Amyloid Oligomers

Capturing the threat between disorder and order

Ortigosa-Pascual, Lei

2024

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BIOCHEMISTRY AND STRUCTURAL BIOLOGY | FACULTY OF SCIENCE | LUND UNIVERSITY

Amyloid Oligomers: Capturing the threat between disorder and order

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Capturing the threat between disorder and order

Lei Ortigosa Pascual

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Science at Lund University, to be publicly defended on Wednesday, 5th of June 2024 at 09:00 in lecture hall A, at the Center for Chemistry and Chemical Engineering.

Faculty opponent
Mireille Claessens

Organization: LUND UNIVERSITY

Document name: Doctoral Dissertation **Date of issue:**

Author(s): Lei Ortigosa Pascual **Sponsoring organization:**

Title and subtitle: Amyloid Oligomers; Capturing the threat between disorder and order

Abstract:

Amyloid proteins are species whose aggregation has been associated with various neurodegenerative diseases. While intrinsically disordered in their monomeric form, they tend to aggregate into highly ordered β-sheet rich fibrils. Nevertheless, many studies indicate that intermediate species known as amyloid oligomers, which are linked to neurotoxicity, may be the connection between amyloid proteins and their pathology. However, oligomers are very transient, non-covalently bound, heterogeneous, and at a very low concentration relative to monomers and fibrils. Due to this, oligomers are challenging to study with conventional methods. and developing and optimizing methods for analyzing oligomers is crucial for the advancement of the amyloid field. In this thesis, we aim to optimize two oligomer analysis methods and use them to improve our understanding of the amyloid system. On one hand, we optimize the Photo-induced cross-linking of unmodified proteins (PICUP) for the study of αSyn, and we use it to identify transient interactions within and between α-synuclein monomers in solution, bound to lipid membranes, and in fibrils. On the other hand, we use microfluid free flow electrophoresis (μFFE) for the study of Aβ42 oligomer populations. Doing so, we learn how oligomer population is affected by the protein production source as well as sheer forces, and we show that amyloid fibrils do not only catalyze oligomer formation but also play a key role in oligomer dissociation.

Key words: amyloid, aggregation, synuclein, α-synuclein, Aβ, oligomers, kinetics, PICUP, μFFE

Classification system and/or index terms (if any) Supplementary bibliographical information

Language: English **Number of pages**: 282

ISBN: 978-91-8096-050-2 (print) ; 978-91-8096-051-9 (digital)

Recipient's notes Price Security classification

Signature Date 2024-04-16

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Author photo

Cover illustration front by Gari Ortigosa Pascual

Cover illustration back by Eki Ortigosa Abdul Hadi

Faculty of Science, Biochemistry and Structural Biology

ISBN: 978-91-8096-050-2 (print)

ISBN: 978-91-8096-051-9 (pdf)

Printed in Sweden by Media-Tryck, Lund University

Lund 2024

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“Eta txori bat pasatzen bada
eta ematen dio pilotari?”
Galder Preguntegi

Table of Contents

List of publications

This thesis is based on the following publications, referred to by their roman numerals:

I. **Photo-Induced Cross-Linking of Unmodified α-Synuclein Oligomers**
L. Ortigosa-Pascual, T. Leiding, S. Linse and T. Pálmadóttir
ACS Chemical Neuroscience, 2023, 14(17), 3192-3205

II. **On the transient interactions of αSyn in different dimensions**
L. Ortigosa-Pascual, N. Ferrante-Carrante, K. Bernfur, K. Makasewicz, E. Sparr and S. Linse
Manuscript

III. **Effect of Aβ42 production source in its kinetics and oligomer formation**
L. Ortigosa-Pascual*, K. Matulewska*, E. Andrzejewska, G. Šneiderienė, A. Dear, T.P.J. Knowles and S. Linse
Manuscript

IV. **The role of shear forces in primary and secondary nucleation of amyloid fibrils**
E. Axell*, J. Hu*, M. Lindberg*, A. Dear**, L. Ortigosa-Pascual**, E. Andrzejewska, G. Šneiderienė, D. Thacker, T.P.J. Knowles, E. Sparr and S. Linse
Resubmitted after revision in PNAS

V. **Aβ oligomer dissociation is catalyzed by fibril surfaces**
A. Dear, D. Thacker, S. Wennmalm, **L. Ortigosa-Pascual**, E. Andrzejewska, G. Meisl, S. Linse and T.P.J. Knowles
Resubmitted after revision in ACS Chemical Neuroscience

*These authors contributed equally to the article.

Publications not included in this thesis

I. **The Bacterial Amyloids Phenol Soluble Modulins from *Staphylococcus aureus* Catalyze Alpha-Synuclein Aggregation**
C. Haikal, **L. Ortigosa-Pascual**, Z. Najarzadeh, K. Bernfur, A. Svanbergsson, D. E. Otzen, S. Linse and J. Li
International Journal of Molecular Sciences, 2021, 22(21), 11594

My contribution to the papers

Paper I: Photo-Induced Cross-Linking of Unmodified α-Synuclein Oligomers

I participated in the planning of experiments. I designed and built the PICUP reaction chamber. I measured, designed, 3D-modeled, 3D-printed and mounted the reaction chamber, as well as writing the program to control it. I expressed and purified the αSyn protein and managed its handling for all experiments. I optimized the PICUP reaction conditions. I performed every PICUP experiment, as well as their analysis. I wrote the first draft of the manuscript and edited according to co-authors' and reviewers' comments.

Paper II: On the transient interactions of αSyn in different dimensions

I took part in the design of the study and planned all the experiments in it. I performed the monomer isolation step for every αSyn variant, as well as their handling and aggregation measurements. I performed every PICUP experiment with every protein variant in the study, as well as analyzing and rationalizing the data. I wrote the first draft of the manuscript and edited according to co-authors' comments.

Paper III: Effect of Aß42 production source in its kinetics and oligomer formation

I took part in the design of the study. I initiated the project with aggregation studies of recombinant and synthetic Aβ42, and then proceeded to guide KM as she continued the kinetic experiments. I guided KM in the kinetic analysis of the data. I purified Aβ(M1-42) and Alexa-488 labelled S8C Aβ(M1-42), and aggregated them, as well as s1Aβ42, for oligomer measurements. I tested the conditions for optimal oligomer measurement. I performed μFFE together with EA and GS. I wrote the first draft of the manuscript together with KM and edited it according to co-authors' comments.

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

I contributed to the oligomer population measurement section of the paper. I purified and aggregated Aβ42 and Alexa-488 labelled S8C Aβ42 for oligomer studies and evaluated the effect of shear forces on this system. I performed μFFE together with EA and GS. I wrote the first draft of the oligomer-population section of the manuscript. I participated in editing the manuscript with co-authors.

Paper V: Aß oligomer dissociation is catalyzed by fibril surfaces

I contributed to the experimental validation of the theoretical claim via μFFE. I purified and aggregated Aβ42 and Alexa-488 labelled S8C Aβ42 for oligomer studies. I separated oligomers from fibrils and performed μFFE together with EA. I participated in editing the section corresponding to the μFFE method.

Acknowledgments

Having spent the last 26 h working on this thesis, without a single minute of sleep, brace yourselves for the rumbling of a sleep-deprived organism that is now 80% coffee. I will do my best to express my gratitude as strongly as many of you deserve, but it probably won't be as eloquently as I would have liked to.

First of all, I am extremely grateful to my supervisor, **Sara Linse**. It is difficult to put into words the gratitude I have for you. You have put the bar of PI so high I genuinely don't think I will ever be as happy with any other supervisor as I have been here. I never thought it was possible to feel ambition for doing great things while taking it calmly and enjoying the experience. At the end of the day, I got into research simply because it is fun, and you have showed me it is possible to keep that attitude to the highest of levels. I am also thankful to my co-supervisors **Thom Leiding** and **Peter Jonson**. Sadly, the thesis project did not end up taking the path that we expected at the beginning, which would have made our paths cross more often. However, I still found your advice and encouragement very inspiring. Also, Thom, thanks for letting me play around with the 3D printer and waste all that material back when I thought the answer to all problems was to 3D print. Special thanks also to **Tuomas Knowles**, who had the generosity to welcome me at his lab for some of the most enriching and productive months of my whole PhD.

Thanks to all past and present members of the Linse lab. First of all, **Tinna**, thanks for introducing me to this wonderful group, and for daring to take me in as a master student, even after seeing how annoyingly stubborn I could be in the course lab. **Rebecca**, thanks for being the order in the chaos, it was reassuring to see I wasn't the only person obsessively organizing their desk. Thanks to you and to **Kalyani** for your quick help when I thought I would lose my sight. Thanks to you two I can now read what I am writing (if I have any time left to do so). **Matthias**, thanks for sharing my addiction of needing sudden urgent walks to the parking while frantically looking at the phone, even if our addictions didn't coincide that much. **Veronica**, thank you for your energy, I loved being the grumpy old man to your over-the-top cheerfulness. **Dev**, thank you for your calming presence, and for those brief football chats (sorry you chose the wrong team). Thanks also to **Eimantas** and **Tanja**, I feel like I learnt most of my amyloid handling tricks by annoying you way too much with questions. Thanks to **Marija** for convincing me to join more socializing events than I would otherwise have the social energy to join. And **Martin**, if someone has had patience with my questions, that is you. Thank you so much for always being so willing to help. And no, I still haven't learnt the numbers for the travel expenses forms. Sorry.

Emil, thank you for being a chaotic influence in the lab. Asko kantsatzen nauzu batzutan, baina hala ere asko eskertzen dut zure energia. Ez dut uste beste inorekin ausartuko nintzenik ezezagun baten kotxean sartzen, gauerdian, Tijuanatik irteten

saiatzen gaudela. Eta bai, euskaraz hitz egiten ari naiz "ikas dezazun", zuk suedieraz egiten duzun bezala. **Max**, thanks for being the jing to Emil's jang. You are the calm where Emil is the chaos, and it's wonderful to have you two always at hand depending on what I feel like. You are the one person I know will always offer help when asked, and it doesn't go unnoticed. **Andreas**, the moment we took the colloidal domain course together I could see you sprinting past me into the horizon. You're going to have a wonderful career, but please, try to control the urge to hit an imaginary tennis ball in the middle of conversations. **Josef**, One Piece of advice: eat fish, man. Also, stay strong, one day Arsenal will also win something important, like Athletic Club just did (I hope this jinxes it and by the time the thesis is printed they have actually won something). **Karolina**, thank you so much for being so patient with me, my chaotic schedule, and my tendency to make meetings turn into conferences. I don't think I would have liked being a project supervisor as much if it wasn't for you. Although I still have nightmares with THE excel file. **Ewelina**, thanks for joining us in Spetses and making it so that, even if you are the latest lab member, I feel like I've known you for years. And of course, thanks for the sheep! **Noemi**, thanks for adjusting to my chaotic schedule, it's always so easy to plan stuff with you and I really appreciate it. To everyone else at CMPS and Kemicentrum, thank you so much for your company, help, and all those things. I'm sorry, my brain is fried from *PhD-ing*, and I don't have the energy to write all your names.

I have to also thank all the collaborators from other labs. **Ewa**, if I said Sara has put the bar unreachably high for a PI, you have done as much or more with the role of a host, a collaborator, and a friend. Working with you didn't feel like working, and well, I guess sometimes it kind of wasn't, as we would spend an hour debating how to pipette a sample, or the ins and outs of the jurisdiction of a president of a municipality. On that note, thanks to you too, **Greta**, for your patience with me, and having the humor even after hours of eternal meetings that ended up questioning the meaning of life itself. On a similar Cambridge note, **Alex**, thanks for making me feel more intelligent by making such advanced concepts so reachable to the *less mentally gifted* such as myself. I envy your balance between quality of work and enjoying your free time as one should. **Caroline**, working with you here was so much fun, but I wouldn't have expected for a "let's throw spaghetti at a wall and see what sticks" type of collaboration to lead to such an unforgettable friendship. Visiting you in NY has definitely been one of the highlights of my PhD, and I will surely visit you around the world as you move! I thoroughly enjoyed our scientific exchanges during my visits, like when you guided me into the art of chopping a rats head off with a suitcase.

Eskerrik asko nire karrerako kide guztiei. Batez ere, eskerrik asko Unai eta Adharari. Nire biokimikarekiko interesa ez litzateke izango orain dena zuengatik ez balitz. Karrera aurretik ezinezkoa irudituko litzaidake hain lagun onak aurkitzea eta, kasualidadez, niri gustatzen zaizkidan txorrada berak ere gustatzea. Eta ostegunetako juerga ostean klasera joatea praktika perfektua da gaur bezalako

egunetarako, lorik egin gabe idazteko energia eukitzeko. Eskerrik asko Markel, Asis y Ana, porque con el cacao que ha sido esta experiencia, no hay nada más relajante que hacer el plan de siempre y recordar qué es lo que de verdad importa en la vida: pillarte el Stockholm – Petrograd.

Azkenik, eskerrik asko nire familiari. Eskerrik asko, lehenik eta behin, **Aita** eta **Amari**. Ez dakit benetan zelan eskertu guztia. Eskerrik asko nahi nuena egitera animatzeagatik, eta, nire hona heldu nintzeneko esperientzia nahasian, erakutsi zenidatelako kasurik txarrenean beti eukiko zaituztedala laguntzeko prest. Eta hau emozio gehiegi da Ortigosa batentzat, beraz beste guztia zuen imajinazioaren esku utziko dut. **Gari** eta **Irari** ere eskerrik asko. Gutxitan batu gara azken urteetan, eta zoritxarrez ez naiz humore onenean egon askotan. Ea behin hau bukatuta gehiago gozatu dezakedan zuen konpainiaz (eta zuen mokordoenaz). And last, but not at all least, thanks to **Anneka**. This would not have been possible without you. And no, it is not a figure of speech. I could fill another book the size of this one with all kinds of gratitude towards you. But given how much you hate any public display of affection, leaving something cheesy in print would probably mean you would use this as leverage for the rest of our lives. So, I'll leave it like that.

Popular Science Summary

We are all familiar with the idea that living organisms are made of atoms and molecules. But not everyone knows that one of those molecules that are crucial for our lives are proteins. And no, they aren't just something we have to eat. Proteins are an essential molecule which form our cells, together with fat, sugars, and nucleic acids. Proteins are the workforce of the cell, carrying out most of the active duties, from moving other molecules, to cutting them or gluing them, giving the cells a "skeleton", and so on. We don't just eat them, we also produce them, as we need them constantly to perform their duties in the cell.

A crucial aspect for a protein to work well is its folding. You can think of it as a sheet of paper that you need to fold into the appropriate shape for its function, like making a paper plane if you want it to fly. Each protein has a specific fold, and that fold is made exactly for the function the protein has. That is the case to such an extent, that some improperly folded proteins are the cause of some diseases. On the other hand, there are some proteins that prefer to be unfolded instead and be a "flat sheet of paper". But if you just have a bunch of flat sheets of paper, isn't it tempting to just stack them neatly on top of each other? Well, that's what these proteins do. These proteins, called amyloid proteins, are proteins we produce in our everyday life, and they perform functions we need for having a normal life. However, they have a tendency to clump together and aggregate into very neat and ordered helix-like structures. These structures are called fibrils. Many neurodegenerative diseases, such as Alzheimer's or Parkinson's, are characterized by the presence of small deposits in the brain, which are formed by amyloid fibrils. And the most interesting thing is that there are many different amyloid proteins, and each of them can be associated to different diseases. This implies that there is something about this aggregation process that they all follow that somehow is connected to the diseases.

This led scientists to study amyloid proteins and their aggregation into fibrils, with the hopes of understanding how the disease is caused and how is it connected to these proteins. Recently, they found out that the proteins alone, one by one, or the fibrils by themselves, don't harm cells too much. Instead, they found that more disordered intermediate species, formed by a bunch of units put together, are actually toxic. This makes the study of these intermediate species, called amyloid oligomers, crucial to understand how the protein behaves and how it is associated with the disease. However, they are just the middle stage of a transition between single proteins and fibrils, so, as one would guess, they are very transient and short lived. Due to that, conventional techniques fail to capture them, and methods have to be developed explicitly for their measurement.

In this thesis, we focus on the optimization of two amyloid oligomer measuring methods, called PICUP and μFFE. For PICUP, we have built a machine to make the

reaction as fast as possible to freeze amyloid oligomers in the position they are in the moment of reaction. In doing so, we have understood a lot of key aspects of the reaction, as well as gaining information on what parts of the protein are in contact with each other when they are in an oligomer together. With μFFE, we can count the number of oligomers on a sample. Using this, we have evaluated different factors and how they affect oligomer population. All in all, we have improved some previously existing methods and/or given them a new niche focus, which has allowed us to learn a lot about oligomers, hopefully taking the amyloid field a small step closer to understanding the connection between protein and disease.

1.Proteins, amyloids, and aggregation

1.1. Proteins

Molecules, bundles of covalently bound atoms, can be easily classified as either organic or inorganic. Organic compounds are those that contain carbon (C) as their core component. They are mostly produced by living beings and are also what living beings are made of. These compounds, often referred to as biomolecules, can be divided into four types: lipids, carbohydrates, nucleic acids, and proteins [1].

The chemistry of proteins

Proteins, possibly the most diverse of the biomolecules, are polymers formed by units known as amino acids [2]. These amino acids are all mostly composed of carbon, hydrogen, nitrogen, and oxygen, but have minor differences between them, leading to the existence of 22 different amino acids (of which only 20 are coded by the human genome) [3] (Figure 1.1). Due to their different atomic structure, amino acids can be hydrophilic (polar) or hydrophobic (non-polar/apolar), and can be neutral, positively, or negatively charged.

Amino acids can be bound to each other via a covalent bond, often referred to as peptide bond. By binding different amino acids together, a peptide chain is formed (Figure 1.2a). Proteins are biomolecules consisting of one or more peptide chains. In all living cells, a molecular machine called *ribosome* is the one responsible for creating proteins by covalently binding amino acids together [1, 2, 4]. Additionally, proteins can be further modified after synthesis by the addition or removal of certain chemical groups, in a process known as post translational modification. With the diversity of amino acids, the potential to chain them in any order and amount, and

all the available modifications, nature has generated a wide array of different proteins.

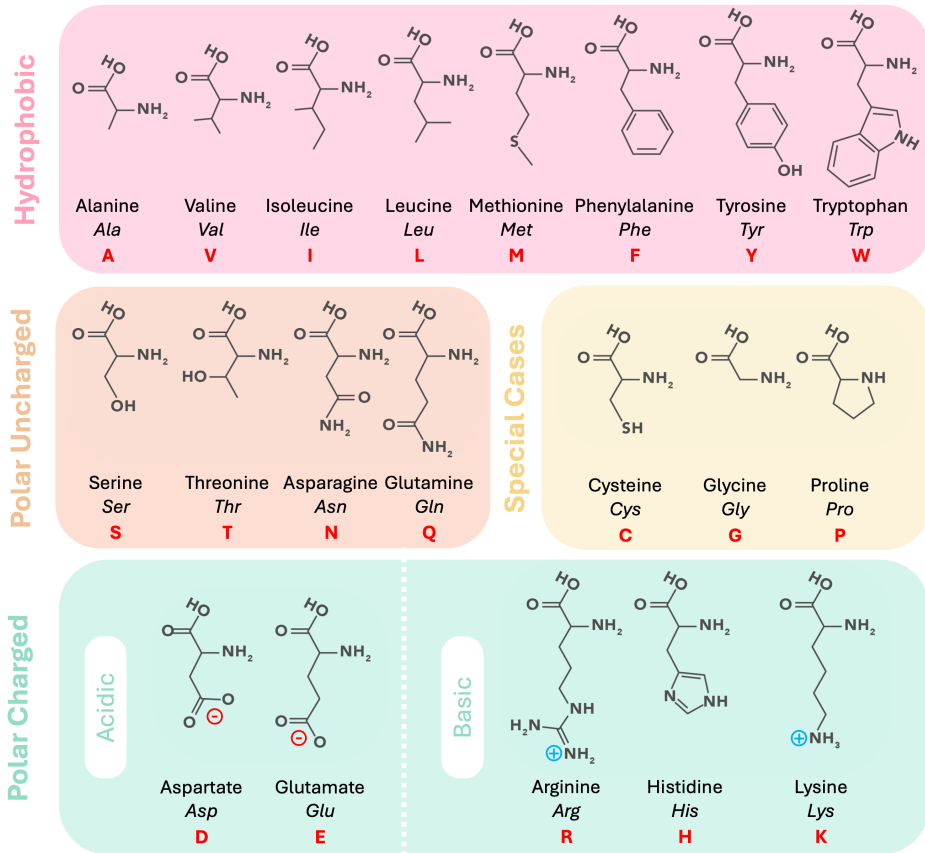

Figure 1.1. The 20 amino acids encoded by the human genome, with full name, three letter abbreviation, and one letter abbreviation

Protein structure and folding

The structure of a protein can be categorized at four different levels [1, 2]. Firstly, the *primary structure* refers to the sequence of amino acids forming the peptide chain (Figure 1.2a). As each amino acid has different chemical characteristics, the order and frequency of each amino acid will dictate how the peptide chain behaves. Some of these amino acids will attract or repel each other and interact differently with the environment they are in. This will lead to them forming small local structures within the chain, referred to as *secondary structures*. Some common

structures found in proteins are α-helix and β-sheets [2, 5] (Figure 1.2b). Since these structures are local, the same peptide chain can contain many parts with different secondary structures. However, most often, the peptide will fold further and combine all its components to form a stable 3D structure, known as its *tertiary structure* (Figure 1.2c). The process of forming a tertiary structure from a peptide chain is also referred to as folding. Finally, there are some proteins that consist of more than one peptide chain combined. The structure these peptide chains form when combined is called *quaternary structure*.

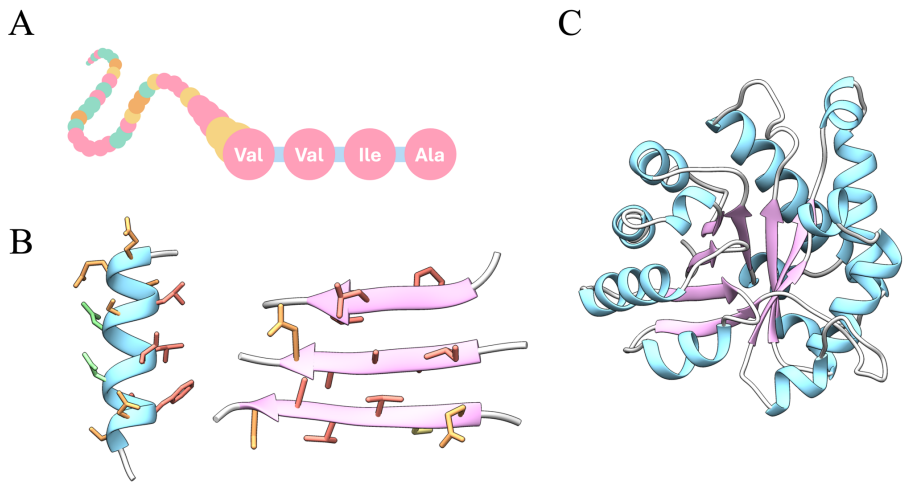

Figure 1.2. Protein structure categories. Primary structure (A), the sequence of amino acids forming a protein. Secondary structure (B), small local structures adopted by the amino acid chain. On the left, an α-helix, on the right, a β-sheet. Tertiary structure (C) of protein Trypanosomal triosephosphate isomerase B (PDB 1AG1), with α-helix (blue), β-sheet (purple) and random coil (grey) regions.

The 3D structure that a peptide chain adopts will dictate what chemical components will be located in certain points in space. The main driving force of protein folding is the hydrophobic effect, the tendency of hydrophobic amino acids to hide in the core of the protein, to avoid coming into contact with the water the protein is in [1]. However, electrostatic interactions also play a crucial role in protein folding. Since the chemical nature of a protein is the key to its function, the correct folding of a protein is crucial for its activity. In fact, incorrect protein folding can lead to loss of protein function and is the origin behind many diseases [6]. However, there are also proteins that prefer to not adopt any particular conformation when alone in solution, which are known as intrinsically disordered proteins [7]. These proteins often take advantage of the flexibility that a lack of structure gives, and usually require this freedom for their correct function. Regardless of their preferred structure when

alone, some proteins can interact with each other, bind together, and form very ordered and highly structured aggregates known as amyloids [8].

1.2. Amyloid proteins

The term *amyloid* was used for the first time by German botanist Matthias Schleiden in the 1840s [9, 10]. In his book titled "Grundzige der wissenschaftlichen Botanik", he describes the use of iodine-sulphuric acid for detecting starch in plants, as their reaction leads to a blue staining. *Amylum* being the Latin word for starch, Schleiden uses the term *amyloid* to refer to "starch-like" structures. The term jumped to the medical literature a decade later, when pathologist Rudolf Virchow identified small deposits in post-mortem nervous systems, which happened to react with iodine-sulphuric acid in a similar manner [11]. With time, new and more specific stains to study amyloid deposits were developed, the most notable one being the use of Congo red, which not only stains the deposits, but also gives them a very particular green birefringence [12]. This birefringence shown by amyloid deposits was a strong indicator of an ordered pattern in the submicroscopic structure. Development of microscopes and dying techniques led to the demonstration that these deposits, be it from animal or human origin, all showed a fibrillar structure [9, 13].

Morphology and Structure

As the available technology improved, so did our understanding of amyloid structure. In 1968, Eanes and Glenner reported amyloid structures obtained from human liver and spleen to have a very particular X-ray diffraction pattern referred to as a cross-β [14-16]. That study marked the start of the description of the amyloid fibrillar structure in the detail with which we understand them today. Although amyloid fibrils vary between them, they all exhibit a few common features: peptide units stacked on top of each other, perpendicular to the axis of the filament, and with all units adopting the same structure (Figure 1.3). These peptides adopt a β-sheet conformation, with 4.7-4.8 Å distance between the strands that form a sheet [5], and 6-11 Å between the β-sheets [17]. The stacking of a multitude of peptide units, or *monomers*, leads to the formation of twisted, thread-like filaments of 10 nm thickness and over 1 μm in length [8, 18]. On top of that, for some proteins, two or more filaments can combine together in parallel [19, 20]. However, the variation between fibrils can be even larger, as the same protein has been observed to form different morphs of fibril depending on conditions of formation [21], or even change morphology at different stages of its formation [22]. Similarly to protein folding, the main driving force of amyloid formation is the hydrophobic effect.

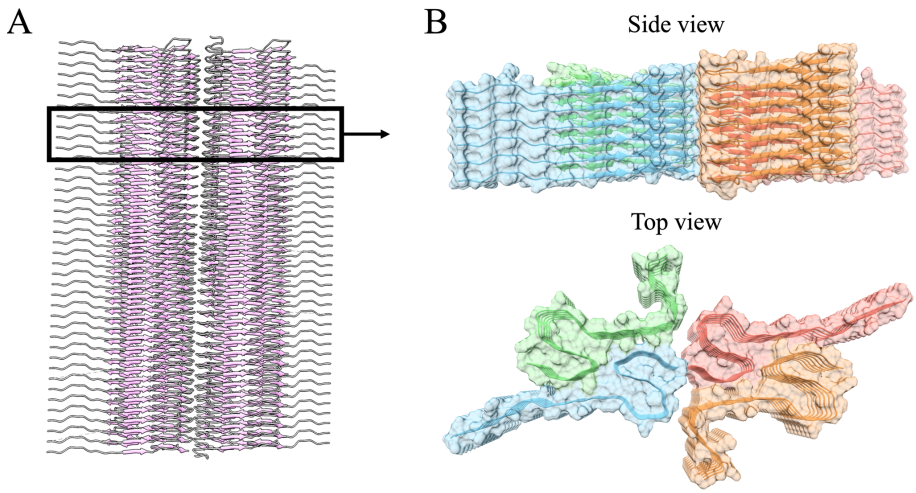

Figure 1.3. Amyloid fibril structure of Aβ42. The fibril is composed of two filaments, each formed by two Aβ42 monomers per segment. (A) Full fibril with secondary structures represented and color coded as in Figure 1.2., showing the richness in β-sheet of amyloid fibrils. (B) Side and top views of a cross section of the Aβ42 fibril. The surface of the protein is colored to showcase how a cross-section is composed of four monomers in this particular fibril structure.

Why are they of interest?

Many proteins have shown the ability to form amyloids *in vitro* when using amyloid-favoring conditions, so much so that it is considered a general feature all proteins have [23]. But amyloid formation is much more than just an interesting physico-chemical trait of proteins. Some proteins adopt an amyloid structure to perform their activity, such as activating antiviral immune response [24], storing hormones [25] or for bacterial biofilm formation [26]. These types of proteins are given the name *functional amyloids* [26, 27]. However, most amyloid formation that occurs in mammals is associated with diseases, referred to as *amyloidosis* [18].

The relationship between amyloids and diseases was made early on in the field, when "fibrous components" were identified in several diseased tissues of different sources [13]. Slowly, amyloid structures formed by different proteins started to be identified in many different diseases [28]. However, this association between disease and the presence of amyloids did not necessarily mean that they were the cause of it. A causal relationship between amyloids and diseases was made when several familial mutations that affected the amyloid proteins' production or aggregation propensity showed an increased risk of developing the disease

associated to them [29]. This finding led to the so-called *Amyloid cascade hypothesis*, first formulated for Alzheimer's disease, which hypothesizes that the deposition of amyloid proteins is the causative effect of the disease they are associated with [30]. Nowadays, over 50 diseases have been identified as being associated to amyloid proteins [8, 18]. Each of these diseases has one or more amyloid proteins whose aggregation into amyloid fibrils has been connected with the pathology. For instance, the two most known amyloid diseases, Alzheimer's disease and Parkinson's disease, are tightly associated with the amyloid proteins called Amyloid-beta (Aβ) and alpha-synuclein (αSyn), respectively [8]. These two proteins are the focus of all the papers in this thesis, with αSyn as the focus of Papers I and II, and Aβ (more specifically the variant Aβ42) for Papers III, IV and V.

Amyloid Beta (Aβ)

In 1907, Alois Alzheimer reported a case of dementia different to any case he had seen before [31]. In this study, he also reported the presence of a "peculiar substance". The disease has come to be known as Alzheimer's disease and is nowadays identified as the leading cause for dementia, accounting for up to 75% of the cases [32]. Alzheimer's disease is a progressive neurodegenerative disease that affects the cerebral cortex and the hippocampus, and its hallmark is the deposition of neurofibrillary tangles of amyloid proteins [33]. It wasn't until 1984 when the "peculiar substance" reported by Alois Alzheimer, and the main component of amyloid deposits, was identified as amyloid beta (Aβ) [34]. It was based on this connection that, in 1992, Hardy and Higgins proposed the *amyloid cascade hypothesis* [30]. Since then, Aβ has been the focus of many studies, and is often used as the model amyloid protein. Due to that, many of the descriptions in this thesis revolve around discoveries done first for Aβ, and later for other amyloids.

Aβ is found *in vivo* as a cleavage product of a transmembrane protein called amyloid precursor protein (APP) [35] (Figure 1.4a). APP is cleaved by β-secretase on the N-terminal side of aspartate 672 of APP, also referred to as aspartate 1 of the Aβ domain [36]. This can be further cleaved by γ-secretase, leading to the formation of various length variants of Aβ with different C-terminus lengths [37-39]. The most prominent cleavage products are Aβ40 and Aβ42, consisting of 40 and 42 amino acids, respectively [40]. Aβ40 is present in the brain at higher concentrations, but Aβ42 has received more attention due to a higher tendency to aggregate and neurotoxicity [41]. The two extra residues in the C-terminus of Aβ42 have been shown to have a big impact in aggregation and fibril morphology [42, 43]. Due to that, Aβ42 has been the protein of choice for Papers III, IV and V.

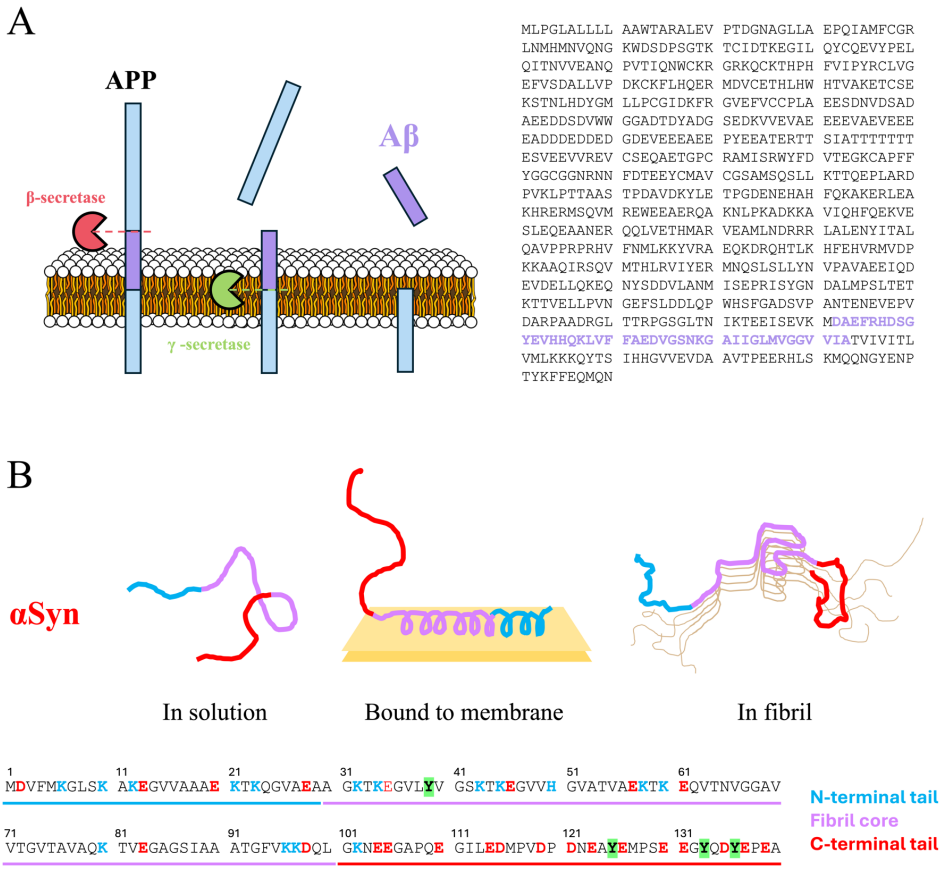

Figure 1.4. (A) Processing of APP leading to the formation of Aβ. β-secretase cuts APP at the N-terminal of Asp 672. γ-secretase cuts the C-terminal of the Aβ domain, with different points of cleavage leading to the formation of the different Aβ variants. In the right, the full sequence of APP, with the sequence of Aβ42 in purple. (B) Cartoon of αSyn in solution (as a random coil), bound to a membrane {first ca. 100 residues in α-helical conformation), or in a fibril (stacked with each other, forming β-sheet rich structures). On the bottom, the sequence of αSyn, with the N-terminal tail in blue, fibril core in purple, and C-terminal tail in red. Acidic and basic amino acids are colored red and blue in the sequence, respectively. Finally, tyrosine residues (Y) are highlighted green. Figure B is adapted from Figure 1 in Paper II.

Alpha-Synuclein (αSyn)

α Synuclein (αSyn) is a protein first identified in the electric ray *Torpedo californica* [44]. A few years later, while studying amyloid plaques from Alzheimer's patients, usually full of amyloid β, a protein referred to as "non-amyloid β component" was identified as αSyn [45]. However, αSyn gained notoriety when a family with familial Parkinson's disease was detected to have an alanine to threonine mutation

in residue 53 of the SNCA gene, responsible for expressing αSyn [46]. Finally, fibrillar αSyn was identified as the main component of Lewy bodies and Lewy neurites commonly found in Parkinson's disease and Dementia with Lewy Bodies [47]. All these and more studies indicated a strong association between several diseases and αSyn malfunction and highlighted the interest of its study.

Protein sequence

αSyn is one of the three isoforms of the synuclein family of proteins, composed of alpha, beta, and gamma synuclein. αSyn is composed of 140 amino acids and is often divided in three segments: the amphipathic N-terminal region (1-60), the central hydrophobic non amyloid β component (NAC) region (61-95), and the C-terminal acidic tail (96-140). The N-terminal region contains many charged residues, but it is slightly more positively charged (10 positive vs 8 negative residues). Due to it being highly conserved through the protein's evolution [48], and the fact that all familial PD mutations are found in this region [46, 49, 51-54], the N-terminal region is considered to play a crucial role in the protein's activity. The NAC region is responsible for the amyloid nature of the protein, as its hydrophobicity has been shown to be the driving force of amyloid formation for αSyn. In the segment of amino acid residues 1-95, αSyn has seven KTKEGV sequence repeats, which play a role in the protein's lipid binding ability [55], [56]. Finally, the C-terminal tail contains 15 acidic amino acids, giving it a strong negative charge in neutral pH. Upon fibril formation, the pKa values increase, which lowers the net charge and electrostatic repulsion in the fibrillar state [57]. Due to the protein's ability to aggregate into amyloid fibrils, its sequence is sometimes divided into three regions defined as N-terminal tail (1-28), fibril core (29-100) and C-terminal tail (100-140) (Figure 1.4b).

Membrane binding

Although its exact native function is still debated, αSyn is associated with vesicle trafficking and dilation of the exocytotic fusion pore [58]. This, along with the finding that Lewy bodies contain lipids in a big proportion [59], has increased the interest of investigating the protein's ability to interact with lipids. Upon binding to lipids, the first 20 to 100 residues of αSyn adopt an amphipathic α-helical structure, with the exact number of residues depending on the available lipid area [60, 61]. The remaining C-terminal residues keep a disordered structure, forming a dense brush of negatively charged tails in solution [62] (Figure 1.4b). The amphipathic α-helix sits parallel to the membrane interface, with the hydrophobic residues pointing down, while the charged residues are exposed to the solution. The adsorption of αSyn to lipid vesicles has been shown to follow a positive cooperativity [63], a behavior which could provide the key to understanding the protein's interaction with lipids and its function in membrane remodeling and vesicle trafficking. Therefore, studying the behavior of αSyn in lipid membranes may provide key insights towards

understanding its function *in vivo* [64]. The ability to aggregate into amyloid fibrils, as well as αSyn's lipid binding capabilities are the reason why this protein is the main focus of Papers I and II.

Given the broad differences between amyloid proteins and the diseases they are associated with, it is a remarkable feature that they all share the formation of a very similar structure, the amyloid fibril, by a process of similar mechanism. Due to that, understanding the process of amyloid fibril formation, which is a form of aggregation, is a crucial step to understanding these diseases, and a necessary effort to learn how to stop them.

1.3. Aggregation and Kinetics

Sigmoidal behavior

In order to understand the amyloid formation that is associated with diseases, we first need to understand how the protein, by itself, aggregates. This is done by *in vitro* studies where we can monitor the behavior of the protein alone, or with minimal additional components. Ideally, one wants to start one's experiments with a pure sample that contains only the specific protein one is interested in, and these proteins in the sample are monomers, i.e., they are by themselves, and not interacting or bound with any other neighboring proteins. As these proteins have a high propensity to aggregate, they are difficult proteins to handle, as we will cover later (see section 3.2. on protein purification). However, improvement in experimental methods has allowed us to understand the aggregation better [65-67]. Several methods can be used to detect the presence of fibrils, such as nuclear magnetic resonance, circular dichroism, fluorescence spectroscopy or scattering [68, 69]. Thioflavin-T (ThT) is one of the most commonly used fibril-detecting molecules, which we will cover later (see section 3.4. on ThT).

This way, if one has a sample with fully monomeric protein, and monitors the appearance of fibrils over time with any of these methods, one will observe the fibril appearance follows an S shape or *sigmoidal curve* (Figure 1.5). The aggregation starts with a *lag phase*, where the system seems to be at a standstill, and we see no visible appearance of fibrils, because their concentration may be below the detection limit of the method used. The lag phase is followed by an *exponential phase*, where fibril growth is accelerated, and the fibril appearance increases sharply. Finally, the fibril formation slows down into what is commonly referred to as a *final plateau*.

The behavior we observe actually consists of different microscopic steps that happen at different rates, and the use of analytical kinetic models has helped us unravel them [67, 70].

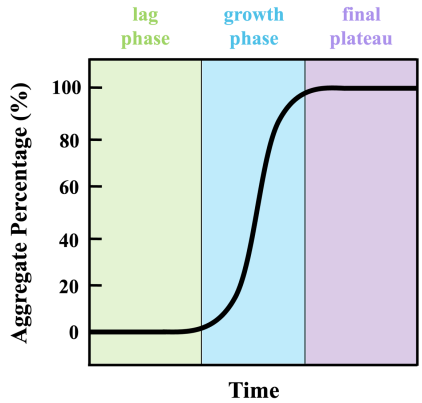

Figure 1.5. Schematic representation of the sigmoidal behavior of amyloid aggregation kinetics.

Primary processes: Primary Nucleation and Elongation

Amyloid proteins have a high tendency to aggregate when present at a concentration above their solubility limit. This means that the protein monomers have an affinity for each other, and they will form large and small species consisting of more than one monomer together [71]. However, the small species are very unstable, so they are very quick to dissolve back to monomers [72]. In fact, they have a higher tendency to dissolve than to form [73]. So, they will try and try, and combine in different numbers, and in different shapes, but then dissociate back to monomers faster than they were formed. This is because the energy cost of forming the right structure, and start the transition, is very big. This is often described as an energy barrier. However, when a species is formed that overcomes that energy barrier, it will then have a bigger tendency to grow than to dissolve back. These species that overcome the energy barrier are called nuclei, and the step that leads to their formation in amyloid proteins is called **primary nucleation** (Figure 1.6a). In other phase transitions that need nucleation for the process to start, the key to overcoming the energy barrier and forming a nucleus is the size of the aggregate. However, for amyloids, the specific structure of the species is also crucial. Due to the big energy barrier that needs to be overcome to form a nucleus, primary nucleation is the slowest process in amyloid formation. This is also the reason for the lag phase one observes when following fibril formation [68] (Figure 1.5). Once the first nucleus

is formed, the rest of the processes can start, while primary nucleation continues in the background and remains at roughly unaltered rate during the entire lag phase.

Once a nucleus is formed, more monomers can join it to make it grow. The addition of a monomer to a nucleus is called **elongation** (Figure 1.6b). As amyloid aggregates into fibrils, this elongation occurs at either end of a fibril. The energy barrier for elongation is lower than the one for primary nucleation, and thus, elongation is a faster process [73]. These two processes together can describe many phase-transitions, such as crystal formation [74] or nanoparticle aggregation [75]. These types of phase-transitions are commonly described as nucleated-growth processes.

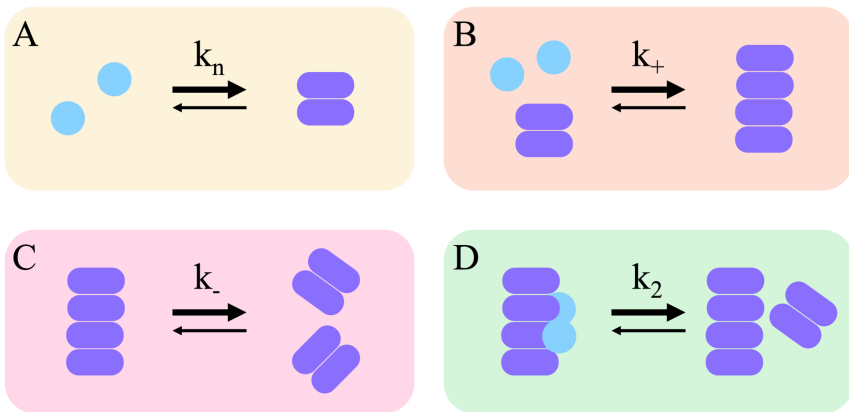

Figure 1.6. The four microscopic steps of amyloid aggregation: primary nucleation (A), elongation (B), fragmentation (C) and secondary nucleation (D). Blue circles represent free monomers, and purple discs represents monomers in fibril conformation.

Secondary processes: Fragmentation and Secondary Nucleation

While primary nucleation and elongation alone can describe many phase transitions, the exponential nature of amyloid aggregation cannot be fully described with only these two processes. The sharp exponential increase that follows the lag phase suggests the presence of another step, that somehow leads to fibrils generating more fibrils. These processes are called secondary processes and are responsible for the exponential growth phase of the sigmoidal curve [67]. But how can a fibril generate more fibrils? One simple way to explain that is through **fragmentation** (Figure 1.6c). If a fibril of a certain size breaks, it can generate two fibrils from one. The

number of proteins in fibrillar form stays the same, but now there will be more fibril ends available for monomers to bind to, which makes elongation stronger, and accelerates the whole aggregation process. This is supported by the finding that if the sample is subjected to strong shaking, fragmentation is accelerated, and that speeds up the aggregation process [67].

The three processes described above could have been enough to describe amyloid formation. However, when evaluating experimental data with a mathematical model for the study of Aβ42 aggregation, it showed that that alone was still not enough to explain the exact behavior of aggregation [67]. That is when the fourth step was identified, that being **secondary nucleation** (Figure 1.6d). Secondary nucleation refers to the formation of a nucleus catalyzed by the fibril surface. In short, similar to primary nucleation, monomers come together and try to form a nucleus. This time, however, they do it while bound to the side of the fibril, and the fibril surface "helps" them perform this transition by lowering the energy barrier. This way, the presence of fibrils leads to the formation of new nuclei, accelerating the aggregation by a lot. Secondary nucleation was a phenomenon first described for sickle cell hemoglobin [76], but it was known in the field of crystallization for a while [77]. Adding secondary nucleation to the aggregation mechanism made the experimental data obtained for Aβ42 match the theory, validating its role in amyloid aggregation [67]. The presence of secondary nucleation in amyloid formation has been confirmed for Aβ42 [67], Aβ40 [43], αSyn [78], IAPP [79] and insulin [80].

Now we have a complete picture, consisting of primary nucleation, elongation, secondary nucleation, and fragmentation (Figure 1.7). These processes can happen simultaneously once the primary nuclei are formed, and they will happen at different rates throughout the different stages of the aggregation. Measuring the rates of these different steps is referred to as studying the *kinetics* of the aggregation. If we monitor the fibril appearance over time, we can measure the duration of the lag phase (lag time), the size of the final plateau (endpoint), or the time it takes to form 50% of the final fibril content (t-half, or $t_{1/2}$), among other things. These parameters can reflect on the dependency the aggregation has on the rates of the different microscopic steps [70]. To do that, it is necessary to formulate a mathematical description of these steps.

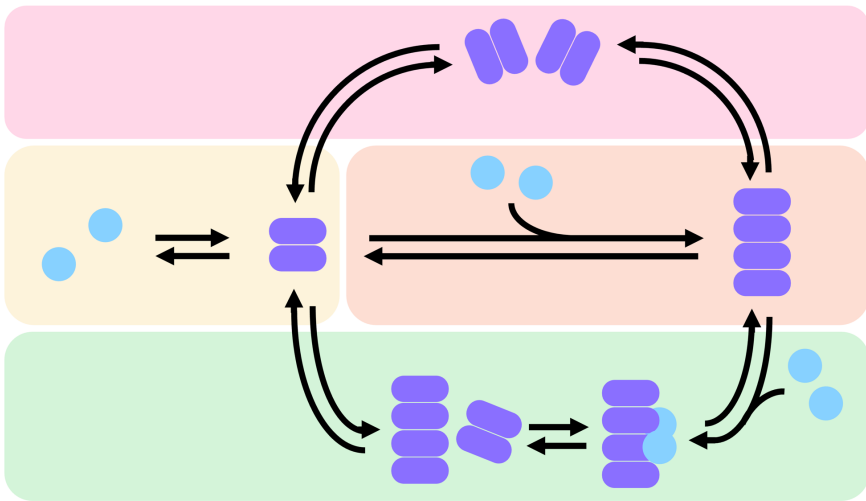

Figure 1.7. Primary nucleation (yellow), elongation (orange), fragmentation (red) and secondary nucleation (green) come together to form the amyloid aggregation mechanism.

Rate equations and Kinetic analysis

The protein that has been the target of kinetic analysis in this thesis, Aβ42, has been demonstrated to follow aggregation dominated by secondary nucleation, and fragmentation plays a negligible role in it [67]. Due to that, and for the sake of simplicity, we will focus on a kinetic model with only primary nucleation, elongation, and secondary nucleation.

To mathematically describe the rate at which a chemical reaction happens, or *reaction rate* (v), we need three components [81]. First, the *concentration* of the substrate of the reaction. As this changes over time, we describe it as a function of time ($x\ (t)$). Secondly, we need the rate constant of the reaction (k). A rate constant is a proportionality constant. When we multiply it by the concentration of the substrate of a reaction, it can give us the rate of that reaction. In very simple terms, it tells us what the speed of the reaction is for a particular substrate concentration. Finally, we need to know the *reaction order* (n). This value depends on the mechanism of the reaction, and it tells us the impact the substrate concentration has on the reaction speed. It is represented as the exponent of the concentration, leading us to the equation:

$$v = k \cdot x(t)^n$$

In some cases, the reaction order acquires a value of 1, making the reaction rate only depend on *x (t)* and *k*. The reaction rate constants are of course different for the different steps of aggregation (Figure 1.6). To distinguish them, we call them:

k_n = primary nucleation rate constant

k_+ = elongation rate constant

k_2 = secondary nucleation rate constant

Our reaction has two main components: monomers and fibrils. Their concentration as a function of time is often described as follows:

m (t) = concentration of free monomers

M (t) = mass concentration of fibrils

P (t) = number concentration of fibrils

The distinction between M and P is very important. Primary and secondary nucleation affect the *number* of fibrils in solution (P) by creating new ones. Meanwhile, elongation doesn't create more fibrils, but it makes the already existing ones grow in *mass* (M).

Now that we have defined the main components, we want to know the rate at which the concentration of each of them is altered. This is mathematically depicted by a partial derivative such as $\partial X/\partial t$. At the beginning of the reaction (*t = 0*), in a fully monomeric sample, the concentration of free monomers (*m(0)*) is whatever concentration we have in the sample, and the concentration of fibrils is zero (*P(0) = M(0) = 0*). Because there are no fibrils, the only reaction happening is the one that only depends on monomer concentration: primary nucleation. The rate at which primary nucleation generates new fibrils is described by this equation:

$$\left(\frac{\partial P}{\partial t}\right)_{primary} = k_n \cdot m(t)^{n_c}$$

where n_c is the primary nucleation reaction order. This equation describes how the number of fibrils (P) increases over time, depending on the concentration of monomer (m) and the primary nucleation rate constant (k_n). Primary nucleation forms new fibrils, increasing their number, but it gives a very small contribution to fibril mass (M). The main contribution to fibril mass comes from elongation. The rate at which elongation generates fibril mass is described by the following equation:

$$\frac{\partial M}{\partial t} = k_+ \cdot m(t) \cdot P(t)$$

As elongation requires a fibril and a monomer to occur, the monomer mass (m) and the fibril number concentration (P) influence it, as represented in the equation. At the same time as creating fibril mass, elongation depletes monomers from the solution. The rate at which monomers are removed from the solution is of course the reverse of the rate by which the new fibril mass is produced. Therefore, the rate at which it does that is described by:

$$\frac{\partial m}{\partial t} = -k_+ \cdot m(t) \cdot P(t)$$

Once fibrils are formed, they can catalyze new fibril formation via secondary nucleation. This is described by the equation:

$$\left(\frac{\partial P}{\partial t}\right)_{secondary} = k_2 \cdot m(t)^{n_2} \cdot M(t)$$

where n_2 represents secondary nucleation reaction order. As all reactions contribute to the mass of different species, one needs to account for them together to predict the behavior of the system. To quantify the contribution of all the microscopic steps to the fibril mass, and use this to calculate the reaction rate constants of each of them, an analytical solution was derived [82] leading to the master equation:

$$\frac{M(t)}{M_\infty} = 1 - \left(\frac{B_+ + C_+}{B_+ + C_+ e^{\kappa t}} \cdot \frac{B_- + C_+ e^{\kappa t}}{B_- + C_+} \right)^{\frac{k_\infty^2}{\kappa k_\infty}} e^{-k_\infty t}$$

where

$$\lambda = \sqrt{2k_+ k_n m(0)^{n_c}}$$

$$\kappa = \sqrt{2k_+ k_2 m(0)^{n_2+1}}$$

$$B_\pm = \left(k_\infty \pm \tilde{k}_\infty\right)/(2\kappa)$$

$$C_\pm = \pm\lambda^2/(2\kappa^2)$$

$$k_\infty = \sqrt{2\kappa^2/[n_2(n_2+1)] + 2\lambda^2/n_c}$$

$$\tilde{k}_\infty = \sqrt{k_\infty^2 - 4C_+ C_- \kappa^2}$$

By using these equations and various expansions of them, one can extract individual rate constants from a reaction, and evaluate how they are affected under different conditions. This is made easier by the online platform Amylofit [70], where one can upload experimental data and analyze it by fitting models based on these equations. By evaluating the quality of the fit between the model and the experimental data, and finding the best fit, one can calculate various kinetic parameters of an aggregation reaction. This type of analysis is the core of Papers III and IV.

If you have a sharp eye, you might have noticed how the whole aggregation description has focused on monomers and fibrils, but I very briefly mentioned the existence of other species that I then proceeded to ignore. At the very beginning of this section, I explained how monomers interact with each other to form very transient species consisting of more than one monomer. The reason why I brushed over them is not because of their little importance, but quite the opposite. In fact, these species happen to be the focus of this thesis, and we call them amyloid oligomers.

2.Amyloid Oligomers

2.1. Definition

Amyloid oligomers are transient species in amyloid fibril formation, believed to be the main species responsible for the pathology of the diseases they are associated with. Their exact definition varies slightly in the literature, often being described based on how their features compare to those of fibrils [83]. Thus, oligomers and fibrils differ in size (smaller vs bigger), in ability to grow (slower vs faster), structure (less ordered vs highly ordered), surface properties (more hydrophobic vs less hydrophobic) and toxicity (toxic vs less toxic) [84-92]. In some studies, these types of comparative features are associated to specific detection techniques, meaning they are operational definitions, leading to the potential disagreement in definitions found in the literature. Additionally, even though these comparative terms apply for the majority of oligomers, they don't necessarily apply to everything referred to as *oligomer* in the literature.

Recent studies have focused on shedding light on oligomer terminology to improve our collective understanding of the association between amyloid oligomers and fibrils, as well as the nature of their toxicity [92, 93]. If we look at the IUPAC Compendium of Chemical Terminology, *oligomer* is defined as "A molecule whose structure comprises a small plurality of units derived from molecules of lower relative molecular mass, whose properties vary significantly with the removal of one or a few of the units" [92, 94]. This definition captures a very heterogeneous group of species, which can vary in size, structure, toxicity, and their propensity to grow into fibrils. Regardless of their heterogeneity, oligomers gained the interest of amyloid researchers due to their connection to toxicity.

2.2. Toxicity

The existence of Aβ oligomers was reported for the first time by *Frackowiak et al* in 1994 [71]. At the time, the amyloid cascade hypothesis was being introduced, and amyloid plaques were believed to be responsible for the pathogenicity of Aβ. Due to that, Aβ oligomers were initially regarded as harmless intermediates of amyloid aggregation [95]. Slowly, many independent studies started to point at the relationship between amyloid oligomers and toxicity. Firstly, it started to be evident that fibrils or fibrillar aggregates did not correlate with pathology as well as initially expected. Studies started showing that species of Aβ formed when inhibiting fibril formation showed neurotoxicity [96], and the presence of Lewy Bodies in Parkinson's disease was not necessarily correlated with pathology [97-99]. Furthermore, samples containing oligomers, but without fibrils, showed the ability to cause neuronal death and affect cognitive function *in vivo* [91, 100, 101]. All of these studies cemented the idea that oligomers were the source of toxicity in amyloids. Amyloid oligomers have since been associated with many pathological effects. For instance, Aβ oligomers have been associated with neural plasticity dysfunction [91, 96, 102, 103], selective neuron death [96, 104-106], impact on astrocytes and microglia [107-110], oxidative stress [111-114], receptor redistribution [115-118], disrupted Ca^{2+} homeostasis [114, 119, 120], synapse deterioration [117, 121, 122], loss of choline acetyltransferase [123, 124], cell cycle re-entry [125, 126], endoplasmic reticulum stress [127, 128], insulin resistance [118, 129-131], aberrant Tau phosphorylation [106, 110, 130-133] and inhibition of axonal transport [134-136], among others.

The specific mechanism of amyloid oligomer toxicity is still unknown. The specifics of their mechanism most likely differ between amyloid proteins, oligomer size and structure, among other features. However, given how many different disease-associated protein oligomers show toxicity, and even some non-disease associated proteins too [137], it is fair to assume they must share a somehow similar mechanism of toxicity [138]. One feature many oligomers share, and thus one of the main suspects to explain their mechanism, is the interaction between oligomers and membranes [101, 139]. Suggestions for the exact mechanism of oligomer toxicity include reactive oxygen species formation [140, 141], increased membrane permeation [142-146], cell membrane pore formation [147-149], binding to nerve receptors [150, 151] and even extracting lipids from the membrane and incorporating them into aggregates [152]. However, to fully understand the role oligomers play in amyloid proteins, one needs to understand their heterogeneity.

2.3. Features of Amyloid Oligomers

As stated above, oligomers are very heterogeneous species that vary in size, structure, or toxicity, among others [84-91]. For a better understanding of these species, it is crucial to establish some parameters and terminologies used in the literature to describe and distinguish them, as well as some of their most characteristic features.

Fibrillar vs non-fibrillar

Oligomers are non-covalently bound transient species. As described when talking about primary nucleation, monomers come together to form oligomers, but they have a high tendency to dissociate back to monomers [72, 73, 93]. Nevertheless, there are those that are capable of rapid elongation through the addition of monomers, similar to how fibrils grow, and have an affinity for fibril-specific antibodies [153, 154]. However, they are shorter than fibrils, even short enough to be colloidally suspended, and they meet the definition of oligomer given above. These species, which share traits with both oligomers and fibrils, have been given the name *fibrillar oligomers* [83, 153]. Since their elongation is faster than the appearance (nucleation) of new fibrils, fibrillar-oligomers are found at lower concentrations than *non-fibrillar oligomers*. Nevertheless, their presence can have a big effect in aggregation kinetics due to their powerful seeding efficiency [85]. Kinetically, most oligomers are non-fibrillar and are incapable of rapid growth [93].

On-pathway vs off-pathway

Up until now, we have talked about oligomers as if they were a transient intermediate of fibril formation. While that can be the case, one can imagine that oligomers could also be formed with a structure so incompatible with that of a fibril, that the only way for the monomers forming that oligomer to become a fibril is to dissociate to monomers and "try again" (Figure 2.1). That hypothetical oligomeric species would not be an intermediate of fibril formation, but rather produced in a side-reaction. Oligomers that are or are not intermediates of fibril formation are often referred to in the literature as on- and off-pathway, respectively [90, 139, 153, 155-157]. This definition comes from early studies of protein folding, where it was hypothesized that proteins folded through a single well-defined "pathway" in the energy landscape, with the steps in between being considered on pathway [158]. However, in the vast network that we now know protein folding and amyloid conformations to be, this vision might not give a full picture of the system [159].

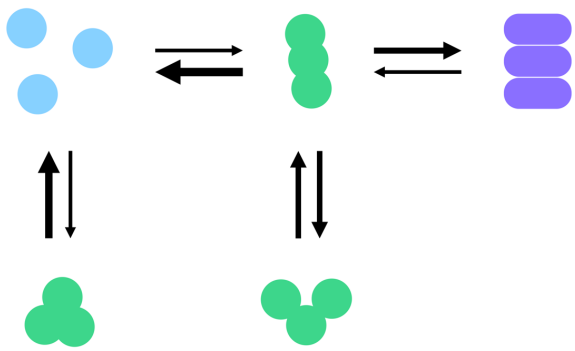

Figure 2.1. Amyloid oligomers (green) can be formed from monomers (blue) as transient intermediates of fibrils (purple). An example of this would be the oligomer with the rod-like shape. Other oligomers are side products, and they cannot turn into fibrils without first dissociating into monomers, as is the case for the globular oligomer at the bottom left. However, the oligomer generation network is even more complex, and can have many additional oligomer steps, as represented by the third oligomer in a V conformation.

Given that every oligomer has a finite, albeit sometimes small, chance of becoming a fibril, a binary description such as on-and off- has been put into question [83]. Instead, *Dear et al.* suggest what they define as *pathway index*, which represents the reduction of the flux to fibrils caused by omitting the reactions that the oligomer takes part in [83]. This can be taken as an indication of the "importance" of a specific oligomer in the fibril formation process. This non-binary definition of on- and off-pathway also considers the fact that the role of an oligomer in fibril formation depends both on the monomer concentration, and the time of the reaction. Regardless, it is important to keep in mind that some oligomers are intermediates in the fibril formation process, whereas others occur as side products, and many of the methods used to study oligomers do not distinguish between the two.

Role of oligomers in fibril kinetics

Given that some oligomers are intermediates of fibril formation, one would expect them to play a key role in fibril kinetics. Although oligomers are not considered in the kinetic model described in section 1.3., they are associated to the processes of nucleation described in that section. We first hinted at them when describing primary nucleation, and this is of course a process that generates oligomers. However, for amyloids such as Aβ42 and IAPP, secondary nucleation has been demonstrated to play a bigger role in oligomer formation than primary nucleation

[73, 160, 161]. To back this up, molecular chaperones targeting secondary nucleation of Aβ42 have been shown to reduce the toxicity in vivo by reducing oligomer formation [160, 162]. When referring to the oligomer formation by these processes, they are sometimes referred to as *primary association* and *secondary association* (Figure 2.2). This connection between fibril and oligomer formation highlights the fragile balance existing between the two, and how targeting one can affect the other. The importance of the role of secondary nucleation in Aβ42 oligomer formation is a central concept of Papers III, IV and V.

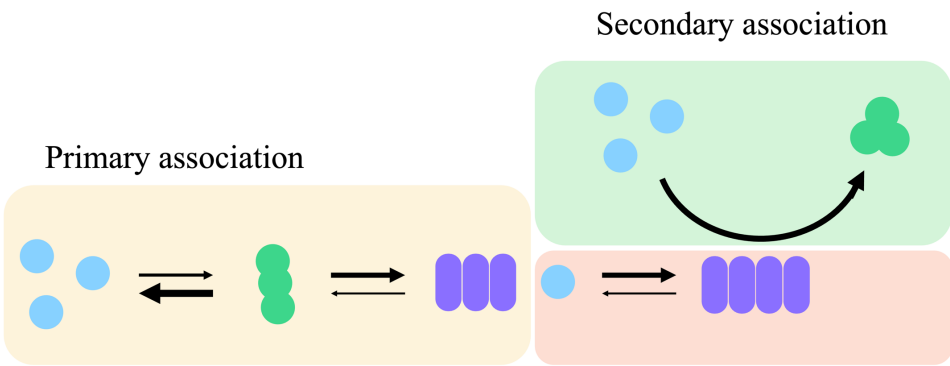

Figure 2.2. Oligomer formation and its connection to fibril formation processes. Oligomers can be formed via primary association, which, when describing fibril formation is referred to as primary nucleation (yellow). However, it has been demonstrated that oligomers form more prominently via secondary association, the formation of oligomers catalyzed by the fibril surface (green).

Productivity

As mentioned above, amyloid oligomers have a high tendency to dissociate back into monomers. However, some of them can turn into fibrils and gain a higher tendency to grow than to dissociate. These constitute the two ways the concentration of oligomers can be affected: by turning into fibrils or turning back into monomers. The *productivity* of an oligomer refers to its propensity to convert into fibrils when compared to its propensity to dissociate to monomers. All amyloid oligomers reported to date show a very low productivity, as mentioned above, due to their high tendency to dissociate back to monomers rather than fibrillate [72, 73, 93]. As an example, αSyn and Aβ42 oligomers, the ones showing the highest productivity of the ones studied, have a productivity rate which barely reaches the 10% mark [93].

Abundance

Amyloid oligomers also differ from each other on their *abundance*. This refers to the maximum concentration of oligomers reached, relative to the monomer concentration of the reaction. For Aβ42 and αSyn, due to their high relative persistence, and them "outliving" the monomers, the theoretical maximum relative concentration is not reached in practice, as monomers disappear from the solution before a steady state is reached [93]. Oligomers with low persistence, however, do reach these values. Interestingly, functional amyloids seem to show lower abundance than that of amyloids associated with toxicity [93].

Persistence and half-life

A way to evaluate the kinetic stability of oligomers is to calculate their *persistence*, or how long do they survive for. By analyzing time-dependent concentration measurements of oligomers and fitting them to a kinetic model, the half-life of different oligomers can be calculated [93]. When doing so, it has been observed that most oligomers have half-lives between 1 and 5 hours. However, αSyn oligomers show a half-life of orders of magnitude longer. This higher stability is theorized to be associated with their structure.

Another interesting parameter to compare regarding oligomers in is their *relative persistence*, or the persistence of oligomers compared to that of monomers. If the relative persistence of an oligomer is high, oligomers stay in solution longer than monomers do, and their disappearance is driven only by oligomer dissociation. In contrast, if the relative persistence is low, oligomers and monomers reach their equilibrium fast, and the disappearance of oligomers from solution is driven by the depletion of monomers. Most oligomers are less stable than their monomers, and thus show a low relative persistence. However, oligomers of Aβ42 and αSyn studied to date have a lifetime that exceeds that of their monomers, and thus a high relative persistence [93]. This means that their disappearance from solution is determined by dissociation. This aspect is central to the study performed in Paper V.

Structure

In the hopes of better understanding oligomers and their role in fibril formation and toxicity, many studies have aimed to elucidate the structure of oligomers. Some oligomers, such as those formed by Aβ40 and Aβ42, have shown to have a generic micellar structure [163, 164]. The fact that Tau and Ure2 oligomers have a similar half-life to Aβ40 and Aβ42 oligomers suggests the possibility of a similar architecture [93]. However, we previously noted that αSyn shows a half-life of

orders of magnitude longer, and this is associated to its structure identified in the literature [90, 165-169].

Overall, αSyn oligomers show higher hydrophobicity than fibrils [90, 167-169]. This hydrophobicity promotes membrane interaction and is argued to be crucial for the toxicity of oligomers [169]. However, some research groups have developed protocols to stabilize αSyn oligomers, which has allowed them to study their structure in higher detail. The most characteristic feature of these αSyn oligomers is that they have an anti-parallel β-sheet rich core, with a more disordered fuzzy outside [165-169]. The less structured region has been identified as being the C terminus for some, and N terminus for other oligomers, and the availability of the N terminus has been shown to play a role in membrane interaction and toxicity of oligomers [169]. Furthermore, some of these oligomers show a ring-like shape [168, 170], which is theorized to be responsible for membrane permeabilization by creating a pore [171].

However, many of these structural studies are performed using stabilized oligomers, which may be representative of only a fraction of the vast heterogeneous population of αSyn oligomers. This leads to a risk of survivor bias, where features attributed to these species are taken as common features of all αSyn oligomers. With that in mind, it is easy to see how αSyn oligomers that were enriched via a stabilizing method will lead to results of higher structure and longer persistence than that of other amyloid oligomers. This risk can be circumvented by the use of different methods, especially important to have when studying such heterogeneous species which can be visible to some, but invisible to other methods. Due to that, the development of methods and techniques to study amyloid oligomers is of crucial importance. This thesis focuses on the optimization and use of two techniques used for amyloid oligomer studies.

3.General Methods

3.1. Protein synthesis

Recombinant protein synthesis

At the dawn of biochemistry as a field, the only way to study proteins was by harvesting big amounts of a sample (tissue or bodily fluid) that contained said protein and isolating it [172]. This is the reason why many initial protein studies involved proteins obtained from easily harvested sources such as milk, blood, or egg white. One example of that is lysozyme, one of the first proteins to be sequenced [173] and to have its X-ray structure determined [174]. As the field evolved, protein studies focused on whichever protein was more easily available. For instance, after the Armour Hot Dog company developed a protocol to purify bovine pancreatic ribonuclease A in bulk in the 1950s [172] and distributed to scientists, RNase A became the main target for protein research. However, to do the same with a human protein expressed in neurons, one can easily see how harvesting human brains to squeeze some protein out of it is probably problematic. But what if we could just ask an organism to produce a protein that it usually doesn't?

As ridiculous as that idea sounds, it was made possible when, in 1973, Stanley Cohen and colleagues managed to build a DNA plasmid *in vitro* that was biologically functional when inserted into *Escherichia coli* (*E. coli*) [175] (Figure 3.1). Later on, *Itakura et al* used this technology to express the human hormone somatostatin in *E. coli*, proving that human proteins could be expressed in bacterial hosts [176]. Since then, methods have developed to allow expression in other systems, such as yeasts [177], baculovirus/insect cell systems [178] or even mammalian cells [179].

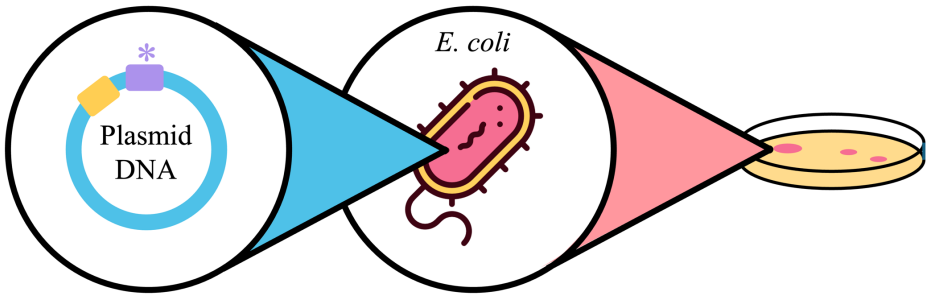

Figure 3.1. Recombinant expression of proteins. A plasmid DNA containing the DNA sequence encoding our protein of interest is inserted in an expression system (in this case, *E. coli*).

With this wide range of options, choosing the right expression system becomes crucial for an optimal protein expression. As one can imagine, human proteins are more faithfully expressed in human cells, as they can more accurately reproduce the natural cellular environment and post-translational modification machinery of the protein [180]. However, due to their complexity and tedious handling, one frequently opts for using simpler organisms to produce their protein. For that same reason, bacteria are still the most commonly used expression system [181], with *E. coli* being the most prominent one due to fast doubling time, ease of growth and ease of genetic manipulation [182].

Peptide synthesis

Cell free protein expression

Given that protein synthesis is a well understood chemical reaction, this same process can be performed in a lab without the use of living organisms. Proteins produced this way are referred to as *synthetic proteins* in this thesis. The first example of such a method is what is known as cell-free protein synthesis, developed by Nirenberg and Matthaei to help elucidate the connection between mRNA and protein expression [183]. The idea behind this method is using the biological machinery of an organism without using a living cell [184]. This method allows better control of the environment of the protein during its production.

Peptide synthesis

However, the system can be simplified even more, by including the minimum components necessary for the formation of a protein: amino acids and the means to covalently bind them together. The first example of such a method is one now referred to as Solution Phase Peptide Synthesis (SPS) [185, 186]. The reaction is centered around the use of "protected" amino acids, amino acids with a chemical modification to stop it from binding covalently to another amino acid [187]. Although many different types of protective groups can be found, the most famous example of a removable temporary amino-protective group is the carbobenzoxy group (Z) [188]. In SPS, an N-terminus protected amino acid is mixed with a C-terminus protected one [189]. The protective ends ensure that once two amino acids react via the un-protected ends, no more amino acids will add to them, forming a dipeptide. After bonding them together, this dipeptide is isolated, purified, and characterized. After removing the N-protective group from the dipeptide, it can be mixed with the third amino-acid of choice. This process is repeated until the protein of interest is synthesized. Due to the need of purification and characterization between every step, the product is purified and evaluated constantly, ensuring high purity. However, the process is very long and tedious, and, for this reason, has been overshadowed by later developed methods.

The most well-known is the one developed in 1963 by Merrifield, nowadays referred to as Solid Phase Peptide Synthesis (SPPS) [190]. In this method, one uses a surface functionalized with hydroxyl or amide groups, which allows for the first amino acid of the peptide to be covalently surface bound. SPPS most often starts with an N-protected C-terminus amino acid being immobilized into the surface via its carboxyl group [191]. After the amino acids that have not bound to the surface are removed, the protective group can be removed from the N-terminus via chemical reaction. Now, the second amino acid of the wanted peptide can be added. By adding one protected amino acid at a time, making it bind, removing the unbound ones, removing the protective group, and adding the next amino acid, one can control the one-by-one elongation of a peptide, until the full sequence is formed. The peptide can then be cleaved from the surface using strong acid, precipitated, and isolated [191]. The solid support makes the isolation of intermediates unnecessary, as well as overcomes the problems of poor solubility of intermediates caused in SPS [186].

Since its creation in 1963, SPPS has been vastly improved by the addition of new solid supports, new linkers, new side chain protection methods and new carboxyl-activating groups [192]. Although many other protein synthesis strategies have been developed since (such as Liquid-Phase Peptide Synthesis or Native Chemical Ligation, among others), SPPS remains the most frequently used method.

Differences of Aβ depending on production route

When it comes to Aβ, several studies have reported a difference in behavior between recombinantly produced protein and that produced through peptide synthesis methods [193-197]. This alarming difference can cause big discrepancies between studies using different sources of protein. However, while the difference has been reported, the mechanistic origin of this is hitherto unknown. The goal of one of the studies of this thesis is to shed light into the origin of this difference, in the hopes of clarifying possible misunderstandings between researchers who choose different protein sources (see Paper III).

3.2. Protein purification

We have covered how one can produce proteins for a study. However, anything that is not our protein and is present in the sample can bias whatever analysis we want to do to it. For this reason, it is common procedure to isolate the protein of interest until the sample contains only our protein. This process is called protein purification.

Steps of purification

Break cells

If the protein has been expressed in cells, the first logical step is to break the cells apart in order to extract the component of interest (Figure 3.2). Cell lysis, as it is called, can be done in many ways: high pressure homogenization, sonication, osmotic shock, and so on [198, 199]. Usually, the method is chosen based on the cell type and the purpose of the lysis, but combinations of several methods can also be used. During the lysis, additional measures are taken to ensure the protein's well-being. For instance, as cell lysis often releases proteases (protein-digesting enzymes) into the media, it is recommended to continue to the next steps quickly and to keep the sample cool in order to reduce its activity. Alternatively, protease inhibitors can be added to the lysate. Another common step is to add DNase to the lysate, in order to degrade the DNA and reduce the viscosity of the sample. Finally, the whole lysis is often done with a buffer that ensures the pH stays at the value desired for the protein of interest.

Isolate proteins

Once the cells are broken, we are left with a sample that contains all the biomolecules present in the organism. The next challenge is to isolate the proteins

from the rest of the biomolecules. A very common first step of purification used to achieve this is centrifugation [198, 199] (Figure 3.2). By applying centrifugal force to our sample, particles of different mass and density will migrate differently. If the necessary force is applied, particles of a certain type will be sedimented at the bottom of the centrifugation tube (referred to as pellet), whereas the rest will be left still suspended in solution (referred to as supernatant). This allows one to separate these components by simply collecting the supernatant and using it for further steps. If the pellet is the one containing the fraction of interest, one can remove the supernatant and resolubilize the pellet to continue with it instead. By playing with different solution conditions, centrifugal forces, and various steps of centrifugation, one can easily enrich the sample with proteins and remove most if not all of the other biomolecules.

Isolate our protein

Now, we have a sample rich in proteins, but we would ideally want to remove all the proteins that aren't ours, which is usually a very high percentage of the sample. At this stage, the molecules we are trying to separate are more similar to each other than in previous steps. Due to that, more meticulous separation methods have to be used, exploiting the different physico-chemical properties of different proteins [198, 199]. One of the most commonly used methods to do this is chromatography.

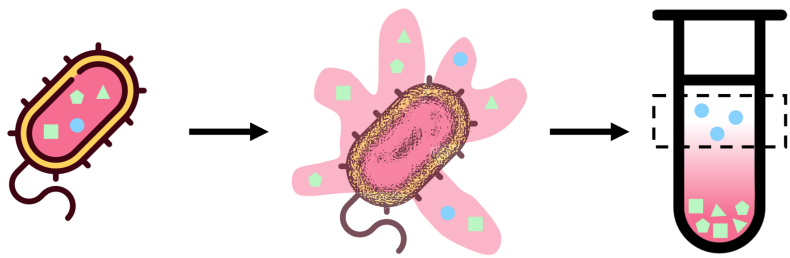

Figure 3.2. Schematic representation of purification procedure. The organism, which has expressed our protein of interest (left) is subjected to cell lysis (middle). The resulting mixture is then centrifuged to separate our protein of interest from the rest.

Chromatography

A chromatography is a separation method, where the sample is dissolved in a solvent (liquid or gas) referred to as the *mobile phase*, and this is made to flow through a solid component (i.e., a column or a sheet), with a material referred to as the *stationary phase* [198, 199]. As different components of the sample have different physico-chemical properties, they will also have different partition coefficients for the mobile versus the stationary phase. This difference in affinity will make the components flow at different speeds, allowing us to separate them. There are different types of chromatographies, different in the nature of both the mobile and the stationary phase, as well as the physico-chemical traits of the proteins they exploit to separate them.

Size exclusion chromatography (SEC) separates proteins based on their size [198-200] (Figure 3.3). This is achieved by using a porous stationary phase, with pores of different sizes. Smaller proteins, being able to squeeze through more pores than the bigger ones, have a bigger available volume in the column, and thus take longer to reach the end of it. On the other hand, bigger proteins will take a shorter time going through the column, as they don't get "engaged" in the pores.

Ion exchange chromatography (IEC) separates proteins based on their charge [198, 199, 201] (Figure 3.3). If the stationary phase has a strong positive charge, negatively charged proteins will stick to it, while positively charged proteins will pass through the column more easily. Of course, the reverse is also possible, where a negatively charged stationary phase can be used to bind positively charged proteins. In order to make the proteins separate from the stationary phase, one can add salt to screen the electrostatic attraction between protein and resin, making the bound proteins come loose and follow the mobile phase instead. Furthermore, adding salt in a slow gradient of increasing concentration allows one to have weakly charged proteins come loose when the concentration is low, and stronger charged proteins follow later, when the salt concentration is higher [199]. This means that not only does IEC separate positive and negatively charged proteins, but it can also separate proteins with different charge intensity. Alternatively, one can use a pH gradient to separate proteins depending on their pKa value differences.

Many other chromatography methods are of course available, such as reverse phase chromatography or affinity chromatography. However, size and charge has been shown to be enough to efficiently purify amyloid proteins [57, 65].

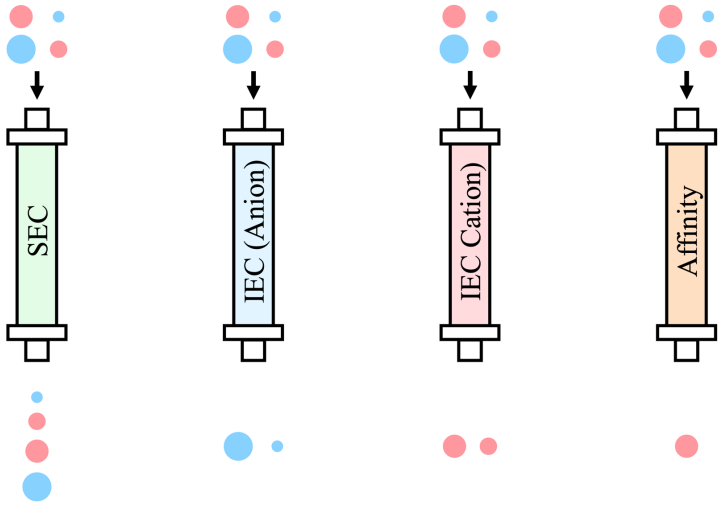

Figure 3.3. Schematic representation of different types of chromatographies. We have a sample with four different proteins of different sizes (circles). Some are positively charged (blue), and some are negatively charged (red). If we use SEC (green column), we will have all the proteins eluting from biggest to smallest. If we use Anion exchange chromatography (blue column), negative proteins will stick to the column and positively charged ones will elute. If we use Cation exchange chromatography (red column), the opposite will occur. Finally, an affinity chromatography (orange column) can bind one or more proteins according to more specific characteristics.

Our purification procedure

Importance of purification

As one can imagine, the purer the sample, the more reliably we can study it. Although this is a general and logical concept, it becomes readily apparent for amyloid self-assembly [66, 69, 202]. The behavior of Aβ is so strongly affected by the smallest quantities of impurities, that its aggregation used to be considered stochastic [203]. Colloquially known as “the protein from hell” for the difficulty of its handling [204], it requires stringent purification to make its aggregation reproducible [65, 66]. The finding that Aβ could indeed lead to reproducible data, and that it was simply a matter of its purity, made a big impact in the field. But how is this process exactly done?

Purification procedure

For αSyn, the cells are broken with sonication and then centrifuged. The supernatant is then poured into boiling buffer and heated to 85 °C. This makes most *E. coli* proteins precipitate, while αSyn remains in solution, which we can then carefully collect to continue with the purification. The sample is then subjected to two IEC and the fraction containing αSyn is freeze-dried until further use. More details on αSyn purification can be found in Papers I and II.

When it comes to Aβ42, however, the recombinantly expressed protein is accumulated in aggregated structures called inclusion bodies, making the purification process slightly different. In this case, after sonicating the sample to break the cells, the inclusion bodies go to the pellet after centrifugation. This way, if we resuspend the pellet, and repeat this process a few times, we can slowly get rid of almost everything except the inclusion bodies. Once this is achieved, the inclusion bodies are solubilized by addition of a high concentration of urea. This sample is diluted and subjected to IEC. The fraction containing Aβ42 is freeze-dried, redissolved in guanidine hydrochloride (GuHCl) and purified with SEC. This last step is repeated again (freeze-dry, redissolve in GuHCl, purify with SEC), and the final sample containing Aβ42 is freeze-dried in aliquots until further use. During all these steps, we monitor where our protein of interest is via 280 nm absorbance and by using SDS-PAGE (see section 3.3). The sample is always evaluated with mass spectrometry at the end of the purification process, and sometimes with NMR spectroscopy. More details on Aβ42 purification can be found in Papers III, IV and V.

Monomer isolation

Sadly, when working with amyloid proteins, it isn't as simple as just having your protein alone in the test tube. As described before, amyloid proteins are very prone to interact with each other very strongly, forming fibrils and all kinds of smaller aggregates. This means that while we do obtain our protein at the end of our purification protocol, we don't know if it is monomeric, fibrillar, or in some stage of an ongoing aggregation. Due to that, and given that most of our studies focus on the aggregation process, we have an additional step to ensure the sample we have is fully monomeric prior to the experiment. This process is in essence the same as the SEC performed during Aβ42 purification, but it is done as an additional last step, for both αSyn and Aβ42, and always directly before the experiments are performed.

The first step of this process is to take the freeze-dried protein, and to dissolve it in 6 M GuHCl. GuHCl is a chaotropic agent, which has a strong capacity to dissolve fibrils and smaller aggregates and reduce whichever structure amyloids are forming

into their monomeric form [65, 205]. However, one has to be aware that this process might not be complete, and some species bigger than monomer may remain. For that reason, the sample is then subjected to a SEC, to separate species by their size (i.e., monomers from potential remaining oligomers or fibrils). The chromatography is performed with the buffer of choice for the upcoming experiment. This way, SEC allows us to not only isolate monomeric protein, but also to exchange the buffer from GuHCl to the one we want to do the following experiment in. This way, we collect a fully monomeric and pure protein sample, which, if handled quickly and at low temperatures, will remain that way until our experiment starts. This monomer isolation process is a crucial step to ensure sample purity in terms of its aggregation order. And it is for that reason that it has been performed prior to absolutely every experiment described in this thesis.

3.3. Electrophoresis and its applications: SDS-PAGE

Electrophoresis

Electrostatic interactions are one of the major interactions governing the behavior of molecules. One such phenomenon, called electrophoresis, describes how a charged particle will move in response to an electric field. This motion depends on the particle's net charge, size, and shape [81]. Proteins, being charged species, are affected by this phenomenon, and thus, it can be exploited to separate or analyze them. In the 1930s, Arne Tiselius pioneered the development of electrophoretic methods for protein studies [206]. Since then, electrophoretic methods, often combined with different protein detection strategies, are a staple of every biochemistry lab. μFFE, the method used to study oligomers in Papers III, IV and V is centered around the use of electrophoresis in buffer solution (see section 4.3.).

SDS-PAGE

How does it work?

Developed in 1970 [207], Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) has become one of the most widely used electrophoresis-based methods for protein studies. To unravel this mouthful of a method, one must start from the end of its name. A polyacrylamide gel electrophoresis is, of course, an electrophoresis performed in a gel, which, to no avid reader's surprise, is made of a substance called polyacrylamide.

Polyacrylamide is a gel-like compound formed by the polymerization of acrylamide under the effect of high temperatures or exposure to UV light [208]. If a sample is injected in a polyacrylamide gel, and an electric field is applied across the gel, proteins with a negative charge will move towards the positive side of said electric field [198]. However, bigger molecules will have a more difficult time passing through the pores of the gel, while the smaller ones will have an easier time, and migrate faster [81]. This way, the method allows us to separate proteins based on their size.

As previously mentioned (see section 1.1.), different proteins have different charges, and will thus migrate differently based on that too, making the separation more complex. A way to work around this is to add sodium dodecyl sulphate (SDS) to the protein sample. SDS is an amphipathic molecule, having both a polar negatively charged component, as well as an apolar one. When SDS is at concentrations below 7-10 mM, it is fully monomeric. However, when that concentration is exceeded, the solution becomes a mixture of coexisting micelles and monomers [209]. The addition of further monomers will only lead to the formation of new micelles. On top of that, in concentrations above 0.1 mM, SDS has the ability to denature proteins, being able to denature most of them when at 1 mM [210] (Figure 3.4a). Although the nature of this interaction is not fully understood [211], SDS binds to most proteins at a rate of one SDS molecule per two amino acids [210, 212]. This interaction masks the intrinsic charge of the proteins, as an excess of negatively charged SDS will lead to all protein-SDS complexes being heavily negatively charged. In fact, these protein-SDS species also have a very similar charge-to-mass ratio. Making all the proteins negatively charged at the same charge-to-mass ratio means that, when subjected to an electric field, all species will migrate to the anode (positive end of the field) and can be separated by mass alone (Figure 3.4b). One should note, as mentioned before, that the shape of the protein can still have an impact in their migration, which is very relevant in analyzing results in Paper I and especially in Paper II.

SDS does not only unfold, or denature, proteins, but it can also break the interactions between monomers of the protein. In the case of amyloid proteins, for instance, SDS has the ability to disaggregate fibrils back into monomers. This means that the outcome of using SDS-PAGE on a fully monomeric sample is the same as using it on a fully fibrillated sample, as long as their concentration is the same (see Paper I).

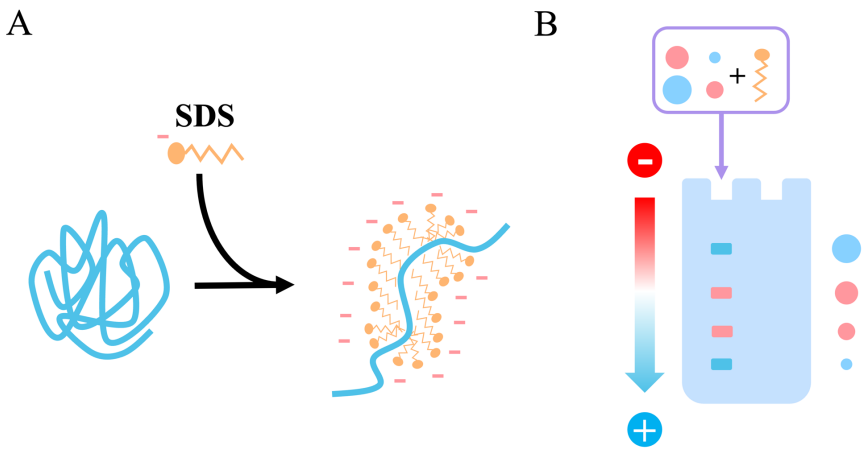

Figure 3.4. (A) When adding SDS, proteins are denatured, and SDS binds to them enough to mask their natural charge and give them an overall negative charge. (B) When mixing a complex sample with SDS and running an SDS-PAGE, proteins will migrate towards the positive end of the applied electric field and will be separated by size.

Detecting the protein

We have established how the proteins are separated. However, as previously mentioned, unless intrinsically colored, another step is necessary to detect said proteins. In the case of SDS-PAGE, this is done by adding a reagent that reacts with proteins and generates color, making the otherwise invisible proteins visible. This is referred to as staining the gel. Many different stains exist that react with proteins with different intensity, sensitivity, efficiency and duration [198]. The most commonly known ones are Coomassie blue [213] and Silver Staining [214]. However, simply being able to see them doesn't tell us much more than how many differently sized molecules we have in the sample (i.e., if all proteins in the sample are the same size and shape, we will see one band, but if there are two different groups, we will see two). A simple way to make this method more informative is by adding a protein sample of known size as a reference. This way, one can use a protein ladder (a mixture of many proteins and peptides of various known sizes) and put it in the gel next to your sample. By comparing the bands of known size with the ones from our sample, one can estimate the size of the proteins forming our sample's bands.

Advantages and disadvantages

SDS-PAGE is a staple of biochemistry research. It is very quick, inexpensive, and efficient. For instance, when a protein is expressed recombinantly, it is common to analyze the cell lysate with SDS-PAGE. If the expression is efficient, one will be

able to see a big band with the size of the expressed protein, hopefully bigger than the other proteins from the expression organism. As one purifies the protein from its contaminants, SDS-PAGE can be used to evaluate how pure the sample is, and thus evaluate the success of the purification. If that was not enough to justify its wide use in making this thesis, SDS-PAGE is also a very efficient method to analyze the outcome of PICUP reactions, as done in Papers I and II.

3.4. Fluorescence and its applications: ThT

General fluorescence

The study of small molecules always involves observing how they interact with different physico-chemical stimuli, and deducing information about the molecule based on that interaction [81]. One very frequently used stimulus is electromagnetic radiation. Electromagnetic radiation exists in different wavelengths. Lower wavelength electromagnetic radiation, such as radio waves, have lower energy, whereas higher wavelength ones, such as X-rays, have higher energy. It is in wavelengths between 400 and 700 nm where we find the visible spectrum, what we commonly refer to as *light*. Light of different colors differ in wavelength (and therefore in energy), and molecules can interact in different ways with them. A lot of molecules in nature can absorb light, which leads to the excitation of electrons in the molecules to orbitals of higher energy. The wavelength and amount of the light absorbed changes from molecule to molecule and can even vary depending on the state the molecule is in. These differences in light absorbance give rise to the different colors we observe with our eyes. However, some molecules have the ability to not only absorb light, but also emit a light of a different wavelength as a consequence. This is what we call fluorescence [81].

When a molecule that is capable of emitting fluorescence (a fluorophore) absorbs a certain wavelength light, it absorbs its energy, and can go into what is defined as an *excited state* (Figure 3.5a). This means that at least one electron in the molecule moves to an orbital of higher energy. The shift in wavelength depends on concomitant excitation of vibrations and the different time scales of events. When the molecule relaxes back to its ground state, it emits light back out. However, the molecule stays in the excited state for a very long time relative to how quickly the vibrational excitation relaxes back to ground state (10^{-10} s vs 10^{-13} s, respectively). Due to this relative long lifetime of the excited state, the molecule has enough time to lose some of that absorbed energy (i.e., vibrating or releasing thermal energy). This means that part of the energy of the light that the molecule absorbed is lost,

and the light emitted when the electron is relaxing back to ground state has lower energy. As previously noted, the difference in energy translates to difference in wavelength. This is why many commonly known fluorescent substances absorb UV light (higher energy and wavelength than visible light), and they emit visible light our eyes can see [81].

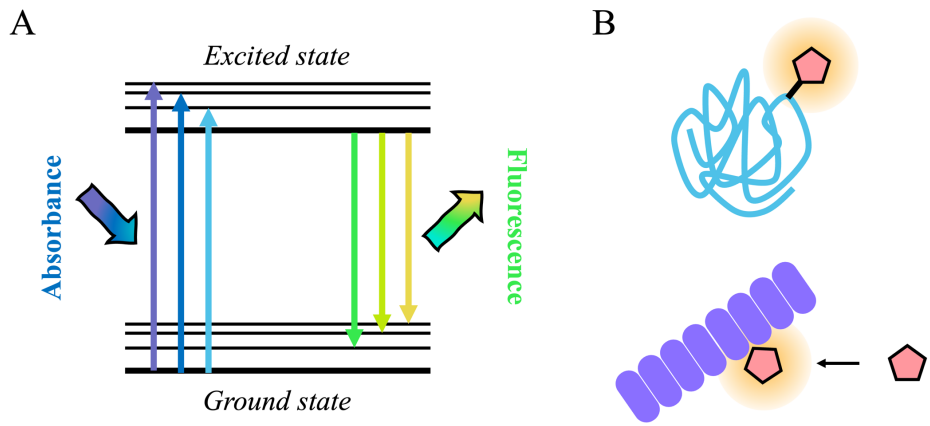

Figure 3.5. Fluorescence and its applications. (A) Absorbance of light of a particular wavelength gets an electron from the ground state to the excited state. When the electron goes back down to the ground state, it emits light with lower energy (higher wavelength). (B) Fluorescence monitoring of samples via covalently bound fluorophore (top), or extrinsic fluorophore (bottom) such as ThT, that increases its fluorescence when bound to fibrils.

Fluorescence to monitor samples

Fluorescent molecules are very convenient for research, as we can use tools with higher accuracy than our eyes to detect light, reaching resolutions down to nanometers or even Ångströms [215]. This way, molecules that emit fluorescence can be more easily studied than those that don't. Sadly, not everybody has the luck to study a protein with fluorescence of its own (referred to as *intrinsic fluorescence*). However, one can chemically link a fluorophore to their target molecule via a covalent bond, allowing one to monitor it as if it were fluorescent itself [216] (Figure 3.5b, top). Albeit a very convenient tool, the covalent addition of a fluorophore alters the chemistry of the studied molecule, and thus runs the risk of altering its behavior. For this reason, one may instead opt for adding an *extrinsic fluorophore*: a fluorophore independent of the target molecule, whose fluorescence changes depending on its interaction with our analyte. This way, one can interpret the state of the target molecule based on how the fluorescence of a second molecule changes when interacting with it. Still, there is significant risk that interaction with the non-

covalent fluorophore shifts the equilibrium or affects the rates of the process. A fluorophore covalently bound to Aβ42 was used for studies using μFFE (see section 4.3), and an extrinsic fluorophore was used for aggregation kinetic measurements in all papers: Thioflavin T.

Thioflavin T

Thioflavin T (ThT) is an extrinsic fluorescent molecule that is very commonly used in amyloid protein studies since its interaction with amyloids was identified [217, 218]. The molecule consists of a benzyl and a benzothiazole ring covalently bound by a single carbon-carbon bond. When ThT is free in solution, the rotation of this bond lets a lot of energy dissipate, meaning that when it absorbs light it will emit very little fluorescence. However, when ThT binds to β-sheet rich structures such as amyloid fibrils, the rotation of said carbon-carbon bond is restricted. This leads to an increase in the fluorescence of the molecule, which has excitation and emission maxima of 440 and 482 nm wavelength, respectively. This means that if amyloid proteins are unaggregated in solution with ThT, we will not detect much fluorescence. However, as the protein aggregates into highly ordered amyloids, ThT will bind to them and increase its fluorescence (Figure 3.5b, bottom). This way, monitoring ThT fluorescence allows one to follow amyloid fibril formation in an indirect way.

One should note that the mechanism by which ThT interacts with amyloid fibrils is much more complex than described here [219]. In fact, ThT can bind differently to different amyloids, and thus give fluorescence to different extents for different species [220]. For that reason, ThT fluorescence data is often normalized, meaning that the fluorescence is plotted relative to the fluorescence at the beginning and the end of the aggregation, showing the relative ThT fluorescence. This is how the kinetic measurements were performed in all papers.

4.Methods to study Amyloid Oligomers

4.1. Types of methods

We have established the importance of oligomers, not only for understanding the amyloid aggregation, but also to fully unravel the connection between amyloid proteins and the diseases they are associated with. However, oligomers are usually species with very low stability, high heterogeneity, are very transient, and at very low concentrations relative to monomers and fibrils [221]. This means that the characterization of specific oligomers cannot be done with common bulk solution methods that rely on averaging the features of all the species in the sample. For this reason, very few methods have the capacity to study oligomers.

Methods used to study oligomers can be divided into two groups [222]. On one hand, there are methods that can detect a single species in solution or distinguish the contribution of individual species to the signal detected. These methods do not rely on altering the system (albeit with some exceptions), and thus can be referred to as *non-perturbing methods*. On the other hand, one can purposefully alter the system to favor the presence of a specific species, reducing the heterogeneity of the sample and allowing for easier analysis. We will refer to these methods as *oligomer enrichment methods*. In a recent review, *Cawood et al.* describe the different types of methods and their strengths and weaknesses [222]. Here, with that paper as inspiration, we provide a short summary of the available methods and their differences.

Non-perturbing methods

Nuclear Magnetic Resonance spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy has been widely used for the characterization of amyloid protein aggregation [223, 224]. Due to its high versatility, many different methods can be used with NMR spectroscopy, which give different information about amyloids. Some methods exploit the fact that some species are NMR-invisible [224, 225]. By looking at the relaxation rates similar to those of monomers, and the exchange between the species, one can monitor small oligomers [226, 227], or look at the exchange with bigger species to study larger oligomers [228]. More advanced methods, such as pressure-jump NMR, allow for changing the energy landscape of amyloid formation in a controlled manner by applying pressure changes, and compare unfolded monomers at high pressure with oligomers formed when decreasing the pressure [229]. NMR can also be used to obtain structural information via PRE studies [223, 230, 231]. These allow one to determine distance restrains, which can then be used in conjunction with molecular dynamic methods to get structural information [232]. However, they require the addition of a paramagnetic spin label, which has the risk of altering the interactions [233]. Other NMR-based methods used to study oligomers include ^{13}C-methyl-TROSY and solid-state NMR [234, 235], although these are more fit for monodisperse samples such as stabilized oligomers.

Single particle methods

This refers to methods that can detect individual species in heterogeneous mixtures. One notable example of that are single particle fluorescence methods. These methods rely on adding a fluorescent dye to the target molecule and using a detection technique precise enough to distinguish single species from each other. One example of this is microfluidic free-flow electrophoresis (μFFE), the method we used in Papers III, IV and V, which we will talk about further in section 4.3. Some single particle fluorescence methods are instead based on labelling with two fluorophores, which can then be used to measure either simultaneous fluorescence or FRET [84, 85, 236-239]. By monitoring the population of a specific oligomer over time, one can also fit the obtained data into kinetic models and determine the rates of oligomer formation [85, 93, 238, 240, 241].

Other methods for single particle studies are also available. For instance, atomic force microscopy (AFM) has previously been used to study oligomers [242]. AFM methods generally focus on dimerization studies, by placing a monomer in a surface and evaluating how it interacts with a monomer placed in the cantilever [243-248]. AFM can also be used as a surface imaging technique [249], having been used for secondary structure imaging when coupled with infrared spectroscopy [250]. The

main limitation of single particle methods is their low structural resolution. However, recent advances in cryo-electron microscopy give hopes of this method having the potential to be used to study structures of single species in the near future.

Oligomer enrichment methods

Sample preparation strategies

Another way to study amyloid oligomers is by skewing the species distribution to favor the generation of a specific oligomer distribution. One way to achieve this is by using particular sample preparation strategies. One very commonly used protocol involves the use of lyophilization, resuspension, and filtration to produce kinetically trapped αSyn oligomers [168, 251, 252]. Production of these types of oligomers has led to many successful studies of the structure, behavior, and toxicity of these oligomers [157, 167-169, 253]. Alternatively, one can obtain similar results with carefully chosen buffers. For instance, there are studies on Aβ peptides prepared in low salt and low temperature [254, 255], or in the presence of detergent micelles [256, 257].

Non-covalent binders

Oligomers can also be stabilized via non-covalent binders. An ideal tool for binding and stabilizing transient amyloid oligomers are antibodies [258]. The most well-known one is the A11 antibody, which recognizes pre-fibrillar toxic oligomers formed by a range of amyloid proteins [138]. Other antibodies which instead bind and stabilize specific amyloids have also been developed [259-261]. Antibody-oligomer complexes have the potential to provide high-resolution structural information. Small molecules other than antibodies can also be used for the same purpose [262-264].

Covalent modification and binders

Covalent modifications of amyloid proteins have also become a common method for modulating their interactions [265-270]. This includes, for example, covalently binding a non-covalent ligand to improve its affinity [265, 271] or modifying the protein sequence [272-275]. These processes are mostly done to improve the stability of certain oligomers. However, they can also be used to destabilize unwanted interactions. For instance, backbone hydrogen bonding, essential for fibril formation [276], can be blocked by chemical modification, blocking the pathway from oligomers to fibrils [277]. This can even be done in a reversible way to tune the time-dependence of oligomer populations [278].

Cross-linking

Finally, forming a covalent bond between the monomers within an oligomer increases oligomer stability. This is known as cross-linking [279]. Stabilizing the species by covalent bond formation makes it possible to purify them, and even allows generation of pure oligomer samples for structural and functional studies [235, 280, 281]. These methods, unlike the previous ones, have the potential to trap oligomers *in vivo*, and even study interactions between oligomers and cellular components. However, this is problematic in practice due to the large number of species that amyloid proteins can be cross-linked with, leading to a low signal-to-noise and very complex data analysis [222]. The most common cross-linking method for the study of amyloid oligomers is the Photo Induced Cross-linking of Unmodified Proteins (PICUP). PICUP, and its use to study αSyn oligomers and transient interactions, are the main focus of Papers I and II (see section 4.2.).

Choice of method

When choosing a method to study oligomers, one should be very aware of the strengths and weaknesses of each procedure and scrutinize which method fits one's protein and question best. At face value, one may think that methods that don't perturb the system would be objectively better. After all, adding any type of tag or change in the protein runs the risk of altering the system. However, non-perturbing methods show a low structural resolution, and are more fit for gaining kinetic information on oligomers. In contrast, oligomer enriching methods are more suited to obtain structural information, and thus may be of better help to unravel the mechanism of oligomer toxicity [222]. This way, one could instead divide the methods as those that are more suited for kinetic information, and those that are better for gaining structural information.

With that in mind, this thesis is focused in two methods used for the studies of amyloid oligomers. The first one, PICUP, is an oligomer enriching method, which we have optimized to obtain structural information about αSyn, its oligomers, and other relevant transient interactions. PICUP of αSyn is the focus of Papers I and II. The second one, μFFE, is a single particle fluorescence technique more suitable for kinetic studies, which we have used as a complementary method to compare Aβ42 oligomer populations between different systems and conditions. μFFE of Aβ42 is utilized in Papers III, IV and V.

4.2. PICUP

As described above, cross-linking is a common method used to stabilize and trap oligomeric species for their study. Cross-linking refers to the formation of covalent bonds between or within molecules [279]. By generating a covalent bond, one can bind species that were previously non-covalently bound, stabilizing them. This way, if the sample subjected to cross-linking contains oligomers, the monomers within an oligomer can be bound together. This makes the previously transient species stable enough to be subjected to analysis that would otherwise alter the system. For instance, one can analyze the sample with SDS-PAGE and, due to covalent bonds not being perturbed by it, measure the oligomer size-distribution of the sample (Figure 4.1a). In order to capture snapshots of transient and short-lived oligomers, fast cross-linking reactions that are indiscriminate in their amino acid preference offer many advantages [282]. Due to that, Photo-Induced Crosslinking of Unmodified Proteins (PICUP) has become a very popular cross-linking method in the amyloid field [86, 281, 283-285].

Reaction

PICUP is a fast oxidative coupling method that can be used without the need for prior modification of the target protein [286]. The reaction revolves around the metal-coordinated complex ruthenium (II) tris-bipyridilcation (Ru(bpy)). When Ru(bpy) absorbs visible light in the presence of an electron acceptor such as ammonium persulfate (APS), it steals an electron from an amino acid. This radical amino acid can then react with another one and form a covalent bond between them (Figure 4.1b) [287]. The amino acid targeted depends on the capacity of the group to stabilize the unpaired electron (most reactive residue being tyrosine, tryptophan, and cysteine) [86, 283, 287, 288], and the distance between reacting residues during the lifetime of the radical [86, 283, 286]. Due to the lack of a standardized machine to perform PICUP, each lab uses their own custom-made apparatus to perform the reaction. Most studies are done with a machine consisting of a common lamp as a light source, and a camera as a sample holder, with the camera shutter controlling the lighting time [86, 283].

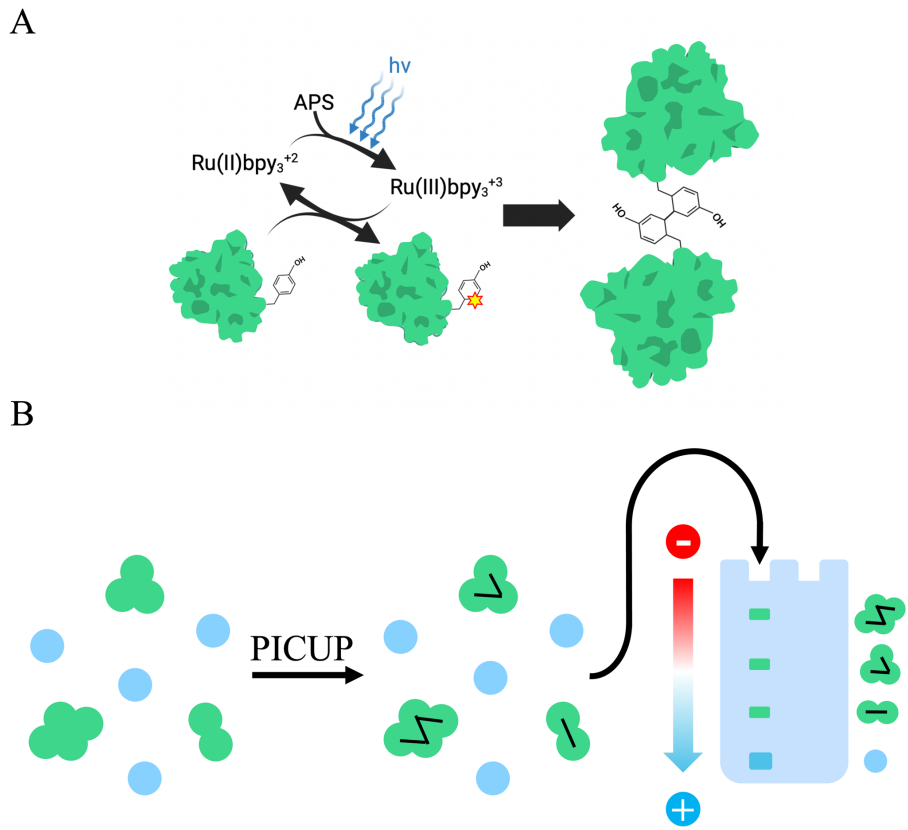

Figure 4.1. Schematic representation of PICUP reaction (A), where Ru(bpy) and APS are used in the presence of light to induce the formation of a covalent bond between two tyrosine residues. Samples can be run in an SDS-PAGE for analysis (B), where they will separate by size.

Advantages

The photo-induced intermediate radicals have a short lifetime, which makes photo-cross-linking methods have a greater specificity when compared to other chemical cross-linking methods [289]. PICUP requires a particularly short reaction time, having been shown to work at irradiation times of under a second [86, 283, 286, 287, 290, 291]. This is particularly important when studying short-lived species like amyloid oligomers, as longer duration reactions run a risk of biasing the system. Additionally, as PICUP requires no modification of the proteins prior to the reaction, it is more likely to capture species formed in native conditions than other methods

which require protein labelling of any sort [292]. On top of that, as PICUP forms a single covalent bond, amino acids must be at a covalent bond distance during the lifetime of the radical to react together [293]. This makes the method very specific to close interactions, making it a very useful tool for structural studies. Finally, the light used for the reaction is in the visible range, making PICUP applicable to cell extracts [287].

Previous uses

Due to all the advantages described above, PICUP has been used for many amyloid oligomer studies. Some examples include comparing oligomer distribution of native and mutant species [86, 284, 291, 294-300], evaluating the effect of inhibitors or other factors on oligomer formation [301-318], and even generating covalent oligomers of defined size [319-324]. The purification of cross-linked samples via an SDS-PAGE extraction method has also been developed, leading to the characterization of the secondary structure, amyloidogenicity and cytotoxicity of PICUP cross-linked Aβ40 oligomers [280, 281]. A similar method has also been applied to Aβ42 [281, 325]. However, most of the literature on PICUP focuses on Aβ, with a minority focusing on αSyn [284, 288, 291, 307, 309, 311, 313, 326, 327].

PICUP in this thesis

Given PICUP's potential for the study of amyloid oligomers, we aimed to optimize the method for the study of αSyn. To do so, in Paper I, we developed a reproducible, easy to modify and inexpensive PICUP reaction chamber with a time resolution down to 1 ms. Using this tool, we demonstrated the outcome of the cross-linking to be oligomers, and not monomers diffusing in close proximity, and we showed the effect various conditions have on the outcome of the reaction. In Paper II, with the help of αSyn mutants, we identify the amino acids partaking in the cross-linking of αSyn when in solution, adsorbed to lipids, or in fibril form. By doing so, we demonstrate PICUP to be a very powerful tool to study transient interactions of αSyn in different environments. More details on PICUP, its strengths and weaknesses and on how to perform the reaction can be found in Papers I and II.

4.3. μFFE

As previously explained, conventional bulk-phase methods have a few shortcomings when analyzing transient short-lived species such as oligomers. One way to study oligomers is by using single-particle methods which can pick out an

individual population or species in a complex sample. However, even some methods that are able to distinguish individual species in a more heterogeneous samples rely on very specific conditions which have the potential to bias the system. Other methods, used to reduce the heterogeneity of the sample prior to measurement, take a relatively long time, increasing the risk of perturbing protein interactions. Therefore, as explained for PICUP, methods that require short reaction or measurement time are crucial to ensure the system is to be as unbiased as possible. Single particle studies have proven very useful for amyloid oligomer studies [84, 85, 236-239, 242, 249, 328]. However, for these types of studies, samples need to be very dilute in order to distinguish between individual particles and diluting prior to analysis can lead to the dissociation of unstable oligomers [236]. This problem can be reduced using microfluidics [237, 329].

Microfluidics

The advantages of using microfluidics come from the different behavior of fluids at the micro scale [330, 331]. In bulk, the main source of mixing comes from chaotic turbulence. In contrast, at the micro scale, there is a low ratio of inertial to viscous forces. This leads to a laminar flow, where viscosity forces dominate, leading to mixing occurring via diffusion rather than turbulence. This way, in the laminar regime, the diffusion (D) of a particle of a spherical shape with a hydrodynamic radius R_H can be described by the Stokes-Einstein-Sutherland equation:

$$D = \frac{k_B T}{6\pi\eta r}$$

where k_B, T and η stand for Boltzmann's constant, temperature, and the viscosity of the fluid, respectively. However, a sample placed on a flowing liquid will also be subjected to advection, the moving of the sample by motion of the fluid. The competition between advection and diffusion is often represented by the Péclet number. This way, a system with a high Péclet number will lead to very slow mixing between molecules. By generating such a condition, one can ensure that diffusion is not the main source of separation of the sample in solution. This is a crucial requirement for performing Microfluidic Free Flow Electrophoresis (μFFE), to, in this instance, avert sample separation through diffusion [332]. When running μFFE, the main phenomenon separating our samples is electrophoresis.

Electrophoresis

In μFFE, an electric field is applied perpendicular to the flow of the sample to promote fractionation [333] (Figure 4.2a). The separation of oligomeric species in the electric field in μFFE is described in detail by *Arter et al* [334]. In simple terms,

the electrophoretic mobility of an oligomer (μ_o) is proportional to the charge of the oligomer (q_o), but inversely proportional to the oligomer's hydrodynamic radius (r_o).

$$\mu_o \propto \frac{q_o}{r_o}$$

Increasing the size of an oligomer, the charge will increase in a linear fashion (i.e. the charge of a dimer is equal to the sum of the charge of two monomers), whereas the hydrodynamic radius increases to a lesser degree. Due to this, oligomers are expected to have a higher electrophoretic mobility than monomers, and this is expected to increase with the oligomer's size [334] (Figure 4.2c). This way, with the means to detect single species, we can identify different species based on their electrophoretic mobility. In µFFE, in order to visualize the sample, fluorescent labelling of the protein is most often necessary.

Fluorescent label

As the detection method of µFFE is based on confocal fluorescence, it is necessary to label the proteins with a fluorescent dye. However, the covalent addition of a dye runs the risk of altering the assembly of the amyloid protein [335]. Due to that, it is important to do thorough evaluation of fluorophore selection and placement. In the case of Aβ42, *Thacker et al*. tested an array of cysteine mutations to evaluate the effect of the addition of the dye in different residues on the kinetics and the morphology of Aβ42 fibril [336]. Based on those results, we used the S8C mutant of Aβ42 labelled with Alexa 488 for our µFFE studies, as it showed the same morphology and similar kinetics as modified Aβ42 wild type. However, because the fluorescently labelled mutant still shows slight differences in kinetics to Aβ42 wild type [336], we opted for using a mixture of labeled and unlabeled protein. This was done at a ratio which ensured wild type-like fibril morphology to accurately emulate protein behavior, without compromising the detection limit of the used setup.

Setup

The microfluidic device used for µFFE is designed with AutoCAD, and a photolithographic mask is printed on an acetate transparency based on it. Using this mask, a SU-8 model is fabricated [337], referred to as the master. The master is then used as a mold to form polydimethylsiloxane (PDMS) devices. The devices are then bonded onto glass slides to form the *chip* that is used for µFFE.

The device used for μFFE has a main electrophoresis chamber where the sample flows through (Figure 4.2a). The sample is flown there together with an auxiliary buffer, consisting of a buffer identical with the conditions of the sample (Figure 4.2a). There are two channels flanking the main chamber. There, 3 M KCl solutions are run as liquid electrodes. These channels are separated from the main electrophoretic chamber so that the generation of electrolysis products does not disturb the flow in the separation area. The liquid electrolyte channels also contain a fluorescent dye to use as reference for calibration of the chamber width in data analysis.

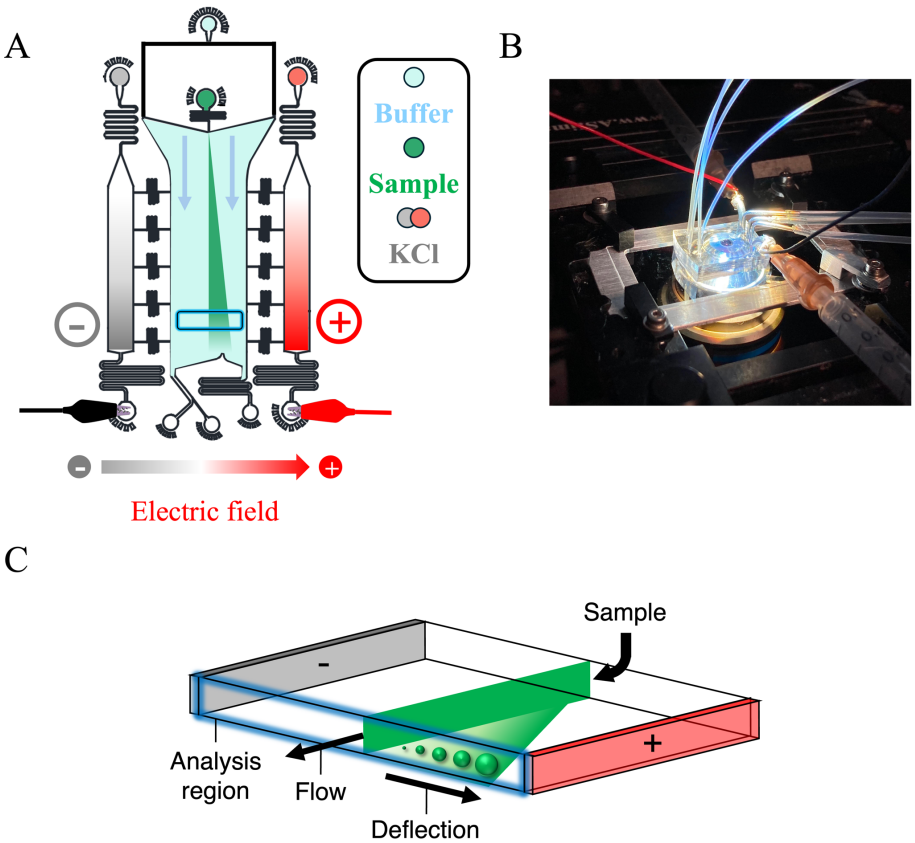

Figure 4.2. Microfluidic free flow electrophoresis (μFFE). (A) Microfluidic device, with an electric field applied perpendicular to the flow of the sample. (B) Confocal setup with the chip, tubing, and syringe. (C) Schematic representation of the deflection of the sample, which, for oligomers, we expect to cause bigger sized oligomers to deflect further, as represented by the size of the spheres. Adapted from Paper IV.

Once the chip is prepared, it is placed on a custom-built single-molecule confocal fluorescence microscopy setup. Tubes are connected to all inlets and outlets, and the flow is rigorously controlled using syringe pumps (Figure 4.2b). Potentials are applied through syringe tips inserted into the electrolyte outlets. A laser beam at 488 nm wavelength is focused on the center of the microfluidic detection channel. The same objective collects the fluorescence, which is focused into a single-photon-counting avalanche diode. A single photon counting module detects the signal at a time resolution of 25 ps, and a custom-written Python code records single-photon events. The chip is mounted in a motorized stage which can be controlled with a custom-made Python-code. Through a movement of the chip mounted on the stage, the confocal volume moves perpendicularly to the sample flow, scanning across the electrophoresis chamber.

An initial data analysis leads to obtaining many single-burst fluorescence measurements, each of them with a fluorescence intensity and a relative placement in the electrophoretic chamber. The electrophoretic mobility is associated with oligomer size as stated above. The fluorescence intensity gives us information about the size of the species by comparison to the fluorescence of a monomer. By defining a lower threshold of fluorescence intensity for what we consider a monomer, and a higher threshold for what we consider a fibril, we can count all the species between these values as being oligomers. This way, we can obtain values of oligomer populations of samples. Further details on setup, operation and data analysis can be found in Papers III, IV and V.

μFFE in this thesis

μFFE, developed at the Knowles lab in the University of Cambridge, has been used for studying αSyn oligomers, their origin, and membrane interactions [334, 338, 339]. For this thesis, we optimized the conditions for the study of Aβ42 oligomers. In Papers III, IV and V, μFFE was used as a complementary method, measuring the Aβ42 oligomer population under different conditions, broadening the understanding of the systems studied in each paper. This allowed us to unravel how Aβ42 oligomer populations are affected by the production source, sheer forces in the aggregation process, and the presence/absence of fibrils in the media.

5.Discussion of Papers

5.1. Paper I

The first paper in this thesis focuses on the optimization of Photo-Induced Cross-linking of Unmodified Proteins (PICUP) as a method to study αSyn oligomers. When looking at the PICUP literature, there is a strong prevalence of studies on Aβ, but very few on αSyn. At the same time, there is no standardized machine one can use to perform PICUP, with each lab building their own custom-made apparatus. With this in mind, we aimed to build a machine to perform PICUP of αSyn and describe the behavior of the reaction under different conditions relevant for amyloid studies.

The reproducibility of the method is tied to the reproducibility of the apparatus used to perform it. Due to that, we aimed to design a reaction chamber that would be as reproducible as possible (Figure 5.1). We designed a 3D-printable case with space for an LED of 450 nm (close to the maximum absorbance of Ru(bpy)), and an opening for a PCR-tube where the sample could be placed 1 mm away from the LED. The lighting time is controlled by an easy-to-use Arduino program with a precision down to 1 ms. Finally, the 3D model of the case is easily modified, giving high versatility to generating variations of this reaction chamber. The material, the 3D model of the case and the program used to control the reaction chamber are made available in the paper, to ensure accessibility. After building the apparatus, we studied the effect of various conditions important for performing PICUP, as well as the nature of the cross-linked products.

Firstly, we prove that the formation of additional bands in the SDS-PAGE gel after subjecting αSyn to PICUP comes from oligomers, and not monomers freely diffusing into proximity to each other. We do this by analyzing the effect of PICUP on lysozyme under the same conditions used for αSyn. Lysozyme has a similar size to αSyn, and thus a similar diffusion rate, and it has an even higher reactivity due to a higher tyrosine content than αSyn. Performing PICUP of lysozyme yielded no cross-linked products, showing that our conditions do not lead to cross-linking by

proximity from diffusion, and thus the species observed for αSyn must represent oligomers.

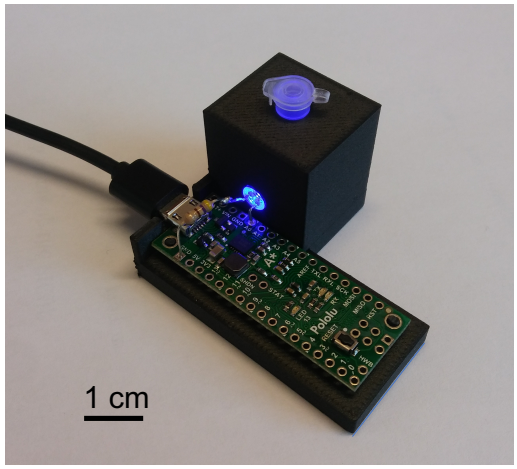

Figure 5.1. Reaction chamber designed in Paper I for PICUP

Secondly, we studied the end of the reaction and the importance of the reaction stopping reagents. First, we show that the time waited between turning the light off and the addition of reaction stopping buffer has no effect on the reaction outcome, suggesting that reagents are being continuously activated by light, but only stay active for short periods of time. This highlights the importance of tight control of the lighting time, as provided with the new reaction chamber built in this paper. We also show that, when analyzing the outcome of PICUP with SDS-PAGE, the use of a reducing agent is not strictly necessary, as other components of the gel loading dye are sufficient to stop the reaction.

Thirdly, we highlight the importance of the lighting time on the outcome of the reaction. We discuss how a short lighting time can bias the system to show smaller oligomeric species than are present in solution. However, a long lighting time runs the risk of biasing the system by generating species that were not present at the beginning of the reaction. This leads us to conclude that PICUP may not be optimal for quantitative studies of oligomer distribution, but can be used for comparative studies, preferably by using a combination of a short and a long lighting time in parallel.

Lastly, the comparative capabilities mentioned above are put into practice by studying PICUP of αSyn under aggregating conditions. We start by showing that

the presence of ThT increases the observed oligomer population, hinting towards the possibility of ThT stabilizing αSyn oligomers. Based on this finding, we choose a ThT concentration low enough to not affect the PICUP reaction, but high enough to detect its fluorescence, and we use that to monitor the aggregation of αSyn. By taking samples throughout the aggregation time-course and doing PICUP at a short and high lighting time in parallel, we monitor the time dependance of the oligomers captured with PICUP (Figure 5.2). We observe that αSyn oligomers captured by PICUP follow the behavior expected of monomers. This, together with the fact that oligomers are detected quickly after monomer isolation, points towards these oligomers being formed directly from monomers.

All in all, this paper leads to the development of a new instrument to perform PICUP and makes the method more efficient, reproducible, and accessible. Additionally, it shows the effect of various parameters relevant to both PICUP and amyloid studies and uses that knowledge to study the behavior of PICUP-visible oligomers during amyloid aggregation, showing that PICUP can play a big role in amyloid oligomer studies.

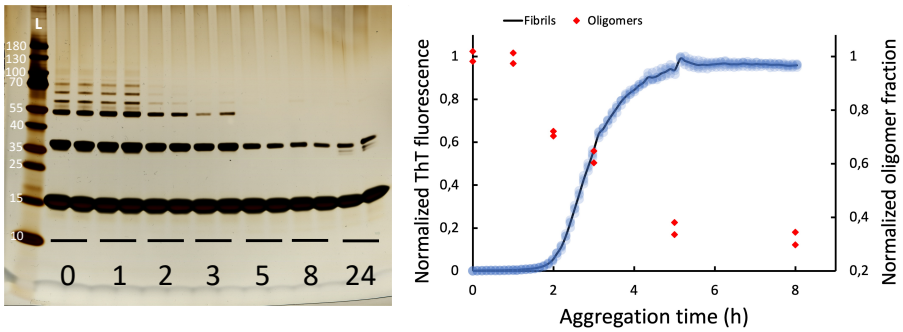

Figure 5.2. PICUP of αSyn at different points during its aggregation. On the left, samples taken at 0, 1, 2, 3, 5, 8 and 24 h of aggregation, cross-linked for 1 s and run in an SDS-PAGE gel stained with silver staining. On the right, normalized ThT fluorescence indicative of fibril formation (black line with blue fading). By analyzing the oligomer bands obtained in the gel to the left, the normalized oligomer fraction at each selected time point is calculated and plotted in duplicate on the right (red dots). The behavior of the oligomeric fraction follows that expected of monomeric αSyn, showing PICUP-visible αSyn oligomers to be species formed from monomers.

5.2. Paper II

This project is a follow up of Paper I, where we expand our understanding of PICUP of αSyn, and show that it is a very powerful tool not only to study oligomers, but also to understand all sorts of transient interactions within and between αSyn proteins. In solution, proteins have the potential to interact with each other in three dimensions. When αSyn is adsorbed onto a lipid membrane, they will be placed on a two-dimensional plane. Finally, when αSyn is forming a fibril, the monomers will be stacked in one dimension. In this paper we study how these different systems affect the outcome of the PICUP of αSyn (Figure 5.3).

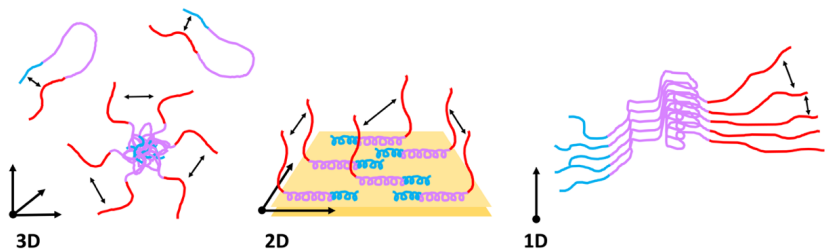

Figure 5.3. αSyn in solution (3D), adsorbed to a lipid membrane (2D) and forming a fibril (1D). The proteins are colored blue for the N-terminal tail, purple for the fibril core and red for the C-terminal tail. Black arrows illustrate the transient interactions observed in this paper.

Given that tyrosine (Tyr, Y) is the most prominent source of cross-linking via PICUP, we expect αSyn to be cross-linking mostly via one or more of its four tyrosines: tyrosine 39 (Y39), close to the N terminus (Nt), and tyrosines 125, 133 and 136 (Y125, Y133 and Y136, respectively) at the C terminus (Ct). Previous studies indicate that phenylalanine (F) has a very low reactivity to PICUP, despite being chemically similar to tyrosine. Based on that, we create an αSyn mutant with the four tyrosines mutated to phenylalanine (4YF). 4YF yields almost no cross-linking, while keeping very similar aggregation and lipid binding capabilities to the wild type (WT) αSyn. This proves that Tyr to Phe mutations are effective tools to inhibit cross-linking while keeping the proteins' self- and co-assembly properties unaltered.

Single point mutations of the four tyrosine residues reveal that, while mutations in the three Ct Tyr do not lead to proteins that look any different from WT αSyn, the Y39 mutant (Y39F) shows a clear change in outcome. The bands obtained for Y39F

are very clear and of distinct sizes, and we do not observe the diffuse bands that we see for WT αSyn. This shows that the presence of Y39 increases the complexity of the cross-linking of αSyn in solution. This effect could come from Y39 participating in inter-molecular cross-linking (between monomers), intra-molecular cross-linking (within a monomer), or both.

To test the role of Y39 in cross-linking of αSyn, we mix mutant Y39F, which only contains the Ct tyrosines, with a new mutant called 3YF, which only contains Y39. This mixture can yield inter-molecular cross-links, but not intra-molecular ones between the N and C termini. Performing PICUP on this sample gives us the same result as obtained for Y39F alone. The fact that removing the ability to generate intra-molecular cross-linking, or removing Y39 completely, yields the same result, i.e. distinct bands, shows that the only role Y39 plays on the PICUP of αSyn in solution is that of cross-linking the N and C termini of the same monomer together, to form what we call intraY39-Ct cross-linking, and we can discard the other option above, i.e. inter-molecular cross-linking. Thus, this shows that there is no Nt-Ct cross-linking between monomers, proving the second cross-linking we observe to be between C-termini (Ct-Ct). The two identified cross-linking patterns reflect on two different behaviors of αSyn in solution.

On one hand, the observed intraY39-Ct is most likely the consequence of the long-range interactions between N and C termini that have been previously reported for monomeric αSyn. The Ct of αSyn plays a self-chaperoning role, with its interaction with the Nt having been shown to play a role on preventing aggregation by "hiding" the aggregation prone NAC region. This is supported by the fact that truncating the Ct or the binding of metal ions lead to faster fibril formation. On the other hand, Ct-Ct cross-linking reports on the interaction of C termini in oligomeric αSyn. Our results indicate that the PICUP-visible oligomers (PvO) are species with Ct exposed to the solution, which makes them susceptible to the reaction. The fact that the C termini can be cross-linked together could hint towards them being in a parallel configuration but could also be a result of the C termini having enough freedom of movement to come into close contact with each other. Regardless, these results give a better insight into the nature or PvO.

We then move onto studying the cross-linking of αSyn when adsorbed to a lipid bilayer (2D) (Figure 5.4, left). We start by doing PICUP of WT αSyn at different lipid to protein (L/P) ratios. We observe that increasing the L/P leads to a decrease in complexity of the obtained SDS-PAGE gel bands similar to that observed when removing intraY39-Ct. This result indicates that Ct-Ct still occurs when αSyn is

adsorbed in the membrane, but intraY39-Ct is blocked upon binding. A calculation of the distance between αSyn proteins in a membrane and the length of the Ct exposed to the solution suggests that the Ct-Ct cross-linking could originate from either specific interactions between C termini, or due to the freedom of movement of the proteins within the fluid membrane.

Finally, we look at PICUP of fully fibrillated proteins (Figure 5.4, right). In line with what we saw in Paper I, fibrillated WT αSyn yields very little cross-linking. By testing the same reaction with all the Tyr→Phe mutants, and considering previously determined αSyn fibril structures, we can conclude that both Y39-Ct and Y39-Y39 cross-linking is blocked in fibrils. Mutants still containing the Ct tyrosines show a stronger cross-linking than those that do not, showing Ct-Ct is still occurring in fibrils. Interestingly, it occurs to a smaller extent than it does in lipid-adsorbed αSyn, despite the Ct being more closely packed. This is yet another indication that the cross-linking in presence of lipids could be reporting on the positioning of αSyn in the membrane.

In summary, this paper shows that PICUP is a very powerful tool to report transient interactions of αSyn. When using it in different systems, PICUP reports on different key components of αSyn's behavior, which depend on the dimensionality of the system.

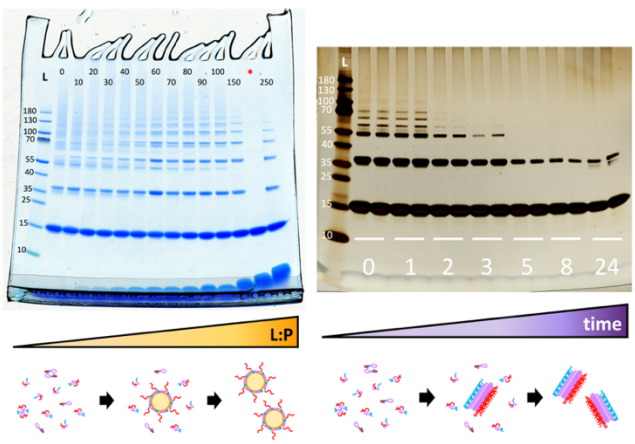

Figure 5.4. The effect of changing the dimensionality of the system on the PICUP of αSyn. On the left, small unilamellar vesicles are added at increasing L/P, leading to the protein adsorbing to the membrane and changing its cross-linking pattern. On the right, we observe how aggregation into a fibril over time alters the outcome of PICUP for αSyn.

5.3. Paper III

The amyloid research community that studies Aβ42 can be divided into two groups: those that use recombinantly produced Aβ42 (rAβ42), and those that use Aβ42 produced through peptide synthesis, referred to in this paper as synthetic Aβ42 (sAβ42). Notably, there are reports in the literature of these two proteins behaving differently, with rAβ42 aggregating faster and showing more cytotoxicity than sAβ42. However, there is no mechanistic understanding of these differences, and, consequently, this makes it difficult to directly compare studies done with one or the other protein, adding an unnecessary hurdle in the communication of the Aβ42 field. In Paper III, we aim to gain a mechanistic understanding of the difference caused by the source of protein production, as well its consequences in kinetic behavior and oligomer formation.

We start by studying the aggregation of two sources of synthetic Aβ42, labelled s1Aβ42 and s2Aβ42, as well as rAβ42. The two synthetic variants show slower aggregation than rAβ42 over the whole range of initial monomer concentration tested (1-6.4 μM) (Figure 5.5). Additionally, we observe that the two synthetic variants of Aβ42 differ from each other but are both consistently slower than rAβ42. Interestingly, the difference in $t_{1/2}$ between synthetic and recombinant seems to remain constant regardless of the concentration. When representing the data in a double logarithmic plot, we can clearly see this behavior by the parallel appearance of the data. This implies all systems follow a secondary nucleation dominated mechanism. Kinetic analysis confirms this and leads to the estimation of the products of rate constants, k_+k_2 and k_+k_n, for the three systems. Given the difference in k_+k_2 being larger than that between k_+k_n values, the results point towards secondary nucleation as the main source of difference between systems.

We then proceeded to study the kinetics in the presence of previously formed fibrils, referred to here as seeds. Seeding each protein with fibrils of the same type showed that sAβ42 has a lower seeding capacity than rAβ42 does, as the addition of the same fraction of seed shows a lower acceleration of the aggregation for sAβ42. We continued with cross-seeding experiments, where rAβ42 monomer was seeded with sAβ42 fibrils, and vice versa. These measurements showed that the sample with synthetic monomer was the one less efficiently accelerated by seeds, regardless of the nature of those seeds. This shows that the low efficiency of seeding of sAβ42 is determined by the nature of the monomer, and not the seed.

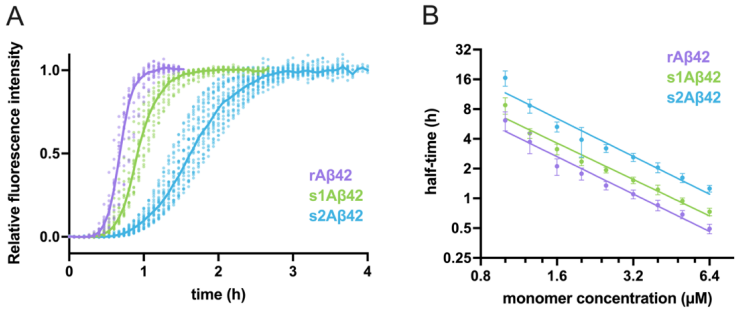

Figure 5.5. Difference in aggregation speed of rAβ42 (purple), s1Aβ42 {green) and s2Aβ42 (blue). (A) Aggregation of Aβ42 with a starting concentration of 5 μM. Dots indicate different measurements, with the line showing the median. (B) The $t_{1/2}$ of each aggregation is plotted against the initial monomer concentration, average and standard deviation over 4-6 replicates from 3-5 different experiments.

We then proceeded to study the kinetics in the presence of previously formed fibrils, referred to here as seeds. Seeding each protein with fibrils of the same type showed that sAβ42 has a lower seeding capacity than rAβ42 does, as addition of the same fraction of seed shows a lower acceleration of the aggregation for sAβ42. We continued with cross-seeding experiments, where rAβ42 monomer was seeded with sAβ42 fibrils, and vice versa. These measurements showed that the sample with synthetic monomer was the one less efficiently accelerated by seeds, regardless of the nature of those seeds. This shows that the low efficiency of seeding of sAβ42 is determined by the nature of the monomer, and not the seed.

When trying to find the source of the difference, previous studies have suggested the potential chemical impurities of sAβ42 to play a role. However, in this study, we made sure to treat all proteins the same way, by freeze-drying, dissolving in GuHCl, isolating monomers, and exchanging the buffer by size exclusion chromatography. Furthermore, we showed how, different batches of s2Aβ42 that showed different kinetics, ended up having the same aggregation behavior after one or two cycles of size exclusion chromatography, both being slower than rAβ42. This shows that, while chemical contaminants can cause differences in aggregation, they are not the origin of the differences between synthetic and recombinant Aβ42.

In the discussion of the paper, we theorize that the sequence inhomogeneity that comes from peptide synthesis could be the source of this difference. Compared to the highly evolved sequence control machinery of living organisms, peptide

synthesis does not show as high of an efficiency in coupling amino acids as the ribosome does. Due to that, peptide synthesis has the potential to generate imperfect Aβ42 peptides with random deletions in the sequence, or with potential D-amino acids instead of the naturally occurring L ones. This would explain many of the observed results. Differences between protein production sources could originate from slightly different synthesis methods. Secondary nucleation being the source of the difference, and the source of the monomer defining the efficiency of the seeding experiments, could both be explained by imperfect Aβ42 monomers binding to the fibril surface and inhibiting secondary nucleation.

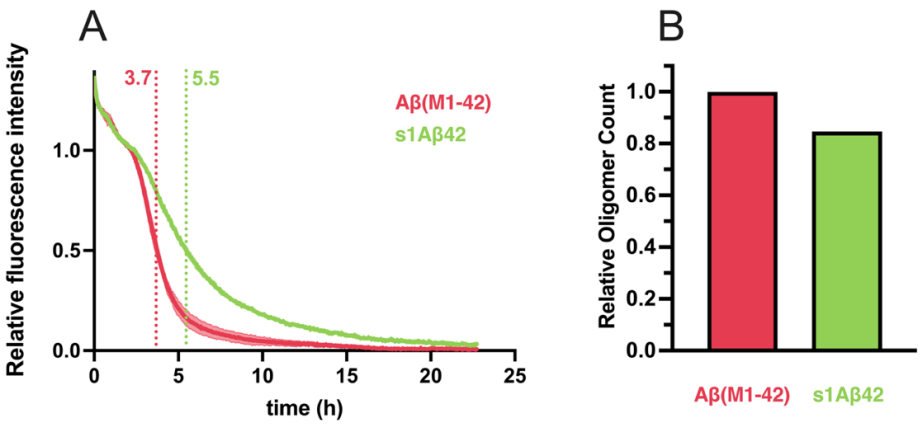

Figure 5.6. Oligomer quantification of recombinant Aβ42 variant with methionine (Aβ(M1-42), red) and s1Aβ42 (green). (A) Aggregation was monitored by fluorescence quenching, and samples were connected at $t_{1/2}$ for μFFE. (B) Data collected from μFFE was used to measure oligomer population for both systems, showing 15 % less oligomers for s1Aβ42

After finding that secondary nucleation is the most likely source of difference between the two systems, and that secondary nucleation is the main source of production of Aβ42 oligomers, we finished the study with measuring whether the protein production source has an effect on oligomer population (Figure 5.6). To do so, we used fluorescently labelled Aβ42 mixed with non-labelled rAβ42 or s1Aβ42. This was done at the lowest possible concentration of fluorescent Aβ42 to disturb the system as little as possible, but without compromising the fluorescence signal for detection. The samples with recombinant and synthetic Aβ42 were aggregated until their respective $t_{1/2}$, collected, and analyzed with μFFE. The results showed s1Aβ42 to have a 15% lower oligomer population than that of recombinant Aβ42. Given that the two systems had a portion of fluorescently labelled Aβ42 of recombinant source, we can infer that the pure recombinant and synthetic systems

would show an even larger difference in oligomer population. This aligns with previous studies showing rAβ42 to be more cytotoxic than sAβ42.

In short, this paper highlights the effect of the source of protein production in Aβ42 kinetics and oligomer formation. Kinetic analysis points towards the difference being caused by a difference in secondary nucleation. In line with this, oligomer population is decreased for synthetic Aβ42. Sequence inhomogeneity of sAβ42 could explain the differences observed. This study sheds light on the important parameters to keep in mind for Aβ42 researchers when choosing their protein source.

5.4. Paper IV

Amyloid aggregation is often monitored in 96-well plates, in devices that monitor the fluorescence by moving the plate relative to the detector in order to measure the samples sequentially. However, this motion introduces a gentle agitation in the system, which has been previously detected for IAPP and Aβ40. In this paper, by taking control of this agitation, we study the mechanistic origin of this phenomenon and further our understanding on the effect of shear forces in amyloid aggregation. We do this by performing the aggregation by either continuous reading (referred to as *agitated* in this paper) or at a reading frequency of 0.05 per min or less (referred to as *idle* in this paper). This way, we identify the effect of agitation to occur also for a fragment of tau, and for Aβ42 (Figure 5.7, left), which we choose as the target of the rest of our analysis in this paper. We then set out to identify the microscopic step of aggregation that is responsible for this difference: is it primary nucleation, elongation, secondary nucleation, or fragmentation?

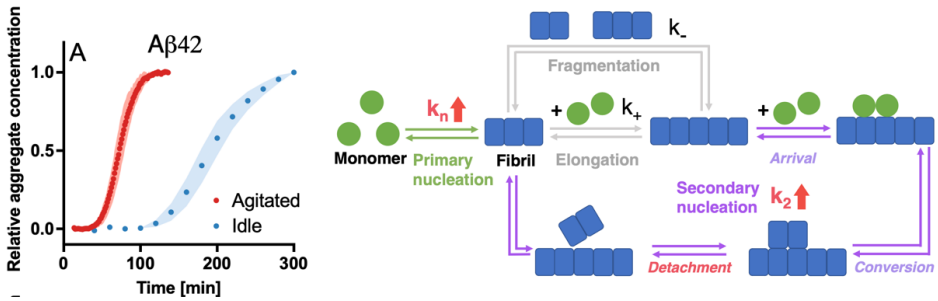

Figure 5.7. Effect of mild agitation by increasing reading frequency. On the left, aggregation of 2.5 μM Aβ42 during continuous reading (agitated), or a reading frequency 0.05 per min. On the right, the conclusion of this paper, revealing that agitation increases both primary and secondary nucleation rates by accelerating the detachment of nuclei from catalytic surfaces.

First, inspecting the double logarithmic plot tells us that fragmentation cannot be responsible for the difference observed. In a secondary nucleation dominated system such as the aggregation of Aβ42, if fragmentation played a big role, it would lead to a log-log plot with a negative curvature, where secondary nucleation would dominate at high monomer concentrations, and fragmentation would dominate at lower ones. Our results do not show this negative curvature, showing that, at all concentrations tested, the fragmentation rate is significantly lower than secondary nucleation, and thus, a decrease in its rate would not describe the difference observed.

Secondly, we tested the aggregation in idle and agitated conditions in the presence of pro-SPC Brichos, a chaperone known for inhibiting secondary nucleation. When this is present, the difference between the two system remains, indicating that one of the two remaining processes, primary nucleation, or elongation, must explain at least part of the observed difference. This experiment also told us that k_+k_n is 12-fold higher for the agitated state, although we cannot distinguish if this contribution comes from primary nucleation or elongation.

Thirdly, fitting the data with a kinetic model, and the knowledge that k_+k_n is 12-fold higher for the agitated system, revealed that the proliferation rate, which is proportional to $(k_+k_2)^{1/2}$ is 2-fold higher for the agitated system. This means that either elongation or secondary nucleation is playing a role on the effect. Following this, taking the fibrils at the end of either an idle or agitated reaction, and using them to seed a second aggregation under the same conditions showed that the fibrils produced under agitated conditions led to faster kinetics. If the difference does not come from elongation, this behavior could only be explained if the agitated reaction led to more, yet shorter, fibrils on average, leading to more fibril ends to elongate on. To validate this, we sonicated the fibrils at the end of either an idle or agitated reaction to make them break them to the same length. Doing this made the subsequent aggregation aggregate the same way regardless of the origin of the fibril, supporting the idea that the difference in seed efficiency comes from the size of fibrils produced under the different conditions. Thus, we conclude that agitation increases secondary nucleation, but not elongation.

Knowing that elongation does not play a role in the difference, we can comfortably assign the 12-fold difference in k_+k_n to primary nucleation. However, this difference in primary nucleation alone cannot explain the difference between the systems, as shown by the misfit of the model to the experimental data. The fit is improved when allowing the idle and agitated conditions to fit k_2 freely, giving further proof that secondary nucleation also plays a role in the effect of agitation. The fitting establishes the finding that mild agitation leads to a 14-fold increase in primary nucleation, and a 4-fold increase in secondary nucleation (Figure 5.7, right).

If primary and secondary nucleation are both increased by mild agitation, it would stand to reason that oligomer populations would also be affected by these conditions. To study that, we follow the aggregation of a mixture of fluorescently labelled and unlabeled Aβ42 and collect the samples at their respective $t_{1/2}$, where we expect the highest oligomer concentration. We then measure these samples with μFFE and observe a clear difference in species' distribution (Figure 5.8). The

agitated sample shows a high population with the behavior we expect from oligomers, whereas the idle sample shows a sharp fluorescence peak at very low deflection, indicative of bigger fibrillar species. The oligomer count reveals that gentle agitation leads to a 25% increase in oligomer population at $t_{1/2}$.

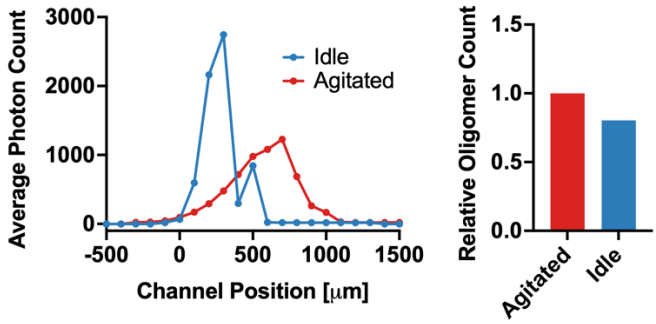

Figure 5.8. The effect of mild agitation in oligomer formation, observed for idle (blue) and agitated (red) systems. On the left, we see the average photon count detected as a function of channel position. On the right, we show that agitation leads to a roughly 25 % increase in oligomer population

Given that a significant amount of primary nucleation occurs in the air-water interface, agitation could increase its speed by increasing the rate of detachment from the interface. This result is supported by the demonstration that increasing the agitation leads to a decreased dependance of the reaction on the available air-water interface, whose ratio to volume is varied through the sample volume per well. Similarly, we show that agitation also accelerates secondary nucleation by speeding up the detachment of nuclei from the surface of Aβ42 fibrils. This is determined by cryo-TEM imaging, where we follow a protocol to form fibrils highly decorated with protrusions that are interpreted to be secondary nucleation intermediates, and we observe how shaking leads to the fibrils being less decorated.

This study shows that mild agitation leads to an increase in both primary and secondary nucleation by speeding up the detachment of nuclei from air-water interface and fibril surface, respectively. This phenomenon also has consequences for the oligomer population. Overall, the results show the importance of tight control of agitation and may aid in the development of specialized kinetic assays aimed to study specific microscopic steps.

5.5. Paper V

As reviewed in this thesis, Aβ42 oligomer formation has been studied in depth. By fitting experimental data on time-dependence of oligomers onto a kinetic model, several key aspects of oligomer dynamics have been established. Firstly, although oligomers can be formed by direct association of monomers (primary nucleation), kinetic model fitting has demonstrated that oligomer formation being catalyzed by fibril surfaces (secondary nucleation) is a necessary step to describe their behavior during aggregation. Secondly, we know that dissociation of amyloid oligomers is a crucial step in the kinetic model, and it is a universal property of most amyloid oligomers that they tend to dissociate to a much faster rate than their conversion to nuclei. However, the exact way this dissociation occurs has not been explored in detail. In this paper, we ponder whether, similarly to oligomer formation, their dissociation is also catalyzed by fibril surfaces, and we provide a theoretical and experimental basis for our answer.

We first establish that fibril surfaces are catalysts of oligomer formation. The IUPAC definition describes the main features of a catalyst, which we show apply for the fibril surface in oligomer formation. Firstly, fibril surfaces are both reactants and products of the reaction they catalyze. Secondly, they do not alter the overall Gibbs free energy change of the reaction. Thirdly, they accelerate the oligomer formation. The extent to which they accelerate the formation, a, is described as the ratio of the rates for oligomer formation through secondary and primary association. Using the newly defined parameter, and kinetic values from the literature, we obtain the acceleration of oligomer formation, $a = xM$, for Aβ42 and Aβ40 to be $x \simeq 30\ \mu M^{-1}$ and $x \simeq 1100\ \mu M^{-1}$, respectively. Thus, we show that oligomer formation is greatly increased by fibril surfaces.

Given that catalysts accelerate reactions by decreasing the height of the free energy barrier, they increase the forward and reverse rates equally (Figure 5.9). This means that oligomer formation and dissociation must be affected to the same extent by the fibril surface. This reasoning applies regardless of whether secondary association is a single-step or a multi-step reaction. Therefore, we conclude that the increase in oligomer dissociation rate is approximately the same size as the increase in formation, a.

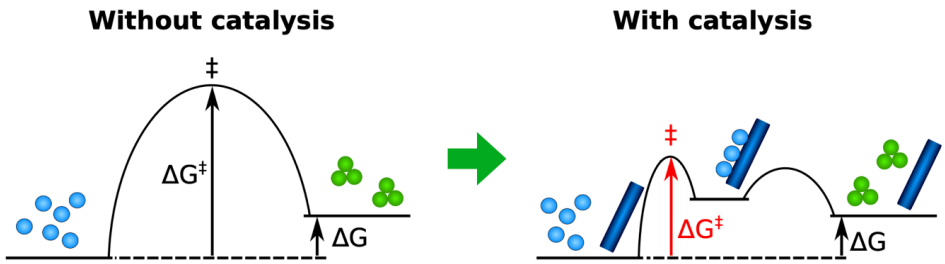

Figure 5.9. Catalytic effect of fibril surfaces on oligomeric reactions. The presence of fibril surfaces does not alter the relative thermodynamic stabilities of monomers and oligomers (ΔG), but it decreases the height of the free energy barrier. This process accelerates the reaction in both directions to the same degree.

In order to validate this claim experimentally, two separate experiments are performed. In the first experiment, fluorescently labelled oligomers are formed, isolated via centrifugation, and then fresh buffer is added either with or without unlabeled fibrils. After five minutes of incubation, fluorescence correlation spectroscopy is used to measure the mass concentration of oligomers and monomers. The result shows that the presence of fibrils leads to a decrease in oligomer concentration and an increase in the monomer population. In the second experiment, samples are aggregated until the start of the final plateau, and half of them are centrifuged to remove fibrils. After three hours of incubation, oligomer and monomer populations are measured with μFFE (Figure 5.10). The results again show that the samples incubated with fibrils show a lower oligomer population. These experiments prove the claim that fibril surfaces indeed catalyze oligomer dissociation. Consistency of this finding with previous kinetic experiments is then verified by re-fitting.

While this reflects the behavior of oligomers *in vitro*, proteostasis in living organisms keeps some proteins at relatively constant monomer concentration. To evaluate how this applies to our new finding, we model a system with constant monomer concentration. Without fibril dissociation, keeping monomer concentrations constant would lead to both oligomer and fibril concentrations to grow exponentially indefinitely. However, when fibril-mediated dissociation is added to the equation, the oligomer concentration follows a sigmoidal behavior. The steady state is reached when the rate of formation is counterbalanced by that of oligomer dissociation. M_c is then described as the concentration of fibril mass required for that steady state to occur. Interestingly, this analysis indicates that, once oligomer concentration has plateaued, drugs that inhibit fibril surfaces will have no effect on oligomer population, as they will block both the formation and dissociation of oligomers. Therefore, fibril-surface inhibiting drugs will only have an effect on

oligomer formation if they are administered while fibril concentration is below M_c and oligomer concentration has not reached its steady-state.

However, when considering *in vivo* systems one has to keep in mind two crucial phenomena: destruction of aggregates (oligomer *clearance*) and removal from areas of high oligomer production (oligomer *transport*). With that in mind, we redefine the mathematical description of M_c, showing that if clearance and transport play a big role, M_c is raised over the values obtained *in vitro*. Additionally, fast enough clearance can raise M_c to values higher than have ever been attained *in vivo*, making fibril-binding strategies more effective. This shows the importance of clearance and the relevance of its consideration during drug development.

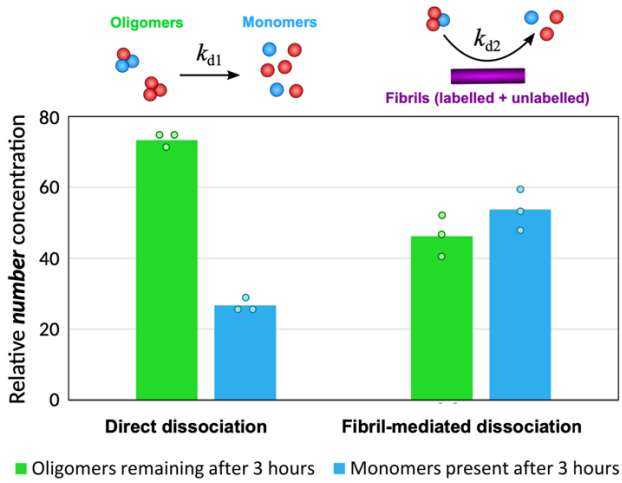

Figure 5.10. Effect of direct dissociation and fibril-mediated dissociation in the oligomer population of Aβ42, measured by μFFE.

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Printed by Media-Tryck, Lund 2024 NORDIC SWAN ECOLABEL 3041 0903

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ISBN 978-91-8096-050-2