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Structure of Unmodified and Pyroglutamylated Amyloid Beta Peptide in Lipid Membranes

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STRUCTURE OF UNMODIFIED AND PYROGLUTAMYLATED AMYLOID BETA PEPTIDE IN LIPID MEMBRANES

by

ROWAN HASSAN

A thesis submitted in partial fulfillment of the requirements for the Honors Undergraduate Thesis Program in Physics in the College of Sciences and in the Burnett Honors College at the University of Central Florida Orlando, Florida

Summer Term, 2021

Thesis Chair: Suren Tatulian, Ph.D.
Abstract

Alzheimer’s Disease (AD) is a devastating neurodegenerative disease that is characterized by brain atrophy, neuronal and synaptic loss, cognitive decline, trouble handling activities of daily life, and ultimately leads to death. Worldwide, at least 30 million people suffer from AD, with 5.8 million suffering in the US alone. Despite extensive basic and clinical research, the underlying molecular mechanisms behind AD remain largely unknown. There are four FDA-approved compounds are used for alleviating symptoms but have no curative potency. The first potentially disease-modifying AD drug, aducanumb, was approved by FDA in June 2021. The main histopathological traits of AD are the Amyloid-beta (Aβ) peptide and the tau protein. Aβ aggregates to form extracellular plaques in brain parenchyma and vasculature while tau forms intraneuronal tangles. Aβ is produced by enzymatic cleavage of the amyloid precursor protein (APP) in the brain. Once APP cleavage occurs, Ab monomers either aggregate extracellularly to form buildups of sticky plaque or embed themselves within the neuronal cell membrane to form pores, causing homeostatic dysregulation and eventually cell death. The mechanism of membrane pores formed by Ab and the pore structure remain to be characterized. This study aims to analyze the structure of four Aβ species in lipid membranes. These are the most abundant form of Aβ, Aβ1-40, and the more cytotoxic form, Aβ1-42, as well as their pyroglutamylated counterparts, pEAβ3-40 and pEAβ3-42, which are hypertoxic. These peptides have been studied using biophysical approaches, i.e., circular dichroism, fluorescence spectroscopy, and Fourier transform infrared spectroscopy. Elucidation of the structure of Aβ membrane pores provides valuable insight into the mechanism of Aβ toxicity and may help develop novel therapies for the lethal mystery that is AD.
Dedications

For those who need proof that they are capable

of achieving what they set out to do.
Acknowledgments

I want to firstly thank Dr. Tatulian for his generosity in providing me access to his lab and providing me guidance as I find my way in research. Thank you to Dr. Hawthorne for being an awesome professor and for advising me to gain experience in the laboratory. I want to also thank Faisal Abedin for his patience in supporting me in the lab, giving me company, and explaining every detail. Also, a big thank you to LEARN for guiding me in my research journey since the beginning. To my family and friends, thank you for seeing me as stronger than I see myself and encouraging me when I need it most. I would not be where I am without you all.
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Chapter 1

INTRODUCTION

A brief overview of AD pathophysiology

Alzheimer’s Disease (AD) is a neurodegenerative disease characterized by neuronal and synaptic loss, cognitive impairment, trouble handling daily life activities, and ultimately leads to death (Lope et al., 2019; NIA, 2017). Worldwide, at least 30 million people suffer from AD, with 5.8 million people suffering in the US alone, making up 60-70% of all global dementia cases (Services, 2020; Silva et al., 2019). Considering these staggering numbers, it is unsurprising that there is great scientific interest and public concern around explaining and treating this life-altering condition. Despite its prevalence, AD pathology is little understood due to many factors, one being the elusive nature of the early stages, which makes diagnosis difficult before significant brain damage has already occurred. Over the years, there has been a considerable lack of success in treating AD in part due to an incomplete understanding of the underlying molecular mechanisms and failed drug trials.

A key histopathological characteristic of neurodegenerative diseases is a buildup of abnormally folded proteins. For AD, this includes the accumulation of Amyloid-beta (Aβ) peptide in the brain in the form of insoluble extracellular plaques and more toxic soluble oligomers (see below) (Benilova, 2012; Selkoe & Hardy, 2016; Amar et al., 2018). Aβ deposits are most commonly found in the hippocampus, which is the area of the brain responsible for memory and cognition (Selkoe et al., 2016; Jack et al., 2016). Synaptic loss is one of the strongest and most identifiable symptoms of AD and is thought to be in part caused by Aβ-
induced pore formation and tau hyperphosphorylation (see below), as has been observed in many studies.

One of the most popular theories of AD etiology is the “amyloid cascade hypothesis”, yet it remains controversial and is still under active debate in the AD research community. The amyloid cascade hypothesis suggests that Aβ peptide initiates a complex pathogenic cascade (yet to be fully understood) that causes AD, such as the accumulation of hyperphosphorylated tau protein, another important player in AD pathology (see below). Aβ monomers build fibrils that lead to insoluble senile plaques. The plaques can then sequester soluble Aβ oligomers until reaching a physical capacity, and afterwards excess oligomers are able to diffuse onto surrounding hydrophobic cell surfaces, especially synaptic membranes, and form pores that lead to neurotoxic effects. (Selkoe & Hardy, 2016) The main argument against the amyloid cascade hypothesis is that it does not provide a complete picture due to nuances in a rarer “familial” subtype of AD, and that plaques are seen in non-AD patients (Selkoe & Hardy, 2016). However, clinically normal (non-AD) patients have a much lower oligomer to plaque ratio than subjects with mild or advanced AD. This denotes that oligomers are more toxic than plaques, and are thus an important area to focus research efforts.

Upon exposure to the brain’s naturally aqueous environment, Aβ peptides become rich in cross β-sheet structures, which allow them to closely agglomerate or form neurotoxic Ca^{2+} permeable pores within neuronal membranes. Specifically, Aβ pores take on a β-barrel conformation as modeled by computer generated images (see Figure 1.2). However, these are only models and have not yet been experimentally confirmed. This thesis attempts to collect
experimental evidence for Aβ pore structure. By observing Aβ’s transition between α-helical and β-sheet structure over exposure to aqueous solution, the structural basis of membrane pore formation and its subsequent toxic effect will be revealed.

**Figure 1.1:** Structures (left) and Aβ_{1-42} (right) monomers determined by solid-state NMR. For Aβ_{1-40}, the N-terminal 8 amino acid residues are not resolved. The backbone is colored blue for beta sheet regions and rose for turn/loop regions. The structure in membranes is not known. (Tyco, 2011; Wälti et al., 2016)

**Spectroscopy**

This study aims to observe the structure of the most prevalent and toxic forms of Aβ in lipid membranes. One of the many ways that protein structure is studied is with spectroscopic techniques. Spectroscopy is used to study the interaction of matter with the electromagnetic field. The structure of the matter determines the interaction it has with electromagnetic radiation, so spectroscopy is used for structural characterization. By measuring the protein’s absorption of electromagnetic radiation, its structure can be characterized. There are many different types of spectroscopic techniques used in the study of protein structure, including vibrational spectroscopy, such as Fourier-transform infrared (FTIR) spectroscopy, which measures the absorption of light at frequencies corresponding to vibrations of chemical bonds, and electronic spectroscopy, such as circular dichroism (CD) and fluorescence, which are based on absorption
and emission of light due to transitions of electrons between energy levels. Light scattering is yet another optical spectroscopy method that evaluates the relative particle size based on emission of light at frequencies of incident radiation. This study uses circular dichroism (CD), fluorescence, FTIR, and light scattering for analysis of Aβ structure in membranes. Further details of each spectroscopic technique will be discussed in Chapter 3.

Figure 1.2: ATR-FTIR spectra of Aβ1-42 in membranes at perpendicular polarization of incident light in dry state (blue) and 4 hours after injection of buffer (red), with assignments of main absorbance bands.
Chapter 2

BACKGROUND/LITERATURE REVIEW

What is Amyloid Beta?

Monomeric Aβ (~4.5 kDa) is a 37-49 amino acid residue peptide derived from the enzymatic cleavage of amyloid precursor protein (APP) (~120 kDa) by proteolytic enzymes β- and γ- secretase (Benilova, 2012; Selkoe & Hardy, 2016; Chow et al., 2010; Agostinho et al., 2015; Chen, 2017). Little is known about APP’s biological function, but researchers posit that it may help with migration of neurons during early development, synaptic formation and repair, intracellular transport, signaling, and other aspects of neuronal homeostasis (MedlinePlus, 2020; Kamboh, 2018). Research is still needed to fully understand APP’s biological role.

AD genetic risk factors are linked to the genes PSEN1 and PSEN2 that encode for the catalytic domain of γ-secretase (Benilova, 2012; Selkoe & Hardy, 2016), as well as the APP gene; mutations in these genes lead to early onset AD that constitutes roughly 5% of all AD cases (Chen, 2017). Another important genetic risk factor is the apolipoprotein E gene, APOE, which is a susceptibility gene for the most common (~95%), late onset AD. APOE occurs in three allelic variants: ε2, ε3, and ε4, of which the ε4 form increases the AD risk up to 15-fold (Chen et al., 2021).
**Forms of Aβ**

Once APP cleavage is complete, Aβ monomers are free to interact with one another and agglomerate into extracellular plaques or into metastable (spreadable) oligomers (Benilova, 2012; Selkoe & Hardy, 2016). The most common forms of Aβ are the 40- and 42-residue species (Aβ1-40 and Aβ1-42), but many shorter forms exist as well due to cleavage by amino- and/or carboxy-peptidases, each with their unique solubility and stability (Benilova, 2012; Beel & Sanders, 2008; O’Brien & Wong, 2011). Depending on the solubility of Aβ monomer in the brain’s naturally aqueous environment, it will either agglomerate into protofibrils which later turn into extracellular plaques or metastable (spreadable) oligomers (Benilova, 2012; Selkoe & Hardy, 2016). Insoluble monomers agglomerate into protofibrils and form plaques. Plaque cores from post-mortem AD brains were shown to be comprised of insoluble forms of Aβ. Soluble monomers of Aβ tend to oligomerize to form dimers, trimers, and various other oligomers and are thought to be more toxic than the plaques and may embed themselves into neuronal membranes to form pores (Amar et al., 2018).

Additionally, more cytotoxic versions of Aβ have been found in AD brains. These N-terminally truncated and pyroglutamylated (at Glu3 or Glu11) forms of Aβ (pEAβ) make up between 10-50% of total Aβ in brains of AD subjects (Mandler et al., 2014). pEAβ is more toxic due to its higher tendency to form β-sheets and is more efficient in forming detrimental pores that disrupt ion flow and consequently damage neuronal function (Dammers et al., 2015; Arispe et al., 1993; Shirwany et al., 2007; Prangkio et al., 2012).
Figure 2.1: 42-residue Aβ (top) vs. pEAβ (bottom). Notice the pEAβ is shorter due to being N-terminally truncated.

Reference: (Wolf, 2012)

_Aβ oligomers and cytotoxicity_

The majority of experimentation on Aβ oligomers is done in vitro, using oversimplified conditions such as an aqueous buffer with or without added organic solvents. This makes it easier to see the characteristics of the peptide but could potentially not explain its biological behavior. Most of the data available on Aβ’s structure is from synthetic Aβ, and it is unclear whether these accurately resemble natural Aβ found in AD brain (Selkoe & Hardy, 2016) In vivo experimentation in human subjects involves biomarkers and brain imaging, and can often be used in presymptomatic patients to begin early treatment. A goal for future AD research is to quantify all Aβ peptides in plasma of presymptomatic and symptomatic AD patients (Selkoe & Hardy, 2016). There is a lack of common agreed upon experimental description of toxic Aβ oligomer that makes interpreting and comparing data from different research groups difficult. Furthermore, no single mechanism explains all aspects of Aβ oligomer toxicity. Nonetheless, it is clear that Aβ oligomers produce a cytotoxic effect, even if the exact mechanism remains unknown.
Not all oligomeric Aβ species affect the same neuronal signaling pathway. For example, Aβ_{56}, a 56-kDa oligomer, was able to selectively activate CaMKIIα receptors in Tg2576 transgenic mice while other purified Aβ oligomer species could not (Amar et al., 2018). Contrary to early belief, it was found that the oligomeric form of Aβ is far more toxic than the fibrillar plaques, as they were shown to impair synaptic function, structure, and number in healthy adult rats (Selkoe & Hardy, 2016). Plaque cores, in contrast, did not affect long-term potentiation (LTP) and dendritic spine structure in the rats, but oligomers did, further providing evidence for oligomer’s higher toxicity (Selkoe & Hardy, 2016). Additionally, soluble oligomers of Aβ_{1-42} isolated from post-mortem AD brains were found to decrease synapse number, inhibit LTP, and enhance long-term synaptic depression in rodent hippocampus (Selkoe & Hardy, 2016). Synaptic loss is one of the strongest quantitative correlates of dementia in AD, so these findings point to oligomers having a large role in AD pathophysiology.

**Tau protein**

The relationship between Aβ and tau is still unresolved, but many lines of evidence suggest they are linked. Aβ is believed to initiate tau hyperphosphorylation (the addition of phosphate groups to a molecule), causing neuritic dystrophy (Benilova, 2012; Amar et al., 2018; Barthelemy et al., 2020; Sato et al., 2018). Microtubule-associated protein tau functions normally to stabilize neuronal microtubules. Tau is normally phosphorylated a post-translational modification, which regulates its normal function in axonal stabilization (Barthelemy et al, 2020). However, hyperphosphorylated tau protein agglomerates and forms intracellular neurofibrillary tangles (NFTs), which could trap vital functional proteins and damage neurons’
axonal transport (Benilova, 2012; Amar et al., 2018; Barthelemy et al., 2020; Sato et al., 2018). There are still many gaps in our understanding of this mechanism.

Distinct oligomeric species of Aβ exert different effects on neuronal signaling and tau biology. For example, tau is hyperphosphorylated with low-n oligomers at different sites than high-n oligomers, such as Aβ₅₆ (Amar, 2018). A study conducted in 2014 found that pyroglutamylated forms of Aβ might initiate tau-dependent cytotoxicity as was seen in post-mortem brains (Mandler et al. 2014).

**Aβ-induced pore formation**

Aβ oligomers tend to insert into neuronal cell membranes and form pores that act as ion channels, which disrupts cellular ionic homeostasis (Dammers et al., 2015; Peters et al., 2016). Disruption of ionic homeostasis is especially devastating for neurons because they rely on a sodium/potassium gradient in order to form action potential. If this gradient is disrupted, then cellular function will greatly reduce or cease altogether. Oligomers were specifically found to physically interact with the membrane lipids cholesterol and ganglioside, both of which are important in maintaining membrane structure around ion channels (Coralie; 2016). Aβ oligomers were shown to form calcium-permeable pores in rat hippocampal neurons, which disrupt synaptic transmission and are thought to be primary contributors to both AD and Parkinson’s disease (Peters et al., 2016). CD and fluorescence spectroscopy revealed that Aβ₂₅-₃₅ (a cytotoxic Aβ fragment) formed the most efficient pores in lipid membranes when in β-sheet conformation (Kandel et al., 2017).
**Current therapies**

Until recently, there have been no disease-modifying drugs available for AD despite rigorous research, in part due to an incomplete understanding of AD’s molecular mechanism, which leads to a lack of drug targets on which to base therapies. Four FDA-approved drugs (donepezil, rivastigmine, galantamine, and memantine) are only remedies for symptoms and have no curative potency. A monoclonal antibody against Aβ aggregates, aducanumab, was recently approved by FDA based on its ability to clear Aβ from brain and retard cognitive decline (see below). In addition to immunotherapy approaches, pharmacological efforts have been directed to suppress Aβ accumulation in the brain by inhibition of β- and γ-secretase, but these endeavors have not been successful thus far mostly because of severe adverse events such as cerebral edema, microhemorrhage, or cancer (Benilova, 2012; Selkoe & Hardy, 2016; Elmaleh, 2019; Alzheimer’s Association, 2019). “Membrane therapies” are being explored to see if alteration of lipid membrane structure can inhibit amyloid pore formation (Coralie, 2016).

As of June 7, 2021, Aducanumab, sponsored by the biotech company Biogen, was approved by the FDA and has been shown to reduce amyloid plaques in clinical studies (Cummings et al., 2021). Aducanumab is a monoclonal antibody that targets aggregated Aβ. Studies have shown that the drug has a high affinity for the neurotoxic oligomeric species of Aβ. Specifically, it binds a linear epitope formed by amino acids 3-7 of the Aβ peptide (Arndt et al., 2018). Amyloid plaques were markedly decreased by the drug, as observed by positron emission tomography (PET). Phosphorylated tau in the cerebrospinal fluid and medial temporal NFTs were also reduced in some patients, which points back to the amyloid hypothesis (Cummings et al., 2021). The Neuropsychiatric Inventory showed 87% reduction in EMERGE scores (a phase 3
study examining cognition, function, and behavior of AD patients) (Haeberlein, 2020). However, the drug is under active debate in the community. Skeptics note that previous clinical trials of Aβ-targeting drugs have been negative, and much criticism is being directed to the FDA for working too closely with Biogen.

Research question

This project will study the structure of the most abundant and toxic forms of Aβ, i.e., Aβ_{1-42}, Aβ_{1-40}, and the hypertoxic pyroglutamylated species pEAβ_{3-42} and pEAβ_{3-40} via multiple spectroscopic techniques. These include CD, fluorescence, light scattering, FTIR, and Attenuated total reflection-FTIR (ATR-FTIR) spectroscopic techniques.

Little is known about Aβ’s pore-forming capabilities. More can be known about the toxic alteration of lipid membranes in AD by analyzing Aβ’s structure in lipid membranes. Since ion-conducting pores formed in neuronal membranes by Aβ contribute to neurotoxicity, this data will reveal the structural basis of membrane pore formation and its subsequent toxic effect.

Comparison of the structure of Aβ peptides in aqueous buffer and when reconstituted in lipid membranes will identify the structural transition of the peptide upon insertion into cellular membranes, such as the plasma, endoplasmic reticulum, or mitochondrial membranes of neurons. It is anticipated that membrane insertion promotes β-sheet structure formation by Aβ peptides, which will imply β-barrel-like pore formation in lipid membranes. This effect is expected to be more pronounced for the hypertoxic peptide species, i.e., pEAβ_{3-42} and pEAβ_{3-40}. In addition, β-barrel pore formation is expected to be facilitated by physiological temperature of 37⁰C as compared to the “room temperature” of 20⁰C.
**Hypothesis:** If Aβ shows that 1. it has β-barrel characteristics in exposure to aqueous environment and 2. embeds itself into lipid membranes, then it is likely that Aβ induces membrane pore formation, leading to neurotoxic effects in AD.

Overall, the collected data will highlight the conformational changes that Aβ peptides undergo in cell membranes, leading to a more thorough understanding of Aβ's role in pore formation and the peptide’s mechanism of neurotoxic action in Alzheimer’s Disease. The uncovered structure of the Aβ-lipid complex may also be important to design new therapeutic techniques and can supplement further experimentation.
Chapter 3

MATERIALS AND METHODS

I. Materials

The peptides, Aβ$_{1-42}$ and Aβ$_{1-40}$ were purchased from rPeptide. pEAβ$_{3-42}$ and pEAβ$_{3-40}$ were purchased from Innovagen (Sweden). The lipids 1-palmitoyl-2-oleyl-sn-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleyl-sn-glycero-3-phosphoglycerol (POPG), and cholesterol were purchased from Avanti Polar Lipids, Inc. (Albaster, AL). The polycarbonate filters used for extrusion were purchased from Avestin, Inc. (Ottawa, Ontario, Canada). The Germanium plate used in ATR-FTIR technique was purchased from Spectral Systems (Irvington, NY). Organic solvents (chloroform, hexafluoroisopropanol (HFIP), methanol), salts, buffers and other chemicals were purchased from Sigma-Aldrich or Fisher Scientific.

![Chemical structures](image)

**Figure 3.1:** The chemical structures of cholesterol (left), POPC (top right), and POPG (bottom right). Reference: Avanti Polar Lipids

II. Methods

Synthetically derived Aβ peptide (of 4 various species previously mentioned) were implanted into lipid membranes consisting of 60% POPC, 30% POPG, and 10% cholesterol. 25 mM NaCl plus 25 mM NaCl plus 25mM Na,K-phosphate buffer at pH 7.2 were used as the
solvent during spectroscopic measurement. In FTIR experiments, D\textsubscript{2}O was used because H\textsubscript{2}O absorbs in the amide I spectral region where the peptide’s structure is analyzed. CD, fluorescence and light scattering measurements was taken at both 20°C and 37°C (room and body temperature, respectively). Fluorescence measurements was taken with excitation at 220 nm and 275 nm. FTIR experiments were conducted at ambient temperature, i.e. 20°C.

The following Table identifies the various roles the spectroscopic techniques have in studying the structure of the peptide:

**Table 3.1**: The various roles of the spectroscopic techniques.

<table>
<thead>
<tr>
<th>CD</th>
<th>Fluorescence</th>
<th>Light Scattering</th>
<th>FTIR</th>
<th>ATR-FTIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>2\textsuperscript{o} structure</td>
<td>3\textsuperscript{o} structure</td>
<td>Presence of vesicles in suspension</td>
<td>2\textsuperscript{o} structure</td>
<td>All that applies to FTIR plus orientation of membrane proteins and lipid structural order</td>
</tr>
<tr>
<td>3\textsuperscript{o} structure</td>
<td></td>
<td></td>
<td>3\textsuperscript{o} structure</td>
<td>Effect of peptide on structure of membrane lipids</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Further details are discussed below in each technique’s respective section.

**Circular dichroism (CD)**

CD is a valuable technique in structural biology. It uses circularly polarized light to measure the ellipticity (the difference of absorption of left- and right-handed circularly polarized light) of optically active molecules (in this case, the Aβ peptide). Ellipticity is calculated with the equation \[\Delta A = A_L - A_R\], where A equals absorbance and L and R equal left and right circularly polarized light, respectively. Data is reported as either differential absorbance (\(\Delta A\)) or in
ellipticity (degrees) \[\theta\]. The spectrum measures dichroism as a function of wavelength. CD is used to observe the secondary structure of proteins (\(\alpha, \beta, \beta\)-turn, and unordered structure). Different secondary structures generate positive or negative CD bands at characteristic wavelengths thus creating unique spectra features, which allows evaluation of the protein’s or peptide’s structure from spectral analysis. Spectral bands can be assigned to various structural features because signals only occur where radiation is absorbed. CD measurements were conducted on a J-810 spectropolarimeter equipped with a fluorescence attachment and a temperature controller (Jasco, Tokyo, Japan). Measurements were taken between 180 nm and 360 nm wavelengths.

**Figure 3.2:** Linear vs. Circularly polarized light

*Fourier-transform infrared spectroscopy (FTIR)*

FTIR is a type of vibrational spectroscopy widely used to analyze protein structure and dynamics. Oscillating dipoles caused by change in internuclear distance or geometry absorb the energy of the incident light. Different chemical groups have vibrations at specific frequencies, so they absorb in different regions of the IR spectrum. FTIR spectra of proteins contain absorbance bands of different structural or functional groups in the mid-IR region, i.e. 4000 cm\(^{-1}\) to 400 cm\(^{-1}\),
where the unit cm\(^{-1}\) simply implies the inverse of the wavelength. Specifically, protein structure is determined by analyzing the amide I and amide II bands. The amide I band (1700-1600 cm\(^{-1}\)) reveals information about the protein’s secondary structure. The amide II band (around 1560-1530 cm\(^{-1}\)) is sensitive to solvent accessibility and protein’s tertiary structure. Using FTIR, the overall secondary and tertiary structures of Aβ peptides can be analyzed. FTIR studies were done using a Vector-22 FTIR spectrometer (Bruker Optics, Billerica, MA, USA) equipped with a liquid nitrogen-cooled Hg-Cd-Te detector.

*Attenuated Total Reflection (ATR)-FTIR*

ATR-FTIR is a useful technique in studying membrane proteins and lipids, protein-membrane interactions, interactions of proteins with drugs, nucleic acids, the structural features of membrane pore forming proteins, and much more. In an ATR-FTIR experiment, the incident infrared light is shone through an internal reflection element (IRE), such as a germanium crystal plate, coated with sample (in this case, Aβ embedded into lipid membrane). The incident light enters the Ge plate through an edge cut at 45 degrees and undergoes a series of total internal reflections, thereby bounces through the crystal until it exits the plate at the other edge and is directed to the detector. At each reflection, an evanescent wave is created outside the Ge plate, allowing the sample deposited on the surface of the plate to absorb part of the light (see Figure 3.3). This way the absorbance spectrum of the sample is recorded as the intensity of the light reaching the detector is less than that of the incoming light. Information about the sample is gathered through the interaction between the wave and the sample and eventually gets shone onto the detector.
This experiment used both parallel and perpendicular polarized light to characterize Aβ’s orientation and structure in the lipid membrane. Polarized ATR-FTIR is especially beneficial in comparison to other FTIR techniques due to its heightened sensitivity and its capability of determining the orientation of membrane bound proteins. Some of the benefits of using ATR-FTIR over other FTIR techniques is that a) small amount of sample can be used to obtain strong signal, and b) the orientation of the peptide can be determined in addition to its secondary and tertiary structures.

![ATR-FTIR setup](https://example.com/figure3)

**Figure 3.3:** ATR-FTIR setup. The grey trapezoid is an internal reflection element (IRE) covered with lipid membranes embedded with proteins. Mirrors (M1-4) direct the incident light through the IRE and into the detector. Reference: (Tatulian, 2019).

**Fluorescence**

Fluorescence spectroscopy has been widely used in structural biology to study proteins. Fluorescence measures the emission of photons by atoms after being excited with UV or visible radiation. When excited with this form of radiation, fluorescent substances (fluorophores) absorb
photons by means of transition of electrons from low (ground) to high (excited) energy levels, then within about 10 ns they return to the ground state and emit photons at lower energy, corresponding to lower frequency and longer wavelength. Part of the energy is lost to the solvent through a process known as solvent relaxation, i.e. reorientation of polar solvent molecules by the dipole moment created by electron transitions. More specifically, fluorescence takes advantage of the intrinsic fluorophores tryptophan, tyrosine, and phenylalanine, which contain an indole, phenol, or benzyl aromatic rings, respectively. These aromatic groups absorb between 260 nm and 280nm and emit between 290 nm and 340nm. Lipid membranes are not typically fluorescent. Fluorescence data is presented as an emission spectrum, i.e., a plot of emission intensity vs. wavelength. Fluorescence measurements were taken with excitation at 220 and 275 nm wavelengths using a J-810 spectropolarimeter with fluorescence attachment.

**Light scattering**

Light scattering occurs when polar or charged groups of molecules or larger particles oscillate and emit radiation when exposed to electromagnetic radiation, including UV or visible light. As the intensity of emission is proportional to the particle size, light scattering can be used to measure particle size, protein-protein interactions, protein-membrane interactions, among other things. Based on this principle, the presence of lipid vesicles, which are ~50 times larger than the Aβ peptides, were observed by this method. Light scattering was done using the same J-810 spectropolarimeter in fluorescence mode, using incident light at large wavelengths, such as 550 nm, to avoid fluorescence emission.
Vesicle preparation

Three lipids were used to prepare unilamellar vesicles: a zwitterionic lipid (POPC), an anionic lipid (POPG), and cholesterol.

A. Vesicle Preparation for CD and Fluorescence:

A mixture of 74.83 µL of 2.00 mM POPC, 37.50 µL of 2.00 mM POPG, 12.50 µL of 2.00 mM cholesterol, and 25.00 µL of 200 µM peptide were combined in a vial, dried with a stream of N₂ gas, and desiccated for 15 minutes. 500 µL of 25 mM NaCl + Na,K-phosphate buffer (pH 7.2) was added to sample, vortexed for 5 minutes, extruded through a 0.1µm Nuclepore polycarbonate membrane 15 times, and collected in a fresh vial. This results in following final lipid and peptide concentrations: 300 µM POPC, 150 µM POPG, 50 µM cholesterol, 10 µM peptide, with total lipid to peptide molar ratio of 50:1. The membrane lipid composition is 60% POPC, 30% POPG, 10% cholesterol.

B. Peptide-Lipid Sample Preparation for ATR-FTIR:

A mixture of 18.75 µL 20 mM POPC, 9.375 µL 20 mM POPG, 3.125 µL 20 mM cholesterol, dissolved in chloroform, and 62.5 µL 200 µM peptide dissolved in HFIP, were combined in a vial, spread onto a Ge plate, and dried via desiccation for 1h before assembly into a flow-through ATR sample cell. FTIR spectra of the peptide-lipid sample, which make a multilayer on the Ge plate, were measured at parallel and perpendicular polarizations of the incident infrared light. Buffer were injected, allowing full hydration of the sample, and spectra at both polarizations were measured. These measurements have allowed determination of peptide structure and
orientation in lipid membranes (see Chapter 4), in both dehydrated and fully hydrated states, which leads to construction of a structural model of membrane pores formed by Aβ peptides.

**Data Analysis**

The orientation of the pore was measured by the β-angle, or the angle between beta strands and the barrel’s central axis (as indicated by Eq (5) in Chapter 4). The angle was measured based on an axis perpendicular to the lipid membrane. Meaningful angles align with 0°, as this means that the channel is completely open on both sides. The angles 0° and 20° were used for the angle of barrel axis relative to the membrane normal during calculations.

Lipid order parameter is another measurement that was considered. This measurement determines how ordered the lipid membrane is, which denotes quality of the lipid bilayer. A lipid order parameter of 0 indicates that there is no bilayer and that the lipids are randomly dispersed throughout the sample, while increasing numbers indicate higher order of bilayers. This is important to consider when studying ion channels because it reveals whether the channel disrupts the membrane order or not.
CHAPTER 4

RESULTS

CD Experiments

For initial structural characterization of Aβ peptides in aqueous buffer and reconstituted in lipid membranes, CD spectra were measured at 20°C and 37°C. These temperatures were chosen for two reasons. Measurements at 20°C were conducted to directly compare CD data with FTIR data, as the FTIR spectrometer does not have a temperature controller and can be used at ambient temperature, i.e. 20°C ±1°C, and measurements at 37°C were conducted to mimic physiological conditions. Far-UV CD spectra of Aβ1-42 in aqueous buffer generated a deep minimum at 218-220 nm at both temperatures, indicating formation of β-sheet structure (Figure 4.1). CD spectra

![CD Spectra](image)

**Figure 4.1:** Circular dichroism spectra of Aβ1-42 in aqueous buffer (25 mM NaCl, 25 mM Na,K-phosphate, pH 7.2) and reconstituted in unilamellar lipid vesicles composed of 60 mol % POPC, 30 mol % POPG, and 10 mol % cholesterol, as indicated, at 1:50 peptide/lipid molar ratio. Blue and red lines correspond to 20°C and 37°C, respectively.
of Aβ₁₋₄₂ in reconstituted in unilamellar lipid vesicles composed of 60 mol % POPC, 30 mol % POPG, and 10 mol % cholesterol display deeper minima at 215-216 nm, again indicating formation of β-sheet structure. The blue-shifted CD bands of the peptide in membranes suggest twisting of β-strands to a larger degree (Wang et al. 2016). Twisted β-strands are typical for β-barrel protein structures, which form pores in cell membranes. Thus, CD data suggest formation of β-barrel-like structure by Aβ₁₋₄₂ in lipid membranes.

**Fluorescence Experiments**

Aβ₁₋₄₂ has fluorescence properties due to the presence of one tyrosine (Tyr) at position 10 and three phenylalanines (Phe) at positions 4, 19, 20. Hence, fluorescence spectroscopy was used to see whether the emission wavelength and/or intensities of these fluorophores change upon incorporation of the peptide in lipid membranes. Both Tyr and Phe can be excited in a wide range of UV radiation, and their emission peaks occur around 310 nm and 287 nm, respectively. It has been identified that Phe can be predominantly excited at 220 nm, whereas Tyr can be selectively excited at 275 nm; both excitation wavelengths were used in this analysis.
Figure 4.2: Fluorescence spectra of Ab<sub>1-42</sub> in aqueous buffer (25 mM NaCl, 25 mM Na,K-phosphate, pH 7.2) and reconstituted in unilamellar lipid vesicles composed of 60 mol % POPC, 30 mol % POPG, and 10 mol % cholesterol, as indicated, at 1:50 peptide/lipid molar ratio. Excitation was at 220 nm (A) or at 275 nm (B). Blue and red lines correspond to 20°C and 37°C, respectively. Fluorescence emission peaks occur at 310 nm for the peptide either in buffer or in vesicle membranes when excited at 220 nm (A). Upon excitation at 275 nm, emission peaks occur at 307 nm at 20°C and at 310 nm at 37°C for the peptide in buffer and at 308 nm for the peptide in vesicle membranes.

Excitation at 220 nm resulted in relatively sharp emission peaks at 310 nm for Ab<sub>1-42</sub> in buffer or in vesicle membranes, with higher intensity in the latter case (Figure 4.2A). This finding indicates that a) Tyr fluorescence obscures that of Phe due to a greater quantum yield (Lakowicz, 1999), and b) the peptide is inserted into the membrane, which protects from fluorescence quenching by water (Li et al., 2011). Stronger signal at 220 nm in the presence of vesicles results from light scattering. Upon excitation at 275 nm, again Tyr emission is detected between 307 nm and 310 nm, with significantly stronger fluorescence signal and stronger scattering of incident light in the presence of vesicles (Figure 4.2B). In all cases, the fluorescence intensity decreases at higher temperature (37°C), as expected (Lakowicz, 1999). Thus, CD and fluorescence
data suggest that Aβ₁₋₄₂ does insert into lipid vesicle membranes and adopts β-sheet structure with twisted strands.

**Light Scattering Experiments**

To obtain additional documentation for the presence of lipid vesicles, light scattering measurements were conducted on same Aβ and Aβ/membrane samples in parallel with CD and fluorescence measurements. Large wavelength (550 nm) for light scattering was chosen to avoid fluorescence effects. As expected, light scattering intensity was much stronger in the presence of vesicles as compared to the Aβ₁₋₄₂ peptide in buffer (Figure 4.3).

**Figure 4.3:** Light scattering at 90 degrees of Aβ₁₋₄₂ in aqueous buffer (25 mM NaCl, 25 mM Na,K-phosphate, pH 7.2) and reconstituted in unilamellar lipid vesicles composed of 60 mol % POPC, 30 mol % POPG, and 10 mol % cholesterol, as indicated, at 1:50 peptide/lipid molar ratio, at 20°C.
**ATR-FTIR Experiments**

The main information on secondary structure and orientation of the peptides relative to the membrane was obtained from ATR-FTIR experiments. These experiments were conducted on peptide-lipid samples in dry state and after addition of aqueous buffer, at 1:50 peptide/lipid molar ratio. The meaning of studying dehydrated (dry) samples is to mimic the Aβ peptide as the transmembrane part of APP, before cleavage and exposure to the aqueous environment. It is known that the Aβ stretch in the context of APP is mostly in α-helical conformation (Barrett et al. 2012) and when cleaved and released into the aqueous medium, acquires β-sheet structure.

ATR-FTIR spectra were measured at parallel (∥) and perpendicular (⊥) polarizations of the incident light, and peak-fitting was applied to identify the spectral components in the lipid C=O and peptide amide I and amide II regions. The overall areas of all components were then determined using both ∥ and ⊥ spectra as follows: $a_i = a_{i,∥} + Ga_{i,⊥}$, where $a_i$ is the area of the $i$-th component and $G$ is a scaling factor, which for present experimental conditions is $G = 1.44$ (Tatulian, 2019; Tatulian et al., 2012). The fractions of α-helix, β-sheet, turn ($t$), and unordered ($ρ$) structures were calculated using the amide I areas of respective components and their extinction coefficients, $ε$, as described earlier (Tatulian, 2019):

$$f_i = \frac{a_{i}}{ε_{i}\left(\frac{a_{α}}{ε_{α}} + \frac{a_{β}}{ε_{β}} + \frac{a_{t}}{ε_{t}} + \frac{a_{ρ}}{ε_{ρ}}\right)}$$

(1)

Previously determined values for the extinction coefficients have been used: $ε_{α} = 7.6 \times 10^7$ cm/mol, $ε_{β} = 6.2 \times 10^7$ cm/mol, $ε_{t} = 5.5 \times 10^7$ cm/mol, $ε_{ρ} = 4.5 \times 10^7$ cm/mol (Venyaminov & Kalnin, 1990). The assignment of amide I components to secondary structure types was done
according to established wavenumber ranges for various protein structures, i.e., turn structures: 1705-1661 cm\(^{-1}\), \(\alpha\)-helix: 1660-1646 cm\(^{-1}\), unordered: 1645-1638 cm\(^{-1}\), \(\beta\)-sheet: 1637-1625 cm\(^{-1}\) (Tatulian, 2019). Amide I components below 1620 cm\(^{-1}\) were assigned to side chains.

![Figure 4.4: ATR-FTIR spectra of A\(\beta_{1-42}\) reconstituted in lipid multilayers composed of 60 mol % POPC, 30 mol % POPG, and 10 mol % cholesterol at 1:50 peptide/lipid molar ratio, dehydrated by desiccation, at parallel (A) and perpendicular (B) polarizations of the incident light. The measured spectrum is shown in solid line colored cyan, and the fitted curve (curvefit) is shown as black dotted line. Spectral components in lipid C=O, amide I and amide II regions are presented as follows: lipid C=O components: black dashed lines; turn structures: solid grey lines; \(\alpha\)-helix; solid green line; unordered: solid blue line; \(\beta\)-sheet: solid red line; side chains: solid brown line; amide II components: solid black lines. Panel C shows the dichroic spectrum, i.e., the ratio of spectra at \(\parallel\) and \(\perp\) polarizations.](image)

Data presented in Figure 4.4A,B show that in dry peptide/lipid samples, A\(\beta_{1-42}\) acquires a combination of \(\alpha\)-helix and \(\beta\)-sheet structures, as indicated by green and red amide I components. The wavenumbers of all amide I structural components, their fractions, and the respective numbers of amino acids for all four peptides in dry state and when hydrated by buffer are presented in Table 1. Data of Table 1 indicate \(\alpha\)-helical and \(\beta\)-sheet fractions in A\(\beta_{1-42}\) in dry
state $f_\alpha = 0.24$ (10 amino acid residues) and $f_\beta = 0.30$ (12.6 amino acid residues). A significant fraction of turn structure ($f_t = 0.41$) and a negligible fraction of unordered structure ($f_\rho = 0.052$) are also present.

**Table 4.1:** Amide I wavenumbers ($\nu$), fractions ($f$), and numbers of amino acid residues ($N$) for $\alpha$-helix, $\beta$-sheet, turn ($t$), and unordered ($\rho$) structures derived from ATR-FTIR spectra.

<table>
<thead>
<tr>
<th></th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>$\alpha$</th>
<th>$\beta$</th>
<th>$\alpha$</th>
<th>$\beta$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry</td>
<td>Buffer</td>
<td>Dry</td>
<td>Buffer</td>
<td>Dry</td>
<td>Buffer</td>
<td>Dry</td>
<td>Buffer</td>
</tr>
<tr>
<td>$\nu_\alpha$ (cm$^{-1}$)</td>
<td>1658</td>
<td>1659-1657 a)</td>
<td>1658-1655</td>
<td>1651-1646</td>
<td>1660-1658</td>
<td>1656-1654</td>
<td>1658-1655</td>
<td>1652-1651</td>
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<tr>
<td>$f_\alpha$</td>
<td>0.24</td>
<td>0.20</td>
<td>0.20</td>
<td>0.25</td>
<td>0.20</td>
<td>0.15</td>
<td>0.22</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.08 b)</td>
<td>0.15</td>
<td>0.13 b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$N_\alpha$</td>
<td>10.0</td>
<td>8.5</td>
<td>8.0</td>
<td>10.0</td>
<td>8.0</td>
<td>6.0</td>
<td>8.3</td>
<td>7.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.2 b)</td>
<td>6.0</td>
<td>5.0 b)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\nu_\beta$ (cm$^{-1}$)</td>
<td>1632-1630</td>
<td>1627</td>
<td>1633-1630</td>
<td>1628</td>
<td>1631-1629</td>
<td>1629</td>
<td>1631-1630</td>
<td>1627-1625</td>
</tr>
<tr>
<td>$f_\beta$</td>
<td>0.30</td>
<td>0.44</td>
<td>0.26</td>
<td>0.37</td>
<td>0.21</td>
<td>0.17</td>
<td>0.26</td>
<td>0.27</td>
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<td>$N_\beta$</td>
<td>12.6</td>
<td>18.2</td>
<td>10.4</td>
<td>14.8</td>
<td>8.4</td>
<td>6.8</td>
<td>10.0</td>
<td>10.3</td>
</tr>
<tr>
<td>( v_\perp ) (cm(^{-1}))</td>
<td>1700-1671</td>
<td>1700-1678</td>
<td>1701-1669</td>
<td>1699-1678</td>
<td>1704-1672</td>
<td>1705-1669</td>
<td>1698-1668</td>
<td>1700-1681</td>
</tr>
<tr>
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<tr>
<td>( f_\perp )</td>
<td>0.41</td>
<td>0.16</td>
<td>0.48</td>
<td>0.30</td>
<td>0.46</td>
<td>0.55</td>
<td>0.45</td>
<td>0.22</td>
</tr>
<tr>
<td>( N_\perp )</td>
<td>17.2</td>
<td>6.9</td>
<td>19.2</td>
<td>12.0</td>
<td>18.4</td>
<td>22.0</td>
<td>17.0</td>
<td>8.4</td>
</tr>
<tr>
<td>( v_\parallel ) (cm(^{-1}))</td>
<td>1645</td>
<td>1643-1642</td>
<td>1645</td>
<td>N/A</td>
<td>1645-1643</td>
<td>1642-1640</td>
<td>1646-1642</td>
<td>1639-1637</td>
</tr>
<tr>
<td>( f_\parallel )</td>
<td>0.052</td>
<td>0.20</td>
<td>0.06</td>
<td>0.00</td>
<td>0.13</td>
<td>0.13</td>
<td>0.07</td>
<td>0.18</td>
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<tr>
<td>( N_\parallel )</td>
<td>2.2</td>
<td>8.4</td>
<td>2.4</td>
<td>0.00</td>
<td>5.2</td>
<td>5.2</td>
<td>2.7</td>
<td>6.7</td>
</tr>
</tbody>
</table>

a) Wavenumbers of \( \perp \) spectra are usually slightly lower than those of \( \parallel \) spectra.

b) These components have been assigned to \( \alpha_{III} \)-helix

The orientational order parameter of \( \alpha \)-helical structure was determined using the formula (Tatulian, 2003):

\[
S = \frac{2B}{(3\cos^2\alpha - 1)(B - 3E_z^2)}
\] (2)

In Eq. (2), \( B = E_x^2 - R E_y^2 + E_z^2 \), \( \alpha \) is the angle between the transition dipole moment and the molecular axis (for \( \alpha \)-helix, \( \alpha = 39^\circ \pm 1^\circ \)), \( E_x = 1.399 \), \( E_y = 1.514 \), and \( E_z = 1.621 \) are the electric vector components of the evanescent wave, and \( R \) is the dichroic ratio, i.e. \( R = a_{II}/a_{\perp} \).

Values of \( R \) for \( \alpha \)-helix have been determined from the dichroic spectrum (Figure 4.4C) and
used to calculate the helical order parameter. The angle of orientation of helical axis relative to the membrane normal, \( \theta \), was calculated from the order parameter through:

\[
\cos \theta = \sqrt{\frac{2S+1}{3}}
\]  

(3)

The orientation of \( \beta \)-strands can be determined from \( \beta \)-sheet dichroic ratio of a protein or a peptide that adopts a structure with a rotational axis of symmetry, such as a \( \beta \)-barrel. Then, the following relationship holds:

\[
\frac{1}{2} (3 \cos^2 \delta - 1) = \frac{2B}{(3 \cos^2 \gamma - 1)(B - 3E_z^2)}
\]  

(4)

In Eq. (4), \( \delta \) is the angle of the transition dipole moment of \( \beta \)-strands with respect to the central axis, and \( \gamma \) is the tilt angle of the central axis of the barrel structure with respect to the membrane normal. The amide I transition dipole is oriented perpendicular to the \( \beta \)-strand axis, which yields the following relationship between angle \( \delta \) and the angle \( \beta \) of orientation of the strand axis relative to the barrel’s central axis:

\[
\delta = \frac{\pi}{2} - \beta
\]  

(5)

The angle \( \beta \) for the proposed barrel structure could be determined through Eqs. (4) and (5) using the measured dichroic ratio for the \( \beta \)-sheet component of the amide I spectrum (Figure 4.4C) if the angle \( \gamma \) was known. In a situation when \( \gamma \) is not known, a practically useful approach is to assume conceivable values, such as \( \gamma = 0 \) degrees or \( \gamma = 20 \) degrees, and determine the
orientations of strands in a β-barrel. This approach has been used here. The resulting orientational parameters, along with α-helical and β-sheet dichroic ratios, are summarized in Table 4.2.

**Table 4.2:** Dichroic ratios and orientational angle for α-helical and β-sheet components of the peptides in dehydrated state and under aqueous buffer.

<table>
<thead>
<tr>
<th></th>
<th>Dry</th>
<th></th>
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<th>Under buffer</th>
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<tr>
<td></td>
<td>Rβ</td>
<td>β (°)</td>
<td>Rα (°)</td>
<td>Rβ</td>
<td>β (°)</td>
<td>Rα (°)</td>
</tr>
<tr>
<td></td>
<td>γ = 0°</td>
<td>γ = 20°</td>
<td></td>
<td>γ = 0°</td>
<td>γ = 20°</td>
<td></td>
</tr>
<tr>
<td>Aβ₁₋₄₂</td>
<td>1.393</td>
<td>25.9</td>
<td>23.6</td>
<td>1.837</td>
<td>59.9</td>
<td>1.710</td>
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<td></td>
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</tr>
<tr>
<td>pEAβ₃₋₄₂</td>
<td>1.456</td>
<td>27.1</td>
<td>25.2</td>
<td>1.458</td>
<td>80.7</td>
<td>2.273</td>
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<tr>
<td>Aβ₁₋₄₀</td>
<td>1.415</td>
<td>26.3</td>
<td>24.2</td>
<td>1.773</td>
<td>62.2</td>
<td>1.931</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>pEAβ₃₋₄₀</td>
<td>1.428</td>
<td>26.6</td>
<td>24.5</td>
<td>1.821</td>
<td>60.5</td>
<td>1.757</td>
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</table>

aData) These spectra display components that can be assigned to α₁-II-helix, see Table 1 for their wavenumbers.

Data of Table 4.2 indicate strand tilt angle of around 25 degrees for Aβ₁₋₄₂ in dehydrated state. This result is consistent with earlier data on the Aβ₂₅₋₃₅ fragment reconstituted in supported
lipid layers of similar lipid composition, where β-strands were tilted by 22±4 degrees from the barrel axis (Kandel et al., 2019). In case of a Bax protein-derived peptide, the proposed β-barrel was characterized with strand tilt angles between 20 and 30 degrees (Tatulian et al., 2012), again in agreement with current data. It is noteworthy that the value of angle β varies within only 2.5 degrees when γ changes from 0 to 20 degrees, indicating the validity of this approach. The orientation of the helical part of Aβ1-42 in dehydrated membranes is around 60 degrees (Table 4.2). It is conceivable that the N-terminal polar part of the peptide forms α-helical structure that lies obliquely on the membrane surface whereas the more hydrophobic C-terminal half embeds into the membrane in the form of β-strands and assembles into a β-barrel structure.

**Figure 4.5:** ATR-FTIR spectra of Aβ1-42 reconstituted in lipid multilayers composed of 60 mol % POPC, 30 mol % POPG, and 10 mol % cholesterol, totally hydrated by a D2O-based buffer (25 mM NaCl, 25 mM Na,K-phosphate, pD 7.2), at parallel (A) and perpendicular (B) polarizations of the incident light. Significance of line type and color is described under Figure 4.4. Panel C shows the dichroic spectrum, i.e., the ratio of spectra at || and \( \perp \) polarizations.

Significant structural transitions occur when an aqueous buffer is injected into the sample cell containing the supported peptide/lipid system (Figure 4.5). First, the presence of the buffer is
manifested by the shift of the lipid C=O band’s components. The lipid carbonyl group stretching vibration generates absorbance band with two components at 1742 cm\(^{-1}\) and 1728 cm\(^{-1}\), where the higher and lower wavenumber components correspond to dehydrated and hydrated C=O groups; H-bonding with water weakens the C=O covalent bond and thereby decreases the vibrational frequency (Blume et al., 1988). Comparison of data in Figures 4.4A,B and 4.5A,B show substantial increase of the low frequency component and decrease of the high frequency component in the presence of buffer. Second, addition of D\(_2\)O-based buffer initiates amide hydrogen/deuterium exchange, which results in a shift of the amide II band from \(\sim 1550\) cm\(^{-1}\) to \(\sim 1450\) cm\(^{-1}\) (the amide II region is not shown in Figure 4.5 because it becomes too noisy). Most importantly, the fraction of \(\beta\)-sheet structure of the peptide increases from 30\% to 44\% mostly at the expense of turn and \(\alpha\)-helix structures, which decline from 41 \% to 16 \% and from 24 \% to 20 \% respectively (Figure 4.5 and Table 4.1). Finally, the orientation of \(\beta\)-strands becomes more tilted (\(\beta \approx 31\) degrees) and the \(\alpha\)-helical axis tilt angle goes from 60 to 65 degrees. The \(\beta\)-strand tilt just above 30 degrees is similar to the respective angle of mitochondrial and bacterial porins, which form canonical \(\beta\)-barrel structure (Páli and Marsh, 2000; Kleinschmidt, 2006; Zeth and Thein, 2010), lending additional support for our conjecture that A\(\beta\)\(_{1-42}\) forms \(\beta\)-barrel structure in lipid membranes.
Figure 4.6: ATR-FTIR spectra of pEAβ3-42 reconstituted in lipid multilayers composed of 60 mol % POPC, 30 mol % POPG, and 10 mol % cholesterol, in dehydrated state, at parallel (A) and perpendicular (B) polarizations of the incident light. Significance of line type and color is described under Figure 4.4. Panel C shows the dichroic spectrum, i.e., the ratio of spectra at ‖ and ⊥ polarizations.

The pyroglutamylated peptide pEAβ3-42 reconstituted in supported membranes exhibits structural properties resembling those of its unmodified counterpart. In dehydrated state, the peptide displays 20% α-helix, 26% β-sheet, 48% turn, and 6% unordered structure (Figure 4.6, Table 4.1). Addition of buffer increases the fraction of β-sheet to 37% and that of α-helix to 25%, while the turn structure declines to 30% and the unordered structure becomes negligible (Figure 4.7, Table 4.1). Interestingly, in the hydrated sample an amide I component emerges in the 1666-1663 cm⁻¹ region, which has been assigned to αII-helix, i.e. a helical structure with identical geometry to the regular α-helix but with slightly tilted amide plane and with weaker helical H-bonding (Torres et al. 1995; Wang and El-Sayed, 2000). This αII-helical structure constitutes around 8% (3.2 amino acid residues, i.e. barely 1 helical turn) of the peptide and is
likely to be located at the edges of the regular \( \alpha \)-helix.

**Figure 4.7**: ATR-FTIR spectra of pEA\( \beta_{3-42} \) reconstituted in lipid multilayers composed of 60 mol \% POPC, 30 mol \% POPG, and 10 mol \% cholesterol, totally hydrated by a \( \text{D}_2\text{O} \)-based buffer (25 mM NaCl, 25 mM Na,K-phosphate, pH 7.2), at parallel (A) and perpendicular (B) polarizations of the incident light. Significance of line type and color is described under Figure 4.4, except that the proposed \( \alpha_{\text{II}} \)-helix component is colored orange. Panel C shows the dichroic spectrum, i.e., the ratio of spectra at \( \parallel \) and \( \perp \) polarizations.

The orientation of \( \beta \)-strands of pEA\( \beta_{3-42} \) in dry state is similar to that of A\( \beta_{1-42} \), but in hydrated state becomes more tilted, i.e. \( \beta = 38-39 \) degrees (Table 4.1). This tilt angle is still in the range of strand tilts of \( \beta \)-barrel porins in mitochondrial and bacterial membranes (Páli and Marsh, 2000; Kleinschmidt, 2006; Zeth and Thein, 2010). The \( \alpha \)-helix in dry state is more inclined to the membrane surface in dry state (\( \theta = 81 \) degrees) and less tilted when hydrated with buffer (\( \theta = 49 \) degrees). The orientation of the \( \alpha_{\text{II}} \)-helix is similar to that of the regular \( \alpha \)-helix, as expected.
Figure 4.8: ATR-FTIR spectra of Aβ1-40 reconstituted in lipid multilayers composed of 60 mol % POPC, 30 mol % POPG, and 10 mol % cholesterol, in dehydrated state, at parallel (A) and perpendicular (B) polarizations of the incident light. Significance of line type and color is described under Figure 4.4. Panel C shows the dichroic spectrum, i.e., the ratio of spectra at || and ⊥ polarizations.*

Aβ1-40 exhibited much milder signal than Aβ1-42 and pEAB3-42. This is potentially caused by it being more hydrophilic, which may have caused a little bit of the sample volume to be washed away when buffer was added and may contribute to a decreased pore-forming capability.

In a dehydrated state, Aβ1-40 peptide displays 20% α-helix (8 amino acid residues), 21% β-sheet (8.4 amino acid residues), 46% turn (18.4 amino acid residues), and 13% unordered structure (5.2 amino acid residues) (Figure 4.8, Table 4.1). After the addition of buffer, structural composition changes to 15% α-helix (6 amino acid residues), 17% β-sheet (6.8 amino acid residues), 55% turn (22 amino acid residues), and 13% unordered structure (5.2 amino acid residues).
Figure 4.9: ATR-FTIR spectra of Aβ_{1-40} reconstituted in lipid multilayers composed of 60 mol % POPC, 30 mol % POPG, and 10 mol % cholesterol, totally hydrated by a D_{2}O-based buffer (25 mM NaCl, 25 mM Na,K-phosphate, pD 7.2), at parallel (A) and perpendicular (B) polarizations of the incident light. Significance of line type and color is described under Figure 4.4, except that the proposed α_{II}-helix component is colored orange. Panel C shows the dichroic spectrum, i.e., the ratio of spectra at ⊥ and || polarizations.

The orientation of β-strands of Aβ_{1-40} in a dry state is about 25 degrees (Table 4.2), and in a hydrated state is about 34 degrees (Table 4.2). This angle is consistent with the strand tilts of mitochondrial and bacterial β-barrel porins (Páli and Marsh, 2000; Kleinschmidt, 2006; Zeth and Thein, 2010). The α-helix tilt angle goes from 62.2 to 59.6 degrees in exposure to buffer.
Figure 4.10: ATR-FTIR spectra of pEAβ₃₋₄₀ reconstituted in lipid multilayers composed of 60 mol % POPC, 30 mol % POPG, and 10 mol % cholesterol, in dehydrated state, at parallel (A) and perpendicular (B) polarizations of the incident light. Significance of line type and color is described under Figure 4.4. Panel C shows the dichroic spectrum, i.e., the ratio of spectra at || and \(\perp\) polarizations.

The pyroglutamylated peptide pEAβ₃₋₄₀ exhibits less structural similarity to Aβ₁₋₄₀ than pEAβ₃₋₄₂ does to its unmodified counterpart. This could be due to the fact that pEAβ₃₋₄₀ is less hydrophilic than Aβ₁₋₄₀. In a dehydrated state, pEAβ₃₋₄₀ peptide displays 22% α-helix (8.3 amino acid residues), 26% β-sheet (10 amino acid residues), 45% turn (17 amino acid residues), and 7% unordered structure (2.7 amino acid residues) (Figure 4.10, Table 4.1). After the addition of buffer, structural composition changes to 20% α-helix (7.6 amino acid residues), 27% β-sheet (10.3 amino acid residues), 22% turn (8.4 amino acid residues), and 18% unordered structure (6.7 amino acid residues).
Figure 4.11: ATR-FTIR spectra of pEAβ3-40 reconstituted in lipid multilayers composed of 60 mol % POPC, 30 mol % POPG, and 10 mol % cholesterol, totally hydrated by a D₂O-based buffer (25 mM NaCl, 25 mM Na,K-phosphate, pD 7.2), at parallel (A) and perpendicular (B) polarizations of the incident light. Significance of line type and color is described under Figure 4.4, except that the proposed α₂-helix component is colored orange. Panel C shows the dichroic spectrum, i.e., the ratio of spectra at II and ⊥ polarizations.

The orientation of β-strands of pEAβ3-40 in a dry state transitioned from about 25 degrees to about 34 degrees after exposure to buffer. Like Aβ1-40, pEAβ3-40 exhibited strand tilts consistent with mitochondrial and bacterial β-barrel porins (Páli and Marsh, 2000; Kleinschmidt, 2006; Zeth and Thein, 2010). The α-helix is 60.5 degrees in a dehydrated state and changes to 57.3 degrees when hydrated with buffer.
**Effect of Aβ peptide on lipid membranes**

If the peptides form β-barrel structure in lipid membranes, they may affect the structural order of the lipid hydrocarbon chains. To test the effect of the peptides on lipid structure, the order parameter of lipid acyl chains in supported membranes has been determined without added peptide and in the presence of each of the four Aβ peptides, in dehydrated and hydrated states. This was accomplished by analysis of the ATR-FTIR spectral region corresponding to methylene (CH₂) stretching vibrations. The CH₂ groups of lipid acyl chains undergo asymmetric and symmetric stretching vibrations and generate absorbance bands at wavenumbers around 2920 cm⁻¹ and 2850 cm⁻¹, respectively (Tatulian 2003) (Figure 4.12). For a hydrocarbon chain in all-trans configuration, the transition dipole moment of CH₂ vibrations is oriented perpendicular to the chain axis. Therefore, the order parameter of lipid acyl chains (S_L) can be determined through Eq. (2) using α = 90°. Dichroic ratios have been determined as \( R_L = a_L// / a_L\perp \), where \( a_L// \) and \( a_L\perp \) are the total areas of lipid CH₂ absorbance bands from 3015 cm⁻¹ to 2810 cm⁻¹.
Figure 4.12: (A): ATR-FTIR spectra of a supported lipid multilayer composed of 60 mol % POPC, 30 mol % POPG, and 10 mol % cholesterol, in dry state (blue) or hydrated by a D$_2$O-based buffer of 25 mM NaCl, 25 mM Na,K-phosphate, pH 7.2 (green), at parallel (solid) and perpendicular (dotted) polarizations of the incident light. (B): Same with reconstituted peptide Ab$_{1-42}$ at peptide/lipid molar ratio of 1:50.
Table 4.3: Lipid acyl chain dichroic ratios ($R_L$), order parameters ($S_L$), and peak wavenumbers of asymmetric and symmetric vibrations of methylene groups ($\nu_{\text{asym}}$, $\nu_{\text{sym}}$) in the absence and presence of four Aβ peptides.

<table>
<thead>
<tr>
<th></th>
<th>Plain lipid</th>
<th>Aβ1-42</th>
<th>pEAB3-42</th>
<th>Aβ1-40</th>
<th>pEAB3-40</th>
</tr>
</thead>
<tbody>
<tr>
<td>$R_L$</td>
<td>1.501</td>
<td>1.379</td>
<td>1.451</td>
<td>1.267</td>
<td>1.568</td>
</tr>
<tr>
<td>$S_L$</td>
<td>0.340</td>
<td>0.441</td>
<td>0.380</td>
<td>0.542</td>
<td>0.287</td>
</tr>
<tr>
<td>$\nu_{\text{asym}}$ (cm$^{-1}$)</td>
<td>2921.1</td>
<td>2922.0-2921.1</td>
<td>2922.6-2922.0</td>
<td>2923.0-2922.0</td>
<td>2922.0-2922.0</td>
</tr>
<tr>
<td>$\nu_{\text{sym}}$ (cm$^{-1}$)</td>
<td>2851.6</td>
<td>2852.6-2851.6</td>
<td>2852.2</td>
<td>2852.6-2851.6</td>
<td>2852.6</td>
</tr>
</tbody>
</table>

a) Dichroic ratios based on total areas between 3015 cm$^{-1}$ and 2810 cm$^{-1}$.

b) Wavenumbers at perpendicular polarization are lower by ~1 cm$^{-1}$ compared to those at parallel polarization.

Data on lipid dichroic ratios, order parameters, and peak wavenumbers of asymmetric and symmetric modes of stretching vibrations are summarized in Table 4.3. As seen from Table 4.3, the order parameter of plain lipid without any peptide is 0.34 in dry state and 0.44 under aqueous buffer. For all-trans hydrocarbon chains oriented strictly perpendicular to the Ge plate, a limiting value of $S_L = 1$ is expected, and for totally unordered chains, $S_L = 0$. Taking into account that both POPC and POPG contain a double bond in their $sn$-2 acyl chains, which adopts cis
configuration and thereby causes significant orientational disorder, these order parameters (0.34-0.44) indicate reasonably well-organized membranes. For comparison, the order parameter of POPC monolayers on Ge plate was 0.05, and that of dipalmitoyl-phosphatidylcholine, a lipid with saturated acyl chains, was 0.7 (Tatulian, 2003). In other studies on POPC/POPG supported membranes, $S_L$ values have been reported in the range between 0.3 and 0.7. The wavenumbers of asymmetric and symmetric CH$_2$ stretching vibrations occur around 2921 cm$^{-1}$ and 2852 cm$^{-1}$. CH$_2$ stretching wavenumbers are sensitive to the physical state of lipids and undergo increase from ~2916 cm$^{-1}$ to ~2924 cm$^{-1}$ upon thermal transition from solid state to fluid state (Mendelsohn and Mantsch, 1986; Mantsch and McElhaney, 1991). Wavenumbers presented in Table 4.3 indicate a fluid-like state of supported membranes, which is due to the unsaturated bonds in POPC and POPG and reasonably models the properties of biological membranes. The presence of peptides in the membranes exerts nontrivial effects on lipid order. In both dry state and under aqueous buffer, A$\beta$1-42 increases the lipid order whereas the other three peptides decrease $S_L$ (Table 3). This finding suggests a unique property of A$\beta$1-42 compared to the other peptides, which may be related to its ability to form ion-conducting pores in membranes. For example, formation of ion channels in plasma membranes of HEK cells has been reported for A$\beta$1-42 oligomers, whereas A$\beta$1-40 failed to form channels in any aggregation state (Bode et al. 2017). Data on membrane pore forming abilities of all A$\beta$ species studied in this work will be required to answer questions about the mechanisms of their differential cytotoxic effects. However, current data provide important information of the structural aspects of interaction of these peptides with membranes and lay groundwork for further studies and discoveries.
Summary

Overall, results indicate that Aβ-induced pore formation is highly plausible. This can be concluded with the evidence of β-barrel structure and the peptide’s insertion into the lipid membrane.

CD data suggest formation of β-barrel-like structure by Aβ₁₋₄₂ in lipid membranes. Aβ in membranes exhibited a blue-shifted spectrum in comparison to Aβ free-floating in buffer, suggesting twisting of β-strands to a larger degree, and pointing towards formation of β-barrel structure when embedded in lipid membranes. Fluorescence data suggest that Aβ₁₋₄₂ does insert into lipid vesicle membranes and adopts β-sheet structure with twisted strands at both excitation wavelengths. Light scattering data confirmed the presence of lipid vesicles.

Out of all 4 peptides, ATR-FTIR data for Aβ₁₋₄₂ shows the strongest transition from alpha helix to beta sheet after exposure to aqueous buffer. The β-strand tilt angle changed from 25 to 30 degrees after exposure to buffer, which is consistent with β-barrel structure of bacterial membrane proteins (Páli and Marsh, 2000; Kleinschmidt, 2006; Zeth and Thein, 2010). Data from Aβ₁₋₄₂ provides reliable evidence for its potential pore forming capabilities. ATR-FTIR data for Aβ₁₋₄₀ showed the same β-strand tilt angle transition from 25 to 30 degrees as Aβ₁₋₄₂, but the transition from alpha helix to beta sheet was the least pronounced out of all 4 peptides. This is perhaps due to Aβ₁₋₄₀’s hydrophilic nature, and a portion of the sample was likely washed away
by the addition of the aqueous buffer. This decreased β-sheet structure may reflect Aβ1-40’s decreased ability to form pores in lipid membranes, but more experimentation is required to fully conclude this. pEAβ3-40 and pEAβ3-42 ATR-FTIR data also indicate β-sheet formation and increased β-strand tilt angle which falls in line with a β-barrel model.

Lipid order structure was also considered to determine the peptides’ effect on lipid bilayer order. This data indicated reasonably well-organized membranes. Aβ1-42 increases the lipid order whereas the other three peptides decrease it, revealing a unique property of Aβ1-42 compared to the other 3 peptides, which may be related to its ability to form ion-conducting pores in membranes.

*Interpretations and Implications*

In order to form β-barrels, β-sheets must first exist, and in order for the β-barrel to function as a neurotoxic ion-conducting pore, it must be embedded in the lipid membrane. In line with the hypothesis, the results reveal that Aβ has β-sheet conformation when exposed to buffer and that it inserts into lipid membranes. This provides further support for the amyloid hypothesis and fits with the theory that Aβ oligomers can induce pore formation in lipid membranes. Because only computer-generated models exist for Aβ-induced pores, these data provide experimental evidence for the structure of Aβ in lipid membranes and strongly point towards β-barrel pore formation in lipid membranes.

These results build on existing evidence that Aβ oligomers disrupt cellular ionic homeostasis (Dammers et al., 2015; Peters et al., 2016) by providing structural evidence for the formation of β-barrels that function as Ca^{2+}-permeable membrane pores. Experimental evidence
for β-barrel structure in lipid membranes is useful in that it provides a good backing for the hypothesis that Aβ oligomers form pores. Aβ showed substantial evidence towards pore formation by revealing β-sheet conformation, falling in line with Kandel et al.’s 2017 study, which reported that Aβ fragments formed the most efficient pores in lipid membranes when in β-sheet conformation.

**Limitations**

The generalizability of the results is limited by lack of data on Aβ1-40, Aβ3-40, and Aβ3-42. CD, fluorescence, and light scattering data was only collected for Aβ1-42 and is needed for the other three peptides in order to best be able to compare their structures in lipid membranes. Significant evidence points towards the existence of β-barrel structures that make up neurotoxic membrane pores. More experimentation is needed to fully conclude Aβ’s mechanism of pore formation.

However, the lack of CD, fluorescence, and light scattering data for on Aβ1-40, Aβ3-40, and Aβ3-42 is a limitation only in the fact that it eliminates the chance to compare the four peptides’ structures via these methods. This lack does not impact the data that was collected for Aβ1-42, nor does it impact that the aims of this study were achieved. Data on these three peptides for CD, fluorescence, and light scattering are necessary for comparison, and the results that were collected are nonetheless valid for the purposes of revealing the structure of Aβ in lipid membranes.

These experiments were done in vitro using a vesicle model, which is much simpler than a biological cell membrane and omits the possibility for Aβ to interact with other membrane
proteins. However, great effort was put into mimicking biological conditions as closely as possible with a vesicle model. Furthermore, this study does not investigate the correlation between β-barrels and neurotoxicity because no in vivo models were used. The data merely just proves the existence of β-barrel structures in lipid membranes which was something only computer generated up to this point. Despite this, it cannot be said from the data collected that these β-barrels directly cause neurotoxicity because this study did not explore that.

**Future Directions**

Data on the pore forming abilities of all Aβ species studied in this work will be required to answer questions about the mechanisms of their differential cytotoxic effects. The pore forming capabilities of Aβ can further be explored using more spectroscopic techniques such as NMR.

Additionally, gaining a visual image of the β-barrel in lipid membrane will provide even more solid evidence for its existence as a neurotoxic pore. Some visual techniques that can be explored to image Aβ structure in lipid membranes include scanning electron microscopy (SEM), which provides picturesque images with high resolution that clearly show the lipid membrane and the embedded β-barrel pores, and electron energy loss spectroscopy (EELS), which can not only allow for visualization of the β-barrel in lipid membrane but also identify the chemical nature of the sample.

Further research is needed to establish a correlation between β-barrel structure and cytotoxicity. Cytotoxicity can be analyzed in biological models such as a cell cultures via cytotoxicity assays, which measure the loss of cell membrane integrity upon cell death. This
technique can be useful to pair evidence of β-barrel formation with cytotoxicity to reveal a correlation between the β-barrel structure and cell death, linking the two together and getting closer to proving that Aβ oligomers are capable of forming pores in lipid membranes.

This data can also provide useful information for novel drug developments and revelation of Aβ’s full toxic pore-forming mechanism in lipid membranes. Currently, treatments for AD are aimed at Aβ plaques and inhibition of cholinesterase and glutamate, but it would be a useful and novel direction to target Aβ pore formation once there is enough evidence for its existence and enough understanding of its mechanism. This way, new and possibly more effective therapeutic directions can be developed to combat AD.
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