Membrane Mediated Aggregation of Amyloid-β Protein: A Potential Key Event in Alzheimer’s Disease

Marcus Bokvist

Akademisk avhandling

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Fakultetsopponent: Docent Lena Måler, Institution för Biokemi and Biofysik Arrheniuslaboratoriet, Stockholms universitet.
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Abstract
The pathogenesis of Alzheimer’s disease (AD), the most common senile dementia, is a complex process. A crucial event in AD is the aggregation of amyloid-β protein (Aβ), a cleavage product from the Amyloid Precursor Protein (APP). Aβ₄₀, a common component in amyloid plaques found in patients, aggregates in vitro at concentrations much higher than the one found in vivo. But in the presence of charged lipid membranes, aggregations occurs at much lower concentration in vitro compared to the membrane-free case. This can be understood due to the ability of Aβ to get electrostatically attracted to target membranes with a pronounced surface potential. This electrostatically driven process accumulates peptide at the membrane surface at concentrations high enough for aggregation while the bulk concentration still remains below threshold. Here, we elucidated the molecular nature of this Aβ-membrane process and its consequences for Aβ misfolding by Circular Dichroism Spectroscopy, Differential Scanning Calorimetry and Nuclear Magnetic Resonance Spectroscopy. First, we revealed by NMR that Aβ₄₀ peptide does indeed interact electrostatically with membranes of negative and positive surface potential. Surprisingly, it even binds to nominal neutral membranes if these contain lipids of opposite charge. Combined NMR and CD studies also revealed that the peptide might be shielded from aggregation when incorporated into the membrane. Moreover, CD studies of Aβ₄₀ added to charged membranes showed that both positively and negatively membranes induce aggregation albeit at different kinetics and finally that macromolecular crowding can both speed up and slow down aggregation of Aβ.

Keywords: Alzheimer’s Disease, Aβ₄₀, Circular Dichroism, NMR, Amyloids, Crowding, Peptide-Lipid Interaction

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Membrane Mediated Aggregation of Amyloid-β Protein: A Potential Key Event in Alzheimer’s Disease

1. LIST OF PAPERS ................................................................................................................. 1

2. INTRODUCTION .................................................................................................................... 2

3. LIPIDS ....................................................................................................................................... 3
   3.1 THE MEMBRANE ................................................................................................................ 3
   3.2 LIPIDS ................................................................................................................................ 6
   3.3 ELECTROSTATICS IN MEMBRANES ................................................................................... 8
   3.4 DIFFERENT LIPID COMPOSITION IN DIFFERENT MEMBRANES ................................. 9
       References .............................................................................................................................. 9

4. ALZHEIMER’S DISEASE ....................................................................................................... 11
   4.1 BACKGROUND ................................................................................................................... 11
   4.2 MOLECULAR PATHOLOGY ................................................................................................. 11
      4.2.1 Amyloid Precursor Protein, APP .................................................................................. 12
      4.2.2 APP Cleavage .............................................................................................................. 13
   4.3 AGING OF MEMBRANE .................................................................................................. 14
   4.4 ESTABLISHED RISK FACTORS ....................................................................................... 15
   4.5 CASCADE HYPOTHESIS OF AD .................................................................................... 16
   4.6 AGGREGATION IN CONNECTION WITH MEMBRANES AND FREE AB ......................... 17
   4.7 TOXICITY .......................................................................................................................... 19
   4.8 THERAPIES ........................................................................................................................ 20
   4.9 FURTHER OUTLOOK ....................................................................................................... 20
       References .............................................................................................................................. 21

5. EXPERIMENTAL TECHNIQUES ........................................................................................... 26
   5.1 CIRCULAR DICHIROISM .................................................................................................. 26
      5.1.1 Basics .......................................................................................................................... 26
      5.1.2 Deconvolution .............................................................................................................. 28
   5.2 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY (NMR) .................................... 29
   5.3 DIFFERENTIAL SCANNING CALORIMETRY .................................................................... 30
       References .............................................................................................................................. 31

6. THE INTERACTION OF AB_{40}-PEPTIDE WITH MODEL MEMBRANES .......... 33
   6.1 AIM OF THE PROJECT ...................................................................................................... 33
   6.2 PAPER I .............................................................................................................................. 33
   6.3 PAPER II ........................................................................................................................... 35
   6.4 PAPER III .......................................................................................................................... 36
   6.5 PAPER IV .......................................................................................................................... 39
   6.6 PAPER V ............................................................................................................................ 40
       References .............................................................................................................................. 41

7. SUMMARY AND FUTURE OUTLOOK ................................................................................. 42

8. ACKNOWLEDGEMENTS ....................................................................................................... 43
References .............................................................................................................. 44
1. List of Papers

This thesis is based on the papers and manuscripts as listed below; which will be referred to in the text as Papers I-V.

I  B. Bonev, A. Watts, M. Bokvist, G. Gröbner
“Electrostatic peptide-lipid interactions of amyloid-beta peptide and pentalysine with membrane surfaces monitored by P-31 MAS NMR”
PHYSICAL CHEMISTRY CHEMICAL PHYSICS 3 (2001) pp. 2904-2910

II  F. Lindström, M. Bokvist, T. Sparrman, G. Gröbner
“Association of amyloid-β peptide with membrane surfaces monitored by solid state NMR”

III  M. Bokvist, F. Lindström, A. Watts, G. Gröbner
“Two Types of Alzheimer’s β-Amyloid (1-40) peptide membrane interactions: Aggregation Preventing Transmembrane Anchoring Versus Accelerated Surface Fibril Formation”

IV  M Bokvist, F Lindström, T Borowik, G Gröbner
Two-dimensional Aggregation of Alzheimer’s β-Amyloid (1-40) on Membrane Surfaces: Two Electrostatically Controlled Alternative Pathways.
Manuscript, To be submitted to Proceedings of National Academy of Science

V  M Bokvist, Gerhard Gröbner
The Impact of Macromolecular Crowding on the Folding Behaviour of Amyloid-β Protein: Membrane Surface Crowding versus Macromolecular Crowding.
Manuscript, To be submitted to FEBS Letters
2. Introduction

Well, here we go, the introduction, the piece of the thesis fairly free in form where the author tries to be different and not too dull and boring; in doing so does like everyone else. Thus fails in his/her purpose, or be objective and fail by default. If I wanted to really make you remember this thesis, dear reader, I could of course quote Terrorizer magazine and ask you to “READ IT OR PROCREATE OFF!” But I guess this is too extreme. Though I have a sneaking suspicion that if your attention is not held by me by now it will never be. Hopefully I have just changed your mind in case you were just planning to read the introduction and acknowledgements and just now changed your mind that the rest of the introduction won’t bore you back to your initial mindset.

At the start of my work in this project it was begun out of a desire to lift a rock to see what lurked beneath it. By now quite a few rocks have been lifted, and although there are more rocks to lift I think it is time to write my PhD-thesis to report what was found beneath those metaphorical stones. This work was done under the prerequisite that what I found there is there whether I look there or not, and secondly if someone else looked they would find the same thing. I won’t go any further into this since this is not a thesis about philosophy of science, and surely anyone interested in this know what fora to visit for such discussions.

So if we go back to the area where I was looking: Alzheimers disease, the most common form of dementia in old people. The mystery of this disease has by no means solved, nor has a cure been found. I have merely examined a small but interesting part of this problem from a biophysical point of view, namely the amyloid β-protein and it’s interaction with membranes, hopefully to use for someone. I won’t go into detail here but leave that to the following chapters. If this sounds interesting enough to start reading the thesis, please go ahead and if not then just go to chapter 8, acknowledgements and see if you find something you like there.
Membrane Mediated Aggregation of Amyloid-β Protein: A Potential Key Event in Alzheimer’s Disease

3. Lipids

3.1 The Membrane

As a boy, a few months before Christmas, mailorder toy companies used to send me their catalogues. Among the many things I found interesting there was “the little chemist”, a small chemistry kit. I was never given that kit for Christmas but later a friend of mine, owning this kit, told me he had mixed all the ingredients in it and then run for his life before the explosion. The truth of this claim might of course doubted but it highlights an interesting phenomenon also present and necessary in the living cell, considering all the different substances and processes in the body: The need for compartmentalization, to prevent everything from reacting at once, by separating the reactants. Separating the reactants of life completely is not good either, since this would mean a quenching of the reactions of life. So what is needed is something that allows controlled separation and transport in and out of the cell, the basic unit of life. For the cell, the plasma membrane performs these tasks [1].

The plasma membrane consists of a lipid bilayer with membrane proteins composed of mainly lipids with their water seeking part facing outward from the bilayer center towards the polar environment and the water hating tails pointing inwards, creating a unpolar inside [1]. The membrane also contains proteins that might cover up to 70% of the surface [2]. They can be divided into many classes due to their properties. One classification is integral and peripheral membrane proteins. Integral proteins are firmly anchored in the membrane and thus difficult to remove. Peripheral proteins on the other hand are more loosely attached to the membrane surface by hydrophobic or electrostatic binding and are more easily removed from the membrane [1]. Because of the asymmetry of the inner and outer side of the membrane the membrane proteins are also asymmetrically distributed on the inward facing and outward facing side [1,3]. Some proteins span the entire membrane cross section eg. Signal proteins, responsible for signaling across the membrane and transport proteins, responsible for transport of components across the membrane in a controlled fashion [1]. With the exception for some simple molecules
like H₂O and O₂, which diffuse in spontaneously, all transport of components across the membranes are strictly controlled by transport proteins [1].

A more elaborate model of the membrane is the Singer Nicholsson model developed approximately 30 years ago [5]. This model postulates that the membrane proteins are sailing on a sea of evenly distributed lipids which have an even thickness and a free and random two-dimensional movement. The lipids do not influence the protein actions, but are merely a liquid-crystalline matrix for the proteins, as can be seen in the top part of Figure 2. The orthodoxy of this model has been modified by later studies where it has been shown that the membrane thickness varies [2,4-5]. The membrane is even quite heterogenous with patches, sometimes called rafts. These highly debated domains contain a high amount of sphingomyelin and cholesterol in the outer leaflet and saturated phospholipids in the inner leaflet [2-4,6]. Rafts also contain high amounts of proteins. These rafts seem to arise from interactions between different lipids but also between lipids and proteins [2-4]. This more complex membrane model is shown in the bottom part of Figure 2. Often in biophysical research, simple biological model membranes are studied, since they are easier to control. This of course brings a risk of loss of relevancy to the biological systems. But in cases where e.g. membrane surface potential and its attraction to peripheral proteins are studied this problem is smaller than in more specific binding cases [3].

The most common membrane lipid components are phospholipids, glycolipids, sphingolipids and cholesterol and among the phospholipids in eukaryotic cells are the zwitterionic Phosphatidylcholine (PC) and Phosphatidylethanolamine (PE) [3]. Other common phospholipids in eukaryotic cells are Phosphatidylinerine (PS), Phosphatidylinositol (PI) and Phosphatidylglycerol (PG). The most commonly used phospholipids in experiments is PC due to its ability to form bilayers [2-3].
Figure 1: The Singer-Nicholsson model, (a) original and (b) modified version, adapted from Engelman [4]. As easily can be seen in (b) compared to (a) the proteins are clumped together in specific groups and the lipids form a rugged surface with an uneven distribution.
3.2 Lipids

A widely accepted definition of what a lipid is does not exist. Dr William W. Christie defined it as follows [8]: “Lipids are fatty acids and their derivates, and substances related biosynthetically or functionally to these compounds”. This definition includes cholesterol and gangliosides and is thus sufficient for this work. Lipids can then be divided into simple and complex ones. A simple lipid will be divided into two different species when hydrolyzed [8].

An interesting group of complex lipids are the sphingolipids, shown in Figure 2. They got their name due to their cryptic nature in the 19th century (The sphinx being a teller of riddles). The main chain is an alcohol called sphingosine. Their signum is a fatty acid linked to a long chain base by an amide bond and also a phosphorous group or a carbohydrate is linked to the terminal hydroxyl group. The sphingolipids are only found in the outer leaflet [6].

A special group of sphingolipids are the gangliosides, sphingolipids with one or more sialic acid groups and polar headgroups and negligible charge at pH 7. Around 6% of the membranes in the brain are made up of these lipids but exist in all levels of animal cells. Growth and differentiation has been showed to be controlled by gangliosides [9].

It has been showed that the levels of phospholipids and gangliosides change as a person gets older as well as during Alzheimer’s disease but this will be dicussed in greater detail in the AD-chapter.
Membrane Mediated Aggregation of Amyloid-β Protein: A Potential Key Event in Alzheimer’s Disease

Figure 2: Structure of A: Ganglioside GM1, B: Sphingomyelin, C: Cholesterol, D: 1,2-Dimyristoyl-sn-Glycero-3-Phosphocholine, DMPC, E: 1,2-Dimyristoyl-sn-Glycero-3-Phospho-1-Glycerol, DMPG. All structures are taken from Avanti Polar lipids homepage.
3.3 Electrostatics in Membranes

Many membrane mediated reactions are controlled by spatial arrangements of the membrane proteins with the membrane lipids acting as cofactors [2]. But it has also been shown that some membrane reactions are simply performed by an electrostatic attraction between the membrane lipid and protein, governing a reaction between a membrane lipid and a protein. McLaughlin Murray showed in 2005 that the MARCKS protein, together with Calmodulin and Ca\(^{2+}\), can control the PIP\(_2\)-lipid, which is important in messenging, entirely through electrostatic interaction. This mechanism is important since electrostatic interaction between the amyloid-\(\beta\) protein and the cell membrane seems to be an important part of AD [10-12].

**Figure 3:** Picture from McLaughlin & Murray [10], electrostatic protein-membrane interaction (a) The MARCKS protein regulate the PIP\(_2\) molecule by electrostatically binding to it: (b) At high Ca\(^{2+}\)-levels a Calmodulin/Ca complex will form and bind to MARCKS and, (c) it will loosen from the membrane surface thus freeing 3 PIP\(_2\)-molecules.
3.4 Different Lipid Composition in Different Membranes

Lipids are constantly produced and broken down in the living body. A balance between synthesis and degradation is necessary for a healthy body. Niemann-Pick’s disease is an example of an illness stemming from an upset in the aforementioned balance. It is a disease where sphingomyelin levels are built up in the brain, spleen and liver due to a defective sphingomyelinase [1].

It is also the case that different membrane types differ in composition with respect to the lipid-protein ratio, lipid composition and protein composition. The level of PC in rat liver changes between 39 and 58 % and the level of PC in human myelin membranes is 10% but is 25% in human erythrocytes is 25%. Also worth noticing is that the amount of PG is low in eucharyotic cells [1,3,13]. The differentiation occurs inside the same membrane, as the lipid composition might differ between the inner and outer leaflet [2]. An example of this separation are the glycolipids which are the main charged component in the outer leaflet, the other main components being SM, cholesterol and PC, which are less abundant in the inner leaflet [3].

The different properties of different lipids comes mainly from three sources; acyl chain structure, polar head structure and acyl chain location. By changing the composition of the membranes these afore mentioned traits will decide the membrane property [14].

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4. Alzheimer’s Disease

4.1 Background

As a young adult I used to work in nursing homes for old people during my summer holidays. Quite a lot of them exhibited quite strange behaviour, mostly due to senile dementia. This is especially true, viewed in the long run as the personality of the disease struck person is completely changed. Senile dementia means the loss of cognitive functions due to old age and the most common form of senile dementia is Alzheimer’s disease (AD) [1,2].

Alzheimer’s disease was first described by the Bavarian psychiatrist Alois Alzheimer in 1906. The patient was a woman in her early 50. At the time it was believed to be a rare presenile disorder and not the most common cause of senile dementia. There were two reasons for this; i) in 1906 most people died of other causes before they could develop AD; ii) there are two types of AD, familiar AD (FAD) which strikes early and sporadic (SAD), the form found in old people. Both forms are characterized by memory loss, cognitive deteriation, altered manners and decline of the language ability [2, 3].

4.2 Molecular Pathology

When the brain of dead AD-patients are studied, amyloid plaques and neurofibrillar tangles (NTF) are found, in the brain and blood vessels. These plaques are abundant in the medial temporal lobe and cortical areas of the brain, as well as dead neurons and decreased amount of synapses (before death) [1-2,4-5]. The NTF’s are twisted helical cytoskeletons and contains hyperphosphorylated tau protein fibers [2-3]. The plaques consist of mainly of aggregated β-amyloid protein (Aβ), a 39-43 long peptide that is the cleavage product of a longer precursor protein, called Amyloid precursor protein (APP) [1,4]. These plaques fulfill the amyloid definition, i.e. they are a protein precipitate that is stained by congo red [1]. There is
no simple correlation between the plaque load and severity of the dementia [1,3]. These plaques place AD in the group of diseases called amyloidoses, all characterized by protein deposits in a body tissue. These deposits are usually self-assembled fibril form of a disease specific protein. The final protein structure of amyloidoses is varied but some type of β-structure is an important part of the intermediate folding of the proteins. Examples of other amyloidoses are Parkinsons disease, Huntingtons disease and Type II diabetes [5].

**4.2.1 Amyloid Precursor Protein, APP**

APP is a 695-770 residue long transemembrane protein found in all mammals, especially in neurons and is part of a protein family called APPL (APP Like proteins) [2,6]. There is some redundancy between the family members as single member knockout mice (SKOM) show little adverse effect while complete family knockout mice (CKOM) die early. Since the CKOM still are being born it indicates that APP is not crucial for the fetal development but for the adult individual and that proteins in the APPL family have overlapping functions [2,6-7]. APP has been shown to attach to many proteins and might possible be a signal protein [6]. Aβ (Figure 1) is produced when APP is cut consecutively by β- and γ-secretase [2,8]. APP is coded on chromosome 21 and persons with Down’s syndrome get AD early in life [8]. APP mRNA transcription increase and Aβ expression have been shown in cases of brain trauma, and are believed to be crucial for the formation of axons and neurogenesis in adult persons. The differentiation decreased when neuronal stem cell were subjected to APP antibodies [8-13]. Recently, work by Salehi et al indicate that the overexpression of APP in Down’s syndrome persons might be the cause of their learning disability by interfering with the nerve growth factor transport [14].

![Amino acid sequence of βAP(1-42).](image)

**Figure 1:** Amino acid sequence of βAP(1-42).
4.2.2 APP Cleavage

There are two main pathways of APP processing, shown in Figure 2, involving α-secretase or β-secretase. α-secretase cuts in the middle of the prospective Aβ sequence into two pieces, the 83 residue C-terminal fragment (CTF-APP-α) and the N-terminal fragment (NTF-APP-α) [2,4,15-16]. The α-secretase cleaves between residue 16 and 17 in the prospective Aβ. When APP is cut by the membrane bound β-secretase into the 99 residue CTF-APP-β and NTF-APP-β and the CTF consecutively is cut by γ-secretase the 39-43 aa Aβ is the result [1-4,8,15]. β-secretase might also cut the CTF shorter (At the 11th residue of a prospective Aβ-molecule) to get the 89 residue CTF-APP-β’[15]. β-secretase is also called BACE (Beta-Amyloid precursor protein Cutting Enzyme) is a aspartyl protease [1,3,15]. The γ-secretase is believed to be a four protein complex consisting of Presenilin1 or 2, nicastrin, PEN-2 and APH-1. BACE is quite specific and only cuts one other protein, while gamma secretase cuts quite a few proteins, among them the NOTCH protein, important in gene transcription [2-3]. The most abundant Aβ species is the 40 aa long version but some of the highly aggregation prone Aβ42 is also present. How the control works is not exactly clear since these different cuts needs to be performed on different sides of the assumed α-helix of APP inside the membrane [1-2,16].

FAD can be caused by mutations in APP to make the Aβ-part more prone to aggregation once cut lose. A second possible cause of FAD is overproduction of Aβ [2-3,8,17]. For example people with Down’s syndrome, who have 3 copies of chromosome 21 (APP is coded on that chromosome), will produce more APP develops AD early in life [3,8]. Another more common cause of FAD is a mutation of the γ-secretase causing it to produce more Aβ42 which will skew the 40/42 rate and induce more aggregation [2,3,7,17].
Figure 2: Adapted from Gandy [1]. A: The cleavage sites for α-, β- and γ-secretase on APP in the membrane. B: The β-amyloid part of APP and the possible cleavage sites for the three secretases on APP in detail.

4.3 Aging of Membrane

As mentioned earlier in the lipid chapter the Aβ interacts electrostatical with the membrane [18-20]. And still there is no SAD until old age. This implies that membranes age, or more correctly; changes in the lipid membrane composition occur with age. This means that a couple of questions needs to be answered; pro primo) does the membrane change with old age? Pro secundo) is there any difference between the non-AD
brain and the AD brain in the membrane respect? Pro tertio) if the answer to 2 is positive, then is the difference between the two kinds of brains there before the onset or is the difference a consequence of AD? Luckily for me and the assumption of my thesis the answer to 1 and 2 are yes [21-24]. When it comes to 3/ the question is not yet fully answered yet since scientists are not allowed to crack open the skull of people at will, so this answer needs to be obtained by other means. To get back to 1 and 2 it has been proven that the levels of both gangliosides and phospho-lipids change during ageing. In healthy brains ganglioside content increases until 50 years. Thereafter their levels will decrease, first slowly and then at an increasing rate, the lifetime change being somewhat banana-shaped [21]. In the case of phospholipids there is no increase but a steady decline. There is no difference between men’s and women’s brains in this aspect [21,23,25]. The AD-brain on the other hand has decreased levels as well as changed proportions of both gangliosides and phospholipids [22-23]. This is important to know since it has been shown that it’s possible to quench the BACE activity, thus quenching Aβ-production, by depleting the membrane of cholesterol and APP is found in cholesterol rich rafts. In any case one has to keep these changed lipid levels in mind since they will be further discussed in the following subchapters [26-27].

4.4 Established Risk Factors

The last cause of FAD is believed to bear some resemblance of the suspected origin of SAD. Evidence seems to indicate that some disturbance of the lipid composition in the ageing brain might alter the 42/40 rate [3,6]. It has been shown that what type of Apoε present is a factor for the risk of getting SAD. Apoε is the body’s major cholesterol transporting protein, ε4 persons having an increased risk compared to the population in general while ε2 persons seems to have a decreased risk of SAD [1-3]. Apoε4, having one less an arginine residue where ε3 has one cystein at position 112, is a least effective antioxidant since it one cysteine residue short, making ε4-persons less resistant to oxidative stress from the Aβ molecules/aggregates [1]. Other risk factors for AD are blood cholesterol, ie the amount of cholesterol in the blood will affect the amount of cholesterol in the membranes [1,28], but this will be more
specifically discussed in later subchapters, physical and mental activity [3], brain reserve capacity [3], and head injury [3,29]. Exactly how a higher physical and mental activity protects against alzheimer’s is still unclear though, but maybe it’s related to the brain reserve capacity, ie the ability of the brain to bypass the dead neurons or it is the mental and physical activity maintains a healthy cholesterol level [3]. But as will shown, if the reader care to read on, membrane composition and change during ageing, seem to play an important role in SAD.

4.5 Cascade Hypothesis of AD

In the last few years it has been suggested that increased levels of Aβ_{42}-release, will cause membrane-associated oxidative stress (MAOS) [4,1]. This in turn will change the lipid composition in the membrane, which in turn will affect the activity of β- and γ-secretase [28]. A pathway suggested by Grimm et al. [30], shown in Figure 3, suggested that an initial Aβ_{42} increase due to ageing induces MAOS which will activate basic and acidic sphingomyelinases (indirect activation) as well as direct activation of the neutral SMase, leading to decreased levels of SM and increased levels of ceramide. The increased levels of ceramide will lead to synaptic dysfunction and more apoptosis. Lower levels of SM, that inhibits presenilin, will cause more γ-secretase activity. At the same time, lower levels of Aβ_{40}, that inhibits the rate limiting step in de novo synthesis of cholesterol, will increase the amount of cholesterol in the membrane. Increased amounts of cholesterol, will increase the BACE- and γ secretase activity and might also further influence the cutting preference of γ-secretase between residue 40 and 41 or between 42 and 43. These changes will be self amplifying [3,30-31].
Figure 3: The pathway suggested by Grimm et al. [30], modified by Mattson et al. [31]. An initial disruption of the APP-cleavage for whatever reason will set of two chains of events that will further disrupt the cleavage balance in a self-amplifying way that in the end will cause nerve cell death.

4.6 Aggregation in Connection with Membranes and Free Aβ

The impatient and alert reader might by now noticed that I have not touched the subject of aggregation yet, except for very briefly in the lipid chapter, and still this is the main focus this thesis. This subchapter will address this.

What needs to be answered is why Aβ that is released in its monomeric form, converts into big fibrillar amyloid plaques [1]. Aβ has been shown
to aggregate in solution [19]. This aggregation is reversible when the aggregates are fresh and the aggregate shape differs with the Aβ42 being more fibrillar [32-33]. Aβ42 is also more aggregation prone than Aβ40 [2]. The concentrations used in these studies are higher than in vivo [34]. To explain the severe aggregation an additional factor or catalyst needs to be added. Studies have shown that when interacting with the lipid membranes Aβ might assemble on the membrane surface, attracted to the surface by electrostatic forces [35-37]. Gangliosides seem to been an important factor by attracting the peptide to the membrane by their charged sialic residues, but data is inconclusive on whether the gangliosides are enough to induce aggregation or if charged phospholipids on the surface are needed for the enrichment on the surface[38-41]. On the surface a sufficient concentration for aggregation is reached, possibly by hydrophobic interaction as is schematically described in figure 4. What is known is that the aggregation is accompanied by a structure change from a unordered structure to a more β-rich structure and thus easy to be followed by Circular Dichroism [18]. The initial aggregates are globular, followed by rodlike structures and finally mature fibers [33]. The aggregation is also prone to seeding, i.e. aggregation is initially slow until a threshold concentration of aggregates is reached after which aggregation accelerates [1,2]. The devil is in the details though since small changes in properties as membrane charge, ion strength, protein concentration and 42/40 ratio and pH easily gives big changes in aggregation behaviour possibly explaining the results of Waschuk et al. [38] where aggregates where formed on plasma-, endosomal- and lysosomal membranes but not on golgi membranes. The mechanism in which the peptides exerts its toxicity is also contested but this will be adressed with in the following subchapter.
Membrane Mediated Aggregation of Amyloid-β Protein: A Potential Key Event in Alzheimer’s Disease

Figure 4: Membrane association of Aβ leads to enrichment on the surface, followed by aggregation into large fibrils. However, the step from membrane enrichment to aggregates might be more complex than indicated here.

4.7 Toxicity

This brings us to the cause of neuronal death. Many causes of the neuronal cell death have been suggested, such as MAOS, reduced membrane plasticity and ion channel formation [3]. The issue is not settled yet, but there seem to be consensus that the big aggregates are not the main problem, but the small oligomeric species of Aβ might be the most toxic [3,28]. Cell death through MAOS is thought to take place via increased oxidation from oligomers which depletes the antioxidant defenses in the brain [3]. This is supported by the fact that the oligomers, the most toxic species is also the most oxidative one [3]. The formation of ionchannels by Aβ, by which important ions might leak through holes in the membrane, is another hypothesis and it has been proven by that Aβ can induce dye-
release in vesicles [42]. Exactly which is the cause of cell death is still unclear though [3].

4.8 Therapies

Many cures have been suggested and as increased understanding of AD is reached more and more therapeutic ways are tried [1,3]. One of the earliest ways of treating AD was to give patients drugs to stimulate the synapses by preventing the breakdown of acetylcholine. In other words, stimulate the cells not yet killed to work faster. This works, but only for a short while, after which too many cells are dead for the remaining neurons to keep up [2]. Other therapies include antibodies against Aβ, which worked OK, except for the tendency to cause inflammation in the brain in about 5% of the test subjects, but new test which will try to overcome this problem are on the way [1,3]. A third way of battling AD is to try to regulate the APP-cutting enzymes. For the alpha-adjusting therapies, the strategy is simply to try to increase the α-secretase activity to prevent Aβ to be formed [1,3]. For BACE and γ-secretase there are two strategies, inhibiting their activity and trying to adjust cleavage site; for BACE both strategies showed promise since it only cuts one other protein and it is possible to make BACE cut at residue 11 of the Aβ-sequence (the β’-cleavage site), but the regulation of BACE requires a rather large molecule [1]. To inhibit the γ-secretase on the other hand is problematic since it cleaves many other proteins too [1,3]. Inducing γ-secretase to cut between residue 38 and 39 to get the less aggregation prone Aβ38 seems to be a promising therapy though [1]. Fibrillisation inhibition, anti-inflammatory drugs and cholesterol lowering drugs have also been tried [1-3].

4.9 Further Outlook

Although the research increasingly moved towards the control of the disrupted cleavage of APP towards Aβ42 and its subsequent self amplification, the crucial part of AD is still the structural changes and
aggregation of Aβ. This is not yet fully understood and needs to be investigated to reveal the molecular mechanism behind AD.

References


5. Experimental Techniques

A popular mystery in the 90-ies was formulated: If a tree falls in the forest and nobody is there to hear it fall, does it then make a sound? The answers and discussions about this problem are numerous enough to write a PhD-thesis on it’s own, so I won’t become engrossed in this too deeply. The important thing is unless you are there to hear or record the sound it’s impossible to know if there was a sound or not, and the same goes for scientific research even though sometimes the techniques used to extract data from experiments might be quite indirect. Thus, this chapter will deal with those techniques used to get the data for this thesis.

5.1 Circular Dichroism

5.1.1 Basics

One characteristic of amino acids in the body, out of many, are that they all, with the exception of glycine, are chiral. A chiral molecule is one that cannot be superimposed on its mirror image [1]. Chiral molecules will rotate planary polarized light, i.e. they are optically active [2]. The optical activity comes from the different refractive indices for clockwise and anticlockwise circularly polarized light, since planary polarized light can be seen as a superposition of two counter rotating circularly polarized waves of light. This can be used to study a molecule’s stereochemistry and is called Optical Rotary Dispersion (ORD) and is shown in the left hand side of Figure 1 [2].

An elaboration of this technique is Circular Dichroism (CD) which are shown in the right hand side of Figure 1 where two waves of light are superimposed $\frac{1}{4} \lambda$ out of phase and 90° angle to each other. This will produce circularly polarized light. Circularly polarized light, will have a constant amplitude but a rotating orientation perpendicular to the propagation direction, as opposed to the linearly polarized light which will
have a constant orientation but shifting amplitude [2]. When the intensity of the lefthand polarized (clockwise) light shone through a sample is compared to the righthand polarized (counterclockwise) light shone through a sample called elipicity is given to [2-4]:

$$\theta = 33(A_L - A_R) \quad (1)$$

A here stands for absorbance ($A = \log_{10}(I_0/I_{after})$) ($I_0$ = intensity before sample, $I_{after}$ = Intensity after passage through sample). $\Theta$ is called the elipicity and are given in millidegrees since earlier spectrometers measured the difference between the vertical absorption and the horizontal absorption and the ellipicity is the tangent between the minor and major axis of the imagined ellipse as shown in eq. (2) [2-4]:

$$\theta = \tan^{-1}\frac{Minoraxis}{Majoraxis} \quad (2)$$

More commonly, results are reported in Mean Residue Ellipicity (deg*cm²*dmol⁻¹). This is done to have a normalization of spectra of different proteins, concentrations and pathlenghts of different experiments []). The conversion is shown in eq. (3). CD is used to resolve protein structure. Since different structure will have different amount of helical structure the ellipicity for these structures will differ [2,4 ].

$$MRE = \frac{CDresult(m\text{deg})}{Pathlength(cm) \cdot Molar\text{ residue concentration}(dmol/1)} \quad (3)$$
CD-spectra of proteins of a single dominant secondary structure are fairly easy to interpret [5-6], but there are some problems associated with CD-spectra. The CD-spectra of a protein does not have distinct and easily separable spectral peaks, but the spectral features are superimposed on each other. Thus any protein with any noticeable amount of mixed structure will be hard to interpret and to quantify the amount of each separate structure [4-7]. A way to try and solve this problem is to deconvolute the CD-spectra. To deconvolute a CD-spectrum, many computer programs are available which with certain selection rules suggest a structure composition based on a linear combination from a set of reference proteins [5-8].

These reference sets contains both the structure and CD-spectra of the proteins in the set. Unfortunately these sets are not completely overlapping i.e. the CD-spectra don’t have completely overlapping wavelength range and if using a big reference set the wavelength range is lacking. Sreerama
et al [7-8] concluded that when deconvoluting and choosing what set to use bigger is better, especially for folding studies. Sreerama et al also studied three different deconvolution programs, CDSSTR, Contin/LL and SELCON3. They concluded that when using a big reference set the CONTIN/LL gave the best result [6-8].

5.2 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR is a technique which uses the orientation of nuclear spin of some nuclei in an outer magnetic field. The Boltzmann distribution describes the relation between two populations at different energy levels as seen in eq(5) [9]:

$$N_\beta / N_\alpha = e^{-\Delta E/kT}$$ (5)

$N_\alpha$ and $N_\beta$: the population of state $\alpha$ and $\beta$ respectively, $\Delta E$: Energy difference between the states, $T$: The thermodynamic temperature and $k$: Boltzmann’s constant. Further if the spin quantum number of the nucleus is greater than 0 (any nucleus with odd number of protons or neutrons) it has a constant magnitude and directional magnetic moment. If such a nucleus is subjected to an outer magnetic field the nucleus will orient itself relative to this field. The H-nucleus for example will either orient itself with or against the outer magnetic field ($B_0$). The energy for these two levels will be eq(6) [9]:

$$E = -\gamma \hbar B_0 m_I$$ (6)

where $\gamma$ is the magnetogyratic ratio and is nuclei-specific, $h$ is Planck’s constant divided by $2\pi$ and $m_I$ is the spin magnetic quantum number (here either $-1/2$ or $1/2$). The energy difference between these two levels then will be eq (7) [9]:

$$\Delta E = \gamma \hbar B_0$$ (7)
and it is easily seen that the lower energy levels will be more heavily populated in a ratio given by eq. (5). radiation of a frequency $\nu$ have the energy given by eq. (8) [9]:

$$E = h\nu \quad (8)$$

that when combined with eq. (7) will give the frequency where it is possible to change the orientation of the nucleus. All this would not be very useful as a spectroscopic technique if it wasn’t for two things, superconducting magnets and the chemical shift [9].

The chemical shift is what makes it possible to distinguish the same nuclei at different chemical environments in the sample. It stems from the interference from the electron cloud around the observed nucleus that will differ and thus the nuclei at different environments will experience different local magnetic fields thus emitting at different frequencies. In short, the sample will be put in a strong superconducting electromagnet, irradiated with a radiofrequency pulse exciting the nuclei and as the sample relaxes back towards the equilibrium the sample will emit radiation in the radiofrequency range that is recorded. The superconducting magnet is important to achieve greater energy difference thus greater resolution of radiofrequencies between the same nuclei of different environment. This is used to study, for example protein lipid interactions without labeling (even though labeling is often necessary in NMR, too) [9].

### 5.3 Differential Scanning Calorimetry

In general, calorimetry is the study of the heat transfer of a chemical or physical progress. Two types of calorimeters are the constant volume calorimeter where the transferred heat is the change in inner energy $\Delta U$ and the constant pressure calorimeter where the transferred heat is the change in enthalpy $\Delta H$. An example of the former is the adiabatic bomb calorimeter, used in many basic level chemistry courses and an example of the latter is DSC, Differential Scanning Calorimetry, used to some extent in this work [10].
In DSC the heat transferred to a constant pressure sample undergoing a physical (e.g., phase change of lipid membrane) or chemical change is recorded and compared to a blank sample (hence the differential part). The scanning part comes from the fact that the sample is scanned over a temperature range and recorded as a thermogram where the heat capacity \( C_p \) or difference in electrical energy \( P \) is plotted against \( T \). If no change occurs (except for the increased temperature) the transferred heat will be eq. (9) [10]:

\[
q_{\text{p,heating}} = C_p \cdot \Delta T \quad (9)
\]

but if there is a change the transferred heat will have to be described as eq. (10) [10]:

\[
q_{\text{p,heating}} + q_{\text{p,change}} = (C_p + C_{p, \text{change}}) \quad (10)
\]

Often peaks might be broad and then the enthalpy might be described as eq. (11) [10]:

\[
\Delta H = \int_{T_1}^{T_2} C_{p,\text{change}}dT \quad (11)
\]

instead. This is a useful technique to track phase changes in model membranes and how they are affected upon protein addition [10].

References

6. The Interaction of Aβ₄₀-Peptide with Model Membranes

6.1 Aim of the Project

There are of course many reasons and inspirations for this work, but the main starting point is the work by Terzi et al. where Aβ₄₀ was shown to aggregate by either increasing concentration enough to induce self aggregation by using a high peptide concentration in the sample or by enriching the peptide on a lipid surface [1]. We tried in greater detail to study the nature of the latter option, membrane enrichment, to see what governs it and to which extent the rate of aggregation is controlled by membrane composition. This was mainly done by studying the peptide aggregation of the samples by CD-spectroscopy but NMR and DSC was also used to determine the interaction type and membrane phase.

6.2 Paper I

Here the interaction between the charged membranes and Aβ₄₀ are studied. When the peptide is added to pure uncharged (DMPC) multilamellar vesicles and studied by ³¹P Magic Angle Spinning (MAS) NMR there was no change in the chemical shift, while for vesicle samples of DMPG/DMPC-mixtures and pure DMPG there was a constant shift change indicating an electrostatic interaction between Aβ₄₀ and the vesicle surface. The shift change for both the uncharged and charged lipids is shown in Figure 1. At the experimental pH (7.8) the peptide has 3 positively charged residues and 6 negatively charged residues giving it a total charge of -3. The charge distribution can be seen in Figure 2. The charge distribution is the same for the peptide at pH 7.8, used in paper I, as for pH 7.4 that is used in the figure. Furthermore there is a decrease in the peak intensity for the PG-peak when subjected to Aβ₄₀ which might be seen as the peptide locating itself near the charged lipid headgroups. This indicates pro primo; that the Aβ₄₀ does not interact with uncharged
membranes but with charged membranes and pro secundo; the interaction is electrostatic with the surface charge of the membrane since the total equal shift change is due to a total reduction of the surface charge of the vesicle surface. Also noteworthy is that no phase transition was observed in the vesicles upon addition of peptide.

**Figure 1:** Isotropic $^{31}$P chemical shift values at 308K for DMPG (top) and DMPC (bottom) as a function of PC/PG lipid molar ratios (relative surface charge before binding) of the multilamellar vesicles before (squares) and after (triangles) addition of Aβ40 at 30:1 lipid to peptide molar ratio. (Adapted from Figure 3 in Paper I)
Figure 2: The charge distribution in Aβ₄₀ at A) pH = 7.4 and B) pH = 5.2. Black color indicates negatively charged residues, light grey positively charged residues and grey uncharged residues. The author would like to point out that the peptide does not necessarily have the secondary structures shown in the figure models [2].

### 6.3 Paper II

The work presented in paper I is here further developed by extending the vesicle types and experimental techniques used. Here the array of membranes was increased to include also a nominally neutral membrane consisting of DMPC:DMPG:DDAB at 3:1:1 molar ratio. Both $^{14}$N and $^{31}$P NMR was used as well as calorimetric studies of the lipid systems and CD spectroscopy monitoring the peptide secondary structure was performed. The initial calorimetry revealed that for DMPC/DMPG vesicles there was a phase transition from gel phase to liquid-crystalline phase at 24.7°C, well below the temperature for the following experiments (308K). When Aβ₄₀ was added to the lipid mixtures there was a broadening of the previous sharp transition and a lowering of the transition temperature to 23.3°C. For the nominally neutral DMPC/DMPG/DDAB there are two sharp, yet overlapping, transitions peaks at 26.5 and 27°C, and in addition one broad peak at approximately 32°C. Thus all examined systems are in
the liquid-crystalline phase at the experimental temperature of 308K (35°C) as seen in Figure 2 in paper II. For the MAS NMR measurements of Aβ40 electrostatic interaction with the DMPC/DMPG, multilamellar vesicles were repeated from paper I for the 31P, i.e. a shift change indicating a electrostatic interaction between the surface and the Aβ40. The 14N results are a bit more inconclusive, but there seems to be indication of a change of the choline group of DMPC upon addition of peptide. Between the nominally neutral membranes and Aβ40 the interaction is visible albeit weaker. This indicates that Aβ40 at pH= 7.8 contains both negatively and positively charged residues that can be attracted to both membrane types and distinguish between the differently charged lipid headgroups. When the secondary structure of the Aβ40 was studied by CD, the structures at different membrane compositions resembled each other although not completely overlapping. To the naked eye the structure suggest a high content of β-structures, something that is confirmed by deconvolution of the spectra, where the in the charged case the peptide structures were 45% β-structure and 49% unordered and for the nominally neutral case 46% and 46%, respectively.

6.4 Paper III

Here the focus lies on studying whether incorporating Aβ40 into or adding it onto the membrane, will cause any difference in its interaction and aggregation behaviour. The peptide was incorporated into the multilamellar vesicles by adding them to the lipid solution before vesicle formation. When studied by 31P MAS NMR, the shift changes differently compared to added peptide for peptide incorporated into the membrane. For samples with incorporated peptide, no real change is seen in the shift values until 40% membrane charge and the shift change is not constant but increasing as the membrane charge increases as can be clearly seen in Figure 3. The shift changes equally for the DMPC and DMPG lipids for the samples where they are both present. The previously presented case in paper I and paper II for 31P MAS NMR on model membranes with added peptide is repeated here with a constant shift change for charged vesicles.
When the samples were studied by CD, there was a clear trend in the incorporated peptide from β-rich secondary structures to more abundant in α-helix from 5% for uncharged membranes to more than 30% for the fully charged membrane, while the amount of unordered structure was slightly reduced as the amount of charge increased on the model membranes. This can be seen in Figure 4 as there is clear trend to alphahelical spectra as the charge of the membrane increases. Figure 4 also shows the structure development for the addition case, the peptide adopts mostly unordered structure, but as the charge increases there is a small decrease of the unordered structure of 5% while there is a small increase in the amount of alphahelical structure from 3% to 9%. The β-structures shows no clear trend as the charge increases from 0 to 100%.

Since the AD progress is a slow procedure, the decision was taken to see if the structure of the peptide changed over time. For the peptide incorporated into 33% charged vesicles (PC/PG 2:1) there was no significant change of structure even after eight weeks and the similar result is repeated when the peptide is incorporated into SDS-micelles, albeit a different secondary structure is retained. When the Aβ_{40} is added to 33% charged PC/PG vesicles there initially very slow structure change, but after day 11 suddenly there is a sudden and quick change of structure from mostly unordered structures to beta structures, while the α-helical content stays close to zero.

The results of this paper leads us to suggest that two possible pathways for Aβ_{40}: 1) If inserted into the membrane it will be anchored there by its hydrophobic part and the more charge, the more deeply anchored it will be, protecting it from aggregation; 2) If added to the membrane surface, attracted to it by electrostatic forces, the peptide will be enriched onto the surface and will aggregate quickly once the first aggregates are formed. These initial aggregates do seem to take some time to form though. What this article does not tackle though is toxicity, that incorporation into membranes protects from aggregation does not mean it protects it from the neurotoxic effect of Aβ_{40} and vice versa.
Figure 3: Isotropic $^{31}$P chemical shift values at 308K for DMPG (top) and DMPC (bottom) as a function of PC/PG lipid molar ratios (relative surface charge before incorporation) of the multilamellar vesicles before (squares) and after (triangles) incorporation of Aβ$_{40}$ at 30:1 lipid to peptide molar ratio. (Adapted from Figure 2, paper III)
Membrane Mediated Aggregation of Amyloid-β Protein: A Potential Key Event in Alzheimer’s Disease

Figure 4: The difference in secondary structure between Aβ40 inserted and added to DMPC/DMPC vesicles as studied by CD-spectroscopy. T= 298K and lipid to peptide ratio is 30:1. (Adapted from Figure 3 in paper III.)

6.5 Paper IV

As already shown in paper II, Aβ40 will interact with nominally neutral membranes. Here we investigated this further by comparing the aggregation of 50µM Aβ40 in presence of negatively charged 3mM DMPC:DMPG (33% charge) vesicles compared to positively charged 3mM DMPC:DDAB (33% charge) vesicles.
When the folding of $\text{A}\beta_{40}$ is studied by CD in the presence of negatively charged vesicles, the process previously described in paper III is repeated; in the CD spectra a structural change can be seen going from mostly unordered to beta structures, but the lagtime is slightly longer before the onset of peptide folding. In the case of folding in the presence of positively charged vesicles there is also folding from mostly unordered structure to beta structures. However, in this case the structure change is immediate. This might be a consequence of the peptide having 6 negative and 3 positive charges at pH 7.4. Moreover, when the results from deconvolution are compared the peptide in negative vesicles goes from 45% $\beta$-structure and 50% unordered structure to 60% and 30%, respectively. In positively the peptide changes from 45% $\beta$-structure and 50% unordered to 55% $\beta$-structure and 40% unordered. Finally, folding seems to cease after 10 days. According to the CD-spectra, the peptide in solution seems to have unordered structure. Over 90 days the peptide gradually adopts other structure as the spectra flatten out. Deconvolution confirm this and indicate that the amount of $\beta$-structure increase at the expense of unordered, but not to the same extent and speed as when charged vesicles are present.

### 6.6 Paper V

For the final paper included in this thesis the effect of macromolecular crowding on aggregation was studied. Macromolecular crowding is the reduced space of the cell from the high amount of diverse biomolecules present, although nominally inert for the studied reaction, but which might induce an effect by reducing the available space available [3].

Here we simulated macromolecular crowding by adding ficoll70, a 70 kDa large polysaccharide, at 3 concentrations (0, 200 and 350g/l) while $\text{A}\beta_{40}$ were to interact with charged DMPC/DMPG vesicles, as well as crowding influence on $\text{A}\beta_{40}$ behavior in absence of vesicles, as monitored by CD.

Three effects of crowding were discovered. The first effect is to induce a faster onset of the aggregation by reducing the free space available for the $\text{A}\beta_{40}$. This can be seen in the crowded vesicle samples, where structure
change of $\text{A} \beta_{40}$ starts earlier, both for the 200 g/l and 350 g/l sample, compared to the uncrowded vesicle sample. The second effect is to retard build up of large aggregates by creating an unfavorable environment for large aggregates, as can be seen later when the amount of folding in the uncrowded sample is greater than for the 350 g/l-sample after 21 days. The uncrowded sample does not fold quicker than the 200 g/l sample though, indicating that the slow down effect of crowding is the weaker effect. The third effect was discovered when the folding was studied in the absence of model membranes but presence of the aforementioned crowding. Then no significant structure change was observed in the peptide, leading to the conclusion that crowding by itself is not means enough to induce aggregation, or to raise the local concentration of $\text{A} \beta_{40}$ enough for that to occur.

References

7. Summary and Future Outlook

At the beginning of the work presented here, AD-research was mainly focused on:

1. Aggregation of Aβ and control of Aβ-aggregation.
2. The nature of Aβ-toxicity in AD.

My efforts have been around point 1 and the main theme for paper I-IV was the attraction between the charged membrane and the charged residues of the Aβ protein. In paper I and II it was confirmed that the Aβ40 interacts electrostatically with charged membranes and that the peptide can discriminate between individual charged lipid headgroups as it interacted with nominally neutral membranes.

Further, in paper III, it was shown that peptide-membrane interactions differ, depending on whether the peptide is incorporated into or added to the membrane and that incorporation might delay aggregation. It was also discovered that aggregation that follows upon addition of the peptide to the membrane surface is delayed, suggesting seed formation is required.

The ability for Aβ to interact and aggregate on both positive and negative surfaces, was discovered in paper IV. Finally, in paper V, the effect of crowding was investigated and it was shown that it might influences aggregation greatly and that the effect is concentration dependent.

For the future, the main focus on the role of aggregation in AD might have move to other aspects of AD, such as the regulation of Aβ levels and aggregation, but since Aβ-aggregation is present in all AD it is still an important part of the disease. There is still much research to be done here, especially in connection with crowding which is largely uncharted research territory.
8. Acknowledgements

If you’re not on this list it’s for one of the two following reasons:

1. I forgot you, and in this case I’m sorry
2. You don’t deserve to be, and in that case I’m not sorry

First of all I would like to thank my parents for making this thesis possible and my wife and future children for making this necessary [1]. Seriously, Father, Mother and Ying, I love you.

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I guess that’s all, if you are not on the list see point 1 & 2.

References

1. Modified quote by Victor Borge.