Capturing transient peptide assemblies associated with Alzheimer's disease

Native mass spectrometry studies of amyloid-β oligomerization

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Abstract
Correct folding of proteins is essential for maintaining a functional living cell. Misfolding and aggregation of proteins, where non-native intermolecular interactions form large and highly ordered amyloid aggregates with low free energy, is hence associated with multiple diseases. One example is Alzheimer’s disease (AD) where the Amyloid-β (Aβ) peptide aggregates into amyloid fibrils, which deposit as neuritic plaques in the brains of AD patients. Nucleation of amyloid fibrils takes place via formation of smaller pre-nucleation clusters, so-called oligomers, which are considered to be especially toxic and are therefore potentially important in AD pathology. Detailed mechanistic molecular knowledge of Aβ aggregation is important for design of AD treatments that target these processes. The oligomeric species are however challenging to study experimentally due to their low abundance and high polydispersity.

Aβ oligomers are in this thesis studied under controlled in vitro conditions using bottom-up biophysics. Highly pure recombinant Aβ peptides are studied, primarily using native ion-mobility mass spectrometry, to monitor the spontaneous formation of oligomers in aqueous solution. Mass spectrometry is capable of resolving individual oligomeric states, while ion mobility provides low-resolution structure information. This is complemented with other biophysical techniques, as well as theoretical modeling. The oligomers are also studied upon modulating intrinsic factors, such as peptide length and sequence, or extrinsic factors, such as the chemical environment. Interactions with two important biological interaction partners are studied: chaperone proteins and cell membranes.

We show how Aβ oligomers assemble, and form extended structures which may be linked to continued growth into amyloid fibrils. We also show how different amyloid chaperone proteins interact with growing aggregates, which modifies and delays the aggregation process. These interactions are shown to depend on specific sequence-motifs in the chaperones and client peptides. Membrane-mimicking micelles are on the other hand able to stabilize globular compact forms of the Aβ oligomers and to inhibit the formation of extended structures which nucleate into amyloid fibrils. This may contribute to enrichment of toxic species in vivo. Interactions with membrane-mimicking systems are shown to be highly dependent on both the Aβ peptide isoform and the properties of the membrane environment, such as headgroup charges. It is also demonstrated how addition of a designed small peptide construct can inhibit formation of Aβ oligomers in the membrane environment.

Keywords: Protein aggregation, amyloid-β, neurodegeneration, Alzheimer’s disease, chaperones, DnaJ, biomembranes, native mass spectrometry.

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CAPTURING TRANSIENT PEPTIDE ASSEMBLIES ASSOCIATED WITH ALZHEIMER'S DISEASE

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The unstructured Aβ peptide aggregates into weakly structured oligomeric forms. These can be stabilized in membrane environments (top), which might be linked to toxic events (pink AD brain). Oligomers can also be captured by cellular defense systems, such as the DNAJB6 chaperone (green healthy brain). Oligomers can also continue into highly structured and ordered fibrillar aggregates (pdb id 2mxu).
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TILL MORMOR

Min släkt är full av hjältar,
decennier utav slit.
Brustna hjärtan, trötta leder,
deras stolthet bar mig hit.
Nu är dom gömda bakom stjärnor,
vid vintergatans kant.
Dom är glömda men dom talar,
genom pennan i min hand.
Och dom bar mig ända hit.

JOAKIM BERG
(Elite, Vapen & ammunition, 2002, kent)
Capturing transient peptide assemblies associated with Alzheimer’s disease

Nicklas Österlund
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ABSTRACT

Correct folding of proteins is essential for maintaining a functional living cell. Misfolding and aggregation of proteins, where non-native intermolecular interactions form large and highly ordered amyloid aggregates with low free energy, is hence associated with multiple diseases. One example is Alzheimer’s disease (AD) where the Amyloid-β (Aβ) peptide aggregates into amyloid fibrils, which deposit as neuritic plaques in the brains of AD patients. Nucleation of amyloid fibrils takes place via formation of smaller pre-nucleation clusters, so-called oligomers, which are considered to be especially toxic and are therefore potentially important in AD pathology. Detailed mechanistic molecular knowledge of Aβ aggregation is important for design of AD treatments that target these processes. The oligomeric species are however challenging to study experimentally due to their low abundance and high polydispersity.

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We show how Aβ oligomers assemble and form extended structures which may be linked to continued growth into amyloid fibrils. We also show how different amyloid chaperone proteins interact with growing aggregates, which modifies and delays the aggregation process. These interactions are shown to depend on specific sequence-motifs in the chaperones and client peptides. Membrane-mimicking micelles are on the other hand able to stabilize globular compact forms of the Aβ oligomers and to inhibit the formation of extended structures which nucleate into amyloid fibrils. This may contribute to the enrichment of toxic species in vivo. Interactions with membrane-mimicking systems are shown to be highly dependent on both the Aβ peptide isoform and the properties of the membrane environment, such as head-group charges. It is also demonstrated how the addition of a designed small peptide construct can inhibit the formation of Aβ oligomers in the membrane environment.
PREFACE

This thesis concludes the research performed by me as a Ph.D. student at Stockholm University between 2017 and 2022. The research topic being explored over these years has been studies of misfolded and aggregated protein species, and their interactions with molecules of relevance to the biology of Alzheimer’s disease. This was made possible thanks to a Ph.D. stipend from the Sven and Lilly Lawski Foundation awarded to me (“Samspelet mellan neuroproteiner och andra cellulära komponenter vid amyloidbildning”).

The biology of Alzheimer’s disease is terribly complex and likely involves multiple important players. I have in this thesis limited my studies and discussions to one such player, the Amyloid-β (Aβ) peptide, and specifically some small aggregates (oligomers) formed by the peptide. This has been explored from a biophysical point of view and is an example of basic science, driven primarily by a curiosity to understand the underlying mechanisms of nature. I hope however that this basic science will also contribute to an increased understanding of molecular processes that could play a part in a cruel disease which currently affects over 50 million patients (as well as their loved ones).

We have in the last few years seen the first signs of what is hopefully a breakthrough when it comes to the development of disease-modifying drugs for Alzheimer’s disease. Many of these promising drug candidates target Aβ oligomers, which motivates further studies on some fundamental aspects of these species and their interaction partners.

The aims of this thesis have been:

1. To detect and study Aβ peptide oligomers in vitro under controlled conditions. (With the challenge being that Aβ oligomers exist in low concentrations and are intrinsically polydisperse. We here use a native mass spectrometry approach to detect oligomers without the need for any labeling or crosslinking.)

2. To investigate in vitro to which extent the detected Aβ oligomers are affected by modulation by selected extrinsic and intrinsic factors. (With a special emphasis on studying how the oligomers can be stabilized or destabilized by some cellular components.)

The research has also involved setting up the technique of native mass spectrometry in our lab. This was greatly facilitated by the COST action BM1403 on Native Mass Spectrometry and Related Methods for Structural Biology, which funded my participation in research schools as well as a short-term scientific visit by me to the University of Antwerp and the group of Professor Frank Sobott.
Seven original research papers which contain my most important scientific findings are attached to this thesis. These papers focus on the structures of Aβ oligomers (Paper I), the interactions of such oligomers with chaperone proteins (Papers II-IV), and interactions with and within membrane environments (Papers V-VII).

The thesis itself will hopefully provide some background which will make it easier to understand the research and how it all fits together. I have tried to write it according to a funnel structure: Life, biology, and disease occur according to the same chemistry and physics as all other things in our universe. The thesis starts with a brief intro to the physics of protein structure (Chapter 1). The biological background for the thesis work (Chapter 2), and the methods used here to study these processes (Chapter 3) are then introduced. My own original research is then briefly summarized (Chapters 4-6), and some overarching conclusions are discussed (Chapter 7).

I hope you find something interesting when reading, or skimming, through this little book and the research papers attached to it. In many ways, I’m very happy with how things turned out. Everything is of course not perfect, I can when I look back find multiple things that I would have perhaps done differently today. A feeling which is nicely expressed in a funny tweet from John M. Mola (@_JohnMola):

"Finishing a PhD is like finishing a group project where your partner made a ton of mistakes at the beginning of the assignment. Except your partner is just you 4 years ago."

But that is fine. It is also nice to see the progress that I have made during these years.

Simma lugnt,

Nicklas,
Sundbyberg, December 2022
Alzheimer’s disease is the most common form of dementia in the world, which is estimated to affect over 50 million individuals. The number of patients is also expected to increase as the population of elderly people in the world is increasing. The disease was discovered at the beginning of the 20th century by the physician Alois Alzheimer who noted fibrillar plaques in the brain tissue of dementia patients. It was during the 1980s established that a small segment from a protein (a peptide) called Aβ was the main component in these plaques, and the hypothesis that clumping (aggregation) of Aβ caused Alzheimer’s disease was formulated.

Today we know that aggregation of Aβ is a complex process with many intermediates. Some of the small structures (called “oligomers”) formed in these intermediate steps are today considered to be more toxic than the mature fibrillar aggregates. The increased toxicity of the oligomers is possibly due to increased reactivity, and that they can be more easily transported within and between cells. One idea is also that the oligomers can interact with the fatty membrane of the cell, resulting in cell leakage.

The fact that Aβ oligomers seem to be particularly toxic makes them interesting for the development of Alzheimer’s disease drugs. A practical problem with oligomer research is however that they exist in very low concentrations (approx. 1% of the total amount of Aβ in a test tube) and are very unstable. This makes it difficult to conduct experiments on how they are stabilized under disease conditions, or how they can be destabilized with drug molecules.

A method called native mass spectrometry is used in this thesis to detect Aβ and its oligomeric states. Mass spectrometry can be described as a way to weigh individual molecules, and enables the detection of each oligomeric state, as these have discrete molecular weights that allow them to be identified. The method is used here, together with other biophysical methods, to study in test tubes how samples with Aβ form oligomers by themselves or in the presence of other molecules. Studies in test tubes, rather than in the cellular environment, enables highly controlled studies where all parameters can be controlled carefully.

In particular, the shape of the oligomers is studied, and how that varies with modifications in the Aβ peptide. Interactions between Aβ and chaperone proteins are also studied. These chaperones exist in the cell to counteract toxic forms of proteins and are a natural safety net against diseases such as Alzheimer’s disease. Knowledge of these natural protective mechanisms increases our understanding of how the disease possibly arise and can give us ideas about how drugs against the disease can be designed. Furthermore, the interaction with the cell membrane is studied, and how this can lead to the formation of oligomers which are able to cause leakage.

The thesis presents techniques and applications of techniques that in the future can be used to study some of the most toxic forms of the Aβ peptide. Which enables increased knowledge about how these structures arise and can be counteracted.
Alzheimers sjukdom är den vanligaste demenssjukdomen i världen med uppskattningsvis över 50 miljoner drabbade. Antalet drabbade förväntas även öka då antalet äldre i världen ökar. Sjukdomen upptäcktes i början av 1900-talet av läkaren Alois Alzheimer som noterade fibrillplack i hjärnvävnaden hos demensdrabbade patienter. Under 1980-talet konstaterades det att en liten proteinbit (peptid) kallad $\text{A}_\beta$ var den främsta komponenten i dessa plack, och hypotesen att ihopklumpning (aggregering) av $\text{A}_\beta$ låg bakom Alzheimers sjukdom formulerades.

Idag känner vi till att aggregeringen av $\text{A}_\beta$ är en komplex process med många mellansteg. Vissa av de små strukturer (kallade "oligomerer") som bildas i dessa mellansteg anses idag vara giftigare än de färdiga fibrillaggregaten. Oligomerernas ökade giftighet beror möjligtvis på att de är mer reaktiva, samt att de lättare kan transporteras inom och mellan celler. En idé är även att oligomererna kan interagera med cellens feta membran, vilket resulterar i att cellerna börjar läcka.

De faktum att $\text{A}_\beta$ oligomerer verkar vara speciellt giftiga gör dem till intressanta inom utvecklingen av läkemedel för Alzheimers sjukdom. Ett praktiskt problem med forskning rörande oligomererna är dock att de existerar i väldigt låg koncentration (ca. 1% av den totala mängden $\text{A}_\beta$ i ett provrör) och är väldigt instabila. Detta gör det svårt att genomföra experiment på hur de stabileras under sjukdomsförhållanden, eller hur de kan destabiliseras med molekyler som kan användas som läkemedel.

I den här avhandlingen används en metod som kallas nativ masspektrometri för att detektera $\text{A}_\beta$ och dess oligomera tillstånd. Masspektrometri kan beskrivas som ett sätt att väga molekyler, och möjliggör detektion av varje enskilt oligomertillstånd då dessa har diskreta molekylvikter som gör att de kan identifieras. Metoden används här, tillsammans med andra biofysikaliska metoder för att i provrör studera hur prover med $\text{A}_\beta$ bildar oligomerer ensamt eller i närvaro av andra molekyler. Studier i provrör, snarare än i den cellulära miljön möjliggör väldigt kontrollerade studier där alla parametrar kan kontrolleras noggrant.

Speciellt studeras oligomerernas form, och hur den varierar med modifikationer i $\text{A}_\beta$ peptiden. Interaktioner mellan $\text{A}_\beta$ och chaperonproteiner, s.k. "molekylära förkläden" studeras även. Dessa chaperoner existerar i cellen för att motverka toksiska former av proteiner, och är ett naturligt skyddsnett mot sjukdomar som Alzheimers sjukdom. Kunskap om dessa naturliga skyddmekanismer ökar vår förståelse för hur sjukdomen möjligtvis uppkommer, och kan ge oss idéer om hur läkemedel mot sjukdomen kan designas. Vidare studeras interaktionen med cell membranet, och hur detta kan leda till bildande av oligomerer som kan orsaka läckage.

Avhandlingen presenterar tekniker och applikationer av tekniker som i framtiden kan användas till att specifikt studera några av de giftigaste formerna av $\text{A}_\beta$ peptiden. Vilket möjliggör ökad kunskap om hur dessa strukturer uppkommer och kan motverkas.
LIST OF PUBLICATIONS

This thesis is based on the following seven original research articles, which are referred to in the text by their respective Roman numerals (I-VII).

Reprints were made with permission from the respective publishers.

I. A HAIRPIN MOTIF IN THE AMYLOID-β PEPTIDE IS IMPORTANT FOR FORMATION OF DISEASE-RELATED OLIGOMERS
Nicklas Österlund, Mohammed Khaled, Isabel Rönnbäck, Leopold L. Ilag, Birgit Strodel, and Astrid Gräslund.
Manuscript in preparation

II. AMYLOID-β OLIGOMERS ARE CAPTURED BY THE DNAJB6 CHAPERONE: DIRECT DETECTION OF INTERACTIONS THAT CAN PREVENT PRIMARY NUCLEATION
Nicklas Österlund, Martin Lundqvist, Leopold L. Ilag, Astrid Gräslund, and Cecilia Emanuelsson.
(doi: 10.1074/jbc.RA120.013459).

III. THE C-TERMINAL DOMAIN OF THE ANTI-AMYLOID CHAPERONE DNAJB6 BINDS TO AMYLOID-β PEPTIDE FIBRILS AND INHIBITS SECONDARY NUCLEATION
Nicklas Österlund, Rebecca Frankel, Andreas Carlsson, Dev Thacker, Maja Karlsson, Vanessa Matus, Astrid Gräslund, Cecilia Emanuelsson, and Sara Linse.
Manuscript in preparation

IV. MASS SPECTROMETRY AND MACHINE LEARNING REVEALS DETERMINANTS OF CLIENT RECOGNITION BY ANTI-AMYLOID CHAPERONES
V. AMYLOID-β PEPTIDE INTERACTIONS WITH AMPHIPHILIC SURFACTANTS: ELECTROSTATIC AND HYDROPHOBIC EFFECTS


VI. NATIVE ION MOBILITY-MASS SPECTROMETRY REVEALS THE FORMATION OF β-BARREL SHAPED AMYLOID-β HEXAMERS IN A MEMBRANE-MIMICKING ENVIRONMENT

Nicklas Österlund, Rani Moons, Leopold L. Ilag, Frank Sobott, and Astrid Gräslund.


VII. THE AMYLOID-INHIBITING NCAM-PrP PEPTIDE TARGETS Aβ PEPTIDE AGGREGATION IN MEMBRANE-MIMETIC ENVIRONMENTS


* These authors contributed equally.

Contributions

I. Initiated and designed the study, supervised all experiments, evaluated all experimental data, wrote the original manuscript draft.

II. Helped design the study. Performed all experiments, evaluated all data, wrote the original manuscript draft.

III. Performed all native MS experiments, evaluated experimental data, generated and evaluated structure models, wrote the original manuscript draft.

IV. Helped design the study. Performed most native MS experiments, generated and evaluated structure models. Reviewed and edited the manuscript.

V. Designed the study, performed all experiments, evaluated all experimental data, wrote the original manuscript draft.

VI. Initiated and designed the study, acquired funding, performed all experiments, generated all structure models, evaluated all data, wrote the original manuscript draft.

VII. Performed all native MS experiments, evaluated data, Reviewed and edited the manuscript.
ADDITIONAL ORIGINAL RESEARCH ARTICLES

- ALZHEIMER’S DISEASE AND CIGARETTE SMOKE COMPONENTS: EFFECTS OF NICOTINE, PAHS, AND CD (II), CR (III), PB (II), PB (IV) IONS ON AMYLOID-β PEPTIDE AGGREGATION
  Wallin, Sholts, Österlund, Luo, Jarvet, Roos, Ilag, Gräslund, & Wärmländer.

- GAS-PHASE COLLISIONS WITH TRIMETHYLAMINE-N-OXIDE ENABLE ACTIVATION-CONTROLLED PROTEIN ION CHARGE REDUCTION
  Kaldmäe, Österlund, Lianoudaki, Sahin, Bergman, Nyman, Kronqvist, Ilag, Allision, Marklund, & Landreh.

- ROLE OF HYDROPHOBIC RESIDUES FOR THE GASEOUS FORMATION OF HELICAL MOTIFS
  Liu, Dong, Liu, Österlund, Gräslund, Carloni, & Li.
  *Chemical Communications*, 55(35), (2019). 5147-5150.

- SOLVENT-ASSISTED PAPER SPRAY IONIZATION MASS SPECTROMETRY (SAPSI-MS) FOR THE ANALYSIS OF BIOMOLECULES AND BIOFLUIDS.
  Riboni, Quaranta, Motwani, Österlund, Gräslund, Bianchi, & Ilag.

- ION MOBILITY-MASS SPECTROMETRY SHOWS STEP-WISE PROTEIN UNFOLDING UNDER ALKALINE CONDITIONS.
  *Chemical Communications*, 57(12), (2021) 1450-1453.

- CHARGE ENGINEERING REVEALS THE ROLES OF IONIZABLE SIDE CHAINS IN ELECTROSPRAY IONIZATION MASS SPECTROMETRY.
  Abramsson, Sahin, Hopper, Branca, Danielsson, Xu, Chandler, Österlund, Ilag, Leppert, Costeira-Paulo, Lang, Tellum, Robinson, Laganowsky, Benesch, Oliveberg, Marklund, Allison, Winther, & Landreh.
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Journal of the American Chemical Society 144.27 (2022): 11949-11954.

- IDENTIFYING THE ROLE OF CO-AGGREGATION OF ALZHEIMERS AMYLOID-β WITH AMORPHOUS PROTEIN AGGREGATES OF NON-AMYLOID PROTEINS

ADDITIONAL REVIEW ARTICLES

- MEMBRANE-MIMETIC SYSTEMS FOR BIOPHYSICAL STUDIES OF THE AMYLOID-β PEPTIDE Österlund, Luo, Wärmländer, & Gräslund.

- METAL BINDING TO THE AMYLOID-β PEPTIDES IN THE PRESENCE OF BIOMEMBRANES: POTENTIAL MECHANISMS OF CELL TOXICITY.
Wärmländer, Österlund, Wallin, Wu, Luo, Tiiman, Jarvet, & Gräslund.

- CELL-PENETRATING PEPTIDES WITH UNEXPECTED ANTI-AMYLOID PROPERTIES.
Österlund, Wärmländer, & Gräslund.
ABBREVIATIONS

βPFO β-barrel pore-forming oligomer.
Aβ Amyloid-β.
AD Alzheimer’s disease.
AF2 AlphaFold2.
AFM Atomic force microscopy.
AmAc Ammonium acetate.
APP Amyloid-precursor protein.
BBB Blood-brain barrier.
CCS Collision cross section.
CD Circular dichroism.
CEM Chain ejection model.
CIA Collision-induced activation.
CMC Critical micelle concentration.
CRM Charge residue model.
CTAB Cetyltrimethylammonium bromide.
CTD C-terminal domain.
DDM n-Dodecyl-β-Maltoside.
DNAJB6 DnaJ homolog subfamily B member 6 isoform b.
DPC Dodecylphosphorylcholine.
EM Electron microscopy.
ESI Electrospray ionization.
fAD Familial Alzheimer’s disease.

H-bond Hydrogen bond.
Hsp Heat-shock protein.
IDP Intrinsically disordered protein.
IM Ion mobility.
JD J-domain.
m/z Mass/charge state ratio.
MS Mass spectrometry.
MS/MS Tandem mass spectrometry.
n/z Oligomeric state/charge state ratio.
NCAM1 Neural cell adhesion molecule 1.
NMR Nuclear magnetic resonance.
pLDDT Predicted local-distance difference test.
qToF Quadrupole-time of flight.
sAD Sporadic Alzheimer’s disease.
SASA Solvent accessible surface area.
SB3-14 Myristyl sulfobetaine.
SDS Sodium dodecyl sulfate.
SEC Size exclusion chromatography.
ThT Thioflavin T.
TWIMS Traveling wave ion mobility.
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II  Amyloid-β oligomers are captured by the DNAJB6 chaperone: Direct detection of interactions that can prevent primary nucleation ...................

III The C-terminal domain of the anti-amyloid chaperone DNAJB6 binds to Amyloid-β peptide fibrils and inhibits secondary nucleation ................

IV  Mass spectrometry and machine learning reveals determinants of client recognition by anti-amyloid chaperones ................................

V  Amyloid-β peptide interactions with amphiphilic surfactants: Electrostatic and hydrophobic effects ........................................

VI Native ion mobility-mass spectrometry reveals the formation of β-barrel shaped amyloid-β hexamers in a membrane-mimicking environment .

VII The amyloid-inhibiting NCAM-PrP peptide targets Aβ peptide aggregation in membrane-mimetic environments ..............................
Chapter 1

PROTEIN PHYSICS

1.1 WHAT IS LIFE?

The difference between an inanimate object, like the thesis that you are holding in your hand, and a living organism, like yourself, is most likely obvious to most readers. So what is life then? Biologists usually approach this question by listing the characteristics of living systems: Living things self-organize, grow, and reproduce. They are able to transform energy and perform useful work in the process, and they can perform specific work as a response to stimuli [1]. We also know on a molecular level that all known living things have a special set of molecules: the basic unit of life is the cell, a discrete water solution separated from its surroundings by a lipid bilayer, the cell membrane. The cell contains a hereditary “genetic” code composed of the polymer deoxyribonucleic acid (DNA), which serves as a blueprint for the production of another type of polymer, proteins. These proteins do in turn function as tiny molecular machines that perform work, and are responsible for most of the processes within the cell that we associate with the life process.

But what are the driving forces in biology? Some sort of non-physical life force, an *élan vital*, which gives objects the characteristic life properties was suggested earlier in history [2]. Better explanations are today fortunately provided by physics. The second law of thermodynamics does for example state that a system will evolve towards its most probable configuration. Or expressed differently: the disorder, entropy, of a system will spontaneously increase over time. The life process does however appear to create order rather than disorder, which is in apparent violation of the second law.

Edwin Schrödinger came to explore this phenomenon in his 1944 book "What is Life?", which in many ways started the field of biophysics. Schrödinger argued that the life processes when creating order in the living system itself must also compensate by creating even more disorder outside of the living system [3]. Living systems are thus *open thermodynamic systems* which import energy and export entropy. The life process is driven by an energy gradient where high energy states are gradually reduced while performing useful work, exporting useless high entropy energy for example in the form of heat. A recent theory even suggests that life processes emerge as they are especially efficient at increasing the entropy of the universe [4].

Further discussions on the peculiarities of life are well outside of the scope of this thesis. But this introduction is meant to emphasize that life processes are not magical, they are governed by the exact same physical laws as any other chemical reactions and can therefore be studied in similar ways. Biological systems are however incredibly complex, and a reductionist approach is often needed where sub-systems are studied in isolation. Such *in vitro* (from Latin "in glass", meaning "in the test tube". In contrast to in the living system *in vivo*, "in the living") will be the topic of this thesis.
1.2 THE POLYPEPTIDE CHAIN

Proteins, the class of biomolecules primarily studied in this thesis, are unbranched polymers formed by condensation of amino acids (Figure 1A). This reaction is energetically unfavorable and the biological synthesis of proteins requires a large catalytic complex known as the ribosome to occur. All natural proteins on earth are formed exclusively from a set of specific L-amino acids. All proteinaceous amino acids but one, proline (P), are also α-amino acids which can be written as H₂N-CαHR-COOH (Figure 1A), with R being a side chain that is characteristic for each amino acid.

These side chains have varying chemical structures and the amino acids are commonly divided into groups based on the physio-chemical properties of their side chains. Such classification is however not always easy. The residue lysine (K) is for example positively charged at neutral pH, but it is also considered to be somewhat hydrophobic due to its long hydrocarbon side chain tail. Figure 1B makes an attempt at classifying the amino acids into groups that can overlap with each other.

The amide bond formed from amino acid condensation is called a peptide bond (Figure 1C, highlighted in orange) and has a certain amount of double-bond character due to the delocalization of electrons over it (Figure 1C, black dashed lines).

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**Figure 1:** A. Condensation of two amino acids into a dipeptide B. The 20 proteinaceous amino acids classified according to physio-chemical properties. Note that an amino acid might be sorted into multiple classes. C. Polypeptide chain with the peptide bonds highlighted (orange spheres, and black dashed lines indicating the delocalisation of electrons). D. Calculated charge of the Aβ peptide as a function of pH.
The double-bond character increases the energy barrier for rotation and therefore stiffens the peptide bond compared to normal single bonds, which are often almost freely rotating. The consequence of this is that the conformation of the peptide backbone is determined only by the HN-Cα (ϕ) and Cα-C=O (ψ) angles (Figure 1C).

The polypeptide chain can be viewed as a linear information string where the combination of amino acids makes up a code, the so-called primary structure. As the protein is synthesized from the ribosome the amino acid chain usually arranges itself in a unique three-dimensional structure. This structure is determined by the chemical nature of the amino acids that make up the chain, and by the physicochemical nature of the environment. The biological environment is most often a polar aqueous solution at moderate pH, ionic strength, and temperature, with the notable exemption of membrane proteins where the environment also includes a non-polar lipid phase. Environmental conditions can furthermore vary between cellular compartments and between species.

The free amine (N-terminal) end, the free carboxyl (C-terminal) end, and some side chains are titratable and their net charge varies with pH (Figure 1D). The structure of a protein is hence not necessarily the same at pH 7 and pH 4, as the physiochemical properties of the polypeptide chain change. Some residues can also be modified after synthesis, which can change the structure and function of the protein. A notable example of this is phosphorylation of some residues, which introduces an additional negative charge in the primary sequence. Modifications such as phosphorylation are important for modulating the activity of proteins during biological processes [5].

1.3 NON-COVALENT INTERACTIONS

Chemistry concerns the breaking and creation of chemical covalent bonds, such as the peptide bond. Not many covalent bonds have however been (intentionally) broken when creating this thesis. Non-covalent interactions are instead central in biophysics, as almost all interactions within and between polypeptide chains are of non-covalent nature. The notable exception is the amino acid cysteine (C) which can form covalent crosslinks within or between polypeptide chains at oxidizing conditions.

Non-covalent interactions are related to perturbations of the electron clouds around molecules. The nature of this perturbation is different in different interactions: Some interactions are very weak while others are very strong, some interactions can act over a long distance, while others are rapidly declining at increased intermolecular separation. Not all molecules are capable of forming all sorts of non-covalent bonds, as this depends on the chemical nature of the molecule. All molecules do however repel each other at very short distances when the electron clouds start to overlap.

A short introduction is here given to some non-covalent interactions. The reader is referred to textbooks in physical chemistry, such as references [6, 7], for more detailed descriptions.
1.3.1 Interactions involving charges

Positive and negative charges attract each other. Two oppositely charged amino acid residues in a protein that are sufficiently close in space can form a favorable charge-charge interaction, a so-called salt bridge. The potential energy for such a charge-charge (coulomb) interaction is described by Equation 1, where $Q_1$ and $Q_2$ are the respective charges of the interacting ions and $r$ is the distance that separates them. The strength of the interaction is also determined by the permittivity ($\varepsilon$) of the surrounding medium. This is most often expressed as the product of the vacuum permittivity ($\varepsilon_0$) and the relative permittivity of the surrounding medium ($\varepsilon_r = \varepsilon / \varepsilon_0$). Water, the biological solvent, has a very high permittivity with an $\varepsilon_r \approx 80$.

$$U_{QQ}(r) = \frac{Q_1 Q_2}{4 \pi \varepsilon_0 \varepsilon_r r}$$  \hspace{1cm} (1)

Coulomb interactions are very strong interactions, which are also long-range due to their $1/r$ dependence. The strength of a coulomb interaction is affected by two important factors: The interaction is weakened by the presence of other ions in the solution, so-called screening. The screening effect increases with the charge and concentration of the screening ion. The interaction is also affected by a change in the permittivity. Notable biological environments with low permittivity are the inside of a folded protein ($\varepsilon_r = 6-7$ [8]), or the inside of a lipid membrane ($\varepsilon_r = 1-2$ [9]). Coulomb interactions in such environments are therefore increased in strength compared to in water.

Molecules without permanent net charge can also interact with charged groups. Electric dipoles are net neutral but have an asymmetric charge distribution (a dipole moment, $\mu$, with a partial negative end, and a partial positive end), which interacts with permanent charges. The energy of an interaction between a charge and a static dipole will depend on the angle between the dipole moment and the interaction direction. The angle dependency is however low in high permittivity environments such as water, as $U$ for all angles is smaller than the available thermal energy, resulting in a rotating dipole. The interaction potential for a rotating dipole does not necessarily average out to zero, as directions are sampled according to the Boltzmann distribution. The energy of a charge interacting with a rotating dipole is given by Equation 2.

$$U_{Q\mu}(r, T) = -\frac{Q^2 \mu^2}{6(4\pi \varepsilon_0 \varepsilon_r)^2} \frac{1}{kT} \frac{1}{r^4}$$  \hspace{1cm} (2)

Note that the distance dependence ($1/r^4$) is stronger than for a pure charge-charge interaction ($1/r$), and that an additional temperature dependence is introduced. The interaction potential approaches zero for high temperatures, as all directions for the dipole moment become increasingly equal in probability.

Even molecules that are not permanent dipoles can interact with charges, as electrons in all molecules are affected (polarised) by external electric fields to some extent. A permanent charge can therefore induce a dipole in a molecule which does not have its own permanent dipole moment.
1.3.2 van der Waals interactions

Van der Waals interactions are a collection of weak non-covalent interactions that all have a $1/r^6$ distance dependence. These include interactions between two rotating dipoles, interactions between a rotating dipole and an induced dipole, and interactions between two induced dipoles (so-called "dispersion"). Dispersion interactions are possible between all pairs of molecules and are caused by fluctuating dipoles in molecule 1 that induce a dipole in molecule 2. Dispersion is very weak but the many interactions can still add up to a non-neglectable energy term, especially in macromolecules such as proteins.

1.3.3 Hydrogen bonds

A hydrogen bond (H-bond) is a non-covalent interaction between a hydrogen linked to a very electronegative element (donor) and another very electronegative atom (acceptor). The hydrogen atom has a high positive partial charge, while the acceptor atom has a high negative partial charge. The resulting interaction (Donor-H···Acceptor) is both unusually strong and highly directional compared to other dipole-dipole interactions.

H-bonds are especially important interactions in biology as the biological solvent is water, which is highly capable of forming H-bonds (O-H···O-H). Proteins also contain multiple amide, amine, carboxyl, and hydroxyl groups which are capable of forming H-bonds between each other and with the surrounding water (for example, N-H···O-H). The strength of an H-bond in water is approximately 20 kJ/mol (in comparison to $RT = 2.6$ kJ/mol at $37^\circ$C, and the binding energy of a covalent bond $\approx 10^2$ kJ/mol). The H-bond is thus an interaction that can both create great stability and be broken much more easily than covalent bonds, which makes them very important in dynamic biological processes. The directionality of the bond furthermore enables the construction of ordered structures in macromolecules.

1.3.4 Self-assembly in water

The favorable interaction energy for the formation of H-bonds has yet another very important implication for biology. The water molecules will always position themselves so that they maximize the number of H-bonds formed, even if a non-polar molecule which is incapable of forming H-bonds is mixed in with the water. Such mixing leads to an increased ordering of water molecules close to the non-polar molecule, to make sure that all water molecules still form the maximum number of H-bonds possible. This arrangement does not change the binding energy of the system much but has a large effect on its entropy, as water has become more ordered. A decrease in entropy is very unfavorable and the system will counteract this by decreasing the contact surface between water and the non-polar molecules. This so-called the hydrophobic effect is very much central to the biophysics of life and underlines how water with its special properties is central to the life process as we know it.
The hydrophobic effect drives many self-assembly processes in biology. The most simple example is perhaps the self-assembly of detergent molecules into spherical aggregates called micelles. Detergent molecules are amphipilic and consist of a polar head group and a non-polar hydrocarbon tail. The hydrophobic effect will favor interactions between the hydrocarbon tails, which is however opposed by the decrease in detergent entropy caused by such aggregation. The assembly can also in the case of detergent head groups with a net charge be opposed by repulsive Coulomb interactions when the detergents are brought close to each other. This results in a thermodynamic trade-off where monomeric detergent molecules are stable until a certain detergent concentration where the gain in water entropy overcomes the entropic and energetic penalty of creating micelles. This concentration where micelles start to form is called the critical micelle concentration (CMC). The CMC is decreased with increased hydrocarbon tail length, and is increased upon increasing the charge of the head groups. Self-assembly is typically also heavily affected by the solution conditions, as the non-covalent interactions are modulated. An increase in ionic strength does for example leads to increased screening of Coulomb interactions, and a decrease in CMC for charged detergents.

Similar self-assembly processes occur for other types of biologically relevant molecules. Lipids assemble into bilayers that form the cell membranes, and hydrophobic amino acid residues are hidden away from the surrounding water in a folded protein.

1.4 FOLDING AND MISFOLDING

A central principle in protein physics is that the primary structure of proteins will spontaneously assemble, fold, into a unique three-dimensional native structure. This relationship between primary structure and higher structure is usually termed Anfinsen’s dogma [10]. One consequence of Anfinsen’s dogma is that the native state must be a minimum in the folding free energy landscape.

So how do proteins minimize their free energy? The major driving force in folding is the hydrophobic effect which makes the protein chain collapse, forming a hydrophobic core hidden away from the aqueous solvent (Figure 2A). The core of proteins is tightly packed, indicating that many van der Waals interactions are formed which decreases the energy of the folded state [11]. Other favorable non-covalent interactions such as salt bridges and H-bonds can also usually form in the folded state. The polypeptide chain will furthermore arrange itself by adjusting $\phi$ and $\psi$ angles so that interchain steric clashes are avoided. It turns out that only two major local structures (secondary structures) are possible for peptide chains: the $\alpha$-helix and the $\beta$-sheet [12]. These secondary structures are stabilized by intrachain H-bonds between the backbone amide groups (Figure 2B). The stabilizing interactions are primarily counteracted by the decrease in chain entropy upon folding, as well as by ionic interactions that can cause electrostatic repulsion [11].
Figure 2: A. Structure model of the SH2 domain (pdb 2cs0) determined by solution state NMR spectroscopy. Hydrophobic residues (I,L,M,F,V,A) are colored orange to illustrate the formation of a hydrophobic core upon folding. B. The same structure model as in A (20 NMR structures), shown in cartoon depiction, colored according to secondary structure: coil = gray, α-helix = red, β-sheet = blue. Secondary structure elements are stabilized by hydrogen bonds (yellow dashed lines). C. Simplified folding free energy landscape for an arbitrary protein. The funneled folding landscape for the native state (left) and the existence of a misfolded state which can form large aggregates (right) are shown. The transition from the native to the misfolded state is in vivo regulated by chaperones.

A vast number of different conformations are in theory available for the polypeptide chain, as a chain with n amino acid residues will have 2(n-1) variable (\(\phi\) and \(\psi\)) backbone angles. It is therefore obvious that the protein does not sample all these angles on its way towards the energy minimum postulated by Anfinsen, as this would lead to extremely slow kinetics for folding [13, 14]. The observed high folding rates for proteins must therefore be the result of a smooth energy landscape where the protein is “funneled” towards the energy minimum (Figure 2C, left). These funnel shaped energy landscapes are probably the result of evolution, as only proteins that are able to properly fold within a reasonable time are successfully conserved [15].
It is however important to note that not all proteins have a single fixed conformation. It is for example normal for proteins to shift between different conformations in response to changes in the environment. This enables proteins to carry out work and transmit signals in response to stimuli. It is also normal for proteins to contain flexible and unstructured segments which do not fold into well-defined structures. Proteins can in fact be thought of to exist on a continuum, from proteins that are folded in completely distinct and stable folds to proteins that are entirely unstructured even in their native environment. This last class, the so-called intrinsically disordered proteins (IDPs) are actually estimated to make up roughly one-third of all eukaryotic proteins [16]. This means that an expansion of Anfinsen's dogma is needed, as the primary structure clearly encodes both structure and disorder. In IPDs this is seen as a higher proportion of hydrophilic residues in their sequences, which makes them unable to form a hydrophobic core. IDPs are also increased in proline residues which are known to break ordered secondary structures [17].

Other states which are higher in energy compared to the native states can also sometimes be populated (Figure 2C), even under native conditions. Misfolding is however in vivo counteracted by various chaperone proteins which guide the folding processes towards the native state. Expression of chaperones is upregulated during cellular stress events, which also gave name to the heat-shock protein (Hsp) group of proteins which was first discovered in Drosophila after exposure to heat stress [18]. So-called holdase chaperones (such as DnaJ/Hsp40, BRICHOS, and Hsp33) recognize and passively bind unfolded segments of proteins and prevent them from forming non-native contacts [19, 20]. This is in contrast to foldase chaperones (such as DnaK/Hsp70, Hsp90, and GroEL) which assist actively in the folding of proteins, in energy-dependent processes which require ATP [21–23]. Holdase and foldase chaperones commonly work together in the cell. Hsp40 does for example bind client proteins, deliver them to Hsp70, and stimulates the ATPase activity of Hsp70 [24].

The population of misfolded states can increase due to various biological alterations, either genetic or environmental. A genetic mutation might for example lead to a protein variant with a destabilized hydrophobic core, which raises the energy of the folded state. Alterations in the cellular environment could lead to protein modifications, changes within the interaction network of the protein, or a loss of chaperone activity. Misfolding of a protein could lead to a partial or complete loss of its biological activity (loss of function), which could lead to various diseases. Some examples of diseases related to misfolding are cystic fibrosis (CFTR), cancer (p53), and sickle cell anemia (hemoglobin) [25–28].

The higher energy of misfolded species also makes such species prone to interact further to reduce their free energy (Figure 2C, right). This often leads to the formation of non-native intermolecular interactions, and non-native and sometimes toxic protein activity (gain of function). The non-native interactions can either be with another copy of the misfolded protein (homotypic), or with another cellular component (heterotypic) [29, 30]. Protein aggregates can furthermore be either ordered or more amorphous in structure, with the aggregate morphology being determined by both intrinsic and extrinsic factors [31, 32].
1.5 THE AMYLOID STATE

Amyloid is a type of highly structured protein aggregate, first described in the 1850s. These aggregates could be stained with iodine and were therefore at the time believed to consist of carbohydrates, hence the name amyloid meaning "starch-like" (from the Latin word for starch "amylum") [33, 34]. It could however be concluded a few years later that proteins, rather than carbohydrates were the main components of amyloid, as seen by the high nitrogen content of the structures [33]. The first criterion for amyloid being adopted was that the structure should display apple green birefringence under polarized light, after staining with the Congo Red dye [33, 35]. This observed birefringence also indicated that amyloid structures were ordered fibrillar structures, which was confirmed using electron microscopy (EM) [36]. Congo Red is still today used for detection of amyloid structures, but has also been complemented with various fluorescent dyes, with the most common one being Thioflavin T (ThT) [37].

A great deal is today known about amyloid structure, with many atomistic structures solved by X-ray diffraction, cryo-EM, or solid-state nuclear magnetic resonance (NMR) spectroscopy (some examples are shown in Figure 3).

Figure 3: Amyloid structures formed by different proteins, all determined by cryo-EM and colored from the N-terminus (blue) to the C-terminus (red). A. The SH2 domain of the PI3-Kinase (pdb 6r4r [38]) B. Serum amyloid A (pdb 6mst [39]) C. Superoxide dismutase 1 (pdb 7vzf [40]) D. Amyloid-β (pdb 5oqv [41]).
All amyloid fibrils are long (µm scale) unbranched structures which could consist of thousands of protein monomers, stacked atop of each other typically separated by approximately 4.7 Å [42]. The fibrils have a characteristic cross-β fold where the β-sheets run perpendicular to the fibril axis (Figure 3). A high morphological polydispersity is however observed, with several structure variants (polymorphs) formed from a single polypeptide sequence both in vitro and in vivo [43]. This for example includes different β-sheet conformations, such as U-shaped and S-shaped conformations within the amyloid core.

Amyloid formation represents a phase transition from the fluid phase into a condensed phase and therefore involves a nucleation event, which is followed by elongation of the formed nucleus [44, 45]. Nuclei, the highest point in the aggregation free energy pathway, are the first species where continued growth of the aggregate is more energetically favorable compared to back-dissociation into smaller aggregates [46, 47]. Formation of amyloid has also been observed to occur via so-called non-classical or multi-step nucleation, where the aggregate size is first increased, resulting in the formation of metastable pre-nucleation clusters, followed by a structural transition into the amyloid state [45, 48, 49]. Nucleation directly from such clusters is often slow, but the presence of pre-formed fibrils (seeds) will greatly accelerate the rate of the nucleation and the conversion into the aggregated state [47, 50]. Seeding with pre-formed fibrils is also known to propagate the specific polymorph present in the seed, both in vivo and in vitro [51–53], indicating that structure templating is taking place. Seeding is also related to prion-like propagation of amyloid in vivo (Section 2.2.1) where misfolded and aggregated species can spread from an affected cell to neighboring cells where they induce aggregation in natively folded species. [54, 55].

The amyloid state is perhaps best known for its connection to diseases, as accumulation of amyloid in various organs such as heart, liver, and kidney is known to cause tissue damage in so-called systemic amyloidosis [56]. Amyloid formation is furthermore also associated with diabetes type II [57], as well as with various neurodegenerative diseases such as Alzheimer’s disease, Parkinson’s disease, Creutzfeld–Jakob disease, Amyotrophic lateral sclerosis, and Huntington’s disease [58]. Non-disease related and highly functional amyloid is however also known to exist. Lower organisms commonly use amyloid to construct structures, such as in bacterial biofilms [59]. Amyloid is also in mammals believed to be involved in important biological processes, for example, hormone storage, epigenetic inheritance, and memory consolidation [60–62]. The amyloid state becomes even more diverse when considering that proteins which are not known to form either disease-related or functional amyloid structures in vivo have been shown to be able to access the amyloid state in vitro under certain solution conditions [63, 64].

The fact that many different proteins with varying sequences, structures, and functions can form the same type of structure indicates that amyloid is a generic state which is available to most polypeptides [65]. It has also been suggested that the amyloid state is the most energetically stable state that a protein can assume [66]. This would mean that the native state of the protein may not be the global free energy minimum, but rather a local free energy minimum which is more or less kinetically trapped under normal cellular conditions [66].
Chapter 2
ALZHEIMER’S DISEASE

2.1 THE AMYLOID-CASCADE HYPOTHESIS

Alzheimer’s disease (AD), the most common cause of dementia, is a neurodegenerative disease characterized by a progressive loss of cognitive functions. The disease often begins with depression and impairment of short-term memory which then progresses with a loss of language, motor function, and long-term memory, disorientation, mood swings, loss of body functions, and ultimately death [67, 68]. The German physician Alois Alzheimer described the disease in his case report "Über eine eigennartige Erkrankung der Hirnrinde" (“About a peculiar disease of the cerebral cortex”) published in 1907 [69]. Alzheimer then extended on his findings in a more well-known 1911 article where he describes the symptoms of a demented 56 year old man and the post-mortem findings in the patient’s brain. Alzheimer there wrote the following about what was observed in the patient’s brain:

"Microscopical investigation showed the cortex to be filled with varying degrees of Fisher’s plaques. Their number in general correspond to the macroscopically recognizable amount of brain atrophy." [70]

"Fisher’s plaques" refers to what is now known as neuritic plaques, discovered by Oscar Fisher a few years earlier. These plaques were around the same time also found to contain amyloid (Chapter 1.5) as they could be stained by Congo Red [33]. The main component of the amyloid fibrils from AD brains was finally in the 1980s determined to be a small peptide, the so-called Amyloid-β (Aβ) peptide [71]. Aβ is however not the only component of neuritic plaques, with multiple proteins and other biological macromolecules being found in plaques isolated from brain samples [72].

It is interesting to note that Alois Alzheimer viewed the plaques that he observed as histological markers, and not as the cause of the disease:

"...we have to conclude that the plaques are not the cause of senile dementia but only an accompanying feature of senile involution of the central nervous system. [70]"

The causative link between amyloid plaques and AD was instead put forward in the early 1990s by John Hardy and Dennis Selkoe who described what came to be known as "the amyloid-cascade hypothesis". The hypothesis states that it is the aggregation of Aβ and the deposition of its aggregates in the brain that is responsible for neuronal cell death and other pathological changes in AD [73, 74]. Disease-related amyloid-deposition is well established in other organs (systemic amyloidosis) where...
The formation of these structures causes progressive and non-reversible tissue damage [56]. The involvement of amyloid-formation in AD does however remain somewhat controversial to this date [75], mostly due to previous failures to develop AD therapies that target amyloid formation.

The strongest argument for the amyloid-cascade hypothesis has been the genetic links between Aβ production and aggregation, and AD. One of the earliest such evidence is the observation that the prevalence of early-onset AD is greatly increased in individuals with Down’s syndrome (trisomy-21) [76]. The brains of adult individuals with Down’s syndrome also display the characteristic Aβ amyloid deposits seen in AD [77]. This increase of Aβ production and aggregation with trisomy-21 lead to speculation that the peptide originates from chromosome 21 [71], which was confirmed with the discovery of the gene encoding the amyloid-precursor protein (APP) on that chromosome [78].

APP is a cell surface receptor and a type-I membrane protein found in most cell types, but at especially high concentrations in the neurons. The protein is processed by extracellular cleavage by either α-secretase (ADAM10) or β-secretases (BACE1) in addition to cleavage in the transmembrane domain by γ-secretase (a protein complex formed by four different proteins). Successive cleavages by α- and γ-secretase cleavage is the predominant route for APP processing, and results in fragments that are considered to be non-toxic [79, 80]. The combination of β-secretase and γ-secretase cleavage is responsible for the production of 38-43 amino acids long disordered Aβ peptides, with the longer variants being more aggregation-prone and considered to be more toxic [81–83].

Mutations in presenilin, the catalytic subunit of γ-secretase is a well-known genetic risk factor for early-onset familial AD (fAD). Such mutations can increase the activity of the secretase and can also increase the production of the more aggregation-prone Aβ variants [84]. Genetic variants of APP furthermore exist which are linked to fAD. Such variants typically have mutations close to the cleavage sites in APP, which leads to increased production of Aβ by β-secretase cleavage [85, 86], or mutations inside the Aβ segment which leads to alterations in the aggregation of the released cleavage product [87, 88]. One known protective APP variant, A673T (corresponding to A2T in Aβ), also exists primarily in Scandinavian populations [89, 90]. The substitution in this so-called Icelandic variant reduces cleavage by β-secretase, which in turn reduces Aβ production by 40% [90].

It should be noted that most cases (approximately 97%) of AD are not linked to genetic variation in APP or APP processing, and are instead classified as sporadic AD (sAD) with a later onset of disease [91]. The familial early-onset genotypes do however give important information about the actors and processes that might be involved in the disease. It is believed that Aβ production is not generally increased in sAD, instead, the clearance of amyloid seems to be affected [92, 93]. This could lead to a gradual increase in aggregation, which is in agreement with the late onset of sAD. Several known genetic risk factors do however exist also for sAD, including mutations in Apolipoprotein E (ApoE) [94], BIN1 [95], and Clusterin (ApoJ) [96]. These proteins could be involved in direct interactions with Aβ which modulates its aggregation behavior, but could also affect other cellular functions which are not directly related to amyloid formation but might disturb cellular homeostasis over time, leading to increased vulnerability.
The assembly pathway for A\(\beta\) involves a gradual transition of monomeric A\(\beta\) peptides with IDP character into the highly ordered amyloid fibrils, via intermediate states (Figure 4), as was introduced in Section 1.5. No universally accepted terminology exists within the amyloid field for these intermediate states [97, 98], and the collection of all such aggregates will will here be referred to as pre-fibrillar aggregates.

A common criticism of the amyloid-cascade hypothesis is the fact that the amyloid-load in the brain correlates poorly with disease severity [75, 99]. This was the observation that already Alois Alzheimer made when he disqualified the plaques as causative agents in the disease. However, a stronger correlation with the disease can be found when considering the concentration of pre-fibrillar A\(\beta\) species, rather than the amyloid species [100–102]. Later research has because of this focused on such intermediate A\(\beta\) aggregates, which seem to show higher toxicity compared the the mature fibrils [103, 104] (Figure 4). A biological example of the apparent link between toxicity and assembly into smaller aggregates is the Arctic A\(\beta\) fAD variant (E22G) which assembles into pre-fibrillar aggregates rather than fibrils [87]. Other A\(\beta\) variants that have been modified to stabilize pre-fibrillar forms also show increased toxicity [105–108] which seems to further strengthen this hypothesis.

Pre-fibrillar A\(\beta\) species are however a diverse class of aggregates and could in theory include on- and off-pathway aggregates from dimers all the way up to large megadalton particles. These aggregates also display large structural polydispersity, with unstructured, \(\beta\)-sheet structured, and helical structured states having been reported [106, 109, 110]. Smaller pre-fibrillar aggregates are usually termed “oligomers”, although this term has also been used for very large species as well [97]. The term oligomer will however in this thesis be reserved for the very smallest aggregates (on the scale of \(10^0\)-\(10^1\) peptide units), which may be metastable pre-nucleation clusters (Section 1.5). Larger aggregates that have grown more fibril-like are instead sometimes termed protofibrils. Fibril properties which start to develop include increased \(\beta\)-sheet structure, an elongated shape as seen by EM, and the ability to bind the amyloid-specific ThT dye [97, 111, 112]. These larger aggregates could either be colloidal fibrils (formed after nucleation), [113–115], or off-pathway aggregates [116, 117].

The work in this thesis is focused on oligomers, as defined above. Studies of pre-fibrillar aggregates seem to suggest that the smaller aggregates are the most toxic states [119, 120], although comparison of different pre-fibrillar species is sometimes difficult due to the varying methods used to detect aggregates, and to test their toxicity [98]. Toxicity for small oligomeric species could however stem from a combination of high chemical reactivity and high diffusion rates [114, 121]. One of the first examples of neurotoxic A\(\beta\) oligomers were the so-called A\(\beta\)-derived diffusible ligands (ADDLs) which were prepared in vitro by co-incubation of A\(\beta\)(1-42) with the chaperone protein clusterin [122]. Such oligomers were found to be non-fibrillar globular structures with molecular masses of 17-42 kDa (4-10 peptides) which were able to inhibit hippocampal long-term potentiation [123, 124], a process that is important for memory formation, and to kill cell cultures at nanomolar concentrations [122, 125–127].
Aβ species go from disordered (blue) to highly ordered (red) upon aggregation. Intermediate species are metastable due to their high free energy, which probably contributes to their toxicity. ThT fluorescence increases upon formation of more fibrillar aggregates B. Monomers assemble into small aggregates, oligomers, which are known to insert into lipid membranes. Larger aggregates are termed protofibrils. Some example structures, a disordered monomer (left, from molecular dynamics simulations), the antiparallel β-sheet structured oligomer formed in a membrane-mimicking environment (middle, from solution state NMR, pdb 6hry [118]) and an amyloid fibril with the typical cross-β structure (right, from cryo-EM, pdb 5oqv [41])

Figure 4: A

Aβ oligomers are detected both in vitro and in vivo [107, 128–131], and are generally dynamic and polymorphic structures. The fact that oligomers formed by different amyloidogenic proteins are recognized by the same antibodies does however indicate that some sort of characteristic structure is found within oligomers [132]. Such characteristics have been suggested to include large hydrophobic surface areas and a propensity to form antiparallel β-sheets [97, 133, 134]. Characterization of oligomers is complicated by the fact that experimental studies are associated with multiple challenges, for example: SDS-PAGE is known to induce artifacts as Aβ interacts strongly with the SDS detergent [135, 136], low concentrations make detection difficult using methods that detect an ensemble average, the low stability means that extraction of oligomers formed in vivo from biological samples is difficult [137], and oligomeric species can easily convert into other structures during the time course of an experiment [138, 139].
2.2.1 Toxicity mechanisms

The reasons for the observed oligomer-related toxicity are not yet fully understood. It has been suggested that oligomers could for example bind to various important biomolecules, such as receptors, and perturb their natural functions [140, 141]. The toxicity could also be due to both intracellular and extracellular Aβ species. Processing of APP can in fact take place both in the plasma membrane and in early endosomes [142]. The low pH of early endosomes is known to promote β-secretase cleavage, and therefore the production of Aβ, while alternative APP processing by α-secretase seems to dominate at the plasma membrane [143, 144]. It is therefore possible that Aβ toxicity originates from intracellular events, and that cells export Aβ aggregates to the extracellular space where they deposit as neurotic plaques [145, 146]. Such trafficking of aggregates is supported by the fact that plaques have been found to contain co-aggregated intracellular proteins [147]. This could also in part be the reason why targeting plaques has failed as therapy for AD, if the most toxic events occur intracellularly and precede extracellular plaque formation.

One theory is that oligomers interact with the plasma membrane and/or organelle membranes, where they could exert their toxic effect by permeabilization of the membrane in various ways. Calcium dysregulation is known to be associated with AD, with observations of altered calcium signaling both in fAD and sAD [148], which could be related to the Aβ-induced weakening of membranes. The influx of calcium into neurons is observed at sites with Aβ deposits [149, 150] and the toxic effects of Aβ can be prevented in cell cultures when Ca\(^{2+}\) is not present extracellularly [151]. Toxicity was also observed to be lowered when Zn\(^{2+}\) was added extracellularly. Localization of Aβ to the inner mitochondrial membrane, Aβ-induced leakage into mitochondria, and mitochondrial dysfunction in AD has also been reported [152–154]. Aβ-induced membrane leakage is further supported by in vitro results using membrane-mimicking systems, such as dye-filled liposomes [155, 156]. Leakage in vitro was found to be either non-specific, caused by amyloid fibrils that destroy the lipid structures, or Ca\(^{2+}\)-specific via presumed pore structures [157]. The latter mechanism could also in vitro be blocked by Zn\(^{2+}\), or molecules which bind to the N-terminal segment of Aβ [157, 158]. The proposed pore model is supported by a solution-state NMR-structure of an Aβ(1-42) tetramer (Figure 4B, purple) which forms an octameric pore [118], and by a cryoEM-structure where the Aβ segment grafted onto α-hemolysin forms a heptameric pore [159]. Various observations of pore-like Aβ structures in lipid membranes using low-resolution microscopy methods have also been made [160–162].

Another observed property of Aβ toxicity is prion-like propagation where toxic species are capable of spreading between cells. It is for example known that injection of homogenates from human AD brains into fAD transgenic mice causes spread of amyloid pathology at the injection site [163]. Amyloid pathology is also known to spread from the injection site throughout the brain [164], and cell to cell propagation of Aβ oligomers has been observed in cell cultures [165]. It has even been shown that AD brain homogenates which are injected into other parts of the mouse body are capable of inducing amyloid pathology in the mouse brain [166].
2.3 THERAPEUTIC STRATEGIES

Four AD drugs are currently approved in Europe, which all affect neurotransmission, are used to manage the symptoms of the disease, but do not affect disease progression [167]. The development of disease-modifying AD therapies that target amyloid formation has been the focus of much research since the amyloid cascade hypothesis was first formulated. Such drugs could in principle target all species along the aggregation pathway that precede the disease-related state.

The production rate of A\(_\beta\) is clearly linked to the development of AD, as was described in Section 2.1. A logical drug target would hence be the secretases that cleave APP. Cleavage by BACE1 is the first step in generating A\(_\beta\) and several BACE1 inhibitors have been investigated as potential anti-AD drugs. One apparent problem is however that BACE1 has many other substrates besides APP and is involved in various important physiological functions [168]. Many clinical trials on BACE1 inhibitors have therefore been canceled due to the observation of severe adverse effects [169–171]. No BACE1 inhibitors are currently in clinical trials for AD, but two enhancers of \(\alpha\)-secretase (MIB-626, and APH-1105) are in Phase II trials [172] (Figure 5). Such drugs could potentially be an alternative way to redirect APP processing away from the A\(_\beta\)-generating pathway.

Another potential problem with modulation of secretase activity is however that the formation of toxic species has already occurred at the observable disease onset, meaning that such therapies would need to be started at an early stage [173]. Modulation of secretase activity could perhaps prove more useful for delaying the onset of fAD in patients with known fAD-related genotypes. Processes that remove toxic species could instead be more attractive drug targets in sAD. The small molecule Thiethylperazine (TEP), currently undergoing a phase II trial, does for example bind and activate the membrane protein transporter ABCC1 which is responsible for clearing A\(_\beta\) and regulating A\(_\beta\) proteostasis in the brain [172, 174].

Pathological species can also be targeted by raising monoclonal antibodies against A\(_\beta\). Four such antibodies are currently in stage III clinical trials: donanemab, ganntenerumab, lecanemab and solanezumab [172] (Figure 5) and one, Aducanumab (sold as Aduhelm), is approved for use against mild AD in the United States. Aducanumab is the first-ever approved disease-modifying therapy for AD and the first new drug approved for AD in almost 20 years [172, 175]. The drug has however been controversial as it first failed two phase III studies, before the data was eventually re-evaluated prior to accelerated approval in the US. [176, 177]. Aducanumab recognizes high-molecular weight aggregates of A\(_\beta\), binds to an N-terminal motif comprising residues 3-7, reduces the amount of A\(_\beta\) oligomers in vitro, and has been shown to reduce the amyloid plaque levels in brains, both in animal models and in humans [178–180]. Modest and conflicting results on cognitive improvements have been suggested to be due to inadequate selectivity for the toxic aggregates, or too low concentrations of the antibody actually entering the brain after administration [181].
Another monoclonal antibody, lecanemab, did recently undergo a phase III trial which indicated a statistically significant reduction of cognitive decline [182]. Lecanemab is raised specifically against pre-fibrillar aggregates which are enriched in the fAD-related arctic Aβ variant, with the hypothesis that targeting such species could benefit the treatment also for sAD cases [179, 183, 184]. These recent breakthroughs using Aβ targeting antibodies are encouraging, and undoubtedly open up a new phase for AD and amyloid research. Several problems with antibodies however exist, including their poor blood-brain barrier (BBB) permeability (0.1-1%), high price, and the need for administration through injections [185, 186].

BBB permeability could possibly be improved by antibody engineering where some moiety which facilitates transfer over the barrier is attached to the antibody [187], e.g. a cell-penetrating peptide [188]. Another approach is to make use of the transferrin receptor which is highly expressed in the cells that make up the BBB. The so-called Brain Shuttle technology attaches a protein sequence to the antibody which is recognized by the transferrin receptor. This facilitates transfer via the receptor, over the BBB [189]. A brain shuttle-gantenerumab construct is currently in Phase II trial [190].

Other types of AD treatment are furthermore being developed, which are summarised in Figure 5. Valitrampirosate is for example a small molecule drug-candidate in phase III clinical trials. The molecule is metabolized in the cell into 3-sulfopranpanoic acid, a naturally occurring substance in the brain that inhibits Aβ oligomerization [191, 192]. Other classes of drug-candidates are also under development, which do not directly target amyloid aggregation. The most common target for the disease-modifying drug candidates is inflammation processes (Figure 5, yellow). Inflammation is probably an important process to target in order to reduce the pathological effects occurring downstream from amyloid formation [193], and such drugs could therefore complement the anti-amyloid drugs.

Anti-Aβ therapies might generally have to be administrated at an early stage, before severe neuronal damage has taken place. Amyloid plaque formation often starts decades before disease onset and diagnosis, and is in individuals with genetic predispositions observed even earlier in life [194, 195]. One possible way to ensure early inhibition of Aβ aggregation would be to develop an AD vaccine where the cell itself develops antibodies against the injected antigen, already before the disease has started to develop. The challenge here would be to design an antigen which is not itself neurotoxic. One attempt to develop an AD vaccine was AN1792 which was stopped in 2003 after a phase I trial showed serious adverse effects in 6% of the tested subjects [196, 197]. Later autopsy findings have however shown that 88% of the studied patients displayed plaque removal and that the vaccine was capable of keeping patients plaque-free for 14 years [198]. Later vaccine candidates have also been developed and tried in phase II studies, but have since been discontinued [199, 200].
Figure 5: AD drug candidates in clinical trials, as of January 5 2021. Reprinted from reference [190] under the CC BY-NC-ND 4.0 licence. Red symbols are drug candidates that target amyloid-formation.
Chapter 3

METHODS

3.1 BOTTOM-UP IN VITRO BIOPHYSICS

Chapter 2 described how Aβ self-assembly occurs in a very complex biological milieu in vivo, which includes multiple interaction partners that probably modulate aggregation in several important ways. Such a molecular environment is often not very suitable for mechanistic studies on how individual components affect the self-assembly process. We therefore in this thesis conduct controlled biophysical experiments using a bottom-up in vitro approach. This means that Aβ is recombinantly produced and purified for test tube studies in isolation under highly controlled conditions. Other components can in such studies be added to Aβ to assay their effects on the peptides structure or self-assembly behavior.

3.1.1 A note on reproducibility

Amyloid formation is under kinetic control and small changes in experimental conditions can therefore have a large consequence on the states populated. The homogeneity and purity of the studied Aβ sample is crucial for obtaining reproducible results. Sequence homogeneity is best obtained by using recombinant peptide production, as biological protein production has higher fidelity compared to chemical peptide synthesis [201]. Care must also be taken to assure an aggregate-free peptide sample as pre-formed aggregates will seed the formation of new aggregates. Lyophilized recombinant peptide samples are therefore first dissolved in a solvent which disfavors aggregation. Such solvents include:

1. Aqueous solutions at high or low pH, which are far from the peptide’s isoelectric point $\mathrm{pK}_\mathrm{i}$, at $\sim 5.3$ (Figure 1D) where electrostatic repulsion makes aggregation less energetically favorable.

2. Organic solvents which increase the solubility of Aβ.

3. Chaotrophic agents such as urea or guanidine hydrochloride which disturb intermolecular interactions between peptides.

Any preformed aggregates are then removed from the solution preferably by size exclusion chromatography (SEC), where only the monomeric peak is collected. Purified samples are kept on ice to slow down aggregation, and should be used immediately. Any agitation will promote aggregation, including repeated pipetting into the solution, flicking the tube or introducing bubbles into the solution. Buffers should furthermore be degassed to reduce air-water interfaces, which promote aggregation [202].
It is important to have full control of all sample conditions during experiments to obtain reproducible results. This includes temperature, pH, ionic strength, and the concentration of peptide and other molecules.

Temperature is a crucial factor as aggregation kinetics are generally increased at higher temperature. It has however been found that the rate of seed-dependent nucleation is increased at lower temperature [203], indicating that also aggregation mechanisms change with temperature. The structure of the peptide is also affected by temperature, with N-terminal folding occurring at low temperatures [204].

The pH value of the buffer controls the charge of proteins, and thus intra- and intermolecular electrostatic interactions. Experiments have here been performed in buffered solutions between pH 7 and 9. Shifting the pH far away from the $K_i$ decreases the amyloid aggregation rate due to increased peptide-peptide repulsion. Salt might sometimes be needed to increase the ionic strength in some experiments. This will however generally lead to an increase in the aggregation rate due to increased screening of ionic interactions [205].

The peptide concentration of samples is determined using near-UV absorption spectroscopy at 280 nm due to the presence of a single tyrosine (Y10) amino acid residue, with an extinction coefficient $\epsilon = 1490 \, M^{-1} \, cm^{-1}$.

### 3.1.2 Structure studies

$\text{A}\beta$ is an IDP in its monomeric form, but can assemble into various structures during aggregation or upon interacting with other molecules. The average secondary structure of $\text{A}\beta$ and other peptides and proteins can be easily measured using circular dichroism (CD) spectroscopy. CD spectroscopy measures the difference in absorbance of left (L)- and right (R)-handed circularly polarised light. It is well known that absorption follows Beer’s law (Equation 3).

$$ A = \epsilon \cdot c \cdot l \quad (3) $$

where $A$ is the absorbance, $\epsilon$ is the molar extinction coefficient, $c$ is the concentration and $l$ is the pathlength of the light. It thus follows that the difference between absorbance of L- and R-handed circularly polarised light depends on the difference in the molar extinction coefficients for the L- and R components (Equation 4)

$$ \Delta A = A_L - A_R = (\epsilon_L - \epsilon_R)c \cdot l \quad (4) $$

The two conditions for CD activity are therefore that the molecular structure must absorb light ($\epsilon \neq 0$) and that the absorbance must be different for L- and R-handed circularly polarised light ($\Delta \epsilon \neq 0$). Only asymmetric (chiral) molecules, or molecules within asymmetric environments have $\Delta \epsilon \neq 0$. Both $\Delta A$ and $\Delta \epsilon$ are possible units for a raw CD signal, but also the older unit ellipticity, measured in degrees or millidegrees (mdeg) is commonly used (Ellipticity (deg) = 32.98$\Delta A$).
Theoretical CD spectra calculated using the webserver pdbmd2cd [206], for an MD structure of a disordered Aβ monomer (blue), an α-helical Aβ monomer from a micellar environment (yellow, pdb 1ba4 [207]), an amyloid fibril of Aβ (red, pdb 5oqv [41]) and an Aβ tetramer from a micellar environment (purple, pdb 6hry [118]).

Figure 6: Theoretical CD spectra calculated using the webserver pdbmd2cd [206], for an MD structure of a disordered Aβ monomer (blue), an α-helical Aβ monomer from a micellar environment (yellow, pdb 1ba4 [207]), an amyloid fibril of Aβ (red, pdb 5oqv [41]) and an Aβ tetramer from a micellar environment (purple, pdb 6hry [118]).

The peptide bond absorbs light in the far-UV range (180-240 nm), and is in asymmetric structures within proteins, which means that CD spectroscopy can be used to study the orientation of the protein backbone. Typical spectral signatures are observed in the far-UV CD spectra for common secondary structures such as α-helix (spectral minima at 222 nm and 208 nm, and spectral maximum at 193 nm), β-sheet (spectral minimum at 215 nm and spectrum maximum at 195 nm) and random coil (spectral minimum at 197 nm) [208]. Most proteins contain a mixture of structures and the resulting CD spectrum will hence be a linear combination of the different spectral signatures for the pure secondary structures.

It is in principle possible to deconvolute an experimental CD spectrum to resolve the relative proportions of secondary structures which make up the spectrum, given that suitable reference spectra for various secondary structures are available and the concentration of the studied protein sample is known [209, 210]. CD spectroscopy can however only resolve the average structure of the entire ensemble. This means that deeper interpretation of CD spectra from heterogeneous populations such as those
formed by Aβ can be difficult. A mixture of random coil and β-sheet signatures in the CD spectrum could for example be interpreted as a mixture of structure within one single state, or as a mixture of multiple differently structured states within the sample. Another way to interpret CD spectra is to calculate theoretical CD spectra from structure models, which can be compared to the experimental spectra. Such theoretical CD spectra for various Aβ models are shown in Figure 6 [206].

CD spectroscopy is in this thesis used in Paper I to measure the average structure of various Aβ variants prior to and after incubation. This shows how the structures of Aβ variants evolve spontaneously over time, which informs us about their relative aggregation propensities.

In Paper III the structure of some chaperone-related protein constructs are measured using CD spectroscopy to monitor their folding state. The structure of the constructs were furthermore monitored upon changing the sample temperature, to determine the melting temperatures of the constructs. The experimental CD spectra could also be compared to the theoretical CD spectra generated from structure prediction to confirm that the constructs were correctly folded.

In Paper V and Paper VI the structure of Aβ was monitored upon the addition of detergent molecules, below and above the CMC. The observed structures were compared to the stabilities (Paper V) and sizes (Paper VI) of the formed assemblies. In Paper VII the structures in such detergent micelles were also studied upon co-aggregation with another peptide.

The mesoscopic structures of larger aggregates were in addition studied using microscopy methods, including atomic force microscopy (AFM) and cryo-EM. These techniques are able to detect the fibrillar morphologies of amyloid aggregates discussed in Chapter 1.5. In Paper III we detect the morphology of Aβ fibrils formed at the end-point of aggregating in the presence and absence of chaperone constructs, and in Paper V and Paper VII we study the morphology of co-aggregates formed from mixtures of Aβ and other molecules.

3.1.3 Aggregation kinetics

Aggregation of amyloid fibrils by the seed-nucleation mechanism has been extensively described by theoretical frameworks where rate equations that describe the assembly process have been developed [211–213]. In brief, the formation of amyloid nuclei can occur in fibril-independent processes, called primary nucleation, as well as in fibril(seed)-dependent processes, called secondary nucleation, with the rates of these processes being determined by their respective rate constants $k_n$ (primary nucleation), $k_2$ (secondary nucleation) and their respective reaction orders $n_c$ and $n_2$. A nucleus that has formed will grow by elongation according to the rate constant $k_+$. An analytical solution to the equation system of underlying rate equations has been formulated in the form of a master equation (Equation 5), which describes the change in the normalized fibril mass fraction with time ($M(t)/M(\infty)$), at varying monomeric starting concentrations ($m(0)$)
\[
\frac{M(t)}{M(\infty)} = 1 - \left( \frac{B_+ + C_+}{B_+ + C_+ e^{\kappa t}} \right) \frac{\kappa^2}{k_\infty} e^{-k_\infty t}
\]

\[B_\pm = (k_\infty \pm \bar{k}_\infty)/(2\kappa) \text{ and } C_\pm = \pm \lambda^2/(2\kappa^2)\]

where

\[
\lambda = \sqrt{2k_+ k_{n} m(0) n_c} \text{ describes primary nucleation, and } \\
\kappa = \sqrt{2k_+ k_2 m(0)^{n_2+1}} \text{ describes secondary nucleation.}
\]

\[k_\infty = \sqrt{2\kappa^2/(n_2(n_2+1)) + 2\lambda^2/n_c} \text{ and } \bar{k}_\infty = \sqrt{k_\infty^2 - 4C_+ C_- \kappa^2}\]

Equation 5 can be fitted to experimental time-dependent aggregation data to estimate the rate constants. This is done in a global fit where data at several m(0) are simultaneously fitted to obtain common (global) kinetic parameters. This can in practice be done using the online fitting software Amylofit [214].

Note that only the combined rate constants \(\sqrt{k_+ k_n}\) and \(\sqrt{k_+ k_2}\) are obtained from Equation 5. Additional experiments under high seed concentrations, where the elongation process dominates, are employed to estimate \(k_+\). \(k_n\) and \(k_2\) can then be calculated when \(k_+\) is known.

**Figure 7:** A. Build-up of amyloid can be described by three microscopic processes. Aggregation kinetic curves are here calculated using Equation 5 and the rate parameters determined in ref [213], for m(0) = 1.0, 1.2, 1.5, 2.0 and 3.0 µM. B. Theoretical amyloid formation kinetics at m(0) = 1.2 µM, when decreasing \(k_n\) (left), \(k_2\) (middle) or \(k_+\) (right).
Figure 7A shows how Equation 5 has been used to calculate theoretical $M(t)/M(\infty)$ curves using the kinetic parameters determined for Ab in reference [213]. Fitting of experimental data can also be used to evaluate what effect different modulators have on the aggregation process, for example, if primary nucleation, secondary nucleation or elongation is mostly affected when the overall rate of aggregation decreases. Figure 7B shows theoretical $M(t)/M(\infty)$ curves calculated using Equation 5 where $k_n$, $k_2$, and $k_1$ are decreased step-wise. It can be seen that a decrease in the primary nucleation rate ($k_n$) prolongs the lag-time without affecting the growth rate, while the opposite is true for a decrease in the secondary nucleation rate ($k_2$).

Several methods can be used to measure $M(t)/M(\infty)$ experimentally, for example CD spectroscopy, where the increase in $\beta$-sheet content is measured over time, or dynamic light scattering where the growth of larger particle sizes is measured over time. The most popular method is however to use fluorescence spectroscopy to monitor the fluorescence of the amyloid-binding dye ThT over time.

The molecular structure of ThT consists of a benzothiazole group and a benzyl group connected by a freely rotating C-C bond. The free rotation becomes hindered once the dye binds to amyloid structures, which leads to a large increase in the fluorescence quantum yield of the molecule [215]. The increase in ThT fluorescence is therefore often related to an increase in amyloid concentration ($M$). Care must however be taken when designing ThT experiments as the dye could affect the assembly kinetics of the aggregates [216]. The addition of some molecules might also affect the ability of ThT to bind amyloid, and/or its fluorescence intensity, leading to false-positive results [217]. It is therefore important to use alternative methods to confirm effects seen in ThT fluorescence, for example by observing structure conversions in CD spectroscopy or by monitoring the end-state of aggregation using microscopy.

ThT fluorescence is induced by exciting the molecule around 450 nm, which results in emission of light around 480 nm [37]. Time-dependent ThT fluorescence experiments can be performed in a highly parallel fashion using a microplate reader, which enables several conditions, concentrations and replicates to be studied at the same time. The intensity of ThT fluorescence is then plotted against time, which most often yields a sigmoidal curve shape which describes $M(t)/M(\infty)$. $M(0)$ should typically be 0 (unless seeds have been introduced) and the plateau of the sigmoidal curve is $M(\infty)$.

We here use time-dependent ThT fluorescence experiments in Paper II and Paper III to evaluate the effect of chaperone variants on Ab-aggregation. In Paper III the data is also fitted to Equation 5 to determine which rate constants are affected by the added chaperone constructs. In Paper V the aggregation kinetics upon addition of different concentrations of detergent is studied, and is related to the structures observed in CD spectroscopy. In Paper VII the effects on Ab aggregation of adding another peptide is studied.
3.2 NATIVE MASS SPECTROMETRY AND RELATED TECHNIQUES

The main experimental technique used in this thesis is native mass spectrometry (MS), an application of MS where very gentle sample and instrument conditions are used to preserve non-covalent interactions in biomolecules upon transfer from solution into the gas phase. An important feature of native MS is that specific non-covalent interactions are being preserved [218–220], meaning that native complexes can be detected without the need for covalent crosslinking, modifications, or labeling. This enables rapid detection of intact complexes at low concentrations and low sample consumption, which can provide information about complex stochiometries [221–223], binding of ligands [224–226], and folding states [227–229].

The technique was in this thesis used for studies of Aβ oligomers as such species are both low abundant and polydisperse (Section 2.2), which makes studies using ensemble averaging techniques difficult. Detection in MS is according to the mass to charge ratio (m/z) of ionized molecules, and different oligomeric and/or ligand-bound states can be detected individually as long as they are separated by a mass difference.

3.2.1 Sample considerations

Native MS is commonly performed using purified protein samples dissolved in aqueous solution close to physiological pH, without organic modifiers. It is important that the protein concentration should be sufficiently low (<50 µM) so that each electrospray droplet contains less than one molecular species on average [230]. Artificially aggregated gas phase species could otherwise show up in the mass spectrum. The sample flow rate should for the same reason be kept low and the capillary tip dimension should be sufficiently small, as smaller droplets will reduce the probability of two non-associated species sharing the same electrospray droplet by chance [231]. High protein concentrations as well as large aggregates (seeds) also greatly increase the rate of aggregation in the electrospray capillary, which can lead to a decrease of spray quality over time or complete clogging of the electrospray capillary. Decreasing the sample concentration and filtrating the sample using a suitable molecular weight cut-off filter often leads to a significant improvement in spectral quality.

The need for controlled experimental conditions was emphasized previously in Section 3.1.1. One problem in native MS is however the lack of MS-compatible buffer systems with good buffer capacity at physiological pH. Organic acids such as carbonates, acetates, formates, and organic bases such as ammonia are volatile and can be used to create MS compatible buffer systems. The obvious choice among these options would be the carbonate ion (pKa = 6.3), for example in the form of ammonium bicarbonate (AmBic). AmBic has however been empirically found to be unsuitable for native MS analysis as electrospray ionization from such solutions can give rise to in-source unfolding [232]. This is probably due to the formation of CO₂ gas in the electrochemical electrospray process [233]. The non-polar CO₂ gas induces unfolding of protein chains as buried hydrophobic groups adsorb to the gas-water interface of bubbles.
Native MS is instead most often performed in ammonium acetate (AmAc) solutions. Aqueous solutions of AmAc have a pH near 7 but do not have any buffering capacity in this pH range as the pK<sub>a</sub> values for acetate and ammonia are 4.8 and 9.2 respectively [234]. The addition of AmAc, especially at higher concentrations, will however give rise to less drastic pH variations compared to a pure water solution also around pH 7. This is important as the electrospray process itself will lead to acidification (in positive ionization mode) of the sprayed solution as protons are accumulated in a shrinking volume [234, 235]. A higher concentration of NH<sub>4</sub><sup>+</sup> in the droplet will thus counteract some of this acidification. Studies of amyloid formation are also sometimes performed at non-neutral pH values. Aβ aggregation is for example commonly studied at pH 8, at which the ammonium component of AmAc has a reasonable buffer capacity.

The AmAc concentration is also used to set the ionic strength. Concentrations as high as 1 M can be used if needed, for example when a pH around 7 needs to be maintained during titration experiments with acidic or alkaline molecules [236]. Increasing the AmAc concentration might also improve spectral quality, as non-volatile ions such as Na<sup>+</sup>, which form adducted (M+xNa<sup>+</sup>) ions, are out-competed by volatile NH<sub>4</sub><sup>+</sup> ions.

### 3.2.2 Instrumentation

The most common type of MS instrument for native MS experiments has been the quadrupole-time of flight (qToF) instrument, due to the high mass range of the ToF detector. High-resolution orbitrap instruments modified for high mass analysis have however also been developed lately [223, 237].

A Synapt G2 qTOF instrument with a traveling-wave ion mobility cell [238] (Figure 8), from Waters Corp was used for all native MS experiments in this thesis. Ions were generated via a nano-electrospray (nano-ESI) source, by offline injection from metal-coated borosilicate emitters loaded with a few µL sample. The generated ions enter the instrument due to a combination of an applied voltage gradient and a pressure gradient. Careful tuning of the voltages and pressures are known to increase transmission of large protein complexes [239]. The instrument has a quadrupole, which acts as a mass filter and allows for selection of specific ions in tandem MS (MS/MS) experiments. This is followed by the traveling wave ion mobility cell which enables separation according to ion shape [240] (Section 3.2.5). The ion mobility cell is in turn followed by the ToF mass detector.

Increasing the potential differences between compartments in the instrument leads to an increase in the kinetic energy of the ions, which in turn can lead to an increase in internal energy upon collisions with gas molecules (collision-induced activation) or surfaces (surface-induced activation). Potential differences are generally kept small in native MS to avoid transfer of large amounts of energy into the proteins, as this could alter native structures. Controlled activation of ions can however yield interesting structural information (Section 3.2.4). Collisional activation is most easily achieved in regions where the gas pressure is higher. In the Synapt instrument these areas are the front of the instrument (sample cone), and just prior to and after the ion mobility cell (trap and transfer cells) where argon gas has been supplied (Figure 8, red).
3.2.3 The electrospray process

ESI is a soft ionization method for liquids, which gives rise to multiply charged ions by adductation with charged species, most often protons (M+xH)\(^{+x}\) but commonly also Na\(^{+}\), K\(^{+}\) or NH\(_3\)^{+}. Multiply charged protein ions will in this thesis generally be annotated by their oligomeric state/charge state (n/z) ratio, as for example seen in Figure 9B.

The liquid sample is injected through a capillary which is held at high voltage relative to the opening of the mass spectrometer. The potential could be either positive or negative, but a positive potential (positive ionization) is by far the most common mode, and also the mode used exclusively in this thesis. The applied voltage will result in the formation of a so-called Taylor cone which emits a spray of droplets. These droplets start out big (μm scale) but rapidly shrink due to evaporation. The shrinking droplets will approach a limit known as the Rayleigh limit, which is the point at which the surface tension that holds the droplet together is exactly matched by the repulsive charge-charge interactions in the droplet. The Rayleigh charge (z\(_R\)) is hence the maximum charge that a charged droplet can hold, and is given by Equation 6.

\[
z_R = \frac{8\pi}{e} \sqrt{\frac{\epsilon_0 \gamma}{\epsilon_0}} r^3
\]

where \(\epsilon_0\) is the vacuum permittivity, \(\gamma\) is the surface tension and \(r\) is the radius of the droplet.
Crossing the Rayleigh limit means that the droplet becomes intrinsically unstable and fission events take place due to charge-charge repulsion. Cycles of evaporation and fission therefore occur which reduce the size of the droplet before complete desolvation of ions occur. Several models for this last step exist with the charge residue model (CRM) and the chain ejection model (CEM) being the relevant models for proteins.

The CRM (Figure 9A) model describes a process where the electrospray droplet continuously shrinks, while always being close to the Rayleigh limit. This is achieved by continuous ejection of small charged ions from the droplet to facilitate continued solvent evaporation [241]. The size of the droplet finally reaches the size of the folded protein itself, which leads to the desolvation of the protein in a compact state closely related to its folded state.

A consequence of the CRM is that a folded protein should not be able to have a higher ESI charge than the $Z_R$ of a droplet with the size of the folded state (Figure 9B). Equation 6 can be modified to convert $r$ into protein molecular weight ($M$), if assuming a spherical shape for the protein. This yields Equation 7 which can be used to calculate an approximate $Z_R$ for a folded protein based on its molecular weight.

$$z_R = \frac{8\pi}{e} \sqrt{\frac{\epsilon_0\gamma^3 M}{4\pi\rho N_A}} \approx 0.0739\sqrt{M}$$

(7)

where $\rho$ is the density of the protein and $N_A$ is Avogadro’s constant.

Observations of protein charge states higher than $Z_R$ (Figure 9B, left) are not in agreement with the CRM and such ions need to be formed in an alternative way. Ionization according to the chain ejection model (CEM, Figure 9A) instead involves a process where one end of the protein leaves the droplet, which leads to the ejection of the protein from the droplet as an unfolded chain. Charge equilibration between the electrospray droplet and the extended chain likely takes place which enables the released ion to acquire the increased gas phase charge [241, 242].

The charge state of an ion can, due to the nature of the ESI process, be considered as a reporter on the solvent-accessible surface area (SASA) of the protein species. High charge states represent extended protein structures (larger SASA), while low charge states represent compact structures (smaller SASA). A well-folded and less dynamic protein will consequently give rise to a narrow charge state distribution with a relatively low average charge (Figure 9B, left and middle), while an unstructured and dynamic protein ensemble will produce a highly charged and wide charge state distribution [243] (Figure 9B, right). The relationship between the mass of a protein ($M$) and its average electrospray charge ($z_{av}$) follows a power law:

$$z_{av} = a \cdot M^k$$

(8)

where the experimental coefficients $a = 0.0141$ and $k = 0.7368$ (unfolded proteins), or $a = 0.0307$ and $k = 0.5753$ (folded proteins) have been determined [244].
Figure 9: A. Overview of the charge residue model for ionisation of folded proteins. Charged species (e.g. H\(^+\)) are shown as red circles. B. Example mass spectra recorded under native conditions for wild-type transthyretin (left) and a monomeric mutant (middle), which are both charged according to the CRM. Wild-type transthyretin at denaturing conditions (right) is charged according to the CEM. Peaks are annotated by their oligomer/charge state (n/z) ratio. The red fields indicate the regions in the mass spectra where z > Z\(_R\). C. Overview of the chain ejection model for ionisation of unfolded proteins. Charged species (e.g. H\(^+\)) are shown as red circles. D. Overview of the sequential events during collision induced activation, upon step-wise increase in collision energy.
3.2.4 Collision-induced activation

Generated gas phase ions can be manipulated within the mass spectrometer using so-called ion activation. The most common method for this is collision-induced activation (CIA). In CIA the gas phase ions are accelerated in a compartment filled with neutral gas atoms or molecules (such as He, Ar, or N₂). The acquired kinetic energy of ions is determined by the voltage applied (V) and the charge of the ion (qₑ) (Equation 9).

\[ E_k = q_e \cdot e \cdot V \]  

Ions will make several discrete low-energy collisions with the neutral gas inside the mass spectrometer. Every single collision will transfer a small amount of energy to the ion, which will redistribute over its internal vibrational modes. A single ion will undergo many thousands of collision events over a microsecond timescale, which gives CIA the characteristics of a slow heating process [245]. Ions will thus only be able to be excited to the lowest excited state available, and CIA will typically proceed via sequential steps, such as: cleaning, rearrangement, unfolding, dissociation, and fragmentation (Figure 9D). This sequential nature of CIA and the resulting effect on intact protein complex ions has been excellently reviewed elsewhere [246].

The amount of activation can be controlled by adjusting the acceleration of ions into the collision cell (the collision voltage), and the gas pressure in the collision cell. Oligomers of higher charge states will be more easily activated as their kinetic energy is higher (Equation 9). Molecules bound non-covalently to oligomers will start to dissociate upon activation. Such molecules can also include detergent and lipids bound to membrane-embedded complexes, which enables the detection of stripped membrane protein complexes [247, 248] (Paper VI and Paper VII).

Oligomers will start to unfold upon increasing the collision voltage. This can be monitored by ion mobility analysis. An unfolded monomer will dissociate from the oligomeric complex upon increasing the collision voltage further. Both the mass and charge of the oligomer is conserved upon dissociation. Charge partitioning will be asymmetric, with the ejected unfolded monomer retaining most of the charge previously carried by the oligomer (Figure 9D, right). For example: collision-induced dissociation of \( n/z = 4/7 \) will result in formation of a highly charged unfolded monomer and a charge-reduced compact trimer. This confirms the oligomeric state and charge state of \( m/z \) signals, but does not necessarily give much information about the architecture of the complex.

The dissociated monomers will start to fragment when increasing the collision voltage even further. This means that fragmentation of folded proteins is not possible using CIA. The use of activation methods where larger amounts of energy are transferred to the gas phase ion over a shorter period of time is needed for dissociation or fragmentation directly from a complex of folded components. One such method is Surface Induced Dissociation (SID), where ions are subjected to one high-energy collision into a surface instead of multiple low-energy gas collisions [249, 250]. This causes dissociation at the weakest interface of a complex, which can for example help to distinguish if a hexamer is a dimer of trimers or a trimer of dimers [251].
### 3.2.5 Ion mobility

Ion mobility (IM) spectrometry is a technique which is often used hyphenated to MS in native MS studies. The mobility (K) of an ion is a proportionality constant that describes how fast the ion moves through a gas upon applying an electric field (E). The mobility of an ion (K) can be calculated using the Mason-Schamp equation:

$$K = \frac{3e}{16N} \sqrt{\frac{2\pi}{\mu kT}} \frac{z}{\Omega} \frac{l}{t_dE}$$  \hspace{1cm} (10)

where e is the elementary charge, N is the gas number density, $\mu$ is the reduced mass of the ion-gas pair, k is Boltzmanns constant, T is temperature, z is the charge of the ion, and $\Omega$ is a shape-factor for the gas-ion pair, commonly referred to as the collision cross section (CCS). K can also in the simplest case be experimentally determined from the drift time ($t_d$), the time it takes for the ion to transverse a fixed length l upon applying a specific electric field E (Equation 10). The most straightforward IM measurements involve measuring $t_d$ for an ion at one or several E in a tube of well-defined l (so-called drift tube IM). The CCS of the ion can then be directly calculated from Equation 10.

Most commercial IM-MS instruments are however not fitted with drift time IM devices. The Synapt G2 instrument used in this thesis instead has a traveling wave IM (TWIMS) device [240] which has better resolution and sensitivity compared to drift tube IM. TWIMS uses a variable electric field which moves through the ion mobility cell and creates an oscillating wave-shaped potential that propels ions forward [240]. Calculations of CCS from first-principle using Equation 10 is consequently impossible using TWIMS data, as the relationship between K and $t_d$ becomes non-linear when varying E. CCS values for ions can instead be obtained from TWIMS by measuring $t_d$ for calibrants of known CCSs followed by fitting the data to a calibration function [252, 253], which in its simplest form can be written:

$$\Omega(t_d) = z \cdot F \cdot t_d^B$$  \hspace{1cm} (11)

where z is the electrospray charge of the ion. F and B are empirical coefficients obtained from calibration. The CCS for an analyte protein ion can then be obtained by inputting its experimentally measured $t_d$, when the coefficients in Equation 11 have been determined. Note that this will only be valid if the instrument settings are not changed between the calibration set and the analyte protein.

It has also been found that the calibrant ions need to be similar to the analyte ion in terms of their chemical structure, molecular weight, and charge state to obtain reliable CCS values in TWIMS [254–256]. This means for example that the CCS of an unfolded protein should be obtained from a calibration set constructed using other unfolded proteins as such species have higher ESI charge states compared to folded proteins (Section 3.2.3). The same is true for membrane proteins, which have lower ESI charge states compared to soluble proteins [257].
The experimental CCS is often compared to the theoretical CCS from structure models, as is described in Section 3.3.3. Experimental CCS values can also be used to determine the shape in which aggregating species grow [258]. It is for example found that a linearly growing aggregate has a CCS proportional to \( n \) (Equation 12), and an isotropically growing aggregate of size \( n \) has a CCS proportional to \( n^{2/3} \) (Equation 13):

\[
CCS_n = CCS_{\Delta n} + CCS_0 \quad \text{(12)}
\]

\[
CCS_n = CCS_x \cdot n^{2/3} \quad \text{(13)}
\]

where \( CCS_x \) is the state which the growth starts from (most commonly \( n = 1 \)), \( CCS_{\Delta} \) is the difference in CCS between states \( n = x \) and \( n = (x+1) \), and \( CCS_0 \) is the "end-cap" CCS of a linear aggregate (\( n = 0 \)). These models were used in Paper I and Paper VI for interpreting the growth of A\( \beta \) oligomers in solution and in micelles.

### 3.2.6 Overlapping oligomeric signals

One practical challenge when interpreting the mass spectrum of an oligomer-forming protein is that signals for oligomeric species easily overlap in the \( m/z \) dimension. This can be understood by realizing that a protein ion with mass to charge ratio \( xm/yz \) will end up in the same position in the mass spectrum as an ion with mass to charge ratio \( 2xm/2yz \). For example: a peak that we annotate as \( n/z = 1/2 \) can consist of dimer (\( z = 4 \)), trimer (\( z = 6 \)), tetramer (\( z = 8 \)) … and so on, components.

Signals with odd \( n \) and/or \( z \) overlap less than signals with even \( n \) and \( z \). These more unique \( n/z \) signals can be used to help annotate MS spectra, as protein species rarely produce electrospray charge state distributions with less than 3 charge states. For example: It is unlikely that an observed \( n/z = 3/5 \) (=6/10) or \( 3/6 \) (=6/12) signal contains a hexamer component if the more unique \( n/z = 6/11 \) and/or \( 6/13 \) signals are not detected (Figure 10A). The hexamer is however likely present if the full distribution, including the odd \( z \)-states, is observed (Figure 10B). The identity of an \( n/z \) signal can also be determined using MS/MS, as was mentioned in Section 3.2.4 (Figure 10C).

The oligomeric state can also sometimes be determined directly from the mass spectrum by inspecting the fine structures of the peaks. The fine structures of MS peaks are due to the incorporation of naturally occurring isotopes, notably \(^{13}\text{C}\) for proteins (1% natural abundance). A distribution of multiple signals that represent proteins with molecular weights that increase in 1 Da increments is hence observed (Figure 10D). The distance between neighboring fine-structure peaks in an \( m/z \) signal is \( \Delta(m/z) \), where \( \Delta m \) in this case is 1. \( \Delta(m/z) \) is consequently the reciprocal charge (1/\( z \)) of the signal. A +2 monomer will for example have a spacing of 0.5 within the fine-structure, while a +4 dimer will have a spacing of 0.25. An overlapping peak with contributions from both monomer and dimer will have two overlapping sets of isotopic distributions (Figure 10D, black). This approach to determine the oligomeric state becomes increasingly difficult at higher charge states as the peak spacing becomes smaller and smaller.
Figure 10: Approaches to resolve overlapping oligomeric states. A. n/z = 3/6 and 3/5 could contain hexameric components, but the lack of odd z-states (n/z = 6/11 and 6/13) makes this unlikely. B. The presence of odd z-states indicate the presence of overlapping n = 3 and n = 6 species. C. The identity of an n/z signal can be determined using MS/MS as the dissociation products in CIA will be a highly charged monomer and a low charged n-1 complex. Note that both the total mass and charge is conserved upon dissociation. D. The fine structure of an MS peak reports on the charge state of the peak. The distance between the isotopic (13C) fine structures is $\Delta(m/z) = 1/z$. The n/z = 1/2 peak for $\text{A}\beta(1-40)$ with n = 1,2,3 components is shown as an example. E. Theory suggests that overlapping (in the m/z dimension) but isotropically growing oligomers should have different drift times in IM. A simulated IM distribution for an n/z = 1/2 peak with n = 1,2,3,4 is shown. F. Experimentally measured n/z = 1/2 peak for $\text{A}\beta(1-40)$.

A third way to deconvolute overlapping signals is to use the IM dimension. The drift time in IM depends on CCS/z (Equations 10,11), meaning that an ion with charge $2z$ will have a smaller drift time compared to an ion with charge $z$, if both ions have the same CCS. An increase in CCS is however expected upon increasing the oligomeric state.

Consider for example an isotropically growing oligomer: n/z = 1/2 has a CCS of $X \text{ Å}^2$ and n/z = 2/4 has a CCS of $2^{2/3} \cdot X \text{ Å}^2$ (isotropic growth, Equation 13). The drift time at n/z = 1/2 will be according to $X/2$, while the drift time at n/z = 2/4 will be according to $(2^{2/3} \cdot X)/4$. It follows from this that the dimer should have a shorter drift time than the monomer (as $2^{2/3}/4 < 2/4$) (Figure 10E), which is also what we observe for $\text{A}\beta$ oligomers in experiments (Figure 10F, Paper I, Paper VI). IM can therefore be used to easily distinguish between overlapping n/z signals.
3.2.7 The effect of the gas phase on protein structure

Transfer of proteins into the gas phase from their native water solvent is a drastic change in environment. This led to early skepticism about native MS as a useful technique for studying native-like protein structures [259]. It has however since been shown using experiments and molecular modeling that overall protein structure can be retained upon ionization and the subsequent gas phase flight through the mass spectrometry [260–262].

The most thermodynamically stable protein structure in vacuo would probably be an inverted structure, as no hydrophobic effect exists without a water solution. This however not a state which is usually observed in experiments, as the number of intramolecular hydrogen bonds increases as the protein surface is no longer able to H-bond with the solvent water. This shift from H-bonding with water to H-bonding on the protein surfaces creates a crosslinking network which locks the protein in its overall solution-state conformation [263]. The strength of all non-covalent interactions also increases as the permittivity of the surrounding medium decreases 40-fold upon transfer to the gas phase (see Chapter 1.3). The activation energy for global unfolding therefore increases, both due to an increase in interaction strength and an increase in the number of interactions, which kinetically traps the proteins in a native-like state over the time frame of a normal MS experiment [263]. This increase in intermolecular interactions can also cause the proteins to collapse slightly [264].

The stability of a native-like gas phase state depends on its electrospray charge and charge-density, as a highly ESI-charged protein is likely to experience destabilizing Coulombic repulsion, which enables gas phase unfolding and subsequent refolding into compact gas phase structures [265, 266]. Low-charged protein ions are therefore often considered to be the most native-like ions. This has for example been shown in an experiment where cytochrome c was stored in an ion trap for up to 30 s. The lower charge states retained their native-like fold over the entire experiment, while higher charge states experienced several structural rearrangement events [267].

The gas phase structure of IDPs is however a special case, as such proteins have many important interactions with the water solvent due to their flexibility. The shift from intermolecular H-bonding in aqueous solution to intramolecular H-bonding in the gas phase is therefore a more drastic change in IDPs. It has for example been shown that the disordered segments of the p53 protein collapse upon transfer to the gas phase [268], and we have shown that Aβ undergoes a transition to a helical structure within the most hydrophobic segments under MS-like conditions [269]. Absolute CCS values for IDPs should therefore be treated with some caution as structural rearrangements can have taken place inside the mass spectrometer [270]. Several studies have however observed that the IDP gas phase structures observed in IM can often serve as informative proxy reporters, which are in reasonable agreement with results from solution state experiments [229].
3.3 COMPUTATIONAL METHODS

Experiments can often be complemented by using computational approaches to build models. Such models can be guided by experimental results but can also help to interpret the experimental data. This is especially helpful when studying species which are difficult to detect experimentally.

A short summary of the computational methods used in this thesis is given here. This is done without too many technical details, as that is beyond the scope of this more experimentally focused thesis.

3.3.1 Structure prediction

Section 1.2 describes how all information about the fold of a protein is encoded within the primary structure. The primary structure should hence be enough in theory to determine the folded structure of a protein. The prediction of protein structures has for a long time however been a challenge [271]. A breakthrough in the prediction of tertiary structure came recently with the development of the machine learning method AlphaFold2 (AF2) by DeepMind, which has enabled structure prediction from the primary structure at near experimental accuracy [272]. This has also enabled structure prediction of essentially the entire human proteome [273]. All these predictions are collected and are available to download from the AlphaFold Protein Database (https://alphafold.ebi.ac.uk). AF2 is also capable of predicting both homo- and heterocomplexes [274], which might enable modeling of interacting protein partners [275].

AF2 was used in Paper I to generate starting structures for molecular dynamics (MD) simulations of peptides where experimental structures are not available. AF2 was also used in Paper III and Paper IV to predict the structure of chaperones and chaperone-client complexes, which are difficult to determine experimentally. AF2 was furthermore used in Paper III to generate structure predictions for some engineered protein constructs. Predicted CD spectra for these AF2 models could be compared to experimental CD spectra to confirm that a properly folded construct was expressed.

Figure 11A shows an example where the structure of the DNAJB6 chaperone has been predicted. The protein is colored according to the predicted local-distance difference test (pLDDT) score generated by AF2. Regions with pLDDT scores >90 (deep blue in Fig. 11) are expected to be modeled with high accuracy, while regions between 70 and 90 (white to blue in Fig. 11) are expected to be modeled well. Predictions with scores between 70 and 50 should be regarded as uncertain. It can be seen that the two folded domains of DNAJB6 are modeled with good pLDDT scores, while the structure of the flexible linker regions have a higher uncertainty. pLDDT scores <50 (deep red in Fig. 11) can in fact be considered as an indication of a disordered segment [273, 276]. AF2 has also been shown to be capable of docking peptides onto folded proteins [277]. This is further explored in Paper IV, and an example is given in Figure 11A where Aβ has been modeled as a 1:1 complex with the C-terminal domain of DNAJB6. The prediction strength is again shown by coloring the structure according to the pLDDT score.
3.3.2 Molecular dynamics simulations

Molecular dynamics (MD) is a method to simulate the movement of molecules over time. This is done by evaluating the forces acting on each atom. Particles accelerate in response to forces according to Newton’s second law of motion (Equation 14, here for the force in the x-dimension, acting on atom i with the mass $m_i$).

$$\frac{F_{xi}}{m_i} = \frac{\delta^2 x}{\delta t^2}$$  \hspace{1cm} (14)

The position ($x$) of atom $i$ is obtained by integration. The force ($F$) acting on each atom is obtained in simulations from a function that describes the potential energy as a function of atom coordinates, the so-called force field. The distribution of positions in space will be according to the respective energies of each position. The goal is to sample enough states to obtain an estimate of the underlying Boltzmann distribution.

A common problem in MD is that all possible states can not be sufficiently sampled in reasonable calculation time. MD simulations of microseconds are today routinely run, which can still be insufficient to capture slow processes such as protein folding (millisecond timescale). This problem is further increased for IDPs, as they display higher flexibility and sample more states compared to folded proteins. Finding a suitable IDP starting structure can also be challenging, as the structure of such proteins are difficult to solve experimentally. MD simulations of $A\beta$ often start from helical structures determined in micelles or non-polar co-solvents [155, 280], in lack of structures determined in aqueous solution. The risk is here that the simulation time is not long enough to completely lose the structural memory of the starting structure. Yet another challenge is that the force fields commonly used in biomolecular MD simulations have been developed for folded proteins, and perform less reliably for IDPs [281].
Different force fields have for example been shown to give rise to different aggregation pathways \[282, 283\]. Capturing the Aβ aggregation process is consequently challenging. Some progress has however been made, for example where 20 full-length Aβ peptides have been allowed to self-interact over a total of 2.5 µs, followed by course-grained transition network analysis to analyze the assembly process for oligomers \[284\].

In Paper I the assembly of Aβ oligomers and their structures were studied by MD. MD was here used as the structure of such species are difficult to determine experimentally. Starting structures of various Aβ segments and variants were here generated using AlphaFold2, and MD simulations of monomers (2 µs), dimers (10 µs), and selected tetramers (10 µs) were run. Dimers were constructed by letting the most populated monomer structures after MD interact. Tetramers were similarly constructed by letting the most populated MD structures of dimers interact. The Charmm36m forcefield was used, which was shown to perform best for modeling aggregation in ref \[282\].

In Paper V the interaction of Aβ monomers with micelles of different surface charges was studied by MD. This was done to try to explain the differences in Aβ structure observed in experiments when micelles of different surface charges were added. Here helical Aβ starting structures were used that were experimentally determined in the micellar environment. The insertion of Aβ in the micelle was beyond the 1 µs simulation time, and several starting structures were instead created where the peptide was placed on the micelle surfaces, partially inserted into the micelles, or fully inserted into the micelle, to study which direction the peptide transitioned towards.

### 3.3.3 Collision cross section calculations

The CCS from experimental ion mobility measurements provides low-resolution structural information and must often be compared to the CCS calculated from structure models. Various methods to make such calculations exist, which differ in their accuracy and computational cost. The trajectory method is an accurate but computationally costly method that takes long-range interactions between ion and buffer gas molecules into account. Long-range interactions are especially pronounced for small molecules, but can often be disregarded for larger molecules such as proteins \[254\]. Methods that disregard long-range interactions include the elastic hard sphere scattering (EHSS) method and the projection approximation (PA) method, which are more commonly used to calculate the CCS of protein models. The PA method also completely omits gas scattering from the calculations and assumes that the CCS of the protein is simply the rotationally averaged projected area of the protein. This simplification leads to a systematic underestimation of the CCS. The PA method can however yield good results when used for proteins, with sometimes just a few percent difference from values generated by the more advanced methods \[258\]. The use of the PA method is therefore often justified, due to the much lower computational cost.

In this thesis the Ion Mobility Projection Approximation Calculation (IMPACT) algorithm was used. IMPACT calculates the CCS of protein structure models according to the PA method and then uses an empirically determined power law \((0.843\Omega^{1.051})\) to adjust the PA CCS to approximately what would be determined by the trajectory method \[285\].
Chapter 4

AMYLOID-β OLIGOMERIZATION

Aβ aggregation starts at an unstructured monomer and populates metastable oligomers, before the formation of an amyloid nucleus. The low concentration of oligomers makes them difficult to study using many biophysical techniques. Native mass spectrometry is here used to directly detect and study the Aβ oligomers. This Chapter will summarize Paper I and discuss the result in the context of other studies on the topic.

4.1 SEQUENCE PROPERTIES

Aβ(1-40) contains two hydrophobic segments, (Aβ(16-22) and Aβ(30-40)), separated by a hydrophilic "hinge region" (Figure 12A). MD simulations in Paper I show that the peptide has the propensity to fold into a hairpin structure where these two hydrophobic segments interact (Figure 12A). Interactions within the hinge, including the D23-K28 salt bridge, are believed to be important for the formation of this structure [286]. We find that truncation of the peptide sequence after residue K28, which removes the C-terminal hydrophobic segment greatly reduces the propensity to self-interact and leads to a more disordered peptide. Earlier studies have also demonstrated how formation of an intramolecular disulfide bridge in the hinge region of the A21C A30C double mutant variant (Aβ(1-40)CC) inhibits the formation of amyloid fibrils [106]. We demonstrate in Paper I that the Aβ(1-40)CC monomer remains as a random coil in solution, meaning that the hairpin state is very unstable in the monomeric state.

Figure 12: A. Sequence hydrophobicity of the Aβ(1-40) peptide calculated according to Eisenberg scale [287]. The two hydrophobic segments are underlined in the sequence. The position of the A21C and A30C substitutions are marked with circles. B MD generated structure of Aβ(1-40) (from Paper I) colored according to hydrophobicity. The N-terminus is marked by a blue circle and the C-terminus is marked by a red circle. the A21, K28, and 30 are pointed out in the structure.
4.2 THE OLIGOMER DISTRIBUTION

We detect a wide distribution of Aβ(1-40) oligomers in Paper I, where oligomer species ranging from dimers to octamers could be confidently annotated. These oligomers are specific for Aβ(1-40), as a scrambled variant of Aβ(1-40)Scr (same amino acid sequence in random order) does not form such oligomers at the same sample and instrument conditions. The highest oligomeric state detected for Aβ(1-40)Scr was instead a dimer.

There is no observed specificity in the oligomer distribution for Aβ(1-40) as no oligomer state seems to be preferred. This is in contrast to for example insulin, a peptide of similar size as Aβ, which can be detected in native MS as a unique hexamer [288, 289]. An earlier IM-MS study suggested that certain specific states were populated, namely n = 2, 4, 6, 12 for Aβ(1-42) and n = 2, 4 for Aβ(1-40) [128], which is in contrast to our data. Another paper has recently reviewed these older results and found that the data had probably been misinterpreted [290]. The wide oligomeric distribution seen here underlines that Aβ(1-40) oligomers in solution exist on a frustrated energy landscape, and that no single state is significantly more energetically favorable than the others.

It is worth noting that the detected oligomers are small assemblies for native MS detection, and it is not entirely clear why no larger species are detected. One explanation could be that such aggregates become increasingly hydrophobic and therefore less water-soluble and/or ionizes less efficiently in ESI. Another possible explanation could be that larger oligomers are held together primarily by hydrophobic interactions which are weak in the gas phase. A third possibility could be that there simply are no larger oligomers to be detected, and that this detected pool of frustrated oligomers convert to more ordered fibrillar aggregates during a nucleation step.

The oligomer distribution could be modulated by modifying the peptide sequence as described in Section 4.1. The Aβ(1-40)CC variant displayed the same oligomeric states, with the same ion mobility conformations, as wild type Aβ(1-40). The CC variant did however display increased oligomer signal intensity, especially for larger (n>4) oligomers. C-terminal truncation was on the other hand shown to reduce the population of larger oligomers, with Aβ(1-28) populating mostly dimeric and trimeric states, and no states higher than the hexamer.

4.3 OLIGOMER SHAPES

The observed ESI charge state for all Aβ oligomers are according to what is expected for folded proteins (Equation 8). The CCS values for oligomeric Aβ(1-40) species were in Paper I shown to increase isotropically ($\Omega \propto n^{2/3}$) between n = 1 and n = 4. The oligomers then continue to grow predominantly according to a linear trend above n = 4 (Figure 13A). Isotropic oligomers were however present up until n = 6, at lower relative intensity compared to the linear conformations. The shift from isotropic to linear growth for larger oligomers indicates that the oligomers start to grow faster in one direction, which could be interpreted as a formation of extended sheets. It is further-
more interesting that it is the larger and linearly growing oligomers that are increased the most in the \( A\beta(1-40)_{\text{CC}} \) variant, as it would indicate that the disulfide bond in the hinge region specifically arranges the peptide into this structure in oligomers, without affecting the structure of the monomeric peptide much. \( A\beta(1-28) \) did on the other hand only populate the isotropically growing oligomers (Figure 13B), further indicating that folding into the hairpin motif is related to aggregation into the extended states.

A recent study fitted the ion mobility data of \( A\beta \) oligomers to growth according to two fibrillar structure models [290]. It should be pointed out that we do not think the extended states represent those types of aggregates for two reasons:

1. It has been found that the growth rate of oligomers is not in agreement with such a model. Elongation is a very energetically favorable process and direct formation of fibrils from elongation of oligomers would result in much lower concentrations of oligomers being detected [129].

2. The \( A\beta_{\text{CC}} \) structure is not compatible with the amyloid fibril structure (Figure 3). It would therefore be expected that the extended IM structures would be absent in \( A\beta_{\text{CC}} \) if they represented fibrillar structures. We instead observe an increase of these extended states in \( A\beta_{\text{CC}} \).

The observed oligomers instead likely represent distinct oligomer structures, which at some point rearrange to the fibrillar fold upon reaching the critical nucleus size.

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**Figure 13:**

**A.** Measured CCS for the detected n/z signals in \( A\beta(1-40)_{\text{CC}} \). Theoretical relationships for isotropic growth starting from the n/z = 1/2 and 1/3 signals are shown as green lines, while the isotropic growth from n/z = 1/4 and 1/5 are shown as gray lines. The linearly growing oligomers are highlighted by the orange field. A top cluster structure for an \( A\beta(1-40)_{\text{CC}} \) oligomer, colored according to hydrophobicity, is shown as an insert.

**B.** Measured CCSs for the detected n/z signals in \( A\beta(1-28) \). Theoretical relationships for isotropic growth starting from the n/z = 1/2, 1/3, and 1/4 signals are shown as purple lines.

**C.** Interpeptide contacts between residues for \( A\beta(1-40)_{\text{CC}} \) dimer (top) and \( A\beta(1-28) \) dimer (bottom).
It is currently not known from experiments what the critical nucleus size for Aβ is, but some simulations have predicted it to be around \( n = 6-14 \) \([48, 291, 292]\), a number which is highly dependent on peptide concentration and other properties \([129, 293]\). The elongated oligomers that we observe could therefore represent species which convert into the fibrillar state. It has on the other hand been shown that Aβ starts to bind the amyloid-specific dye ThT at \( \sim n = 60 \) \([112]\), which would suggest that this is the point at which amyloid starts to form. This could however be explained by quick growth after forming a nucleus of high free energy. Another recent study has shown that aggregates with \( n < 9 \) bind to the Brichos chaperone domain, which inhibits secondary nucleation \([294]\). This could indicate that these small aggregates have already converted into a fibrillar state which is secondary nucleation competent, and that the chaperone is able to capture these before they grow any further.

Nucleation from the elongated oligomeric states provides an explanation for why we do not observe any significantly larger oligomers in the mass spectrum. The inability of AβCC to convert into a fibrillar fold and nucleate into amyloid would also explain why the populations of these states are increased in this peptide variant.

**4.4 SECONDARY STRUCTURE**

MD simulations were in Paper I used to monitor how the structure-content of the different Aβ variants evolved upon increasing the oligomeric state, something which is very difficult to study experimentally.

The Aβ(1-40)Scr variant, which was experimentally found to only form dimers, was in MD shown to fold into an amphipathic helix. This structure seemed to be able to form a somewhat stable dimer. This is in contrast to the β-hairpin motif in Aβ(1-40) oligomers, which provides an obvious mechanism for how the growth of aggregates takes place. The observed β-hairpin motif in Aβ(1-40) (Figure 12B) is weak in the monomeric state but enables the addition of multiple monomers, which creates a larger antiparallel β-sheet. Increasing the oligomeric state led to an increase in β-sheet content in the MD simulations, as the hairpin motif in Aβ became increasingly stabilized. The disulfide bond in AβCC is also compatible with such an antiparallel structure (Figure 13C, top), which is in agreement with the increase of AβCC oligomers in the mass spectrum.

Aβ(1-28) which do not have hairpin propensity within the monomer did not assemble into β-sheet oligomers in MD simulations, and did instead remain unstructured (Figure 13C, bottom). This is in agreement with low aggregation propensity as seen by CD spectroscopy, as well as the lack of large \( n>6 \) and extended oligomers in IM-MS. This would indicate that the isotropic conformation family might be related to more unstructured aggregates which do not nucleate into amyloid as effectively.

It should be noted that IM-MS does not report on secondary structure. An earlier study using gas phase infrared spectroscopy coupled to IM-MS did however show that a deviation towards CCSs larger than those expected for isotropic growth was correlated with an increase in β-sheet content for some other amyloidogenic peptides \([295]\). The results of Paper I indicate that this correlation could hold true also in the Aβ system.
Chapter 5

ANTI-AMYLOID CHAPERONES

It was in Chapter 1 suggested that the native state of a protein is not always the global free energy minimum, and that the amyloid state might be a thermodynamically more stable state. Organisms have therefore needed to evolve molecular systems that control amyloid formation and its related toxic mechanisms in vivo. This is in part done by molecular chaperone proteins that are able to recognize and bind amyloidogenic species and prevent their aggregation.

This chapter will discuss the results of Papers II-IV where the focus is a human chaperone, the DnaJ homolog subfamily B member 6 isoform b (DNAJB6). The results on DNAJB6 will also be discussed in the context of other chaperones and non-chaperone proteins which delay amyloid formation.

5.1 DNAJB6

DNAJB6 is a DnaJ-type (Hsp40) chaperone that displays a very high anti-amyloid activity both in vivo and in vitro. The anti-amyloid activity of DNAJB6 was first discovered when searching for natural suppressors of polyglutamine (polyQ) aggregation related to Huntington’s disease [296]. Anti-amyloid activity towards both αSyn and Aβ aggregation has since been observed [297–299]. Analysis of the aggregation kinetics (Section 3.1.3) has shown that DNAJB6 delays amyloid formation by strongly suppressing the primary nucleation rate of amyloid formation, as seen by an increase in the lag time (Figure 14A, Figure 7B, left). This is done at remarkably low chaperone:client ratios. The chaperone to a lesser extent also suppresses secondary nucleation in addition to the strong effect on primary nucleation [297].

The most simple explanation for the inhibition of primary nucleation is binding to monomeric Aβ species, which makes these monomers unavailable for aggregation. The very low binding stoichiometry does however suggest that DNAJB6 rather binds to low abundant pre-nucleation oligomers and hinders their further aggregation. Consider for example the concentrations of DNAJB6 in Figure 14A. These represent only 0.08 % - 2.5% of m(0) (3 µM), which is on the same order of magnitude as the concentration of Aβ oligomers formed during an in vitro aggregation experiment [129]. Our ThT aggregation results in Paper II also indicate that the chaperone is able to suppress Aβ(1-40) even more efficiently (at lower chaperone:client ratios) than what has been previously shown for Aβ(1-42). This is probably due to the lower primary nucleation rate of Aβ(1-40) and its lower concentration of oligomers [300].
The binding of DNAJB6 to Aβ oligomers was examined in Paper II, where native MS was used to directly detect the oligomers and the effect of pre-incubation with DNAJB6 on their signal intensity. Such pre-incubation did indeed lead to a decrease in Aβ oligomers relative to monomers (Figure 14B). This could either mean that DNAJB6 binds the oligomers with high affinity, effectively removing them from the pool of Aβ species, or that DNAJB6 disaggregates the oligomers. Earlier results have shown that DNAJB6 can be found in the Aβ fibrils at the end of in vitro aggregation assays [297], pointing towards high-affinity binding between Aβ and chaperone, in a so-called "holdase" mechanism. The observed anti-oligomer effect was also larger for higher oligomer states (Figure 14C).

DNAJB6 has two important structural features that seem to be important for its anti-amyloid activity:

1. It has a functionally important disordered region rich in serine (S) and threonine (T)-residues. This is a unique structural motif that DNAJB6 shares with its closest homologs but which is absent in the canonical DNAJB1 chaperone. Substituting these residues with alanines (A) in DNAJB6 results in a loss of anti-amyloid activity [297].

2. It forms large polydisperse megadalton aggregates. This is also in contrast to DNAJB1 which functions as a dimer [301]. The S/T-residues seem to play a role in the formation of the chaperone aggregates, as deletion of the S/T-rich region produces a monomeric DNAJB6 variant (DNAJB6 ΔS/T) [278, 297].

We also find in Paper II that an S/T-A substituted variant of DNAJB6 does not bind Aβ(1-40) oligomers, and that crosslinked wildtype DNAJB6 loses its anti-amyloid activity, indicating that an exchange between the large aggregate and smaller chaperone species seems to be of importance. Both 1. and 2. above are therefore clearly important for the function of the chaperone.

5.1.1 What is the client binding site in DNAJB6?

DNAJB6 consists of two folded domains (Figure 14D): The J-domain (JD) is a highly conserved domain found in all DnaJ-proteins which facilitates binding to and transfer of the bound client to DnaK/Hsp70 proteins for ATP-dependent refolding. The other domain, the C-terminal domain (CTD) is specific for DNAJB6 and consists of a single β-sheet. The two domains are linked by the disordered and flexible linker which contains the S/T-rich segment, close to and in the first β-strand of CTD. The CTD is critical for activity in vivo [296], and has been considered the putative client-binding domain, due to its proximity to the functionally important S/T-residues. Crosslinking mass spectrometry also finds crosslinks between Aβ and lysine residues in the CTD, but no crosslinks between Aβ and the JD [302].

The CTD was hence expressed and studied in isolation in Paper III to determine if it could mimic the anti-amyloid activity of the full-length chaperone. The CTD formed a well-folded protein with significantly less polydispersity compared to the full-length protein, with monomeric, dimeric, and tetrameric states populated but no higher molecular weight aggregates. S/T-A substitutions in β-strand 1 (β1), destabi-
lized the structure of the construct somewhat, and shifted the protein ensemble towards the monomeric state. The CTD did interestingly not inhibit primary nucleation of Aβ as the full-length chaperone, but instead only inhibited secondary nucleation (Figure 14E). Modulation of secondary nucleation is most easily understood as an interaction with the fibril surface, which was confirmed by surface plasmon resonance with immobilized fibrils. No binding to free Aβ oligomers could be detected.

The CTD suppressed secondary nucleation approximately to the same extent as what has earlier been observed for full-length DNAJB6 [297]. This indicates that the ability to suppress secondary nucleation is retained by the isolated CTD, while the strong suppression of primary nucleation seems to require also additional parts of the chaperone in addition to CTD. ST-A substitutions in CTD did not affect the anti-amyloid activity of the construct much, in contrast to the large effect of such substitution on the activity of full-length DNAJB6 [303]. The effect of the CTD on Aβ aggregation is also qualitatively similar to the monomeric DNAJB6 ΔS/T variant, which also causes a decrease in Aβ growth rate without any change in lag time [297]. This would suggest that it might be the S/T residues in the disordered linker which are important for binding of smaller aggregates and inhibition of primary nucleation. Inhibition of secondary nucleation does on the other hand not seem to be dependent on this structural motif.

5.2 COMPARISON WITH NON-SPECIFIC PROTEIN INTERACTIONS

The altered interaction mode of the CTD raises the question if its interaction with Aβ is specific or non-specific. The interaction with the full-length DNAJB6 is clearly specific as very low concentrations (nM, 0.0003:1 client:chaperone ratio) are needed to delay aggregation for tens of hours in vitro (Paper II and [304]). The CTD however requires much higher concentrations (µM, 1:1 ratio) for inhibition (Paper III).

Interactions between specific client:chaperone pairs and non-specific peptide-protein pairs were compared in Paper IV. A generally observed feature in non-specific binding is complementary non-covalent interactions, such as binding of hydrophobic segments of Aβ to hydrophobic protein patches, or binding of negative residues to positive protein patches. We show in Paper IV that Aβ often binds to subunit interfaces of proteins, as these are easily accessible hydrophobic surfaces. We have also shown elsewhere that denatured proteins suppress Aβ aggregation more efficiently than native proteins, probably due to a higher proportion of exposed hydrophobic residues [305]. Earlier studies have also shown that positive net charge generally decreases Aβ aggregation [306]. The CTD does indeed carry a positive net charge (+3 at pH 8), which could mean that the observed effect might be non-specific.

In contrast, the binding of a client peptide to the anti-amyloid chaperone domain Brichos involves specific recognition of sequences with β-strand propensity (Paper IV). This leads to the formation of a complex via complementary strand-strand interactions that create a larger β-sheet. We notice that this is also very similar to how AF2 suggests that Aβ interacts with the CTD of DNAJB6 (Figure 11) via folding of Aβ into a
hairpin structure which interacts with the first $\beta$-strand ($\beta_1$) of the CTD. Such an interaction would instead suggest a more specific interaction mode. This mechanism for specific inhibition of amyloid nucleation is interesting considering the discussions in Chapter 4 where the oligomerization propensity of A$\beta$ was linked to the formation of $\beta$-sheets that can easily grow by addition of new monomeric units.

The possible role of $\beta_1$ in binding A$\beta$ was tested in Paper III by producing a protein construct containing only the two first $\beta$-strand strands ($\beta_1 + \beta_2$) in CTD. This CTD-derived hairpin was grafted onto the very stable S100G protein to facilitate proper folding. A similar anti-amyloid activity was observed for this S100G-CTD$\beta_1-2$ construct as was observed for the CTD (Figure 14F). A control protein where the S100G-grafted sequence for $\beta_1 + \beta_2$ was scrambled was also produced (S100G-CTD$\beta_1-2$Scr). This negative control protein displayed very little anti-amyloid activity (Figure 14G), despite having the same charge as S100G-CTD$\beta_1-2$. This indicates that the effect of CTD is likely due to more specific interactions instead of general and non-specific charge-interactions. These interactions could in DNAJB6 be via a combination of strand-strand interactions and interactions with the unstructured S/T-rich segment (Figure 14H), similar to what was shown for Brichos in Paper IV.

### 5.3 THE "UNHAPPY" CHAPERONE

If the CTD contains the client-binding site, why does the CTD not fully replicate the anti-amyloid activity of the full-length chaperone? One thermodynamic explanation concerns the high polydispersity of DNAJB6, which is linked to a high chemical potential. This high chaperone potential could drive the formation of co-complexes with A$\beta$, if the co-complex decreases the energy of the chaperone. This can perhaps even be seen in Paper II where DNAJB6 monomers, dimers, and trimers suddenly become detectable in MS upon adding A$\beta$ to the sample.

We have already concluded earlier in the thesis that the amyloid state is energetically very favorable, and it might be difficult for an A$\beta$-chaperone co-complex to reach such low energies. Creation of the co-complex could however still be energetically favorable if the drop in energy for the chaperone when creating the co-complex compensates for the increase in energy between the amyloid state and the co-complex state. This means that chaperones and other molecules that form favorable co-complexes with A$\beta$ should preferably be aggregation-prone themselves [307].

The isolated CTD and the S100G-CTD$\beta_1-2$ construct are both happily folded proteins without aggregation propensity and are considerably less prone to co-aggregate with A$\beta$, as they have no drive to decrease their free energy. This could possibly also explains why the S/T residues are functionally important, as they seem to be important for formation of the polydisperse DNAJB6 aggregates [278, 303]. NMR studies of the CTD have shown that the S/T-A substitutions lead to a change in backbone motion, which affects the monomer-oligomer equilibrium [279, 308]. This change of edge-strand behavior in the CTD could be imagined to be able to both affect the client binding directly, or indirectly via a change of chaperone assembly state and exchange dynamics.
Figure 14: A. ThT kinetics for 3 µM Aβ(1-42) alone (black) and upon addition of increasing concentrations of DNAJB6 (4.7 nM to 75 nM).

B. Aβ(1-40) tetramer intensities in native MS after pre-incubation with (light blue) and without (dark blue) DNAJB6 (Data from Paper II).

C. The decrease in oligomer intensity after pre-incubation for each oligomeric state (Paper II).

D. AF2 model of full-length DNAJB6 colored from N-terminal (blue) to C-terminal (red).

E. ThT kinetics for 3 µM Aβ(1-42) alone (black) and upon addition of increasing concentrations of CTD (0.38 µM to 3 µM) (Data from Paper III).

F-G. ThT kinetics for 3 µM Aβ(1-42) alone (black) and upon addition of increasing concentrations of β1 + β2 from the CTD grafted to S100G (S100G-CTDβ1-2) (F) or a scrambled version of β1 + β2 grafted to S100G (S100G-CTDβ1-2Scr) (G) (0.38 µM to 3 µM) (Data from Paper III). The AF2 models of the engineered S100G-CTDβ1-2 and S100G-CTDβ1-2Scr constructs are also shown colored according to their pLDDT score (blue to red).

H. AF2 model of Aβ(1-42) (gray) docked to CTD. Functionally important and conserved S/T residues in DNAJB6 are shown as pink spheres, while the crosslinked lysines (K16 in Aβ and K196 in DNAJB6) are shown as purple spheres (Paper III).
Chapter 6
EFFECTS OF MEMBRANE ENVIRONMENTS

The energetically frustrated oligomer population described in Chapter 4 probably indicates a high propensity to co-aggregate with other amphiphilic molecules to form more stable structures. Such interactions were also shown for non-specific proteins in Paper IV. Aβ is known to also associate with biological membranes, which could be linked to neurotoxic mechanisms (Section 2.2.1). Papers V-VII in this thesis aimed to study such interactions, using biophysical studies in membrane-mimicking systems. Detergent molecules, which form micelles above their CMCs (Section 1.3.4), were in all these studies used as simplified model membranes.

Many different types of interactions within a membrane environment are possible. Paper V focuses on interactions between detergent molecules and Aβ, Paper VI examines Aβ-Aβ interactions within detergent micelles, and Paper VII finally illustrates how Aβ can interact with other biomolecules within detergent micelles.

6.1 THE ROLE OF ELECTROSTATICS AND HYDROPHOBICITY

The most commonly used detergent for biophysical studies of Aβ is the anionic detergent sodium dodecyl sulfate (SDS), which is known to solubilize and stabilize Aβ in a monomeric and α-helical state (Figure 6, yellow). This has for example enabled the determination of structure models of Aβ monomers using solution-state NMR [207, 309, 310]. Lower concentrations of SDS have however also been shown to cause co-aggregation with Aβ, where the peptide folds into a β-sheet-rich state [311, 312].

Such studies were in Paper V extended to also include detergents with other head group charges (cationic, zwitterionic, and non-ionic). The studied cationic detergent was cetyltrimethylammonium bromide (CTAB) which contains a positively charged tertiary amine group. The zwitterionic detergent was myristyl sulfobetaine (SB3-14), which has both a sulfonate group (like in SDS) and a tertiary amine group (like in CTAB). The non-ionic detergent employed in the study was Dodecyl β-D-maltoside (DDM), which has a non-charged but highly polar maltoside sugar headgroup.

CD spectroscopy showed that no tested concentration (0.1-20X CMC) of non-ionic DDM detergent was capable of inducing structure in Aβ(1-40), in contrast to SDS, CTAB and SB3-14. The addition of DDM was however shown to decrease the aggregation propensity of Aβ, which could be attributed to interactions between DDM molecules and the two hydrophobic segments within the peptide sequence. Such interactions compete with the formation of hydrophobic peptide-peptide interactions and thus provide the peptide with an alternative favorable interaction to aggregation.
This difference in behavior was furthermore seen in MD simulations, where an Aβ(1-40) monomer had a higher propensity to insert as an α-helix in an anionic SDS micelle, while the peptide interacted mostly on the surface of the uncharged DDM micelle, in a more unstructured conformation.

CTAB and SB3-14 both induced a transition of Aβ from its IDP state into structured states when titrated to the peptide solution. The effect was largest for CTAB which induced the formation of β-sheets and co-aggregates already at 0.1X CMC. The peptide then rapidly transitioned into an α-helical state upon further increasing the detergent concentration. The zwitterionic detergent SB3-14 induces structure formation at slightly higher concentrations, with a concentration close to 1X CMC needed for formation of the β-sheet state. The β-sheets eventually converted to an α-helical structure when the micelle:peptide ratio was increased above 1.

These results indicate that the detergent head group charge is related to the ability of detergents to induce structure in Aβ. The net positively charged CTAB readily interacts with the net negatively charged Aβ peptide, while the net neutral SB3-14 required micelle formation to induce β-sheet structure in the peptide. The β-sheet structured state formed at intermediate detergent concentration is an aggregation-prone stage for SDS, CTAB as well as SB3-14, indicating that this is a state where the detergent has facilitated formation of a co-complex of multiple Aβ peptides that is capable of forming even larger aggregates. Dilution of the peptide in a larger pool of SB3-14 micelles however shifts the structure from this aggregated β-sheet to an α-helical state which is associated with the Aβ monomer. This behavior, where Aβ is in a random coil state at c < CMC; a β-sheet state at c > CMC and micelle:peptide ratio < 1; and a α-helical state at c > CMC and micelle:peptide ratio > 1, was confirmed using a shorter detergent (SB3-12) which has a ten times higher CMC compared to SB3-14.

The here observed stabilization of Aβ due to hydrophobic interaction with detergent tails (as in DDM, and SB3-14 below the CMC) competes with the interactions between charged groups in the detergent head group and Aβ which are capable of inducing structure in Aβ (as in CTAB, and SB3-14 above the CMC). Interactions with cellular membranes will probably also be able to act both stabilizing and destabilizing, depending on the composition of the membrane, and other conditions.

6.2 PORE FORMATION

The β-sheet structure observed in the presence of zwitterionic micelles, at low micelle:peptide ratios, is especially interesting. This state likely represents an aggregated state where multiple peptides might co-exist in a single micelle. ThT aggregation assays also show that this state is the most aggregation-prone state formed upon titration with SB3-14. The aggregation propensity is however still lower than that observed for Aβ(1-40) without detergent (Paper V). This could indicate that β-sheet structured intermediate aggregates are somewhat stabilized in this environment. This can for example be imagined to be due to a stabilization of hydrophobic surfaces in Aβ by the detergent hydrocarbon tails, and/or an increased interaction strength for
electrostatic interactions, such as the D23-K28 salt bridge, in the low permittivity environment inside the micelle (Section 1.3). The β-sheet state was therefore further examined to learn more about these membrane-induced intermediate Aβ species.

A previous study has assayed multiple detergent conditions and shown that efficient stabilization of intermediate β-sheet aggregates could be achieved by mixing Aβ(1-42) with the zwitterionic detergent dodecylphosphorylcholine (DPC) at a 0.5:1 micelle:peptide ratio [313], which agrees with our findings in Paper V. This so-called β-barrel pore-forming oligomer (βPFO) state, was also found to induce leakage over model membranes [313]. We therefore in Paper VI used the conditions described in ref [313] to study if we could detect the formation of pore-like oligomers using native IM-MS. This was done by co-incubating Aβ(1-42) with DPC at 37°C for 24 h at pH 9, followed by isolation of the βPFO fraction by SEC. SEC also served to desalt the samples and buffer exchange into a solution of MS-friendly AmAc salt and DDM detergent.

Native IM-MS analysis of intact micelle-peptide co-clusters was performed and CIA was applied to remove the detergent molecules (Paper VI). This revealed that Aβ(1-42) formed a distribution of oligomers between n = 2 and n = 6 in this micellar environment, with much higher intensities than what is normally observed for Aβ oligomers without micelles. The charge states for the monomers and oligomers were lower than the corresponding charge states in samples without micelles, which could indicate that parts of the peptides are inserted in the micelle, which would decrease their SASA. The final ESI-charge of micelle-embedded proteins can, however, also depend on charge-equilibration between protein and detergent during CIA [257]. This effect should in our case be minor, as DDM is not a charge-reducing detergent [257, 314].

Fractionation of the sample using a 30 kDa molecular-weight cut-off filter supported the theory that the oligomers detected in native MS are in fact part of bigger co-complexes in solution, as the oligomers (which all have molecular weight <30 kDa) could only be detected in the filter retentate (Mw > 30 kDa) and not in the filtrate (Mw < 30 kDa) (Paper VI). SEC also indicated that the Aβ(1-42) samples incubated in micelles formed some sort of intermediate aggregate, and CD spectroscopy revealed that the micelle-induced state was β-sheet structured (Paper VI).

IM measurements of the micelle-induced oligomers revealed isotropic growth for all detected oligomers (between n = 1 and n = 6). The linear conformation family observed above n = 4 in the absence of micelles (Paper I) was however not present. This indicates that the micelle is able to stabilize the oligomer in such a way that the growth of the elongated oligomeric states is not favorable. This agrees with the increase in the oligomer population in micelles, assuming that the elongated oligomers are on-pathway for amyloid formation as was discussed in Chapter 4.

The shorter, less hydrophobic, and less toxic Aβ(1-40) variant formed mainly dimers and trimers in micelles, and a scrambled variant of Aβ(1-42) did not form any oligomers at all under the same conditions. Structure modeling showed that at least a tetramer would be needed to form some sort of pore-like complex which could perhaps facilitate leakage over the membrane. This difference between the peptide variants is interesting as it provides a possible link between oligomerization propensity in the membrane environment and intrinsic peptide toxicity.
6.3 MODULATION OF OLIGOMERIZATION IN THE MEMBRANE

Detection of changes in the oligomeric distribution is interesting if the smaller oligomeric states can be thought of as less toxic than the larger states. This has support in literature where for example oligomers stabilized by intermolecular crosslinks show an increase in toxicity with oligomeric state [107]. One extrinsic factor which could modify the distribution of oligomers in a membrane is the lipid composition of the membrane. Studies of membrane proteins have for example shown that different specific lipids can stabilize specific oligomeric states [315]. Specific lipid binding has also been observed for Aβ, for example to anionic lipids, cholesterol, and gangliosides [316–319]

In Paper VI we show as a proof-of-principle that the micelle-stabilized oligomers could be detected also after titrating zwitterionic phosphocholine lipids to the samples. Specific lipid binding was in fact observed to oligomers after detergent-removal in CIA, indicating some degree of specific binding between the oligomers and the lipids. The oligomer distribution was also shifted towards larger oligomers upon increasing the lipid concentration. This could either be due to a lipid-induced stabilization of oligomeric species in solution, or by an increase in the (kinetic) gas phase stability of peptide-micelle co-complexes when adding lipids to the micelle. Future studies should clarify the role of different classes of lipids in stabilizing membrane-embedded Aβ oligomers.

Aβ(1-42) was in Paper VII incubated in micelles together with another peptide with known anti-amyloid properties. This peptide is a chimeric construct consisting of a N-terminal signal peptide sequence from a membrane protein (the neural cell adhesion molecule 1, NCAM1) and a C-terminal cationic hexapeptide derived from the mouse PrP protein (KKRPKP, mPrP(23-28)). The resulting NCAM1(1-19)-mPrP(23-28) construct (NCAM-PrP) has been shown to decrease both prion propagation and amyloid toxicity in cell culture [320–322]. The exact mechanism of action is not known, but the NCAM-PrP peptide has been found to enter cells, to co-localize with Aβ intracellularly, and to clear Aβ from the cell to the extracellular space [321]. We hypothesize that the membrane-environment might be a relevant environment for interaction as both peptides are membrane-active.

Native IM-MS of the co-incubated Aβ(1-42)/NCAM-PrP/micelle mixture revealed that the NCAM-PrP formed multiple heterooligomers with Aβ. Formation of these heterooligomers also shifted the entire oligomer distribution towards lower oligomeric states, which may be less toxic. Co-incubation of the two peptides without micelles however lead to the rapid formation of large co-aggregates, possibly due to the strong electrostatic attraction of the negatively charged Aβ peptide and the positively charged NCAM-PrP peptide. Co-incubation of a scrambled Aβ(1-42) peptide with NCAM-PrP and micelles did not lead to formation of any heterooligomers, likely due to the fact that the scrambled Aβ(1-42) peptide does not oligomers in micelles (Paper VI).
Chapter 7

CONCLUDING REMARKS AND OUTLOOK

What have we been able to detect?

Aβ oligomers between 2 and 8 peptides can be detected with certainty for the wild-type Aβ(1-40) peptide using native MS. This is extended to n = 12 for the Aβ(1-40)CC peptide. One feature of these detected oligomers is that no single oligomeric state seems to be significantly favorable in solution. This is however slightly altered in detergent micelles where more preferred oligomeric states can be observed. The fact that Aβ does not populate preferred oligomeric states in solution could also be the reason why oligomers are enriched in micelles, as this environment provides a way to reach more energetically favorable states by hydrophobic peptide-detergent interactions.

The absence of the linearly growing oligomers in the presence of micelles (Paper VI and Paper VII) indicates that a membrane environment may be capable of stabilizing disease-related peptides species, with inhibition of fibril formation as a result. This is supported by the fact that the zwitterionic micelles delay amyloid aggregation at the employed detergent concentrations (Paper V), and by the observation that the less aggregation-prone Aβ(1-28) peptides do not form the elongated ion mobility conformations (Paper I). It is therefore reasonable to suspect that the elongated oligomers between n = 4 and 12 are on-pathway species that can convert into fibrilar species.

These structures do however require more studies in general, for example using gas phase spectroscopy which provides experimental information about the secondary structure of the oligomers. Studies of other amyloidogenic peptides have for example been able to link the formation of β-sheet-rich extended oligomers to the ability to form fibrilar amyloid structures on the mesoscale [323]. The βPFO oligomers formed in micelles are however also β-sheet structured in solution, but do only form isotropic structures as seen by IM. It is therefore possible that isotropically growing oligomers can be both unstructured (as suspected for Aβ(1-28)) and structured (as suspected for the βPFO state).

We have finally also shown that important interactions between Aβ and other biomolecules can be studied using native IM-MS both in solution (Papers II-IV) and in micelles (Paper VII). The studies in Paper I and Paper IV also highlight how modeling and native IM-MS can be used in combination to study transient Aβ interactions. Mass spectrometry does for example provide excellent detection of the entire peptide ensemble, which is currently difficult to capture using MD. MD does on the other hand provide high-resolution information about the structure and dynamics of oligomers, which is not easily obtained by experiments. The use of AF2 furthermore provides structure models, at considerably lower computational cost compared to MD, which can be experimentally validated using IM-MS (Paper IV).
How are these results relevant in biology?

It is not known how these detected in vitro oligomers are related to oligomers observed in vivo. Aβ peptide concentrations in vivo are approximately 3 orders of magnitude lower than what has been used in these studies, and the peptides exist in an environment where aggregation is highly regulated. The very efficient binding of Aβ by the DnaJ B6 chaperone illustrates how the cell makes sure that energetically frustrated peptide oligomers are not allowed to react freely with other cellular components. These toxic oligomers are therefore probably not a problem under normal cellular conditions, the control systems could, however, lose some of their capacity with age, leading to a gradual buildup of Aβ oligomers. Such a slow buildup is in agreement with the development of sAD at higher age.

It is interesting to note on a physiochemical level that the oligomers can be concentrated, rather than dispersed in a hydrophobic membrane-like environment. The membrane environment can therefore perhaps serve as a site where the local concentration of Aβ could be increased in vivo. It is worth pointing out that we detect micelle-bound oligomers reaching up to n = 6 in Paper VI, up to n = 4 in Paper VII, while other studies have found oligomers specifically at n = 8 [118], n = 4 [324] and at n = 7 [159]. The micellar environment thus seems to narrow the oligomer distribution considerably, but the exact aggregation number that is stabilized depends strongly on the sample conditions. It is likely that membrane properties such as lipid composition, bilayer thickness, and fluidity, and protein content are important determinants for Aβ co-aggregation also in vivo. Further studies should examine the effect of specific lipid environments on the stabilization of Aβ oligomers. Aβ is for example known to localize to the inner mitochondrial membrane [152], which has a high negative net charge due to its bacterial origin. Increased cholesterol levels as a risk factor in AD could also be interesting to consider in this context. Intracellular oligomerization in endosomes and lysosomes is also interesting, as these compartments provide a low-pH environment (pH 5.5-4.5) which could trigger Aβ aggregation also at lower concentrations as inter-peptide repulsion is decreased [117]. Further in vitro studies of Aβ oligomerization at lower pH values should therefore be of interest.

It would in general be helpful to be able to correlate observed structural effects to cell toxicity when possible. This has in some cases been possible in this thesis. The NCAM-PrP peptide in Paper VII is known to reduce cell toxicity, which makes the reduction of micelle-embedded oligomers interesting. The same is true for the toxicity difference of Aβ(1-40) and Aβ(1-42), which also correlates with the formation of larger oligomers in micelles (Paper VI). The AβCC variant is also more toxic than the wild-type peptide, and is here shown to form increased amounts of oligomers in solution. A study where the effects on toxicity in vivo upon addition of modulators are systematically compared to structure effects on oligomers in vitro (in aqueous solution and in micelles) would be interesting to further draw conclusions on the biological relevance of these observed species.
What can these findings be used for?

A lot of progress in AD research is now being made with the first-ever disease-modifying drugs being approved, which also target Aβ aggregation (Section 2.3). There is increased consensus that pre-fibrillar species are important toxic species that many of the drug candidates in clinical trials target. The methodologies demonstrated here could be useful tools in mapping interesting species and their interaction with drug candidates, as has been suggested before [325]. It is for example worth noticing that the interaction of the phase III drug candidate valitrampirosate with Aβ oligomers was studied using native IM-MS at the pre-clinical stage [192]. An obvious extension to such earlier studies would be to monitor the effect of potential drug-candidates also on oligomers in membrane-like environments, as was done for the NCAM-PrP peptide in Paper VII.

The studies in this thesis also provide some mechanistic information on how oligomers form, which could be used for the rational design of AD therapies. The lessons learned from nature’s own anti-amyloid systems do for example provide some insight into how oligomers are targeted in vivo. Both DNAJB6 and Brichos are here suggested to bind their amyloidogenic clients via strand-strand interactions which create antiparallel β-sheets (Paper IV). This further highlights the possible importance of β-sheet formation in growing oligomers as was highlighted in Paper I, and points out the possibility to specifically inhibit oligomeric growth by providing complementary strand-strand interactions that can stabilize the energetically frustrated oligomers.

We still need to learn much more about the oligomers and the next step for the studies described here would be to introduce more perturbations in the systems to observe how the different states change. fAD-related variants, such as the Arctic (E22G) variant, could possibly inform us about what properties might be important in AD toxicity. The fAD variants generally have substitutions in the hinge region, which could hint at an important role of this segment in oligomer formation. It could for example be easily imagined that such substitutions modulate the formation of the hairpin motif. Do fAD variants increase oligomerization? Do they increase or decrease the population of extended linear conformation states? Do they interact strongly in the micelle environment? Answers to these questions could take us a few steps closer to understanding Aβ oligomers and their roles in AD toxicity.
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