into the phospholipids layers at the air/water interface [29, 30, 31]. Most of these experiments used BSA as the protein solution in the sub phase and different lipids on the surface and observed the adsorption of protein into the phospholipids layer at air/water interface. The adsorption of protein was observed as the change in the molecular area compared to the molecular area of pure lipid. These studies claim that the BSA will tend to adsorb more at air/water interface when the head groups of phospholipids are anionic and less adsorption is observed when they are zwitterionic [29]. The adsorption of BSA into the phospholipids layer at air/water interface will also depend upon the structure of head group of the lipids [31]. Cho et al. did the surface pressure measurements using mixed monolayers of lecithin and BSA, with ratios ranging from 0.5 to 2.0 [30]. Spreading of the mixed monolayers is done using Turner’s monolayer spreading method. At high pressures the isotherm of mixed monolayer of BSA and lecithin was close to the isotherm of pure lecithin possibly due to the surface pressure and the steric exclusion effects.
1.10 Direction of research

It is clear from liposome and whole cell experiments that AFPs can interact with plasma membranes, in some cases providing a benefit for cells stored at low temperature. However, if AFP is to be used for this purpose, a better understanding of the mechanism by which the membrane is stabilized. In this thesis we use surface pressure balance measurements to complement the studies that have been done using the liposomes and whole cell models. These preliminary data are a start toward quantifying changes that occur in the membrane, so as to be able to predict the ability of AFPs to stabilize plasma membranes with specific compositions. We have characterized interactions of several lipid-AFP combinations to gain a better understanding AFP-membrane interaction. With further characterization, we believe that this system can be used to help develop more effective additives for storage of tissues.
CHAPTER II
MATERIALS AND METHODS

2.1 Summary
In order to study protein-lipid interactions, we characterized Type I and Type III AFP interaction with two different phospholipids. Cultures for expression and purification of Type I [32] and a two-domain Type III AFP [33, 34] were available in the laboratory. However, a single domain Type III AFP was desired to compare to previous studies, so we used standard molecular biology techniques to produce a single domain of the two-domain protein. Here the procedures used to prepare the proteins and characterize their interactions on a Langmuir trough will be described.

2.2 Synthesis of Type III AFP (RD3N)

2.2.1 Single Domain RD3 (RD3N) Gene
We prepared the gene for a single domain of type III antifreeze protein by polymerase chain reaction (PCR) amplification from the gene for the two-domain RD3 protein [35] using a PCR Sprint Thermocycler (Thermo Electron Corporation). The program we used holds the sample at 94°C melting temperature for 1 min followed by
two sets of 15 cycles, in the first holding the sample at 57°C annealing temperature for 30 sec and elongation at 72°C for 1 min and in the second using two annealing temperatures 57°C and 62°C for 30 sec each and elongation at 72°C for 1 min, and finishing with an elongation at 72°C for 6 min. The PCR reaction volume contains primers (concentrations of 10µM), RD3 template DNA and PCR master mix (Promega). We did a PCR of RD3 gene by using a forward primer (GGCCCGCATATGAATAAAGCTTCC) and a reverse primer (GCGGGATCCCTATTCGTAGTTTTT) which amplified the N-terminal domain of RD3 and contained restriction enzyme cut sites for insertion into the vector. We ran 2% Agarose gel (Appendix A) to separate the amplified RD3N gene from the full RD3 gene. We cut the RD3N from gel and purified it by using the Wizard SV Gel and PCR Clean Up System (Promega) and eluted the DNA in 30µL of nuclease free water.

2.2.2 Digestion and Ligation of RD3N and pET 20b

The enzymes used for digestion are BamH I and Nde I (New England Bio Labs). BamH I will cut RD3N at one end and leaves a 5’ GATC overhang with a sticky end GGATCC. Nde I will cut the other end of RD3N and leaves a 5’ TA overhang with a sticky end CATATG. The total reaction volume for the digestion is 30.8 µL which is calculated for digestion of 10 µL (10^{10} M) RD3N. This reaction volume is incubated at 37°C for 1 hour. We ran a 1.5% Agarose gel and separated the digested piece of DNA from the enzymes, reagents and cut pieces of DNA. We purified the gel and eluted in 30µL of nuclease free water. Similarly, we double digested pET 20b vector (Novagen). We used T4 DNA ligase (Promega) to ligate both double digested RD3N and double digested pET 20b. A 2:1 ratio of insert to vector was used to calculate the reaction volume. We incubated the reaction volume for 20 minutes at room temperature.
2.2.3 Transformation and Plasmid Extraction

We used 10 µL of the ligated product in the transformation reaction. After mixing it with BL21 competent cells we placed it on ice for 30 minutes and heat-shocked it for 40 seconds in a 42°C water bath. We added 250 µL of room temperature SOC medium (Novagen) to above reaction volume and incubated at 37°C for one hour with shaking (250 rpm). Then we spread the reaction volume on LB agar plates which has antibiotic (ampicillin) and then incubated the plate at 37°C overnight. The colonies grew on the LB agar plate and we selected a few individual colonies to grow 6 ml starter cultures in LB medium (Appendix A) with ampicillin in it. The cultures were incubated at 37°C overnight with shaking (300 rpm). We made 1 ml frozen stock of the starter culture and stored in a -70°C freezer. The remaining 5ml starter culture is transferred into the 15 ml falcon tube and centrifuged at 6000 x g for 15 min and pour of the supernatant and retained the bacterial pellet. We purified the plasmid DNA of the obtained bacterial pellet and eluted it in 30 µL of nuclease free water. This DNA was sequenced in DNA Analysis Facility, Science and Research Bldg (Cleveland State University) demonstrating that we have the correct sequence for RD3N.

2.2.4 Expression of RD3N

We made 5 ml of starter culture from the frozen stock of RD3N and mixed in 1 L of LB medium with 0.1 mg of ampicillin in it. We took a 2 L flask and transferred the entire solution into it and incubated at 37°C with shaking at 300 rpm for 6 hours. When the optical density of the culture reached 0.6 using a UV spectrophotometer (set at 600nm
wavelength) and to induce the expression we added 120 mg of isopropyl-beta-D-thiogalactopyranoside (IPTG) (Fisher Biotech). We continued the incubation for another 6 h and transferred the solution into four 250 ml centrifuge bottles and balance them. We centrifuged the balanced bottles at 7000 rpm for 15 min and removed the supernatant and stored the pellet at -70°C.

2.2.5 Extraction and purification of RD3N

We resuspended the frozen pellets in 10 ml BPER (Pierce) and centrifuged them at 27000g for 15 min and retained the pellet. We again resuspended the pellet in 10 ml BPER and added 200 µL of 10 mg/ml solution of egg white lysozyme (ACROS) and incubated on ice for 5 min and then added 20 ml of 1:10 diluted (in ddH2O) BPER. We centrifuged at 27000g for 15 min poured off the supernatant and gel like layer and again resuspended the pellet in 30 ml of 1:20 diluted (in ddH2O) BPER and centrifuged at 27000g for 15 min and poured off the supernatant. We did this until we got a white pellet and resuspended the final pellet in 10 ml of 6M Guanadine HCL (Appendix B). We added 190 ml of refolding buffer (Appendix A) to the pellet dissolved in Guanadine HCL and chilled the solution for 4 days at 4°C.

We cut two 20 cm lengths of the dialysis tube (SPECTRUM) of MWCO 1000 for 200 ml of protein solution and soaked them in distilled water for 30 min. We then poured the protein solution into the dialysis tubes and placed them in a bucket containing 2.5 L of chilled 50 mM acetic acid buffer (Appendix A) solution of pH ~ 3.7 and kept at 4°C overnight. We replaced the acetic acid buffer solution twice. After final dialysis we poured the dialysis solution in to Oakridge tube and centrifuged at 27000g for 15 min and
retained the supernatant and stored at 4°C. We shell froze the supernatant in a 600ml Labconco flask and lyophilized it using Lyph-Lok 4.5 Litre Freeze Dry System. We lyophilized the sample for a long time in order to make the protein soluble. After lyophilizing we had 50 mg of RD3N protein in powder form which was stored at room temperature.

2.2.6 High performance Liquid Chromatography (HPLC)

Protein was purified using reverse phase HPCL using a gradient of 60% acetonitrile solution. We used a Shimadzu liquid chromatograph to purify RD3N. The column we used is ultra C-18 5 µm 150 x 10 mm (Restek) which is hydrophobic and the two solvents we used are solvent A (50 ml acetic nitrile, 950 ml H₂O, 1 ml TFA) and solvent B ( 600 ml acetonitrile, 300 ml 2-propanol, 100 ml H₂O, 0.75 ml TFA). We injected RD3N through the injector and it is adsorbed to the column and we allowed the solvents to pass through the column and the protein came out at 40% of solvent B. The protein was detected by a UV detector attached to the HPLC. We collected the sample when we observed the peak and sent it to the Department of Pathology at Case Western Reserve University for Mass Spectrophotometry (MALDI Spectrophotometer).

2.2.7 Thermal Hysteresis

Thermal hysteresis is the difference between melting and freezing temperatures of the ice crystal in the presence of antifreeze proteins (AFPs). We calculated the thermal hysteresis of single domain RD3 using a nanoliter osmometer made up of copper (built in
the laboratory of Prof. Art Heuer at CWRU by Alan McIlwain). It has an aluminum sample stage with five wells drilled in it. This is a temperature controlled device working by Peltier principle creating temperature difference between the two ends of the metal when voltage is applied and is connected to a computer where the temperature data is stored. Cold water was used as circulating fluid to remove excess heat accumulated at the bottom of the osmometer. Osmometer was attached to an optical microscope to visualize any morphological changes of the ice crystals. Immersion oil was placed in one of the wells and single domain RD3 protein was suspended in it using a Nanoliter injector. The sample was frozen by decreasing the temperature of the sample stage down to -40°C. Then, temperature was slowly increased until a single ice crystal was obtained. This temperature was recorded as melting point temperature. Ice crystal growth was observed by decreasing the temperature and the temperature at which the crystal starts growing was recorded as freezing temperature. This difference between melting and freezing temperatures was determined as thermal hysteresis.

### 2.3 Surface Pressure-Area Isotherms

We measured surface pressure isotherms of pure lipids and pure AFPs (Type III, Type I) using a computer-controlled Langmuir Mini Trough (NIMA 112D). The maximum volume of the trough is 50 ml. The trough and two moving barriers are made up of polytetrafluroethylene (PTFE). The two PTFE barriers apply symmetric compression to the surface, while the surface pressure is measured using a Wilhelmy plate with a resolution of 1 µN/m. The temperature of the trough can be controlled by circulating heated or chilled water through channels in the aluminum support of the trough. This water does not mix with the protein solution but controls the solution.
temperature by changing the trough temperature. All the data collected were saved as text files and plotted using Microsoft Excel.

To obtain accurate, reproducible measurements, it is important that the trough is thoroughly cleaned first with chloroform using kimwipes and then fill it with pure water and aspirate the surface to empty the trough. A isotherm of pure water (Millipore water 18.2 MΩ-cm) is run to make sure that the surface is clean. We zero the surface pressure with the barriers open and run the isotherm if the surface is clean the surface pressure should not increase. If the surface pressure increases then we need to clean the trough again. Once the trough is clean we set the trough temperature to 21°C by circulating water through the trough. We then put the lipids on the surface of pure water with a nonoliter syringe (Hamilton) and wait half hour for the chloroform to evaporate and then perform the isotherm cycles. With the Nima software, we set the speed of the barriers at 10 cm²/min.

The lipids we used were 1, 2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine (DMPE) which are from Avanti Polar Lipids. We used 4 µL of each lipid on the surface of pure water whose concentrations are 1.25 M and the molecular weights are 635.86 g/mol (DMPE) and 734.05 g/mol (DPPC). The surface area of the trough is 80 cm² when the barriers are open and 20 cm² when they are closed and the time taken for each compression and expansion cycle is 12 min. The lipids are in gas like phase structure when the barriers are opened and they go from gas phase to liquid like-phase and to solid like-phase when the barriers are compressed. The surface pressure was recorded using a tensiometer while the compression and expansion of the barriers was taking place and surface pressure versus
area/mol was plotted. We made 10 ml stock solution of Type I and Type III (RD3, RD3N) AFPs by adding 10 mg of protein (lyophilized product) in 5 ml of Millipore water. The surface pressure isotherms of RD3, RD3N (Type III) and Type I are measured using 0.5 ml of their solution (concentrations of 2 mg/1 ml) in the sub phase. We did the isotherm of pure bovine serum albumin (BSA) using 0.5 ml of albumin standard (Pierce) whose concentration is 2 mg/ml. We also did the isotherms with AFPs and BSA in the subphase and lipids placed on the surface.
CHAPTER III
RESULTS

3.1 Protein Expression, Purification, and Characterization

A key part of this thesis is obtaining sufficient protein for performing surface pressure experiments. RD3, a two domain Type III AFP, and a Type I protein were readily available in the lab. For direct comparison to previous studies, a single domain Type III protein was needed. This section describes the results of the expression, purification, and characterization of this protein.

3.1.1 DNA Sequencing Result

The sequence of the N-domain RD3 (RD3N) is the same as RD1, except in the place of the 39th amino acid it has asparagine (AAC) instead of lysine (AAA or AAG). The DNA sequencing results showed that our RD3N DNA has the correct sequence for the N-domain of RD3 (Table 1).
TABLE 1

<table>
<thead>
<tr>
<th>Protein (Type III AFP)</th>
<th>Amino Acid Sequence of Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>RD3N</td>
<td>AATAAAGCTTCCGTTGTTGCTAACCAGCTGATCCCGATCAACACCGCTC TGACCCCTGATCATGATGAAAGCTGAAGTTGTTACCCCGATGGGTATCCCGGC TGAGAAATCCCGGAACCTGGTTGGTATGCAAGTTAACCCTGGCTGTTCCGCTG GGTACCACCCCTGATGCCGGACATGGTTAAAAACCTACGAA</td>
</tr>
<tr>
<td>RD1</td>
<td>AATAAAGCTTCCGTTGTTGCTAACCAGCTGATCCCGATCAACACCGCTC TGACCCCTGATCATGATGAAAGCTGAAGTTGTTACCCCGATGGGTATCCCGGC TGAGAAATCCCGGAACCTGGTTGGTATGCAAGTTAACCCTGGCTGTTCCGCTG GGTACCACCCCTGATGCCGGACATGGTTAAAAACCTACGAA</td>
</tr>
</tbody>
</table>

3.1.2 Thermal Hysteresis

We made 0.25 mM solution of RD3N to do the thermal hysteresis measurements. One property of the AFP is they bind to ice crystal and inhibit their growth and change the morphology of the ice crystal.
In this experiment we find that the single ice crystal grown in the presence of RD3N did not grow for small range of temperature and the crystal structure was changed to a hexagonal to bipyramidal (Figure 5). This shows that RD3N binds to the ice crystal and inhibits its growth, which leads to a change in the morphology of the crystal structure. This shows that the protein is functionally active.

3.1.3 HPLC and Mass Spectrophotometry

HPLC was done to check whether the protein sample is pure and to separate it from any other impurities. We found that RD3N is pure by obtaining a single peak. This peak was sent for mass spectrometry to find out the exact molecular weight.
Figure 6. This is a plot from the MALDI mass spectrophotometer showing the molecular weight of RD3N Type III AFP as 7135.2 Da.

The result from mass spectrophotometer (Figure 6) gives the molecular weight of RD3N as 7135.2 Da. The calculated molecular weight of RD3N is 7023.40 Da. It is unclear where the additional weight of 111.8 Da comes from, but we assume that the protein might be associated with some additional water molecules.
3.2 Isotherms of Pure Proteins

3.2.1 Isotherms of Type III (RD3N, RD3) AFPs

![Isotherms of Pure RD3N and RD3](image)

**Figure 7.** a) Isotherm of RD3N has desorption surface pressure ~ 18 mN/m and isotherm of RD3 has desorption surface pressure ~ 23 mN/m. The rates of compression and expansion for all the isotherms are 10 cm$^2$/min. The arrow going towards left indicates compression and coming towards right indicates expansion of the isotherm cycle.

The surface collapse pressure is the pressure at which a monolayer of protein/lipid collapses, losing some quantity into the sub-phase. Surface pressure-area isotherms are formed by compressing the protein/lipid present on the surface to form a monolayer. The surface pressure of RD3N gradually increased by closing the Teflon barriers and a monolayer of RD3N is formed at a surface area of 25 cm$^2$ and by closing barriers beyond surface area of 25 cm$^2$ the protein on surface starts desorbing and its corresponding desorbing surface pressure is 18 mN/m (Figure 7). The RD3 isotherm starts from ~3 mN/m which suggests that more protein is adsorbed at the air-water interface compared to RD3N. The surface pressure of RD3 also increased by closing the barriers and a
monolayer is formed at a surface area of 45 cm$^2$. The protein started desorbing as we compress the monolayer below surface area of 45 cm$^2$ and the corresponding pressure where the monolayer of RD3 starts desorbing is 23 mN/m.

**3.2.2 Isotherm of Type I AFP**

![Itherm of Pure Type I AFP](image)

**Figure 8. Isotherm of Type I AFP which desorbs at a surface pressure of ~33 mN/m.**

In the Figure 8, the isotherm starts at zero surface pressure and as we compress the surface the surface pressure starts increasing up to a pressure of ~33 mN/m and stays constant. At this point the protein on the surface is desorbing into sub-phase that is why there is no increase in the surface pressure.
3.2.3 Isotherm of Pure BSA

Figure 9. Isotherms of BSA which has a surface collapse pressure of ~ 20 mN/m.

From the Figure 9 we see that the isotherm starts at 5 mN/m which means that there is a reasonable amount of BSA on the surface interacting with each other by decreasing the surface tension resulting in a positive surface pressure. When we start the compression cycle the surface pressure increases and it appears that it has a phase transition at 20 mN/m but after this transition the surface pressure does not increase much as we compress the surface indicating that BSA is desorbing to the sub-phase. This means that the phase transition is nothing but desorption of BSA in to sub-phase.
3.3 Isotherms of Pure Lipids

3.3.1 Isotherm of DPPC

Figure 10. Isotherm of pure lipid DPPC 4 µL spread on 50 ml of pure water which has a phase transition at ~ 6.5 mN/m.

Figure 10 shows a phase transition which is happening at ~6.5 mN/m in the compression cycle of DPPC. After this phase transition we see that there is a rapid increase in surface pressure for a small change in surface area. No collapse of pure DPPC occurs up to a surface pressure of 50 mN/m. We also see that there is a hysteresis even though there is no loss of DPPC during compression cycle. The reason for this might be the rearrangement of lipids. The lipids need some time to reach equilibrium, and to give this time we need to run the isotherms at very slow speed. Unfortunately the software can only run at 2 cm²/min, which is not slow enough.
3.3.2 Isotherm of Pure DMPE

![Graph of Isotherm of DMPE]

**Figure 11.** Isotherm of pure lipid DMPE (4 µL) spread on 50 ml of pure water which has a phase transition at ~6 mN/m. In this we see that there is no collapse of the monolayer but it still has hysteresis.

From the Figure 11 we can see there is a phase transition which is happening at ~6 mN/m in the compression cycle. After this phase transition we see that there is a rapid increase in surface pressure for a small change in surface area. No collapse of pure DMPE occurs up to a surface pressure of 50 mN/m. We also observe a hysteresis without loss of DMPE during compression cycle, as in DPPC.
3.4 Isotherms of DPPC with Proteins

3.4.1 Isotherm of DPPC with BSA

![Isotherm of DPPC with BSA](image)

Figure 12. A) Isotherm of pure lipid DPPC 4 µL spread on 50 ml of pure water which has a phase transition at ~ 6.5 mN/m. B) The Isotherm of DPPC (4 µL) spread on surface of BSA protein solution (1mg/50ml) which has a phase transition at ~ 20 mN/m.

In the compression cycle, the isotherm of DPPC with BSA (sub-phase) has a plateau starting at a surface area of 140 Å²/molecule, occurring at a surface pressure of 20 mN/m. The surface pressure does not increase much as we compress the surface from 140 to 80 Å²/lipid. The isotherm of DPPC with BSA overlaps the pure DPPC isotherm when we compress the surface below 80 Å²/molecule (Figure 12).

In the expansion cycle, the isotherm of DPPC with BSA (sub-phase) follows the pure DPPC curve down to a surface pressure of 20 mN/m and then deviates from the pure DPPC curve (Figure 12).
3.4.2 Isotherm of DPPC with Type III (RD3N) AFP

Figure 13. A) Isotherm of pure lipid DPPC 4 µL spread on 50 ml of pure water which has a phase transition at ~ 6.5 mN/m. B) The Isotherm of DPPC (4 µL) spread on surface of RD3N solution (1mg/50ml) which has a phase transition at ~ 20 mN/m.

The isotherm of DPPC with RD3N has a plateau starting at a molecular area of 170 Å²/lipid during compression cycle and this plateau is occurring at a surface pressure range of 18 mN/m. The isotherm of DPPC with RD3N (sub-phase) has an increase in surface pressure when we compress the surface from 140-70 Å²/lipid and it overlaps with pure DPPC when we compress the surface below 75 Å²/lipid (Figure 13). During the expansion cycle the isotherm of DPPC with RD3N behaves as pure DPPC down to a surface pressure of 28 mN/m and below that pressure it deviates from the pure DPPC (Figure 13).
3.4.3 Isotherm of DPPC with Type III (RD3) AFP

Figure 14. A) Isotherm of pure lipid DPPC 4 µL spread on 50 ml of pure water which has a phase transition at ~ 6.5 mN/m. B) The Isotherm of DPPC (4 µL) spread on surface of RD3 solution (1mg/50ml) which has a phase transition at ~ 24 mN/m.

The isotherm DPPC with RD3 also has a plateau starting from a molecular area of 160 Å²/lipid and the corresponding surface pressure is 24 mN/m. In compression and expansion cycles the isotherm of DPPC with RD3 deviates from pure DPPC at all surface pressures. This isotherm exhibits same surface pressures for greater molecular areas when compared to pure DPPC. In the expansion cycle it has a plateau at a surface pressure of 24 mN/m (Figure 14).
3.4.4 Isotherm of DPPC with Type I AFP

The isotherm of DPPC with Type I also have a plateau starting from a molecular area of 140 Å²/lipid and the corresponding surface pressure is 35 mN/m. In compression cycle, the surface pressure does not change much as we compress the surface from 150-70 Å²/lipid. The isotherm of DPPC with Type I seem to be identical with pure DPPC at surface pressures above 40 mN/m and in the expansion cycle up to a surface pressure of 20 mN/m and below that pressure it deviates from pure DPPC (Figure 15).
3.5 Isotherms of DMPE with Proteins

3.5.1 Isotherm of DMPE with BSA

In the compression cycle, the isotherm of DMPE in the presence of BSA has a plateau starting from a molecular area of 130 Å²/lipid. This plateau is occurring at a surface pressure of 20 mN/m. There is not much increase in surface pressure as we compress the teflon barriers from the molecular area 130-70 Å²/lipid and when we compress below 70 Å²/lipid the pure isotherm of DMPE and DMPE with BSA overlap with each other. In the expansion cycle, the isotherm of DMPE with BSA follows the pure DMPE curve up to a surface pressure of 20 mN/m and then deviates from the pure DMPE curve (Figure 16).

Figure 16. A) Isotherm of pure lipid DMPE 4 µL spread on 50 ml of pure water which has a phase transition at ~ 6 mN/m. B) The Isotherm of DMPE (4 µL) spread on surface of BSA protein solution (1mg/50ml) which has a phase transition at ~ 20 mN/m.
3.5.2 Isotherm of DMPE with Type III (RD3N) AFP

Figure 17. A) Isotherm of pure lipid DMPE 4 µL spread on 50 ml of pure water which has a phase transition at ~ 6 mN/m. B) The Isotherm of DMPE (4 µL) spread on surface of RD3N solution (1mg/50ml) which has a phase transition at ~ 18 mN/m.

During the compression cycle the isotherm of DMPE with RD3N has a plateau starting from a molecular area of 140 Å²/lipid and this plateau is occurring at a surface pressure of 18 mN/m. The surface pressure of DMPE with RD3N increases significantly as we compress the surface area from 120-60 Å²/lipid and the isotherm overlaps on pure DMPE as we compress the molecular area below 60 Å²/lipid. In the expansion cycle the isotherm of DMPE with RD3N follows the pure DMPE and deviates from it below 30 mN/m (Figure 17).
3.5.3 Isotherm of DMPE with Type III (RD3) AFP

![Isotherm of DMPE with Type III (RD3)](image)

Figure 18. A) Isotherm of pure lipid DMPE 4 µL spread on 50 ml of pure water which has a phase transition at ~ 6 mN/m. B) The Isotherm of DMPE (4 µL) spread on surface of RD3 AFP solution (1mg/50ml) which has a phase transition at ~ 24 mN/m.

The isotherm DMPE with RD3 has a plateau starting at a molecular area of 130 Å²/lipid and the corresponding surface pressure is 24 mN/m. The surface pressure doesn’t change much as we compress the surface from 130-80 Å²/lipid. In compression cycle, the isotherm of DMPE with RD3 behaves as pure DMPE at surface pressures above 45 mN/m and in the expansion cycle it behaves as pure DMPE up to a surface pressure of 25 mN/m and below that pressure it deviates from pure DMPE (Figure18).
3.5.4 Isotherm of DMPE with Type I AFP

The isotherm of DMPE with Type I have a plateau starting from at a molecular area of 140 Å$^2$/lipid and the corresponding surface pressure is 35 mN/m. In compression and expansion cycles the isotherm of DMPE with Type I deviates from pure DMPE at all surface pressures. The surface pressure doesn’t change much as we compress the surface from 140 to 70 Å$^2$/lipid. This isotherm exhibits same surface pressures for greater molecular areas when compared to pure DMPE (Figure 19).

Figure 19. A) Isotherm of pure lipid DMPE 4 µL spread on 50 ml of pure water which has a phase transition at ~ 6 mN/m. B) The Isotherm of DMPE (4 µL) spread on surface of Type I AFP solution (1mg/50ml) which has a phase transition at ~ 35 mN/m.
CHAPTER IV
DISCUSSION

4.1 Surface Pressure Area isotherms

4.1.1 BSA with DPPC and DMPE

The plateau present in DPPC with BSA and DMPE with BSA isotherms (Figures 12 and 16) occurs at a surface pressure of ~20 mN/m which is the surface pressure at which the monolayer of pure BSA desorbs (Figure 9). This shows that the BSA present on the surface is forced to go in to the sub-phase as we compress the mixed monolayer of lipid with BSA to the collapse surface pressure of pure BSA. During this compression cycle the surface pressure does not increase after this plateau because at this point the BSA is going in to sub-phase. From Figure 12 and Figure 16, we observe that after this plateau the surface pressure starts increasing when it reaches the point where there is no BSA on the surface. If there is no BSA on the surface then it is same as the pure lipid. But during the expansion cycle of the isotherm the lipid with BSA isotherm deviates from the pure lipid curve below the surface pressure of 20 mN/m. This is happening because the surface pressure where it is deviating from the pure lipid is lower than the desorbing pressure of BSA monolayer so BSA present in the sub-phase is coming on to the surface.
The BSA is desorbing at ~20 mN/m and coming on to the surface below the 20 mN/m and behaving like pure BSA no matter which lipid is present on the surface.

From this discussion it is clear that BSA is behaving independently, which means that there is no interaction or weak interaction between BSA and the lipids DPPC and DMPE.

4.1.2 RD3N AFP with DPPC and DMPE

In Figures 13 and 17, the isotherms of RD3N with DPPC and RD3N with DMPE also have a plateau which happens at surface pressure of 18 mN/m where the monolayer of pure RD3N desorbs (Figure 7). After this plateau the surface pressure increases as we compress the monolayer of RD3N with both DPPC and DMPE but this surface pressure corresponds to an increase in molecular area compared to pure lipids. This increase in molecular area indicates that some RD3N is present on the surface even at surface pressures above the collapsing pressure of the pure RD3N. With this we can assume that there is some interaction between the lipids and RD3N which is holding the protein on the surface. But at high surface pressures above 45 mN/m the molecular areas are same for the isotherm of RD3N with lipids and pure lipids, indicating that the interaction between them is not strong enough to hold the RD3N on the surface at those high pressures. During the expansion cycle the isotherm of RD3N with lipids deviates from the isotherm of pure lipid below the surface pressure of 24 mN/m, which is higher than the collapse pressure of pure RD3N. This suggests that there is some interaction between RD3N and the lipids, which is driving RD3N back to the surface above the collapse pressure of pure RD3N.
From this discussion we can say that there is some interaction between the lipids (DPPC, DMPE) and RD3N but this interaction is too weak for the lipids to hold RD3N on the surface at high pressures above 45 mN/m. We can also say that the interaction between the lipids (DPPC, DMPE) and RD3N is stronger than the interaction of lipids with BSA.

4.1.3 RD3 AFP with DMPE and DPPC

In Figure 18, the isotherm of DMPE with RD3 has a plateau that occurs at a surface pressure where the monolayer of pure RD3 collapses (Figure 7). After this plateau there is an increase in the surface pressure as we compress the mixed monolayer of RD3 and DMPE and their corresponding molecular area is greater compared to pure DMPE indicating that there is some RD3 present on the surface due to the interaction between DMPE and RD3. At high surface pressures above 45 mN/m this isotherm behaves like pure DMPE that is there is no RD3 on the surface. The interaction between the DMPE and RD3 is not strong enough to hold the RD3 on the surface at high pressures above 45 mN/m.

This shows that there is some interaction between the DMPE and RD3 and it is too weak for the lipid to hold RD3 on the surface at high pressures above 45 mN/m but this interaction is stronger than the interaction between DMPE and BSA.

In Figure 14, the isotherm of DPPC with RD3 also has a plateau at surface pressure where the monolayer of pure RD3 collapses (Figure 7). After the plateau there is increase in the surface pressure as we compress the mixed monolayer of DPPC and RD3 and their corresponding molecular area is greater compared to pure DPPC. Even at high surface pressures above 45 mN/m, the isotherm of DPPC with RD3 has greater molecular
area when compared with the molecular area of pure DPPC indicating that RD3 is present on the surface along with DPPC due to the interaction between them. This shows that the interaction between them is strong enough to hold the RD3 on the surface even above the collapse pressures of RD3 monolayer. In the expansion cycle we see a plateau for the isotherm of DPPC with RD3, which is occurring at 22 mN/m, below the collapse pressure of pure RD3.

With this we can say that there is some interaction between DPPC and RD3 that is strong enough to hold RD3 on the surface at high surface pressures above 45 mN/m. We can also say that this interaction is stronger than the interaction between RD3N with DPPC and BSA with DPPC.

### 4.1.4 Type I AFP with DMPE and DPPC

In Figure 19, the isotherm of DMPE with Type I also has a plateau at surface pressure where the monolayer of pure Type I collapses (Figure 8). After this plateau there is increase in the surface pressure as we compress the monolayer of DMPE with Type I and the corresponding molecular area is greater than pure DMPE. At surface pressures above 35 mN/m, the isotherm of DMPE with Type I has the same surface pressures as the pure lipid (DMPE) but at a greater molecular area when compared to pure lipid. This means that Type I is present on the surface along with DMPE due to interaction between them and it is strong enough to hold the Type I on the surface even above the surface pressures of 35 mN/m. In the expansion cycle, there is a phase transition which occurs at 20 mN/m, which is below the collapse pressure of pure Type I indicating that Type I is returning to the surface.
From the above discussion we can say that the interaction between Type I and DMPE is stronger than the interaction between RD3N, RD3 and BSA with DMPE.

The isotherm of DPPC with Type I has a plateau (Figure 15) which occurs at surface pressure where the monolayer of pure Type I collapses (Figure 8). After this plateau there is no increase in the surface pressure as we compress the monolayer and the corresponding molecular area decreases until it reaches the molecular area of pure DPPC indicating that all the Type I present on the surface is going into sub-phase because of no interaction between the Type I and DPPC. At high surface pressures like 45 mN/m the isotherm of DPPC with type I behave as pure DPPC, which is observed by comparing their molecular areas. Type I comes on to the surface at surface pressures below 20 mN/m during the expansion cycle which is below the collapse pressure of Type I monolayer.

This shows that there is no or some interaction between the DPPC and Type I which is not strong enough to hold Type I on the surface and it is also weaker than the interaction between RD3N or RD3 with DPPC.

4.2 Models explaining the possible interactions of proteins with lipids

Figures 20, 21, and 22 represent the surface interaction of lipids with proteins and AFPs. Each figure illustrates three different possible cases of lipid-protein interactions at high surface pressures which are (c) protein staying with lipid on the surface, (d) protein is desorbed in to the water sub-phase leaving the lipid on the surface of pure water, (e) pulling the protein along with the lipid in to the water sub-phase. If case c happens then
the corresponding lipid-protein isotherm should have the same surface pressure as the pure lipid for a higher surface area/molecule.

![Diagram showing different layers of lipids and proteins](image)

**Figure 20.** Schematic showing a) Lipids at A/W interface b) lipids present with Type III (RD3) AFPs at A/W interface c) compressed layer with AFPs and lipids d) compressed monolayer layer of lipids with AFPs in sub-phase e) Lipids pulled in to sub-phase along with AFPs

For case d to happen the lipid-protein isotherm should overlap on top of pure lipid isotherm at high pressures. In case e, the surface pressures of lipid-protein isotherm should be same as the pure lipid isotherm for a smaller surface area/molecule.

From Figures 12, 16, 13, 17, 15, and 18 the BSA-DPPC, BSA-DMPE, RD3N-DPPC, RD3N-DMPE, Type I-DPPC, RD3-DMPE isotherms overlap on top with their corresponding pure lipid isotherms at high pressures. This corresponds to the case d in Figures 20, 21, and 22 where the protein is desorbed in to the sub-phase and only lipid is present on the surface so these isotherms should behave like pure lipids.
Figure 21. Schematic showing a) Lipids at A/W interface b) lipids present with Type III (RD3N) AFPs at A/W interface c) compressed layer with AFPs and lipids d) compressed monolayer layer of lipids with AFPs in sub-phase e) Lipids pulled in to sub-phase along with AFPs.

From Figures 14 and 19, the RD3-DPPC, Type I-DMPE isotherms has the same surface pressures as their corresponding pure lipids but for a greater surface area/molecule. This interaction corresponds to case c where the protein stays on the water surface along with the lipid at high pressures.
Figure 22. Schematic showing a) Lipids at A/W interface b) lipids present with type I AFPs at A/W interface c) compressed layer with AFPs and lipids d) compressed monolayer layer of lipids with AFPs in sub-phase e) Lipids pulled in to sub-phase along with AFPs.

In case e where the protein pulls the lipid molecules into the sub-phase of water during high pressures and due to loss of lipids into the sub-phase the lipid-protein isotherms should have the same surface pressures as the pure lipid for a smaller surface area/molecule and this behavior is not observed in any of the results. So we can say that with using lipids (DPPC, DMPE) on surface and proteins (BSA, Type III (RD1, RD3), Type I) in sub-phase we can have only two possible cases (c, d) of interactions.
CHAPTER V

CONCLUSIONS

The RD3N which is produced by cutting one domain of RD3 showed weak interaction with lipids DPPC and DMPE while RD3 showed good interaction with DPPC and weak interaction with DMPE. RD3 has better interaction with lipids than N-RD3 even though both are Type III AFPs. These two AFPs behave differently and the reason for this is not known. Type I AFP showed strong interaction with DMPE when compared with the interaction of RD3 with DMPE. BSA showed weak interactions with both lipids DPPC and DMPE. We observed that RD3 has good interaction with DPPC and Type I has good interaction with DMPE. We can conclude that RD3, RD3N and Type I AFP have stronger interaction with lipids DPPC and DMPE than BSA with lipids DPPC and DMPE. These results suggest that not all antifreeze proteins behave similarly with any particular type of lipid. These surface pressure-area measurements are used to find the interactions between the different lipids and AFPs. These experiments are repeatable and reproducible and they are less time consuming.

Our future plan is to compare these isotherms with liposome leakage studies and predict which proteins are good preservation agents, so that we will be able to develop a
protocol by which one will be able to determine a priori whether a compound will be a successful membrane protector.
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APPENDIX A

1) 2% Agarose Gel

Take 5 g of genetical analysis grade and add it to 250 ml of TAE gel running buffer. Heat it while stirring until the solution is transparent. Wait for it to cool and add 25 µL of ethidium bromide and pour it on the gel box.

2) 1000X Ampicillin

Add 100 mg of ampicillin in 1ml of ddH₂O.

3) 10X TAE gel Running Buffer

Tris base ------ 48.4 g
Glacial Acetic Acid------11.42 ml
Na₂EDTA₂H₂O-------- 7.44 g
ddH₂O…………… to 1 L

4) 1L of LB Medium

i. Add 5 g NaCl to flask
ii. Add 10 g peptone to flask
iii. Add 5 g Yeast Extract to flask
iv. Add ddH₂O to 1 L mark on flask
v. Mix. Can swirl by hand or use magnetic stirrer.
vi. Cover with foil

Put autoclave tape on foil then autoclave flask and contents
5) **Miniprep**

   6) **6 M Guanadine HCL**

   Add 5.7 g Guanadine HCl to 10 mL ddH$_2$O

6) **50mM Acetic Acid buffer**

   Add 125mL of 1M acetic acid to 2.375L of dH$_2$O.

7) **Refolding Buffer**

   a. For 500 mL stock sol’n.

      i. Find 500 mL bottle or other container with a lid and volume markings.

      ii. Add 2.9 g NaCl (on shelf, will be chunky).

      iii. Add 43.3 g $K_2$PO$_4$ (on shelf, also chunky).

      iv. Fill to 500 mL level. Mix until homogenous.

      v. Check pH, should be ~10. Adjust if necessary.

      vi. Label, cap, and store at 4°C.
APPENDIX B

Expression and Purification of RD3 and RD3N

Starter Culture

1. Make 1 Liter LB (Luria-Bertani) in a 2 L Erlenmeyer flask

2. Put 5 mL LB into 15 mL culture tube.
   vii. add 5 µL of 1000X ampicillin to the 5 mL of LB

3. Get RD3/RD3N frozen sample from -70°C freezer

4. Scrape a small chunk (very small) of the frozen RD3 and put it into the 15 mL falcon tube with the LB-A.

5. Place cap on Falcon tube and label.

6. Put in 37°C shaker/incubator for ~12 hours.

Protein Expression

1. Add 1 ml of 1000X amp stock to 1 L of LB if not already added.(from ‘fridge)

2. Remove at least 4 mL of LB from flask and put it in a 15 mL culture tube. This is for use in the UV spec later.

3. Get starter culture from shaker/incubator.

4. Put entire starter into flask and either
   a. Put in 37°C shaker/incubator for 4-6 hours.

5. Wait appropriate amount of time!

6. Get culture from shaker or magnetic stir plate and test Optical Density (OD).
   a. Run blank: Put 2 mL LB-A (not your culture) into UV cuvette.
b. Set cuvette in UV spectrophotometer.

c. Read at 600nm.

d. Press “set ref”. This will set baseline.

e. Remove blank and insert cuvette with 2 mL of your culture.

f. If the reading is near 0.6 or higher Add 120 mg IPTG (from `fridge) to 1 L flask of culture bringing it to 0.5 mM. It is important to thoroughly mix the IPTG in about 1mL of culture prior to adding it to the flask. This will induce expression.

7. After adding IPTG you can either

   Put in incubator/shaker for 6 hours.

8. After appropriate amount of time remove culture from shaker/incubator or stir plate.

9. Find 250 mL centrifuge bottles (in drawer).

10. Evenly split culture amongst an even number of bottles.

11. Centrifuge the balanced bottles at 7000 RPM for 15 minutes.

12. Pour off supernatant and freeze the pellet (the cells) at -70°C for as long as we want.

**Protein Extraction**

2. Get pellets from -70°C freezer.

   a. Keep everything on ice.
b. Resuspend each pellet in 10 mL BPER.

3. Transfer to 30 mL centrifuge tubes.

4. Balance tubes and centrifuge at 27000g for 15 minutes.

5. Pour off supernatant and gel-like layer above the pellet as much as possible.

6. Resuspend pellet in 10 mL BPER.

7. Add 200 µL of 10 mg/mL solution of lysozyme (in ddH$_2$O) to the solution.
   a. Mix well and incubate on ice for 5 minutes.

8. Add 20 mL 1:10 diluted (in ddH$_2$O) BPER.

9. Centrifuge at 27,000g for 15 minutes.

10. Pour off the supernatant and remove as much of the gel-like layer above the pellet as you can. (There is no protein in the gel layer this is observed by running the gel.)

11. Resuspend the pellet in 30 mL 1:20 diluted (in ddH$_2$O) BPER.

12. Centrifuge at 27,000g for 15 minutes.

13. Repeat steps 9-11 at least two more times.
   a. A bright white pellet should be visible with sufficient repetition of these last 3 steps.

14. Resuspend final pellet in 10 mL of 6M Guanidine HCl.

15. Add 190 mL of refolding buffer to pellet dissolved in Guanadine HCl.

16. Chill solution for 2-3 days at 4°C. (Can chill for up to 2 months.)

**Purification and Desalting**

1. Get dialysis tubing (MWCO 1,000) and clips.
2. Determine amount of tubing needed for your protein sol’n.
   a. Tubing holds 6.4 mL/cm
   b. i.e. 200 mL sol’n: Two 18 cm tubing pieces.
3. Cut appropriate amount of tubing.
4. Soak tubing in ddH\textsubscript{2}O for 30 minutes.
5. Make 1M acetic acid stock if necessary
   a. Add 28 mL to 500 mL dH\textsubscript{2}O
6. Make 2.5 L of 50 mM acetic acid buffer sol’n for dialysis
   a. Add 125 mL of 1 M stock to 2.375 L of dH\textsubscript{2}O.
   b. pH should be \(\sim 3.7\) (3-4 is okay).
   c. Adjust pH if needed.
   d. Chill in ‘fridge until cold.
7. Get refolded protein sol’n from ‘fridge.
8. Retain 100 \(\mu\)L of sol’n in a microcentrifuge tube for running the gel.
9. Clip one end of tubing.
10. Pour protein sol’n into dialysis tubing.
11. Clip the open end of tubing.
12. Place dialysis bag(s) in 2.5 L buffer sol’n.
13. Place into cold room for a few hours or overnight.
14. Remove dialysis bags and change buffer solution at least twice.
   a. Be sure to use cold solutions.
15. After final dialysis, empty dialysis bag(s) into Oakridge tubes.
16. Centrifuge at 27000 g (15000 RPM) for 15 minutes.
17. Retain *supernatant* for lyophilizing.

18. Keep supernatant in labeled falcon tubes at 4°C until needed.