Biochemical and Biophysical Studies of Vision Loss: Cataract and Macular Degeneration

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ABSTRACT

BIOCHEMICAL AND BIOPHYSICAL STUDIES OF VISION LOSS: CATARACT AND MACULAR DEGENERATION

Michael C. Vega, PhD
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Northern Illinois University, 2019
Elizabeth R. Gaillard, Director

Age-related biochemical and biophysical changes to the structural properties of ocular lens membrane proteins and retinal pigment epithelium (RPE) cells are thought to play an underlying role in the etiology and progression of age-related cataract and macular degeneration. This dissertation work studies biophysical properties of biomimetic membranes to aid in further understanding of the role of aquaporin-0 in cataract pathogenesis and the biochemical composition of age-related pigmented granules, melanolipofuscin, and their implications in macular degeneration.

Cataract is the leading cause of blindness globally and is characterized by the opacification of the ocular lens. As a key optical component of the eye, the lens must remain transparent and elastic throughout life which requires proper water homeostasis through lens specific integral membrane protein aquaporin-0 (AQP0). Structures of AQP0 lack protein-lipid bilayer structural information. Since AQP0 function depends on its lipid bilayer environment,
this work investigated structural properties of oriented biomimetic lipid membranes using x-ray reflectivity and compared the results to literature. The data were fit with physically meaningful parameters such as molecular area and bilayer thickness. Results from the fits show a general decrease in molecular area which is consistent with the condensation effect of cholesterol on lipid bilayers. Some parameters for the highest cholesterol mol% tended to deviate from what was expected based on general trends. This may possibly be due to phase separation of cholesterol into cholesterol-rich domains as observed in lens fiber cell membranes. These data provide important information to understanding AQP0/lipid bilayer structural properties upon insertion of AQP0 into the biomimetic membranes.

Age-related macular degeneration (AMD) is the leading cause of vision loss in developed countries including the United States. AMD presents in two distinct forms, exudative and non-exudative, with the exudative form being the less common but most severe form. Both forms are characterized by the accumulation of membrane-bound fluorescent pigment granules in the retinal pigment epithelium (RPE), a monolayer of cells that line the back of the retina and form the blood-retinal barrier. The pigment granules form from lysosomal uptake and storage of undigested material that is of photoreceptor origin and are termed lipofuscin. The lipofuscin granules appear to correlate with retinal degeneration and have received considerable attention in the literature. Over time, some of the lipofuscin granules fuse with the protective melanin containing vesicles (melanosomes) to form melanolipofuscin which is potentially very harmful to the tissue because it likely inhibits the ability of melanin to protect against oxidative and photooxidative stress. However, almost nothing is known about the chemical composition or reactivity of these granules. Therefore, we have undertaken a systematic analysis of the organic soluble portion of human retinal melanolipofuscin using liquid chromatography-mass
spectrometry. A2E is a by-product of the visual cycle, a known component of retinal lipofuscin, and implicated in oxidative stress in the RPE due its ability to produce reactive oxygen species. A2E was identified in organic soluble melanoplipofuscin based on characteristic absorption maximum around 440 nm and tandem mass spectrometry fragments. Two abundant ions with m/z 618 and 646 were investigated. Tandem mass spectrometry data suggest that m/z 618 and 646 could result from the reaction of an aldehyde derivative of oxidized A2E with lysine and arginine, respectively. The proposed structures for m/z 618 and 646 will be synthesized and analyzed with these methods to confirm this hypothesis.
BIOCHEMICAL AND BIOPHYSICAL STUDIES OF VISION LOSS: CATARACT AND MACULAR DEGENERATION

BY

MICHAEL C. VEGA

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A DISSERTATION SUBMITTED TO THE GRADUATE SCHOOL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY AND BIOCHEMISTRY

Doctoral Director:
Elizabeth R. Gaillard
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CHAPTER 1

INTRODUCTION

The human eye (Figure 1.1) is a complex organ whose function is comparable to that of a camera. It receives, focuses, and transmits light through a lens to create an image of its surroundings; an image that is ultimately decoded by the brain. To do so, it is heavily exposed to the environment and light and is therefore subject to high levels of oxidative stress. Oxidative stress is believed to play a key role in various eye pathologies, for example, cataract and age-related macular degeneration (AMD) (Kruk, Kubasik-Kladna, & Aboul-Enein, 2015).

Cataract is the clouding of the ocular lens which prevents clear vision (Figure 1.2). It is often age-related (Figure 1.3.a) though children can be born with cataract (congenital cataract) or cataract can develop due to injury, inflammation, or other diseases. According to the World Health Organization (WHO), cataract is a leading cause of vision loss accounting for 51% of world blindness as of the last assessment in 2010. It is the leading cause of blindness in developing countries in part due to the lack of availability of cataract surgery, the gold standard in treating cataract. As life expectancy increases, the occurrence of cataract is expected to increase (Figure 1.3.b) (“World Health Organization,” n.d.).
Age-related macular degeneration (AMD) is a degenerative retinal disease that affects the macular region of the retina; the region responsible for sharp central vision (Figure 1.4.a). The World Health Organization reports AMD to be the third leading cause of blindness globally (“World Health Organization,” n.d.). It is the leading cause of vision loss among the elderly in developed countries (de Jong, 2006) including the United States. The National Eye Institute reports that about 2.07 million Americans were diagnosed with AMD in 2010 and that number is expected to more than double to 5.44 million by 2050 (“National Eye Institute |,” n.d.). AMD projections are illustrated in Figure 1.4.b.

Figure 1.1: Basic anatomy of the human eye (“Human Eye Anatomy - Parts of the Eye and Structure of the Human Eye,” n.d.).
Figure 1.2: Depiction of cataract and the resulting blurred vision. (a) Normal lens (left) and cataractous lens (right) (b) Normal vision (left) and vision impairment due to cataract (right) ("National Eye Institute |," n.d.; “Saroya Eye Hospital,” n.d.).
Figure 1.3: (a) Prevalence rates for cataract by age and race in 2010. (b) Projections for cataract to year 2050 (“National Eye Institute,” n.d.).
Figure 1.4: Depiction of vision impairment due to macular degeneration and projections to the year 2050. (a) Normal vision (left) and vision impairment as a result of advanced AMD (right) (b) Projections for AMD until 2050 (“National Eye Institute |,” n.d.).
The Lens

The ocular lens is an avascular organ located in the anterior chamber of the eye. Together with the cornea, it comprises the optical element of the eye. Two critical features of healthy ocular lenses necessary for its proper function are transparency and accommodation (illustrated in Figure 1.5). Transparency is important so that incoming light is focused onto the retina rather than being scattered. Accommodation refers to lens elasticity or the ability for the shape of the lens to change to effectively change the focal length of the lens. Age-related changes often lead to cataract and presbyopia which is the loss of ability to see objects close up (Boyd, 219AD).

Figure 1.5: Illustration of the critical features of a healthy lens. (a) Schematic of the transmission and focusing of radiation through a transparent lens (b) Schematic of lens accommodation ("Your Baby and Kids Room Idea and Furniture | Yamsixteen," n.d.; "ZEISS Microscopy Online Campus | Microscopy Basics | Introduction," n.d.)
The lens continues to grow throughout the life of an individual. It is comprised of two main cell types. Throughout life, a monolayer of epithelial cells on the outside of the lens cortex undergo mitosis and the daughter cells will differentiate into elongated secondary fiber cells. Ultimately, the secondary fiber cells will produce large amounts of crystallin proteins, develop many membrane undulations, lose their cellular organelles, and become concentrically packed as mature fiber cells making up the bulk of the lens (Augusteyn, 2010). Lens development and the concentric packing of fiber cells is depicted in Figure 1.5. Notably, the bulk of lens cells lack cellular machinery for synthesizing new protein. Therefore, lens cells and proteins accumulate biophysical and biochemical changes with age and the cells and proteins in the lens core are as old as the individual.

The lens has developed a system to reduce aberrations and maintain transparency to be able to function properly. It utilizes a gradient of refractive index (GRIN) which is achieved by differential protein expression and concentrations across fiber cells. The concentration gradient requires that fluid balance is properly maintained (Pierscionek & Regini, 2012). Accommodation requires that the lens be stretched or relaxed which requires fiber cell volume changes (Gerometta, Zamudio, Escobar, & Candia, 2007). Fiber cell membranes must be highly water-permeable which is presumably achieved through aquaporin transmembrane proteins (Schey, Wang, L. Wenke, & Qi, 2014), the predominant family of water transport proteins.
Figure 1.6: Illustration of lens development and a micrograph of fiber cell packing. (a) Schematic of lens development (b) Micrograph of fiber cell packing (Adler & Hart, 1992; “Embryonic development of human eye,” n.d.).
Aquaporins

Aquaporins (AQPs) are a family of transmembrane proteins found ubiquitously in the body and responsible for transporting water across the plasma membrane. The first, aquaporin-1 (AQP1), was isolated from red blood cells by Peter Agre in the early 1990s (Preston, Carroll, Guggino, & Agre, 1992; Zeidel, Ambudkar, Smith, & Agre, 1992); a discovery that earned Agre the 2003 Nobel Prize in Chemistry (“The 2003 Nobel Prize in Chemistry - Popular information,” n.d.) along with Roderick MacKinnon for his work with ion channels. There are now 13 known mammalian aquaporins, AQP0 to AQP12, some of which are observed to transport other small molecules such as glycerol (so called aquaglyceroporins) (Schey et al., 2014) and possibly small gas molecules like O$_2$ (Herrera & Garvin, 2011).

All aquaporins have the same basic structure of two tandem repeats. Each repeat has three transmembrane α-helices and a hydrophobic-loop containing a highly conserved asparagine-proline-alanine (NPA) motif (Fu et al., 2000; Gonen, Sliz, Kistler, Cheng, & Walz, 2004; Harries, Akhavan, Miercke, Khademi, & Stroud, 2004a; Murata et al., 2000; Ren, Reddy, Cheng, Melnyk, & Mitra, 2001; Savage, Egea, Robles-Colmenares, O’Connell, & Stroud, 2003; Sui, Han, Lee, Walian, & Jap, n.d.). Loops B and E, the NPA-containing loops, fold back into the membrane forming short α-helices that line the pore. The NPA motifs are important for a proton exclusion mechanism and a constriction site composed of an aromatic and arginine residue (ar/R site) is important for water selectivity (de Groot & Grubmüller, 2005; Gonen et al., 2005).
Most of the aquaporins are expressed throughout the eye (Figure 1.7). Lens epithelial cells express mostly AQP1 with AQP5 and AQP7 also contributing to membrane protein concentration. Significant changes in AQP expression occur when epithelial cells differentiate to mature fiber cells.

Figure 1.7: Diagram of the lens showing localization of aquaporin expression (Schey et al., 2014).
Aquaporin-0 (AQP0)

Upon differentiation, AQP1 expression is replaced with AQP0 expression. AQP0, formerly known as major intrinsic protein (MIP), is lens specific and comprises more than 50% of lens membrane proteins (Alcala, Lieska, & Maisel, 1975). AQP0 is a unique member of the AQP family. It suits the needs of the lens by making lens membranes water permeable, but it is also the only AQP known to form membrane junctions in vivo (Costello, McIntosh, & Robertson, 1989). Therefore, in addition to conducting water across the fiber cell membranes, it has important structural functions in the lens (Colom, Casuso, Boudier, & Scheuring, 2012; Fotiadis et al., 2000; Kumari & Varadaraj, 2009; Lindsey Rose et al., 2006; Liu, Xu, Gu, Nicholson, & Jiang, 2011; Nakazawa et al., 2011; Simon et al., 1982; Zhen Wang & Schey, 2011; Yu, Yin, Lafer, & Jiang, 2005). The importance of AQP0 to the proper functioning of the lens is highlighted in several studies which have identified novel mutations that lead to cataract formation.

Interestingly, AQP0 has 40 times less water permeability compared to AQP1 when expressed in Xenopus oocytes (Chandy, Zampighi, Kreman, & Hall, 1997). However, AQP0 water permeability may be pH dependent. Structures for AQP0 have been determined using electron and x-ray crystallography (Gonen et al., 2005; Gonen, Sliz, et al., 2004; Harries, Akhavan, Miercke, Khademi, & Stroud, 2004b). AQP0 monomers are arranged as homotetramers in fiber cell membranes (Figure 1.8).
Figure 1.8: A structure of AQP0 from the Protein Data Bank. The smaller inset shows the structure colored by chain. PDB ID: 2B6P (H. Berman, Henrick, & Nakamura, 2003; H. M. Berman et al., 2000; Gonen et al., 2005; Rose et al., 2018)
Differences in pore lining residues are thought to account for AQP0 poor water permeability. Tyr-23 and Tyr-149 in sheep AQP0 are replaced with Phe-24 and Thr-157, respectively, in AQP1. Molecular dynamics simulations suggest these tyrosine residues cause poor water permeability in AQP0 (Hashido, Ikeguchi, & Kidera, 2005; Jensen et al., 2008; Qiu, Ma, Shen, & Guo, 2010). Saboe et al. showed experimentally that Tyr-23 and Tyr-149 both contribute to poor water permeability of AQP0 with Tyr-23 being the major contributor (Saboe et al., 2017). His-40 and His-66 are implicated in the pH dependent behavior of AQP0 (Németh-Cahalan & Hall, 2000; Németh-Cahalan, Kalman, & Hall, 2004; Varadaraj, Kumari, Shiels, & Mathias, 2005) but not all studies have shown pH dependence (Virkki, Cooper, & Boron, 2001; Zeuthen & Klaerke, 1999). Water conduction for AQP0 is slightly pH dependent with 1.5 to 2 times greater conductance at pH 7.5 compared to 6.5 (Saboe et al., 2017).

The importance of AQP0 in lens transparency is indicated through several animal models for cataract. For example, AQP0 null mice have cataract and altered fiber cell packing (Al-Ghoul et al., 2003; Shiels et al., 2001) and expressing AQP1 in these mice only partially restores lens transparency and packing (Shiels et al., 2001). Novel mutations in the AQP0 sequence have been linked to cataract formation (Berry, Francis, Kaushal, Moore, & Bhattacharya, 2000; Francis et al., 2000; Geyer et al., 2006; Gu et al., 2007; Jiang et al., 2009; Jin, Jiang, Wang, & Yao, 2010; W. Wang et al., 2010). Two more recently discovered mutations are the D150H and Y219* mutations which were identified in four generations of a Chinese family (Shentu, Miao, Tang, Yin, & Zhao, 2015; Song, Wang, Liu, & Xiao, 2015). These mutations all lead to congenital cataract but natural mutations in AQP1 have no observable adverse effects (Preston, Smith, Zeidel, Moulds, & Agre, 1994) and AQP5 null mice show no cataract formation (Kumari,
Varadaraj, Yerramilli, Menon, & Varadaraj, 2012; Ma et al., 1999). Further highlighting the unique importance of AQP0 in lens structure and function.

Membrane Protein-Lipid Bilayer Structure

Mature fiber cells lack organelles, so the plasma membranes of fiber cells comprise most of the lipid composition of the lens. Bulk membrane properties (including diffusion barriers) are determined by the bilayer composition (Raguz, Mainali, O’Brien, & Subczynski, 2014). Studies also show that membrane proteins can be affected by the lipid bilayer (Reichow & Gonen, 2009; Tong, Briggs, & McIntosh, 2012; Tong, Canty, Briggs, & McIntosh, 2013). Tong et al. (2013) demonstrated that the water permeability of AQP0 is dependent on its lipid bilayer environment (Tong et al., 2013). Therefore, the structure of membrane protein-lipid complexes and its effect on protein function are of interest.

Most structural studies focus on membrane proteins and the lipid bilayer separately. It is difficult to get structural information on the interactions of membrane proteins and lipids. Most of the structures of membrane proteins available have been determined from three-dimensional (3D) crystals, typically prepared from detergent-solubilized membrane proteins, using x-ray crystallography, but native lipids rarely co-crystallize (Hite, Gonen, Harrison, & Walz, 2008). X-ray crystallographic structures of AQP0 from 3D crystals have been reported from detergent-solubilized tetramers (Harries et al., 2004a). Two-dimensional (2D) crystals where isolated membrane proteins are reconstituted into artificial lipid membranes can be studied with x-ray crystallography (Hite, Raunser, & Walz, 2007). Two electron crystallographic structures from
2D crystal structures have been determined, namely bacteriorhodopsin (bR) (Mitsuoka et al., 1999) and aquaporin-0 (Gonen et al., 2005). Therefore, AQP0 is a model membrane protein with structural data that can be used to compare differences between using detergents or lipids. What is lacking is structural information on the interactions between AQP0 mutants and lipid bilayers. Differences in mutant protein-lipid interactions compared to native protein-lipid interactions may provide more insight into cataract pathology.

**Lens Fiber Cell Bilayer Composition**

The plasma membrane of lens fiber cells comprises most of the lipids in the ocular lens. The lipid composition of these plasma membranes varies by species and is different than plasma membranes found elsewhere in the body. Like other plasma membranes, fiber cell membranes contain mostly phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (SM) (D Borchman, Tang, & Yappert, 1999; Douglas Borchman & Yappert, 2010; Yappert, Rujoi, Borchman, Vorobyov, & Estrada, 2003), however; SM is present in higher concentrations compared to other plasma membranes (Douglas Borchman, Yappert, & Afzal, 2004; Yappert & Borchman, 2004). Additionally, lens fiber cell membranes contain very high concentrations of cholesterol (Chol). When attempting to mimic properties of lens plasma membranes, it is important to consider four key factors. They 1) are highly saturated (Deeley et al., 2008; L. K. Li, So, & Spector, 1985; Yappert et al., 2003), 2) contain high concentrations of sphingolipids, mainly dihydrosphingomyelin (DHSM) (D. Borchman, Byrdwell, & Yappert, 1994; Yappert et al., 2003), 3) have very high Chol concentration (L.-K. Li, So, & Spector, 1987;
L. K. Li et al., 1985; Rujoi, Jin, Borchman, Tang, & Yappert, 2003; Zelenka, 1984), and 4) have a high density of membrane proteins (Bassnett, Shi, & Vrensen, 2011; Gonen, Cheng, Kistler, & Walz, 2004; Kistler & Bullivant, 1980; L.-K. Li, Roy, & Spector, 1986; L. K. Li et al., 1985).

Age-related Macular Degeneration (AMD)

Following our discussion of cataract, the leading cause of vision loss globally, we turn to discussing age-related macular degeneration, the leading cause of vision loss in developed countries like the United States. Early signs of AMD are characterized by the build-up of drusen, deposits ranging from white to yellow in color as seen in an ophthalmoscope, from unknown origin (de Jong, 2006). Progression of AMD can lead to two distinct forms; non-exudative (dry) and exudative (wet) AMD. Schematic representations of a normal retina compared to non-exudative and exudative AMD retinas are shown in Figure 1.9. Dry AMD, also known as geographic atrophy, is characterized by the slow death of photoreceptor cells (“National Eye Institute |,” n.d.). About 90% of patients diagnosed with AMD have dry AMD. Vision loss is typically very slow for these patients and there are no approved treatments available for dry AMD. Persons with wet AMD, the debilitating form of the disease, account for the remaining 10% of AMD patients. Neovascularization is characteristic of wet AMD; new blood vessels will grow into the retinal space and since these vessels are often weaker than normal blood vessels, blood will leak into retina (de Jong, 2006; “National Eye Institute |,” n.d.). Anti-VEGF eye injections can be administered to treat wet AMD, but these injections are typically very expensive and require monthly injections which makes patient compliance problematic.
Wet and dry AMD can occur in any combination. One or both eyes can be diagnosed with either dry or wet AMD or one eye with wet and one with dry. Dry AMD can develop into wet AMD and vice versa (de Jong, 2006). There is debate on whether non-exudative and exudative AMD are in fact two forms of the same disease or if they are in fact two distinct diseases (Zarbin, 2004). What is known, regardless of the type of AMD, is that the retinal pigment epithelium is a central element in age-related macular degeneration pathogenesis.

Figure 1.9: Schematic representations of a normal retina (left), non-exudative AMD retina (center), and exudative AMD retina (right) (“Age-related Macular Degeneration,” 2016).
The retinal pigment epithelium (RPE) is one of the layers of the retina, the light sensitive tissue that lines the back of the eye. The RPE is a single layer of cuboidal, post-mitotic cells positioned between the photoreceptors and Bruch’s membrane (Figure 1.10). They are so named due to the high density of pigment-containing (melanin-containing) organelles, melanosomes, in the cytoplasm (de Jong, 2006). RPE cells play many critical roles in healthy retina function. They phagocytose and digest spent photoreceptor outer segments which is critical for photoreceptor renewal. Regeneration of the visual pigment, rhodopsin, is carried out in RPE cells (Figure 1.11). Tight junctions between the RPE and the underlying Bruch’s membrane form the blood-retinal barrier. Transport of fluid and ions between the photoreceptors and the choroid are thus regulated by the RPE (de Jong, 2006). The RPE is characterized by very high metabolic activity and is subject to a high partial pressure of oxygen from the choroid. Thus, the RPE cells are subject to oxidative stress and age-related changes. Despite the debate of whether dry and wet AMD are two distinct diseases or two forms of the same disease, a common link between the two is the accumulation of age-related pigment granules in RPE cells.
Figure 1.10: Schematic showing the location of the retinal pigment epithelium in relation to other tissues in the eye. **A:** Overview of the eye with a portion of the retina boxed in. **B:** Zoomed view of the boxed in region in (A). The RPE is the final layer of the retina which is between photoreceptors and the underlying Bruch’s membrane and choroid (Keeling, Lotery, Tumbarello, & Ratnayaka, 2018).
Figure 1.11: The Classical Visual Cycle. All-trans-retinal is released by opsin in the rod outer segments (OS) and reduced to all-trans-retinol. After reduction, all-trans-retinol enters the RPE. 11-cis-retinal is regenerated in three enzymatic steps and returns to the OS ("The Visual Cycle," n.d.).

RPE Lipofuscin

Lipofuscin is a term used to refer to autofluorescent lysosomal storage bodies that accumulate in post-mitotic cells with age. The accumulation of RPE lipofuscin is implicated in
retinal pathologies including Best’s macular dystrophy, Stargardt’s disease, and AMD (Delori, Goger, & Dorey, 2001; Dorey, Wu, Ebenstein, Garsd, & Weiter, 1989; L Feeney-Burns, Hilderbrand, & Eldridge, 1984; Gaillard, Atherton, Eldred, & Dillon, 1995; Lopez, Maumenee, de la Cruz, & Green, 1990; Rabb, Tso, & Fishman, 1986; Weingeist, Kobrin, & Watzke, 1982) and has received considerable attention in the literature. RPE lipofuscin is a yellow-brown, electron dense age-related pigment with a heterogenous composition. In the RPE, lipofuscin is believed to result from incomplete digestion of spent photoreceptor outer segments in lysosomes (Figure 1.12) (Boulton, McKechnie, Breda, Bayly, & Marshall, 1989; L Feeney-Burns & Eldred, 1983; Feeney, 1978). It is potentially toxic to RPE cells because studies have shown that it is photoreactive, producing reactive oxygen species (ROS) such as singlet oxygen, hydrogen peroxide, and superoxide anions (Avalle, Dillon, Tari, & Gaillard, 2005; Davies et al., 2001; Gaillard et al., 1995; Różanowska et al., 1998; Wassell, Davies, Bardsley, & Boulton, 1999).

Eldred identified the first known fluorophore of RPE lipofuscin, the bis-retinoid N-retinylidene-N-retinylethanolamine (A2E) (Figure 1.13.a) (Eldred & Lasky, 1993). A2E is a pyridinium bis-retinoid with a proposed biosynthesis utilizing 2 equivalents of all-trans-retinal (Vitamin A) and 1 equivalent of phosphatidylethanolamine (PE) (Figure 1.13.b) (Parish, Hashimoto, Nakanishi, Dillon, & Sparrow, 1998). Photooxidation of A2E has been shown to produce highly reactive aldehydes and ketones (Zhen Wang, Keller, Dillon, & Gaillard, 2006). Small molecule chemical analysis studies utilizing LC-MS/MS indicated that higher molecular weight derivatives of A2E may result from reactions of A2E with the photoinduced reactive aldehydes and ketones (Murdaugh et al., 2011). Therefore, besides A2E, there a numerous other components of RPE lipofuscin that are structurally related to A2E. In addition to photoreactive toxicity, RPE
lipofuscin can account for up to 19% of the cytoplasmic volume of RPE cells which has been shown to inhibit its phagocytic capacity (Sundelin, Wihlmark, Nilsson, & Brunk, 1998).

Figure 1.12: Schematic representation of the proposed mechanism of RPE lipofuscin formation.

(1) RPE phagocytose photoreceptor outer segments (OS) which are (2) ingested by lysosomes forming phagolysosomes. In young eyes (under 10 years), the phagolysosomes become smaller. In older eyes (over 10 years), phagolysosomes fuse with pre-existing lipofuscin (Feeney, 1978).
Figure 1.13: Structure of A2E and its proposed biosynthesis. (a) Structure of A2E, a fluorescent component of human RPE lipofuscin and a byproduct of the visual cycle. (b) Proposed biosynthesis of A2E (Parish et al., 1998; Tolleson et al., 2005).
Melanolipofuscin

Melanolipofuscin is another type of age-related pigment that accumulates in RPE cells with age. Structurally, melanolipofuscin granules have a melanin-like core with a lipofuscin-like periphery as seen in the micrograph in Figure 1.14 (Biesemeier, Schraermeyer, & Eibl, 2011). As a result of this, melanolipofuscin is hypothesized to form by fusion of lysosomes containing undigestible ROS debris with melanosomes (the melanin containing organelles of RPE cells). Melanosomes are specialized organelles of the RPE that play a dual protective role; by reducing chromatic aberration, which improves visual acuity and by acting as an antioxidant which protects RPE cells from oxidative and photooxidative damage (Hu, Simon, & Sarna, 2018; Ostrovskii & Dontsov, n.d.; Z. Wang, Dillon, & Gaillard, 2006). Therefore, the proposed fusion of melanosomes and lipofuscin to form the complex melanolipofuscin granules could be deleterious to the RPE as it may inhibit the protective function of melanin.

The amount of melanin, the protective pigment in melanosomes, decreases in RPE cells with age (usually starting at around age 40) (L. Feeney-Burns et al., 1984; Weiter, Delori, Wing, & Fitch, 1986). It is reported that human RPE cells contain 36% more melanin in the 20-30 year age group compared to the 60-90 year age group (Rózanowski et al., n.d.). Concomitantly, the volume occupied by melanosomes decreases from about 8% (under 20 years old) to about 3.5% (in the age group 41-90 years) (L. Feeney-Burns et al., 1984). Specific mechanisms of melanosome degradation are still debated. However, Dontsov (2017) reports that superoxide radicals at physiological pH degraded melanin from various sources (human and bovine RPE melanosomes, squid ink bag, and synthetic DOPA-melanin). Furthermore, the amount of
melanin in melanolipofuscin granules was less compared to melanosomes. Therefore, it is possible that the generation of superoxide from the lipofuscin-like periphery of melanolipofuscin granules directly degrades melanin in the core of said granules (Dontsov, Sakina, & Ostrovsky, 2017). Despite Dontsov’s work very little is known of the mechanisms of melanin degradation or the reactivity of melanolipofuscin. Additionally, very little is known of the chemical composition of these complex granules.

Figure 1.14: Micrograph showing RPE melanosomes (M) and the two age-related pigments, lipofuscin (L) and melanolipofuscin (ML) (Biesemeier et al. 2011).
Cataract and age-related macular degeneration (AMD) are two leading causes of blindness worldwide. Cataract is the clouding of the ocular lens which causes light scattering (rather than light focused to the retina) resulting in blurred vision. AMD is a retinal disease that affects the macula, the region of the retina responsible for sharp central vision. The Centers for Disease Control consider AMD to be a public health crisis and expect cataract to become a greater burden to health due to the aging population (“Centers for Disease Control and Prevention,” n.d.). These two eye pathologies affect different areas of the eye, but they share oxidative stress as an underlying cause. Age-related changes that cause biochemical and biophysical changes in the different locations likely lead to onset and progression of cataract and AMD.

The ocular lens is a key optical element of the eye and must remain transparent and elastic throughout life for proper vision. Proper water homeostasis is crucial for lens transparency. Lens-specific AQP0 regulates water homeostasis, however, the mechanisms by which it functions remain unclear. AQP0 is one of the few membrane proteins whose structure has been determined using x-ray crystallography. However, these structures lack protein-lipid bilayer information, therefore, they lack important structural information because AQP0 function depends on the lipid bilayer environment. To solve this problem, we aim to reconstitute AQP0 in biomimetic membranes and examine protein-bilayer structure using x-ray scattering techniques such as specular x-ray reflectivity. To that end, we have probed physical properties of biomimetic supported lipid bilayers using specular x-ray reflectivity and compared our results to
those of physical properties and phase diagrams in the literature. These data provide physical parameter information necessary to understand protein-bilayer physical characteristics.

AMD presents in two distinct forms, exudative and non-exudative, with the exudative form being the less common but most severe form. Both forms are characterized by the accumulation of membrane-bound fluorescent pigment granules in the retinal pigment epithelium, a monolayer of cells that line the back of the retina and form the blood-retinal barrier. The pigment granules form from lysosomal uptake and storage of undigested material that is of photoreceptor origin and are termed lipofuscin. The lipofuscin granules appear to correlate with retinal degeneration and have received considerable attention in the literature. Over time, some of the lipofuscin granules fuse with the protective melanin containing vesicles (melanosomes) to form melanolipofuscin which is potentially very harmful to the tissue because it likely inhibits the ability of melanin to protect against oxidative and photooxidative stress. However, very little is known of the chemical reactivity and almost nothing is known about the chemical composition of these granules. Therefore, we have undertaken a systematic analysis of the organic soluble portion of human retinal melanolipofuscin using liquid chromatography-mass spectrometry. Comparison of these data to our extensive database for lipofuscin has allowed us to identify known vitamin A derivative byproducts of the visual cycle, namely A2E and oxidized A2E.
All the chemicals used in this dissertation work were of the highest grade available or as indicated. Cholesterol (≥99 %), HEPES, potassium chloride, sucrose, formic acid (HPLC grade) 2-mercaptoethanol, ethanol (95%) for substrate cleaning, glacial acetic acid, sodium chloride, ethylenediaminetetraacetic acid (EDTA), and ethylene-bis(oxyethylenenitrilo)tetraacetic acid (EGTA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (Acros HPLC grade for sample prep), 0.1- and 0.2- μm polycarbonate filters and drain discs (Whatman), chloroform (HPLC grade), calcium chloride, Tris base, hydrochloric acid, gradient SDS tris-glycine gels (Invitrogen), prestained molecular weight ladder (Invitrogen), NuPAGE SDS sample buffer and reducing buffer (Invitrogen), glacial acetic acid were purchased from Thermo Fisher Scientific (Pittsburg, PA, USA). Ethanol (100%) was purchased from Ultrapure (Darien, CT, USA) for use with ethanol injection. n-Octyl-β-D-glucoside was purchased from Anatrace (Maumee, OH, USA). 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC, >99%) was
purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). Unless otherwise stated, all nitrogen and argon gas used was compressed and $\geq 99.9\%$ (Airgas). All water used was purified using a Millipore Milli-Q Plus Purepak 2 water purification system (Bellerica, MA, USA) with a resistivity of 18.2 MΩ cm at 25 °C unless stated otherwise. Methanol used on the LCMS-8045 system were LCMS grade from Burdick and Jackson and purchased from Thermo Fisher Scientific (Pittsburg, PA, USA).

Instrumentation

Liquid Chromatography Mass Spectrometry (LCMS)

All melanolipofuscin extracts were analyzed using a Shimadzu Nexera X2 ultra-high performance liquid chromatography (UHPLC) system coupled to a Shimadzu triple quadrupole mass spectrometer (LCMS-8045) (Columbia, MD, USA). The HPLC system included an SPD-M30A photodiode array for UV-Vis detection. The LCMS-8045 was equipped with a dual ion source (DUIS) which allowed for the introduction of ions via electrospray ionization (ESI) and/or atmospheric pressure chemical ionization (APCI).

Mass spectrometers detect mass-to-charge ($m/z$) ratios of analytes; the key factor being that the analytes must carry charge (i.e. that they are ions). ESI and APCI are techniques used to generate ions from liquid phase samples, both of which are performed at atmospheric pressure. With ESI, solvent, containing dissolved analytes, is continuously flowed through a capillary with a high potential (typically 3 to 4 kV) applied at the capillary tip relative to the counter-electrode.
(the walls of the surrounding atmospheric pressure area). A Taylor cone of solvent forms at the tip. A strong electric field (created by the potential difference between the capillary tip and the counter-electrode) causes solvent to break away from the Taylor cone as a fine mist of solvent droplets. Solvent evaporation is assisted by the flow of nitrogen gas and elevated temperatures. With sufficient evaporation, ions are ejected from charged droplets into the gas phase in the atmospheric-pressure region. With APCI, solvent, containing dissolved analytes, is flowed through a capillary coaxially with nebulizing and sheath gas (typically N₂) at elevated temperatures (350 to 500 °C). A fine mist of droplets sprays from the capillary and is converted to a gas stream by the gas flow and high temperature. A discharge electrode (known as a corona tip or corona needle) is held at high voltage (typically 2 to 3 kV) with respect to the counter-electrode. Analyte ions are believed to form by a series of three steps: [1] nitrogen and water ions are formed by the corona-created electrons with the buffer gas, [2] charge transfer reactions with water, methanol, or acetonitrile in the buffer generates solvated charged species, and lastly [3] proton transfer from the solvated charged species to the analytes generates [M + H]⁺ analyte ions. Overview depictions of ESI and APCI are shown in Figures 2.1 and 2.2, respectively. ESI is applicable to a wide variety of analytes while APCI is more applicable to relatively less polar and thermally stable compounds such as steroids, pesticides, and pharmaceutical drugs (Dass, 2007).
Figure 2.1: Schematic of electrospray ionization (Shimadzu Corporation, 2016).

Figure 2.2: Schematic of atmospheric-pressure chemical ionization (Shimadzu Corporation, 2016).
After ions are formed, they are guided into the mass spectrometer and sorted by their $m/z$ ratios. A triple quadrupole mass spectrometer was used in this dissertation work. A schematic of the LCMS-8045 analysis unit is shown in Figure 2.3 and a side view a typical quadrupole is depicted in Figure 2.4. Q1 and Q3 of triple quadrupole instruments function as “true” quadrupoles. They consist of four identical metal rods parallel to one another. Ions enter and travel through the quadrupole along the Z-axis and will vibrate along the XY plane in an oscillating electric field as a result of applying direct-current (dc: U) and radio-frequency (rf: V cos $\omega t$) potential across the metal rods. The motion of ions in the XY plane is described by Mathieu coordinates $a_u$ and $q_u$ and are given by the equations 2.1 and 2.2

$$a_u = \frac{8zeU}{m\omega^2r_0^2} \quad Eq \ 2.1$$

$$q_u = \frac{4zeV}{m\omega^2r_0^2} \quad Eq \ 2.2$$

where $U$ is the dc potential, $V$ is the amplitude, $\omega$ is the angular frequency, $m$ is the ion mass, $z$ is the ion charge, and $r_0$ is the inscribed radius (half of the distance between opposite rods). Figure 2.5 depicts stable regions for ions of different masses. If the values of $U$ and $V$ are varied along scan line 1 such that the $a/q$ ratio is constant (i.e. $\frac{a}{q} = \frac{2U}{V}$ is constant), then $m_1$, $m_2$, and $m_3$ will pass through the quadrupole one at a time in the order of $m_1$, then $m_2$, and lastly $m_3$. Therefore, mass spectra are obtained by varying $U$ and $V$ potentials but keeping the $a/q$ ratio constant.
Figure 2.3: Schematic of the quadrupole analysis unit (Shimadzu Corporation, 2016)
Figure 2.4: Side view of typical quadrupole mass spectrometer (Shimadzu Corporation, 2016).

Figure 2.5: Stable regions for a quadrupole mass filter (Shimadzu Corporation, 2016).
q2 is an rf-only quadrupole. In this case, V is scanned with U = 0, so the mass scan line falls on the x-axis in Figure 2.5. This results in a wideband mass filter which essentially allows all ions to pass through. In a triple quadrupole instrument, q2 functions as the collision cell. Ions that are passed into q2 from Q1 can undergo collision induced dissociation (CID) by being collided with inert gas molecules (typically helium) forming so-called product ions from the parent or precursor ions. q2 passes the product ions to Q3 which can be scanned to collect mass spectra of the product ions. The use of a fragmentation method to collect product ions from parent ions is tandem mass spectrometry (MS/MS). Fragmentation is characteristic of the species fragmentated and can thus be used to determine chemical structures.

Ions are passed from Q3 to the detector, which consists of a conversion dynode and electron multiplier (Figure 2.6). Ions passed to the detector are accelerated to collide with conversion dynode electrode which releases ions and secondary electrons which in turn are accelerated to the electron multiplier. The secondary electrons are detected and amplified by the electron multiplier and the amplified signal is sent to the pulse count detection system. Pulses that exceed a threshold value are converted to readable spectrum.

In addition to the triple quadrupole mass spectrometer, high-resolution mass spectrometry data was collected using a Bruker Maxis Plus quadrupole time-of-flight (Q-TOF) hybrid mass spectrometer equipped with an ESI source. The hybrid mass spectrometer consisted of two quadrupoles, Q1 and q2, which function in the same manner as Q1 and q2 of the triple quadrupole instrument. In the hybrid instrument, Q3 is replaced with a time-of-flight (TOF) mass analyzer which detects the m/z of ions essentially based on the differences in the amount of time it takes the ions to travel the flight tube path and is mathematically represented by \( KE = e z V = \)
\[ \frac{mv^2}{2} \] where \( z \) is the integral number of units of charge on the ion, \( e \) (in C) is the fundamental unit of charge, \( V \) (in Volts) is the ion acceleration voltage, \( m \) (in kg) is the mass of the ion, and \( v \) (in m/s) is the velocity of the ion. This gives

\[ \frac{m}{z} = \frac{2eV}{v^2} = \frac{2eV}{(\text{flight tube distance})} \times \left(\frac{\text{time-of-flight}}{\text{flight tube distance}}\right). \]

Figure 2.6: Schematic of an electron multiplier and the pulse count detection system. a) Schematic of the electron multiplier, b) Schematic of the pulse count detection system (Shimadzu Corporation, 2016).
Specular X-ray Reflectivity (XRR)

Specular X-ray reflectivity (XRR) measurements were performed on supported lipid bilayers at beamline station 33-BM-C at the Advanced Photon Source (APS) at U.S. Department of Energy’s Argonne National Laboratory (ANL) (Lemont, IL). The APS provides ultra-bright, high-energy x-ray beams to more than 5,000 users (researchers from universities, industry, medical schools, and other research institutions). New ideas and research brought to the APS by users spans an array of scientific fields: materials science, biology, chemistry, environmental, geological, planetary, and fundamental physics. A couple of examples of benefits accruing at the APS include better materials for lithium-ion batteries and other energy technologies as well as clues into the causes of and treatments for diseases such as AIDS or toxic threats like anthrax (The Advanced Photon Source, n.d.).

When visible light travels through a material, it does so at a speed less than that of the speed of light in a vacuum. This gives rise to the refractive index, n, of visible light through matter which is represented by equation 2.3 (Eq 2.3)

\[ n = \frac{c}{v} \quad Eq\ 2.3 \]

where \( c \) is the speed of light in a vacuum and \( v \) is the speed of light through some material. As visible light encounters a boundary between two materials (i.e. the refractive index changes), it can be reflected from the boundary or refracted into the new material. Snell’s law describes this phenomenon as shown in Figure 2.7 and is represented by equation 2.4 (Giancoli, 2009).

\[ n_1 \sin \theta_1 = n_2 \sin \theta_2 \quad Eq\ 2.4 \]
When x-rays are incident on a surface, similar phenomenon occur as with visible light and the refractive index is the important parameter. For x-rays (wavelengths on the order of 1 Å), the refractive index is given by $n = 1 - \delta - i\beta$ where $\delta$ and $\beta$ are given by equations 2.5 and 2.6, respectively (Tolan, 1999; Vaclav Holy, Pietsch, & Baumbach, 1999).

$$\delta = \frac{\lambda^2}{2\pi r_e \rho_e} \quad Eq \ 2.5$$

$$\beta = \frac{\lambda}{4\pi \mu_x} \quad Eq \ 2.6$$

where $r_e$ is the classical electron radius ($2.818 \times 10^{-15}$ m), $\rho_e$ is the electron density of the material, $\lambda$ is the x-ray wavelength, and $\mu_x$ is the absorption length for x-rays through the material. Values for $\delta$ range from $10^{-5}$ to $10^{-6}$ and $\beta$ values are even smaller. Therefore, $n$ is
slightly less than 1 which results in a phenomenon called total external reflection. When the incident angle ($\alpha_i$) of an x-ray beam is less than the critical angle ($\alpha_c$) with respect to the sample surface, the x-rays are totally reflected from the surface. The critical angle is given by equation 2.7.

$$\alpha_c = \sqrt{2\delta} \quad Eq \ 2.7$$

Since $\beta$ values are exceedingly small, the $\beta$ term in equation 3 is sometimes neglected resulting in the average electron density of the material being the key parameter in determining $n$. Therefore, x-rays are reflected from a surface as a function of incident angle and changes in average electron densities (Fujii, 2016; Krassimir Stoev, 1997; Yasaka, 2010).

In x-ray reflectivity, the reflected intensity of x-rays from a surface is collected as a function of incident angle (Figure 2.8). Figure 2.9 depicts a system of one interface between two layers, in this case a silicon substrate in water. Total external reflection is observed below $\alpha_c$ (0.096° for Si in water) and the reflected intensity decreases as a function of $1/\theta^4$ at higher incident angles as shown in Figure 2.9.

Figure 2.8: Geometry of an x-ray reflectivity experiment (“X-ray Reflectivity,” n.d.).
Figure 2.9: Reflectivity profile from a bare silicon substrate (data collected in-house).

Upon depositing a layer of interest on the substrate, the layer thickness, interface and surface roughness, and average electron densities of the materials change the reflectivity profile. Reflected x-rays from the substrate surface that are out-of-phase with reflected x-rays from the layer surface results in “dips” or fringes in the reflectivity profile. As layer thickness increases, \( \Delta \theta \) between fringes decreases. The depth of the fringes is indicative of electron density. The
more smooth the surface and interface boundaries are (i.e. the lower the roughness), the greater the detail in reflectivity profile features. The effect of layer thickness, surface and interface roughness, and electron density on reflectivity profiles is illustrated in Figure 2.10.

Figure 2.10: Illustration of the effect of surface roughness, film thickness, and electron density on reflectivity profiles (“X-ray Reflectivity,” n.d.).
Methods

Preparation of Large Unilamellar Vesicles (LUVs)

LUVs of DPPC with 0 mol%, 10 mol%, 20 mol%, 33 mol%, or 40 mol% cholesterol were prepared via an ethanol injection method. Briefly, 10 mg of DPPC and the appropriate amount of cholesterol (Chol) (see Table 2.1) were dissolved in 0.25 mL of 200 proof ethanol to a final phospholipid concentration of 40 mg/mL. Then the ethanol solution was injected at a flow rate of 4.5 mL/hr with moderate stirring into 10 mL of buffer (20 mM HEPES, 150 mM KCl, pH 7.4) so that the final phospholipid concentration was 1 mg/mL. Excess ethanol was removed by bubbling argon gas through the LUV suspension. The resulting vesicle solution was extruded through a 0.2-μm polycarbonate filter and then 10 times through a double layer of 0.1-μm polycarbonate filters. For ethanol injection and extrusion, the temperature was held between 50-55°C, above the thermotropic phase transition (T_m) for pure DPPC of 41°C. During the ethanol injection, the round-bottom flask was suspended in a water bath. For extrusion, the LUVs were kept in a 50°C oven and a water circulator set to 50°C was attached to the thermobarrel of the extruder. The final LUV solutions were stored at 4°C and used the next day to prepare the supported lipid bilayers (SLBs).

Table 2.1: Appropriate Masses of DPPC and Cholesterol

<table>
<thead>
<tr>
<th>Cholesterol Fraction</th>
<th>Mass of DPPC (mg)</th>
<th>Mass of Cholesterol (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
<td>10.0</td>
<td>0.0</td>
</tr>
<tr>
<td>0.10</td>
<td>10.0</td>
<td>0.6</td>
</tr>
<tr>
<td>0.20</td>
<td>10.0</td>
<td>1.3</td>
</tr>
<tr>
<td>0.33</td>
<td>10.0</td>
<td>2.6</td>
</tr>
<tr>
<td>0.40</td>
<td>10.0</td>
<td>3.5</td>
</tr>
</tbody>
</table>
Substrate Cleaning

The silicon substrates were 5 x 15 mm pieces diced from single side polished 2” diameter N type silicon (1,1,1 crystal orientation) by the NIU Microelectronics and Cleanroom facility. The silicon substrates were cleaned by sonication in ethanol, then chloroform, and finally water. The substrate was rinsed with water between sonication steps. After sonication in water, the substrate was dried with a stream of nitrogen gas and the surface treated with UV-ozone for five minutes. Cleaned substrates were stored in water until use.

Preparation of Supported Lipid Bilayers (SLBs)

SLBs were prepared using a vesicle bursting method. Briefly, vesicle solutions (5 mL of 0.2 mg/mL) and the substrate were separately pre-warmed to ~50°C in 10-mL beakers. The vesicle solutions were supplemented with 5 mM calcium chloride and then the substrate was submerged in the vesicle solution and incubated for 60 minutes. The samples were cooled to ambient temperature and then excess vesicles and salts were diluted away with water by first carefully submerging the beaker in a basin of water (~1.8 L). The beaker was then submerged in a second basin of water (~1.8 L) to place the substrate in an in-house made sample chamber. The second basin of water was supplemented with 10 mM 2-mercaptoethanol (BME) as an antioxidant for the x-ray experiments.
X-ray Reflectivity

X-ray reflectivity (XRR) measurements were performed on supported lipid bilayers at beamline station 33-BM-C at the Advanced Photon Source at Argonne National Laboratory (Lemont, IL). The x-ray beam energy was 13.5 keV (0.0919 nm wavelength) and the beam size had a width of 1.5 mm and a height of 0.1 mm. XRR data was collected from incident angles of 0.04° to 3°. This corresponds to a wavevector transfer (q_z) of 8.1 x 10^{-4} to 7.2 nm^{-1} as calculated using \( q_z = \frac{4\pi}{\lambda} \sin \theta \). Reflection was collected using a Pilatus 100K area detector located 1200 mm from the sample.

Each SLB described above was measured at 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C. The temperature was controlled with a thermoelectric temperature controller (TTC) (Melcor Thermal Solutions, Trenton, NJ). The sample chamber was mounted onto a copper plate which in turn was bolted to a second copper plate with the peltier element between the two copper plates. The thermometer was bolted to the sample chamber opposite the location of the peltier element. The temperature was set, and the entire system was allowed to heat up and stabilize which typically required about five minutes. After temperature stabilization, the entire system was allowed to equilibrate for 20-30 min to allow the SLB to thermally equilibrate. The sample was aligned in the incident beam and then XRR data was collected in segments from 0.04° to 3°. Each XRR measurement took a total of about 90 s. Due to the possibility of x-ray damage, the sample stage was translocated to collect XRR data on a fresh spot along the substrate.
Preparation of Crude Bovine Lens Membranes

The procedure for preparing crude lens membranes and isolating aquaporin-0 was adapted from works done by Gonen and Walz (Gonen, Cheng, et al., 2004; Gonen, Donaldson, & Kistler, 2000; Gonen, Sliz, et al., 2004; Saboe et al., 2017). Bovine lenses were obtained from Aurora Packing Company Incorporated (Aurora, IL) on the day of slaughter. On the same day, the bovine lenses were excised by cutting an incision along the sclera approximately midway between the front (cornea) and back (optic nerve) of the eye with a razor long enough so that the lens could be removed. Excised lenses were placed in ice-cold homogenization buffer (5 mM Tris, 5 mM EDTA, and 5 mM EGTA, pH 8) until all lenses had been excised. The softer lens cortex and harder lens nuclear tissue were processed separately. After excision, the lenses were decapsulated and the soft cortical tissue was scraped away from the tougher nuclear tissue. Cortical tissue or nuclear tissue from three bovine lenses was homogenized in ice-cold homogenization buffer with 20-30 strokes. The cortical homogenates were pooled, and the nuclear homogenates were pooled. The homogenates were centrifuged at 18000g and 4°C for 22 min. The crude membranes were washed twice by removing the supernatant, resuspending the pellet in ice-cold homogenization buffer, and centrifuging under the same conditions.

Isolation of Aquaporin-0 from Crude Bovine Lens Membranes

Lens membrane proteins were solubilized by gentle stirring of crude bovine lens membranes at 4°C for one hour in solubilizing buffer (10 mM Tris, 4% octyl glucoside (OG), pH
8). Insoluble material was removed by centrifugation at 4°C and 48400g. One milliliter of the resulting supernatant was loaded onto a 5-mL HiTrap Q High Performance anion exchange column equilibrated with 10 mM Tris at pH 8 with 1.2% OG. AQP0 was eluted with 10 mM Tris at pH 8 with 1.2% OG and 300 mM NaCl.

**SDS-PAGE Analysis of Isolated Aquaporin-0**

SDS-PAGE analysis was performed on the isolated aquaporin-0 following the standard reducing procedure provided by Invitrogen with the Novex Wedgewell 8-16% Tris-Glycine Gels. Briefly, twenty microliters of the ion-exchange eluant was mixed with 20 μL of the Tris-glycine SDS sample buffer and 4 μL of the NuPAGE Reducing Agent. The sample was heated at 85°C for 2 min and allowed to cool to ambient temperature. Twenty microliters of the sample were loaded onto a gel along with the 20 μL of the SeeBlue Pre-Stained Standard. The gel ran at 225 V for about 45 min. The gel was removed from the cassette and destained by gentle agitation on a Belly Dancer (Stovall Life Science, Greensboro, NC) in destaining solution (methanol/glacial acetic acid/water (3:1:6)).

**Preparation of Organic Soluble Melanolipofuscin from Human Donor RPE Cells**

Melanolipofuscin granules were isolated from previously obtained human RPE cells following methods described by Feeney-Burns (L Feeney-Burns & Eldred, 1983) as depicted in 2.11. The RPE cells were homogenized and the homogenate was centrifuged at 4°C and 100g for
5 minutes using a Beckman J2-HS centrifuge and a JA-20 rotor to pellet cellular debris. Sucrose gradients were built bottom-to-top using 3 mL each of sucrose solutions of 2.25, 1.37, and 0.63 M. About 1 mL of the homogenate supernatant was carefully layered on the top of the sucrose gradients. The gradients were then centrifuged at 4°C and 8,500g for 20 min. The interface between the bottom two layers was collected and lyophilized. The sucrose fractions containing melanolipofuscin granules from three donors in their 70s were pooled and subjected to a Folch extraction using a 2:1 ratio of chloroform/methanol (CHCl₃:CH₃OH) and water to wash the organic phase. The organic soluble portion was dried under argon gas and reconstituted in 500 μL of 50:50 water/methanol containing 0.1% formic acid. Each sample was carefully handled and protected from light during the entire process.

Mass Spectrometry Analysis of Organic Soluble Melanolipofuscin

The reconstituted organic soluble fraction from melanolipofuscin was placed in autosampler vials and kept at 15°C in the autosampler rack/chamber of the LCMS-8045 system. Ten microliters of sample were injected onto the column. The column used was a Restek Force Biphenyl 1.8 μm particle size of dimensions 50 x 2.1 mm and the column oven temperature was set to 40°C. Solvents A and B were water and methanol, respectively, each containing 0.1% formic acid. The gradient consisted of a flow rate of 0.40 mL/min of isocratic 50% B from 0-2 minutes, increase to 100% B from 2-10 min, isocratic 100% B from 10-15 minutes, and re-equilibration of the column from 15-17 min at 50% B. The PDA output was collected from 190 – 700 nm over the entire 17-min gradient. The ion source was set to use the DUIS functionality (i.e. ESI and APCI were used to generate ions). The nebulizing, heating, and drying gas flows
were set to 2 L/min, 10 L/min, and 10 L/min, respectively. The interface temperature was 300°C. The desolvation line (DL) and heat block temperatures were 250°C and 400°C, respectively.

Figure 2.11: Separation of melanolipofuscin (MLF) from human RPE cells and a schematic of a Folch extraction. (a) Separation of melanolipofuscin from human RPE cells (L. Feeney-Burns & Eldred, 1983). (b) Schematic of a Folch extraction where organic soluble melanolipofuscin is extracted by agitating the granules in 2:1 chloroform/methanol and then washing the organic phase three times with water.
The triple quadrupole mass spectrometer was initially set to collect all ions in the m/z range of 100 – 2000 for the time range 0.01 – 15 minutes by employing the Q3 scan set-up whereby Q1 and q2 are set to pass all ions to Q3 and Q3 is scanned to collect mass spectra of individual ions. In the postrun analysis software in LabSolutions, the mass spectra were averaged across peaks in the base peak chromatogram (BPC) and a list of ions of interest (typically of ion intensity ~1,000,000 or greater) was generated for further analysis with tandem mass spectrometry using the product ion scan capabilities of the LCMS-8045.

High-resolution data was collected by diluting 80 μL of the melanolipofuscin extract sample to a final volume of 280 μL by adding 200 μL of methanol and infusing the sample directly into the hybrid mass spectrometer. Due to the complex composition of the sample, several narrow mass windows were selected to focus on specific ions of interest. Multiple reaction monitoring (MRM) was employed to gather fragmentation data on some of these ions.
CHAPTER 3

INVESTIGATING THE EFFECT OF CHOLESTEROL ON THE STRUCTURE OF SOLID-SUPPORTED MODEL PHOSPHATIDYLCHOLINE LIPID BILAYERS

Introduction

The ocular lens is an avascular tissue that, together with the cornea, focuses light onto the retina. For proper vision, it is crucial that the lens remain transparent and elastic throughout the life of an individual which requires proper water homeostasis. Water homeostasis is thought to be maintained throughout the human body by a family of transmembrane proteins known as the aquaporins (AQPs). Aquaporin-0 (AQP0) is lens specific and is the major membrane protein found in mature lens fiber cells (Schey et al., 2014). Studies show that AQP0 serves [1] to make lens fiber cell membranes permeable to water and [2] as a structural protein (Colom et al., 2012; Fotiadis et al., 2000; Kumari & Varadaraj, 2009; Lindsey Rose et al., 2006; Liu et al., 2011; Nakazawa et al., 2011; Simon et al., 1982; Zhen Wang & Schey, 2011; Yu et al., 2005). Its importance to proper lens function is also highlighted in several studies that link AQP0
mutations to lens pathology, namely congenital cataract (Berry et al., 2000; Francis et al., 2000; Geyer et al., 2006; Gu et al., 2007; Jiang et al., 2009; Jin et al., 2010; W. Wang et al., 2010).

AQP0 structure has been determined using x-ray crystallography (Gonen et al., 2005; Harries et al., 2004a). But x-ray crystallography studies on membrane proteins often lack protein-lipid structural information. This is a problem because several studies show that membrane protein function is influenced by the surrounding lipid environment (Raguz et al., 2014; Reichow & Gonen, 2009; Tong et al., 2012, 2013). There is a need for experimental techniques that can probe membrane protein-bilayer structure at the molecular level in order to better understand the functional interplay between these two critical biological structures.

Biological membranes are complex structures that are essential for many biological functions: cell protection, regulating nutrient transport, compartmentalizing metabolic processes, and signal transduction (Deleu, 2010). Once proposed to consist of two leaflets of homogenous, fluid lipid composition containing proteins and glycoproteins (Singer & Nicolson, 1972), there is now no doubt that lipids phase separate into microdomains, called lipid rafts, first proposed by (Simons & Ikonen, 1997) and (Brown & London, 1997). Since the proposal of lipid rafts, a surge of studies have been conducted to better understand membrane structure as well as to develop new techniques for doing so.

Supported lipid bilayers (SLBs) are biomimetic model membranes that consist of a flat lipid bilayer supported on a solid surface; mica, glass, and silicon oxide surfaces are most common. As shown in Figure 3.1, single SLBs consist of two flat lipid sheets. The polar head groups face opposite each other with one layer (leaflet) facing the substrate and the hydrocarbon chains of each leaflet meeting in the center of the bilayer. SLBs have many advantages compared
to free-floating liposomes (Loose & Schwille, 2009). SLBs can be prepared easily and are more stable compared to liposomes which can fuse and aggregate over time. Tamm and McConnell (1985) showed that lateral mobility and rotation of individual lipids is retained in SLBs. Furthermore, a wide variety of surface sensitive techniques can be used to characterize SLBs such as atomic force microscopy (AFM) (Goksu, Vanegas, Blanchette, Lin, & Longo, 2009; Lin, Blanchette, Ratto, & Longo, 2007; Mingeot-Leclercq, Deleu, Brasseur, & Dufrêne, 2008), secondary ion mass spectrometry (SIMS) (Galli Marxer, Kraft, Weber, Hutcheon, & Boxer, 2005; Kraft, 2006), fluorescence microscopy (Crane & Tamm, 2007), optical ellipsometry (Puu & Gustafson, 1997), quartz-crystal microbalance (Keller & Kasemo, 1998), neutron reflectivity (Wacklin & Thomas, 2007), grazing-incidence small-angle x-ray scattering (GISAXS) (Gumí-Audenis et al., 2018), and specular X-ray reflectivity (Miller, Majewski, Gog, & Kuhl, 2005; Miller, Majewski, & Kuhl, 2006; Nováková, Giewekemeyer, & Salditt, 2006; Reich et al., 2008; Schubert et al., 2008; S. T. Wang, Fukuto, & Yang, 2008).

Figure 3.1: Schematic representation of single supported lipid bilayer (SLB). Schematic was drawn using Inkscape™.
Specular X-ray reflectivity (XRR) is a surface sensitive technique where x-rays (0.01 to 10 nm, 124 to 0.124 keV) are incident on a surface at grazing angles (0.1 – 3°) and the reflected x-rays are counted under the condition of $\theta_{\text{reflected}} = \theta_{\text{incident}}$. The reflectivity profile is dependent on structural parameters of the layer including surface and interface roughness, thickness, and electron density (“X-ray Reflectivity,” n.d.). Fitting of XRR data gives electron density profiles (EDPs) through a thin-layer sample that is normal to the substrate surface. The length scale of x-rays can allow atomic level resolution of structural features of a thin-film. Changes in EDPs as a function of temperature or composition could reveal effects of components of biological membranes on biomimetic SLBs.

Cholesterol is an important component of eukaryotic membranes. It can alter bulk membrane properties such as fluidity, permeability, and hydrophobicity and can change local lipid composition by inducing phase separation or domain formation in lipid bilayers (Subczynski, Pasenkiewicz-Gierula, Widomska, Mainali, & Raguz, 2017). In binary lipid mixtures, cholesterol is known for inducing changes to the lamellar phase behavior of its pure lipid counterpart. Figure 3.2 illustrates the main phase behaviors of lipid bilayers (Deleu, 2010). Bilayer phases are characterized by lateral organization, molecular order, and mobility of individual lipids in the plane of the bilayer. Bilayers composed of a single lipid have a characteristic thermotropic phase transition ($T_m$) which increases with hydrocarbon chain length and degree of saturation (Cevc, 1991). The extreme phases are the gel phase ($S_o$) below the $T_m$ and the liquid-disordered phase ($L_d$) above the $T_m$. Gel phases are characterized with all-trans conformations of the hydrocarbon chains resulting in elongated chains and tight packing of the lipids. Consequently, lipid mobility is significantly reduced with lateral diffusion on the order of
(C_D \sim 10^{-11} \text{ cm}^2 \text{ s}^{-1}) \text{ (Deleu, 2010)}. In the L_d phase, the hydrocarbon chains adopt trans-	extit{gauche} isomerization which reduces hydrocarbon chain length. Tight packing is also lost and rotational and lateral mobility (C_D \sim 10^{-8} \text{ cm}^2 \text{ s}^{-1}) of individual lipids is increased. Some membrane lipids, like phosphatidylcholines, undergo transition in two steps through the ripple phase intermediate (P_{\beta}). The addition of cholesterol imposes another phase behavior called the liquid-ordered phase (L_o) which shares characteristics of the gel and liquid-disordered phases. Phase diagrams for DPPC/Cholesterol mixtures have been deduced from various studies utilizing NMR, DSC, 2D-ELDOR, and molecular dynamics. Figure 3.3 shows one such phase diagram from (Vist & Davis, 1990) and updated based on data from (Davis, Clair, & Juhasz, 2009).

Figure 3.2: Illustration of phase behavior adopted by lipid bilayers in aqueous solution (Deleu, 2010).
Figure 3.3: DPPC/Cholesterol phase diagram determined from NMR and DSC studies of liposomes (Davis et al., 2009; Vist & Davis, 1990). Note that L$_{\alpha}$ and L$_{d}$ are often used interchangeably to denote the liquid-disordered or fluid phase and L$_{\beta}$ is often used to denote the S$_o$ phase.
The overall effect of cholesterol on lipid bilayers is especially important in the ocular lens where the highest ratios of cholesterol to phospholipid are found: 2 to 1 in the cortex and up to 4 to 3 in the nucleus (L.-K. Li et al., 1987; L. K. Li et al., 1985). With such high cholesterol concentrations, membrane raft domains are favored in aged and cataractous fiber cells. The domains are believed to interfere with aggregation of alpha-crystallin, the major structural protein of the lens, at fiber cell membranes thereby maintaining lens transparency. (Widomska, Raguz, Dillon, Gaillard, & Subczynski, 2007) were able to use ESR probes to study the hydrophobicity and oxygen transport parameters in fiber cell extracts from calf lenses and POPC/cholesterol model membranes. Overall, they found a hydrophobic barrier to small polar compounds in the bilayer center and strong barrier to molecular oxygen in the head groups. These effects are attributed to the high ordering and lipid immobilization effects of cholesterol on membranes. These observations supply evidence that cholesterol affects lipid packing in bilayers and may aid in interpretation of changes in EDPs as a function of cholesterol concentration in biomimetic membranes.

Overall, the purpose of this work is to use specular x-ray reflectivity to study the effect of cholesterol (Chol) on supported lipid bilayers (SLBs) composed of dipalmitoylphosphatidylcholine (DPPC). Studying these SLBs is of interest due to the importance of lipid membrane structure on the function of membrane proteins, specifically native aquaporin-0 and its role in lens pathology. SLBs are planar and therefore lack bulk curvature observed in biological membranes. However, XRR has better resolution compared to other x-ray techniques like small-angle x-ray scattering which can be applied to free floating liposomal systems.
Data Fitting

The data were fit to simulated reflectivity by modeling each set as a single lipid bilayer on top of a silicon substrate with a native oxide layer. The model consisted of a series of slabs corresponding to a water super phase, two slabs for outer (water-facing) and inner (substrate-facing) phospholipid head groups, two slabs for outer and inner hydrocarbon chain (tail) regions, one slab for the methyl trough where the hydrocarbon chains meet, one slab for a thin water layer between the bilayer and substrate, one slab for the native oxide layer, one slab for the silicon substrate, and two slabs corresponding to cholesterol in the inner and outer leaflets of the bilayer. A schematic of the slabs is shown in Figure 3.4. Each slab was defined by a value of roughness, thickness, and electron density to generate a calculated reflectivity profile.

In order to reduce the number of floating parameters for each sample, bare silicon substrates were first fit to a 3-slab model of a water, an oxide, and a silicon layer by varying roughness and thickness values directly. The resulting silicon-oxide interface roughness (0.11 nm) and oxide thickness (1.50 nm) were input into the lipid bilayer model and not allowed to vary. The silicon, silicon oxide, and water layers had electron densities (e\(^{-}\) nm\(^{-3}\)) of 699.2, 629.2, and 334.3, respectively.

The phospholipid bilayer is defined by four thicknesses; the bilayer thickness (d\(_B\)), the head group thickness (d\(_H\)), the methyl trough thickness (d\(_M\)), and the tail thickness (d\(_C\)). d\(_B\) was constrained to fit between values of 3.0 – 6.0 nm which is justified since reported thickness values range from around 3.5 nm to around 5.3 nm (see Figure 3.5). DPPC has a phosphatidylcholine head group which is reported to have a thickness ranging from 0.5 – 1.0 nm
(Miller et al., 2005, 2006). To further reduce the number of floating parameters, $d_H$ was fit for the pure DPPC bilayer at 25°C constrained between 0.5 – 1.0 nm and that best fit value (0.78 nm) was used for all subsequent fits. $d_M$ was constrained to values to 0.0 – 0.8 nm (Nagle et al., 1996). $d_C$ was calculated from the other three thickness parameters according to

$$d_C = \frac{d_B}{2} - d_H - \frac{d_M}{2}.$$

Figure 3.4: Schematic of the slab model for fitting lipid bilayer x-ray reflectivity data.
Figure 3.5: A compendium of (a) area per lipid and (b) bilayer thickness values for DPPC bilayers from the literature (Hartkamp et al., 2016; Inoko & Mitsui, 1978; Janiak, Small, & Shipley, 1976; Katsaras, Yang, & Epand, 1992; Kučerka, Nieh, & Katsaras, 2011; Lis, McAlister, Fuller, Rand, & Parsegian, 1982; Lyubartsev & Rabinovich, 2011; Rand & Parsegian, 1989; Ruocco & Shipley, 1982; Schubert, Schneck, & Tanaka, 2011; Sun, Tristram-Nagle, Suter, & Nagle, 1996b, 1996a; Tardieu, Luzzati, & Reman, 1973; Tjörnhammar & Edholm, 2014; Y. Wang, Gkeka, Fuchs, Liedl, & Cournia, 2016; Wiener, Suter, & Nagle, 1989). The volume values in panel (c) were calculated from the values in (a) and (b) by $V = \frac{A_L (D_B)}{2}$. These data come from various SAXS measurements (often multilayered films) and MD simulations. It is apparent in panels (a) and (b) that significant differences are reported throughout the literature as also pointed out by Poger et al (Poger, Caron, & Mark, 2016). The red squares in panels (a) and (b) represent the values from our fits.
The position of cholesterol in lipid bilayers depends on the length of the hydrocarbon chains (Marquardt, Heberle, et al., 2016). Therefore, a cholesterol position parameter relative to $d_B$ was included and is denoted $Z_{\text{chol}}$ and depicted in Figure 3.4. Values for $Z_{\text{chol}}$ range from 0.0 - 1.0: 0.0 indicates that cholesterol is outside the bilayer and 1.0 indicates that cholesterol is between the two bilayer leaflets. The cholesterol length (i.e. thickness; $d_{\text{chol}}$) was fixed at 1.7 nm based on (Marquardt, Kučerka, Wassall, Harroun, & Katsaras, 2016). Cholesterol was assumed to displace phospholipids, thus; the overall contribution of electron density to the bilayer regions were adjusted by the mole fraction of cholesterol, $\chi_{\text{chol}}$ (see Equations 3.1 – 3.3).

Each phospholipid and cholesterol slab are defined by some cross-sectional area value. In the program, it is called the area per head group ($A_H$). The number of electrons (e$^-$) per slab was calculated based of the chemical formula for DPPC and cholesterol; H denotes the head region, T denotes the tail region, PC refers to the phosphatidylcholine head group, DP refers to the C16:0 hydrocarbon chains, and chol denotes cholesterol. The head region of DPPC includes the carbon atoms of the glycerol moiety, therefore $e_{H,PC} = 164$ electrons. The remaining N_e$^-$ are $e_{T,DP} = 242$ electrons and $e_{\text{chol}} = 216$ electrons. Lastly, the number of water molecules ($N_{\text{water}}$) interacting with the PC head group was considered in calculating the electron densities and $e_{\text{water}} = 10$ electrons. Therefore, the electron densities for the phospholipid and cholesterol slabs were calculated using equations 3.1 – 3.3. Lastly, each interface was roughened by a global roughness factor denoted $\sigma$. Overall, the bilayers were defined by seven floating parameters: $A_H$, $d_B$, $d_H$ (fit for one data set and then fixed), $d_M$, $N_{\text{water}}$, $Z_{\text{chol}}$, and $\sigma$.

$$\rho_{H,PC} = \frac{(e_{H,PC} + (e_{\text{water}} \times N_{\text{water}}))(1 - \chi_{\text{chol}})}{(A_H)(d_H)} \quad (Eq \ 3.1)$$
\[
\rho_{T,DP} = \frac{(e_{T,DP})(1 - \chi_{chol})}{(A_H)(d_C)} \quad (Eq \ 3.2)
\]
\[
\rho_{chol} = \frac{(e_{chol})(\chi_{chol})}{(A_H)(d_{chol})} \quad (Eq \ 3.3)
\]

The best fits could not be achieved by using just the parameters described above (see Figure 3.6). Two additional parameters were incorporated to account for asymmetry (\(\eta\)) in the bilayers; position (layer spacing) asymmetry (\(\eta_Z\)) and roughness asymmetry (\(\eta_\sigma\)). The supported membranes are prepared from liposomes. Differences in curvature between the outer leaflet (positive curvature) and the inner leaflet (negative curvature) of the liposomes could lead to [1] unequal partitioning of cholesterol between the leaflets (Marquardt, Kučerka, et al., 2016) or [2] differences in the packing of phospholipid and cholesterol molecules. In principal, both could occur and possibly transfer to the orientated conformation. The substrate-facing leaflet could be more immobilized due to substrate-bilayer interactions compared to the water-facing leaflet. In principal, differences in substrate-bilayer interactions compared to water-bilayer interactions could lead to asymmetries in the bilayer. Therefore, the use of \(\eta_Z\) and \(\eta_\sigma\) is justified. Figure 3.7 shows an example of the effect of the asymmetry parameters on the bilayer model. The asymmetry parameters modified their respective parameter, \(y\), by \[y = y_0 \eta z\] where \(y_0\) is the value for the parameter at the edge of the substrate-facing leaflet, \(\eta\) is the fit value for the asymmetry parameter, \(z\) is the position along the bilayer, and \(d_B\) is the bilayer thickness. Therefore, values of \(\eta_Z > 1\) and \(\eta_\sigma > 1\) would imply the water-facing leaflet is longer and rougher compared to the substrate-facing leaflet.
Figure 3.6: Best fit to the 20 mol% data without position or roughness asymmetry (red) and fitting position and roughness asymmetry (green). Data sets and fits are multiplied by successive factors of 1000 for clarity.
Figure 3.7: Example of the bilayer model showing the bilayer parts without including asymmetry (a) and including asymmetry (b). The examples are of the 20 mol% data at 35°C where $\eta_\sigma = 1.33$ and $\eta_Z = 0.94$. The gray and red lines represent the effect of $\eta_\sigma$ and $\eta_Z$, respectively.
Results and Discussion

Single x-ray reflectivity measurements on silicon SLBs composed of DPPC containing 0 mol%, 10 mol%, 20 mol%, 33 mol%, or 40 mol% cholesterol were taken at beamline 33-BM-C at the Advanced Photon Source. For brevity, the SLBs are referred to by their mol% cholesterol. The x-ray wavelength was 0.0919 nm (13.5 keV) and data was collected from 0.04 – 3.00° which corresponds to a wavevector of $0.1 < q_z < 7.2 \text{ nm}^{-1}$ ($q_z = \frac{4\pi \sin(\theta)}{\lambda}$). The detector used was a Pilatus 100K area detector with square pixels of 172 μm and positioned 1200 mm from the sample.

Figures 3.8 – 3.12 show the fits to the reflectivity measurements. Successive data are offset by multiples of 1000 for clarity and increase in 5°C steps from 25°C to 50°C top-to-bottom. Fractional error ($\sigma_{\text{fractional}}$) was calculated using equation 3.4; the values are tabulated and averages in Table 3.1. The fits are the result of fitting the data over the entire q range. Generally, the data is fit well in the range of $2.5 < q < 5 \text{ nm}^{-1}$. However, it was not always possible to simultaneously capture the first oscillation ($1 < q < 2 \text{ nm}^{-1}$) while also fitting the data beyond $q = 5 \text{ nm}^{-1}$. Due to the difficulty in fitting experimental data to high q (Miller et al., 2005), our fractional errors are calculated out to $q = 5 \text{ nm}^{-1}$ and the overall average is $0.33 \pm 0.05$.

$$\sigma_{\text{fractional}} = \sqrt{\frac{\sum_{i=1}^{N} \left( \frac{I(q)_i - I(q)_{i, \text{fit}}}{I(q)_i} \right)^2}{N}} \quad (Eq \ 3.4)$$
Figure 3.8: Best fit (red) to the 0 mol% data (black). From top to bottom: 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C. Data sets and fits are multiplied by successive factors of 1000 for clarity.
Figure 3.9: Best fit (red) to the 10 mol% data (black). From top to bottom: 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C. Data sets and fits are multiplied by successive factors of 1000 for clarity.
Figure 3.10: Best fit (red) to the 20 mol% data (black). From top to bottom: 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C. Data sets and fits are multiplied by successive factors of 1000 for clarity.
Figure 3.11: Best fit (red) to the 33 mol% data (black). From top to bottom: 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C. Data sets and fits are multiplied by successive factors of 1000 for clarity.
Figure 3.12: Best fit (red) to the 40 mol% data (black). From top to bottom: 25°C, 30°C, 35°C, 40°C, 45°C, and 50°C. Data sets and fits are multiplied by successive factors of 1000 for clarity.

Table 3.1: $\sigma_{\text{fractional}}$ values, averages, and standard deviations for $q < 5 \text{ nm}^{-1}$

<table>
<thead>
<tr>
<th>Temp. (°C)</th>
<th>$X_{\text{chol}}$</th>
<th>0.00</th>
<th>0.10</th>
<th>0.20</th>
<th>0.33</th>
<th>0.40</th>
<th>$\sigma_{\text{fractional}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.0</td>
<td>0.24</td>
<td>0.28</td>
<td>0.37</td>
<td>0.35</td>
<td>0.27</td>
<td>0.30 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>30.0</td>
<td>0.26</td>
<td>0.31</td>
<td>0.41</td>
<td>0.34</td>
<td>0.27</td>
<td>0.32 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>35.0</td>
<td>0.39</td>
<td>0.37</td>
<td>0.36</td>
<td>0.37</td>
<td>0.29</td>
<td>0.36 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>40.0</td>
<td>0.25</td>
<td>0.34</td>
<td>0.38</td>
<td>0.32</td>
<td>0.36</td>
<td>0.33 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>45.0</td>
<td>0.27</td>
<td>0.43</td>
<td>0.35</td>
<td>0.37</td>
<td>0.30</td>
<td>0.35 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>50.0</td>
<td>0.33</td>
<td>0.31</td>
<td>0.35</td>
<td>0.37</td>
<td>0.41</td>
<td>0.36 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>$\sigma_{\text{fractional}}$</td>
<td>0.29 ± 0.05</td>
<td>0.34 ± 0.05</td>
<td>0.37 ± 0.02</td>
<td>0.35 ± 0.02</td>
<td>0.32 ± 0.05</td>
<td>0.33 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>
Figures 3.13 – 3.17 show the electron density profiles (EDPs) for the fits shown in Figures 3.8 – 3.12, respectively. Figures 3.13, 3.14, and 3.15 show the EDPs for the 0, 10, and 20 mol% fits, respectively. There is a clear, abrupt thinning of the membrane with increasing temperature as evidence by the left shift of the water-facing head group peak. For 0 mol% cholesterol, the shift appears to stop between 40°C and 45°C which is consistent with the $T_m$ for DPPC of 41°C (McElhaney, 1982). $T_m$ for 10 and 20 mol% cholesterol is lower than that of pure DPPC, occurring between 35°C and 40°C. The decrease in $T_m$ with the addition of 10 mol% or 20 mol% could be due to cholesterol disrupting the hydrophobic packing of the C16:0 chains. Overall, the 33 mol% and 40 mol% membranes thin with increasing temperature, but the thinning is more gradual compared to the other three membranes as shown by the smaller leftward steps of the water-facing head group peak in Figures 3.16 and 3.17. The gradual thinning could indicate that the membrane does not exist in multiple distinct phases which is consistent with Figure 3.3 showing that the Lo has no phase transition.
Figure 3.13: Electron density profiles for 0 mol% cholesterol.

Figure 3.14: Electron density profiles for 10 mol% cholesterol.
Figure 3.15: Electron density profiles for 20 mol\% cholesterol.

Figure 3.16: Electron density profiles for 33 mol\% cholesterol.
Figure 3.17: Electron density profiles for 40 mol% cholesterol.

For most of the fits, $\eta_\sigma$ is greater than one (see Figure 3.26.b) meaning that the roughness increases across the bilayer from the substrate- to the water-facing side. This is observed in Figure 3.13 for the 45°C and 50°C fits where the water-facing tail is more smoothed out (less defined) compared to the substrate-facing tail. Another consequence of $\eta_\sigma > 1$ is a lower electron density for the water-facing head group compared to the substrate-facing head group which is also observed in Figure 3.13. These features are also evident in, for example, Figures 3.14 – 3.17 for the 25°C EDPs. On average, $\eta_\sigma$ approaches unity as temperature increases (see Figure 3.26.b). The trend in $\eta_\sigma$ is reasonable. The roughness is, at least in part, due to the
mobility of the chemical components of the membranes. It is reasonable to assume that the 
substrate-facing components would be more immobilized due to van der Waals interaction with 
the substrate and thus the roughness of that side is influenced by the roughness of the oxide 
layer. The energy for a thermal undulation likely decreases the further away the interface is from 
van der Waals field of the substrate. Hence, the water-facing components may be comparatively 
more fluid manifesting as greater roughness. As temperature increases, the substrate interactions 
may be disturbed giving the substrate-facing leaflet greater mobility.

Figures 3.18 – 3.23 depict the EDPs plotted as a function of mol% cholesterol rather than 
temperature. Figure 3.18 shows the EDPs at 25°C. The membrane seems to slightly thicken with 
the addition of cholesterol but the amount of thickening (~0.3 nm) is the same regardless of 
mol% cholesterol. The EDPs of the 10 mol% and 20 mol% bilayers are nearly identical 
suggesting similar phase behavior and overall packing of the components. The phase diagram 
shown in Figure 3.3 indicates a L\(\beta\) + L\(\alpha\) coexistence region in this mol% cholesterol from about 
30-37°C. These data suggest that the phase boundaries for this coexistence region could be 
extended downwards to 25°C. This is reasonable as a pure DPPC bilayer would exist in the L\(\beta\) 
phase in this temperature range.
Figure 3.18: Electron density profiles at 25°C as a function of mol% cholesterol. The profiles are offset by adding multiples of 75 for clarity in the bottom figure.
Figure 3.19: Electron density profiles at 30°C as a function of mol% cholesterol. The profiles are offset by adding multiples of 75 for clarity in the bottom figure.
Figure 3.20: Electron density profiles at 35°C as a function of mol% cholesterol. The profiles are offset by adding multiples of 75 for clarity in the bottom figure.
Figure 3.21: Electron density profiles at 40°C as a function of mol% cholesterol. The profiles are offset by adding multiples of 75 for clarity in the bottom figure.
Figure 3.22: Electron density profiles at 45°C as a function of mol% cholesterol. The profiles are offset by adding multiples of 75 for clarity in the bottom figure.
Figure 3.23: Electron density profiles at 50°C as a function of mol% cholesterol. The profiles are offset by adding multiples of 75 for clarity in the bottom figure.
At 33 mol% cholesterol, there is an overall decrease of electron density at 25°C. (Vist & Davis, 1990) observed L_0 phase behavior above ~25 mol% cholesterol at all temperatures measured. An L_β + L_0 to L_0 phase behavior shift would result in overall less order due to the loss of order from gel to liquid-ordered phase shift. It is reasonable that the components would occupy more space with greater disorder and roughness would increase. Both factors would result in a decrease in electron density. The head group region of the 40 mol% data shows an increase in electron density compared to the 33 mol% data and the hydrocarbon region has an overall increase in electron density compared to all other samples. The increase in electron density is likely due to the fact that more cholesterol is present, much of which is in the hydrocarbon region and the bilayer center according to the increase in electron density. The EDPs at 30°C are shown in Figure 3.19. These EDPs are remarkably similar to those at 25°C. If the phase boundary lines separating the L_β + L_0 region from the other regions are extended downwards in Figure 3.3 to 25°C, these results make sense.

The EDPs at 35°C are shown in Figure 3.20. The profiles are overall much more similar to each other than at any of the other temperature. This could be due to the coexistence of L_β, L_α, L_0 phases. The decrease in electron density in the head group region with X_{chol} is likely due to the disruption of ordered head packing in gel phase DPPC by the continued addition of cholesterol. There is increased electron density in the hydrocarbon region and bilayer center for the 40 mol% data which is likely due to cholesterol descent into the bilayer.

Figure 3.21 shows the EDPs at 40°C. The pure DPPC bilayer has melted into the L_α phase. This is evident from the increase in roughness across the bilayer which is expected as mobility of individual lipids increases with temperature. The electron density also decreases as
the lipids become more disordered and thus occupy more volume. At 10 mol\%, the electron density in the hydrocarbon region increases probably due to the ordering effect cholesterol has on fluid phase hydrocarbon chains. The electron density further increases in the hydrocarbon region with addition of 20 mol\% possibly due to the more ordering of the DPPC chains and the crossover into the L_α + L_o coexistence region based on Figure 3.3. Cholesterol could also be moving into the bilayer center as evident by the prominent “bumps” of electron density in the hydrocarbon region. The 33mol\% bilayer probably exhibits L_o phase behavior which would imply overall increase in chain ordering and concomitant increase in electron density. This is true compared to the pure DPPC bilayer, however, electron density is lost compared to 10 and 20 mol\% probably because the bilayer is thicker and thus the components occupy greater volume. This behavior is expected of the 40 mol\% as it also exhibits L_o phase behavior under these conditions. However, the 40 mol\% EDP profile does not closely resemble that of the 33 mol\%.

Furthermore, the 40 mol\% bilayer exhibited greater electron density in the hydrocarbon region compared to the cholesterol-containing membranes at lower temperatures. The opposite is true at 40°C. The mobility of individual lipids increases with temperature. Perhaps this suggests that with greater mobility cholesterol could begin to form cholesterol-rich rafts at around 40 mol\% in these artificial SLBs.

The EDPs of the 0, 10, 20, and 33 mol\% SLBs are similar with respect to each other at 45°C compared to 40°C as shown in Figure 3.22. The major difference seems to be a slight thinning of the SLBs at 45°C which could be due to some intercalation of the hydrocarbon chains with increased mobility. The two major differences for the 40 mol\% membrane is [1] the essential loss of the head group peaks possibly due to a complete disruption of head group
packing by cholesterol and increased thermal energy and [2] an overall decrease in electron density through the hydrophobic region of the SLB which would happen if the components occupy more space as a function of increased mobility.

The relationship among the 0, 10, 20, and 33 mol% EDPs at 50°C are again similar as shown in Figure 3.23. The behavior of the 40 mol% SLB is interesting. At 50°C, the head group features have re-appeared with a concomitant loss of hydrocarbon chain features. The electron density at the bilayer center of the 40 mol% membrane is about the same at 45°C and 50°C but increases on either side of the center at 50°C compared to 45°C. Packing density changes of the head groups may account for an apparent sudden reappearance of head group feature perhaps as a result of domain formations.

A more direct investigation of the effect of cholesterol on DPPC/cholesterol SLBs is found by plotting the values for the fitting parameters as a function of cholesterol fraction. These are represented in Figures 3.24 – 3.27. Figure 3.24.b shows the change in bilayer thickness as a function of mol% cholesterol. Bilayer thickness generally increases with mol% cholesterol with a notable exception: when the temperature was set to 35°C. At this temperature, the SLBs may have exhibited variable phase behavior or microdomain behavior due to phase boundary conditions (see Figure 3.3). Thermotropic phase transitions or in other words, the melting from one distinct phase behavior to another, is also evident by large differences in bilayer thickness between low (T ≤ 35°C) and high (T ≥ 40°C) temperatures up to and including 20 mol% cholesterol. Less dramatic fluctuations in bilayer thickness occur at 33 mol% cholesterol suggesting more uniform phase behavior. With 40 mol% cholesterol, fluctuations in bilayer
thickness increase compared to 33 mol%; could this be the result of the formation of cholesterol-rich domains remains to be answered.

The trends in area per molecule, plotted in Figure 3.24.a, share similar characteristics to the trends in bilayer thickness. Overall, we observe a net decrease in area per molecule with increasing mol% cholesterol which is consistent with the known condensation effect cholesterol imposes on phospholipid bilayers (Miyoshi, Lönnfors, Peter Slotte, & Kato, 2014; Y. Wang et al., 2016). Thermotropic phase transitions are evident by significant differences in area per molecule between low and high temperatures up to and including 20 mol% cholesterol. Like with the bilayer thickness, changes in area per molecule are reduced with 33 mol% cholesterol and greater differences occur with 40 mol% cholesterol.
Figure 3.24: Parameters from the best fits to the reflectivity data. (a) Area per molecule (nm$^2$) (b) Bilayer thickness (nm).
Figure 3.25: Parameters from the best fits to the reflectivity data. (a) Volume (nm$^3$) (b) Cholesterol position.
Another useful physical parameter to characterize lipid bilayers that is commonly reported is molecular volume. While it is not a fitting parameter directly in our work, it is calculated from a few fitting parameters by \( V = \left( \frac{d_B - d_M}{2} \right) A_H - N_w V_w \) where \( d_B \) is the bilayer thickness (nm), \( d_M \) is the methyl trough thickness (nm), \( A_H \) is the area per head group, \( N_w \) is the number of water molecules per headgroup (which for clarity are shown in Figure 3.27.b), and \( V_w \) is the molecular volume of water which is calculated from mass density of water at the relevant temperature (“Water - Specific Volume,” n.d.). Values for molecular volume of the SLBs is shown in Figure 3.25.a. Since molecular volume is calculated from bilayer thickness and molecular area, it is not surprising that overall trends in molecular volume sync nicely with bilayer thickness and molecular area.

Cholesterol position (\( Z_{\text{choi}} \)) within the bilayer is depicted in Figure 3.25.b. As a function of mol% cholesterol, there is not a real trend with cholesterol position. Interesting points are observed as a function of temperature. Cholesterol tends to move towards the bilayer center (values approach 1) with increasing temperature up to and including 20 mol% cholesterol. This is consistent with observations that cholesterol descends towards the bilayer center in shorter chain lipids (Marquardt, Heberle, et al., 2016). In other words, as 0, 10, and 20 mol% SLBs melt into more fluid (i.e. shorter hydrocarbon length), cholesterol moves towards the bilayer center. Less movement is observed at 33 mol% cholesterol which could due to more uniform phase behavior (e.g. \( L_o \)). The drastic movement of cholesterol towards the head groups with 40 mol% cholesterol at 50°C could be indication of cholesterol-rich bilayer domains (Marquardt, Heberle, et al., 2016).
Figure 3.26: Parameters from the best fits to the reflectivity data. (a) Global roughness, $\sigma$, in nm.

(b) Asymmetry in global roughness, $\eta_\sigma$. 
The global roughness ($\sigma$) and roughness asymmetry ($\eta_\sigma$) parameters are plotted in Figure 3.26.a and 3.26.b, respectively. Values for $\eta_\sigma > 1$ implies an increase in $\sigma$ across the SLB from the substrate-facing to the water-facing side and vice versa. This is depicted in Figure 3.6 (gray lines) where $\eta_\sigma = 1.00$ (Figure 3.6.a) and 1.33 (Figure 3.6.b) for 20 mol% at 35°C. To reduce the number of fitting parameters, the global roughness was originally fixed and the roughness across the SLB modified with the asymmetry parameter. However, at higher temperatures ($T \geq 40^\circ$C), the best fits could not always be achieved by only floating $\eta_\sigma$, therefore, $\sigma$ was floated at higher temperatures. In these cases, $\sigma$ increased indicating overall greater bilayer roughness which is reasonable because the individual molecules would exhibit greater thermal motion at higher temperatures. There are two main observations regarding $\sigma$ and $\eta_\sigma$: [1] most values for $\eta_\sigma$ are greater than 1 and [2] $\sigma$ and $\eta_\sigma$ seem to be inversely related. Substrate-facing SLB components are probably more influenced by substrate-bilayer interactions and are hence more immobilized whereas water-facing components probably exhibit greater mobility. This is observed in main observation [1] where bilayer roughness increases from substrate to water. If considering $\sigma$ in terms of overall SLB component mobility, main observation [2] follows nicely. As temperature is increased, the mobility of the individual molecules would increase (increase in $\sigma$), thus reducing the effect of substrate-bilayer interactions. The bilayer leaflets would behave more similarly, hence the concomitant decrease in $\eta_\sigma$. 
Figure 3.27: Parameters from the best fits to the reflectivity data. (a) Asymmetry in layer spacing, $Z_{\sigma}$. (b) Number of water molecules per head group.
Figure 3.27.a shows the values for the position asymmetry ($\eta_Z$). Values less than one indicate a shorter water-facing leaflet compared to no asymmetry ($\eta_Z = 1$). This is represented in Figure 3.7 by the red lines. A possible rationale for $\eta_Z$ changes is variable $trans/gauche$ conformations about the C-C bonds in the hydrocarbon chains. The observed values of $\eta_Z < 1$ implies thinner water-facing hydrocarbon chains which would be true of greater $gauche$ conformations (i.e. more fluid like phase behavior). One way to induce variable phase behavior between the two leaflets is unequal partitioning of cholesterol. For $T \leq 35^\circ C$, a value of $\eta_Z < 1$ implies less $L_\beta$ behavior in the water-facing leaflet which could be achieved by more cholesterol in the water-facing leaflet. For $T \geq 40^\circ C$, a pure DPPC bilayer would melt into the $L_\alpha$ phase. The water-facing leaflet would exhibit greater $L_\alpha$ behavior based on the $\eta_Z$ values being less than one at these temperatures which implies redistribution of cholesterol from the water-facing leaflet to the substrate-facing leaflet. This is plausible given increased mobility of the molecules at higher temperatures and supported by the overall loss in $\eta_o$ with temperature.

Phase diagrams are another useful representation of the parameters describing the SLBs and select phase diagrams are represented in Figure 3.28. The condensation effect of cholesterol on bilayers is represented in Figure 3.28.a by the overall decrease in area per molecule with increasing mol% cholesterol which is consistent with (Y. Wang et al., 2016). The phase diagram for bilayer thickness (Figure 3.28.b) is similar to that for the area per molecule in that the greater amount of variation at mol% cholesterol $\leq 20\%$ compared to $\geq 33\%$ is consistent with phase behavior predicted by (Davis et al., 2009; Vist & Davis, 1990) and even the $Z_{chol}$ phase diagram (Figure 3.28.d) is consistent with this as well. The volume per molecule is somewhat
representative of multiple phase behaviors at mol% cholesterol ≤ 0.20 but more erratic at higher cholesterol concentrations as evident from the volume phase diagram (Figure 3.28.c).

Conclusion

Our long-term goal is to study the structure of lens-specific native and mutated aquaporin-0 – lipid bilayer complexes to better understand the bilayer influence on aquaporin-0 function and hence its implications in lens pathology, namely cataracts. To that end, we have reduced the plasma membrane of lens fiber cells down to a two-component system composed of dipalmitoylphosphatidylcholine and cholesterol and investigated the effect of cholesterol on silicon supported lipid bilayers of this model system using specular x-ray reflectivity. The data were fit to a model comprising a series of slabs representing portions of the chemical constituents of the model membranes. The electron density, thickness, and roughness of each slab was determined from the chemical formula of the constituents and fitting a few physically meaningful parameters (e.g. area per molecule, cholesterol thickness, hydrocarbon chain thickness, etc.).

We observed the well-known condensation effect cholesterol has on phospholipid bilayers as seen in Figure 3.24.a where the area per molecule tends to decrease with increasing cholesterol fraction. Bilayer thickness generally increased with increasing mol% cholesterol. There were greater differences in area per molecule and bilayer thickness between T ≥ 40°C and T ≤ 35°C for mol% cholesterol ≤ 20% than at 33% or 40% which is indicative of the thermotropic phase transitions at lower cholesterol fractions.
Figure 3.28: Phase diagrams from select fitting parameters. (a) Area per lipid (nm$^2$), (b) Bilayer thickness (nm), (c) Volume per lipid (nm$^3$) calculated as $V = \left(\frac{d_B - d_M}{2}\right)A_H - N_{water}V_{water}$, and (d) Cholesterol position. The colorbars are false coloring scales for the magnitude of the fitting parameters where in each case, blue to yellow indicates low to high magnitude, respectively. The phase diagram from Vist (1990) is overlaid in each with the phases labeled in (a) for reference (Davis et al., 2009; Vist & Davis, 1990).
Our data seem to indicate unequal partitioning of cholesterol between the bilayer leaflets suggested by the position and roughness asymmetry values. The position asymmetry values of less than one implies shorter hydrocarbon chains in the water-facing leaflet which could occur if that leaflet exhibited more fluid-like phase behavior. This is possible if greater cholesterol content is present there at lower temperatures and redistributes to the substrate-facing leaflet as temperature increases. This notion is also supported in two ways: [1] by the decrease in roughness asymmetry with increasing temperature, as the leaflets become more uniform in composition and the possible diminution of substrate-bilayer interactions and [2] by the cholesterol position tending to increase towards a value of one with increasing temperature, which implies the movement of cholesterol towards the bilayer center. It is reported that in liposomes, cholesterol prefers to locate in the cytosol-facing leaflet of plasma membranes probably due to its affinity for the high negative curvature of that leaflet (Marquardt, Kučerka, et al., 2016). Additionally, other work from our group and collaborators probed the location of cholesterol in liposomes composed of egg-derived phosphatidylcholines and cholesterol using small-angle x-ray scattering (SAXS). Their data suggested unequal partitioning of cholesterol but, due to the symmetry factor of SAXS, they could not assign to which leaflet (unpublished work). These points may suggest a reason for the unequal partitioning in non-spherical samples like SLBs as they lack global curvature. The SLBs were prepared from liposomes via a vesicle bursting method. It remains unanswered if the cholesterol was unequally partitioned in the liposomes and upon rupture the overall composition between leaflets remained until redistribution at higher temperatures.
A couple of discrepancies that need to be addressed are [1] the “erratic’ behavior of the volume per molecule as depicted in Figure 3.25.a and [2] the sudden movement of cholesterol outwards from the bilayer center at high cholesterol concentration and temperature. MD simulations suggest a steady linear decrease in volume with temperature for DPPC/Cholesterol membranes (Y. Wang et al., 2016). However, we observe increasing volumes compared to what would be expected at 33 mol% and 40 mol% cholesterol at higher temperatures. These data may suggest the formation of rafts in the SLBs. Lipid rafts or microdomains of different lipid composition in biological membranes have been studied for years and cholesterol-rich Lo is thought to provide the platform (Marquardt, Kučerka, et al., 2016). (Armstrong et al., 2013) have observed cholesterol induced lipid domains in a single bilayer component system. Our observed movement of cholesterol towards the bilayer leaflets could represent the formation of cholesterol bilayer domains (CBDs) in our model SLBs. Our apparent unequal partitioning could provide such a concentration at higher overall cholesterol content (e.g. 33 and 40 mol%) and increased mobility at higher temperatures may provide the lateral movement needed for cholesterol molecules to combine into a CBD. In any event, this is an important consideration since lens plasma membranes contain extremely high concentrations of cholesterol and CBDs have been proposed in them (Widomska, Subczynski, Mainali, & Raguz, 2017).

Our fitting routine essentially treats the SLBs as homogenous mixtures of DPPC/Cholesterol and the overall area per individual molecule is adjusted based on the mole fraction of cholesterol (see equations 3.1 – 3.3). Our overall highest fractional error in the fits were for the 40 mol% samples which are likely not homogenous due to CBDs. The second highest fractional errors were to the fits at 35°C where the systems likely exhibit mixed bilayer
phase behavior. These fractional errors and the deviant behavior of the volume and cholesterol fraction suggest that fitting the data as a homogenous mixture is less than ideal and that we should explore a more sophisticated model and work to incorporate it into the fitting routine. Furthermore, our fitting routine occasionally produces large error bars for the fitting parameters (error bars not shown). It remains unanswered if this could be due to the direct influence of one parameter on another during fitting iterations. More detailed analysis of the error calculations is needed to assess the reliability of our fitting routine.
CHAPTER 4

ISOLATION OF AQUAPORIN-0 FROM BOVINE LENSES

Introduction

Our x-ray reflectivity studies on biomimetic supported lipid bilayers have provided us with information on membrane organization which is important to our long-term goal of investigating membrane protein-lipid bilayer structure. Our main interest is in the structure of the aquaporin-0/lipid bilayer complex due to its implication in cataract development which is the leading cause of blindness worldwide. As discussed in Chapter 1 (page 11), AQP0 is a lens-specific aquaporin that accounts for more than 50% of the fiber cell membrane protein fraction (Alcala et al., 1975).

The primary function of AQP0 is believed to be maintenance of water homeostasis in the lens, a function that is critical for lens transparency and flexibility (Schey et al., 2014). Studies have presented strong evidence that the surrounding bilayer modulates the function of aquaporins (Tong et al., 2012, 2013).

de Groot (Briones, Aponte-Santamaría, & de Groot, 2017) published work recently investigating the localization and ordering of lipids around AQP0. Figures 4.1 and 4.2 depict
their calculated images of the AQP0/lipid bilayer complex and some important physical parameters in describing phospholipid organization, namely area-per-lipid (APL) and phosphate-phosphate distance (a measure of bilayer thickness). The APL simulations are shown in Figure 4.1 with average APL and standard deviations in Å² of 58.8 ± 0.8 for (a), 48.5 ± 0.2 for (b), 56.2 ± 0.8 for (c), and 58.8 ± 0.7 for (d). The calculations for phosphate-phosphate thickness (ThkP-P) shown in Figure 4.2 indicate a thickening (color change to red from blue) for DMPC lipids adjacent to AQP0. Bilayer thickening adjacent to AQP0 may be the result of reducing hydrophobic mismatch between the membrane protein exterior and the hydrophilic environment. This work highlights some important characteristics of membrane protein/lipid bilayer complexes which are characteristics we are interested in for the AQP0/lipid bilayer complex. However, de Groot’s work is simulation/calculation based. We are developing techniques to probe AQP0/lipid bilayer organization experimentally utilizing x-ray scattering techniques and work by de Groot, Walz, Shulten, and Tong provide results to compare with.

One of the difficulties with our work is that membrane proteins are notoriously difficult to isolate without destroying the functional 3D structure of the protein. However, Walz’ research group has published several papers detailing their procedures (Gonen et al., 2000; Gonen, Sliz, et al., 2004; Saboe et al., 2017). Bovine lenses are an attractive source of aquaporin-0 (AQP0) and bovine eyes are readily available from Aurora Packing Company (Aurora, IL).

In this chapter, we describe our method development for the isolation and characterization of AQP0 from bovine lenses. We have modified the procedures described by Walz to include a single chromatography step, ion-exchange chromatography. Additionally, our data indicates that we can eliminate unnecessary elution steps, thereby, making the overall
isolation of AQP0 more sustainable. Isolated protein obtained by this protocol will be used to prepare AQP0/bilayer complexes. We will probe the structural organization of these complexes and the protein-bilayer interactions with X-ray scattering techniques and microscopy techniques, namely atomic force microscopy (AFM).

Figure 4.1: Local area-per-lipid (APL) parameter around AQP0. The lipid used was dimyristoylphosphatidylcholine (DMPC) (Briones et al., 2017). The color bar shows the range of APL spanned with dark blue representing 50 Å² and dark red representing 70 Å². The dashed lines on the color bar represent the average APL for a pure DMPC bilayer. (a) Simulation performed at T = 310 K; fluid-phase DMPC. (b) Simulation performed at 280 K; gel-phase DMPC. (c) Simulation performed at 310 K with forcing DMPC into gel-phase behavior. (d) Simulation performed at 310 K while restraining AQP0.
Figure 4.2: Phosphate-phosphate thickness ($\text{Thk}_{\text{P-P}}$) parameter around monomers of the AQP0 tetramer (Briones et al., 2017). The lipid used was dimyristoylphosphatidylcholine (DMPC). The color bar shows the range of $\text{Thk}_{\text{P-P}}$ spanned with dark blue representing 3.0 Å and dark red representing 4.0 Å. The dashed lines on the color bar represent the average $\text{Thk}_{\text{P-P}}$ for a pure DMPC bilayer. (a) Simulation performed at $T = 310$ K; fluid-phase DMPC. (b) Simulation performed at 280 K; gel-phase DMPC. (c) Simulation performed at 310 K with forcing DMPC into gel-phase behavior. (d) Simulation performed at 310 K while restraining AQP0.
Figures 4.3 – 4.5 illustrate results from our initial attempt at isolating aquaporin-0 from bovine lenses. The procedure was adapted from work by Gonen and Walz (Gonen et al., 2000; Gonen, Sliz, et al., 2004). Initially, ~30 whole decapsulated bovine lenses were homogenized and the crude membrane pelleted by centrifugation. The membrane proteins were solubilized in 100 mL of buffer containing 4% octyl-glucoside (OG) and the insoluble material removed by centrifugation. Five milliliters of the total solubilized proteins were loaded onto a HiTrap Q High Performance anion exchange column and eluted with 150 mM NaCl. All SDS-PAGE samples were prepared using a ratio of 16 μL of lens membrane protein sample to 40 μL of total SDS-PAGE sample work-up.

It is clear from the elution profile (Figure 4.4) that the anion exchange column was overloaded. The greatest absorbance at 280 nm ($A_{280}$) occurred with the column void volume. Since AQP0 is the major intrinsic membrane protein of the lens, the greatest $A_{280}$ would theoretically have occurred with 150 mM NaCl (fractions 16 -20). In fact, the second highest $A_{280}$ did occur with 150 mM NaCl supporting the assumption that AQP0 was bound to the column and later eluted off. Qualitatively, these results are encouraging, however, overloading a chromatographic column hinders separation likely leading to less than desired purity.

The SDS-PAGE results shown in Figures 4.4 and 4.5 are encouraging regardless of the overloading of the HiTrap Q column. Lane A of Figure 4.4 and arguably lane B as well were overloaded probably due to the sample being the total soluble lens membrane proteins with no chromatographic separation at all. However, none of the other lanes were overloaded and nearly
all the aliquots tested showed proteins at about 28 kDa against the molecular weight ladder, the molecular weight of AQP0. It is also reported that AQP0 is truncated in older lens fiber cells which may shift its function from primarily a water pore to primarily a structural protein that forms gap junctions between fiber cells. This may account for the presence of two bands around 28 kDa.

Figure 4.3: Elution profile of lens membrane proteins. Various concentrations of chloride (sodium chloride) are introduced in a stepwise gradient to compete for binding sites on the HiTrap Q High Performance column. Fraction 0: final ~1 mL of flow through from column loading, Fractions 1-5: 0 mM, Fractions 6-10: 50 mM, Fractions 11-15: 100 mM, Fractions 16-20: 150 mM, Fractions 21-25: 200 mM, Fractions 26-30: 250 mM, and Fractions 31-40: $10^3$ mM
Figure 4.4: SDS-PAGE gel of lens membrane proteins corresponding to aliquots taken from fractions shown in Figure 4.3. (A) Total soluble lens proteins (5X), (B) Total soluble lens proteins (1X), (C) effluent from ion-exchange column loading (1X), and (D) effluent from ion-exchange column loading (2X).
Figure 4.5: SDS-PAGE gel of lens membrane proteins corresponding to aliquots taken from fractions shown in Figure 4.3. (A) Column wash, (B) 50 mM NaCl, (C) 100 mM NaCl (center fraction), (D) 100 mM NaCl (last fraction), (E-I) 150 mM NaCl; (E) first fraction to (I) last fraction, (J) 200 mM NaCl; first fraction, (K) 200 mM NaCl; last fraction, (L) 250 mM NaCl, (M) 10^3 mM NaCl

Saboe and Walz (2017) (Saboe et al., 2017) reported the elution of AQP0 with 300 mM NaCl compared to 150 mM NaCl as reported by Gonen and Walz.(Gonen et al., 2000; Gonen, Sliz, et al., 2004) Due to column overloading in our first attempt, it was unclear what elution peaks corresponded to “true” separation and which were the result of overloading. Furthermore,
octyl-glucoside is an expensive non-denaturing detergent (~$500 per 25 g) and is required in large quantity between the 4% solubilization buffer and the 1.2% gradient elution buffers. Therefore, in our second attempt, the number of gradient steps was reduced, and the concentrations of sodium chloride were chosen to incorporate both 150 mM and 300 mM NaCl. Overall, the second attempt is as described in chapter 2.

Figure 4.6: Elution profile of lens membrane proteins following a revised isolation protocol adapted from (Saboe et al., 2017). Various concentrations of chloride (sodium chloride) are introduced in a stepwise gradient to compete for binding sites on the HiTrap Q High Performance column. Fractions 1-2: flow through from column loading, Fractions 3-7: 0 mM, Fractions 8-13: 75 mM, Fractions 14-19: 150 mM, Fractions 20-25: 300 mM, Fractions 26-31: $10^3$ mM.
Figure 4.7: SDS-PAGE gel of lens membrane proteins corresponding to aliquots taken from fractions shown in Figure 4.6. The values along the top refer to the concentration of chloride (sodium chloride) in mM added to the buffer to compete for binding on the column. To the left of the central ladder, the loaded sample is 2X the sample loaded to the right of the central ladder. Consistent with the first separation, 16 μL of sample was used to prepare 40 μL of total SDS-PAGE sample work-up. Every 16-μL aliquot was taken from the fraction with the greatest A_{280}. 
We observe essentially no protein in the column loading effluent or in the washing of the column with 0 mM NaCl. There are some faint bands of protein at 75 and 150 mM NaCl. It is with 300 mM NaCl that the greatest intensity bands appear at around the 28 kDa mark. Overall, our second attempt at isolating AQP0 was more successful and it is evident that we require 300 mM NaCl to elute AQP0 from the HiTrap Q column. The SDS-PAGE results are very promising, however, a protein digest of purified AQP0 and analysis of the resulting peptides using LC/MS/MS would confirm or deny the isolation of AQP0.
Figure 4.9: Base peak chromatograms corresponding to the peptides from the excised gel band boxed in red. (a) Scanning the mass-to-charge (m/z) range of 128 – 1000. (b) Scanning the m/z range of 1000 – 2000.
To confirm our SDS-PAGE results, we excised bands from the gel shown in Figure 4.8 and performed an in-gel tryptic digest protocol (Thermo Scientific In-Gel Tryptic Digestion Kit: 89871X). The resulting peptides (band boxed in red) were reconstituted in a total volume of 1 mL and analyzed on the triple quadrupole instrument in the NIU Molecular Analysis Core Facility. There was very little signal change from injecting 10-50 μL of the peptide solution compared to running a null injection (data not shown). SDS-PAGE analysis is typically best with about 20 μg of total protein loaded per well. Assuming 20 μg of protein per gel band and 95% recovery of peptides, the total peptide concentration is estimated to be ~19 μg/mL, therefore, it is possible that the concentrations of individual peptides are below the detection limit of the instrument. In order to test this hypothesis, the peptides from the bands boxed in green (Figure 4.8) were combined, diluted to final volume of 300 μL and a 10 μL aliquot was injected onto the column. The base peak chromatograms (BPCs) are shown in Figure 4.9. While there is some signal change at lower % organic, the two profiles are rather similar. Therefore, the question of whether the issue is due to concentration or perhaps a failed in-gel digest or both remained unanswered.

To answer these questions, 2 mg of standard bovine serum albumin (BSA) were digested using an in-solution tryptic digest protocol. The desalted peptides were reconstituted in 400 μL of water and 1 μL was injected onto the system and the BPC is shown in Figure 4.10. There are intense signals above the instrumental background in these data. The PDA output at 214 nm and 280 nm is overlaid on the BSA peptides BPC in Figure 4.11.a and 4.11.b, respectively. Absorbance at 280 nm is classical for observing tyrosine and tryptophan residues and 214 nm is probably better for monitoring total peptides. The PDA output indicates the presence of peptides
throughout the 5 to ~14-min mark, some of which are not obvious in the base peak chromatogram alone.

Figure 4.10: Base peak chromatograms corresponding to the BSA peptides (red) and null injection (black). The m/z range scanned was 100 - 2000.
Figure 4.11: The PDA output at (a) 214 nm and (b) 280 nm is overlaid on the base peak chromatogram for the BSA peptides.
Averaging mass spectra across the obvious peaks in the BPC, a list of m/z ratios whose ion intensity was about 25% relative to the most abundant ion was generated and these are shown in Figure 4.12. The BSA amino acid sequence (UniProt: P02769) (Bairoch & Apweiler, 1997; Consortium, 2019) was digested using trypsin with the following settings: [1] show m/z from 0 – 2000, [2] minimum peptide length of 1, [3] use the predefined “frequent” modifications to proteins, and [4] report multiple charges (Baker, P.R. and Clauser, K.R., n.d. http://prospector.ucsf.edu). Table 4.1 lists the peptides (including amino acid numbers and sequence) predicted with MS-Digest that could match the data shown in Figure 4.12. To first order, the peptides listed in Table 4.1 correspond to 271 amino acid residues and the P02769 sequence contains 607 amino acid residues which implies a sequence coverage of ~45%. The results shown in Figure 4.12 and Table 4.1 are encouraging, however, they are hardly conclusive in confirming BSA digestion. To be conclusive, the ions need to be selected as precursor ions and fragmented with collision induced dissociation (CID). This will generate so-called b/y ions which will help in confirming amino acid sequence and protein coverage as commonly reported with digestion results. Tandem mass spectrometry of these peptides is in progress.
Figure 4.12: List of precursor ions from the BSA digestion for analysis with tandem mass spectrometry.
Table 4.1: Peptides predicted from MS-Digest using Protein Prospector (Baker, P.R. and Clauser, K.R., n.d. http://prospector.ucsf.edu)

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Discussion

Overall, we have had success in adapting procedures (Gonen et al., 2000; Gonen, Sliz, et al., 2004; Saboe et al., 2017) to isolate and purify bovine lens AQP0. Bovine eyes are relatively inexpensive and readily available making the overall cost of the protein relatively low. However, the non-denaturing detergent octyl glucoside (OG) is used and is expensive. Our data in Figures 4.6 and 4.7 suggest that a single step of ~150 mM NaCl may suffice to wash the HiTrap Q column after column loading thereby reducing the overall amount of OG needed per isolation.

To verify the results of the SDS-PAGE analysis of isolated AQP0, gel bands were excised and tryptic digested to analyze via LC/MS/MS. However, this experiment did not work either as result of low concentration or a failed in-gel digest. Errors in the LCMS-8045 software are not likely because bovine serum albumin (BSA) was tryptic digested using an in-solution protocol and we observe significant peptide signal in the PDA at 280 nm and in the base peak chromatogram. A list of ions of interest with m/z ratios matching those predicted from MS-Digest in Protein Prospector (http://prospector.ucsf.edu) suggest that the digestion was successful and imply sequence of ~45%. However, LC/MS/MS analysis of b/y ions from these peptides is needed to confirm this. Overall, the BSA digest was useful in ruling out software setting(s) as possible issue with the AQP0 digest. Furthermore, the BSA digest could serve as a standard for users of the LCMS-8045. The next step to verify the results of the SDS-PAGE is to tryptic digest AQP0 in solution and analyze the peptides using the BSA procedures.
CHAPTER 5

INVESTIGATING THE CHEMICAL COMPOSITION OF HUMAN RPE MELANOLIPOFUSCIN

Introduction

Thus far, we have discussed in this dissertation work pertaining to cataract, the leading cause of vision loss globally that affects the ocular lens (“World Health Organization,” n.d.). We now shift our focus to the retina and the leading cause of vision loss in developed countries, age-related macular degeneration (AMD) (de Jong, 2006). As discussed in chapter 1 (pages 15-16), late stages of AMD present in two distinct forms: exudative or wet AMD and non-exudative or dry AMD (de Jong, 2006). Several risk factors have been correlated with the onset and progression of AMD such as drusen deposits, chronic inflammation, and neovascularization (Figure 5.1) (Nowak, 1978).

AMD onset and progression are multifactorial (Figure 5.1). As discussed in Chapter 1 (page 16), there is evidence that wet and dry AMD may not be two forms of the same disease but rather two distinct diseases (Chong et al., 2005; Hageman et al., 2001). Despite the debate, a common attribute of the ageing retina is the accumulation of auto fluorescent pigment granules
in the retinal pigment epithelium (RPE) cells; RPE lipofuscin and melanolipofuscin (Feeney-Burns & Eldred, 1983; Feeney-Burns et al., 1984).

Figure 5.1: Risk factors associated with the onset and progression of AMD (Nowak, 1978).
RPE lipofuscin has received considerable attention in the literature. It forms from indigestible rod outer segment (OS) material in RPE lysosomes after phagocytosis (Feeney-Burns & Eldred, 1983) and its composition is heterogeneous (Murdaugh et al., 2010, 2011). RPE lipofuscin could be toxic to RPE cells because studies show that it is photoreactive (Avalle et al., 2005; Davies et al., 2001; Gaillard et al., 1995; Rózanowska et al., 1998; Wassell et al., 1999) and can inhibit the phagocytic ability of RPE cells (Sundelin et al., 1998).

RPE melanolipofuscin has received little attention in the literature. Melanolipofuscin is thought to form by the fusion of lysosomal storage bodies (lipofuscin) with the specialized melanin-containing organelles, melanosomes. This could have deleterious effects on RPE cells as this fusion may inhibit the protective function of melanin. Furthermore, (Dontsov et al., 2017) hypothesizes that melanin content of melanolipofuscin granules is less than melanin content of melanosomes due to direct degradation of the melanin in the core of melanolipofuscin by reactive oxygen species (ROS) generated by the lipofuscin-like periphery.

The proposed mechanism of melanolipofuscin formation is depicted in Figure 5.2 (for more information refer to Chapter 1, pages 23-25). This mechanism coupled with (Dontsov et al., 2017) proposal for melanin degradation in RPE cells provide starting points for investigating the much less understood chemical composition of melanolipofuscin. Our goal is to investigate the composition of organic soluble RPE melanolipofuscin utilizing LC/MS to help shed some light on the mechanism of melanolipofuscin formation and mechanisms of melanin degradation and to identify biomarkers for the early detection of AMD. To aid in mining the data, we have considered current knowledge of the composition of RPE lipofuscin and melanin degradation to be able to identify common species.
Figure 5.2: Diagram depicting the proposed overall relationship between RPE lipofuscin formation and melanolipofuscin formation. RPE cells phagocytose rod outer segments (OS) which are up taken by lysosomes. Incomplete digestion of OS results in lipofuscin formation which continues to accumulate with age. Around age 40, melanolipofuscin granules begin to accumulate as a result of the proposed mechanism where melanosomes fuse with the accruing lipofuscin.
Results

Organic soluble melanolipofuscin (MLF) extracts from three donors in their 70s were pooled and subjected to a Folch extraction as described in Chapter 2. The organic fraction was dried under a stream of Argon gas and reconstituted in 500 μL of starting mobile phase (50:50 water/methanol with 0.1% formic acid). The base peak chromatogram (BPC) from organic soluble melanolipofuscin is shown in Figure 5.3: Figure 5.3.a shows the gradient overlaid on the BPC and Figure 5.3.b shows the absorbance from 300 – 500 nm overlaid on the BPC. It is evident from the BPC that several hydrophobic species exist in the MLF extract which is consistent with observed chemical compositions of human RPE lipofuscin (LF) (Murdaugh et al., 2010, 2011).

Our group is also interested in potential biomarkers for the early diagnosis of AMD and oxidation products that may provide additional information on the mechanisms involved in oxidative stress in the RPE. Some of the molecules, such as 3-nitrotyrosine and levodopa, tend to be less hydrophobic and elute sooner. The BPC shows an intense signal around 0.50 – 1.00 mins which is within the void volume calculated for the column (0.46 mL) and indicates little or no retention. In future studies, a modified gradient that starts with a lower organic composition and is more shallow would be better suited for investigating both major groups of compounds: i.e., the less hydrophobic potential biomarkers and oxidation products and the more hydrophobic, potentially A2E-related components.
Figure 5.3: Base peak chromatogram (BPC) of organic soluble melanolipofuscin from three pairs of eyes pooled from human donors in their 70s (black) and (a) the gradient in terms of percent organic composition in red and (b) the absorbance from 300 – 500 nm in red.
An ion with m/z 592 was detected in the triple quadrupole mass spectrometer. This m/z matches that of A2E, a known component of RPE lipofuscin (Avalle, Wang, Dillon, & Gaillard, 2004; Murdaugh, Avalle, et al., 2010; Sparrow, Parish, Hashimoto, & Nakanishi, 1999; Zhen Wang et al., 2006). Figure 5.4 shows the structure of A2E and depicts commonly observed tandem mass spectrometry (MS/MS) fragments and absorption maximum used to identify A2E (Murdaugh, Avalle, et al., 2010; Murdaugh, Wang, Del Priore, Dillon, & Gaillard, 2010). To determine if m/z 592 is A2E, absorption data and MS/MS data were collected and analyzed. The triple quadrupole mass spectrometer was used to collect absorption data with its inline photodiode array and a Bruker Maxis quadrupole time-of-flight hybrid mass spectrometer was used to collect the MS/MS data.

Figure 5.4: Chemical structure of A2E which has a theoretical m/z of 592.4518. Characteristic absorption maxima and tandem mass spectrometry fragments are also depicted.
Absorption at 440 nm is overlaid on the extracted ion chromatogram for m/z 592 in Figure 5.5. Two peaks are observed for m/z 592 which probably corresponds to A2E and iso-A2E (Parish et al., 1998). Absorption at 440 nm suggest that m/z 592 is A2E.

Figure 5.5: The extracted ion chromatogram for m/z 592 (black) shown with the absorbance at 440 nm (red). The occurrence of two peaks is suggestive of A2E and iso-A2E as observed by (Parish et al., 1998).
Fragmentation data for m/z 592 was collected with 60.0 eV collision energy with the high-resolution hybrid mass spectrometer. The MS/MS data is displayed in Figure 5.6. The parent ion, m/z 592, is most abundant. However, fragments with m/z of 376, 404, 418, 442, and 468 were detected which correspond well to commonly observed fragments for A2E as illustrated in Figure 5.4. Overall, the absorbance data and MS/MS data support that m/z 592 is A2E. The occurrence of a double peak in the extracted ion chromatogram and matching absorption maximum around 440 nm suggest that A2E and iso-A2E are present in the melanolipofuscin extract.

Figure 5.6: Fragmentation data from the precursor ion m/z 592 from the melanolipofuscin extract. Data was collected at 60.0 eV on the ESI-Q-TOF hybrid mass spectrometer.
Figure 5.7: 3D representation of the absorption from the melanolipofuscin extract at retention time 0 – 10 min (x-axis) and wavelength 300 – 700 nm (y-axis). The arbitrary absorption units are plotted along the z-axis. The color scheme runs from low (purple: -4 mAU) to high (red: 15 mAU) as shown in the color bar. (a) The absorption maximum around 440 nm and retention time ~9.5 min corresponding to A2E and iso-A2E. (b) An ion with m/z 646 has an absorption maximum around 330 nm. (c) The most abundant ion in the extract with m/z 618 exhibits absorption in the 300 – 350 nm range.
Figure 5.7 displays the absorption data represented in a 3D plot with retention time (0 – 10 min) and wavelength (300 – 700 nm) on the x, y plane and absorption intensity (mAU) on the z-axis. Figure 5.7.a points out the absorption of A2E and iso-A2E with a maximum at ~440 nm and retention time of ~9.5 min. An additional ion of interest (Figure 5.7.b) with m/z 646 absorbed with maxima ~330 nm. One very abundant ion with m/z 618 absorbed ~330 nm as depicted by Figure 5.7.c. Extracted ion chromatograms for the ions corresponding to Figure 5.7.b and 5.7.c are shown in Figure 5.8 with the absorbance at 330 nm (red) overlaid.

Figure 5.8: Absorbance at 330 nm (red) overlaid on the extracted ion chromatograms for m/z 618 and m/z 646.
Figure 5.9: (a) Structure of A2E with cleavage sites indicated and masses for the resulting reactive aldehydes, (b) structure of oxidized A2E (m/z 608) with cleavage sites indicated for the resulting reactive aldehydes, and (c) the structure for the reactive aldehyde with m/z 488.
A2E is known to undergo autooxidization to form products such as furano-A2E, bisfurano-A2E, and peroxy-A2E (Dillon, Wang, Avalle, & Gaillard, 2004; Kim, Jockusch, Itagaki, Turro, & Sparrow, 2008; Murdaugh et al., 2010; Thao, Renfus, Dillon, & Gaillard, 2014a; Zhen Wang et al., 2006). The structures for A2E and furano-A2E are shown in Figure 5.9a and b, respectively. (Zhen Wang et al., 2006) observed a series of reactive aldehydes and ketones derived from oxidation and photoxidation of synthesized A2E. Cleavage sites along the polyene chains of A2E and oxidized A2E corresonding to the aldehydes (Zhen Wang et al., 2006) are shown in Figure 5.9a and b. To better understand the role of A2E in protein modifications in Bruch’s Membrane (BM), (Thao, Renfus, Dillon, & Gaillard, 2014b) reacted a fragment of fibronectin with A2E under oxidative conditions and were able to identify sites of A2E-derived aldehyde modifications to lysine and arginine residues using LC/ESI/MS. Therefore, it is possible that our observed ions with m/z 618 and 646 may be the result of A2E-derived aldehydes reacting with amino acids, namely lysine and arginine. Two proposed structures involving the A2E-derived aldehyde with m/z 488 (Figure 5.8.C) are discussed here.
Figure 5.10: General proposed reaction scheme for the reaction of A2E-derived aldehydes with
(a) lysine and (b) arginine.
The ion with m/z 618 could possibly be accounted for by the reaction of the A2E-derived aldehyde m/z 488 (Figure 5.9.c) with lysine according to the proposed scheme shown in Figure 5.10.a. The MS and MS/MS data for m/z 618 are shown in Figure 5.11 a and b, respectively. The proposed structure for m/z 618 is shown in Figure 5.12. Also shown in Figure 5.12 are cleavages that may account for the fragment ions observed. In some cases, multiple cleavages could account for observed fragments and are therefore color coded accordingly.

The ion with m/z 646 could possibly be accounted for by the reaction of the A2E-derived aldehyde m/z 488 (Figure 5.9.c) with arginine according to the proposed scheme shown in Figure 5.10.b. The MS and MS/MS data for m/z 646 are shown in Figures 5.13 a and b, respectively. The proposed structure for m/z 646 is shown in Figure 5.14. Also shown in Figure 5.14 are cleavages that may account for the fragment ions observed. In some cases, multiple cleavages could account for observed fragments and are therefore color coded accordingly.

It is important to remember that lipofuscin is essentially indigestible material from rod outer segments that is sequestered in RPE lysosomes, so-called lysosomal storage bodies (Feeney-Burns, Berman, & Rothman, 1980; Feeney-Burns & Eldred, 1983). The primary function of lysosomes is to degrade macromolecules such as proteins and resulting amino acids are transported out (Pisoni & Schneider, 1992). Therefore, it is reasonable to conclude that A2E or any of its oxidation products may react with amino acids to produce structures like the ones proposed here. Furthermore, these reactions may well be carried out in RPE lipofuscin, therefore, our observation of them in organic soluble extracts from RPE melanolipofuscin support the hypothesis that RPE melanolipofuscin is a complex granule formed by the fusion of RPE lipofuscin with RPE melanosomes.
Figure 5.11: (a) MS data for m/z 618 and (b) MS/MS data for m/z 618 collected at 40.0 eV.
Figure 5.12: Proposed structure for observed ion with m/z 618 generated by reaction of an oxidized A2E aldehyde (m/z 488) with lysine. Proposed cleavage sites are indicated for fragment ions. In some cases, multiple cleavages may result in an observed fragment ion, therefore, the cleavages are color coded accordingly.
Figure 5.13: (a) MS data for m/z 646 and (b) MS/MS data for m/z 646 collected at 55.0 eV.
Figure 5.14: Proposed structure for observed ion with m/z 646 generated by reaction of an oxidized A2E aldehyde (m/z 488) with arginine. Proposed cleavage sites are indicated for fragment ions. In some cases, multiple cleavages may result in an observed fragment ion, therefore, the cleavages are color coded accordingly.
Discussion

Organic soluble extract from RPE melanolipofuscin has been investigated with LC/MS. The base peak chromatogram indicates that, like RPE lipofuscin, RPE melanolipofuscin is a complex mixture. Elution of several ions at higher organic concentration in the mobile phase has been observed by our group in lipofuscin studies of which some of those ions were determined to be A2E-related (Murdaugh et al., 2010, 2011). At higher organic concentration, we observed A2E in the melanolipofuscin extract by detection of m/z 592 and confirmed its presence by absorption at 440 nm and analyzing MS/MS data.

Two abundant ions with m/z 618 and 646 were detected and investigated further with tandem mass spectrometry. Analysis of MS/MS data suggests that m/z 618 and m/z 646 could potentially be the result of the reaction of an A2E-derived aldehyde with lysine and arginine, respectively. These reactions are possible because lipofuscin, and hence A2E, is sequestered in RPE lysosomes where digestible proteins are degraded to amino acids. Therefore, these results may provide support for the hypothesis that fusion of RPE lipofuscin with RPE melanosomes produces the complex granule, RPE melanolipofuscin.

Follow up studies are needed to confirm these results. A2E will be synthesized following the protocol from (Parish et al., 1998) and oxidized according to work by (Zhen Wang et al., 2006). The resulting oxidation products will be reacted with lysine and arginine and analyzed using the methods described here. Further studies on additional organic extracts from RPE melanolipofuscin are needed as well.
CHAPTER 6

CONCLUSIONS AND FUTURE WORK

The ocular lens and retina undergo biochemical and biophysical changes with age which can ultimately lead to loss of vision. In this dissertation work, two-component biomimetic membranes were investigated with X-rays to understand the overall organization of the bilayer components which provides necessary background information for understanding membrane protein/bilayer complexes. We describe our method development for the isolation of lens-specific aquaporin-0 (AQP0) from bovine lens; protein isolated via these protocols will be used to prepare AQP0/bilayer complexes to study the overall organization with X-ray scattering techniques. Ageing retinal pigment epithelial (RPE) cells accumulate autofluorescent pigment granules which are implicated in the pathogenesis of the retina. In this dissertation work, we investigated the chemical composition of the complex granule, melanolipofuscin, to better understand its possible relation to RPE lipofuscin, possible mechanisms of melanin degradation, and to identify biomarkers for the early detection of age-related macular degeneration (AMD).

The ocular lens is a key optical component of the eye which focuses light onto the retina. Therefore, it is crucial that it maintains transparency and elasticity throughout life which requires proper water homeostasis. The transmembrane family of proteins, the aquaporins, are believed to
maintain water homeostasis throughout the body. Lens-specific AQP0 accounts for more than 50% of the fraction of membrane proteins by mass in the ocular lens (Alcala et al., 1975) and is presumably specialized to the needs of the lens. Several novel mutations in AQP0 have been identified in four generations of a family with congenital cataract which provides strong evidence for AQP0 role in cataractogenesis (Shentu et al., 2015; Song et al., 2015). Studies have indicated that aquaporin water permeability is dependent on its lipid bilayer environment (Tong et al., 2013; Tong, Wu, Briggs, Schulten, & McIntosh, 2016) but much of this work is computational. The available experimental knowledge of AQP0 structure and function, like many membrane proteins, is limited.

Therefore, we are developing methods to probe AQP0/bilayer complex structure using X-ray scattering techniques. To that end, we have investigated the overall organization of cholesterol-containing biomimetic supported lipid bilayers of dipalmitoylphosphatidylcholine (DPPC) using x-ray reflectivity (XRR). This biomimetic system is attractive because [1] of the availability of literature on DPPC and DPPC/Cholesterol bilayers from computational work (Feller, Venable, & Pastor, 1997; Smondyrev & Berkowitz, 1999; Y. Wang et al., 2016; Zhang et al., 2016; Zhang, Lervik, Seddon, & Bresme, 2015) and experimental work (Belička, Gerelli, Kučerka, & Fragneto, 2015; Chiang, Costa-Filho, & Freed, 2007; Davis et al., 2009; Gumí-Audenis et al., 2018; Huang, Lee, Das Gupta, Blume, & Griffin, 1993; Shaghaghi, Keyvanloo, Huang, Szoka, & Thewalt, 2017) using techniques such as nuclear magnetic resonance (NMR), differential scanning calorimetry (DSC), and x-ray diffraction (XRD), [2] lens fiber cell membranes are highly saturated (L. K. Li et al., 1985; Yappert et al., 2003) and DPPC is fully
saturated, and [3] the highest cholesterol to phospholipid mole fractions are found in the ocular lens (L.-K. Li et al., 1987; L. K. Li et al., 1985; Yappert et al., 2003).

Our x-ray reflectivity results from the biomimetic membranes show general trends that are consistent with research on DPPC/Cholesterol bilayers mentioned above. Cholesterol has a well-known condensation effect on phospholipids (Hung, Lee, Chen, & Huang, 2007; Marquardt, Kučerka, et al., 2016) meaning the phospholipids occupy less molecular area in the presence of cholesterol. Molecular area was a fitting parameter in this work, and it tended to decrease with increasing mol% cholesterol. Overall bilayer thickness tended to increase with mol% cholesterol. With mol% cholesterol ≤ 20%, the molecular area, bilayer thickness, molecular volume, and cholesterol location in the bilayer tended to exhibit greater differences at T ≥ 40°C compared to T ≤ 35°C which suggests that the biomimetic bilayers exhibited distinct phase behavior changes with temperature at those mol% cholesterol. This is consistent with phase diagrams for DPPC/Cholesterol mixtures determined from NMR and DSC studies (Vist & Davis, 1990). The reduced amount of variation with mol% cholesterol ≥ 33% may suggest more uniform phase behavior which is also consistent with phase diagrams for DPPC/Cholesterol. Membranes have a characteristic thermotropic phase transition (T_m) above which the hydrocarbon chains melt (Deleu, 2010), and the bilayer thins. Cholesterol has been shown to reside more towards the bilayer center of thinner membranes (Marquardt, Heberle, et al., 2016) and in our fits, cholesterol tended to move towards the bilayer center with increasing temperature. The fits to the 40 mol% data resulted in parameters that seemed to deviate from the general trends which may indicate the formation of cholesterol-rich bilayer domains as has been postulated for lens fiber cell membranes (Widomska et al., 2017).
Overall, the general trends in the fitting parameters seem consistent with expected phase behavior of these supported biomimetic membranes. It is important to note that limitations due to beam size and the number of samples, these results are based on fits to single measurements made at the Advanced Photon Source (APS) (Argonne National Laboratory, Lemont, IL). There are two courses of action that can help to verify these results. The first is to fit these data with another program. These fits were generated with fitting code written by our collaborators. Using a commercially available fitting program can help to verify these results. The second is to measure additional samples under the same conditions if we can obtain additional beam time at the APS.

Our XRR results have provided important information on the organization of our biomimetic membranes which is critical to understand AQP0/bilayer complex organization. One of the issues in working with a membrane protein is that they are notoriously difficult to isolate while maintaining its functional structure. Walz research group has published papers (Gonen, Cheng, et al., 2004; Gonen et al., 2000; Saboe et al., 2017) detailing their procedures and we describe in this dissertation our efforts in adapting those procedures in our lab. SDS-PAGE results indicate that we have fairly pure AQP0 (~28 kDa) as indicated by the presence of protein bands around 28 kDa relative to the molecular weight ladder. Our attempt at digestion of AQP0 via an in-gel tryptic digestion protocol and subsequent verification of the SDS-PAGE results by analyzing the peptides with LC/MS was inconclusive, probably due to peptide concentrations below the detection limit of the mass spectrometer.

The next steps in this dissertation work is to prepare an in-solution digest of AQP0 and analyze the resulting peptides. This will help us to verify the results of SDS-PAGE. Lastly,
AQP0 isolated via these protocols will be used to prepare AQP0/bilayer complexes. We will study the overall organization and structure of these complexes with X-ray and light scattering techniques and microscopy such as atomic force microscopy.

Similar to how biophysical and biochemical changes in the lens can lead to development of cataract, the most prevalent loss of vision globally, biophysical and biochemical changes can lead to the most prevalent loss of vision in developed countries, AMD. An attribute of the ageing retina is the accumulation of autofluorescent pigment granules, termed lipofuscin and melanolipofuscin, in the retinal pigment epithelium (RPE) (L Feeney-Burns & Eldred, 1983; L Feeney-Burns et al., 1984). RPE lipofuscin has received considerable attention in the literature and results from the build-up of undigestible material that is shed from rod outer segments (OS) (Boulton et al., 1989; L Feeney-Burns & Eldred, 1983; Feeney, 1978). Retinal lipofuscin [1] has been shown to produce reactive species that can damage cellular components (Avalle et al., 2005; Davies et al., 2001; Gaillard et al., 1995; Różanowska et al., 1998; Wassell et al., 1999) and [2] inhibit the phagocytic ability of RPE cells (Sundelin et al., 1998). Much less is known of RPE melanolipofuscin which is proposed to form by fusion of lysosomal storage bodies with the specialized RPE melanosomes. Furthermore, it is proposed that reactive species generated by the lipofuscin- like portion of melanolipofuscin directly degrade RPE melanin (Dontsov et al., 2017). This could have deleterious effects on the RPE and ultimately the retinal tissue.

In this dissertation, we have investigated the chemical composition of RPE melanolipofuscin to better understand its relationship to RPE lipofuscin, its possible role in melanin degradation, and to identify biomarkers for the early detection of AMD. RPE melanolipofuscin is isolated from human donor globes and subjected to a Folch extraction. The
organic soluble melanolipofuscin is collected and analyzed via LC/MS. A known fluorophore of the ageing retina, A2E, was detected and confirmed by monitoring absorption at 440 nm and analyzing MS/MS fragmentation data.

Additionally, two ions at m/z 618 and 646 were detected with m/z 618 producing the greatest signal from the melanolipofuscin extract. Absorption data showed that these ions absorb around 330 nm. Fragmentation data was analyzed and suggests that m/z 618 may be the result of an A2E-derived aldehyde reacting with lysine and m/z 646 may be the same reaction with arginine. These results need to be verified by analyzing additional RPE melanolipofuscin organic-soluble extracts. Furthermore, A2E will be synthesized and oxidized. The resulting oxidation products will be reacted with lysine and arginine and analyzed with our methods.
REFERENCES


