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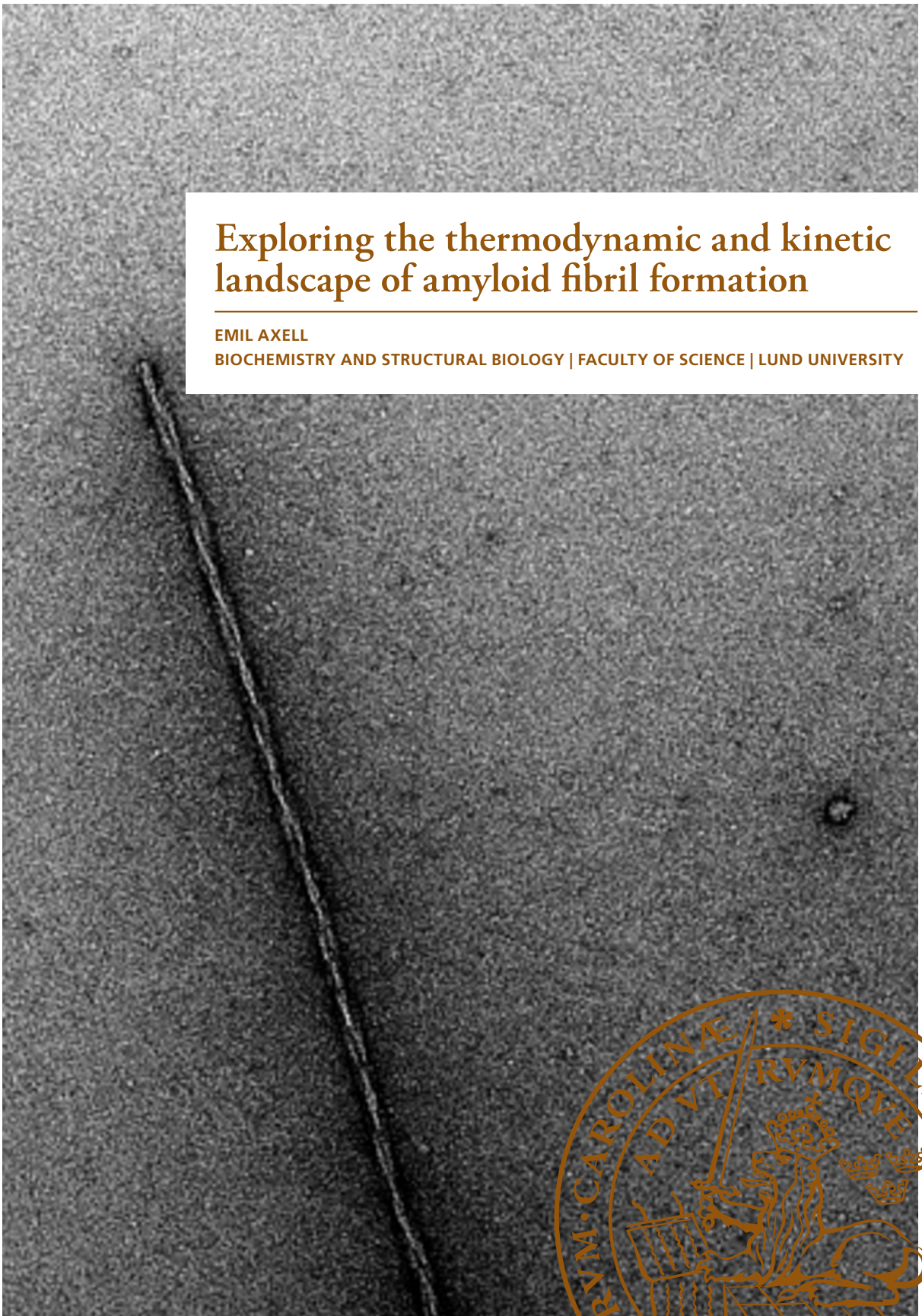

Exploring the thermodynamic and kinetic landscape of amyloid fibril formation

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Emil Axell

LUND
UNIVERSITY

Thesis for the degree of Doctor of Philosophy
Thesis advisors: Prof. Sara Linse, Prof. Emma Sparr
Faculty opponent: Prof. Andreas Barth

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Abstract:

Misfolding, self-assembly, and subsequent deposition of amyloid β plaques and tau tangles are closely associated with Alzheimer's disease (AD) and its neurodegenerative processes, driving healthy neurons into a diseased state. At present, AD is the most widespread form of dementia, causing profound personal and familial hardship as well as imposing a substantial societal economic burden. Currently, there is a lack of effective therapeutic interventions and the number of people affected by the disease is expected to continue to grow. This calls for extensive investigations of all aspects of the disease and its associated underlying molecular participants.

The purpose of the work that forms the basis of this thesis has been to investigate fundamental properties of amyloid β and tau self-assembly and their solubility. The results of this research are presented as five research articles. In Paper I, a solubility assay for determining the solubility of amyloid proteins is developed and its use demonstrated for the Aβ40 peptide. Paper II finds that tau truncated of its flexible regions adjacent to the amyloid core, (tau 304-380C322S) spontaneously forms fibrils without the use of external inducers and that the proliferation of fibrils occurs through secondary nucleation. Next, in Paper III, the methodology developed in Paper I is applied to the fragment of tau from Paper II, and its solubility is established. Paper IV explores how fluid shear forces influences the microscopic steps of fibril formation and find that primary and secondary nucleation is increased by mild shear, while elongation and fragmentation is unaffected for Aβ42. The increase in secondary nucleation, in turn increases the oligomer population. Paper V explores one of the body's own defense mechanisms against protein misfolding, the molecular chaperone DNAJB6b, and how its assembly state affects the potency of Aβ42 aggregation inhibition.

Collectivly, this work provides new insights and avenues for continued research that hopefully one day may help improve the prospects and quality of life for the individuals suffering from AD.

Key words: amyloid beta, self-assembly, alzheimer's disease, Aβ42, tau, protein solubility, shear forces, secondary nucleation, DNAJB6b, AD, protein aggregation, neurodegenerative diseases

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Dedicated to uncover the secrets of life and unravel the mysteries of nature.

Contents

Abbreviations

Aβ	Amyloid Beta
AD	Alzheimer's Disease
AFM	Atomic Force Microscopy
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid beta Precursor Protein
CBD	Corticobasal Degeneration
CNS	Central Nervous System
Cryo-TEM	Cryogenic Transmission Electron Microscopy
CSF	Cerebrospinal Fluid
CTE	Chronic Traumatic Encephalopathy
EDTA	Ethylenediaminetetraacetic Acid
FTD	Frontotemporal Dementia
IEX	Ion Exchange Chromatography
MALDI-TOF MS	Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry
MTBR	Microtubule Binding Region
NMR	Nuclear Magnetic Resonance
PHF	Paired Helical Filaments
PSP	Progressive Supranuclear Palsy
RELION	REgularized LIkelihood OptimizatioN
SEC	Size Exclusion Chromatography
ThT	Thioflavin T

List of publications

This thesis is based on the following publications:

I **A label-free high-throughput protein solubility assay and its application to Aβ40**
Max Lindberg, **Emil Axell**, Emma Sparr, Sara Linse
Biophysical Chemistry 307 (2024): 107165.

II **Proliferation of Tau 304–380 Fragment Aggregates through Autocatalytic Secondary Nucleation**
Diana Camargo, Eimantas Sileikis, Sean Chia, **Emil Axell**, Katja Bernfur, Rodrigo L. Cataldi, Samuel I. A. Cohen, Georg Meisl, Johnny Habchi, Tuomas P. J. Knowles, Michele Vendruscolo, and Sara Linse
ACS Chemical Neuroscience 12, no. 23 (2021): 4406-4415.

III **The solubility of the amyloidogenic core of tau**
Emil Axell, Andreas Carlsson, Emma Sparr, and Sara Linse
Manuscript

IV **The role of shear forces in primary and secondary nucleation of amyloid fibrils**
Emil Axell, Jing Hu, Max Lindberg, Alexander J. Dear, Lei Ortigosa-Pascual, Ewa A. Andrzejewska, Greta Šneiderienė, Dev Thacker, Tuomas P. J. Knowles, Emma Sparr, and Sara Linse
Proceedings of the National Academy of Sciences 121, 25 (2024): e2322572121.

V **The Ability of DNAJB6b to Suppress Amyloid Formation Depends on the Chaperone Aggregation State**
Andreas Carlsson, **Emil Axell**, Cecilia Emanuelsson, Ulf Olsson and Sara Linse
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Publications not included in this thesis:

A Comparison of the Transglycosylation Capacity between the Guar GH27 Aga27A and Bacteroides GH36 BoGal36A α-Galactosidases
Mathias Wiemann, **Emil Axell** and Henrik Stålbrand
Applied Sciences 12(10) (2022): 5123

On the reversibility of amyloid fibril formation
Tinna Pálmadóttir, Josef Getachew, Lei Ortigosa-Pascual, **Emil Axell**, Jiapeng Wei, Ulf Olsson, Tuomas PJ Knowles, Sara Linse
Biophysics Rev (2025) *Accepted*

My contribution to the articles

Paper I: A label-free high-throughput protein solubility assay and its application to Aβ40
I participated in the design of the study, purified peptides, optimized and performed solubility experiments. I contributed to the writing of the manuscript.

Paper II: Proliferation of Tau 304–380 Fragment Aggregates through Autocatalytic Secondary Nucleation
I expressed and purified the ^{15}N isotope labeled variant of tau. I contributed to the design of oligomer source experiments, performed control kinetic experiments, optimized the purification protocol by incorporating two sequential SPHP ion exchange steps and designed and carried out the experiments investigating the effect of X34 concentration on the signal intensity and the aggregation kinetics.

Paper III: The solubility of the amyloidogenic core of tau
I conceptualized and initiated the study and played an important role in the design and execution of the experiments. I wrote the initial draft of the manuscript and revised it after comments from my co-authors.

Paper IV: The role of shear forces in primary and secondary nucleation of amyloid fibrils
I conceptualized and initiated the study and played an important role in the design and data analysis of all experiments. I performed the aggregation across the different amyloid systems, I carried out the dose-response, did the elongation/re-seeding, conducted the cryo-EM imaging exploring morphological differences and performed the concentration dependent aggregation of Aβ42. I wrote the initial draft of the manuscript and revised it together with my co-authors.

Paper V: The Ability of DNAJB6b to Suppress Amyloid Formation Depends on the Chaperone Aggregation State
I conceptualized and initiated the study and played an important role in the design and execution of the experiments. I purified Aβ42, performed kinetics with DNAJB6. I contributed to the writing of the manuscript.

Populärvetenskaplig sammanfattning

Förbättrade levnadsvillkor leder både till en ökande och en åldrande befolkning. I takt med att befolkningen blir äldre ökar även andelen individer som drabbas av åldersrelaterade sjukdomar. Bland de vanligast förekommande sjukdomarna är demenssjukdomar och ungefär hälften av alla som lider av demens är drabbade av Alzheimers sjukdom. Idag finns det varken effektiva botemedel eller snabba, billiga och pålitliga sätt att säkert ställa en Alzheimer diagnos.

Allt eftersom sjukdomen utvecklas kan symtomen bli värre och det kan till slut bli svårt att bo kvar i hemmet. I senare skeden kräver allt fler patienter kvalificerad vård på särskilda demensboenden som har kapacitet till och kan hantera de svåra utmaningarna vid Alzheimers sjukdom. Kostnaden för denna typ av vård är skenande och den samhällsekonomiska bördan, samt det individuella lidandet för patienten och dess anhöriga är avsevärd. Det finns hopp, men trots att vi det gångna året har fått godkännande för nya läkemedel för att behandla och bromsa utvecklingen av Alzheimers sjukdom, finns det idag inga läkemedel som fullständigt kan stoppa sjukdomsutvecklingen eller ännu bättre, reversera skadan som uppstått. Därför krävs det i hög grad, fortsatt forskning och förståelse för sjukdomen, för att en dag se till att vi kan utrota den helt. Just att sjukdomen kan anamma olika karaktär hos olika individer och att vi inte riktigt än vet alla de underliggande sjukdomsmekanismerna innebär att vi är i behov av fortsatt forskning, för att utveckla effektiv diagnostik och botemedel mot sjukdomen.

Gemensamt för alla som drabbats av Alzheimers sjukdom, är att proteinet amyloid β klumpar ihop sig utanför hjärnans celler, och proteinet tau bildar trådar innuti cellerna. Denna process av att proteiner klumpar ihop sig, skapar förbigående protein sammansättningar som kallas för oligomerer. De varierar i storlek och det är just dessa och denna process många forskare tror är det skadliga som leder till att hjärnans celler och särskilt nervcellerna dör. När fler och fler neuroner dör och inte längre fungerar som de ska, leder detta till att nervsignaler får allt svårare att ta sig fram. Något som i sin tur ger upphov till många av de klassiska symptomen på sjukdomen.

Forskningen i denna avhandling fokuserar just på amyloid β och tau. Frågeställningarna har en fundamental karaktär och har som syfte och mål att skapa ny och förbättrad förståelse för dessa proteiners egenskaper. Hoppet är att denna kunskap en dag ska kunna bidra till insikter som i sin tur leder till att bota sjukdomen.

I avhandlingen handlar en del av denna förståelsen om att mäta och förstå hur snabbt proteinerna amyloid β och tau klumpar ihop sig, samt hur en annan klass proteiner, chaperoner, bidrar till att bromsa processen.

Vidare har avhandlingen utvecklat metodologi för att mäta hur mycket protein som kan klumpa ihop sig, genom att mäta hur mycket protein som finns kvar i lösning efter att processen har skett. Detta kan förstås genom en vardaglig liknelse med salt och vatten; om du fortsätter tillsätta salt i ett vattenglas, kommer till slut inget mer salt att kunna lösas i vattnet. Det kommer istället att fälla ut, och sedan sedimentera till glasets botten. Det samma gäller för proteiner, men proteiner har en förmåga att hålla sig kvar i lösning trots att de egentligen rent termodynamiskt inte skulle kunna göra det över en längre tid. Denna förmåga att hålla sig kvar i lösning kallas metastabilitet och du har kanske sett det i andra vardagliga sammanhang, som till exempel i handvärmare man aktiverar med hjälp av en liten metallbricka, eller när du gjort sockerlag. Denna metastabilitet kan vara väldigt hög hos proteiner och att få dem att nå jämvikt kan vara en utmaning.

Just att hjälpa proteinerna att nå jämvikt och sedan mäta hur mycket som finns kvar i lösningen har varit en central del av mitt arbete. Även här kommer chaperonerna in, då de ändrar och påverkar andra proteinerns förutsättnignar att hålla sig kvar i lösningen. Denna typ av grundläggande förståelse, skapar förutsättningar för att bygga vidare på och slutligen öka vår kunskap och förståelse kring de proteiner som är en central del av sjukdomsförloppet i Alzheiemrs sjukdom. I slutändan är det yttersta målet att dessa insikter en dag leder till effektiva botemedel som kan gynna hela mänskligheten.

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Someone once told me that who your office mate is, is more important than any other co-worker. Toward this end, you have been all I could have asked for **Max**. Starting on the very same day and working on similar projects have enabled us endless discussions which has been a big part of developing and cementing my understanding on many topics. Your willingness to interrupt your own work to help others with small or large things has not gone by unnoticed.

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Being a good office mate of course also applies to you **Lei**. I know we have had our differences when it comes to setting the office thermostat and whether or not to keep the door closed, but I think our relationship has endured and been forged by this. I hope you wear a

shirt with a breast pocket for the next party, in that case I will supply Ouzo and Mastikka, I do not recall which one or if it was the combination that made you master the coin and made the whole amyloid meeting go skinny dipping in Greece. Algunas cosas es mejor discutirlas en idiomas que no todos entienden. Ez dut inoiz ahaztuko gure safari txikia kartelaren lurraldera. Jan dudan tako eta kale-janari onena izan zen, baina zapore mingotsa geratu zitzaidan ordu batzuk lehenago ibili ginen kale berberean ikusi genuena ikusi genuenean. Oraindik ezin dut ulertu nola ausartu ginen ezezagun batzuekin favela barrena gidatzera, askatasunaren lurraldera itzultzeko gure ahaleginean. Espero dut egunen batean suedieraz hitz egiten ikastea, behar bezala komunikatu gaitezen. Jag gjorde mitt bästa.

Dev, thank you for all your patience during our cryo imaging sessions, I look forward to having you back in the lab and continuing to work on cryo projects with you! **Jing**, seeing your hard work, determination, and how quickly you learned new techniques has been a source of inspiration. I am very happy to see that you enjoy climbing so much! **Alex**, your intuition for amyloid assembly goes without saying, I am very glad to have worked with you and I am very grateful for the mentorship you provided while being in the group and still continue to offer. **Tinna** our discussions about thesis writing and defense have been a great support at the end. Thank you also for all you thought about alpha synuclein when I was completely new in the lab many years ago. **Erik** thank you for being the sherif, keeping an operational and clean lab space. **Ewelina**, thank you for always caring and putting up with things that sometimes appear on your lab bench. Thank you **Johan** for all the interesting discussions and perspectives, I hope we get to do some NMR one day! **Egle**, thank you for all the time we spent teaching together. Thank you **Thom**, witnessing you start a company and building instruments has been a source of inspiration. Thank you **Martin** for keeping everything running smoothly in the lab and for your time spent supervising me years ago, the good time I had in the lab during my undergraduate years greatly factored in when choosing to start my PhD in the group.

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Part I

Research context

Prologue

Embarking on the PhD journey has been a fascinating experience and endeavor that forever will alter my perspectives. It has certainly both challenged and developed my knowledge and understanding, as well as equipped me with the tools to continuously search for growth and development.

Before setting the stage for my doctoral research, I would like to begin with a brief summary of what I consider to be the most important conclusions and findings obtained in each study.

Paper I: Developing an inexpensive and robust methodology to investigate the solubility of amyloid peptides.

Paper II: Establishing an *in vitro* aggregation model of tau that readily self-assembles and forms amyloid fibrils in the absence of external inducers and demonstrating that the proliferation of new aggregates occurs through autocatalyic secondary nucleation.

Paper III: Exploring the remarkable metastability and solubility of the tau amyloid core fragment and developing methodology for reaching its equilibrium.

Paper IV: Revealing the effect of shear forces on the microscopic steps of amyloid fibril self-assembly and how this can influence the rate limiting steps of the mechanism.

Paper V: Showing that it is the smaller subunits of the chaperone DNAJB6b that is the active component in inhibiting the aggregation of the Aβ42 peptide rather than the large assemblies.

Chapter 1

Introduction

For proteins to acquire and maintain their biological function they are often required to fold their poly-peptide chain into specific ordered three-dimensional structures. To aid in this process there is a special class of proteins, chaperones, whose main purpose is to assist other proteins to adapt and maintain their correct folding. Protein folding is a complex and delicate process and when it goes wrong, the consequences can be severe. Many human diseases are the result of protein misfolding, this aberrant phenomenon along with how chaperones serve to prevent it is the main focus of my research, forming the foundation of the research papers on which this work is based. More specifically, this thesis and research papers attempt to interrogate and address fundamental properties of protein misfolding related to Alzheimer's disease (AD). In Chapter 1, we will establish a framework, and I will provide you with the context in which I comprehend the findings of my research. In Chapter 2, I present the methods used to obtain the data on which my findings are based. Finally, in Chapter 3, the findings of my research will be discussed in more detail. Lastly, I briefly share my thoughts about future research directions in Chapter 4. Before we dive deeper into the intricacies of AD and its related proteins, let us first examine general aspects, commonalities and differences among the multitude of amyloid-forming proteins in the next couple of sections.

1.1 Amyloid proteins

Amyloid refers to a state of protein in which monomers normally found in solution are precipitated in protein inclusions. We will examine their architecture in more detail in Section 1.2 and look at the mechanism of their assembly in section 1.3 before narrowing in on AD in section 1.4, where we will explore in more depth the two proteins, amyloid beta (Aβ) and tau, the two proteins towards which much of my PhD research has been devoted.

Amyloid is often associated with various neurodegenerative diseases that affect the brain but is also found in other organs. There are also amyloids that are not disease-related, in which proteins adopt the amyloid structure and play a functional role in biological systems. These amyloids are not the topic of this thesis but deserve to be mentioned [1]. There are other protein deposition diseases that do not involve amyloid, see reference [2] for a comprehensive review.

To date, more than 40 disease-related amyloid proteins have been identified in humans, and the list is continuously growing [3]. Table 1.1 lists some common, but also more rare, diseases caused by amyloid-forming proteins to highlight the diversity of organs affected and the proteins involved.

Some of the diseases are common, while others are extremely rare. This group of proteins shares many common traits, but in terms of their amino acid sequence and the pathologies they cause, they make a diverse and heterogeneous group. In many cases, cleavage from a precursor protein precedes fibril formation; we will examine this in more detail for the Aβ peptide later on. Some proteins form amyloid the way they are translated, while other proteins may form amyloid only after post-translational modifications. Generally, aggregation of one peptide leads to one disease, but some peptides are associated with multiple diseases, such as in the case of tau, which we will examine in later sections. Cryogenic transmission electron microscopy (Cryo-TEM) of patient-derived amyloid fibrils has begun to reveal previously unknown secrets of amyloids. When patient-derived amyloids are analyzed, many other proteins and molecular players are often found associated with the deposits. Recently, an interesting odd case emerged, in which two different peptides, a fragment of TDP-43 and ANXA11 were found to be co-assembled in the amyloid fibril [16]. This seems to be the only case, yet identified, in which two different peptides together make up the amyloid fibril. Although AD plaques seem to contain a plethora of Aβ length variants [17].

Some amyloid proteins aggregate intracellularly, while others do so extracellularly. Certain protein mutations are inherited and enhance amyloid formation only in specific families, whereas others occur sporadically. Some proteins form deposits in the organ where they are expressed, while others deposit in completely different parts of the body. Table 1.1 gives an overview of some occurring amyloid diseases, the order in which they are presented should not be viewed as a strict hierarchy of their occurrence, as this vary across populations, demography and age groups. See reference [3] for a comprehensive collection of human and animal amyloids identified to date. Lastly, albeit rare, four iatrogenic amyloids comprised of hormonal drugs accumulated at the site of their injection has been reported [3]. These ”exogenous” amyloids are among the most recently identified amyloids in humans and are likely a growing class as a result of the rapidly increasing number of biological therapeutics available [18].

Table 1.1: Overview of selected amyloid proteins, affecting multiple different organs, associated with a variety of human diseases.

Fibril Protein constituent	Main affected organs	Disease
Aβ	CNS	Alzheimer's disease [4]
Tau *	CNS	Alzheimer's disease, FTD, Pick disease and others [5]
α-synuclein *	CNS	Parkinson's disease, Dementia with Lewy bodies, Multiple system atrophy and others [6]
Transthyretin	Heart, lung and ligaments	Senile systemic amyloidosis and others [7]
Serum amyloid A protein	All organs except CNS	AA amyloidosis [8]
Immunoglobulin light-chain amyloidosis	All organs except CNS	Light chain amyloidosis [9]
β2-microglobulin	Musculoskeletal system	Dialysis-related amyloidosis [10]
Islet amyloid polypeptide	Pancreas	Type II diabetes and Insulinoma [11]
Prion protein *	CNS	Creutzfeldt-Jakob disease, Fatal insomnia, Spongiform encephalopathy, kuru and others [12]
Lysozyme	Kidney	Lysozyme amyloidosis [13]
Apolipoprotein A	Heart, liver, kidney, skin and other	ApoA amyloidosis (multiple) [14]
Insulin, Enfurvitide, GLP-1, IL-1RA	Injection site	Injection localized iatrogenic amyloidosis [15]

** Fibrils of these amyloid proteins form intracellularly.*

1.2 Amyloid fibril architecture

The amyloid structure exhibits a high degree of molecular order with a repeating pattern in one dimension along the fibril axis. The molecular organization of protein in the amyloid structure gives it remarkable properties, including a very high stability. The structure also gives the proteins the ability to serve as a template for monomers in solution, enabling the propagation of specific amyloid folds depending on the seed origin.

Because of the large size and low tumbling rate of amyloid fibrils, solution NMR spectroscopy is not suitable for their structure elucidation. Furthermore, amyloid fibrils do not readily form protein crystals, except in the case of selected short peptide fibril model systems [19],[20], making X-ray crystallography less suitable as well. This excludes the two most common strategies for the structure elucidation of macromolecules and made amyloid fibrils elude high-resolution structure determination. Solid-state magic angle spinning NMR spectroscopy delivered breakthroughs in the early 2000s by enabling the high-resolution atomic models of amyloid fibrils to be solved [21]. However, this approach remains limited to *in vitro* assembled fibrils due to the large sample and isotope requirements [22]. Nevertheless, seeding recombinant isotope-labeled monomeric solutions with amyloid extracted from biological sources could enable determination of native *in vivo* structures through templating [23] [24] [25].

The rapid cryo-EM resolution revolution [26] in combination with helical reconstruction in the software RELION [27] yielded the first cryo-EM derived *ex vivo* amyloid structure a decade later [28]. This structure of tau from AD was a significant milestone and marks the beginning of an exciting era, where it is now possible to use this method to solve atomic resolution structures of amyloid extracted directly from tissues. This could provide clues that may aid in the rational design of drugs and inhibitors with the potential to mitigate and treat disease. Cryo-EM has become the standard method for elucidating the high-resolution molecular structure of amyloid fibrils and is actively revealing more of their secrets.

Amyloid fibrils are composed of monomers stacked on top of each other, held together and stabilized by hydrophobic interactions of multiple side chains buried in the core, while the extended β-sheet conformation satisfies most hydrogen bonding donors and acceptors along the peptide backbone (Figure 1.1). Initially, high resolution structural information was obtained through X-ray fibre diffraction. When examined through fibre diffraction, amyloids give rise to a sharp meridional reflection at ≈ 4.75 Å and a diffuse ring at ≈ 10 Å [29]. The first reflection corresponds to the distance between the strands in the fibril (Figure 1.1C) and the second reflection to the distance between the filaments in the fibril. The periodic nature of fibrils gives rise to a characteristic reflection pattern that has led amyloids to be described as having a cross-β structure. Multiple filament cores may bundle

together and yield a varying number of monomers per plane, this number is specific for each amyloid protein and may even vary between morphs of the same protein [30] [31].

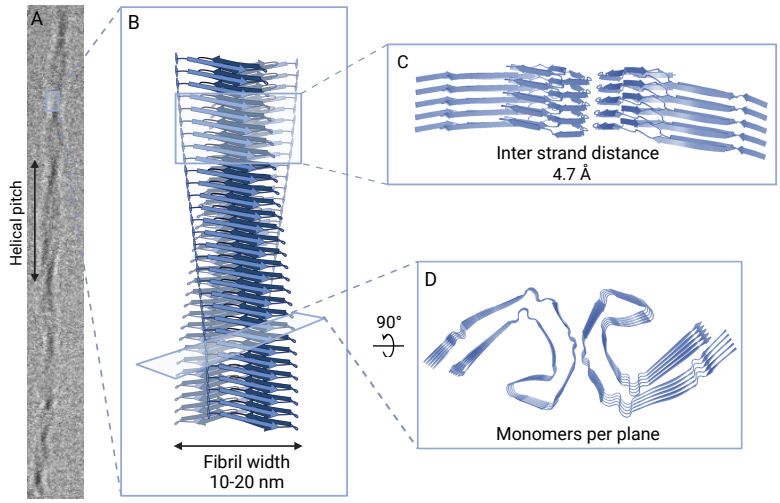


Figure 1.1: Cryo-EM image illustrating the helical nature of amyloid fibrils (A). The fibril is made up of individual monomers stacked upon each other in a β-sheet configuration, (B) with an inter strand spacing of approximately 4.7 Å (C). Multiple filaments may be bundled together. Looking at a cross section of the fibril down its axis (D), the core may be made up of one or multiple monomers per plane. This number varies between different proteins and morphs, in this specific example, the core consists of two monomers per plane (*ex vivo* AD tau, 5O4L). The dimensions/distances indicated in the figure should be considered typical and may differ between different proteins.

The β-sheet stacking of monomers is slightly misaligned and offset, generating a periodic twisting around the axis of the fibril. When looking at amyloid fibrils at the nanometer scale using TEM or AFM, this twisting becomes evident and enables characterization of its helical pitch. The helical pitch varies between proteins and between morphs of the same protein, and the fibrils can be practically straight with periodicity of hundreds of nanometers to very tightly twisting with a periodicity of just a few nanometers. The existence of a periodic twist is currently a prerequisite for 3D reconstruction of amyloid fibrils through single particle analysis of cryo-EM data, which currently makes solving the structure of ”straight” fibrils with longer periodicity particularly challenging. Although many architectonic elements are shared between amyloids, they have particular ways of packing their fibrillar core, allowing the same protein to have many different polymorphs, something we will look closer into in the case of tau later in this chapter. First, we will examine in greater detail the driving forces and mechanisms underlying amyloid fibril formation.

1.3 Amyloid self-assembly

How do soluble proteins end up as insoluble amyloid deposits, and can we prevent this from happening?

Much is known about the molecular mechanisms of amyloid fibril self-assembly and the architecture of amyloid fibrils. Yet, amyloid still holds many secrets and much is unknown about the onset and propagation of the self-assembly process *in vivo*. Key challenges in the field include identifying which steps of the self-assembly process or which species along the assembly pathway are toxic and harmful to the cell. Equally important is determining how to intervene with this process to protect the organism. Understanding which species and steps of the process contribute to cytotoxicity is essential to develop strategies and therapeutics to mitigate and prevent the resulting damage. To obtain this level of understanding, we may first begin to investigate their behavior and driving forces in simple model systems. To this end, it is an effective strategy to employ recombinant proteins in pure buffer systems; this way, multiple physiochemical properties may be investigated in a bottom-up approach and complexity introduced into the system in a systematic and controlled manner.

When not in the amyloid state, the precursor protein may be folded, bound to another protein or intrinsically disordered. In healthy individuals, these proteins typically remain in their harmless native state and avoid forming amyloid throughout their life time. However, under certain conditions and in some individuals, specific proteins self-assemble into amyloid fibrils. These proteins, before fibril formation, may have existed in a metastable state, stabilized by chaperones and high kinetic barriers to nucleation.

In vitro studies of otherwise non-amyloidgenic proteins have shown that numerous of these proteins, under specific conditions, may form amyloid [32] [33]. These observations have led to the proposal that the ability to form amyloid and adopt the amyloid structure is universal for all amino acid-based polymers [34] [32]. This seemingly general ability of proteins to adopt the amyloid structure further introduces questions regarding the driving forces of protein folding. Is the folding of proteins into well defined structures merely finding a metastable local minimum, protected from amyloid formation by kinetic barriers in the local energy folding landscape? If the global energy minimum is represented by the amyloid structure, would that imply that globular protein folds are nothing more than kinetically trapped metastable states, with amyloid formation being the thermodynamically favored? Perhaps this somewhat ambiguous behavior of proteins under certain conditions is a fundamental property of the protein energy landscape [35]. Given this perspective, the free monomer concentration is of significance when investigating protein free energy landscapes, because of its effect on the chemical potential. Above the solubility limit S, the protein will exist in a supersaturated solution with a chemical potential higher than that achievable in a fibril, giving rise to a thermodynamic drive of fibril formation. We will

elaborate on this, and its implications in more detail when we explore the thermodynamics of fibril formation in section 1.3.3.

Amyloid aggregation is often associated with high age, which is a known risk factor. Of the roughly 100 000 proteins expressed in the human body, relatively few have been reported to form amyloid in their biological context. One may imagine that there has been strong evolutionary pressure to select against protein aggregation and fibril formation during the reproductive phase of life. Perhaps fibril formation at high age, occurring outside the window of reproductive fitness might explain why it could have evaded the evolutionary pressure.

Before examining the thermodynamics of fibril formation in more detail, let us first take a closer look at the underlying molecular steps involved in amyloid fibril assembly.

1.3.1 The microscopic steps of amyloid fibril assembly

The self-assembly of free monomers into amyloid fibrils can be viewed as a phase transition [36], meaning that the processes involved can be understood and described through classical thermodynamics. This perspective has led to the development of master equations that are capable of describing many of the observed features of amyloid fibril formation. This has also led to the development of open access software able to fit to the data, the solutions of coupled differential equations used to describe the various microscopic steps involved in amyloid fibril formation [37]. Before looking in more detail at the master equations, let us first examine the microscopic steps through which fibrils form and proliferate, and introduce some notations used when describing the rate constant of each step mathematically (figure 1.2).

For monomers in solution to begin to form fibrils, first of all they need to be at a sufficiently high concentration (above the solubility limit, *S*) where it is energetically favorable for fibril formation to occur. Next, more than one monomer needs to come together in order to form the first nuclei. When the growth of these first nuclei happens faster than the dissociation back into monomers, primary nucleation has occurred. Once the first fibril has emerged through primary nucleation, monomers in solution can add on to the fibril ends through the process of elongation. In a system with fibrils present, the surfaces of the fibrils act as a catalysing surfaces on which new nuclei can form, this nucleation is refereed to as secondary nucleation. Lastly, a fibril may break into two through fragmentation. The rate of these microscopic steps may be described through rate constants where primary nucleation is typically denoted k_n, elongation k_+, secondary nucleation k_2 and fragmentation k_-.

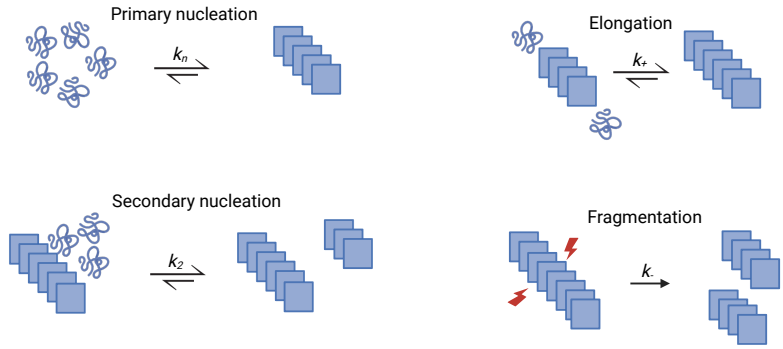


Figure 1.2: The macroscopic growth of amyloid fibrils typically involves four main microscopic steps; Primary nucleation, in which monomers come together in solution to form a new amyloid fibril. Elongation, in which monomers add on to the ends of already existing fibrils. Secondary nucleation, the fibril interface provides catalytic surface on which monomers can nucleate. Lastly, fragmentation in which fibrils break in solution, doubling the number of fibril ends, on which new elongation may occur.

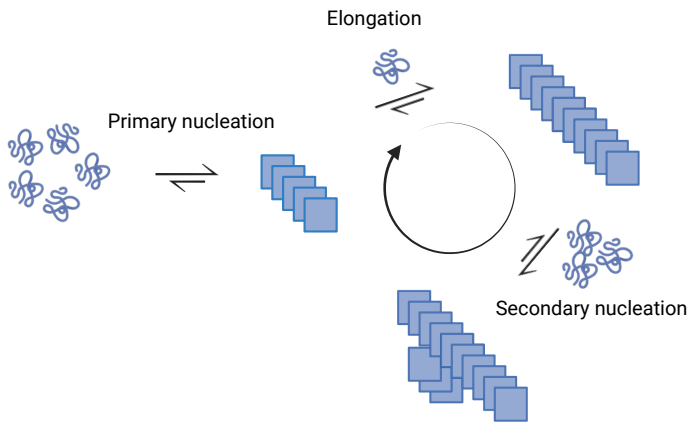


Figure 1.3: The microscopic steps of amyloid fibril formation act in conjunction and the growth of fibril mass is autocatalytically amplified by elongation and the proliferation of new nuclei by secondary nucleation.

The microscopic steps act in a concerted manner (figure 1.3), where secondary nucleation and elongation gives rise to an autocatalytic proliferation of fibrils that typically gives rise to a sigmoidal fibril growth curve (figure 1.4). For many amyloid forming proteins, primary nucleation is very slow relative to secondary nucleation and elongation [38]. As a result of this rate difference, the proliferation of new nuclei occurs predominantly through secondary nucleation, rather than primary nucleation and fragmentation.

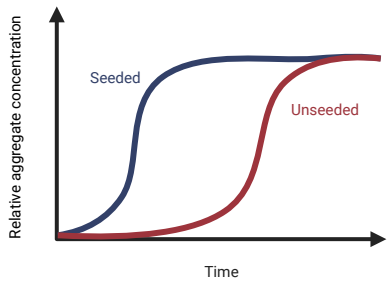


Figure 1.4: Slow primary nucleation and the autocatalytic nature of elongation and secondary nucleation typically gives rise to sigmoidal fibril growth curve. The introduction of pre-formed seeds largely bypasses rate limiting steps of primary nucleation.

1.3.2 Kinetics of amyloid fibril assembly

Once the first primary nucleation event has occurred, all other microscopic steps also begin and continue throughout the aggregation process but at different reaction rates. The reaction rates are governed by the monomer concentration $[m]$, the total fibril mass concentration $[M]$, the number of fibril ends $[P]$ and the respective rate constants of each of the microscopic steps involved in fibril formation. Typically, the kinetics of fibril formation is followed by the increase in fibril mass, and it is thus convenient to express the reaction rate in terms of the mass concentration of fibrils with respect to time, $M(t)$. However, since not only the total fibril mass governs the overall kinetics, but also the number of fibril ends, it is useful to introduce an additional term to describe the reaction rate in terms of the fibril number concentration $P(t)$ [37]. We now have two descriptions of how the system changes with respect to time, one that describes the changes in overall fibril mass and one that describes the changes in number of individual fibrils. Let us now look at how we can express these changes in the system and formulate reaction rates using the rate constants of the individual microscopic steps.

The reaction rate of primary nucleation is only dependent on the monomer concentration $[m]$, therefore the reaction rate of primary nucleation can be expressed as $k_n[m]^{n_c}$ where

the exponent n_c denotes the reaction order. Since primary nucleation gives rise to new fibril ends, it is useful to describe this change in terms of the aggregate number:

$$\frac{dP}{dt} = k_n m(t)^{n_c} \tag{1.1}$$

The reaction rate of elongation depends both on the monomer concentration and the number of fibril ends and can be expressed in terms of $k_+[P][m]$. Elongation consumes monomers and gives rise to increased fibril mass and is thus suitably expressed with respect to fibril mass concentration:

$$\frac{dM}{dt} = k_+ P(t) m(t) \tag{1.2}$$

or expressed as the change of monomer:

$$\frac{dm}{dt} = -k_+ P(t) m(t) \tag{1.3}$$

Secondary nucleation rate is dependent on the overall fibril surface and the monomer concentration and gives rise to new fibril nuclei, thus it is conveniently expressed as $k_2[M][m]^{n_2}$ with respect to the aggregate number concentration:

$$\frac{dP}{dt} = k_2 M(t) m(t)^{n_2} \tag{1.4}$$

Lastly, fragmentation is only dependent on the fibril mass concentration and gives rise to new fibril ends, and can thus be expressed as $k_-[M]$ with respect to the aggregate number:

$$\frac{dP}{dt} = k_- M(t) \tag{1.5}$$

Now we have expressions that can be used to describe the rates of the microscopic steps of fibril formation. These steps and differential equations are intertwined and to model the time evolution of fibril formation a set of master equations that couple the differential equations must be employed [39, 40]. This enables fitting, which accurately capture the macroscopic proliferation and behavior of experimental data. The above presented equations and mathematical formalism is the foundation underlying the online fitting platform amylofit [37], a user friendly software onto which kinetic data may be uploaded and fitted using for instance a secondary nucleation dominated model. By performing aggregation kinetics with various starting monomer concentrations and with pre-formed seeds, many of the underlying global rate constants can be estimated and insights about the amyloid system in question may be obtained. The fitting of experimental data using different models and the evaluation of the quality of the fits helps to decipher and further strengthen insights into the mechanistic details of the self-assembly reaction. This framework and approach has been instrumental in developing our current mechanistic understanding of amyloid fibril formation and continues to yield new insights, for example, evaluating the effect of

aggregation inhibitors, familial mutations, or environmental factors on the assembly rate. Fitting with amylofit has the potential to resolve which microscopic steps are effected by a certain perturbation, as well as the power to bridge experimental data and theoretical models.

1.3.3 Thermodynamics of amyloid fibril assembly

The formation of amyloid fibrils can be viewed as a phase transition, from highly disordered free monomers to highly ordered fibrils. For monomers to self-assemble into amyloid fibrils, there must be a thermodynamic driving force to do so, implying the Gibbs free energy change ΔG, over the self assembly reaction must be negative, a criteria which is fulfilled for a supersaturated solution.

Unless two components are fully miscible, the solubility of a solute in a solvent is finite. For amyloid fibrils (or any other system for that matter), this means there is a maximum concentration that can exist in solution at equilibrium. We can denote this well-defined monomer concentration S. For supersaturated (metastable) solutions, the concentation is $> S$ and ΔG<0, at equilibrium the monomer concentration is equal to S and ΔG=0.

In the solution phase, a homogeneous solution of monomers [m] may remain supersaturated, at concentrations above their solubility limit S for a prolonged time, without forming amyloid because of the high energy barrier for forming the initial nuclei (figure 1.5) [41].

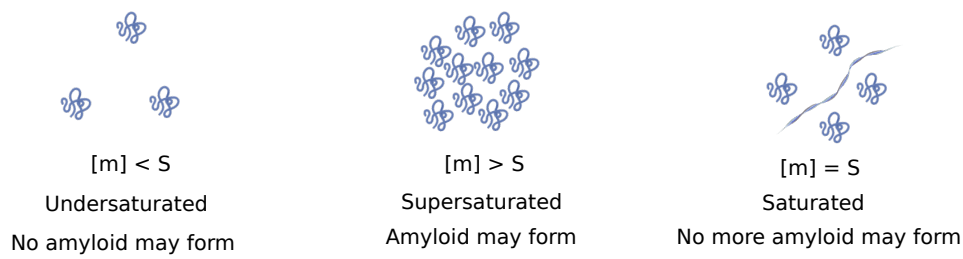


Figure 1.5: Monomers present at or below the solubility limit, [m] $\leq$ S, will remain monomeric and any fibrils present will dissolve. Above the solubility limit, [m] $>$ S, monomers may nucleate and form a fibril phase. At equilibrium above the solubility limit there is coexistence of monomers and fibrils, the concentration of free monomer in solution is equal to the solubility limit, [m] = S.

Once the formation of an amyloid phase has occurred through primary nucleation, the out of equilibrium monomer phase will continue to shrink and the amyloid phase will continue to grow, through all microscopic processes, until the chemical potential of all components is equal in all phases and no more change in free energy is observed. At equilibrium the

system consist of two phases, one solution phase of monomers, in equilibrium with the solid fibril phase. The monomers contribution to the free energy can be expressed per molar by taking the partial molar derivative of the Gibbs free energy with respect to the number of moles of monomer at constant temperature (T), pressure (P) and moles fibril (N_f). This gives us the chemical potential of the monomer μ_m:

$$\mu_m = \left(\frac{\partial G}{\partial N_m}\right)_{T,P,N_f} \tag{1.6}$$

The chemical potential of the monomer in an ideal solution can be written as:

$$\mu_m = \mu_m^\circ + k_B T \ln[m] \tag{1.7}$$

where μ_m° denotes the standard chemical potential in the solution, k_B the Boltzmann constant, T temperature and $[m]$ the monomer concentration. The fibril phase has a fixed composition, meaning that its internal energy and chemical potential do not change with the monomer concentration, unlike the chemical potential of the monomer in the solution phase, which changes with concentration. The chemical potential of a monomer in the fibril phase is constant and is generally defined as the standard state of the pure component phase and can be written as μ_f=μ_f° [42].

At equilibrium, ΔG=0, the chemical potential of a monomer in solution is equal to the chemical potential of a monomer in the fibril, μ_m=μ_f [42]. Substituting this into equation 1.7, gives:

$$\mu_f^\circ = \mu_m^\circ + k_B T \ln[m] \tag{1.8}$$

At equilibrium, the free monomer concentration [m] is equal to the limit of solubility S, rearranging 1.8 and taking the exponential of both sides gives:

$$[m] = S = \exp\left(\frac{\mu_f^\circ - \mu_m^\circ}{k_B T}\right) \tag{1.9}$$

Experimentally, one way to investigate the solubility of amyloid forming proteins is to measure the concentration of monomers remaining in solution after amyloid fibril formation has occurred, and the system has been given sufficient time to reach equilibrium. Knowing when equilibrium has been reached is practically challenging but can be interpreted as reached when change can no longer be observed in the system. When investigating the solubility of amyloid proteins, slow fibril formation kinetics is a challenge, but can be mitigated and overcome by speeding up secondary processes through agitation or seeding. The equilibrium is independent of the assembly pathway. It is important to keep in mind

the reversibility of the reaction, if the system is perturbed at equilibrium, it will react in the direction that minimizes the difference in chemical potential between the two phases according to Le Chanteliers principle. Either by forming more fibril if more monomers are added, or by dissolution of monomers from the fibril ends if diluted upon reaching equilibrium.

After this general introduction of amyloid, let us dive further into the specifics of AD and its related proteins.

1.4 Alzheimer's disease

Alios Alzheimer, a German physician, took great interest in a 51 year old woman with strange symptoms affecting her behavior, especially her memory. Upon her death, Alzheimer had arranged to receive and study her brain. After dissecting it and using the recently developed Bielschowsky silver staining method, Alzhemier was able to stain brain slices and identify many of the pathological traits we today recognize as hallmarks of Alzheimer's disease (AD) [43] (for an english translation [44]). In particular, deposits of intracellular neurofibrillary tangles and extracellular plaques were discovered throughout her brain. Alzheimer also noted other traits we today clinically associate with AD, including brain atrophy and observable changes to the vasculature. This discovery made more than a century ago has shaped the whole field's perspective on the disease, leading to the disease being viewed as and characterized by the presence of plaque and tangle deposits. Furthermore, these initial observations highlight and established the connection between physical changes to the brain and the clinical symptoms of the disease. A century later, many unanswered questions remain, and our understanding of the underlying disease mechanisms is yet not complete. Nevertheless, the last century and especially the last decades has been a golden age for amyloid research, in particular for AD. Today we know and recognize the amyloid β peptide (Aβ) and tau as the main protein constituents of plaques and tangles found in the AD brain, respectively [4],[45],[46]. As we explored in Section 1.2, we begin to have a good understanding of the aggregation, and more structural information of fibrils extracted directly from affected patients from various amyloid diseases, including AD [47]. During the last few years, we have had the privilege of witnessing the approval of the first anti-amyloid drugs for AD. Through continued research, we begin to translate more insight and knowledge into development that, in the end, will lead to better prospects for the individuals suffering from the disease. Before going into more detail on AD, let us first have a closer look at the two protein constituents of plaques and tangles, Aβ and tau, which are the two proteins much of this thesis is devoted towards.

1.4.1 Amyloid-β

Amyloid-β (Aβ) is a group of peptide fragments produced by sequential cleavage of the larger amyloid precursor protein (APP) by β- and γ-secretase (figure 1.6). Various mutations, both in the involved secretases and in the APP itself, affect the distribution and level of Aβ production[48] [49]. Many of the mutations associated with early onset and familial AD increase the overall Aβ production or increase the Aβ 42:40 ratio. The different length variants of Aβ are all found in AD plaque deposits [50], but are found to varying degrees, with Aβ42 and Aβ43 being the most prominent in plaques [51], while Aβ40 being the predominant product of APP processing and constituting 60% of the total Aβ found *in vivo* in CSF [52]. The major remaining Aβ variants, Aβ37, Aβ38 and Aβ42 make up 8 %, 15 % and 10 %, respectively, of the total Aβ in CSF of healthy individuals [53].

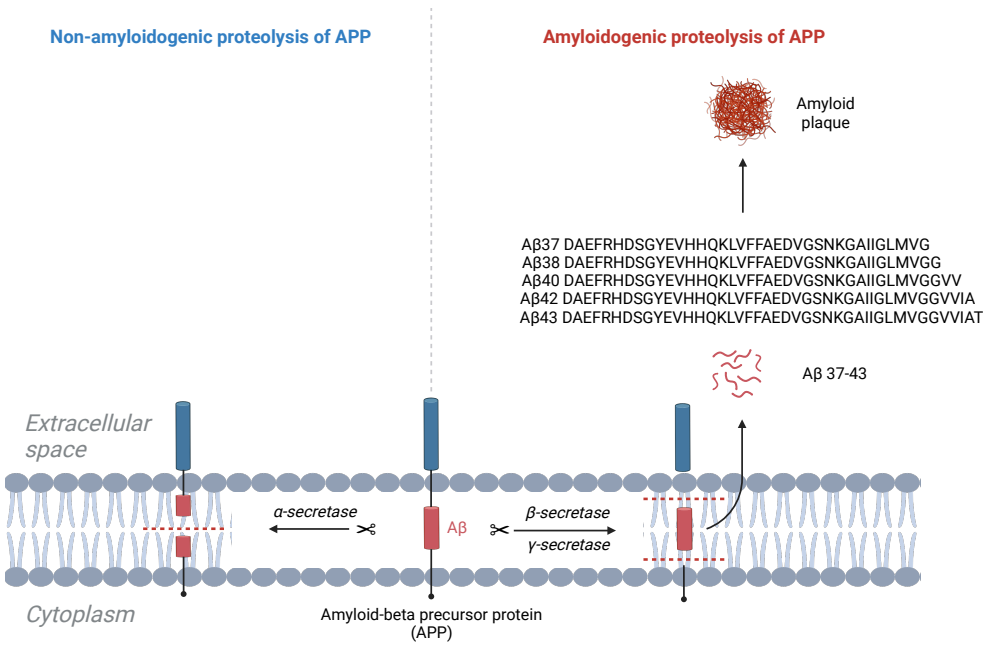


Figure 1.6: Proteolytic processing of the amyloid β precursor protein (APP) results in various Aβ peptides. Cleavage by α-secretase (left) results in non-amyloidogenic peptides that are further processed by the proteolysis machinery. When instead cleaved by β-secretase and γ-secretase (right) amyloidogenic peptides of Aβ 37-43 are generated.

This discrepancy between the total available Aβ variants *in vivo*, and their proportion found in the plaques, could be explained by differences in the aggregation tendency, self-assembly speed and their hydrophobicity. Among the variants, Aβ42 and 43 are the most hydrophobic and the most prone to aggregation [54] and aggregate much faster than $A\beta 40$, 38 and 37 [53].

The level of different Aβ variants in CSF, especially the 42:40 ratio is currently used as an AD biomarker. Interestingly, in AD brain the Aβ42 concentration is greatly reduced compared to that in healthy controls [55]. This is possibly explained by the presence and formation of amyloid plaques in AD [56].

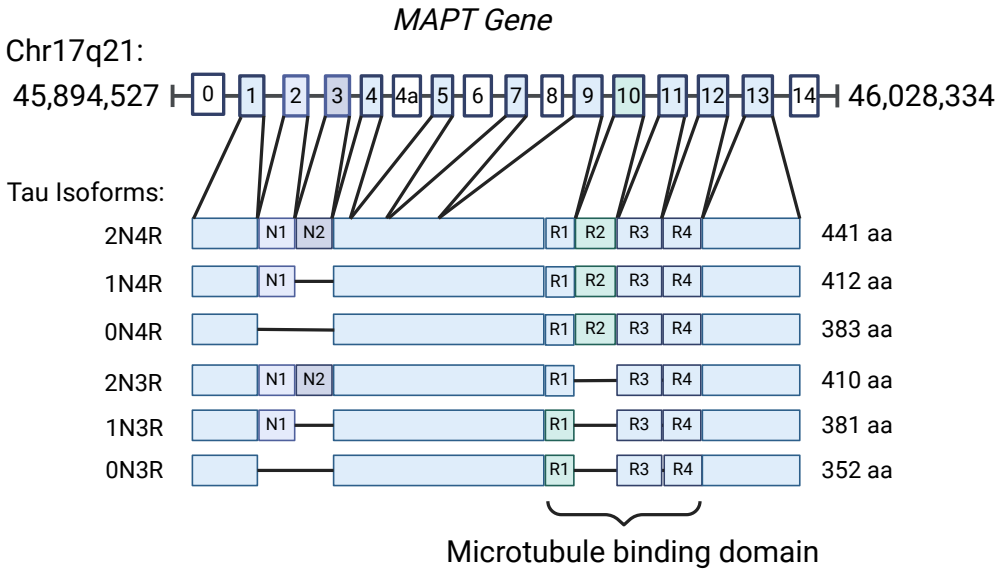
1.4.2 Tau

Tau refers to a group of protein isoforms encoded by the *MAPT* gene. In the developed central nervous system (CNS), alternative splicing of exons 2, 3 and 10 results in the expression of six different isoforms (Figure 1.7) of tau [57]. Tau is predominantly expressed in neurons [58] and the cellular concentration vary around 0.5-2 μM [59] in healthy neurons, while the level of expression and cellular location may vary across different regions of the CNS [60]. Nevertheless, tau is also found in other cell types, such as oligodendrocytes and astrocytes at concentrations lower than those typically observed in neurons [61]. Other isoforms than the six exist, in the fetal brain, a shorter isoform [57] is found and a larger variant is expressed in the peripheral nervous system [62], sometimes called big tau or PNS tau.

Tau was first isolated and characterized as a heat stable protein in the microtubule fraction of porcine brain in 1975 [63]. Its heat resistance is a trait that we exploited in **Paper I** in the purification process after recombinant over expression in *E. coli*. Boiling denatured many of the endogenous *E. coli* proteins, while tau remained soluble and could be further purified from the soluble fraction after removal from the denatured proteins by centrifugation.

Initially, much focus of tau research evolved around its physiological role and in association with microtubles. Tau is believed to serve important roles in the assembly, maintenance and stability of microtubles [63]. It is with the repeat domain that tau exhibits affinity toward microtubles and the 4R isoforms exhibit higher affinity than the 3R isoforms [64]. Interestingly, its the same repeat region of tau that has affinity for microtubles that make up the amyloidogenic core in tau fibrils. The fact that it is the same protein region that makes up tau-tau interactions in fibrils as the tau-microtuble interaction has suggested microtubles acting as both tau reservoir and chaperone and could potentially prevent aberrant tau configurations [65].

After identification of tau as the main protein constituent of neurofibrillary tangles in the AD brain in 1985 [66] [67], another avenue of tau research was initiated. Tau is subject to many post-translational modifications [68], the most investigated being phosphorylation. In AD tau has been found to be hyperphosporylated [69] and the level of phosphorylated tau in blood and CSF is promising biomarkers for AD diagnosis [70]. When tau is phosphorylated, its affinity towards microtubles is typically reduced [71], and as a consequence, less tau is bound to microtubules and more free tau becomes available in the cell to aggregate. Tau is not only involved in AD, but is also found aggregated in other neurodegenerative diseases, collectively referred to as tauopathies.



Amino acid sequence of 2N4R:

MAEPRQEFEVMEDHAGTYGLGDRKDQGGYTMHQDQEGDTDAGLKESPLQTPTEDGSEEPGS
ETSDAKSTPTAEDVTAPLVDEGAPGKQAAAQPHTEIPEGTTAEEAGIGDTPSLEDEAAGHV
TQARMVSKSKDGTGSDDKKAKGADGKTKIATPRGAAPPGQKGQANATRIPAKTPPAPKTPP
SSGEPPKSGDRSGYSSPGSPGTPGSRSRTPSLPTPPTREPKKVAVVRTPPKSPSSASRLQT
APVPMPDLKNVKSKIGSTENLKHQPGGGKVQIINKKLDLSNVQSKCGSKDNIKHVPGGGSV
QIVYKPVDLSKVTSKCGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGSLDNITHVPGGG
NKKIETHKLTFRENAKAKTDHGAEIVYKSPVVSGDTSPRHLSNVSSTGSIDMVDSPQLATL
ADEVSASLAKQGL

Figure 1.7: Alternative splicing of exons 2, 3 and 10 in the transcript of the *MAPT* gene results in six different tau isoforms, ranging from 352 - 441 amino acids in length. The amino acid sequence of 2N4R is shown with 304-380 marked red. Inclusion or exclusion of exon 2 and 3 in the N-terminal protrusion region, and of the R2 repeat in the microtuble binding region has resulted in nomenclature typically used to refer to the different tau isoforms. Gene coordinates according to ENSG00000186868, located on chromosome 17q21, 45,894,527-46,028,334 forward strand GRCh38:CM000679.2.

Interestingly, the protafilament fold of tau fibrils varies across the different tauopathies (Figure 1.8) and the fibril morphology is disease specific.

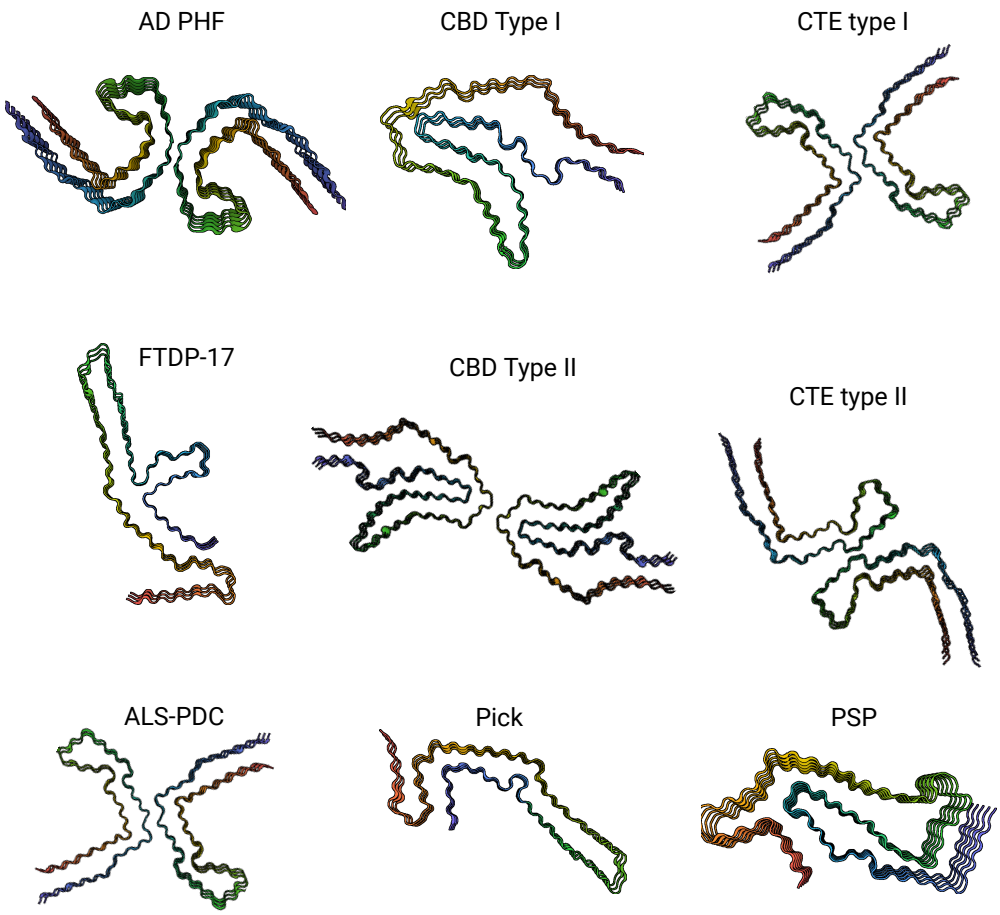


Figure 1.8: Atomic structure models of *ex vivo* tau fibrils from different tauopathies. Tau display a wide range of different polymorphs and vary across different tauopathies. AD PHF (5O3L), CBD Type I (6NWP), CTE Type I (6NWQ), FTDP-17 (9GG0), CBD Type II (6TJX), CTE Type II (9ERN), ALS-PDC (8OTG), Pick (6GX5), PSP (7P65)

1.4.3 The Amyloid cascade hypothesis

In early stages of AD, Aβ aggregation and plaque formation typically precedes tau tangle formation. Interestingly, molecular interactions between Aβ oligomers and kinases that phosphorylate tau has been identified [72] [73] and support the amyloid cascade hypothesis, a hypothesis that has been central in the AD field and links the two molecular players Aβ and tau.

The amyloid cascade hypothesis proposes Aβ plaque deposition as the main pathogenic driver in AD. The deposition of Aβ plaque in turn sets of a cascade of events including generation of toxic Aβ oligomers, causing neuronal damage, tau hyperphosporylation and subsequent tau tangle formation, activation of the brain's immune cells, microglia and asrocytes, leading to chronic inflammation and progression of AD. The hypothesis has been one of the leading perspectives on AD onset and progression in the field, since its introduction in the early 90's [74]. Although also being subject of much debate and skepticism. One of the main arguments against the hypothesis is the lack of a strong correlation between Aβ plaque load and cognition in AD [75] [76]. Furthermore, emerging data from clinical trials, both successful and failed ones, and the effects of approved antibodies paint a picture that suggests that the story of AD and its treatment is more complicated than just removing amyloid plaque. The removal of amyloid plaque by anti amyloid antibodies has largely failed and does not restore cognition [77]. Apart from the FDA approved aducanumab and lecanemab, the amyloid cascade hypothesis has not yet led to drugs or disease modifiers that significantly alters the clinical outcome of AD [78]. Regardless of the outcome of thousands of clinical trials the amyloid cascade hypothesis continues to shape our thinking about AD. Other perspectives exists, like the loss of function of soluble Aβ and tau, rather than the gain of a toxic function by aggregation, but receives limited attention.

1.4.4 Innate defense against AD

Molecular chaperones, mentioned in the very first paragraph of this chapter are important for proteins to adapt and maintain their correct folding. A part of this job extends also to prevent protein misfolding and aggregation. Multiple molecular chaperones are expressed in the CNS where they play an important role in the prevention of protein aggregation onset.

Correct expression levels and activity is crucial for maintaining proteostasis. Thus, they can be seen as one of the body's own defense mechanisms against protein misfolding and amyloid formation. Many of the chaperones achieve this function, "Up the thermodynamic ladder" through active mechanisms involving ATP hydrolysis. One chaperone expressed in the CNS and neurons, which does not rely on ATP hydrolysis for its function, is DNAJB6b. It acts as a co-chaperone with one of its functions being the recruitment of substrates to the

ATP dependent HSP70 complex. In addition to recruitment to HSP70, DNAJB6b also exhibits chaperone activity on it own. It has been shown to be a potent inhibitor of Aβ42 aggregation through interference with primary and secondary nucleation, as well as elongation [79]. Furthermore, DNAJB6b has been shown to increase the apparent solubility of Aβ42 [80], possibly by forming co-aggregates with a higher Aβ42 chemical potential compared to that achieved in pure Aβ42 fibrils [81]. The ability of DNAJB6b to both slow down the aggregation and raise the apparent solubility of an amyloid peptide, illustrates a promising property of molecular chaperones. The delayed self-assembly onset and reduced fibril formation speed, as well as the increase in apparent solubility could very well be all the proteolysis machinery needs to keep the balance in check and prevent protein aggregation diseases. These properties emphasize the therapeutic potential of effectively targeting multiple of the microscopic steps of amyloid fibril formation. Future therapeutic strategies might just be successful by having such multifaceted capacity and could very well require different approaches depending on where in the disease development the individual patient is. A better understanding of the evolutionary endogenous defense mechanisms against protein misfolding and amyloid fibril formation, could aid in future therapeutic development and hopefully be able to mitigate this terrible disease.

Chapter 2

Methods

During my PhD studies, I have been fortunate to be able to use and learn an array of different methods and techniques, as well as be able to develop new methodologies. This chapter will not cover them all exhaustively, but I will highlight core methods used in many of the thesis papers.

2.1 Recombinant protein production & purification

Paper I, II, III, IV and V

All work in this thesis rely on pure recombinant proteins. The generation of recombinant proteins to work with often start with overexpression in *E. coli* before various purification strategies. In brief, this is made possible by first codon optimizing the gene of interest for expression in *E. coli*, before insertion of the gene into a suitable expression plasmid. The plasmid is then transformed into an expression strain and the protein is overexpressed. The *E. coli* cells are harvested and broken open. Now you have a mixture of your protein and the thousands of endogenous *E. coli* proteins. Next follows various purification strategies and methods in order to isolate your protein of interest from all the *E. coli* proteins. Separation may be achieved through utilizing the various physicochemical properties of proteins, to give two examples I will mention the first steps of purification after lysing *E. coli*, during the purification of tau and Aβ42, both used in this thesis. A large proportion of tau is found in the soluble fraction after cell lysis and centrifugation. The next step involves boiling the supernatant, in which a large proportion of the soluble endogenous proteins precipitate, while tau remains in solution. Already at this point a large majority of the proteins present at start has been removed. On the other hand, Aβ42 is highly aggregation prone and forms inclusions in *E. coli*, this is a trait we utilize and purify the peptide from these precipitates, through dissolving them in Urea. Next follows multiple steps of ion exchange chromatography (IEX) and size exclusion chromatography (SEC) (Figure 2.1) in

which proteins may be separated from each other depending on their surface charge and size. By playing around with pH while performing different types of IEX, using either positively or negatively charged resins, proteins of various surface charge may be separated from each other. The degree of separation is often monitored through the elution profiles obtained by surveying the fractions absorbance at 280 nm (Figure 2.1 C) or by running the eluted fractions on an SDS-PAGE gel. Doing so gives insights into sample purity and effectiveness of the separation steps. High sample purity is crucial for reproducibility when performing amyloid fibril formation kinetics, and trace amounts of contaminants may cause irreproducible results. This also applies to sample inhomogeneity due to the presence of self-assembled species, a crucial component of reproducible amyloid kinetics is being able to start with a very homogeneous monomer solutions at the beginning of experiments. A philosophy that permeates our lab when its comes to protein purification is to always sacrifice yield for purity, and whenever possible attempt to develop tag free purification strategies rather than using strategies relying on affinity tags.

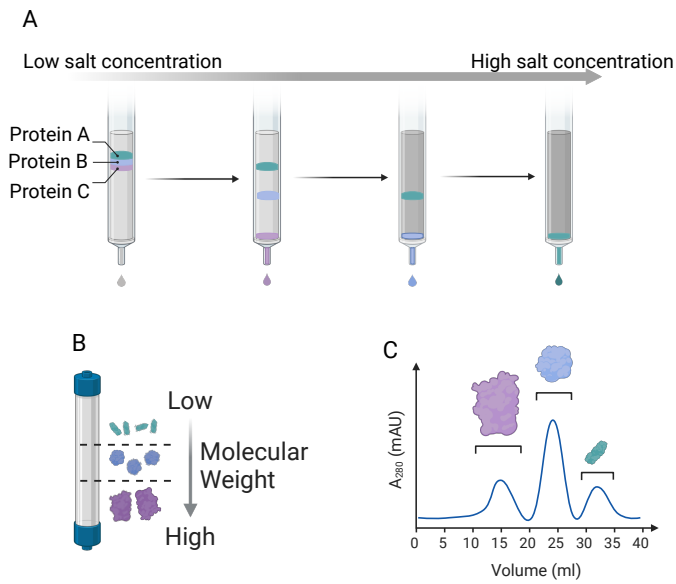


Figure 2.1: Proteins in complex sample mixtures may be separated from each other utilizing their physicochemical properties (A) illustrate the principle of ion exchange chromatography, proteins are separated based on their surface charge, often on a resin of opposite charge and commonly eluted through a salt gradient to achieve sharp elution profiles. (B) Shows the principle of size exclusion chromatography, another commonly employed separation method, in which proteins are separated based on their relative size and shape rather than any interaction. Particles of larger size travel faster through the column. (C) illustrates the obtained chromatogram when monitoring the elution profile of (A) & (B).

2.2 Amyloid binding reporter dyes

Paper I, II, III, IV and V

Aggregation kinetic data obtained in this thesis rely on the fluorescent signal obtained by the dyes thioflavin-T (ThT) and X34 upon binding to amyloid fibrils. In solution both dyes have large rotational freedom and low fluorescence, upon binding to fibrils the rotational freedom is restricted, and the fluorescent emission signal is both enhanced and red shifted. The fluorescent signal enables the detection of amyloid fibrils through fluorescence microscopy, which has found practice in histology and postmortem diagnosis. The shift in the emission signal has also enabled the detection of fibrils in a time-resolved manner, in bulk assays, through fluorescence spectroscopy. Performing time resolved kinetic measurements of fibril formation has been invaluable in establishing the microscopic steps of amyloid fibril formation discussed earlier in this thesis. In my work, being able to perform "on the fly" assays using ThT and X34 flourescence has been crucial. I have used these dyes for both quality control purposes, to ask questions like whether or not my sample has formed fibrils yet, and in more systematic ways for entire studies where these dyes have enabled following the self-assembly reactions as a function of time by reporting on the increase of fibril mass through the increase in fluorescence signal (figure 2.2).

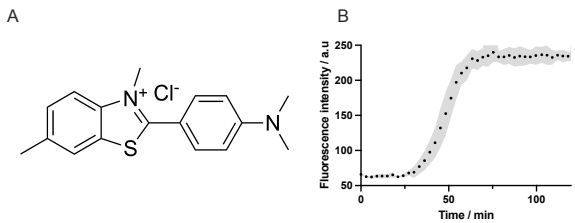


Figure 2.2: The chemical structure of the amyloid binding dye thioflavin-T (A) and the increase in fluorescence intensity as 5 μM Aβ42 self-assembles and form fibrils in the presence of 2 μM ThT

X34 a Congo red derivative also binds amyloid fibril in a similar way to ThT, but yield slightly higher intensity than ThT when binding to tau fibrils.

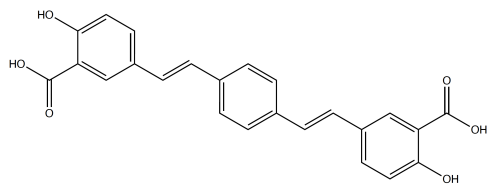


Figure 2.3: The chemical structure of the amyloid binding dye X34.

2.3 Solubility studies: separation of monomer and fibrils and subsequent monomer quantification

Paper I & III

When investigating the solubility of amyloid proteins, there are three major obstacles that must be overcome in order to get good results. First, the self-assembly process needs to reach equilibrium on a time scale where the peptide does not have time to be broken down by proteases or bacterial growth. This becomes an issue especially when working at 37 °C, but can be mitigated by working as cleanly as possible, by sterile filtering the buffers and by supplementing with 0.02 % Na_2N_3 to prevent bacterial and fungal growth. To this end, for the solubility study on tau, the self-assembly mechanism was accelerated by mechanical stirring.

When equilibrium is reached, the precipitated fibrils must be separated from the solution phase in order to quantify the soluble monomers accurately. In the solubility studies, both centrifugation and filtration were explored and found to be viable strategies for separating monomers from fibrils. An array of different filters and tube materials was explored in order to maximize separation efficiency and minimize artifacts introduced by surface adsorption of the monomer to the filter material and tube walls.

Lastly, when the monomers and fibrils have been separated the remaining monomer must be quantified. In this work, the proteins intrinsic absorbance, the fluorescence obtained through derivatization and radioactivity through labeling has been used. The investigated fragment of tau and the Aβ40 peptide contain one tyrosine each, which gives rise to the absorbance at 280 nm with an extinction coefficient of approximately 1490 M^{-1} cm^{-1}, this gives rise to a detectable signal, but at lower concentrations detection becomes challenging. In addition to absorbance at 280, the deep UV backbone absorbance of the peptide backbone has been used. For quantification purposes, absorbance has mainly been used after reverse phase HPLC, and this has enabled the detection of potential degradation of the sample during incubation. Another advantage of HPLC is the possibility of quantifying your sample in a more complex sample matrix. Furthermore, larger sample volumes may be injected and lower the limit of quantification.

Another strategy that is less time consuming and less dependent on expensive instrumentation, and a way to enhance the limit of quantification of monomers in solutions is to derivatize them with o-phthalaldehyde (OPA) (2.4). OPA reacts with primary amines, which include the lysine sidechains and the N-terminus of peptides. The unreacted dye itself is non-fluorescent, while the derivatized amino acid product becomes fluorescent and allows quantification across many orders of magnitude down to the nanomolar range. This enables accurate and sensitive quantification of peptides. One large drawback of the method

is that it measures total protein concentration and thus requires that the samples are pure, and a standard curve is needed for quantification.

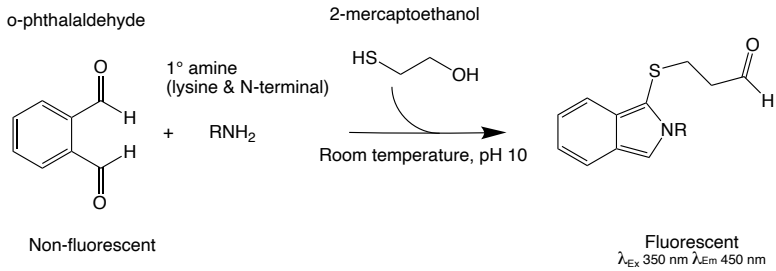


Figure 2.4: The mechanism of peptide o-pthaldehyde derivatization.

Lastly, peptide quantification has also been done through liquid scintillation counting (LSC). This procedure is overall more time consuming but with the advantage that quantification is possible in complex sample matrices, without the need of sample purification (figure 2.5). This method requires radiolabeling of your protein of interest, in this work with ^{3}H, achieved through expressing the protein in *E. coli* using minimal media supplemented with tritiated glucose as sole carbon source.

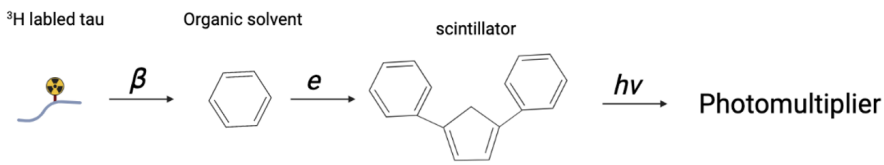


Figure 2.5: Schematic overview of liquid scintillation counting. Quantification is possible through using a radiolabled peptide. The emitted particle by radioactive decay interacts and excites the solvent, which in turn transfers its energy to a scintillator molecule. When the electron of the scintillator returns to the ground state the emitted photons is counted thorugh a photomultiplier. This allows for sensitive quantification of the radiolabeled peptide through a standard curve.

Combined these three strategies of protein quantification have offered a versatile tool case through which different aspects of protein solubility has been interrogated.

2.4 Cryo-TEM

Paper II & IV

Cryogenic transmission electron microscopy (cryo-TEM) provides a window into the protein world and allows us to get a glimpse down on the nanometer scale. Sample preparation with your protein of interest captured in vitrified ice is a prerequisite for imaging with cryo-TEM. This is achieved through flash freezing thin layers of your protein sample deposited on small grid supports (figure 2.6)

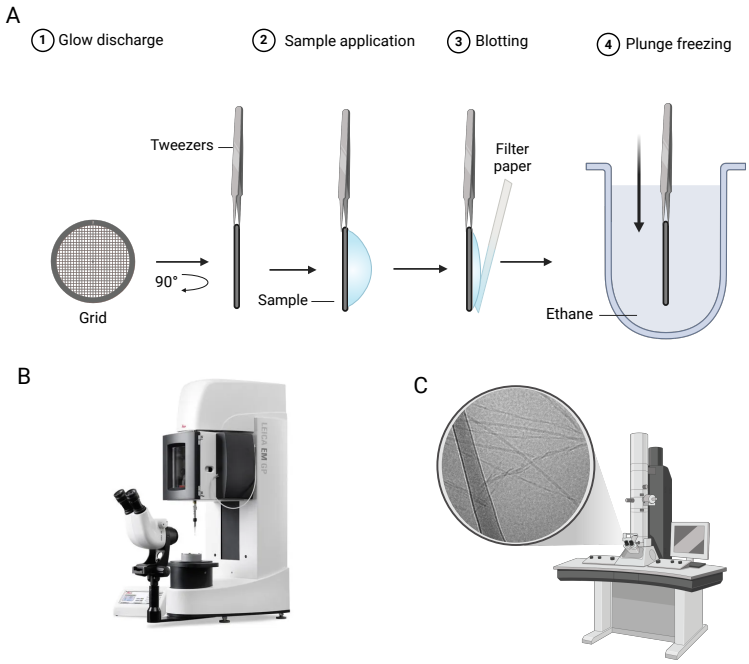


Figure 2.6: Entrapment of the sample in vitreous ice is crucial for cryo-TEM imaging. This is achieved through flash freezing the sample as illustrated by the schematic workflow in (A). The sample preparation is achieved semi automatically using a plunging robot illustrated in (B). Lastly the grids are loaded and imaged in an electron microscope (C) which enables imaging of amyloid fibrils on the nanometer scale.

This process is semi-automated to happen so rapidly that the thin film is frozen so quickly that the water molecules do not have time to nucleate to form a crystalline phase, but rather form vitreous ice. The sample solution is applied to glow discharged grids and surplus sample is blotted away. Next the grid is rapidly submerged in liquid ethane cooled by liquid nitrogen. Next imaging may be performed in an electron microscope using focused accelerated electrons to image your protein of interest.

Chapter 3

Summary & Discussion of papers

3.1 Paper I: A label-free high-throughput protein solubility assay and its application to Aβ40

This work was initiated in order to develop a platform on which intrinsic and extrinsic factors of amyloid solubility could be investigated. Efforts have been made in the literature to investigate the solubility of amyloid proteins, but many of these endeavors suffer from being complicated, expensive and requiring specialized infrastructure and knowledge. Furthermore, there is a large discrepancy among the reported values of solubility for Aβ40 solubility in the literature [42][82][83][84][85][86].

Methodology developed and employed in the lab prior to this project had already reported on the solubility of the Aβ [42] [87], which made it the perfect peptide to use in order to develop new methodology.

As mentioned in section 2.3, measuring solubility of amyloid proteins can be divided into three distinct obstacles that must be overcome. First, the supersaturated monomers must self-assemble into fibrils and the equilibrium between monomers in solution and fibrils must settle. Our operational interpretation is that equilibrium has been reached when we can no longer observe changes in the monomer concentration over time. Nevertheless, we can never be sure that equilibrium has been reached, unless the same concentration is approached from the opposite direction, namely, by dissolving fibrils. We define the observed value as "*apparent solubility*" as we cannot be completely certain that equilibrium has been reached, as well as what is measured are all soluble species, monomers, dimers, oligomers etc. Secondly, once equilibrium has settled, in order to measure the equilibrium monomer concentration, one must separate the free monomers from the fibrils. Lastly, the monomers must be quantified. In short the three obstacles outlined are overcome successively (Figure 3.1). First, by a temperature jump, monomers of various starting concentrations kept

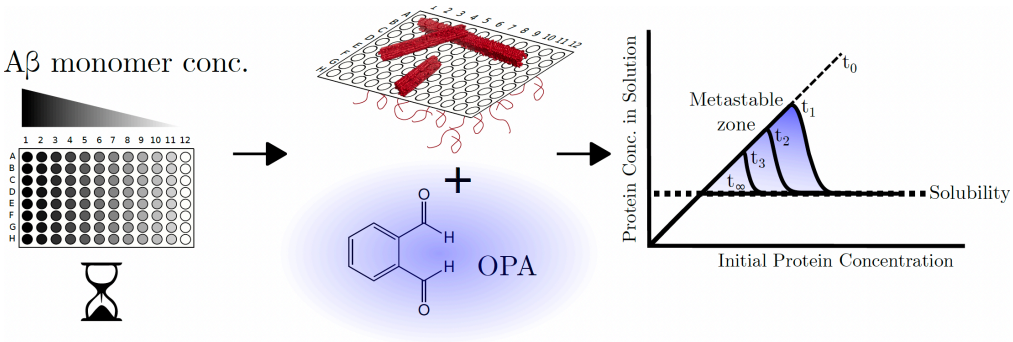


Figure 3.1: The solubility of the Aβ40 peptide is determined by allowing monomers to form fibrils and the equilibrium between them to settle. Thereafter, monomers are separated from fibrils through filtration. Lastly, remaining monomers are quantified through fluorescent intensity after derivatization with OPA.

on ice were rapidly moved into metastable conditions at 37 °C where they are provided time to nucleate and form fibrils until no change in monomer concentration is observed. As a function of time along the aggregation pathway, monomers and fibrils are separated through filtration, before being derivatized with OPA to enable quantification through fluorescence intensity detection.

After optimization of the methodology, we carried out a careful evaluation of filter efficacy and investigation of the limit of quantification the quantification strategy. The developed methodology was employed to investigate the effect of temperature, salt and the effect of the initial methionine in the sequence, on the final solubility of the peptide (figure 3.2).

The developed methodology provides an inexpensive and high throughput platform through which the effects of intrinsic properties, such as mutations, as well as extrinsic factors, including temperature, pH, salts, and potential drug candidates, on the solubility may be explored. One significant drawback of the methodology is that the quantification method relies on pure peptide and will not work if other primary amines are present. The methodology is versatile and the principles used should be able to be applied to any amyloid system.

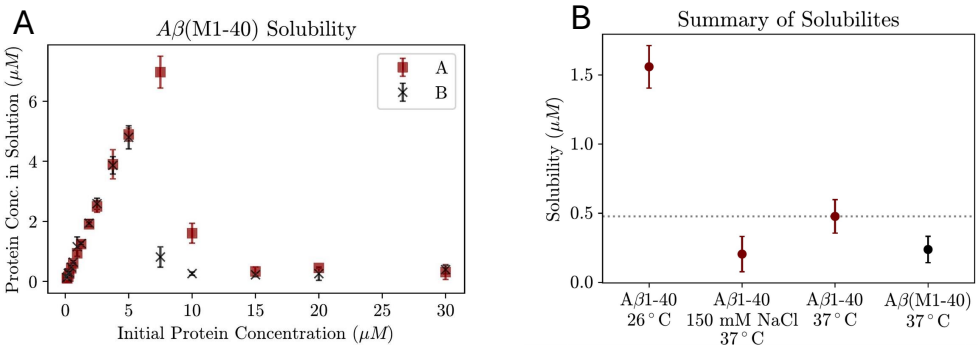


Figure 3.2: The apparent solubility of the Aβ40 peptide as a function of initial monomer concentration after incubation at 37 °C for 25h in 20 mM sodium phospate, 0.2 mM EDTA, pH 7.4 (panel A) red squares and black cross denoted A and B represent identical experiments performed on two different days, error bars = SD, N=4. (Panel B) Solubilities of Aβ40 at 26 °C, in the presence of 150 mM NaCl, without and with the methionine start codon.

3.2 Paper II: Proliferation of Tau 304–380 Fragment Aggregates through Autocatalytic Secondary Nucleation

The high solubility of *in vitro* full-length tau has hampered research efforts that focus on the underlying molecular mechanisms leading to tau tangle fibril formation. To accelerate amyloid formation and overcome high nucleation barriers, negatively charged polyanions like heparin or RNA are commonly added to trigger the fibril formation of tau. Here we take another approach and remove the flexible regions flanking the amyloid core in AD, and find that this fragment of tau (304-380C322S) spontaneously self-assembles and forms amyloid fibrils under mild buffer conditions, without the use of external inducers, within reasonable laboratory time frames (Figure 3.3 C). This enabled further studies of the rate constants of the microscopic steps of fibril assembly.

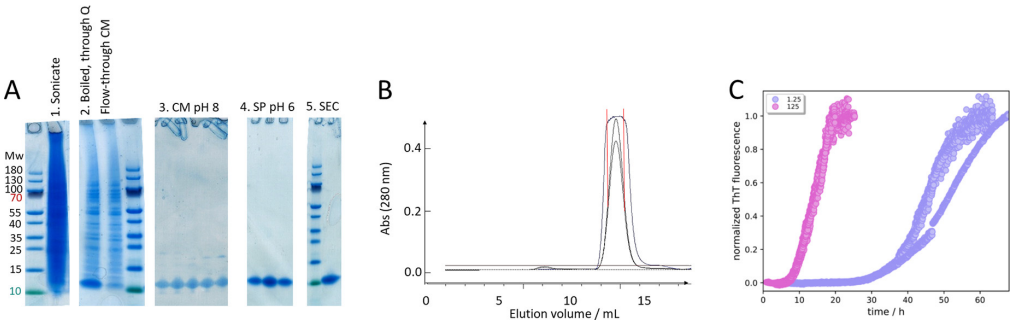


Figure 3.3: Tau304-380C322S is readily overexpressed in *E. coli* and may be purified efficiently without any protein tags. (A) shows the SDS-page gel after each purification step, (B) the chromatogram after monomer isolation by size exclusion chromatography and (C) the kinetic traces monitored with 2 μM reporter dye X34.

First we developed a tag free purification strategy that yields large amounts of ultra pure tau 304-380C322S (Figure 3.3). We found that this fragment of tau readily self-assembles and forms fibrils under mild buffer conditions (20 mM sodium phospate, 0.2 mM EDTA pH 8) without the use of external inducers.

This enabled a systematic study of the microscopic steps of tau amyloid fibril formation in the absence of any external inducing molecules. The aggregation of the tau fragment displays monomer concentration dependent aggregation, with decreasing half-times ($t_{1/2}$) as initial monomer concentration increases (figure 3.4).

Fitting of the kinetic traces using a primary nucleation elongation model (figure 3.4 B) yields fits that describe the data poorly. While models containing secondary nucleation or fragmentation, both yield good fits (figure 3.4 C & D). When kinetic experiments were performed in the presence of preformed seeds, the quality of the fits was similar when using

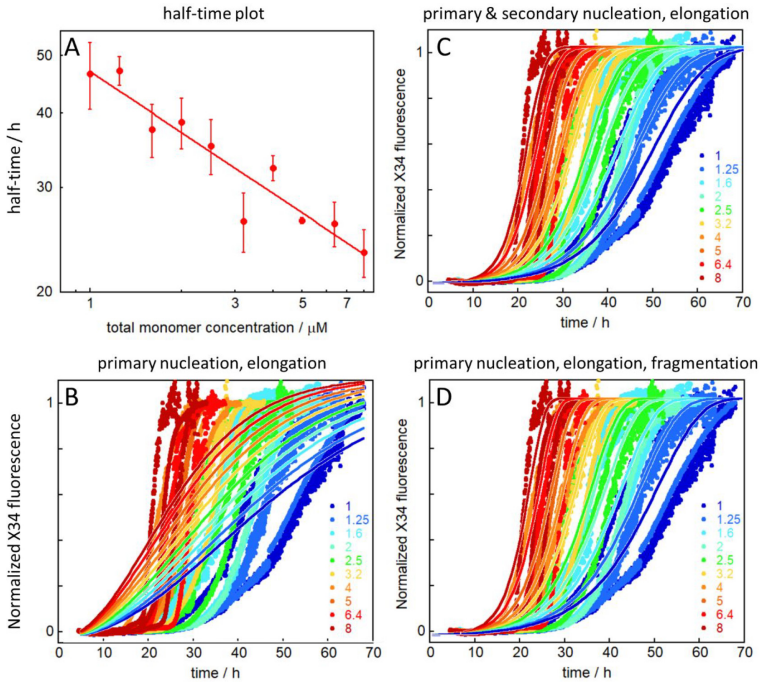


Figure 3.4: Tau 304-380C322S readily self-assembles and forms fibrils under mild buffer conditions, 20 mM sodium phospate, 0.2 mM EDTA, pH 8. (A) shows the $t_{1/2}$ as a function of initial monomer concentration, with a fitted power function with an exponent γ=-0.65. The rest of the panels contain normalized kinetic traces of triplicates with varying initial monomer concentration with the solid lines represented by global fits obtained using, (B) a primary nucleation K_n, elongation K_+ model, (C) primary nucleation K_n, multi step secondary nucleation K_2 and elongation K_+ model, lastly (D) using primary nucleation K_n, elongation K_+ and fragmentation K_- model.

either the secondary nucleation or fragmentation models. Therefore, the relative contribution from secondary nucleation and fragmentation could not be discerned from these kinetic experiments alone.

In order to distinguish whether the secondary processes of aggregate proliferation occur through secondary nucleation or fragmentation we performed experiments using a mixture of isotope labeled ^{15}N and unlabeled tau. This enables the determination of the origin of smaller species and oligomers, whose generation could be catalyzed by secondary nucleation or the fragmentation of already formed fibrils.

Unlabeled ^{14}N monomers were supplemented with labeled ^{15}N seeds and allowed to form fibrils until reaction half-time was reached. The formed fibrils were then separated from the oligomers and monomers by centrifugation. Subsequently, the remaining monomers and oligomers in the supernate were further separated by size exclusion chromatography (refer to 3.5 for a schematic of the experimental procedure).

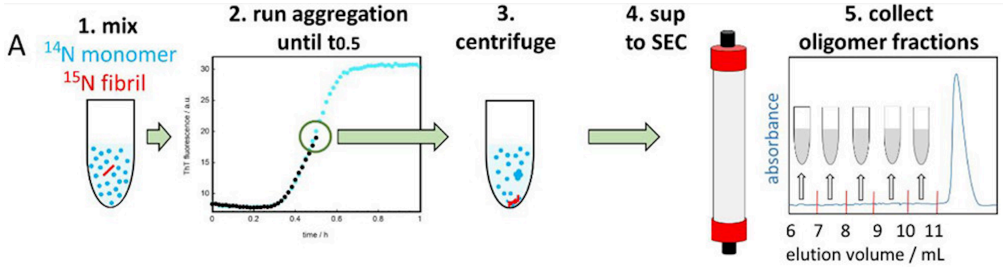


Figure 3.5: To determine the origin of oligomers during tau aggregation, 10 µM N14 monomers were supplemented with 1 µM N15 labeled seeds. The self-assembly reaction was allowed to proceed to $t_{1/2}$ upon which the formed fibrils was removed from the supernatant through centrifugation. The remaining supernatant was subjected to size exclusion chromatography, and fractions along the elution profile was collected and analyzed through MALDI-TOF mass spectrometry.

Fractions of larger size eluting before the remaining ^{14}N monomers were collected and analyzed with MALDI-TOF MS. In the oligomer fractions eluting before the monomer, we only detect ^{14}N tau. Thus, oligomer species has originated from secondary nucleation rather than fragmentation. Although the absence of evidence is not evidence of absence, given the high sensitivity of MALDI-TOF MS, and the lack of observable ^{15}N signal, we conclude that negligible amounts of the tau in the oligomers originate from the ^{15}N labeled seeds. This finding, or rather the lack thereof, suggests secondary nucleation of monomers on the fibril surface, rather than fragmentation of existing fibrils, is the major origin of oligomers. Insight into the mechanism of oligomer formation could provide clues about potential drug targets in the prevention of toxicity in AD and other tauopathies. The lack of ^{15}N species also suggest that the observed proliferation of tau aggregates occurs predominantly through autocatalytic secondary nucleation and not through fragmentation.

In conclusion, this work provides valuable strategies for the purification and kinetic characterization of tau. This in turn creates a foundation that could be extended for the characterization of other tau fragments or even full-length tau variants, whenever good strategies for their aggregation onset is established. Furthermore, aspects such as potential seeding potency of the current tau fragment *in vivo* but also in seeding other variants of tau remain yet to be explored.

3.3 Paper III: On the solubility and metastability of the amyloidogenic core of tau

The methodology developed to investigate the solubility of amyloid fibrils in **Paper I**, combined with the identification of a tau fragment that readily self-assembles into amyloid fibrils in **Paper II** now made it possible to examine the solubility of this fragment. Unlike the results obtained when measuring the solubility of Aβ40 in **Paper I** , tau showed much greater variability in the observed apparent solubility. Sometimes, even after being incubated for extended periods of time, it seemed as if the reactions were far from reaching their equilibrium. This somewhat ambiguous behavior of tau obstructed this project but also served as the inspiration for initiating the work that led to **Paper IV**.

One strategy of accelerating amyloid fibril formation is through agitation. Following the free monomer concentration of tau during the aggregation process, accelerated by stirring and starting from two different supersaturated solutions, led both of them to converge towards ≈ 6.1 nM (figure 3.6). The free monomer concentration was measured with HPLC-UV, after removal of fibrils by centrifugation. This approach resulted in a considerably lower apparent solubility than if the system instead was seeded under more quiescent conditions.

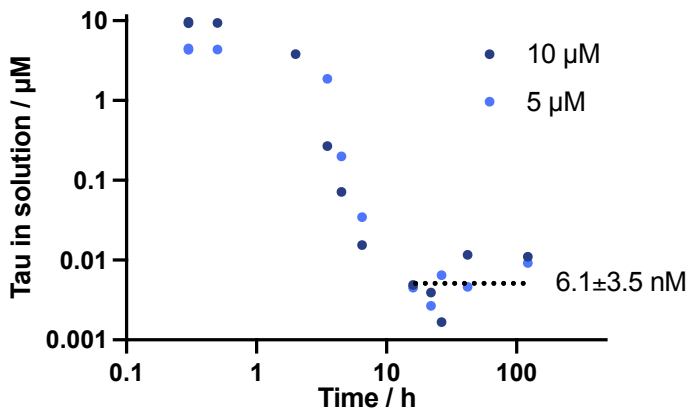


Figure 3.6: Convergence of tau monomers toward equilibrium accelerated by stirring. The concentration of free tau monomer as a function of time on log-axis. Starting from 10 µM (dark blue) and 5 µM (light blue) freshly purified tau monomers in 20 mM sodium phosphate, 0.2 mM EDTA, 0.02% NaN_3 pH 8.0 at 37 °C. The free monomer concentration is quantified by HPLC-UV after removal of fibrils by centrifugation. The plateau concentrations observed after 16-122 h were used to calculate the apparent solubility and standard deviation of 6.1 ± 3.5 nM (n=10).

Next, we tried to figure out how stirring could have such a dramatic effect on the apparent solubility compared to seeding. The monomer system was incubated with or without stirring at 37 °C and the free monomer concentration was followed as a function of time (Figure 3.7b). After 240 hours of incubation, the stirring was stopped in the sample stirred from the beginning and stirring was started in the initially idle sample. In a separate sample, an idle sample were supplemented with 1% seeds and kept idle, see figure 3.7a for a schematic illustration of the experimental setup.

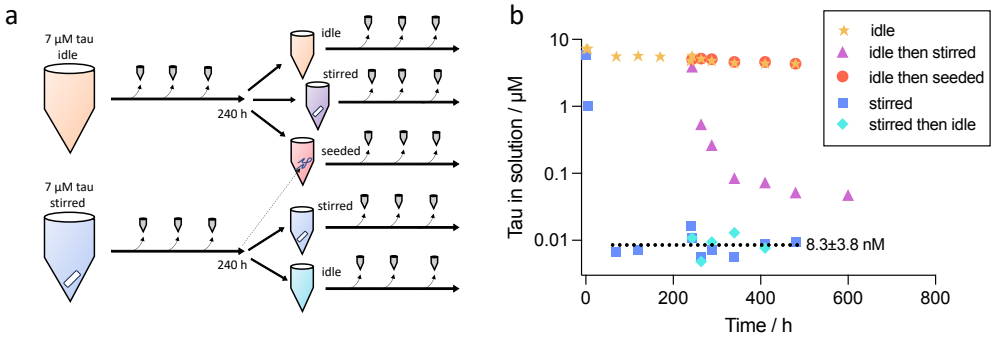


Figure 3.7: The solubility of tau and the effect of agitation through mixing. (a) schematic outlining the experimental setup: 7 µM tau was incubated at 37 °C with or without stirring. Samples were removed as a function of time for free monomer quantification seen in figure (b). After 240 h the idle sample were divided into three samples: one continued idle, in another stirring was initiated and a third was also kept idle, but 1 % seeds were introduced. The initially stirred reaction was divided into two: one was kept stirred and the other was now kept idle. (b) Quantification of the free tau monomer using HPLC UV-absorbance. The last 9 time points of the continuously stirred sample were used to calculate the mean free monomer concentration of 8.3 ± 3.8 nM.

The idle samples largely remained supersaturated during the duration of the experiment. Surprisingly, this also seems to be the case when seeds are introduced in the idle reaction (red circles, figure 3.7b). If stirring was initiated in the from the beginning idle reaction, the metastable regime collapses, though it appears to plateau at a higher apparent free monomer concentration than if stirred from the beginning of the reaction. One reason for the higher apparent free monomer concentration in the initial idle then stirred reactions could be degradation or other chemical modification that alters the system as a consequence of prolonged incubation at 37 °C. The degradation time is limited when samples are stirred from the beginning and the apparent equilibrium is reached quickly.

Different ways of quantifying the monomer concentration after separation from fibrils were explored (Figure 3.8). One notable difference between the quantification methods is that the lower value obtained with HPLC only uses the integral of the intact tau monomer peak,

while the OPA and liquid scintillation counting (LSC) methods quantify the total protein in solution and do not discriminate between the intact tau monomer or otherwise modified or degraded protein. This probably contributes to the discrepancy between the values of the different methods. The separation with HPLC prior to quantification excludes any signal originating from species with different retention times than that of the unmodified tau monomer. Small peaks not present at the beginning of the reaction were observed in the chromatogram after prolonged incubation. The different strategies of quantification described so far, all have drawbacks as well as advantages. Using OPA for quantification is very quick and inexpensive, but a requirement is pure samples, since it will react unspecifically with any primary amine present in the system. LSC requires isotope labeled proteins and in the same manner as OPA it does not discriminate between intact or otherwise modified monomer. However, the use of isotope labeled protein enables quantification in the presence of other molecular players or in more complex sample matrices like blood or CSF. Lastly, HPLC requires more optimization but also enables quantification in more complex matrices and has the advantage that it is able to detect potential modifications and degradation in the sample.

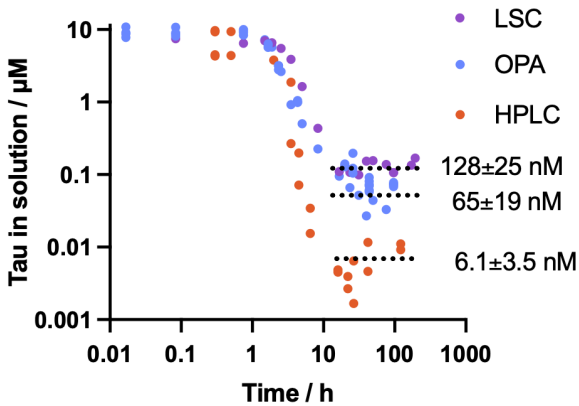


Figure 3.8: Different strategies of free monomer quantification after removal from fibrils. The free monomer concentration of tau was followed starting from 10 µM monomers in (purple) quantified through LSC, (blue) through OPA and orange with HPLC

The developed methodology in **Paper I** as well as the additional strategies for quantification and reaching apparent equilibrium introduced here in **Paper III** serves as a platform through which further insights regarding both extrinsic and extrinsic factors may be explored. Especially in the case for tau, one may wonder how large and what effect the fuzzy coat, the flexible regions flanking the amyloidogenic core has on the solubility of the fibril.

3.4 Paper IV: The role of shear forces in primary and secondary nucleation of amyloid fibrils

The work that ultimately led to "**Paper IV**" started as an odd observation during the work on a fragment of tau spanning amino acids 304-380. There was a growing suspicion that the aggregation of tau was occurring at a lower rate or not at all in samples for which the aggregation was not actively being monitored in a plate reader. The focus of the tau project in **Paper III** was to investigate the sample at the end stage after fibril formation had occurred. Therefore, the samples were in several cases not monitored in a plate reader during the aggregation reaction, but were instead just incubated. Initially, the observation that only samples that had been studied in the plate reader formed fibrils was attributed to surface materials in the 96-well plates versus the tubes, which could potentially cause differences in heterogeneous primary nucleation, and possibly also to the presence of fluorescent dyes in the reaction mixture.

To identify the origin of the phenomenon, we came up with a simple experiment in which freshly purified tau was incubated for 72 hours completely quiescent at 37 °C in an incubator, before being placed and monitored in a plate reader. Another sample of the same preparation was monitored directly in a plate reader (see Figure 3.9 for a schematic overview of the experiment). Two identical solutions of tau monomers are expected to self-assemble and form fibrils within the same time frame if the reaction rates in the two solutions are the same. However, the results in Figure 3.9 suggest that the fragment was still in a monomeric state after being incubated for 72 hours. When moved to a plate reader after incubation, a typical sigmoid of fibril formation was observed.

Many colleagues in the group were pursuing projects focusing on the kinetics of amyloid fibril formation. When figure 3.9 was presented at a group meeting, the finding sent a small shock wave through the room, and not all attendees were fully convinced. The kinetic traces told an indisputable story that fueled us with curiosity and guided us down a path of discovery. Soon after, any doubts about the effect being only tau specific was ruled out when the effect was detected across other amyloid systems (Figure 3.10). After an extensive literature search, it became evident that vigorous agitation was commonly employed to speed up fibril formation, but less was known about the very mild shear unintentionally introduced when 96-well plates are being moved on its stage past the fluorescent detector in plate readers. This was not just an artifact, but rather a generic physical phenomena that affected the self-assembly of multiple different amyloid proteins. Systematically studying it, in turn helped us better understand the self-assembly reaction. When it became evident that the phenomenon had previously been described for fibril formation of the islet amyloid polypeptide and Aβ40 [88], but that a detailed description of the origin of the effect and its consequences for the microscopic steps of amyloid fibril formation was missing, we were determined to investigate it further.

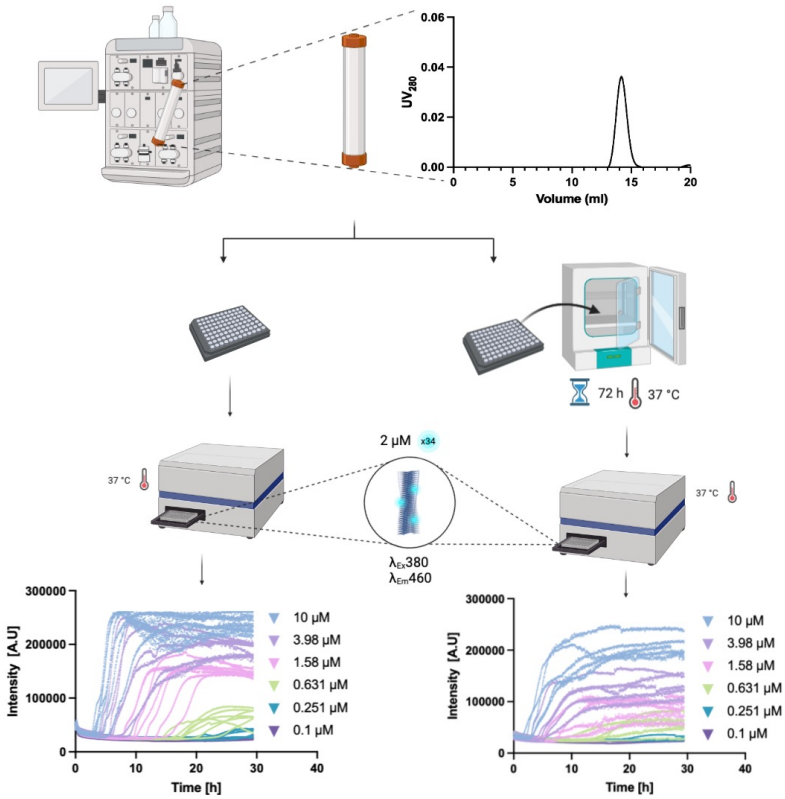


Figure 3.9: The aggregation of freshly purified tau monomers was either monitored directly (left) or first incubated quiescently for 72 hours at 37 °C in an incubator before being moved to a plate reader where the fluorescent intensity was monitored (right).

After having obtained data and realized that shear seems to affect multiple different amyloid systems and identified a way to control the level of shear, a systematic study was possible. The kinetics of Aβ42 fibril formation is quick and reproducible as well as sensitive to small environmental changes. This made Aβ42 the perfect probe to further interrogate the effect of shear on amyloid fibril formation. By asking yes/no questions about whether shear affects each microscopic step of amyloid fibril formation (Figure 3.11), a series of experiments was carried out in order to interrogate each microscopic step in isolation and determine to what extent each step was affected.

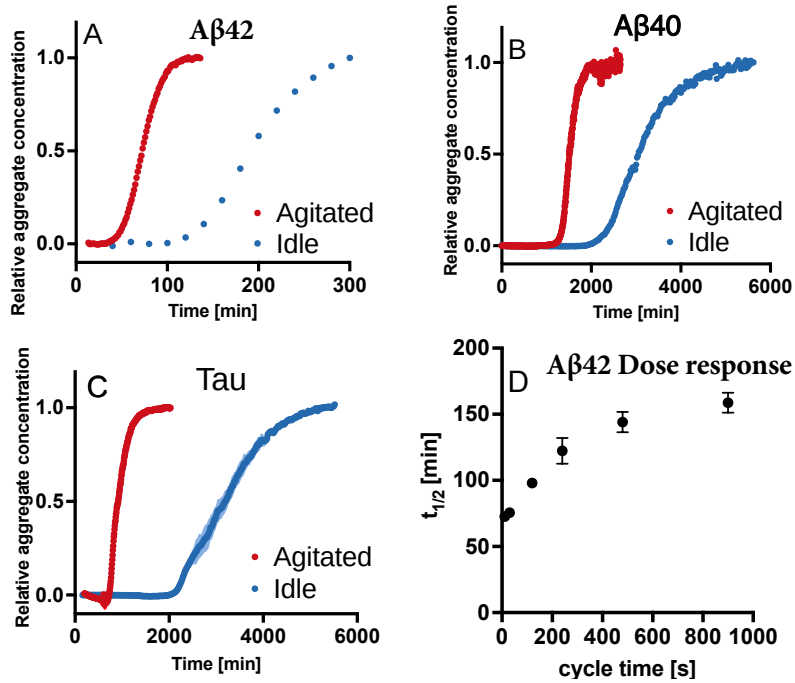


Figure 3.10: Mild shear introduced by gentle agitation influence the aggregation rate of multiple amyloid systems. (A) The kinetic traces of 2.5 μM Aβ42, (B) 2.5 μM Aβ40 and (C) 5 μM tau, under agitation (red) and under idle conditions (blue), introduced by altering the reading frequency through varying the cycle time. The level of shear can be varied in a controlled manner by altering the cycle time of the plate reader program as illustrated by the fluorescent intensity intensity half time of 2.5 μM Aβ42 (D).

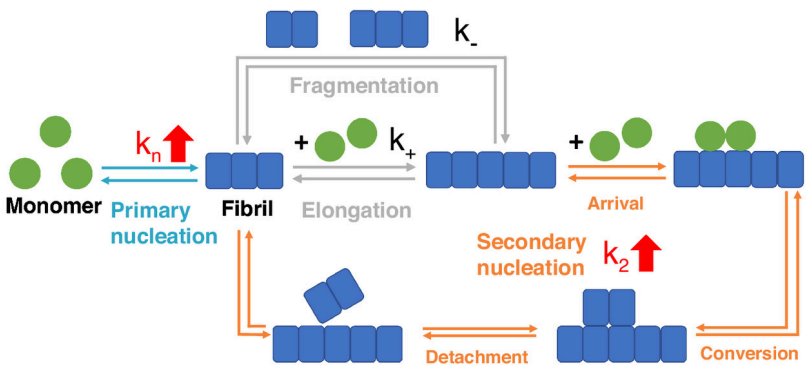


Figure 3.11: Schematic overview of the microscopic steps of amyloid fibril formation. Red arrows indicating which steps found to be affected by mild shear.

Vigorous agitation by stirring or shaking is known to accelerate fibril fragmentation. Therefore, first the effect of mild shear on fragmentation was explored. This was done through performing kinetics experiments starting from various monomer concentrations. From previous studies, it is known that Aβ42 fibril proliferation is dominated by secondary nucleation [38]. The fragmentation rate is independent of monomer concentration, while the secondary nucleation rate depends on it. In a system where both secondary nucleation and fragmentation are present, the scaling exponent manifests itself as having a negative curvature with a kink. When both of these processes are present, at lower monomer concentrations, the scaling exponent will be similar to that expected for a system rate limited by fragmentation. When the monomer concentration is increased, the scaling exponent will adopt a slope expected for secondary nucleation rate limited systems. This competition between the processes gives rise to the kink when the scaling exponent is plotted. The scaling exponent may be obtained from the slope when the log of $t_{1/2}$ is plotted versus the log of the initial monomer concentration. A negative scaling exponent is not observed under mild agitation conditions, hence a dominating effect from increased fragmentation does not explain the difference in kinetics observed between idle and agitated conditions.

Having excluded a dominant effect on fragmentation from the microscopic steps (figure 3.11), the effect on primary nucleation, secondary nucleation and elongation remain. To simplify the system and attempt to further deconvolute the effect on the individual microscopic steps, we performed kinetics experiments for Aβ42 in the presence of the secondary nucleation inhibitor Brichos. As Brichos significantly reduces the occurrence of secondary nucleation events, the rates of the primary nucleation, and elongation, can instead be explored.

If the effect of mild shear forces induced by mild agitation, was only affecting the secondary nucleation, one would expect that the differences between agitated and non-agitated samples would diminish in the presence of Brichos. Interestingly, the opposite was observed, as the difference was instead amplified. Through fitting of the kinetic traces using a primary nucleation/elongation model, it was found that there is a 12-fold increase of the $k_n k_+$ under agitated condition compared to the idle. So there must be an effect of mild shear on the primary nucleation and/or elongation. Next, the effect on elongation was then explored. Fibrils formed under mild agitation showed a higher seeding potency than seeds formed under idle conditions when used to seed fresh monomer solutions at 1:1 seed:monomer ratio. This suggests that seeds formed under agitated conditions contain more fibril ends onto which monomer can elongate. Having excluded an effect on fragmentation, more fibril ends could only originate from increased secondary nucleation. If agitation would significantly accelerate elongation, this would increase the average fibril length and subsequently decrease the number of fibril ends. The opposite is observed and a significant effect on elongation can not be established. This suggest that the 12-fold increase in the rate constant product $k_n k_+$, is due to an effect on primary nucleation, rather than elongation.

Next, kinetic traces of samples in a concentration range was fitted while restricting primary nucleation to be 12-fold higher under agitated conditions. The resulting fits did not describe the behavior of the data well. Obtaining inadequate fits while imposing restrictions on only primary nucleation, suggests that an effect only on primary nucleation is unlikely. When in addition allowing secondary nucleation rate constant, k_2, to vary freely, the fits are in good agreement with the experimental data and around 4-fold higher for agitated compared to idle conditions. Increased secondary nucleation during fibril formation leads to the generation of more oligomers. To investigate if this was the case under agitated conditions and to reinforce that secondary nucleation was increased. The oligomer population formed under idle and agitated conditions was investigated at $t_{1/2}$ using free flow electrophoresis. Indeed, more oligomers were detected in the agitated samples compared to the idle ones.

Lastly, to gain a deeper mechanistic understanding of how the shear introduced by gentle agitation affects primary nucleation and secondary nucleation we looked in more detail on whether the arrival of monomers, or detachment of oligomeric species was sped up. If majority of the primary nucleation of Aβ42 occurs at the air-water interface, the detachment of newly formed oligomeric species from the air-water interface could become rate limiting. We find that decreasing the ratio between the interfacial area and the sample volume leads to a reduced effect of agitation on the aggregation process and that the effect of agitation is largest in samples where the area:volume ratio is low. From this we can argue that the detachment of nuclei from the air-water interface is rate limiting and accelerated by the shear introduced from agitation. To gain insight into the detachment of secondary nuclei, a model system in which Aβ42 fibrils become decorated by what are believed to be secondary nucleation intermediates [89] yet to detach. Through cryo-TEM we find that the shear imposed by vortexing is sufficient to reduce the number of smaller species decorating the fibril surfaces, but not enough to fragment the fibrils. This is interpreted as an ultrastructural indication that shear leads to increased oligomeric detachment from the fibrils.

In conclusion, we provide support that shear speeds up the detachment of oligomeric species in both primary and secondary nucleation. This provides new opportunities during assay development, if any of the microscopic steps should be targeted in isolation. Personally, it has been humbling to have had so many people take interest in what started as an odd observation, and I am happy to have had the opportunity to explore and understand the observation to my satisfaction. When I started this project, I knew that amyloid fibril formation was very sensitive to small environmental changes, but I was unaware that this also applied to things like cycle time or reading frequency. This has really affected my way of thinking about sample measurement and always make me think twice whether the measurement itself could perturb the system.

3.5 Paper V: The Ability of DNAJB6b to Suppress Amyloid Formation Depends on the Chaperone Aggregation State

Chaperone activity may be regulated in response to cellular stress and is often associated with specific assembly states [90]. The potent anti-amyloid chaperone DNAJB6b self-assembles into larger oligomeric assemblies in a concentration dependent manner and little is known about the activity of the ensemble of species. The aim of this study was to investigate the chaperone activity of the different assembly states on the self assembly of Aβ42 .

Above a threshold concentration of ≈ 10 μM the size stops growing and all higher concentrations adopt a mean particle size with hydrodynamic radius (R_h) of ≈ 12 nm. Below 100 nM, a concentration independent regime is present, where the mean assembly size has a hydrodynamic radius of $R_h \approx 5$ nm. Upon dilution from concentrations above 10 μM with $R_h \approx 12$ nm, to concentrations below 100 nM, a gradual decay of the mean particle size from 12 nm to 5 nm starts (in blue, Figure 3.14), requiring around 3 days to reach the lower equilibrium size at room temperature [91]. The relatively slow dissociation rate of larger DNAJB6b assemblies into smaller oligomers after dilution below subunit solubility, combined with the relatively fast self-assembly kinetics of Aβ42 monomers into amyloid fibrils, was instrumental for the study in **Paper V**. The discrepancy between the dissociation and association rates allowed investigation of the inhibitory power of the DNAJB6b oligomer ensemble along its dissolution pathway, on the self-assembly of Aβ42. This was achieved by pre-incubating DNAJB6b for different lengths of time after dilution, before initiating amyloid self-assembly reactions with the addition of freshly purified Aβ42 monomers.

A schematic overview of the experimental design is given in Figure 3.12. Crucial for reproducibility was to dilute the starting DNAJB6b from a significantly high concentration, to ensure an effective dilution of any smaller species present in equilibrium with the larger oligomers and minimize their presence at the beginning of the reaction.

Strikingly, immediately upon dilution the larger oligomeric species present had no inhibitory effect on Aβ42 fibril assembly (Figure 3.13). Only after longer pre-incubation times does DNAJB6b develop its inhibitory power.

Only when DNAJB6b approaches its smaller assembly size, does it achieve its full suppressive capacity (Figure 3.14). In combination with the observation that freshly diluted DNAJB6b, before having had time to dissociate into its smaller constituents, has yet to acquire its amyloid-suppressive properties, this suggests that it is the smaller subunits, rather than the large assemblies, that carry the inhibitory capacity.

These findings opens up interesting questions; is there interaction between larger oligomers and Aβ42, or is the lack of inhibitory capacity due to a lack of interaction? Furthermore,

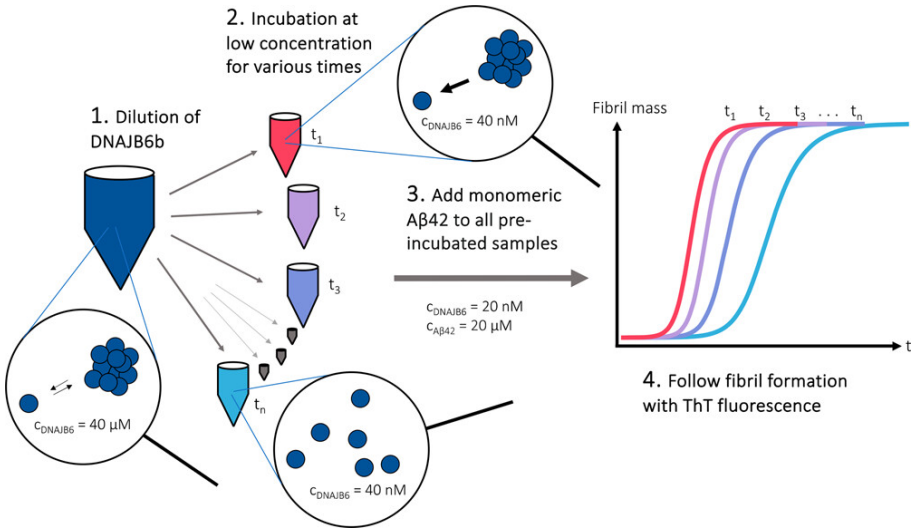


Figure 3.12: Schematic overview of experimental setup. 40 µM DNAJB6b was diluted to 20 nM and preincubated for various times before being mixed with Aβ42 monomers supplemented with ThT to probe the inhibitory power of DNAJB6b along its dissociation pathway.

it remains to establish the exact size distribution of DNAJB6b below 100 nM. Does it dissolve completely into monomers? Hopefully, single-molecule techniques might be able to answer these questions in the future and reveal if the inhibitory capacity resides in the monomer, dimer, tetrameter or even larger assembly?

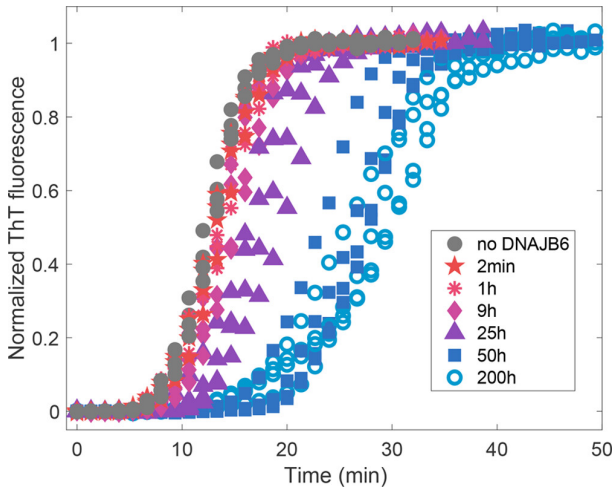


Figure 3.13: Kinetic traces of 20 µM Aβ42 monomers in the presence of 20 nM DNAJB6b. DNAJB6b was diluted from 40 µM and allowed to pre-incubate at room temperature for various time, as denoted by the legend, before the reaction was started by addition of Aβ42 monomers.

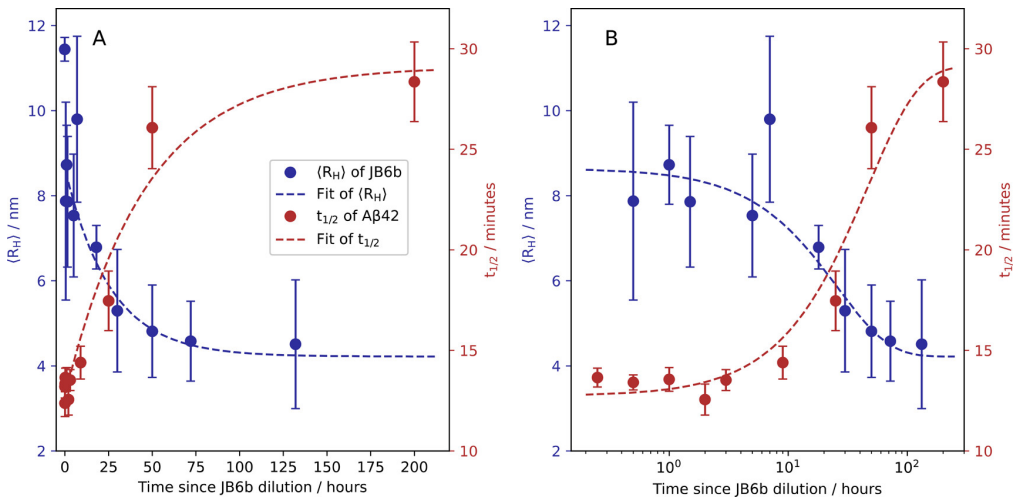


Figure 3.14: Upon dilution of DNAJB6b from above to below its CMC, begins a gradual dissociation of oligomeric assemblies. The ensemble of species has varying Aβ42 inhibiting capacity. In blue, the mean R_h after dilution of 6 µM DNAJB6b to 100 nM. In red the $t_{1/2}$ of 20 µM Aβ42 in the presence of 20 nM DNAJB6b diluted from 40 µM with varying incubation times before the amyloid reaction was started. (A) represented on a linear time axis, (B) on a logarithmic time axis.

Chapter 4

Future perspective

Our understanding of amyloid aggregation and the underlying molecular mechanisms is constantly expanding. As this wealth of knowledge is further refined and the finer details take form, a more complete picture will inevitably unfold. From a comprehensive understanding of all aspects of the diseases caused by amyloid aggregation, strategies of disease intervention that we may not even have thought of yet will emerge. Translation of basic research takes time, the knowledge needs time to mature and to begin to permeate our way of thinking.

Attacking a complex disease like AD as an engineering problem could be one way to find a solution. Dividing the multiple challenges and obstacles into smaller pieces will hopefully eventually enable us to tackle this devastating disease. First, we need inexpensive and accurate diagnostic tools capable of detecting the early manifestations of the disease. Without an effective way of diagnosing, even if we had the "silver bullet" against the disease, it would be of limited value if we did not have the means to identify the right individuals to give it to. The recent developments by Dr. Hansson on blood-based tests for AD detection [70] could be what is required. Furthermore, improved diagnostics will greatly facilitate recruitment to clinical trials, which in turn will support any drug development effort. Therefore, developing diagnostics is as important as finding an effective treatment.

Toward this end, seed amplification assays (SSA's) might yet have the potential to play a role. They have the ability to detect minuscule amounts of amyloid and are already being used in clinical practice for the diagnosis of prion diseases [92]. Furthermore, they have demonstrated considerable promise, especially for the detection of aberrant variants of α-synuclein in Parkinson's disease [93]. Specifically, the finding of a truncated variant of tau (**Paper II**) that self-assembles without inducers, and its increased aggregation rate when seeded with preformed fibrils could make it and other truncated variants of tau suitable substrates in SAA. Evidence exists in the literature that fibrils derived from AD patients ac-

celerate the self-assembly kinetics of a similar recombinant truncated variant of tau, further strengthening the idea of its use as substrates in SAA [94]. Therefore, it is not impossible to imagine that other truncated variants of tau also might have this potential. The recent advances in cryo-TEM and the structure determination of multiple tau filaments from different tauopathies [95] have shown that they have different fibril morphologies. This might give these tau fibrils different seeding capacities and could potentially be utilized not only to diagnose AD, but also with the additional benefit and potential to distinguish between different tauopathies.

The strategies developed in **Paper II** also provide a simple framework and model system in which tau aggregation and the effect of potential inhibitors can be studied. The slow primary nucleation rate and the striking metastability illustrated in **Paper III** & **Paper IV** raises questions regarding tau aggregation onset. The finding that mild shear forces significantly accelerates the primary nucleation rate poses questions like; could the shear inside the cell or outside in the interstitial fluid exert enough force to trigger aggregation onset? Extending the amyloidogenic core of tau with flexible regions on the N and C-termini likely leads to increased solubility and increased energetic barriers for primary nucleation. Could protein aggregation onset be the result of unfortunate proteolytic processing of the full-length peptide, into shorter more aggregation prone fragments that spark the cascade? Are the seeds of truncated fibrils capable of seeding longer unmodified variants? Or are other co-factors and the combination with post-translational modifications required? Many questions remain and there are many interesting avenues to explore. Having developed methodology for studying both kinetics and solubility, simply adding amino acids one by one and investigating the effect on kinetics and equilibrium would yield interesting insights. We know that the onset of the aggregation of full-length tau is very slow. If the fuzzy coat is systematically extended, at what point will the primary nucleation barriers become so high that aggregation occurs beyond practical laboratory time scales? Could these energy barriers be overcome by using seeds with a specific morphology? These are all small curiosities that deserve further investigation and need to be overcome in order to obtain better systems to study tau aggregation.

One of the bodies own defense mechanisms against protein aggregation are the chaperones. Drawing inspiration from nature and harnessing the power of evolution could be a highly effective strategy which could be employed when trying to create disease interventions. The potency of DNAJB6b in particular in suppressing amyloid formation is remarkable. It was recently shown to also influence tau aggregation in a cell model system [96]. Could increasing the levels of certain chaperones in humans be sufficient to keep amyloid diseases in check? A more detailed molecular understanding of the effect of DNAJB6b on tau aggregation is needed. The observation in **Paper V** that smaller oligomers rather than larger DNAJB6b assemblies possess the inhibitory power raises a question; does the larger inert DNAJB6b assemblies form inside cells and how does the cellular environment affect

the effect? The possible combined effect of DNAJB6b on both Aβ and tau aggregation is promising. This implies that the combinatorial space contains more molecules with this capacity. A more detailed understanding of how the observed effects are exercised is necessary in order to fine-tune and further develop molecules to gain the desired properties, which could lead to the therapeutics of tomorrow.

I see many directions and natural continuations of my research, and I have found that when answering one question, many new questions often arise. Furthermore, one often stumbles upon interesting observation by serendipity if one is observant. I hope to continue following and satisfying my curiosity.

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Part II

Research Papers

About the author

EMIL AXELL began his academic journey in Lund studying physics and mathematics. After two semesters, he continued studying chemistry, where he was fascinated and drawn towards the intersection between physics, medicine, and biology. Captivated by this interdisciplinary field, he completed his undergraduate studies in chemistry before spending a year at the National University of Singapore, specializing in chemical biology, protein engineering, and biophysics. After returning to Lund, he earned a degree in biochemistry. Upon graduation, he joined Sara Linse's research group to pursue his PhD in biochemistry. During his postgraduate studies, he has been investigating the biophysics of amyloid proteins involved in Alzheimer's disease. His research explores fundamental properties of protein self-assembly and solubility, with a particular focus on amyloid-forming proteins and chaperones implicated in neurodegenerative diseases.

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