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The Interaction Between Amyloid Beta Peptide and Phospholipids

By

Xin Ma

THE UNIVERSITY of EDINBURGH

Thesis submitted for the degree of doctor of philosophy at the University of Edinburgh

2015
Disclaimer

I, Xin Ma, performed all of the experiments presented in this thesis unless otherwise clearly stated in the text. No part of this work has been or is being submitted for any other degree or qualification.

Signed:

Date:
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Abstract

The aim of the thesis project was to examine what form(s) of Amyloid beta (Aβ) (25-35) peptide interact with phospholipids in vitro and the implications of this for the mechanism of Alzheimer’s Diseases (AD). The mechanism of AD is thought to involve protein folding and misfolding. An increasing amount of evidence has shown that protein misfolding plays an important role in the biological and pathological processes of AD. Although seen as the biomedical markers of those diseases, the roles of amyloid aggregates themselves are still not fully understood. Whether the aggregates, or the monomer, or some other intermediates of Aβ cause AD is still unknown. In order to investigate the membrane-interaction of Aβ and its implications for AD, two forms of Aβ, namely levorotary and dextrorotary (L- and D-) Aβ isomers were used. Evidence has shown that L- and D- peptide can each form aggregates in a humid environment. However, when mixed together, L- and D- peptides tend not to form any aggregates. Using the mixtures of L- and D- peptides at different proportions and as well as using L- and D- alone can help us to determine the toxic form of Aβ.

Phospholipids have been used to mimic membrane bilayers. Biological membranes in vivo are a complicated system. They contain three types of lipids, namely phospholipids, glycolipids, and steroids. Different types of cells and different membranes have different proportions of those lipids. Studying the interaction between Aβ and membranes in vivo can be extremely difficult. Artificial membranes, which only contains one kind of lipids, on the other hand, are a useful tool for the study of molecular interactions. Phospholipids are the most abundant type of membrane lipid and thus that can be seen as representative of cell membranes. The interactions of Aβ and different kinds of phospholipids have been investigated in this project. This thesis discusses the secondary structure of Aβ in different environment, the interaction between Aβ and phospholipids at the air-water surface, and the location of Aβ in membranes.
during the interaction. The study provides useful information of the mechanisms and the origin of AD. At the end of the thesis, a discussion chapter analyses the difficulties of studying Aβ and AD and the potentials and inadequacies of this research.
Lay Summary of Thesis

The lay summary is a brief summary intended to facilitate knowledge transfer and enhance accessibility, therefore the language used should be non-technical and suitable for a general audience. (See the Degree Regulations and Programmes of Study, General Postgraduate Degree Programme Regulations. These regulations are available via: http://www.drps.ed.ac.uk/.)

Insert the lay summary text here - the space will expand as you type. Your lay summary must be contained on this side.

Amyloid beta peptide is a potential agent that causes Alzheimer’s Disease. This peptide accumulates into fibrils when humid. In the process of fibril formation, a few forms of amyloid peptide occur. My research aimed to find out which form of peptide is toxic to cell membranes. Phospholipids are used to mimic cell membranes in my study because they are the most abundant type of membrane lipids. Techniques such as Langmuir trough, Brewster Angle Microscopy and Neutron Scattering were used to investigate the interactions between Amyloid beta peptide and phospholipids and the location of Amyloid in membranes during the interaction. Fibril growth and Circular Dichroism were used to study the structure of Amyloid and its ability to form fibrils.
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<tr>
<td>Å</td>
<td>Angstrom</td>
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<tr>
<td>AD</td>
<td>Alzheimer's Disease</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazole</td>
</tr>
<tr>
<td>APOE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
</tr>
<tr>
<td>Aβ</td>
<td>amyloid beta peptide</td>
</tr>
<tr>
<td>BACE-1</td>
<td>beta-site APP cleaving enzyme</td>
</tr>
<tr>
<td>BAK</td>
<td>Bcl-2 homologous antagonist killer</td>
</tr>
<tr>
<td>BAM</td>
<td>Brewster Angle Microscopy</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>D-</td>
<td>right-handed isomer</td>
</tr>
<tr>
<td>DOPC</td>
<td>dioleoylphosphatidylcholine</td>
</tr>
<tr>
<td>DOPG</td>
<td>dioleoylphosphatidylglycerol</td>
</tr>
<tr>
<td>DPPC</td>
<td>dipalmitoylphosphatidylcholine</td>
</tr>
<tr>
<td>ELISAs</td>
<td>enzyme-linked immunosorbent assays</td>
</tr>
<tr>
<td>EMR</td>
<td>Electromagnetic radiation</td>
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<tr>
<td>GAGs</td>
<td>glycosaminoglycans</td>
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<tr>
<td>IAPP</td>
<td>islet amyloid polypeptide</td>
</tr>
<tr>
<td>L-</td>
<td>left-handed isomer</td>
</tr>
<tr>
<td>MCI</td>
<td>mild cognitive impairment</td>
</tr>
<tr>
<td>MME</td>
<td>membrane metallo-endopeptidase</td>
</tr>
<tr>
<td>Acronym</td>
<td>Full Form</td>
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<tr>
<td>NDP</td>
<td>nucleation-dependant polymerization</td>
</tr>
<tr>
<td>NIDDM</td>
<td>noninsulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-d-aspartate</td>
</tr>
<tr>
<td>PC</td>
<td>phosphatidylcholine</td>
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<tr>
<td>PE</td>
<td>phosphatidylethanolamine</td>
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<td>PG</td>
<td>phosphatidylglycerol</td>
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<td>PI</td>
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<td>PSEN1</td>
<td>presenilin 1</td>
</tr>
<tr>
<td>PSEN2</td>
<td>presenilin 2</td>
</tr>
<tr>
<td>PTFE</td>
<td>polytetrafuloroethylene</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>TFA</td>
<td>trifluoroacetic acid</td>
</tr>
<tr>
<td>TFE</td>
<td>2,2,2-Trifluoroethanol</td>
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<td>ThT</td>
<td>Thioflavin T</td>
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Chapter 3: Langmuir trough

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Chapter 1

Introduction to Alzheimer’s disease and biomedical activities of amyloid beta peptide
1.1 Alzheimer’s disease

1.1.1 Prevalence of Alzheimer’s Disease

Alzheimer’s Disease (AD) is the most common type of dementia. In 2001, about 24 million people worldwide had dementia. It is expected that by 2040, about 81 million people are going to have dementia. Among those, about 60% of the cases of dementia are AD. According to Forecasting The Global Burden of Alzheimer’s Disease, the number of the population who suffered from AD in 2006 was 26.6 million. In the UK, the incidence of AD is 1.95 per 1000 person-years. The primary risk factor is aging. People who are aged more than 65 years are more likely to have AD. It has also been suggested that women have higher risk developing AD, especially those who are older than 85.

The first case of Alzheimer’s Disease was reported by German psychiatrist Alois Alzheimer in Tubingen, Germany in 1906. A few years later, Emil Kraepelin first described the disease and named it Alzheimer’s Disease.

3. Forecasting the global burden of Alzheimer's disease Ron Brookmeyer, Elizabeth Johnson et all John Hopkins University Department of Biostatistics working papers Year 2007, Paper 130
1.1.2 Symptoms

AD is a slowly progressive neuron degenerative disease. A definitive diagnosis cannot be made based only on the clinical symptoms. The early clinical phase is called mild cognitive impairment (MCI), in which patients suffer from mild memory loss and impairment of learning. Patients often have problems remembering recent events and learning new information. However, these symptoms can be mistakenly related to a normal aging process or just stress. The onset of neurodegeneration can occur a long time (20-30 years) before the first symptoms show. As AD processes, patients have more problems dealing with daily activities. For example, speaking problems, difficulties in executive functions and apathy can be observed. MCI is also observed in people who undergo the normal aging process, as well as in vascular dementia. This is why diagnosing AD can be difficult, as it can be easily confused with aging or other types of dementia.

1.1.3 Diagnosis

The diagnosis for AD is normally based on the medical history and clinical examination. Neurological and psychiatric examinations can also be used to help with the diagnosis.

The National Institute of Neurological and Communicative Diseases and Stroke and the Alzheimer’s Disease and Related Disorders Association (NINCDS-ADRDA) published the criteria for diagnosis of AD in 1984. It has been widely used for diagnosis of AD. The criteria follow a combination of diagnosed dementia and clear clinical features of AD. The other commonly used criteria are found in Diagnostic and Statistical Manual of Mental Disorders, fourth edition. (DSM-IV-TR)\(^7\). To meet these criteria, the suspected AD patients have to present with memory loss and impairment of more than one cognitive domain in the brain. The NINCDS-ADRDA criteria are not very precise. Their specificity is about 70% and their sensitivity about 80\%.\(^8\) The figures are even lower in patients who have mild or early stage AD. The only accurate and definite diagnosis so far only relies on neuropathology.

Neuropathological techniques are used to examine AD involves by the use of neuroimaging, CT and MRI. The application of these techniques can rule out the

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possibilities of other kinds of dementia. CT and MRI can help to visualise the
degeneration of the brain or a brain tumour. Neuroimaging is used to diagnose
cerebrovascular disease.

1.1.4 Genetic factors

AD is both a sporadic and familial disease. The prevalence of the familiar AD is
below 0.1%. The familial form is observed in patients who are younger than
65 years. The identified affected genes are the APP gene on chromosome 21,10
the PSEN1 gene and the PSEN2 gene. Other research has shown that the
apolipoprotein E (APOE) ε4 allele plays an important role in sporadic AD.11
ApoE has been suspected of promoting amyloid fibrillation. As a cholesterol
transporter, ApoE4 is less active in transferring and reusing membrane lipids as
well as in repairing neurons. The APOE ε4 allele in heterozygotes increases the
risk of AD by 3 times and in homozygotes by 15 times.121314

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expressing mutant tau and APP. Science 2001;293:1487-91.
1.1.5 Characteristics of Alzheimer’s disease

The medial temporal lobe is where the first pathological changes happen in AD. According to evidence from CT and MRI, hippocampal impairment occurs in the disease. Such hippocampal atrophy as revealed by MRI may distinguish AD from non-AD disease.\(^\text{15}\) [Fig 1.1.5.1] However, such impairment also occurs in the hippocampi and entorhinal cortex in other types of dementia sufferers.

![Normal brain scan (left) and AD patient brain scan (right)](http://www.sth.org.hk/medical_service.asp?lang_code=en&dept_code=NKS)

Fig 1.1.5.1 Normal brain scan (left) and AD patient brain scan (right)

Tangles and plaques found in the central neuron system are characteristic of AD. [Fig 1.1.5.2] They first appear in the medial temporal lobe and cortical regions. Among these tangles and plaques, dead neurons can be seen. Other types of damage, such as the degeneration of white matter, oxidative damage and amyloid angiophathy can also be found.\(^\text{16}\)

\(^\text{15}\) Jagust W. Positron emission tomography and magnetic resonance imaging in the diagnosis and prediction of dementia. Alzheimers Dementia 2006; 2: 36–42.

Fig 1.1.5.2 Plaques and tangles are shown in the brain
(http://ladulab.anat.uic.edu/)

Neurofibrillary tangles are also found in the pyramidal neurons of the medial temporal lobes. They are found in AD as well as some other neurodegenerative diseases. The number of tangles indicates the severity of the AD. The tangles are caused by the hyperphosphorylation and aggregation of tau protein, whereas the plaques (or fibrils) are present in the neocortical terminal areas. The major components of the plaques are amyloid beta (Aβ) peptides, where they are present in the fibril form.

1.1.6 Treatment

1.1.6.1 Aβ fibrils conformation inhibitors

Some studies have proven that Aβ fibrillisation can be interrupted by certain chemicals by preventing the formation of β sheets. For example, glycosaminoglycans (GAGs) have been proved to bind with Aβ. Some drug candidates are designed to interfere the binding between GAGs and to prevent further GAG-induced accumulation of Aβ. Alzhemed is one of these candidate drugs.

Metal chelators are potential drug candidates that work by interfering with the association between Aβ and Cu²⁺ and Zn²⁺ that potentially prevent AD progressing. A number of chelator candidates are being studied, such as desferrioxamine, bicyclam analogue JKL169 and triethylenetetramine. They all show some therapeutic potential by their high affinities to the ions. However, further research is required to evaluate these candidates better.

1.1.6.2 Antioxidants

The links between Aβ and oxidative stress are well studied. It has been reported that the progress of AD can potentially be slowed down by the intake of antioxidants because of the effects they have on cognitive impairment in the brain caused by oxidative stress. Some studies have shown the benefits of
dietary intake of Vitamin E. However, the effects are only very modest. Vitamin C and gossypin are under study as well, but their effects are subtle. Melatonin has displayed an ability to protect neurons against Aβ plaques in in vitro experiments, and has shown its potential for improving cognitive functions in model mice. Some recent studies have drawn attention to curcumin, a widely used Indian spice as well as a food preservative. Curcumin is believed to prevent amyloid formation. Fewer brain amyloid plaques were found in amyloid loaded mice after administration of curcumin by injection or by mouth. It has also been reported to serve as a metal chelator due to its ability to bind Cu and Fe ions.

Although showing potential therapeutic value, none of the drug candidates mentioned above have been clinically proved to be an effective treatment for AD. So far, there is no medication available to cure AD. Although some of them have the potential to delay the progress of this disease.

1.2 Amyloid β peptide

Amyloid proteins, or amyloids are a group of proteins that share similar structural properties. Amyloids form aggregates that later on develop to fibrils and present in body. Quite a few human diseases are caused by abnormal amyloid aggregation, such as Alzheimer’s Disease, Parkinson’s Disease and Diabetes mellitus type 2, whose featured proteins are respectively Amyloid β, Alpha-synuclein, and Islet Amyloid Polypeptide (IAPP). The main topic of this thesis is the potential cytotoxicity of Aβ. Hence that is the focus of this chapter.

1.2.1 APP and the generation of Aβ

The presence of amyloid deposits, formed from mis-accumulated protein in amyloid diseases underpins the amyloid hypothesis of AD\textsuperscript{23-24}. In this hypothesis, Aβ peptide is the membrane active agent that triggers AD. Aβ peptide is the product of a series enzymatic actions on amyloid precursor protein (APP). APP consists of 695-770 amino acids and has a large hydrophilic N-terminal extracellular domain, a single hydrophobic transmembrane domain and a small C-terminal domain, which is immersed in the cytoplasm [Fig

\begin{thebibliography}{99}
\end{thebibliography}
1.2.1.1. The cleavage of APP involves three enzymes. In the non-amyloidogenic pathway, α-secretase initiates the cleavage of APP within the Aβ fragment of the long chain of APP. This releases an ectodomain of APP (sAPPα) and a C-terminal domain (C83), which is later digested by γ-secretase. Thus, this process prevents the formation of Aβ25. However, in an alternative, amyloidogenic pathway, APP is cleaved by β- and γ-secretase. β-secretase 1, which is also known as beta-site APP cleaving enzyme (BACE-1), cleaves APP to form two segments, the N-terminal and the C-β terminal of APP. The C-β terminal fragment is the target of γ-secretase, cleavage of which produces a number of isoform peptides of 36-43 amino acid residues in length (Aβ peptide) and AICD, a 50 amino acid residue. The residues of Aβ peptide locate partly within the ectodomain and partly within the transmembrane domain of APP26 [Fig 1.2.1.2], and AICD is released to after a chain action by γ-secretase.

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Aβ peptides are normally 40-amino-acids in length (Aβ_{40}) and 42 residues in length (Aβ_{42}). In central neuron system, monomers of Aβ_{40} are more dominant than Aβ_{42}. However, Aβ_{42} is the prevalent isoform found in the amyloid plaques. It is more hydrophobic and has a greater tendency to aggregate into fibrils than Aβ_{40}. Both of Aβ_{40} and Aβ_{42} are prone to aggregate. 

1.2.2 Amyloid Hypothesis

Aβ peptides were initially thought to be abnormal protein. Recent studies have shown that Aβ is the constituent product during normal cell metabolism, as explained before. Therefore, it is not just the origin but also the failure of the clearance of Aβ that generates AD. Under normal circumstances, the clearance of Aβ involves neprilysin, also known as membrane metallo-endopeptidase (MME), peptidases, insulin-degrading enzyme and endothelin-converting enzyme. For Aβ transferring through the blood-brain barrier, the clearing process is under the mediation of the receptor related proteins which control the metabolism of low-density of lipoprotein and complex glycation end products. The amyloid cascade hypothesis states that the imbalance of the generation and clearance of Aβ initiates the accumulation of the peptides that eventually causes the degeneration of neurons and dementia.

In a typical Aβ clearing process, microglia are actively involved and Aβ peptides are engulfed through phagocytosis in the neuron system. Astrocytes also

participate in this process. They are activated by certain receptors and facilitate of the transfer of Aβ from brain to circulation of the whole body.\textsuperscript{30}

It is worth mentioning that the balance of generation and clearance of Aβ is also affected by the Aβ\textsubscript{42} / Aβ\textsubscript{40} ratio. The level of Aβ\textsubscript{42} is elevated by genetic mutations, in particular, the mutation of genes of APP, presenilin 1 (PSEN1) and presenilin 2 (PSEN 2). The direct influence of enhanced levels of Aβ\textsubscript{42} results in more oligomers, which results in more fibrils in the brain.\textsuperscript{31}

\textbf{1.2.3 Amyloid fibril formation}

In the aqueous environment, the process of formation of amyloid fibrils can be described as nucleation-dependent polymerization (NDP), which occurs through several steps. The process follows a nucleation growth mechanism. This mechanism can be depicted as three stages, namely the lag phase, the exponential growth and the equilibrium phase. The lag phase is a relatively slow process in which the nucleus is being formed. Such a nucleus is a thermodynamic nucleus, which requires monomers to be bonded together, and is limited in size. The nucleus is the only structure able to promote further aggregation. The formation of this nucleus is subject to an activation energy


\textsuperscript{31} Christian Haass and Dennis J. Selkoe Soluble protein oligomers in neurodegeneration: lessons from the Alzheimer’s amyloid β-peptide Nature volume 8 February 2007
barrier. Once the nucleus is formed, the energy barrier is overcome and the fibril growth proceeds rapidly, which characterises the next stage. During this process, many intermediate species are generated, which are quasi-stable \(^\text{12}\). Eventually, as the concentration of monomers in solution falls, an equilibrium phase is reached. [Fig 1.2.3] \(^\text{13}\)

![Diagram of amyloid fibril formation by nucleation-dependant polymerization (NDP)](image)

**Fig 1.2.3 Amyloid fibril formation by nucleation-dependant polymerization (NDP)**

**1.2.4 Tau protein**

Another pathological marker of AD is neurofibrillary tangles. Those tangles can be found in pyramidal neurons. The number of the tangles also indicates the
severity of AD, along with the number of amyloid fibrils. Such tangles are composed of aggregated hyperphosphorylated tau protein. Binding to microtubules, tau is an axonal protein that normally promotes the stability and assembly of microtubules. It also enhances vesicle transport. The phosphorylation of tau is regulated by some kinases and phosphates. When the balance of the regulation is disturbed, tau protein becomes hyperphosphorylated. In this form, tau has the tendency to self-associate and thus become insoluble.

The insoluble form of tau loses its links to microtubules and causes the disassembly of microtubules. As a result, it further damages axonal transport and the functions of neurons and the synapse. The total amount of tau in cerebrospinal fluid and the abnormal high levels of phospho-tau amino acids T181 and T231 can be used as a biological marker in examining AD. There is some evidence to suggest that tau aggregation is related to the accumulation of Aβ. The presence of Aβ can trigger further tau accumulation.

1.3 Biomedical activities of Aβ

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Aging itself has impact on synapse loss. Brain scans of AD patients who have MCI show that the number of hippocampal synapses decline. Also, the remaining synapses are bigger in size as a compensatory response. As AD develops, the communication between neurons cannot be achieved by synapses. Brain slices of transgenic mice that over-express APP and have numerous amyloid plaques show impairment of basal transmission and long term potentiation.\textsuperscript{35} The latter serves as an indicator of memory function in synapses. As a result, the molecules responsible for sending and receiving signals are affected and the function of those molecules is inhibited. Also, endocytosis of \textit{N}-methyl-\textit{d}-aspartate (NMDA) and \textit{α}-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid surface receptors at synaptic plasma membranes inhibits the release of presynaptic neurotransmitters and disrupts the ion currents of postsynaptic glutamate receptor.\textsuperscript{36} The endocytosis of AMPA also reduces synapse currents after high frequency stimulus activities.

The abnormal amount of Aβ accumulation in the central neuron system of AD sufferers raises the question of whether Aβ introduces the impairment of neurons and eventually causes the loss of memory. Although substantial amounts of Aβ can be found in healthy elder people, the form of the Aβ is not amyloid fibrils. Mass spectrometry with of Western blotting and with enzyme-linked immunosorbent assays (ELISAs) provide a detailed and accurate

\textsuperscript{35} Larson J, Lynch G, Games D, Seubert P. Alterations in synaptic transmission and long-term potentiation in hippocampal slices from young and aged PDAPP
evaluation of Aβ. Both the quantity presented in brain and the forms of Aβ can be examined. It suggests that the soluble forms of Aβ oligomers have more influence on the severity of memory loss and cognitive impairment than amyloid fibrils.\textsuperscript{37,38,39} Also, the size of Aβ oligomers makes them easier to immerse into the synaptic clefts than fibrillar plaques. Although it is not yet known if the large forms of aggregates are inert to the neurons, or even protective to them, many researchers have suggested that neuronal dysfunction is induced by Aβ oligomers.\textsuperscript{40} The toxicity of fibrils is supported by the amount of fibrils found surrounding neurons. However those fibrils are typically found close to neuritis. This again indicates that the smaller sized oligomers may cause neuronal impairment.

1.3.1 Impact of soluble Aβ peptide on neurons and synapses

Many research groups have studied the potential mechanism of the soluble oligomers binding to synaptic plasma membranes. Some results showed BACE is stimulated by neuronal electrical activity and thus that it enhances or further generates Aβ. Such increased levels decrease the synaptic activity and synaptic

\textsuperscript{37} Naslund, J. et al. Correlation between elevated levels of amyloid β-peptide in the brain and cognitive decline. JAMA 283, 1571–1577 (2000).
transmission is interrupted. Moreover, Cirrito et al used *in vivo* microdialysis probes to demonstrate that interstitial fluid Aβ concentrations correlate with the synaptic activity in APP transgenic mice. It is possible that soluble Aβ oligomers interfere with signalling pathways of certain NMDA or AMPA receptors at synaptic plasma membranes.

1.3.1.2 Mitochondrial failure

Aβ is reported to be toxic to mitochondria. The first indicator of Aβ toxicity on mitochondria is the failure of glucose utilization in the brain of AD patients. The activities of key enzymes in isolated mitochondria are inhibited by Aβ. In vivo experiments have revealed the accumulation of Aβ in mitochondria of brain cells. The possibility of Aβ toxicity on mitochondria is supported by the evidence of membrane destabilization induced by Aβ. Mitochondrial failure can be explained by Aβ interactions with mitochondrial proteins. Cytochrome c is a key protein in electron transport. Enhanced levels of cytochrome c are

present when Aβ oligomers introduced to HT-22 cells and targeting Bcl-2 homologous antagonist killer (BAK) in mitochondria. Further more, Aβ oligomers have been proved to bind to BAK and to accelerate the formation of apoptotic pores. Some other evidence of Aβ toxicity on mitochondria is provided by the abnormal respiratory chain enzyme activities caused by Aβ accumulation, as well as the interactions between Aβ and components of mitochondria that are important to metabolic and antioxidant actions.48 Aβ is capable of damaging mitochondrial DNA from its oxidative abilities. All this indicates a potential role of Aβ in oxidative stress in brain. Some reports supported this by stating that Aβ is the initiator of some reactive oxygen species (ROS) and reactive nitrogen species (RNS) that triggers the oxygenic damage of the brain.49

1.3.1.3 Metals, oxidative stress and Aβ

Maintaining the oxidative balance of the brain is one of the requirements of metabolic activities of the body. The balance is mainly regulated by antioxidants. Regulated active metals such as Cu and Fe take part in redox reactions in the brain and interact with oxygen. This results in the production of ROS. Several enzymes, such as cytochrome c oxidase and amine oxidase, then interact with

the activated oxygen and take part in the ROS recycling process. Aβ has been shown to associate with Cu²⁺, Zn²⁺, and Fe³⁺ and reduce the ions to Cu¹⁺, Zn¹⁺, and Fe²⁺. It thus interrupts the electron transfers in oxygen cycling in the brain. The binding site of the ions in Aβ is located between amino acid position 6 and 28.⁵⁰ Aβ affiliation to Cu and Fe is reportedly to generate ROS such as H₂O₂, although this process requires the presence of the active ions.⁵¹

1.4 Biomedical activities of amyloid peptides Aβ(25-35)

Many fragments of the full length Aβ have been investigated, among which, Aβ(25-35) is most studied. Aβ(25-35) resembles the full length peptide in structural characteristics as well as its biological toxicities. It is also the smallest peptide that remains both neuronal toxicity and peptide aggregation. Thus, Aβ(25-35) serves as a good amyloid model to study the full length Aβ peptide.

1.4.1 Structure

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Aβ(25-35) contains 11 amino acids GSNKGAIIIILM. It comes from the transmembrane site of APP with C-terminus inserting into the membrane. Naturally Aβ(25-35) cannot be produced from APP processing. It has an amphiphilic nature. In water, it has a lower tendency to solubilise compared to its much higher tendency to self-aggregate. The structure of Aβ(25-35) is not well defined and it has the potential to develop into different conformations. One study shows that monomeric Aβ(25-35) has a β-hairpin conformation and random coils. The arrangement of dimers to trimmers, and the ability of adopting the hairpin conformations into β-sheets decides the possibility of fibrillization.

### 1.4.2 Biological activities

Aβ(25-35) resembles the full length peptide in its biological activities. A study of the effects Aβ(25-35) has on mitochondria shows decreasing activities of the respiratory chain complex in neuron culture after treatment with Aβ(25-35), as well as decreased ATP concentration and mitochondria loss. As a result,

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mitochondrial failure leads to the generation of $O_2^-$ and an enhanced level of ROS.\textsuperscript{56} Aβ(25-35) is also found to facilitate the activity of monoamine oxidase which is a mitochondrial enzyme that can generates ROS.\textsuperscript{57} Moreover, the effect that Aβ(25-35) has on mitochondria also includes an ability to increase the activity of cysteine protease calpains, which results in synaptic transmission failure and memory impairment.\textsuperscript{58}

Aβ(25-35) also demonstrates its effects on the nucleus. DNA fragmentation happens after treatment of Aβ(25-35) in vivo.\textsuperscript{59} Polymerase, a multifunctional enzymes that repairs DNA damage is over expressed by the presence of Aβ(25-35) both \textit{in vivo} and \textit{in vitro}.\textsuperscript{60,61} An excessive amount of polymerase increases the toxicity by glutamate and may also drain ATP pool that leads to cell death.

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Tau phosphorylation produces neurofibrillary tangles in neurons, which is the other major character of AD found in AD patients. There is evidence showing that exposure to Aβ(25-35) activates tau protein kinase TPK I/GSK-3ngl The high concentration of enzyme enhances the tau phosphorylation in vitro. Thus, Aβ(25-35) increases tau phosphorylation.\(^{62}\)

1.5 L- and D- Amino acids

1.5.1 Optical isomers

The chemical term used to describe two molecules that are exactly mirror images of each other is ‘optical isomers’. If the mirror image of one molecule exists and is composed identically then this molecule or its mirror image is chiral. The two molecules are called optical isomers or enantiomers.\(^{63}\) Optical isomers contain identical numbers of identical atoms but may exhibit different physical, chemical or biological characteristics. On the contrary, if the result of a rotary reflection of a molecule is itself, then this molecule is achiral. The rotary reflection is a combination of a rotation around an axis and a reflection in a plane. Chirality of molecules is widely used, for example, in the fields of

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chemistry, physics and biophysics. Fig 1.5.1 gives an example of the optical isomers of alanine.

Fig 1.5.1 L- and D- Alanine


1.5.2 Naming

1.5.2.1 Optical character

When polarized light travels through an isomer, the transmitted light changes its rotation of plane. If the light rotates clockwise after passing by an isomer then this isomer is named (+), or d-. If the light rotates counter clockwise, then the isomer is named (-), or l-. The use of d- and l- comes from Latin words dexter and laevus, respectively.
1.5.2.2 Structural character

More commonly, the naming of optical isomers is by their special configuration related to chiral molecule of glyceraldehyde. The two isomers of glyceraldehyde are named D- and L-, in smaller capitals. The structure of molecules is compared to the isomers of glyceraldehyde and being named as L- or D- isomers.

The use of optical isomers is essential in my research. The structure of one isomer is different from the other. When small molecules form into larger peptides they obtain more complicated structures. For example, if D-peptides form into larger peptides and have a β-shaped structure, then the rotation of the β plane is different from that of L-peptides. Using optical isomers is very useful in studying the fibrillization, because hypothetically when mixed the two isomers the progress of fibrillation is slowed down by the lower chance of the same type of structure (D- alone or L- alone) elongating and extending to form a larger structure. If it is true, using isomers can be the key in studying which type of structure of Aβ causing the membrane toxicity. In this hypothesis, more fibrils exist in the system of L- peptides alone or D- peptides alone than in the mixture of the two. Using the system of one type of isomers and the mixture of the isomers on membrane helps to decide membrane active species.
1.6 Aim of my research

My research focuses on the membrane activities of \( \text{A} \beta \). Amyloid diseases such as AD and noninsulin-dependent diabetes mellitus (NIDDM) are reported to be related to cell damage and cell death in different tissues and organs. This leads to our hypothesis of the common pathogenic mechanism of \( \text{A} \beta \), the potential membrane dysfunctions caused by \( \text{A} \beta \). The first question my work aim to answer is whether \( \text{A} \beta \) interact with membrane.

There is a growing body of evidence that shows that the mature amyloid fibrils do not have membrane activities. Instead, the amyloid intermediates formed by a variety of different amyloidogenic peptides are membrane-active\(^{64}\). It has been shown in previous studies of amyloid diseases that the membrane active agents are the amyloid intermediates. For example, studies about islet amyloid polypeptide (IAPP) illustrated that non-fibril forms of IAPP penetrate into voltage clamped planar bilayers, monomeric IAPP and IAPP fibrils can not. Also, IAPP oligomers cause the death of pancreatic beta cells by apoptosis and necrosis.\(^{65,66}\) In previous studies, researchers used Langmuir films and laser

light scattering\textsuperscript{67} to support that non-fibril IAPP has membrane activities on both monolayer and membrane bilayers. Also, studies have shown the time scale of intermediates IAPP peptides penetrating lipids monolayers. The previous research provided fundamental information of amyloid membrane activities and it corresponds to the hypothesis suggest by Ashley et al. of the probability of such activities. The binding site of amyloid might be the exposed hydrophobic domain at amyloid’s carboxyl terminal, which gives the amyloid opportunity to insert into lipid bilayers.\textsuperscript{68} The previously studied membrane activities of the amyloid intermediate has suggest the key role it plays in pathogenesis of amyloid disease. That leads to another question this work focuses on. Are the fibrils or the intermediates are membrane active agents? How do the interactive species affect membrane?

The use of optical isomers is essential in my research. Using optical isomers is very useful in studying the fibrillization, because hypothetically when mixed the two isomers the progress of fibrillizaion is slowed down by the lower chance of forming the same type of structure (D- alone or L- alone). The fibrils are formed by the repeated $\beta$ structures. When there are mixtures in the solution, it is less possible to form fibrils than using the same type of isomers. By comparing the different effect on membranes caused by one type of isomers and the mixtures,


\textsuperscript{68} Ashley, R.H., et al., \textit{Autoinsertion of soluble oligomers of Alzheimer’s Aβ(1–42) peptide into cholesterol-containing membranes is accompanied by relocation of the sterol towards the bilayer surface}. BMC Structural Biology, 2006. \textbf{6}: p. 21-21.
it is easier to tell if the fibrils are the more active species or the intermediates are.
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Chapter 2

Fibril growth
2.1 Protein misfolding

Central to any study of the mechanism of Alzheimer’s Diseases is the study of protein folding and misfolding. More and more evidence has shown that protein misfolding plays a fundamental role in the biological and pathological processes of AD. For example, fibrillar assemblies of peptides that characterize a β sheet structure have been discovered to have different physiological functions.

Amyloid fibrils are found in central nervous systems of patients suffering from many amyloid related diseases such as AD, Prion disease, Parkinson’s disease and type II diabetes, etc.1 Although generally accepted as the biomedical markers of those diseases, the roles of amyloid aggregates are still not fully understood. This chapter mainly discusses the driving forces, mechanism and progress of amyloid fibril formation, and the potential role fibrils play in AD.

2.1.1 Protein structure and stability

Proteins are large molecules that contain one or more than one polypeptide

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chains of amino acid residues. The range of size, in terms the number of amino acids residues can be from tens to thousands. Different sequences of amino acids define different proteins. The same protein can have alternative 3-dimensional structures, which are referred as conformations of the protein. Due to the physical size of proteins, which is between 1-100 nm, they are classified as nano-particles. The size of protein and different groups of amino acids make proteins a complex subject to study. One protein can undergo complicated structural changes. For example, some peptides can form aggregates and the process can sometimes be reversible. Such changes in structure may also reflect in the protein's biological function.

Aβ peptides are normally 40-amino-acids in length (Aβ (1-40)); a less abundant form is 42 residues in length (Aβ (1-42)). However, Aβ (1-42) is the prevalent isoform found in the amyloid plaques. It is more hydrophobic and has a greater tendency to aggregate into fibrils than Aβ (1-40), therefore Aβ (1-42) is considered to be more toxic to neurons. Among the most studied fragments of Aβ (1-42), Aβ (25-35) is claimed to represent the biologic activity of Aβ since it is believed to retain the toxicity of the full length Aβ peptide. Some studies indicate that Aβ (25-35) aggregate into β sheets in a process similar to that of

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Aβ (1-42). However, other researchers have focused on the study of Aβ (31-35) and conclude that this shorter fragment may trigger the apoptotic process in neurons. In some other studies, Aβ(16-20) is believed to be the central hydrophobic area causing Aβ self-accumulation.

2.1.2 Fibril formation

The process of protein fibril formation is related to protein stability. There are two types of protein degradation, namely chemical degradation and physical degradation. Chemical degradation happens when covalent bonds are modified, such as the process of oxidation, deamidation, or disulfide bond shuffling. Physical degradation refers to any other kinds of protein unfolding, including protein aggregation and undesirable adsorption to surfaces. Non-native

aggregation may happen repeatedly in the process of folding and refolding, purification, and other processes. Hence, it can be particularly difficult to study. Sometimes aggregation occurs in solution environments under neutral pH and under thermodynamically favored temperature (37°C), and sometimes even without the presence of stresses. Non-native protein aggregation is usually an irreversible process. The final aggregates often contain high concentrations of intermolecular-sheet structures.9 The onset and aggregation rate of proteins have proved to be strongly dependent on the environment of the solution. For example, the temperature, pH, salt type and concentration and presence or absence of surfactants highly influence the process of aggregation.10

The onset of the protein mis-folding is a process called denaturation, during which the protein acquires the ability to propagate infinitely into aggregated structures.

A few studies have shown that the size of intermediate aggregates ranges from dimers to complexes of a million Dalton, or even bigger.1112 Electron and atomic

force microscopy have been used to study the sizes of soluble spherical aggregates of a great number of types of amyloids. These aggregates tend to be about 3–10 nm in diameter.\textsuperscript{13} These aggregates, sometimes called micelles or protofibrils,\textsuperscript{14} are considered to be the intermediates of the pathway of the formation process and therefore have been the focus of much research effort. Some spherical units may combine together to form more curvilinear aggregates, which are seen as the origin of the fibrils, and are therefore called protofibrils. However, to form protofibrils requires a much longer aggregation time.\textsuperscript{15} Most of the aggregates that have been observed have a cross-β structure. (Fig 2.1.2) These β structure aggregates are the final stages of the protein’s conformational change. Those final products are 6-10nm in size and usually have a smooth surface, if not helical.\textsuperscript{16} Atomic force microscopy reveals similarities in the spectrum and structure of different types of amyloid aggregates.

\textsuperscript{13} Anguiano M, Nowak RJ, Lansbury Jr PT. Protofibrillar islet amyloid polypeptide permeabilizes synthetic vesicles by a pore-like mechanism that may be relevant to type II diabetes. Biochemistry 2002;41:11338–43.  
Fig 2.1.2 An example of cross beta structure

(http://www.humpath.com/IMG/jpg_amyloid_fibrils_04_1.jpg)

2.1.3 Nucleation growth mechanism

Amyloid fibril formation is explained in 1.2.3. Another theory is the Lumry-Eyring framework that is a well-known model that helps to explain and analyze the fibril aggregation pathway. Formula 2.1.3 is an illustration of Lumry-Eyring framework. It involves two stages of aggregation, a structural change of protein, which is reversible, and an irreversible stage of non-native species aggregation assembling, following the first stage.\(^{17,18}\)

1) N ⇔ TS ⇔ A

2) A_i + A_m = A_{m+1}  

N represents the native stage of protein, TS is the transitional state of the protein which leads to the formation of a further aggregation A_i. A_i is seen as the intermediate. A_m are the aggregates containing m molecules and A_{m+1} are aggregates that have m + i molecules. As mentioned before, the rate of the series of reactions is determined by the thermodynamics and kinetics. This theory involving the transitional state TS can be graphically explained in Fig 2.1.3.2.

![Diagram of protein aggregation](image)

**Fig 2.1.3.2** The kinetics of protein aggregation

The arbitrary free energy y-axis represents N, the free energies of reactant, TS, the transitional state, and A_n and A_m, the intermediates and final aggregates. On
the x-axis, the process of each step of the series of reactions is explained.

Thermodynamically favored final products $A_m$ have the lowest free energy and are thus are at the bottom of the y-axis. The curved lines are energy barriers that exist throughout the whole event. Between reactants and products the energies required to produce each different molecular configuration are different. Hence the energy lines are at different levels. $\Delta G$ is the activation free energy, which is the difference between the transition state and reactant. The transition state has the maximum energy configuration. The factor that decides the rate of the protein aggregation in the multiple step reaction is the step that needs the highest $\Delta G$. An irreversible reactions follow a traditional reversible state. The order of the aggregation reaction depends on the step that requires highest $\Delta G$.

The aggregation transition state determines the conformational stability of the protein native state and the whole process of fibrils aggregation depends on this state. At the conclusion of this process, the native protein losses its structures and aggregation starts. The half-unfolded proteins are easier and more prone to aggregate and they subsequently go through the transitional state. In this state, some of the native assemblies are combined with structurally-changed species and enter the next and final stage of fibril formation procedure. Protein molecules can also assemble to form higher order aggregates in addition to the
formational changes happening during aggregation. The process of protein assembly happens as a result of interactions of attractive intermolecular.

The number and size of aggregates both increase when the native protein population is diminished following the onset of aggregation. Aggregates become visible after their growth and precipitate out of solution and can be visually detected and observed. As in the first theory explained before, a lag phase is shown from the aggregation of some proteins. In this case, the loss of native protein cannot be detected and the solution containing proteins is still clear. Later on, a rapid loss of native protein is usually detected. On the other hand, there is evidence that the presence of protein seeds has been shown to reduce the lag time dramatically and also helps to promote the aggregation process.¹⁹

In both theories, the process of protein aggregation is actually a nucleation and growth event that the aggregates first accumulate and then exceed their solubility and eventually precipitate out from solution. An energy barrier existing in the nucleation always causes the lag phase in the aggregation process. The free energy required to produce the intermediates creates the barrier. The size of the aggregates also interferes with the process.²⁰ The nucleus only

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grows when the size of the next phase is higher than the energy barrier.

2.2 ThT binding

2.2.1 The use of ThT

Thioflavin T (ThT) is a benzothiazole salt. ThT was first discovered to show enhanced fluorescence after binding to amyloid fibrils in tissues by Vassar and Culling. The spectroscopic properties of amyloids change dramatically after the binding, giving rise to a wide range of studies of conformational change in amyloid fibrils. Congo red has been widely used to detect the presence of amyloid fibrils in tissues. However, it is much less sensitive than ThT, so it is no longer the stain of choice for studies of amyloid fibrils. ThT has been proved to be highly specific for the detection of amyloid compared to other dyes such as Congo red, crystal violet and van Gieson. ThT is widely used not just in amyloid diagnosis in vivo, but has also been used to provide information about

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amyloid fibril formation *in vitro*, and for monitoring amyloid fibril growth. Combining the use of fluorescence spectroscopy and ThT gives insight into amyloid fibril growth and allows the visualization of fibril formation.

### 2.2.2 ThT structure

ThT is a benzothiazole dye whose structure is shown in Fig 2.2.2. The structure of ThT can be viewed as a hydrophobic group consisting of a phenyl group with a dimethylamine attached to it, and a hydrophilic benzothiazole group that has a polar N and a polar S. The benzylamine and benzathiole rings can rotate freely at the carbon–carbon bond in solution, which suppresses photons emission and gives rise to low level of ThT fluorescence.\(^{24}\) When ThT binds with amyloid fibrils, the freedom of carbon–carbon bond rotation is much limited because of the presence of ThT- amyloid binding site. \(^{25}\) As a result, the ThT fluorescence is triggered and enhanced when the amyloid fibrils extend.

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2.2.3 Mechanism of ThT binding amyloid fibrils

There have been a few hypothetical explanations of how ThT binds to amyloid fibrils. The widely accepted view is that ThT binds with the surface of the cross β section of amyloid fibrils. In amyloid β fibrils, the side chains form a ladder like structure in which each ‘strand’ of the chains is parallel to other strands along the long axis of the fibrils (Fig 2.1.2). ThT binding sites are parallel to the long axis of amyloid fibrils as revealed by polarized fluorescence microscopy\textsuperscript{26}. It is not difficult to conclude that the side chains might be the bonding sites. Further evidence shows that the side chains form ‘channel like’\textsuperscript{27} structures along the fibrils that are exposed to solvents. These structures can bind to ThT

regardless of the peptide sequence.\textsuperscript{27,27,28} This is supported by molecular
dynamics stimulations.\textsuperscript{29} It has also been suggested that there are particular
sites in fibrils that have high affinity to ThT such as aromatic residues.\textsuperscript{27} Studies
have also shown that ThT does not bind with highly charged fibrils, \textsuperscript{30} or to
cross $\beta$ sessions with fewer than 4 aromatic-hydrophobic residues.\textsuperscript{27}

Another hypothesis of ThT binding describes the interactions between ThT
micelles and amyloid fibrils. ThT is composed of a hydrophobic group and a
polar group, which allows ThT molecules to form micelles with the polar group
facing outside in aqueous solution. Khuranna et al concluded that the enhanced
ThT fluorescence corresponds to the formation of micelles and the binding is
the hydrophobic interactions between micelles and fibrils.\textsuperscript{25}

The wide usage of ThT staining for studies of amyloid fibrils is also due to its
high specificity. ThT does not interact with any other forms of protein, such as
folded or partially folded monomers of soluble oligomeric proteins. While it has
not been proved that ThT definitely does not bind to such complexes, no

\textsuperscript{28} Biancalana, M., et al., Aromatic Cross-Strand Ladders Control the Structure and Stability of
\textsuperscript{29} Wu, C., et al., The Binding of Thioflavin T and Its Neutral Analog BTA-1 to Protofibrils of the
Alzheimer’s Disease A beta(16-22) Peptide Probed by Molecular Dynamics Simulations. Journal
\textsuperscript{30} Leonid Breydo, N.M., Iliia V Baskakov, Methods for conversion of prion protein into amyloid
changes in ThT fluorescence have been observed.

2.3 Fibril growth experiment

2.3.1 Materials

All reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise noted. Water for all solutions was deionized with Synergy 185UV Ultrapure Water System (Millipore Corporation, Billerica, MA).

1. Phosphate-buffered saline (PBS) buffer (pH = 7.4)
2. Thioflavin T (ThT) from Invitrogen (Carlsbad, CA)
3. Before experiment premake 1 mM stock in water and store at room temperature in the dark, avoiding light.
4. 3/32-in. diameter Teflon spheres from McMaster-Carr (Los Angeles, CA)
5. 96 well flat bottom non-treated polystyrene assay plates from Corning Life Sciences (Acton, MA).
6. Mylar plate sealers from Thermo Labsystems (Franklin, MA).
7. Micro-plate fluorescence reader from Fluoroscan Ascent CF, Thermo Electron
8. Corporation (Waltham, MA) with excitation at 444-nm and 485-nm emission filters.

9. 1.5 ml plastic tubes from Fisher Scientific. (Pittsburgh, PA)

10. Previously monomerised amyloid beta (25-35)

2.3.2 Methods

2.3.2.1 Monomeric peptide preparation

The literature records a number of different procedures for the production of monomeric Aβ (25-35). In this experiment I used the method published by Jao et al. In brief, the process involves dissolving Aβ in trifluoroacetic acid (TFA) to give a concentration of 1 mg/ml in a glass tube. A few minutes of sonication is required, until the solution goes clear. The peptide is then dried for a few minutes under nitrogen in a fumehood. In TFA, Aβ can remain monomeric. In principle, small aliquots can be dried and kept at 4°C until needed. However, it is better to prepare fresh material before each experiment.

TFA is very easily oxidized. It is best is to buy it in small aliquots of 1 ml each, rather than leaving a bottle open over several weeks or months.

2.3.2.2 Conversion of Aβ into Amyloid Fibrils

1. The process of Aβ conversion into amyloid fibrils was observed from a semi-automated setup in triplicate to ensure reproducibility. The method has previously been described by Leonid Breydo et al in Methods for conversion of prion protein into amyloid fibrils. Three Teflon spheres were added to each well of a 96-well plate before any solution was added. PBS buffer and thioflavin T were mixed in a plastic tube to make ThT stock with the final ThT concentration 0.01mM. When mixing the solutions the tube should be covered to avoid light. Stocks of solution of Aβ were finally mixed in the tube to make the Aβ solutions at desired concentrations. The tube was shaken thoroughly and 160μl added per well. The plate was covered with a plate sealer. The mixing process should be as fast as possible, as that the progress of fibril formation takes place soon as the Aβ peptide is hydrated. In my experiment, different

concentrations of Aβ solutions were examined, namely 250μM, 125μM, and 62.5μM. The sample of each concentration of Aβ solutions was examined three times and the solutions of the same sample were added in three wells next to each other.

2. The plate was inserted into the incubation shaker. The incubation set up was: Temp: 37°C; shaking speed: 900 rpm; shaking diameter 1 mm; fluorescence measurements every 5 minutes; excitation at 444 nm and emission at 485 nm.

3. The data were transferred to Origin from OriginLab Corp. (Northampton, MA) after the experiment and fitted to the following equation:

\[ F = A + B + c \times t \]

where A is the starting point of ThT fluorescence, B is the change in ThT fluorescence during the experiment, and c is an empirical parameter. It indicates the changes of fluorescence emission after fibril formation.

The experiment lasted for 24 hours in total, and 288 readings for each well were recorded.
2.3.3 Results

Each concentration of Aβ solutions was examined three times in triplicates to assure the reproducibility. It took more than 20mins for the ThT fluorescence intensity begin to grow (from 0 to 1), followed by another 20mins for the intensity to grow to its maximum, for most of the Aβ solutions that examined. Fig (2.3.3.1-6) show the ThT fluorescence for PBS control and each Aβ solution at different concentrations. Table 2.3.3 compares the maximum fluorescence for each Aβ solution at different concentrations. Fig (2.3.3.7-10) show at each concentration the average ThT fluorescence for different Aβ solutions.

1. For the same concentration of the peptide, L- or D- amyloid alone has higher intensity than the mixture. The L-/D- 50/50 mixture had the lowest intensity compared to others, except for one recording for Aβ concentration at 0.5mM. (Fig 2.3.3.7) The mixture of L-/D- 75/25 and mixture of L-/D- 25/75 has slightly more intensity than L-/D- 50/50, but much less that L-/D- alone. (Fig 2.3.3.1-5)

2. It took much longer for the peptide at low concentration (0.0625mM) to reach to the maximum intensity, especially for L-/D- 50/50. (Fig 2.3.3.10)
3. For the same concentration of Aβ solutions, the maximum fluorescence of the triplets happened at different time. (Fig 2.3.3.1-5)

4. The maximum ThT fluorescence of the same peptide at the same concentration is slightly different from one to another triplet. (Fig 2.3.3.1-5)

It is safe to conclude that in the solution, the mixtures of L- and D- amyloid obviously form less fibril. For the same concentration of the peptide, the average maximum intensity for L-/D- 50/50 is much less than the intensity of L-amyloid or D-amyloid alone. The progress of fibril formation is complicated and involves a few stages. The lag phase that initiates the formation usually takes much longer time compared to the following stages. The progress of fibril growth depends on the energy barrier in the solution and the chance of the molecules encountering each other to form nucleus and to elongate the peptide. It takes longer time for the mixture of L- and D- peptide to form fibrils because it has less structure of the same kind to form fibrils. When the different structures (L- or D-) of Aβ encounter each other, they tend not to elongate to form the fibrils.

In different solutions, even with the same peptide at the same concentration, the
chances of the molecules running into each other and elongating to fibrils are different. It is possible that the fibril formation starts at different time in different solutions and in different solutions, the final amount of fibrils may not be the same. The chance of β sheets exposing their bonding spots to ThT and finally binding with ThT can be different from one solution to another, even when the concentration of Aβ is the same. The maximum fluorescence depends on the amount of the fibrils in the solution and the exposure of the bonding points of β sheets. Hence in the experiment the maximum fluorescence of the three triplets happened at different time, and from one well to another the maximum fluorescence is different. In general, for the same peptide, the higher the concentration was, the higher the fluorescence intensity collected. (Fig 2.3.3.1-5)

After it reached the maximum ThT fluorescence intensity, the signal of the intensity started to go down. As the fibril elongates, the weight of the fibril become heavier. As a result, the fibril drops to the bottom of the solution when it is too heavy. Also, as the fibril elongates, the fibrils pile up. The binding point of the fibril and ThT might be covered by other fibrils or other parts of its own. As the fibrils grow, the structures that give rise to the fluorescence are covered or sink to the bottom of the wells to make weaker fluorescence signal or to make the signal difficult to collect.
Fig 2.3.3.1 L-Aβ fibril growth. The collected maximum ThT fluorescence for L-Aβ at concentration 0.5mM, 0.25mM, 0.125mM and 0.0625mM is 203.7, 167.8, 48.4, and 4.76. The maximum fluorescence occurred respectively at 2700s, 3600s, 5100s, and 21300s after reading.
Fig 2.3.3.2 D-Aβ fibril growth. The maximum ThT fluorescence for D-Aβ at 0.5mM, 0.25mM, 0.125mM and 0.0625mM is 195.4, 145.9, 34.93 and 8.078. The average maximum fluorescence occurred respectively at around 900s, 1200s, 1800s, and 3600s after reading.
Fig 2.3.3.3 L-/D-Aβ fibril growth. (L-/D- 50/50) The maximum ThT fluorescence for L-/D-Aβ at 0.5mM, 0.25mM, 0.125mM and 0.0625mM is 129.0, 56.72, 12.43 and 3.375. The average maximum fluorescence occurred respectively at around 3300s, 3000s, 4200s, and 7500s after reading.
Fig 2.3.3.4 L-/-D- Aβ fibril growth. (L-/D- 75/25) The maximum ThT fluorescence for L-/D- Aβ at 0.5mM, 0.25mM, 0.125mM and 0.0625mM is 117.7, 72.15, 16.09 and 4.90. The average maximum fluorescence occurred respectively at around 3600s, 3300s, 3900s, and 13800s after reading.
Fig 2.3.3.5 L-/D-\( \alpha \)-fibril growth. (L-/D- 25/75) The maximum ThT fluorescence for L-/D-\( \alpha \) at 0.5mM, 0.25mM, 0.125mM and 0.0625mM is 126.5, 74.46, 21.14, and 4.916. The average maximum fluorescence occurred respectively at around 1500s, 1800s, 3000s, and 4500s after reading.
Fig 2.3.3.6 PBS control. The maximum ThT fluorescence for PBS is 0.7227.
<table>
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<tr>
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<th>0.5mM</th>
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<td>L-</td>
<td>203.7</td>
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<tr>
<td>D-</td>
<td>195.4</td>
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<td>L-/D- 50/50</td>
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<td>L-/D- 75/25</td>
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<td>L-/D- 25/75</td>
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<td>PBS Control</td>
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Table 2.3.3  Maximum ThT fluorescence intensity for Aβ solutions at each concentration. The unit is in Normalized Intensity (a.u.).
Fig 2.3.3.7 Aβ peptide fibril growth at Aβ concentration 0.5mM, the data for each sample is the average of the measurements of three repeats.

Fig 2.3.3.8 Aβ peptide fibril growth at Aβ concentration 0.25mM, the data for each sample is the average of the measurements of three repeats.
Fig 2.3.3.9 Aβ peptide fibril growth at Aβ concentration 0.125mM, the data for each sample is the average of the measurements of three repeats.
Fig 2.3.3.10 Aβ peptide fibril growth at Aβ concentration 0.0625mM, the data for each sample is the average of the measurements of three repeats.
2.4 References list


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Chapter 3

Langmuir Trough
3.1 Introduction

3.1.1 Langmuir trough

The Langmuir trough is a laboratory instrument that measures the surface pressure and the surface area of a monolayer of amphiphilic molecules such as lipids at a water-air interface (Fig 3.1.1). How the film of molecules responds to expansion and compression at an interface of two media is a key factor in determining the formation and stability of colloidal systems. The Langmuir trough is an excellent tool that was invented to monitor the change of surface pressure (interfacial tension) upon contraction or expansion. It can be useful to study the kinetics of interaction between different kinds of molecules. The information can be gathered from either a single monolayer or bilayers of insoluble film.

Fig 3.1.1 A monolayer of lipids at the air-water interface of a Langmuir trough
The basic idea behind the trough instrument came from Dr. Irving Langmuir in 1917. The method has since been used to develop a related technique called the Langmuir-Blodgett method, based on Langmuir's observation that a solid substrate can be used as the base of a water-surface layer observed and the subsequent discovery by Dr Katharine Blodgett, in 1935, that a few of such films could be piled together from one top of another. Based on those facts, the troughs that may be used to produce either a single monolayer or bilayers were invented and used for a great range of physical or physiochemical experiments.

The components of the Langmuir trough instrument include the trough itself, a barrier, and a Whilhelmy plate. The Laugmuir trough is typically constructed from polytetrafuloroethylene (PTFE), which has a hydrophobic nature. The trough is filled with water, or buffer, and a monolayer of lipids is allowed to self-assemble on the liquid surface. In order to control the surface area of the monolayer, a barrier runs from one side of the trough to the other side, until the lipids on subphase (water/buffer) are closely-packed and well organized. The Whilhelmy plate is used to measure the surface pressure. A software package makes it possible to keep the surface area constant while measuring changes in surface pressure, or vice-versa.
The aim of a trough experiment is to study a monolayer amphiphilic molecules, which have a both hydrophobic and a hydrophilic region.

3.1.2 Langmuir trough experiment

In a typical experiment, a liquid surface is prepared and cleaned. A monolayer of amphiphilic molecules is prepared on top of the liquid. When placed on the surface, the layer of molecules may be compressed by opening or closing the barriers. The properties of amphiphilic molecules can then be studied by measuring the change of surface area or pressure, as monitored by a detector and a sensor. A Wilhelmy plate is always used as a sensor.

In a typical experiment, phospholipids are usually dissolved in a volatile solvent first, then are deposited on the surface of liquid (a subphase). The liquid is often water or buffer. Because of their amphiphilic nature, the molecules of phospholipids are arranged as a monolayer between the two media of air and subphase.
When closing the barrier, the surface pressure of the phospholipid monolayer is changed due to the compression of the surface layer. By monitoring the changes of the surface pressure against the area it is possible to generate what is known as a pressure-area isotherm. This will be further explained later in this chapter. Information about the phospholipid layer on the interface can be achieved by analyzing the isotherms.

Plots of the surface pressure against area per molecule at a constant temperature often contain sharp bends which indicate phase transitions in the two-dimensional layer. At low surface pressure, the phospholipids will be in the gaseous phase. With increasing surface pressure, next comes the liquid phase, followed by the solid phase. Finally at a higher pressure the monolayer becomes unstable and collapses. (Fig 3.1.2)
Fig 3.1.2 A typical isotherm of a phospholipid monolayer, displaying the different phases and the collapse point.

(http://nano.uib.no/Langmuir.php)

3.2 Cell membranes and phospholipids

3.2.1 Membranes

The cell membrane, or the plasma membrane, or phospholipid bilayer, is a
semi-permeable lipid bilayer that exist in all cells. Proteins are embedded in the phospholipid layer, or attached to its surface. This cell membrane separates the intracellular components from the extra cellular environment, and is selectively permeable and able to regulate what enters and exits the cell, thereby protecting the cells and enabling the transport of materials needed for survival.

The cell membrane consists of three classes of amphipathic lipids: phospholipids, glycolipids, and steroids. The relative proportions of these lipids depends on the type of cell, but in most of the cases phospholipids are in the majority.

3.2.2 Phospholipids

Phospholipids have hydrocarbon tails that are hydrophobic, whereas the polar head is hydrophilic. As the plasma membrane faces watery solutions on both sides, its phospholipids accommodate this by forming a phospholipid bilayer

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with the hydrophobic tails facing each other. (Fig 3.2.2.1)

![Phospholipid Bilayer Diagram]

Fig 3.2.2.1 Illustration of phospholipid bilayer

The cell membrane therefore prevents hydrophilic solutes from passively diffusing across the band of hydrophobic tail groups, allowing the cell to control the movement of these substances via trans-membrane protein complexes such as pores and gates.

According to the fluid mosaic model of S. J. Singer and Garth Nicolson\(^3\), the biological membrane can be considered as a two-dimensional liquid where all lipid and protein molecules diffuse more or less freely in two dimensions.

The head groups of phospholipids in biological membranes are mainly phosphatidylcholine \((\text{PC})\), phosphatidylethanolamine \((\text{PE})\),

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phosphatidylglycerol (PG), phosphatidylinositol (PI), and phosphatidylserine (PS).

The fatty chains in phospholipids usually contain an even number of carbon atoms, typically between 14 and 24. 16- and 18-carbon fatty acids are the most common. Most phospholipids in the bilayer have two fatty acid chains, so are said to be diacyl. The fatty acids may be saturated or unsaturated, with the configuration of the double bonds nearly always cis.

The length and the degree of unsaturation of fatty acids chains have a profound effect on the fluidity of a membrane, as the double-bond in unsaturated lipids creates a kink in the chain, preventing the fatty acids from packing together as tightly, thus decreasing the melting point or increasing the fluidity of the membrane.

3.2.2.1 DPPC

Dipalmitoylphosphatidylcholine (DPPC) is a good model for biological phospholipids. Studying DPPC in the laboratory addresses several technical
difficulties owing to the very high surface pressures achievable and the highly-stable films it produces under pressure *in vivo*. For the effective study of DPPC, optimisation of the Langmuir film apparatus is necessary, in order to provide maximum stability of the floating monolayer. By minimising collapse (monolayer area loss whilst held at constant pressure) brought about by interactions between the monolayer and the Langmuir trough surfaces, the true properties of the monolayer can be observed. The structure of DPPC is depicted below. (Fig 3.2.2.1.1)

![Structure of DPPC](image)

**3.2.2.2 DOPG and DOPC**

To further understand the influence that Aβ has on lipid membranes, lipids monolayer mixed of dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylglycerol (DOPG) was under study in the trough experiment.
DOPG has a negative charge and the mixture of DOPC and DOPG is a good model of charged membrane. The structures of DOPC and DOPG are shown below. (Fig 3.2.2.2.1, Fig 3.2.2.2.2)

Fig 3.2.2.2.1 Structure of DOPC

Fig 3.2.2.1.2 Structure of DOPG with a negative charge

3.2.3 Properties of Aβ
Aβ peptides are of amphiphilic nature, commonly possessing a hydrophilic domain near the N terminal and a hydrophobic segment close to the C terminus. However, native unstructured Aβ is soluble in aqueous media. Studies have shown that unstructured Aβ molecules will transform into α-helical and/or β-sheet conformations at various rates depending on the different solution environment. Aβ tends to form fibrils from monomers and other intermediates in a liquid environment. Though the amphiphilic property of Aβ has been demonstrated by a few studies and Aβ is loosely characterized as a surface-active molecule, it is unclear what conformation and which intermediate is surface active. Therefore, investigations on how Aβ adsorbs into phospholipids at an interface and the structural characterization of the aggregates should provide insights into the Aβ aggregation process and pathogenesis of AD.

Phospholipid monolayers at the air-liquid interface have properties that mimic a cell membrane. Therefore, it is of both fundamental interest and biological relevance to examine the adsorption of Aβ species at such an interface. I measured the adsorption kinetics of Aβ by monitoring the surface pressure change in a Langmuir trough.
3.3 Langmuir trough studies

3.3.1 Surface tension and surface pressure

Surface tension is the force that is needed to expand the surface of a liquid isothermally and it is often measured by unit per area. At 20°C water has the surface tension of 72.8 dyne/cm. Dyne is a unit of force. In bulk solution, the forces exerted on any molecule by neighbouring molecules are evenly distributed in all directions. Hence that the net force on the molecule is zero. However, at the air surface of the solution, the force caused by adhesion to the molecules in the air and the force caused by cohesion of the water molecules are not equal. As a result, and because the cohesion force is stronger than adhesion force, there exists an inward net force at the air-water interface. The resulting net force gives rise to surface tension. Surface tension tends to drag the liquid molecules towards each other at the surface and, therefore, prevents the surface from expanding. Water normally has an unusually large surface tension.

When amphiphiles, such as phospholipids are introduced to a polar liquid subphase, such as the lipid monolayer at air-water interface, after the solvent has evaporated into the air, two major forces are applied to the amphipiles. There is an ionic force because of the hydrogen bonding between the polar head groups and the subphase, and an intermolecular force, which is van der Waals force. The latter is far weaker than the ionic force so that the head groups of lipids monolayers can immerge into the subphase. When the monolayer is
compressed, the distance between the molecules becomes smaller and, as a consequence of this, the van der Waals force becomes bigger. As a result, the net force (surface tension) on the surface becomes smaller. Surface pressure is the measurement of the change of surface tension. By compressing the monolayer, surface pressure will increase.

3.3.2 Isotherm

Surface pressure is measured by a Whilhelmy plate and the typical characteristics of a monolayer at the air-water surface can be read by a pressure-area isotherm. That is, the surface pressure measured against area per molecule at a constant temperature.

3.3.3 Surfactants

Surfactants are amphiphilic organic molecules that contain both a hydrophobic part and a hydrophilic part. Linking together two parts with completely different natures in the same molecule give surfactants certain characteristic

properties. They can absorb at air-water interfaces because the hydrophilic group can dissolve in the water, whereas the hydrophobic part repulses the water and therefore tends to extend out of the water towards the air. At water-oil boundaries they form an interface between the water and the oil. Surfactants can reduce the surface tension of water because the fact that they adsorb at the air-water surface.

The properties of surfactants are highly dependent on the balance between the two groups. When the hydrophobic group is not hydrophobic enough, the detergent molecules have a tendency to dissolve in water. Alternatively, if the hydrophilic part is not soluble enough then the molecules might evaporate into the air. The balance of the relative sizes of the two parts of amphiphiles controls the properties of surfactants. Only when they are well balanced can a monolayer at an air-water surface can be achieved.

The term Langmuir film is often used to refer to insoluble films that formed by lipid molecules. Such lipid molecules are amphiphiles, so the lipid monolayer as used in this study may be considered to be surfactants. The two parts that form amphiphiles are hydrophilic head groups and hydrophobic tails. For example, PC has choline as a headgroup and glycerophosphoric acid and two fatty acid
tails, often one saturated fatty acid and one unsaturated fatty acid. Whereas PE has ethanolamine as the headgroup. The head groups prefer water and the tail group repulse water. As a result, such molecules will self-aggregate into a 2 dimensional system at the air-water surface.

The Langmuir trough is well suited to the study of the ordering of lipid molecules at the air-water interface as it provides an excellent monolayer model system. The thermodynamic variables are easily controllable. The surface pressure is simply controlled by moving the barrier on top of the trough and the lipid molecules stay at one side of the trough, while water freely flows underneath. The temperature of the system is easy to control by connecting a temperature controlled water tank to the trough.

**3.3.4 Phases of monolayer and phase transitions**

At the air-water surface, a surface pressure vs. temperature phase diagram of a lipid monolayer may effectively be replicated by measuring an isotherm of surface pressure against molecular area. Before or at the beginning of compression, the molecules of a lipid monolayer act as two-dimensional gas and
can be described by

\[ \pi A = kT \]

Where \( \pi \) refers to surface pressure, A the molecular area, and K the Boltzmann constant \( 1.3806488(13) \times 10^{-23} \text{ J/K} \).

Further compression leads the monolayer to go into a liquid expanded phase where the head groups are transitionally disordered.\(^5\) As the compression continues the molecules of the monolayer get closer together and become more ordered, that is when the monolayer is going to liquid phase. The steep linear part at monolayer isotherm often reflects a liquid phase. A kink can be observed upon further compression and after the kink the monolayer is less compressible. This is when the monolayer is at solid state. The phases of a Langmuir monolayer is illustrated in Fig 3.1.2.

X-ray diffraction studies show that at liquid phase, the tails of amphiphiles are tilted at a certain angle to the surface. A decrease in surface area results in a decrease in tilted angle. In the solid state the tails are nearly perpendicular to

the surface. As the surface pressure continues to go higher, the monolayer will eventually lose its form and collapse. The molecules cannot be compressed in a two dimensional form because of the very strong outside force. The collapse often happens either near the edge of the barrier or the corner of the trough, or by the Wilhelmy plate. The collapse pressure is the maximum pressure the monolayer can withstand.

The order and number of phase transitions have long been an issue of controversy, until it was solved by combining Langmuir trough experiments with optical measurements.

3.4 Materials and methods

All the lipids used in this experiment were purchased from Avanti Polar Lipids (Alabama, USA). The peptide used was first restored to monomeric form. The method was described in previous chapter. The Langmuir trough was first

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cleaned using chloroform and Kimwipes. After this had been completed the
trough was filled with PBS at pH7.4 until the meniscus protruded above the
surface by 2 or 3mm. A piece of filter paper was hung on the surface pressure
sensor from an electronic wire so that the filter paper extended into the PBS.
The pressure sensor gave an initial surface pressure reading of 0mN/m. To
make sure the surface was clean, the surface of PBS was compressed a few times
by the barrier until a very low surface pressure (< 0.3mN/m) was achieved. If
the resultant isotherm was not acceptable the surface was cleaned using a
suction pump and the baseline retested until acceptable. After this had been
completed the compression rate was adjusted by setting the single barrier to
move at a rate of 20 cm²/min, for the Langmuir trough that had a maximum
area of 92cm², or a rate of 100cm²/min, for the Langmuir trough that had a
maximum area of 164cm². The relevant details of the intended monolayer
spreading solution were inputted into the NIMA program manager.

Once an acceptable baseline had been elucidated, a certain amount of the
phospholipid monolayer spreading solution was spread across the subphase
using a Hamilton syringe. This was then left for around 10 minutes to allow the
chloroform to evaporate. After this period had expired the surface pressure
sensor was zeroed to 0mN/m and an isotherm measured. All monolayer
experiments were run at a room temperature. Pressure control data was
obtained by keeping the pressure at a constant 25mN/m, whereas the isotherm data was collected by keeping pressure below a maximum of 30mN/m. These pressures were maintained by constantly altering the area of the trough.

3.5 Results

3.5.1 Isotherm

3.5.1.1 Isotherm of DPPC and mixed monolayer

The isotherm for a DPPC and DPPC monolayer mixed with Aβ peptide was recorded on a smaller Langmuir trough that has a maximum area of 92cm². A monolayer of 10μl DPPC (1mg/ml) was spread on 50ml PBS buffer for the DPPC isotherm. A monolayer of 10μl DPPC mixed with 0.5μM Aβ was used for examination of the isotherm of the mixed layer. A trace of TFA, used when making the monomeric Aβ, was kept when mixing the peptide with the DPPC to make a well-mixed peptide-lipid solution. Fig 3.5.1.1 shows the isotherms for
DPPC and the mixed layers. From the isotherms, it is clear that the plateau of the liquid phase is less obvious in all the peptide-lipid monolayers than in that of DPPC. When compressed to the same surface pressure, each of the mixed monolayers occupied more area than the DPPC monolayer. Aβ has a clear effect on the DPPC.

Fig 3.5.1.1 Isotherms of DPPC and DPPC with peptide mixed monolayer. 10μl
DPPC (1mg/ml) was used in DPPC isotherm. DPPC mixed with 0.5μM Aβ was used in all other monolayers containing either L-, D- or (L-/D- 50/50) peptide. All experiments were performed in room temperature. (20°C)

3.5.1.2 Isotherm of DPPC on PBS with Aβ in the subphase

The isotherm of DPPC on different subphases was measured, with the aim of examining the ability of Aβ to interact with the lipid from a water-based environment. The DPPC isotherm was examined in a cycle by opening and closing the bar after the first isotherm. (Fig 3.5.1.2-5) The subphase that contained Aβ peptide was prepared 1 hour before the isotherm. The DPPC isotherm displays a typical 3 phase shape, namely a gas phase, a quite flat liquid phase, and a solid phase. The phase transitions for those 3 phases are obvious (Fig 3.5.1.2). Interestingly, the phases of DPPC on a peptide mixed subphase were not obvious. The transition from gas phase to liquid phase nearly disappears, indicating the existence of a quite disordered monolayer even under a high pressure. (Fig 3.5.1.3-5) The gaps between the starting points of 2 isotherms in a cycle for DPPC on peptide containing subphase further indicated the disorder and instability of the monolayer under the influence of Aβ peptide.
The difference between the isotherm on 3 kinds of subphase with different peptide was subtle.

Figure 3.5.1.2 DPPC isotherm on PBS. DPPC (1mg/ml) 10μl, PBS 0.01M, pH 7.4, this experiment was performed in room temperature. (20°C)
Figure 3.5.1.3 DPPC isotherm on (PBS + 0.5mg/ml L-). DPPC (1mg/ml) 10μl, PBS 0.01M, pH 7.4, this experiment was performed in room temperature. (20°C)
Figure 3.5.1.4 DPPC isotherm on (PBS + 0.5mg/ml D-). DPPC isotherm on PBS. DPPC (1mg/ml) 10μl, PBS 0.01M, pH 7.4, this experiment was performed in room temperature. (20°C)
Figure 3.5.1.5 DPPC isotherm on PBS + 0.5mg/ml (Lr/D- 50%/50%) DPPC isotherm on PBS. DPPC (1mg/ml) 10μl, PBS 0.01M, pH 7.4, this experiment was performed in room temperature. (20°C)

3.5.2 Pressure control experiment

To further understand the influence Aβ peptide has on the lipid monolayer, the monolayer of phospholipid was kept at a certain pressure and then Aβ peptide
was added underneath the monolayer. In this experiment, a Langmuir trough that has a maximum working area of 164cm^2 was used. 15μl of lipid (10mg/ml) was spread on a 190ml PBS subphase. 5μM (about 1mg) Aβ peptide was added after the pressure reached 15mNm^{-1}. Measurements for each type of peptide were repeated 6 times.

Fig 3.5.2.1-3 are the graphs of the expansion of area after the peptide was introduced. All plots start at the moment the peptide was added. The pressure stayed unchanged for at least 10 minutes. Fig 3.5.2.4 shows the surface area of the monolayer after PBS was added. Table 3.5.2.1-3 records the measurements of the changes of surface areas per molecule and the time taken before the changes started to happen.

All three types of peptide (L-,D- (L-/D- 50%/50%)) expanded the monolayer. Among them, (L-/D- 50%/50%) peptide had the most predominant influence upon the monolayer. The average expansion of the area per molecule of DPPC on the subphase containing (L-/D- 50%/50%) is 3.1nm^2*10^3, which is nearly twice high as L- or D- peptide alone (1.8nm^2*10^3, 1.5nm^2*10^3 respectively). It also took much longer for (L-/D- 50%/50%) peptide to start its influence on the monolayer too. PBS control shows no influence on DPPC. The method of inserting peptide underneath the monolayer is valid.
The mixture of (L-/D- 50%/50%) peptide contains less fibrils and more soluble forms of the peptide, and also have more influence on the monolayer. The trough experiment suggests that the fibrils have less influence on the monolayer than the other forms of the peptide. The soluble forms of Aβ peptide are more membrane active.

Fig 3.5.2.1 Langmuir trough 15mNm-1 pressure control. 5μM L- peptide was added in 10mins after DPPC monolayer reached to pressure 15mNm-1. The beginning point is when peptide added in. DPPC (10mg/ml) 15μl, PBS 0.01M, pH 7.4, this experiment was performed in room temperature. (20°C)
Fig 3.5.2.2 Langmuir trough 15mNm-1 pressure control. 5μM D-peptide was added in 10mins after DPPC monolayer reached to pressure 15mNm-1. The beginning point is when peptide added in. DPPC (10mg/ml) 15μl, PBS 0.01M, pH 7.4, this experiment was performed in room temperature. (20°C)
Fig 3.5.2.3 Langmuir trough 15mNm-1 pressure control. 5μM (L-/D- 50%/50%) peptide was added in 10mins after DPPC monolayer reached to pressure 15mNm-1. The beginning point is when peptide added in. DPPC (10mg/ml) 15μl, PBS 0.01M, pH 7.4, this experiment was performed in room temperature. (20°C)

Fig 3.5.2.4 Langmuir trough 15mNm-1 pressure control. 1ml PBS was added in 10mins after DPPC monolayer reached to pressure 15mNm-1. The beginning point is when peptide added in. DPPC (10mg/ml) 15μl, PBS 0.01M, pH 7.4, this experiment was performed in room temperature. (20°C)
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Table 3.5.2.1 The changes of surface area after L-peptide added in and the time (in seconds) for the changes start to happen in 6 experiments.

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Table 3.5.2.2 The changes of surface area after D- peptide added in and the time (in seconds) for the changes start to happen in 6 experiments.

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Table 3.5.2.3 The changes of surface area after (L-/D- 50%/50%) peptide added in and the time (in seconds) for the changes start to happen in 6 experiments.

A mixture of DOPG/DOPC (10%/90%) was also used to test the influence of the peptide on a charged monolayer. Such monolayer is less stable than DPPC and the surface area tends to reduce as the monolayer breaks down. The L- peptide was added once the monolayer reached to 15 mNm⁻¹. The gradient of change of the area was recorded in comparison of without adding the peptide and is shown in Fig 3.5.2.5. The gradient of the slope was changed after adding the peptide, from -0.0327 to -0.0233, suggesting an influence of the peptide.
Fig 3.5.2.5 Langmuir trough 15mNm-1 pressure control. 5μM (L-/D- 50%/50%) peptide was added in after DOPG/DOPC (10%/90%) monolayer reached to pressure 15mNm-1. The beginning point is when peptide added in. DOPG/DOPC (10%/90%) (10mg/ml) 15μl, PBS 0.01M, pH 7.4, this experiment was performed in room temperature. (20°C)

The trough experiment showed clearly the interaction between the Aβ peptide and lipid monolayer. The peptide is able to interact from a water environment to lipid, indicating the potential ability of Aβ (25-35) peptide interrupting lipid layers in the nervous system. We learnt from the fibril growth experiment that the mixed peptide doesn't form fibril as well as pure L- or pure D- peptide. In the through, the mixture of (L-/D- 50%/50%) peptide contains less fibrils and
more soluble forms of the peptide. The trough experiment suggests that the fibrils have less influence on the monolayer than the other forms of the peptide. The soluble forms of Aβ peptide are more membrane active. Further experiments were carried out to assure this conclusion, and explained in later chapters.
3.6 References list


7. M.Szablewski, P.M.a., Tensiometers and langmuir-biodgett troughs operating manual 1999. 47.

Chapter 4

Brewster Angle Microscopy
4.1 Introduction

The use of the Langmuir trough makes it possible to determine the properties of amphiphilic monolayers, such as their surface tension and surface phase transitions, as well as to study the interactions between the monolayers and other components, such as membrane active proteins. Brewster Angle Microscopy (BAM) has been used in membrane physics studies due to its ability to allow researchers to visualize monolayer surface and to study the morphology of the monolayer. The combination of the Langmuir trough and BAM is used widely to study amphiphilic lipid monolayers

4.1.1 Theory

4.1.1.1 Polarized light/ polarization

Electromagnetic radiation (EMR) has properties of both waves and particles. EMR is often referred to light in physics. To better explain it, it is possible to use light as an example. Light, as electromagnetic radiation, can be viewed as
travelling waves, which are self-propagating and oscillate in more than one direction. At the same time, it can also be considered to be discrete photons that carry radiant energy when they travel (Fig 4.1.1.1.1). The property of waves that allows for different planes of oscillation is called polarization. If the electric field $E$ and the magnetic field of the light $H$ are perpendicular to the direction of propagation of the waves then such waves are called transverse waves (Fig 4.1.1.1.2). $E$ and $H$ are also perpendicular to each other. Light can be described as transverse waves. Natural light consists of different waves that have different properties, such as wavelengths and polarization states, and is therefore not polarized. To achieve polarization, a polarizer may be used to filter out the unwanted waves and achieve a polarization in a certain direction. If $E$ and $H$ are each aligned single planes then the light is linearly polarized. When the polarization direction of this light has the same plane as the propagation of the light then this light is p polarized (Fig 4.1.1.1.3).
Fig 4.1.1.1.1 Light can be viewed as both particles and waves.

Fig 4.1.1.1.2 The electric field and the magnetic field are perpendicular to each other and also perpendicular to the direction of propagation.

(http://www.physast.uga.edu/~rls/1010/ch5/ovhd.html)
The Brewster angle is the angle of incidence at which plane polarized light is not reflected from an interface between two media. The light is completely transmitted through the interface at the Brewster angle.

If the refractive index of the two media $n_1$ and $n_2$ respectively then when light travels from medium 1 to medium 2 we have

$$n_1 \sin(\theta_1) = n_2 \sin(\theta_2)$$
\( \theta_1 \) and \( \theta_2 \) are angle of reflection and angle of refraction

Also, in order to generate p polarized light, the refracted light should be perpendicular to the incident light

\[ \theta_1 + \theta_2 = 90^\circ \]

Now we have

\[ n_1 \sin \theta_1 = n_2 \sin(90 - \theta_1) = n_2 \cos \theta_1 \]

So

\[ \frac{n_2}{n_1} = \frac{\sin \theta_1}{\cos \theta_1} = \tan \theta_1 \]

\[ \theta_1 = \tan^{-1} \left( \frac{n_2}{n_1} \right) \]

4.1.2 Brewster Angle Microscope (BAM) experiment

The BAM technique exploits the principle about light reflection explained above.

A BAM experiment is normally carried out at the surface of a Langmuir trough,
with a laser p polarized light beam source and a camera that receives the reflected light from the Langmuir monolayer surface (Fig 4). A BAM instrument is connected to a computer and is usually mounted onto a Langmuir film balance controlled by the computer.\(^1\) At the Brewster angle, p polarized light is transmitted through the interface of two media. For a beam of incidence p polarized light there is will be no reflected light from a clean surface (such as pure water or PBS) thus no reflection signal will be received by the camera. However, there will be a change of reflection signal when the surface is changed from water to a lipid monolayer, because this will affect the refractive index. This change can therefore be visualized (Fig 4.1.2.1). By the same principle, when the surface is perturbed by membrane active proteins, or when there are non-uniform domains (possibly caused by monolayer-associated aggregates) on the surface of monolayer, the optical properties of the system will change again, thus that reflected signal changes and this can be collected and monitored.

Fig 4.1.2.1 Brewster angle on the surface Left: the incident beam travels through the surface at the Brewster angle (no reflection). Right: (partial) reflection occurs when the surface is perturbed (http://www.sciencephoto.com/media/97801/view).

BAM is very useful for studying lipid monolayers in a Langmuir trough. It is a good tool for studying the morphology of lipids as well as lipid monolayer phase changes. Reflection from membrane surfaces visualized by BAM can be used to observe membrane topography, including as domain structure, with and without any peptide-induced interference. In order to study membrane active proteins and to visualize peptide-induced surface changes, it is important to understand the basic lipid domain structures under various surface pressures and in different (air, liquid and solid) phase.

4.1.2.1 BAM images of lipids (morphology study)
The topography of a Langmuir monolayer can be visualized by BAM. BAM can be used to distinguish between different molecular arrangements at the air-water surface, and can also give information about the inner textures of lipid monolayers. Studies have shown that the lipids with C_{14}–C_{16} alkyl chains (chains of between 14 and 16 carbons) produce the best-defined domain structures.\textsuperscript{1} Lipid monolayers spontaneous assemble at air-water surfaces. The alignment of molecules generates polarization normal to the monolayer surface, leading to storage of electrostatic energy in the monolayer. When the lipid monolayer is equilibrated, the shape of lipid domain depends on the balance of line tension and electrostatic energy.\textsuperscript{2} At this point, the shape free energy tends to be minimized. Round-shaped domains are preferred by line tension, and at the same time the electrostatic energy enlarges the domain shape. Some well-studied chemical compounds have circular structure, for example, fatty acids ethyl and methyl groups, which have the potential to develop well-defined structures. Phospholipids are able to form different phases with different structures. Such changes and the growth of domain can be visualized by BAM. For example, monolayers of DPPC, one of the most studied lipids, show changes of the shape of its domain under different surface pressures, from

circular-shaped domains in its gas phase to S-shaped domains in gas-liquid phase, and then to a cover shaped domain in its liquid phase, with the size of domain increasing.\textsuperscript{3}

Non-equilibrium structures can be visualized by BAM. When the energy level of the equilibrium phase of a monolayer cannot reach to its minimum, and at the same time the growth kinetics are much faster than the time needed to reach equilibrium, the monolayer will be in a non-equilibrated state. The time it takes for the monolayer to relax to the original equilibrated state differs from one type of molecule to another, and can vary from a few minutes to more than an hour.\textsuperscript{4} Some of the structures with long chains may stay indefinitely in a pre-equilibrated state.\textsuperscript{5}

When the monolayer is not equilibrated, the pattern of lipid domain tends to be ‘fractal-like’. A higher rate of compression produces a more fractal structure. A lower rate of compression or a very slow compression produces round-shaped lipid domains.

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BAM is also able to reveal the structure of the inner domain of certain chemicals, which leads to the possibility of analysis of the geometric structure of these chemicals. This usage of BAM is based on the packing of the molecules of the target molecules and the consequential change of reflection of p-polarized light. Ethyl stearate is a good example to explain this. In solid phase, ethyl stearate produces round-shaped domains, as observed by BAM. The domain is divided by straight lines into 6 segments that exhibit different uniform brightness. The differences in brightness can help to determine the orientation of the chains. The darkest regions in the pictures indicate that there is no signal received by the camera. This means there is no reflection of the light from the surface to be captured by the camera. At the Brewster angle, the incident p polarized light is only transmitted when the surface of the medium is perpendicular to the plane of incident light. This suggests that the tilt of the chains is perpendicular with the plain of the incident p-polarized light in the darkest area of a domain. On the contrary, the brightest area of the domain suggests the chains are parallel with the plane of incidence light. (Fig 4.1.2.2)

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Fig 4.1.2.2 The arrow shows the brightest part of the domain of DPPC suggesting this part is the tails of DPPC which are parallel with the incident plane polarized light.

4.1.2.2 Phase transition study
BAM is a very useful tool for the study of lipid monolayer phase transitions. Using a Langmuir trough isotherm to study lipid monolayers in gas phase, liquid phase and condensed phase, and the phase transitions, all of these can be visualized by BAM during compression of monolayer. BAM may also used to help to define the three different phases. The Langmuir method can provide certain information about monolayer characteristics and isotherm information. However, the exact point of phase transition cannot be determined by the Langmuir trough alone. BAM can help to define each phase by depicting the shapes of the lipid domains and the change of phases by showing the differences in light contrast. For example, the tilt of lipid tails group indicates the phase of the monolayer. At the same time, the different angles of tilt of the phospholipids tails give rise to different light contrast. As explained in previous chapter, the main difference between lipid monolayer in condensed phase and solid phase is in the tail groups. At a liquid condensed phase, the tilt of the tails is at a certain angle relative to the surface (i.e. not perpendicular to the surface), and the angle changes as the compression proceeds. When the monolayer reaches the solid phase, the tails are perpendicular to the surface. By combining the Langmuir trough and the use of BAM, a better understanding of the physic properties of phospholipids and lipids monolayer can be achieved.
4.1.2.3 BAM studies of membrane active proteins

The differences in head groups between phospholipids give rise to different interactions between water-soluble membrane active molecules and lipid monolayers. The head groups of DPPC and DPPE are zwitterionic and thus have fewer opportunities to interact with positively-charged molecules in the subphase than DPPG or DPPS, which are negatively-charged. When membrane active molecules are at present at the air-water surface, the two-dimensional structure of the lipid monolayer is disrupted. As a result, the morphology of the lipid monolayer will change. Using different kinds of phospholipid monolayers to study target membrane active proteins under BAM can provide useful information about the activity of the proteins.

4.2 Materials and Methods

DPPC was purchased from Avanti Polar Lipids (Alabama, USA). Aβ (25-35) was purchased from ALMAC (Gladsmuir, UK). The peptide used was first converted back into monomers. The measurements were taken on a Brewster angle microscope (NFT, Germany) mounted on a Nima Langmuir trough. The microscope provides Ångstrom resolution and a lateral resolution on the scale of micrometer. BAM gives insight into a liquid monolayer from the reflection of
plane polarized light.

The trough was first cleaned and an isotherm of 15μl DPPC (10mg/ml) on pure water was performed as described in previous chapter. Then the surface pressure was fixed as 10mN/m. 10mins after 5μM Aβ peptide was added in the subphase and the pictures of BAM were taken every few minutes to record any potential changes on the lipid subphase.

4.3 Results

Fig 4.3.1 shows the BAM image of the surface pure water. Fig 4.3.2(a-c) show BAM images of the surface of DPPC at different surface pressures. The domains of DPPC do not appear when there is no surface pressure. However, when the pressure increases, the domains start to become more obvious. It is difficult to see the individual shape of each domain in this experiment.

Figs 4.3.3-4.3.6 show BAM images of the surface of DPPC after pure water or the peptide added in. The domain areas become more and more obvious after the surface pressure reaches to 5 mN/m. However, the surface remains undisturbed in all the pictures. The domains of DPPC molecules are evenly distributed under different pressures measured. The sizes of the areas of the domains in different
pictures do not look the same but there is no visible sign of interaction on the surface of the monolayer after L- Aβ, D- Aβ or (L-/D- 50/50) added in the subphase. This is interesting because the trough experiments have shown the expanding effects the peptide has on DPPC monolayer.

A possible explanation of why BAM does not show any morphology change of DPPC monolayer could be that, although Aβ interacts with the monolayer, it does not penetrate from the subphase of water to the very surface of the DPPC. BAM enables the visualization of the surface of the monolayer on the trough, however, it can not visualize the bottom of the lipid, which is head group. Aβ might interact with the head groups of DPPC, but not go further into the tails, thus that there is no visible changes from BAM. In order to see possible surface changes, further experiments could be carried out with a much lower surface pressure. It would be easier for the peptide to penetrate into the membranes when it has less pressure resistance to overcome.
Fig 4.3.1 BAM image of pure water on Langmuir trough. Image size 531 × 531 μm. Bar length 50 μm.
Fig 4.3.2a BAM image of the monolayer of DPPC on a Langmuir trough when the pressure=0mN/m. Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
Fig 4.3.2b BAM image of the monolayer of DPPC on a Langmuir trough when the pressure=5mN/m. Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
Fig 4.3.2c BAM image of the monolayer of DPPC on a Langmuir trough when the pressure=10mN/m. Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
4.3.3.a BAM image of DPPC monolayer 10mins after 1mg water added in the subphase.

Pressure=10mN/m, Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
4.3.3.b BAM image of DPPC monolayer 30mins after 1mg water added in the subphase.

Pressure=10mN/m, Temperature= 20°C, image size 531x531μm. Bar length 50 μm.
4.3.3.c BAM image of DPPC monolayer 60 mins after 1 mg water added in the subphase.

Pressure = 10 mN/m, Temperature = 20°C, image size 531 × 531 μm. Bar length 50 μm.
4.3.3.d BAM image of DPPC monolayer 120mins after 1mg water added in the subphase.

Pressure=10mN/m, Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
4.3.4.a BAM image of DPPC monolayer 10 mins after 1mg L-Aβ added in the subphase.

Pressure=10mN/m, Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
4.3.4.b BAM image of DPPC monolayer 30mins after 1mg L-Aβ added in the subphase.

Pressure=10mN/m, Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
4.3.4.c BAM image of DPPC monolayer 60mins after 1mg L-Aβ added in the subphase.

Pressure=10mN/m, Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
4.3.4.d BAM image of DPPC monolayer 120 mins after 1 mg L-\(\alpha\)β added in the subphase.

Pressure = 10 mN/m, Temperature = 20°C, image size 531 × 531 µm. Bar length 50 µm.
4.3.5.a BAM image of DPPC monolayer 10mins after 1mg D-Aβ added in the subphase.

Pressure=10mN/m, Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
4.3.5.b BAM image of DPPC monolayer 30mins after 1mg D-Aβ added in the subphase.

Pressure=10mN/m, Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
4.3.5.c BAM image of DPPC monolayer 60mins after 1mg D-Aβ added in the subphase.

Pressure=10mN/m, Temperature= 20°C, image size 531×531µm. Bar length 50 µm.
4.3.5.d BAM image of DPPC monolayer 120mins after 1mg D-Aβ added in the subphase.

Pressure=10mN/m, Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
4.3.6.a BAM image of DPPC monolayer 10mins after 1mg (L- /D- 50/50)-Aβ added in the subphase. Pressure=10mN/m, Temperature=20°C, image size 531×531μm. Bar length 50μm.
4.3.6.b BAM image of DPPC monolayer 30mins after 1mg (L-/D- 50/50)-Aβ added in the subphase. Pressure=10mN/m, Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
4.3.6.c BAM image of DPPC monolayer 60mins after 1mg (L-/D- 50/50)-Aβ added in the subphase. Pressure=10mN/m, Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
4.3.6.d BAM image of DPPC monolayer 120mins after 1mg (L-/D- 50/50)-Aβ added in the subphase. Pressure=10mN/m, Temperature= 20°C, image size 531×531μm. Bar length 50 μm.
4.4 References list


Chapter 5

Circular Dichroism
5.1 Introduction

Circular Dichroism (CD) was used to examine the secondary structure of L- and D- Aβ (25-35) in different environments. The study of secondary structure of the peptide is important to understand its folding and mis-folding. The secondary structure of Aβ peptides is reported to be β sheet by previous studies.\(^1\) However, the structure of the fragment Aβ (25-35) remains unclear. It is known that the secondary structure of a peptide is not just defined by the primary sequence. The environment also plays an important role in determining the peptide’s secondary conformation.\(^2\) For example, using sodium dodecyl sulfate (SDS) is one way to promote the formation of secondary structure. In this experiment, I examined the effect of different environments on the secondary structure of Aβ (25-35).

5.1.1 Peptide Secondary structure

The primary structure of a peptide is its sequence of amino acids. The secondary structure is the spatial arrangement of the backbone of the peptide chain into

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regularly repeating structural motifs. Such spatial arrangements will contribute to the peptide’s physical and chemical characters. Secondary structure is stabilised by the hydrogen bonds between amino-groups and keto-groups of the peptide bonds. For a polypeptide, the secondary structure is formed mainly because of the rigid and planer peptide bond, so that the peptide’s spatial movement is restricted. The types of amino acid residues and the peptide bonds within the molecules highly affect the peptide’s secondary structure.

The most well characterised polypeptide secondary structures are namely the α-helix and β-sheet and are shown in Fig 5.1.1.2.

An α-helix is a right-handed spiral conformation, where the main chain helix is maintained by regular H-bonds between backbone carboxyl groups and amino groups. Every amino group (N-H) in the backbone gives up a hydrogen bond to a carboxyl group (C=O), which is four amino acids away along the chain. Projected onto the helix axis, each residue is 1.5 Å rising up from its neighbours, and rotated by 100°. This results in 3.6 amino acids per turn of the helix. The helix turns 360° every 5.4 Å. The α-helix always rotates counter-clockwise, when the peptide chain contains L-amino acids. If you hold your right hand with the thump pointing from the N- to the C-terminus, the tips of the curled fingers move anticlockwise. So a α-helix is called right-handed.

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The structure of β-sheet is less common than α-helix. β-sheets consist of fully extended polypeptide units called β-strands. Each β-sheet has β-strands connected laterally, with no hydrogen bonding happening between C=O and N-H in neighbouring amino acids; in each strand, H-bonding is connected with N-H and C=O groups from another polypeptide chain. The distance between each two adjacent amino acids is 3.5 Å.

Fig 5.1.1.1 Left: Hydrogen bonding in α-helix; Right: Hydrogen bonding in β-sheet

A random coil structure indicates a kind of conformation that lacks regular secondary structures. Unlike a α-helix or a β-sheet structure, in a random coil structure, the directions of the bonds of the subunits of polypeptide are random. (Fig 5.1.1.2)
Fig 5.1.1.2 A random coil structure (yellow) is compared with $\alpha$-helix (pink) and $\beta$-sheet (blue) (http://www.psc.edu/science/Darden/bpti_spline_dense2.GIF)
5.1.2 Theoretical background to Circular Dichroism

Light can be considered to be a travelling electromagnetic wave that consists of an electric vector and a magnetic vector. Light polarisation only relates to the electric component of light. The definition of polarised light was explained in Chapter 4. In unpolarised light, the electric vector vibrates in all the directions that are perpendicular to the direction of propagation. When unpolarised light passes a special filter, which only allows the lights with the vectors that vibrate parallel to the optical axis of the crystal to pass through, the light becomes plane polarised. This is illustrated in Fig 5.1.2.1. The vibrations of electric vector are confined to one plane, which is orthogonal to the direction of light traveling.

Plane polarised light occurs when the electric field vector oscillates in only one plane. However, circularly polarised light happens when the electric field vector rotates as the light propagates, thus that it forms a helical ‘wave train’. If the vector rotates clockwise then the circular polarised light is right handed, and if the vector is anti-clockwise then the light is left handed. (Fig 5.1.2.2) Right and left circularly polarised light of equal amplitude can be considered to form plane polarised light. (When the amplitudes are not equal, then it forms elliptically polarised light).
Fig 5.1.2.1 When unpolarised light passes by a polariser (filter), the plane polarised light is produced.

Circular dichroism is a technique that measures the differential absorption of left and right handed polarised lights. When a plane-polarised light passes by a modulator, its right and left circular components are usually reduced to different extent. The modulator can be a piezoelectric crystal such as quartz,
which is subjected to 50KHz alternating electric field. After the modulator, the elliptically polarised light is produced when the two beams recombine, if the two circular components are absorbed to different degree. If the two components are not absorbed or they are absorbed to the same level, then the combination results in a radiation polarised in the same plane as the original light.

A CD instrument does not combine the two components but monitors the two separate components. In this case, θ is used to describe the displayed dichroism at a given wavelength of radiation. It defines the ellipticity as an angle whose tangent is the ratio of the minor axis b and major axis a of the resultant ellipse. The absorption of the two polarisations is measured, as a difference of absorption, yielding the CD spectra of the sample. CD measurements depend on the concentration of the sample. The ellipticity is as a result defined as molar ellipticities. In most of the biological work, the ellipticities are of the order of 10 millidegrees. Therefore, the experiment must be carried out carefully to obtain useful results.

The observed ellipticity θ (in degree) can be described at a given wavelength as

$$[\theta]_{\text{mrw}, \lambda} = \theta \times \text{MRW/10.d.C}$$

Where MRW is the mean weight of the residue of the dichroic solute. C is the concentration of the sample. Its unit is g mL\(^{-1}\). d is the distance of optical path. Its unit is cm.

When dichroism is plotted as a function of wavelength, a CD spectrum is obtained. The two circularly polarised components must pass through an asymmetric medium in order to observe the dichroism. For that reason, the sample medium is designed to have an intrinsic chromophore and exhibit chirality. As the circular components are asymmetric inherently, the interactions with the chromophore are different. The chromophore in the case of polypeptides is the peptide bond, whose electronic absorbance is at 180-240nm. The spectra of peptides at far ultraviolet regions are determined by transitions of amide groups from nèπ* to πèπ* and the structures of peptide backbones.\(^5\)

The chromophores around chiral centres of a polypeptide have unique spatial orientation, which gives rise to CD spectrum. Since the naturally occurring amino acids are asymmetric, the chiral centres in polypeptides lead to an asymmetric secondary structure. The chiral centres of L- and D- peptide exhibit different chirality so that L- and D- are supposed to have different CD spectrum.

5.2 Materials and methods

5.2.1 Materials

All the lipids and peptides used in this experiment were purchased from Avanti Polar Lipids (Alabama, USA). Thioflavin T (ThT) was from Invitrogen (Carlsbad, CA). PBS, SDS and TFE were from Sigma-Aldrich (St. Louis, MO).
5.2.2 Methods

CD experiments were carried out at chemistry department in Glasgow University. Jasco J-180 was used to record CD spectra. The last experiment was conducted at Roslin institute. CD spectroscopy in the far-UV spectral region (180-260 nm) was used to determine the secondary structure of the peptide. The obtained CD spectra are compared with reference spectra so the types of secondary structures can be known. Thus analysing the far-UV CD spectrum, we can determine the approximate fraction of each secondary structure type. The signature spectra of the reference secondary structures is shown below. The spectra gained from our experiment are compared the reference spectra. A typical alpha-helix structure features a very high starting point over zero point (at wavelength 180 to 190nm), a very high peak, and a ‘w’ shape below zero. The spectrum of a beta-sheet has relatively lower starting point just above zero, a relatively low peak over zero then followed by a dent below zero. The other shapes indicate the protein measured is unstructured, or a mixture of different structure.
Fig 5.2.2 CD reference spectra of protein signature secondary structures

http://cnx.org/contents/334b4223-4339-4d31-82e6-549b23df1741@2/Circular-Dichroism-Spectroscopy

5.2.3 Sample preparation
5.2.3.1 Peptide in different solutions

Monomeric Aβ stock was prepared first and then added to different solutions.

5.2.3.2 Lipid Vesicles Preparation

The effects of the lipids on peptide conformation were determined by adding an aliquot of stock peptide solution to lipid suspended in PBS. To prepare the suspensions, the lipids were first redissolved in chloroform (1mg/ml), and then dried under a stream of nitrogen, and resuspended in PBS at a concentration of 1 mg/ml. Lipid suspensions were carried through 3 cycles of freeze-thaw in liquid nitrogen, which promotes formation of both bilayer and multilayer lipid sheets. The lipid suspensions were then bath sonicated for 15–20 min to ensure formation of vesicles. The final peptide concentration was 10mM.

5.3 Results and Discussion
The secondary structures of L- and D- peptide were examined in different environments. Fig 5.3.1 shows the CD spectra of the freshly made L-, D- and L-/D- (50%/50%) in PBS. Fig 5.3.2 compares the CD spectra of L-, D- and L-/D- (50%/50%) in lipid vesicle for 5mins and a period of 24 hours. Fig 5.3.3a-b shows the comparison of the CD spectra of freshly made L- and D- peptides in different environments, namely PBS, TFE and SDS. Fig 5.3.4 describes the CD spectrum of L- Aβ (25-35) in PBS after 24hours.

The spectra of L- and D- are nearly mirror images of each other (Fig5.3.1-2). This can be explained by the chiral centres in their structures. L- and D- peptide are optical isomers, so that their chiral centre structures are mirror images of each other. The CD signals are the results of the chiral centres of the peptide thus that the CD signal reflects the optical characteristics of their chiral centres. Also, the L-/D- (50%/50%) mixture gives nearly a flat line in the CD spectrum. The same amount of L- and D- peptide in the system means that the same amounts of radiation of light from the two peptides compromise each other, which leads to an almost zero CD signal.

Both freshly made L- and D- peptide have structures of random coils in all the environments measured. Comparing the obtained CD spectra to reference spectra, for example, in Fig 5.3.3a, the CD signal of L- peptide starts around 0 at wavelength 190nm, slightly going down then going up again at wavelength 200nm, and then becomes more flat towards the end. It is a typical random unstructured CD curve (Fig 5.2.2). There is no substantial difference of CD
spectra in PBS, TFE and SDS. Another experiment was conducted aimed to examine the secondary structure of fibril. D- Aβ (25-35) was dissolved in PBS a day ago before the CD experiment. Fibril growth experiment has shown that Aβ (25-35) starts to form fibrils after several minutes. This gave enough time for fibril formation. After 24 hours, D- peptide in PBS shows a typical β sheets structure. (Fig 5.3.4). The results show that the random structure of Aβ (25-35) evolves into β sheets during the process of formation of fibrils.

The CD spectra of L- and D- in lipid vesicles do not show differences between vesicles formed after 5mins and after an incubation of 24 hours. In a period of 24 hours, the Aβ (25-35) peptide might not form fibrils in a lipid environment, as Aβ (25-35) peptide does in PBS.

Fig 5.3.1 Far UV CD spectra of L-peptide in PBS (pink), D-peptide in PBS (green) and D+L peptide in PBS (blue). Peptide concentration approximately 1mM,
although the intensity differences suggest that the L- β-amyloid is probably more concentrated.

Fig 5.3.2 Far UV CD spectra of L-peptide in lipids (T=5min (black) T=24hrs (red)), D-peptide in lipids (T= 5 mins (blue) T= 24hrs (green)) and D+L peptide in lipids (pink). 24hrs incubation at 4 °C.
Fig 5.3.3a Far UV CD spectra of L-peptide in PBS (pink), 50% TFE (red) and 0.05% SDS (blue). Peptide concentration is 600uM.

Fig 5.3.3b Far UV CD spectra of D-peptide in 50% TFE (pink) and 0.05% SDS (blue). Peptide concentration approximately 600uM.
Fig 5.3.4 Far UV CD spectra of D-peptide in PBS after 24 hours at 4°C. Peptide concentration approximately 600uM.
5.4 References list


Chapter 6

Neutron diffraction
6.1 General introduction

Neutron scattering techniques have been widely used for the study of the structure of biological membranes. Neutrons were first predicted to exist by a British physicist, Ernest Rutherford, in the 1920s. However, due to the neutron’s uncharged nature, the proof of its existence was quite difficult to obtain. In 1932, the English physicist James Chadwick proved that neutrons did exist by identifying the new radiation generated from the interaction of α particles with beryllium nuclei. When the radiation passed through paraffin wax, protons were produced and detected.

\[ \alpha + ^9\text{Be} \rightarrow ^{12}\text{C} + n \]

A controlled nuclear reaction was achieved in the first nuclear reactor by Enrico Fermi in 1942, allowing the development of the neutron scattering technique. However, it was not until 1945 that nuclear reactors were accessible for scientific research at Argonne National Laboratory, Illinois.
6.1.1 Neutrons for biological science

The neutron is a non-charged, elementary particle that found in all atomic nuclei except for hydrogen. It has a mass similar to that of a proton, a nuclear spin of $\frac{1}{2}$, and a magnetic moment. The neutron beams used for biological experiments can be generated by either nuclear reactors or spallation sources.

For example, in Chalk River Laboratories at Canada, a neutron reactor was used. A series of collisions with deuterium atoms thermalize the fission of $^{235}\text{U}$ atoms, which results in the production of neutrons. After exiting from the reactor, neutron beams are then being monochromated from a crystal monochromator, and can be used for biological studies.

Researchers travel to institutes that have either a spallation source or a neutron reactor to use the neutron beams. When the beam time is granted, under the help of local scientific and technical expertise, researchers carry out the scattering measurements. The institutes that possess neutron sources are few.

and often far away and the application process for beam time can be long and complicated. The successful applicants usually have to wait months after the application of the beam time to initiate their experiments.

### 6.1.2 Diffraction and Properties of Neutrons

Diffraction can be defined as ‘the deviation of a wave from its natural direction of propagation when it encounters an object or a medium with spatially varying transmittance’. Max von Laue first observed ordered X-ray diffraction from a crystal. An aperture can cause diffraction from a wave. To have the maximum effect, the wavelength should be close to the size of the aperture. When a series of apertures are carefully placed and the distance between the each opening is fixed, the encountered wave will be diffracted and there will be interactions between the diffracted waves. In crystallography, a crystal, rather than a set of apertures causes the diffraction. The angle of diffraction and the kind of crystal decide the intensity of the interference which can be measured to give insight into the structure of crystal.

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Neutrons have the ability to penetrate deep into condensed matter because there is no electrostatic force to overcome due to their uncharged nature. When neutrons interact with atomic nuclei, the complex interaction in between a nuclear spin and a magnetic moment is present. The power of an atom to scatter neutrons does not lie in its position in the periodic table, unlike X-ray. The ability to scatter neutrons even differs between different isotopes. $\text{H}_2\text{O}$ and $\text{D}_2\text{O}$, for example, have different scattering power, or different scattering length densities.

### 6.1.3 Theory of neutron diffraction experiment

When a series of monochromatic beams encounter a sample, only at Bragg angle, the diffraction can happen. This is described by Bragg's law in 6.1.3.

$$n\lambda = 2d \sin \theta.$$  

Where $n$ is an integer (called the order of diffraction), $\lambda$ is the wavelength of the
beam, \( d \) is the repeated space between the membrane bilayers, and \( \theta \) is \( 1/2 \) of the diffraction angle of Bragg reflection. The distance between the scattered waves is related to the wavelength. This will only happen at Bragg angle.

If the scattering centers are aligned, it will lead to constructive interference. On the contrary, if the centers are not aligned, destructive interference will happen, which means we will not be able to observe any diffraction. Neutrons have very weak interactions with the component atoms of phospholipid. In order to measure good intensity, a scattering sample needs to contain many hundreds or thousands of membrane units. In an experiment, samples of good quality are required. A good quality of the sample means highly aligned multi-bilayer 'lattice stacking' units.
Fig 6.1.3.1 A lattice stacking structure, which consists of hundreds of repeated units is required in order to employ neutron diffraction.

The sample is placed in between a monitor and a detector. The angles of scan are fixed before each experiment. The V1 membrane diffractometer in Berlin has a monochromator providing wavelength between 0.4 to 0.6 nm and a Be filter that can remove unnecessary second order wavelengths. It provides the scattering angles between -10° to 120°. The scheme of the diffraction geometry is shown below. (Fig.6.1.3.2)

![Diffraction Scheme](image_url)

Fig 6.1.3.2 When the neutron beam encounter the sample at a certain angle, the
first order of the diffraction is produced. The scattering centers are at a distance ‘d’ apart.

6.2 Methods

6.2.1 Sample preparation

DOPC was purchased from Avanti Polar Lipids. Aβ and deuterated Aβ (d-Aβ) were purchased from ALMAC (Gladsmuir, UK). The deuterated label was at Alanine (L-Alanine-D4). 20mg of DOPC was dissolved in 1g chloroform and then mixed with 3%(mol/mol) monomeric Aβ with trace of TFA. The mixture was added to an artist’s airbrush and then deposit evenly onto a quartz slide under air or nitrogen. In order to remove all trace of the solvent, the sample slides were dried under the air for a few minutes and then placed in a vacuum for 6-8 hours. The samples then were placed into the cans for neutron scattering with a suitable humidity for equilibration. The Aβ used in the experiments is either pure L- or D- peptide, or the mixture of L- and D-, as in (1/3 L- + 2/3 D-), (2/3 L- + 1/3 D-).
Neutron diffraction data were collected on the V1 membrane diffractometer at Helmholtz Zentrum Berlin. The humidity of the sample was controlled by changing the salts in the sample cans. The temperature of the sample was held at 37 °C by a thermostatic water bath that circulated water though each sample can. The humidity for samples at 8% D₂O was 98%, 94% and 84% and the humidity for samples at 25% D₂O was 94%. The salts that were used to achieve humidity at 98%, 94% and 84% were K₂SO₄, KNO₃ and KCl, respectively. All the samples were measured in sequence from the highest humidity to the lowest. Each sample was equilibrated to the desired temperature and humidity for at least 10 hours. A programme of consecutive θ-2θ scans were carried out. The angles for the scans in this experiment were from 1.5° to 20°.

The CARESS software was used to collect the data and convert it from pixel response to a linear spectrum. Igor Pro was then used to fit each peak and subtract background noise. For each sample, 5 orders of diffraction with different peak intensities are visible and measured. The Gaussian distributions were fitted to Bragg reflections. The information of the position of each peak, its width and area were recorded. Fig 6.2.1.1-6.2.1.2 give examples of Gaussian fitting and scattering patterns.
Fig 6.2.1.1 An example of Gaussian fitting to the first and second order of a DOPC bilayer
Fig 6.2.1.2 Diffraction pattern of DOPC bilayer with L- Aβ peptide at 37°C with 8% D$_2$O at 98% humidity

6.2.2 Phase determination

In order to determine the phase (+ or -) of each order of diffraction, and to produce more detailed structural information, the stacks of phospholipids from the samples were hydrated and the d-repeat was progressively changed by controlling the humidity of the sample can, as described above. The percentage of D$_2$O in the humidity was also varied, in order to determine the water profile between adjacent bilayer in the multi-bilayer stack. At 8% D$_2$O, the neutron scattering length density is 0, so it is “invisible” to neutrons. At each order of neutron diffraction, when the scanning angle meets the Bragg geometry, the value of structure factor was given. This method works well on neuron
scattering for lamellar lipids. It also allows the same sample to be scanned a few times because of the very small amount of radiation damage from the neutrons.

Structure factors amplitudes were given by formula 6.2.2.1

\[ |F(q)| = \sqrt{C_{\text{abs}}C_{\text{lor}}}I \] 6.2.2.1

Where \( C_{\text{abs}} \) is the correction factor, and \( C_{\text{lor}} \) is Lorentz correction factor

As for the data collected at 8% D\(_2\)O, the structure factors were phased in reciprocal space from using the continuous form factor, as described in Formula 6.2.2.2

\[ F(h) = \sum_{H=0}^{H_{\text{max}}} F(H) \frac{\sin(\pi Dh/d - \pi H)}{(\pi Dh/d - \pi H)} \] 6.2.2.2

Where \( F(h) \) is structure factor, which corresponds to a \( d \) repeat of \( D \).
These methods have been described in detail in Darkes & Bradshaw 2000 and Bradshaw et al 2006.

6.3 Results and discussion

An example of neutron scattering density profile for DOPC is displayed in Figure 6.3.1. The hydrophilic part of the lipid bilayer locates at the outer region of the profile. A sketch of half of a DOPC bilayer is also shown to assist the interpretation of the profile. The phosphate head groups locate at (+/-) 18Å. At 0Å is the terminal of methyl groups of the fatty acids of DOPC.


Fig 6.3.1 Bilayer profile of DOPC. The horizontal axis is distance across the bilayer, in Å, and the vertical axis is neutron scattering length density, in arbitrary units.

Fully corrected and scaled structure factors for DOPC and DOPC with Aβ peptide are shown in Table 6.3.1. The errors of the sets of structure factors were determined by least squares fitting when structure factor amplitudes were plotted in quasi-continuous transforms. Difference profiles were produced by subtracting DOPC bilayers profile from the profiles of DOPC with Aβ peptide.
and are displayed in Figure 6.3.3. A water distribution profile is shown in Figure 6.3.2.

<table>
<thead>
<tr>
<th></th>
<th>F (1)</th>
<th>F (2)</th>
<th>F (3)</th>
<th>F (4)</th>
<th>F (5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DOPC</td>
<td>3.955</td>
<td>-2.191</td>
<td>-1.554</td>
<td>-0.763</td>
<td>0.483</td>
</tr>
<tr>
<td></td>
<td>±0.0025</td>
<td>±0.026</td>
<td>±0.0040</td>
<td>±0.0084</td>
<td>±0.034</td>
</tr>
<tr>
<td>DOPC+ L- Aβ</td>
<td>4.9548</td>
<td>-2.34</td>
<td>-1.728</td>
<td>-0.528</td>
<td>0.576</td>
</tr>
<tr>
<td></td>
<td>±0.0060</td>
<td>±0.097</td>
<td>±0.055</td>
<td>±0.025</td>
<td>±0.024</td>
</tr>
<tr>
<td>DOPC+ dL- Aβ</td>
<td>5.2785</td>
<td>-2.8129</td>
<td>-1.955</td>
<td>-0.74865</td>
<td>0.40595</td>
</tr>
<tr>
<td></td>
<td>±0.30</td>
<td>±0.17</td>
<td>±0.0019</td>
<td>±0.081</td>
<td>±0.083</td>
</tr>
<tr>
<td>DOPC+(1/3)D- +(2/3)L- Aβ</td>
<td>4.31496</td>
<td>-2.2776</td>
<td>-1.29792</td>
<td>-0.52</td>
<td>0.59592</td>
</tr>
<tr>
<td></td>
<td>±0.20</td>
<td>±0.21</td>
<td>±0.084</td>
<td>±0.037</td>
<td>±0.011</td>
</tr>
<tr>
<td>DOPC+(1/3)D- +(2/3)dL- Aβ</td>
<td>5.208</td>
<td>-3.0576</td>
<td>-2.2176</td>
<td>-0.5936</td>
<td>0.7616</td>
</tr>
<tr>
<td></td>
<td>±0.084</td>
<td>±0.15</td>
<td>±0.0027</td>
<td>±0.071</td>
<td>±0.037</td>
</tr>
</tbody>
</table>

Table 6.3.1 The structure factor amplitudes for DOPC bilayers and DOPC with amyloid peptides at 37°C, and 8% D₂O.
Figure 6.3.2 Water profile and distribution compared to DOPC. The horizontal axis is distance across the bilayer, in Å, and the vertical axis is neutron scattering length density, in arbitrary units.

The difference profiles describing the shape of the water distribution between adjacent bilayers in the multi-bilayer stack show clear differences when peptide is present. Figure 6.3.2 shows that the presence of peptide, either all L- or mixtures of D- and L-, allows water to penetrate into the surface layers of the
bilayer. This may represent water of hydration binding loosely to the peptide, or it may be deuterons exchanged for protons on the peptide surface. Whichever is correct, it is clear that the peptide must be penetrating into the surface of the lipid bilayers. There are subtle differences in the water distributions between the all L and (1/3D-+2/3L-) peptides but it is not possible to say how significant these differences are.
Figure 6.3.3 DOPC profile and difference profiles showing the distribution of peptide. The horizontal axis is distance across the bilayer, in Å, and the vertical axis is neutron scattering length density, in arbitrary units.

In Figure 6.3.3, the distribution profiles of the peptide suggest interaction between Aβ peptide and DOPC lipid. Although there are changes in detail between the all-L and(1/3D+2/3L-) peptides, both show penetration deep into the non-polar regions of the bilayer. Once again, it is difficult to say whether the differences between the profiles of the two types of peptide are significant or not.

In order to gain more detailed information about the interaction of the peptide with the phospholipids, it is necessary to use specifically-deuterated peptide. This is because the difference profiles calculated by subtracting lipid alone from lipid plus peptide include other features apart from the peptide itself. I have already shown (above) that the water profile changes when peptide is added to DOPC bilayers. This difference in water distribution will also contribute to the peptide distribution profiles, as will any changes to the lipid profiles caused by
molecular displacement by peptide molecules.

The use of specifically-deuterated peptide has two advantages. Firstly, by subtracting lipid plus undeuterated peptide from lipid plus deuterated peptide, the only difference is the presence of the deuterons on the labeled amino acid. The resulting difference profiles, therefore, will not include any changes in water distribution or molecular rearrangements. Secondly, the label distribution approximates to a simple Gaussian distribution, allowing more precise interpretation of the differences by model fitting. This is particularly accurate if the fitting process is carried out in reciprocal (diffraction) space, since this avoids any artifacts caused by termination error.

The profiles of deuterated peptide are shown in Fig 6.3.4. The difference of the peptide profiles between deuterium peptide and non-deuterium peptide gives rise to the profiles of deuterium label distribution, which is shown in Fig 6.3.5. The two Gaussian distributions of all the diffraction profiles are given and fitted by least squares refinement to difference structure factors. The Gaussian fitting profiles give the information of the center, position and distribution of the
peptide. (Table 6.3.2) The Gaussian fitting profiles of the label distributions for L-peptide and \((2/3L+1/3D^-)\) peptide are displayed in Table 6.3.3.

Fig 6.3.4 Profiles of distribution of deuterated peptide
6.3.5 The profiles of deuterium label distribution. Profiles obtained by subtracting profiles of non-deuterium peptide from deuterium peptide. The horizontal axis is distance across the bilayer, in Å, and the vertical axis is neutron scattering length density, in arbitrary units.
Table 6.3.2 The location of peptide. The centre, width and occupancy of Gaussian distributions fitted in reciprocal space to difference structure factors.

<table>
<thead>
<tr>
<th>Gaussian</th>
<th>DOPC+ L-αβ</th>
<th>DOPC+ dL-αβ</th>
<th>DOPC+(1/3)D-+(3/2)L-αβ</th>
<th>DOPC+(1/3)D-+(2/3)dL-αβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position</td>
<td>25.45 Å</td>
<td>25.66 Å</td>
<td>25.66 Å</td>
<td>25.45 Å</td>
</tr>
<tr>
<td>Width</td>
<td>6.27</td>
<td>0</td>
<td>4.11</td>
<td>6.07</td>
</tr>
<tr>
<td>Occupancy</td>
<td>46.3%</td>
<td>0</td>
<td>40%</td>
<td>24.3%</td>
</tr>
<tr>
<td>Position</td>
<td>14.79 Å</td>
<td>17.49 Å</td>
<td>12.85 Å</td>
<td>15.3 Å</td>
</tr>
<tr>
<td>Width</td>
<td>5.1</td>
<td>7.2</td>
<td>6.17</td>
<td>5.1</td>
</tr>
<tr>
<td>Occupancy</td>
<td>53.7%</td>
<td>100%</td>
<td>60%</td>
<td>75.7%</td>
</tr>
</tbody>
</table>

Sharp peaks from the deuterium label show that the interaction of peptide and lipid is specific. In terms of the deuterated peptide, the peaks are smaller in the mixtures than the peaks of samples contain all deuterated peptide, reflecting the fact that there is less deuterated label present in the mixtures. The mixture of L- and D- peptide clearly penetrates deeper into the lipid than L- peptide. Table

Table 6.3.3 The location of deuterium label. The center, width and occupancy of Gaussian distributions fitted in reciprocal space to difference structure factors.

<table>
<thead>
<tr>
<th>Gaussian</th>
<th>Deuterated label in L-</th>
<th>Deuterated label in (1/3)D-+(2/3)L-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position</td>
<td>18.13 Å</td>
<td>16.58 Å</td>
</tr>
<tr>
<td>Width</td>
<td>6.53</td>
<td>5.1</td>
</tr>
<tr>
<td>Occupancy</td>
<td>100%</td>
<td>100%</td>
</tr>
</tbody>
</table>
6.3.2 provides more detailed information of the position of the deuterium label.

The mixture goes deeply into the center (hydrophobic part) of bilayers. L- Aβ lies around just below the glycerol group. Since previous studies of fibril growth verified that there are fewer fibrils in the mixture of peptide than in the pure peptide (either L- or D- alone), and that the mixtures have more impact on DOPC, it is not difficult to draw a conclusion that it is some intermediate forms of Aβ, rather than mature fibrils that have a greater influence on lipids. This finding agrees with the previous research by Ashley et al of the penetration of soluble forms of Aβ into bilayers. The water profiles indicate that water does not go into the center of the bilayers even with the interactions of the peptide. The peptide does not go across the bilayers. The width of water peaks is consistent with sub-glycerol penetration of the peptide.

The shapes of the profiles of samples containing deuterated peptide look alike and the shapes of the profiles of samples containing non-deuterated peptide look quite similar. However, the two kinds of samples show different shapes of profile. Also, the samples that contain non-deuterated peptide lie closer to the center of the bilayers than the samples that contain deuterated peptide. It is difficult to interpret the difference of membrane interruption between samples.
that have deuterated and non deuterated peptide.

One study by D’Ursi et al. shows the conformation of Aβ (25-35) in a simulated lipid environment (80/20 HFIP/water) is highly ordered and folded in C-terminal.\(^5\) Which means that the beginning of Aβ (25-35) is less hydrophobic and less prone to form fibrils. According to our results, the toxic species of Aβ (25-35) are not likely to be the fibrils. If the results from the study by D’Ursi et al are a true representation of reality, the toxic component of Aβ (25-35) could be the starting fragment of the sequence. Fig6.3.6 is to illustrate its structure and membrane active residue. This agrees with former studies about membrane active residue being within Aβ(25-28).\(^6,7\)


Fig 6.3.6 Aβ (25-35) structure in 80/20 HFIP/water environment that mimics a lipid environment and its membrane active region.
6.4 References list

1. T. A. Harroun, G. D. Wignall, J. Katsaras Neutron Scattering for Biology Neutron Scattering in BiologyBiological and Medical Physics, Biomedical Engineering 2006, pp 1-18


7. Kar, S., et al., Amyloid beta-peptide inhibits high-affinity choline uptake and acetylcholine
Chapter 7

General conclusion
My PhD work focused on three major questions. The first was to study the structure and self-assembly phenomena of Aβ. Secondly, to answer the question whether Aβ (25-35) interacts with phospholipids? And thirdly, if it does, what kind of interaction does it have with the lipids? The method of mixing two types of isomers L- and D- peptide was used to help differentiate between the possible interactive species of Aβ (25-35).

To answer the first question, the CD experiment and fibril growth experiment were conducted. CD provided insight into the secondary structure of Aβ (25-35). It was important to study the secondary structure of this peptide, since Aβ is able to self-assemble, leading to the formation of fibrils. The study of secondary structure makes it possible to study the basic structure of fibrils and gives information about the mechanism of fibril formation. The purpose of the fibril growth experiment was to observe and study the self-accumulation of Aβ (25-35), as well as to determine whether mixtures of L- and D- peptide are able to form fibrils in the same way as either L- or D- peptide alone, since the mixtures have contradictive structures with the potential to slow down the fibril formation. The results of these studies benefited later experiments whose aims were to find out the toxic species (or forms) of Aβ (25-35) on phospholipid.
In order to study the interaction between Aβ (25-35) and phospholipids, Langmuir experiments, BAM and neutron experiments were carried out. The aim of the Langmuir trough experiment was to look at the isotherm and surface pressure change of a phospholipid monolayer under the influence of Aβ (25-35). This experiment design was straightforward, and sensitive to any changes that happened at the monolayer surface. The observation of changes in the monolayer surface area or pressure led me to try BAM experiments, whose aim was to visualize any topological changes at the Langmuir monolayer, that had the potential to explain the changes. In order to confirm the results from Langmuir trough, as well as to give more detailed information of the interactions, such as the location of peptide at monolayer when any interaction happens, neutron diffraction experiments were conducted.

All these three experiments mentioned above also provided information about the toxic forms of Aβ (25-35). During the process of changing from monomers to fibrils, under a biological environment, Aβ (25-35) peptide undergoes a few structural changes. In order to determine whether it is the final product of fibril formation process, or monomers, or intermediates that is membrane active, is the key aim of this study.

The fibril growth experiment had previously proved that the mixture of isomers
has reduced tendency to form fibrils. By conducting these three experiments using a single peptide, and with mixtures of both, to see the different level of interaction with the phospholipid, I hoped to be able to determine what was the toxic form of Aβ (25-35).

The CD measurement showed that the secondary structure of Aβ (25-35), either mixed with lipids, or in PBS, is random when Aβ is freshly made. Later on, the secondary structure of Aβ evolves into β sheets. This result makes it interesting to study the mechanism of the structure change during fibril formation. The CD signal from mixture of L- and D- (50%/50%) was nearly a flat line, since the secondary structures of L- and D- are contradictory to each other. The fibril growth experiment proves that L-, or D- peptide alone forms twice the amount of fibrils as that in the mixtures. This gave me a basis for studying the toxic species of Aβ.

Aβ (25-35) is a membrane active peptide. In Langmuir experiments, it expands the phospholipid monolayer when the surface pressure is fixed. It can interact with the monolayer either from the PBS subphase below, or by simply exposing itself in the lipids. This is shown by isotherms of lipids mixed with Aβ (25-35). Although the Langmuir experiments show that the peptide causes changes to monolayers on the air-water interface, BAM experiments show no visible
changes. A possible explanation is that the Aβ (25-35) peptide may only interact with the head group region of DPPC, and does not go deep into the lipid layer, beyond the head groups, into the tails. Thus there is no change of the reflected light from the surface of DPPC. Fig 7.1 shows the possible interaction between Aβ (25-35) and DPPC on the surface of Langmuir trough.

Fig 7.1 Aβ (25-35) peptides stay close to the head groups of DPPC

The neutron experiments give more information about the location of Aβ (25-35) peptide in DOPC bilayers. The introduction of deuterated label provides more precise details of the location of the peptide. L- Aβ (25-35) sits just below the level of the phosphate group, whereas mixtures of L- and D- Aβ (25-35) peptide penetrate deeper and reach beyond the glycerol group. Figure 7.2(a-b) illustrates the position of Aβ (25-35) peptide on DOPC.
Fig 7.2 a L- Aβ (25-35) sits round phosphate group
Fig 7.2 b L-/D- Aβ (25-35) penetrates deeply and reaches to glycerol group

This observation, that mixtures of peptide penetrate deeper into the bilayer indicates that Aβ fibrils prevent bilayer interaction, since fibril growth is inhibited in the mixtures. These results show the ability of Aβ (25-35) to interact with phospholipid membranes, and indicate that fibril form is less
membrane-active than the intermediate species of Aβ (25-35).

The membranes of neuron cells are complex and consist of many kinds of phospholipids and proteins. The use of model of bilayers containing only phospholipid provides insight into the possible action of Aβ (25-35) on neuron cells, but it would be quite difficult to use the same technique to study complicated biological membranes. Aβ (25-35) is a fragment of its full version Aβ (1-40,42). Its toxicity is demonstrated by this PhD study. Further possible studies involving Aβ (1-40,42) and comparisons of this peptide to the shorter one used in my study may be beneficial to our understanding of the biological activities of this peptide.