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Polymorphic protein aggregation in tauopathies

Alexander Sandberg

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Department of Physics, Chemistry and Biology
Linköping University

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Cover: Transmission electron micrograph of Tau 0N3R fibrils induced by heparin and seeded with human brain material. The cover image is approximately 2 μm across.

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Abstract

Alzheimer's disease(s) comprises one of the most common and costly neurodegenerative diseases. With a larger population and an increasing life expectancy, amyloid diseases (with age as one of the most prominent risk factors) will generate an even larger burden on healthcare. We know that protein misfolding is involved in the disease process but lack a complete understanding of the mechanism behind these diseases, both the sporadic and hereditary variants. It is not always known whether it is a gain-of-toxic function or loss-of-function that causes the neurodegeneration. To determine the correct diagnosis is a major challenge. If diagnosed, only a few amyloid diseases can be treated today.

Amyloids are highly ordered filamentous protein aggregates with a β -sheet structure. From identical or similar amino acid sequences, a large variety of structures can be formed by different secondary and tertiary structures and by different packing of the individual filaments. This is known as fibril polymorphism.

This work focuses on characterization on two proteins involved in Alzheimer's disease and other neurodegenerative diseases, namely Amyloid- β ($A\beta$) and microtubule associated protein tau (τ). In order to investigate the properties of these proteins *in vitro* it is important to have protocols for production of recombinant protein that enables characterization of these aggregation prone proteins. We present protocols for recombinant expression, purification and non-denaturing fibrillation assays used in our lab to produce and analyze $A\beta$, τ and the prion protein.

Development of new ligands for characterization of fibrils is an important way of investigating different fibrillary structures and characterizing and distinguishing between the different polymorphs of aggregates. We showed that the central benzene ring of the amyloid ligand X-34 can be exchanged for other heterocyclic motifs and still retain targeting of the "Congo red" binding site. The compounds do not compete with the Pittsburgh compound B (PiB) binding site on recombinant $A\beta$ fibrils.

We also characterized tau fibrils formed from seeding with tau aggregates from patients diagnosed with different neurodegenerative tauopathies. We use aggregation kinetics to test the seeding activity on two different sequence isoforms of tau, 0N3R and 0N4R. Fibrillation kinetics, an array of recently developed ligands (including the X-34 analogs) and electron microscopy were used to characterize different polymorphs of the tau aggregates formed by seeded templating from patient derived seeds. Our data showed that brains contain seeds with different morphologies even with in patients diagnosed with the same disease.

Investigations of the rare tau mutant G273R found in a patient with a presumed tauopathy also highlights the problem with proper diagnostics. Our results reveal that *in vitro* this mutation change the binding properties of 0N4R tau to the cytoskeletal proteins microtubules and F-actin. Furthermore, we could show that when seeded, the fibril formation seeding activity followed a sequence similarity dependent manner. In fibrils formed during heparin-induced aggregation we could distinguish between wild type and mutant tau as they form fibrils with different thickness. Our *in vitro* biophysical data support that the G273R mutant is causing a 4R tauopathy.

The work in this thesis increase our knowledge in the field of tau aggregation and tau fibril polymorphism.

Populärvetenskaplig sammanfattning

En av de vanligaste och mest kostsamma sjukdomarna är den nervdödande Alzheimers sjukdom. Med en större population och ökad förväntad livslängd kommer amyloida sjukdomar, som har ålder som den viktigaste riskfaktorn, att generera en ökad börda för sjukvården. Vi saknar en fullständig förståelse för mekanismerna bakom dessa sjukdomar både för de sporadiska och ärftliga varianterna. Man vet att felveckade proteiner är inblandade i dessa sjukdomar. Det är inte alltid känt hur den felveckade formen av ett protein alstrar en toxisk funktion eller om det är en förlust av dennas funktion som orsakar nervdöden. Att kunna fastställa en korrekt diagnos är en stor utmaning för forskarvärlden idag. Även när en korrekt diagnos kan ställas är det endast ett fåtal amyloida sjukdomar som kan behandlas idag.

Amyloider är mycket välordnade filamentösa proteinaggregat med β -flakstruktur. Från identiska eller liknande aminosyrasekvenser kan ett stort antal strukturer bildas med olika sekundär- och tertiär struktur och olika packning av individuella filament. Vi kallar detta för strukturell polymorfism.

Det här arbetet fokuserar på karakterisering av två proteiner involverade i Alzheimers sjukdom och andra neurodegenerativa sjukdomar nämligen Amyloid β ($A\beta$) och mikrotubuli associerade protein tau (tau). För att kunna undersöka egenskaperna hos dessa proteiner är det viktigt att ha protokoll för produktion av rekombinant protein för att kunna karakterisera dessa aggregeringsbenägna proteiner. Vi utvecklade protokoll för rekombinant uttryck, rening och icke-denaturerande fibrilleringsanalyser som används i vårt labb för att producera och analysera $A\beta$, tau och prionproteinet.

Utveckling av nya ligander för karakterisering av fibriller är en viktig väg för att undersöka olika fibrillstrukturer och för karakterisering och för att kunna särskilja mellan olika polymorfer av aggregat. I det här arbetet visas att den centrala bensenringen hos amyloidliganden X-34 kan bytas ut mot andra heterocykliska motiv och fortfarande behålla sin specificitet mot "Congo röd" bindnings-sätet utan att konkurrera med Pittsburgh compound B (PiB) bindnings-säte på rekombinanta $A\beta$ fibriller.

Vi karaktäriserade också tau fibriller bildade via ympning, så kallad seeding, med tau aggregat isolerade från patienter diagnosticerade med olika nervdödande taupatier. Vi använder aggregerings kinetik för att testa seedningsförmåga på två olika sekvens isoformer av tau. Nyligen utvecklade ligander (inkluderat X-34 analoger) och elektronmikroskopi användes för att karakterisera de olika polymorferna av tau aggregaten. Våra data påvisar att olika patienter bär på olika seeds, det vill säga olika polymorfer. Även mellan patienter med samma diagnos finns skillnader.

Undersökningar av den ovanliga tau mutationen G273R understryker också problemet med fastställandet av korrekt diagnos. Våra resultat från provrörsexperiment avslöjar att den här mutationen ändrar bindningsegenskaperna av 0N4R tau till cytoskelettproteinerna mikrotubulin och F-aktin. Vi kunde ytterligare visa att när fibrilleringsreaktionen seedades så följde det en sekvenslikhetsberoende mekanism. Fibrerna som bildas under heparininducering kan skiljas åt mellan normalt och muterat tau genom att de har olika tjocklek. Våra biofysikaliska data stödjer att G273R tau mutationen kan orsaka en 4R taupati.

Arbetet i denna avhandling ökar vår kunskap inom området tau-aggregering och tau fibril-polymorfism.

List of papers included in the thesis

In this thesis the following papers are included, which are referred to in the text by Roman numerals (I-IV) (* Authors contributed equally)

- I. Sandberg A, Nyström S. **Purification and Fibrillation of Recombinant Human Amyloid- β , Prion Protein, and Tau Under Native Conditions**. Methods Mol Biol. 2018 1779:147-166.
- II. Zhang J*, Sandberg A*, Konsmo A, Wu X, Nyström S, Nilsson KPR, Konradsson P, LeVine H 3rd, Lindgren M, Hammarström P. **Detection and Imaging of A β 1-42 and Tau Fibrils by Redesigned Fluorescent X-34 Analogues**. Chemistry. 2018 24(28):7210-7216.
- III. Sandberg A, Lindberg, M, Zhang, J., Wu, X, Konradsson, P, Shirani, H, Nilsson, KPR, Svensson, S, ,Svensson S, Svenningsson P, Nyström S, Hammarström P. ***In vitro* propagation of patient specific Tau fibril polymorphs** (manuscript)
- IV. Sandberg A*, Ling H*, Gearing M, Dombronski B, Cantwell L, R'Bibo L, Levey A, Schellenberg G, Hardy J, Nyström S, Svensson S, Hammarström P, Revesz T, Mok K. **Fibrillation and molecular characteristics are coherent with clinical and pathological features of 4-repeat tauopathy caused by MAPT variant G273R** (submitted)

Contribution report

Paper I: Alexander Sandberg (AS) developed already published protocols, made improvements, developed new fibrillation protocols and used the protocols to produce protein and study fibrillation kinetics, wrote two of the protocols.

Paper II: AS Produced protein, participated in measurements, technical and theoretical input during measurements and data analysis. Contributed to the writing process.

Paper III: AS Performed the measurements done with recombinant protein, data analysis and interpretations. Wrote a draft of the manuscript.

Paper IV: AS Performed the measurements done with the recombinant protein. Performed most of the measurements, data analysis and interpretation. Wrote parts of the article.

Papers not included in this thesis

Zhang J, Konsmo A, **Sandberg A**, Wu X, Nyström S, Obermüller U, Wegenast-Braun BM, Konradsson P, Lindgren M, Hammarström P. Phenolic Bis-styrylbenzo[c]-1,2,5-thiadiazoles as Probes for Fluorescence Microscopy Mapping of A β Plaque Heterogeneity. *J. Med. Chem.* 2019, 62(4):2038-2048.

Zhang J, Wang J, **Sandberg A**, Wu X, Nyström S, LeVine H 3rd, Konradsson P, Hammarström P, Durbeej B, Lindgren M. Intramolecular Proton and Charge Transfer of Pyrene-based trans-Stilbene Salicylic Acids Applied to Detection of Aggregated Proteins. *Chemphyschem.* 2018, 19(22):3001-3009.

Jonson M, Nyström S, **Sandberg A**, Carlback M, Michno W, Hanrieder J, Starkenberg A, Nilsson KPR, Thor S, Hammarström P. Aggregated A β 1-42 Is Selectively Toxic for Neurons, Whereas Glial Cells Produce Mature Fibrils with Low Toxicity in *Drosophila*. *Cell Chemical Biology.* 2018, 25(5):595-610.

Zhang J, **Sandberg A**, Wu X, Nyström S, Lindgren M, Konradsson P, Hammarström P. *trans*-Stilbenoids with Extended Fluorescence Lifetimes for the Characterization of Amyloid Fibrils. *ACS Omega.* 2017, 2(8):4693-4704.

Supervisors

Main supervisor

Per Hammarström, Professor
Div. of Chemistry
Dept. of Physics, Chemistry and Biology
Linköping University, Sweden

Co-supervisor

Sofie Nyström, Docent
Div. of Chemistry
Dept. of Physics, Chemistry and Biology
Linköping University, Sweden

Thesis Committee

Faculty Opponent

Wei-Feng Xue, Senior Lecturer
School of Biosciences
University of Kent

Committee Board

Elin Esbjörner Winters, Associate Professor
Chemical Biology
Biology and Biological Engineering
Chalmers University of Technology

Greta Hultqvist, Associate Senior Lecturer
Protein Drug Design
Dept. Pharmaceutical Cell Biology
Uppsala Universitet

Per Nilsson, Assistant Professor
Div. of Neurogeriatrics
Department of Neurobiology, Care Sciences
and Society
Karolinska Institutet

Caroline Brommesson, Senior Lecturer
Div. Molecular Surface Physics and Nano
Science
Dept. Physics, Chemistry and Biology.

List of Abbreviations

3R	Three repeat tau
4R	Four repeat tau
A β	Amyloid β
A β PP	Amyloid β precursor protein
AD	Alzheimer's disease
AFM	Atomic force microscopy
AICD	Amyloid β precursor protein intracellular cytoplasmic/C-terminal domain
CBD	Corticobasal degeneration
CMA	Chaperone-mediated autophagy
cryo-EM	Cryogenic electron microscopy
Da	Dalton
DNA	Deoxyribonucleic acid
DTT	Dithiotreitol
ECL	Electrochemical luminescence
E. coli	Escherichia coli
E1	Ubiquitin-activating enzymes
E2	Ubiquitin-conjugating enzymes
E3	Ubiquitin ligase enzymes
F-actin	Filamentous actin
FTD	Frontotemporal degeneration
FTLD	Frontotemporal lobar degeneration
FUS	Tumor associated protein fused in sarcoma
ICT	Internal charge transfer
IPTG	β -D-1-thiogalactopyranoside
kDa	Kilo dalton
K18	Tau fragment containing amino acid 244-368 corresponding to the 4R region of tau
K19	Tau fragment containing amino acid 244-368 excluding 275-305 corresponding to the 3R region of tau
LB	Luria Broth
LCO	Luminescent conjugated oligothiophene
LVF	Linear variable filters
mRNA	Messenger Ribonucleic acid
MAPT or tau	Microtubule associated protein tau
MSD	Mesoscale discovery
NG2	Nerve/glial antigen 2
NFT	Neurofibrillary tangles
NPF	Narrow Pick filaments
PDB	Protein data bank
PHF	Paired helical filaments
PrP	Prion protein
PSEN-1	Presenilin-1

PSEN-2	Presenilin-2
PSP	Progressive supranuclear palsy
PWT	Pseudo wild type
RAGE	Receptor for advanced glycation endproducts
sA β PP	Soluble amyloid β precursor protein
ssNMR	Solid state nuclear magnetic resonance
SF	Straight filament
TDP-43	Transactive response DNA binding protein of 43 kD
TEM	Transmission electron microscopy
TICT	Twisted internal charge transfer
TPA	Tripropylamine
UPS	Ubiquitin proteasome system
WPF	Wide Pick filaments
Å (10^{-10} m)	Ångström

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INTRODUCTION

Proteins

The central dogma of molecular biology

The central dogma of molecular biology explains how sequential information is transferred in life. DNA can be replicated thereby allowing the cell to multiply and copy the genetic information to the daughter cells. DNA can be transcribed to RNA. One of the variants is called mRNA where three nucleotides form a codon and each codon translates to an amino acid. The mRNA sequence is translated into an amino acid sequence. Francis Crick 1957 [1] stated that this sequential information can never go from protein to protein or protein to nucleotide.

It has been commonly acknowledged that nucleic acids are the sole carriers of phenotypic inheritance. The prion hypothesis has stated that this is not true and proteins can transmit phenotypic information.

Amino acids

Proteins are involved in all forms of life as we know it. They direct metabolism, signaling or movement in all organisms and act as structural components in all life forms from simple single cell organisms to the largest complex multicellular species. Most proteins have a specific fold, the native fold, to be able to perform their function. Proteins are built by amino acids connected to each other. All amino acids have the common feature that they have carboxylic acids and amines as functional groups allowing them to be connected together in a sequence like beads on a string.

Figure 1: The amino acids; A: Glycine, B: Tryptophan, C: Arginine and D: Glutamic acid. E shows the four amino acids linked together via the peptide bond forming a four amino acid long peptide.

Different side groups on the amino acids determine their specific properties, for examples see Figure 1. There are 20 naturally occurring amino acids composing proteins. The amino acids can be divided into groups depending on their properties. Each amino acid has its unique properties but can be more or less similar to another. Some amino acids are charged, positive and negative. Others are hydrophilic/polar while some are hydrophobic/non-polar. They have different sizes and are prone to align at different angles to their neighbor's when connected to each other. Amino acids are linked together by amide bonds formed from the amino group and carboxylic group forming polypeptide chains. Polypeptide sequences in the range of 2-50 amino acid are generally called peptides and longer chains are called proteins [2].

Protein folding

In 1962 it was suggested that the primary sequence determines the fold of the native protein since it is the most thermodynamically stable configuration due to side-chain functional groups (R-groups) [3]. When the protein folds, the interactions between the R-groups dictate the formation of the native fold.

Depending on the amino acids and their properties the two major types of secondary structure, α -helices and β -sheet [4, 5], form. Hydrogen bonding between the backbone of the polypeptide chain stabilize these structures.

α -helices are formed by hydrogen bonding between peptide bonds every fourth amino acid in a spiral, between amino acid n and $n+4$, resulting in a corkscrew like structure (see Figure 2) of the amino acid backbone with approximately 3.6 amino acids per turn.

β -sheets are formed from extended β -strands (see Figure 2) that are parallel or antiparallel, meaning that the strands run in the same orientation or run in the opposite direction respectively. The β -sheet is stabilized by hydrogen bonding between the peptide bonds of the strands and is held together in a rigid structure.

Figure 2: Cartoon illustration of two secondary structure elements α -helix (top) and β -sheet (bottom), PDB ID: 4R4L and 6FWW.

Other helical structures also exist but α -helices are the most common. Myoglobin was the first protein to be structurally determined, reported in 1958 [6]. To date (October 18, 2019) there

are over 140 000 protein only structures in the protein data bank [7], with about 10 000 structures being added annually in recent years [8].

Some amino acids have a preference for a hydrogen bonding conformation resulting in β -sheet or helices, but most are more or less flexible, so the structure is dependent on the sequence of surrounding amino acids and the environment. Other structural motifs can also be shorter turns between sheets or helices, or loops and random coil. Random coil is the lack of any clearly defined rigid secondary structure by bonding; longer random coils can be highly flexible areas of or entire proteins.

It is suggested that hydrophobicity is the main driving force in the folding of globular proteins. Since amino acids have different hydrophobicity, this is what determines the fold. When proteins are in an aqueous solution they will collapse into a structure where hydrophobic parts are hidden from a hydrophilic environment. This means that the secondary structure can just as well be a by-product of the tertiary structure as what causes it. This collapse also reduces the number of possible interaction partners for each amino acid to form the structure of the protein [9-11].

Super secondary and tertiary structure describes how the secondary structure elements are oriented in relation to each other and by R-group interactions. This can be by a continued extended hydrogen bonding network between β -sheets or α -helices, charged or polar amino acids interacting or columbic interactions, where dipole moments are induced by temporary electron density fluctuations within hydrophobic amino acids. Quaternary structure is when proteins interact with each other, forming protein complexes which can be formed from different proteins with specialized functions in to heteromers or homomers.

Quality control of proteins

For a protein to be able to perform its function it is often required to have a specific conformation. For an introduction to protein function with an emphasis on enzymes and folding, see reference [12].

Chaperones are proteins that interact with other proteins that have not yet been folded correctly or that have become misfolded. The chaperones interact with misfolded proteins, unfold them and are able to promote structural changes towards the correct fold or let them spontaneously refold. Chaperones can also recruit ubiquitin ligase proteins that target the misfolded and aggregated proteins for degradation. It has been shown that the ability to maintain an efficient proteostasis system declines with age, which can lead to aggregation induced toxicity [13-15].

Post translational modification by ubiquitination of proteins can participate in different pathways (for examples, see Figure 3). Two of these are degradation via the lysosome or proteasome. Ubiquitylation is initiated by ubiquitin-activating enzymes (E1s), Ubiquitin-conjugating enzymes (E2s) and ubiquitin ligase enzymes (E3s) [16].

Ubiquitin can be bound to lysine residues or the N-terminal of proteins, and from this more ubiquitin monomers are linked, forming chains of ubiquitin linked to the targeted proteins. There is a code for how the ubiquitination chain is linked. It has not been completely determined how this chain coding mediates the exact fate of the targeted molecules. One major function is to send proteins for degradation [17].

Degradation can occur through different pathways, the ubiquitin proteasome system (UPS), or some of the lysosomal degradation pathways like chaperone-mediated autophagy (CMA) or autophagy.

Figure 3: Some of the pathways of degradation for proteins. Ubiquitin molecules (black stars) are covalently attached to proteins by E1, E2 and E3. This ubiquitin chain is the signal for degradation which can occur through the proteasomal pathway A1-2, or endocytosis B1-B4, or autophagy C1-C3. Both the endocytosis and autophagy pathway result in a lysosome where the proteins/aggregates are degraded.

The proteasome is a large protein complex that degrades proteins in the cytosol and nucleus. This differs from the lysosome system by not being compartmentalized. The proteasome degrades proteins into short peptides which are further broken down in the cytosol to amino acids by aminopeptidases [18]. The proteasome complex consists of a catalytic core built up by two outer rings consisting of β -rings and two inner α rings, with each ring consisting of seven sub units forming a barrel [19].

The β -rings have catalytic activity and when the proteasome is non-active, the α -rings are closed forming a physical barrier hindering proteins from reaching the active site. Regulatory proteins consisting of two parts, called the lid and the base, are attached to the α -ring ends. These regulatory proteins are responsible for: recognition, deubiquitinating, unfolding the captured proteins and changing the conformation of the α -rings to an open state allowing the unfolded protein chain to enter the catalytic β -ring [20].

CMA is mediated by chaperones in the cytosol, misfolded proteins interact with chaperones and are ubiquitinated. Chaperones in the cytosol unfold targeted proteins which are translocated through the membrane of the lysosome where the protein is degraded [21].

Plasma membrane proteins are degraded in lysosomes where mono-ubiquitination is the signal for the start of compartmentalization towards an endosome. Ubiquitination also occurs at endosomal membranes to direct towards the lysosomal pathway after internalization.

The autophagosome is the degradation pathway where larger substrates, like organelles, microbes and protein aggregates can be cleared. Protein aggregates can be tagged with ubiquitin which recruits proteins necessary for the autophagosome to be initiated, the protein aggregate is engulfed by a double membrane and the autophagosome is then fused with a late stage endosome or lysosome where the proteins can be degraded [22, 23].

Misfolding of proteins into amyloids

There are several different types of protein aggregates but in this thesis, aggregates related to amyloid proteins are discussed.

There are more than 30 proteins in human classified as amyloids, as recognized by the Amyloid nomenclature committee [24]. To be called an amyloid there are definitions for how to characterize the protein. The protein must be characterized by protein sequence analysis, deposited in tissue and have affinity for Congo red and show green birefringence when viewed with polarization microscopy.

The aggregation of amyloidogenic proteins into fibrils is thought to go through several steps. This process can be simplified into three steps *in vitro* where there is a limited amount of monomers: lag phase (nucleation phase), growth phase and stationary phase (see Figure 4). During the lag phase protein undergoes conformational changes allowing the protein to oligomerize. Within these oligomers, a fibril nucleus can be formed. This primary nucleation contains β -rich structure where monomers can add to start to form a fiber. This phase is rate-determining for the formation of fibrils due to a series of thermodynamically unfavorable steps [25].

Figure 4: A schematic image of fibril formation *in vitro*. During the lag phase monomers can misfold and/or oligomerize. Nuclei can form where more monomers attach. In the growth phase nuclei develop into proto-fibrils and mature in to proto-filaments and fibrils. Stationary phase equilibrium between dissociation and association of monomer to fibrils is reached. For a more thorough explanation of fibril formation and definitions of the different species present, see [26].

The fibrils are elongated by the addition of monomers to the ends. As the fibrils grow longer, they fragment thereby creating new ends where monomers can adhere. There is also the possibility for secondary nucleation to take place at the surface of the formed fibrils [27]. The growth phase is followed by the stationary phase where, mature fibrils are formed. There is no clear definition of what a mature fibril is and, even in the stationary phase, rearrangements and bundling of fibrils are going on [28]. Seeds are preformed fibrils that when added in small amounts to monomers in solution reduce the lag time for the conversion of monomers to fibrils. Seeding bypasses the slow process of forming nuclei. Seeding is thought to occur through different pathways that can run in parallel one being addition of monomers to the fibril ends, elongating the fibril until it breaks creating new ends for elongation.

Secondary nucleation is the aggregation of monomers to the surface of fibrils. The fibril surface catalyzes the formation of the nucleus of fibrils [27]. The seed structure can template the structure of the newly formed fibrils. This allows for the generation of strains with different morphology. Different seeds can have different characteristics and conformations which determine their seeding potency [29].

Amyloid polymorphism

Polymorphism of amyloid fibrils is a term used to describe structural differences within fibrils formed from proteins or peptides with the same amino acid sequence [30]. Depending on the conditions under which the fibrils were made, there is a morphological difference. There can also be a difference in morphology between fibrils formed under the same conditions. These differences vary from different packing of fibril filaments to differences in how single amino acids are oriented in the folded monomer within the fibril.

Polymorphisms can be identified using a variety of techniques: antibodies [31], fluorescent ligands [32], solid state nuclear magnetic resonance (ss-NMR) [33], transmission electron microscopy (TEM), atomic force microscopy (AFM), cryogenic electron microscopy (cryo-EM).

Prions are a group of transmissible amyloid diseases [34]. It has been shown that PrP can form different strains that give distinct phenotypical characteristics [35]. These strains maintain their structure and properties even if passed through several hosts. These strains have a defined structure that is a template, in other words a strain is dependent on its morphology [36]. If a mutant is introduced that makes the protein incapable of adopting a certain morphology, this mutation is protective towards this strain of prion disease [37]. Other amyloid proteins such as α -synuclein [38] and tau [39] have also been suggested to have prion characteristics.

With the recent advances in cryo-EM, several different kinds of polymorphs of tau fibrils have been suggested. This polymorphism can be on different levels. Preparations from an Alzheimer's disease (3R and 4R tau) brain revealed two distinct polymorphs of filaments: paired helical filaments (PHF), 70-150 Å width and 650-800 Å crossover distance, and straight filaments (SF), 100 Å width and 700-900 Å crossover distance (Figure 5 A) [40]. Both these structures contain paired structurally similar protofilaments and have the same secondary and tertiary structure. The difference between PHF and SF lies on an ultrastructural level and quaternary in nature, meaning the way the protofilaments are packed in relation to each other.

Two types of filaments were also observed for Pick's disease (3R tau) where Narrow pick filament (NPF), 50-150 Å width and crossover distance 1,000 Å, and wide pick filament (WPF), 50-300 Å width and approximately 1,000 Å crossover distance, were found and in this case both filament types contained the same secondary and tertiary structure (Figure 5 B) [41]. The morphological differences in these filaments depend on whether they are composed of one protofilament, NPF, or two protofilaments, WPF.

Chronic traumatic encephalopathy (CTE) is also a tauopathy with a mix of 3R and 4R tau in the fibrils, showing an ultrastructural morphology, similar to that of the AD derived fibrils. The CTE filaments show differences at a secondary and tertiary level (Figure 5 C) [42].

There have also been reported structures on heparin induced filaments of 4R tau *in vitro*. Filaments called Snake, 40-100 Å width and crossover distance 650 Å, Twister, 80 Å width and 250 Å crossover, Hose and Jagged, 50-90 Å width and 450 cross over (Figure 5 D) [43]. These morphologically different filaments differ from each other in both secondary and tertiary structure.

Figure 5: Different kinds of polymorphs of tau, found in a variety of tauopathies. From *in vivo* and *in vitro* conditions. Several different cryo-EM structures for tau fibrils can be found in the literature A, 5o3l PHF and 5o3t SF from AD brain [40] B, 6GX5 NF from Pick's disease brain [41] C, 6NWP Type 1 and 6NWQ Type 2 from CTE brain [42] D, 6QJH, 6QJM, 6QJP and 6QJQ [43] recombinantly expressed tau induced by heparin.

Amyloid- β (A β)

The amyloid beta peptide originates from the transmembrane amyloid beta precursor protein (A β PP) (see Figure 6). The A β peptide is generated by the cleavage by β -secretase [44] on the extracellular side and γ -secretase (presenilin-1 or -2, which are subunits of γ -secretase complex) in the transmembrane region generating A β fragments 38-43 aa long with A β 1-40 and 1-42 being the most abundant.

Mutations in the A β PP gene or Presenilin-1 or -2 shifting the cleavage position so that the A β 42/40 ratio is increased are related to hereditary AD [45]. A peptide not involved in the amyloidogenic pathway can also be generated if A β PP is cleaved by α -secretase, which cleaves 16 amino acids into the A β -peptide [46]. Most of the A β PP is located at the membrane of the golgi apparatus and a small fraction is located at the plasma membrane. At the plasma membrane A β PP can be cleaved by α -secretase, resulting in a non-amyloidogenic pathway [47].

A β PP at the plasma membrane is internalized from the plasma membrane and delivered to endosomes which can be recycled to the golgi or plasma membrane or degraded in lysosomes. β -secretase is located in the golgi and late endosomes where cleavage can occur [48]. γ -secretase is active in several compartments and it is thought that A β is produced in lipid rafts in the trans-golgi network and endosomes [49, 50]. There are today around 60 mutations in the A β PP, over 300 mutations in PSEN-1 and around 50 PSEN-2 mutations listed at Alzforum [51]. Most are related to hereditary AD.

How pathogenic A β spreads within the brain is debated. A β oligomers have been shown to be able to spread through exosomes [52]. The A β PP A673T mutation [53] has been shown to be protective against AD. The mechanism is thought to be due to lowered A β production due to less cleavage by β -secretase compared to the wild type A β PP [54].

Figure 6: Schematic image of the A β PP processing by β -secretase and γ -secretase. The full length A β PP is cleaved by β -secretase and γ -secretase generating the soluble A β PP ectodomain (sA β PP) A β peptide and intracellular C-terminal fragment (AICD).

Tau

Microtubule associated protein tau (MAPT) is mainly located in axons [55] where it stabilizes microtubules and crosslinks microtubules with F-actin in the axon initiating segment [56-58]. Tau exists in six different isoforms in the human brain [59]. These variants are generated through alternative RNA splicing of exon 2, 3 and 10. Exon 2 and 3 are located N-terminally, generating 0N 1N or 2N tau and exon 10 is located in the repeat region, generating 3R or 4R tau (Figure 7).

Phosphorylation regulates the binding of tau to microtubules [60]. Abnormal phosphorylation is detected in NFT [61]. Attempts have been made to distinguish tauopathies on their different phosphorylation patterns [62], but this is not yet a reliable universal tool for diagnostics. Several other post translational modifications have also been found in tau.

Methylation of lysine residues in tau has been found [63]. These modifications are not thought to regulate binding to microtubules, but could be a part of crosstalk with other posttranslational modifications and were shown to suppress tau aggregation *in vitro* [63].

Tau is also glycosylated. It has been shown that O-GlcNAcylation appears to have a regulatory role for phosphorylation of tau. Down regulation of the glycosylation resulted in an up regulation of phosphorylation of tau, and it was shown that O-GlcNAcylation was lower in brain material derived from AD brain compared to control [64].

Acetylation has also been shown to be a post translational modification of tau which regulates phosphorylation [65]. Some acetylation competes for phosphorylation sites on tau and if acetylated tau is less aggregation prone. These sites were found to be hypoacetylated in AD patient derived brain material [65].

It has been suggested that the aggregation of tau generates a failure of micro tubular homeostasis in the axons leading to neurodegeneration [66]. *In vitro* experiments on repeat region fragments of tau K18 (4R) and K19 (3R) have shown that intramolecular disulfide bonds in K18 fragments inhibit or slow down aggregation of tau, but intermolecular disulfide bonds dimerizing K19 fragments promote aggregation [67]. Other studies have shown that a higher order of oligomers and aggregates form more rapidly under oxidizing conditions. In the same study it was shown that reduction resistant and reduction sensitive oligomers could be found *in vitro* and in cell cultures and mouse models [68].

Figure 7: Schematic image showing all six isoforms of the tau protein. The exons subjected to alternative splicing to generate the six isoforms are marked Exon 2, Exon 3 and Exon 10. Each isoform is written above the schematic sequence with the number of amino acids in each isoform.

Neurodegenerative Diseases

Neurons

Neurons are electrochemically excitable cells, which transmit the signal from one cell to the next via synapses. Neurons have dendrites branching out from the soma. These dendrites can be in contact with axon terminals from other neurons. When a neuron is excited, an action potential is initiated and sent through the axon to the axon terminal.

At the axon terminal, the action potential is converted to a chemical signal via neurotransmitters which are released at the synapse. Synapses can be connected to other neurons, sending the signal further, or to another cell type, generating a response from the receiver cell (Figure 8).

There is a wide distribution of neurons, and they are divided into classes, subclasses, types and subtypes exceeding the scope of this thesis. It has been shown that different neuron types are differentially vulnerable to various neurodegenerative diseases [69, 70].

Figure 8: Two neurons with dendrites, soma and axon connected via synapses, and an astrocyte participating in the tripartite synapse. Oligodendrocyte forming isolating myelin sheets at the axon of a neuron. The oligodendrocyte precursor NG-2, and a differentiated microglia phagocytosing a protein aggregate.

Glial cells

Glial cells is a collective name for several different cell types that can be divided in to four major groups: microglia, astrocytes, oligodendrocytes and their progenitor NG-2 glia [71]. It is generally acknowledged that glial cells perform far more specialized tasks in the brain than merely being the glue for the neurons. Each glial subtype has its own distinct functions within the brain (Figure 8).

Microglia are thought to be dormant in the healthy brain but have the ability move and react (become activated) in response to any insult on the brain by moving fine filopodia, and act as the nervous system's immunocompetent and phagocytic cells [71, 72].

Astrocytes are a group of cells with diverse functions in the brain. Some of these support functions are the homeostasis of water and ions, participation in the tripartite synapse and maintenance of the blood brain barrier (BBB) [71, 73].

Oligodendrocytes are the myelinating cells, creating an insulating layer around axons allowing rapid conduction and trophic support [74].

NG-2 glia are precursors to the oligodendrocytes that generate these myelinating cells. *In vivo* experiments show that as soon as one NG-2 glial cell is lost due to differentiation or cell death it is replaced, leading to a constant number of NG-2 cells [75]. They are considered a lineage due to diverse functions, one of them being their ability to form functional synapses with neurons. This characteristic is not fully understood and NG-2 glia are only able to receive neuronal signals but not transmit them further, suggesting that they may be monitoring firing patterns of surrounding neurons [76].

Neurodegeneration

Neurodegenerative diseases are a class of diseases caused by the degeneration and ultimately death of neurons, resulting in compromised motor control and/or cognitive function due to progressive neural damage and neuronal loss. For many neurodegenerative diseases it is difficult to perform a correct diagnosis due to the overlap of symptoms characteristic of the different diseases. *Post mortem* autopsy is the most certain, though not always completely accurate, way of evaluating neurodegenerative diseases [77]. Therefore, there is a need for less invasive accurate diagnostic techniques that are applicable early in the disease progression.

There are different hypotheses for how misfolded proteins can induce neurotoxicity. One hypothesis is the loss-of-function, where neurodegeneration is due to depletion of the protein into aggregates, thereby rendering it unable to perform its native function [78]. Other hypotheses involve some sort of gain-of-toxic-function. This could be mediated from extracellular deposits of aggregated proteins interacting with cell surface receptors like A β , which have been shown to interact with the RAGE receptor [79].

Intracellular aggregates could also recruit factors in the cell, chaperones and proteasomes, leading to a depletion of these viable proteins and hindering them from performing their function due to being locked to the aggregates, which they are not able to unfold/degrade [80, 81].

A β oligomers have been suggested to inhibit proteasome activity by stabilizing a closed conformation of the α -subunit of the proteasome. Due to common structures it is also suggested that oligomers from other aggregation prone proteins could have a similar mechanism [82].

Toxicity could also be mediated by pores in the cellular membrane allowing ions to pass through, altering the homeostasis and eventually leading to cell death. This has been shown for A β and ion channels with various pore sizes have been detected [83]. It has also been shown that oxidative stress is increased in neurodegeneration, which could be caused by the aggregated proteins [84].

Tauopathies

When looking at neurodegenerative diseases in general, tau is the most commonly found aggregated protein and has been identified as the primary cause of disease in several disorders (Table 1) [85]. The spreading of neurofibrillary tangles in the human brain can be staged according to the Braak staging, which divides the spreading of NFT in to six (6) stages, where the location of NFT may vary with different tauopathies. For more about staging, see the heading Alzheimer’s disease or reference [86].

There are several different suggested mechanisms for spreading of tau. For example, it has been suggested that microglia could mediate tau propagation [87], or that it can be propagated through synapses [88]. This could be through tunneling nanotubes between cells [89] and several other mechanisms have been suggested, ectosomes, vesicles etc [90].

Figure 9. A: Schematic image of 2N4R tau with some disease associated phosphorylation sites [91] indicated above the tau scheme. Below the tau scheme is a list of the disease associated amino acid substitution mutations [51]. B: Sequence of repeat 1-3 with the location for the G273R mutant indicated by the asterisk. Arrow pointing up indicating G304 and arrow pointing down G335. Highlighted **Bold** = F-Actin binding site, Underlined = microtubule binding site, **Bold and Underlined** = microtubule and F-Actin binding site [58].

Spreading of aggregated tau has been shown in cell cultures. Whereas full-length tau expressed in cell cultures did not aggregate spontaneously, it did so if pre-aggregated tau was added to the culture media. Aggregates were taken up by the cells and fibrillation of endogenous tau was initiated [92].

Mice that are intracerebrally injected with preformed tau fibrils show a spread of aggregation. The spread in the brain to distant regions follows neuroanatomic connections, indicating active induction and spreading of pathology [93]. It has also been shown that fragments of tau, in this

case the repeat region, propagated as amyloid strains in cell cultures could maintain their strain characteristics in seeding experiments passing through three inoculation generations of mice and back in to cell lines [39]. It was also shown that tau was able to propagate in different brain regions in mice depending on which strain the seed originated from. Different strains also showed altered spreading rates in mouse brains [94].

Table 1: Primary and secondary tauopathies and whether they are considered 3R or 4R pathologies or mixtures of 3R and 4R [95-98].

Primary tauopathies	Secondary tauopathies
Aging-related tau astroglipathy (4R)	Alzheimer's disease (3R & 4R)
Argyrophilic grain disease (4R)	Amyotrophic lateral sclerosis/parkinsonism-dementia complex (3R & 4R)
Chronic traumatic encephalopathy (3R & 4R)	Anti-IgLON5-related tauopathy
Corticobasal degeneration (4R)	Down's syndrome (3R & 4R)
Diffuse neurofibrillary tangles with calcification	Familial British dementia
Familial frontotemporal dementia and parkinsonism (FTDP-17) (3R, 4R or both)	Familial Danish dementia
Globular glial tauopathies (4R)	Gerstmann-Sträussler-Scheinker disease
Guadeloupean parkinsonism	Huntington's disease
Pick's disease (3R)	Meningio-angiomas
Postencephalitic parkinsonism	Myotonic dystrophy
Progressive supranuclear palsy (4R)	Neurodegeneration with brain iron accumulation
	Niemann-Pick disease, type C (3R & 4R)
	Non-Guamanian motor neuron disease with neurofibrillary tangles
	SLC9A6-related mental retardation
	Subacute sclerosing panencephalitis
	White-matter tauopathy with globular glial inclusions

There are over 100 mutations (some listed in Figure 9) listed in the Alzforum data base of known mutations in Tau [51]. Not all are associated with disease. Tau mutations are not linked to hereditary AD but have been linked to several other tauopathies, mostly Frontotemporal dementia and also to PSP. Mutations may lead to alternated expression ratios of different isoforms, alternations in microtubule assembly and/or the propensity to aggregate.

Frontotemporal lobar degeneration (FTLD)

FTLD is the third most common cause of early onset dementia after AD and vascular disease [99]. FTLD is divided into three different major molecular classes depending on which pathologic protein is found: FTD-Tau which is comprised of aggregated tau [100]. Transactive response DNA binding protein of 43 kD (TDP-43) [101] or Tumor associated protein fused in sarcoma (FUS) [102].

These classes then contain sub-categories based on lesion distribution and inclusion distribution [103]. These classes are further divided in to subtypes where for example CBD and PSP are two members of the FTLD-tau class [104]. Approximately 5% of all FTD cases are caused by mutations in the MAPT gen, 10% of all FTD cases are inherited in a dominant manner and 40% of all cases have a family history of FTD [105]. Common to all FTLD variants is protein aggregation in the frontal and/or anterior temporal cortices [106].

Figure 10, showing schematic image of tau stabilizing microtubules (left). Right Blue star indicating some alteration of tau could be mutation or phosphorylation generating tau with less affinity towards microtubules leading to microtubule depolymerization.

FTD generating mutations within the MAPT gene can either effect the splicing of mRNA, resulting in an overproduction of 4R tau, in turn resulting in an increase of tau amyloid fibrils, or mutations resulting in amino acid substitutions or deletions, leading to a reduced ability to interact with microtubules (see Figure 10). This can result in tau protein aggregation and thereby gain a toxic function [107].

Progressive supranuclear palsy (PSP)

PSP has some characteristic propagation of aggregated tau in the brain: Early stages: Basal ganglia. Middle stages: Posterior frontal lobe, brain stem, cerebellum. Late stage: association cortex [108]. PSP shares common features with other FTLN but has some characteristic pathology: Globose neurofibrillary tangles [109] and Tufted astrocytes (Figure 11) [110].

Figure 11: Black arrow showing a tufted astrocyte, normally a pathological characteristic of PSP. Red arrow showing astrocytic plaque, normally a characteristic feature of sporadic CBD. Image from Paper IV Figure 1.

Corticobasal degeneration (CBD)

The characteristic spreading of tau aggregates in CBD is: Early stages: basal ganglia. Middle stages: primary motor cortex, frontal lobes gray matter, and white matter. Late stages: continuous spread in gray and white matter [111]. Characteristic pathological features: Ballooned neurons and CBD specific astrocytic plaques (Figure 11) [112].

Alzheimer's disease (AD)

AD is a progressive neurodegenerative disease and the most common cause of dementia, estimated to account for 60-80% of all people suffering from dementia. Alzheimer's Disease International (ADI) estimate that 50 million people world-wide live with dementia and this number will increase to over 150 million by 2050. AD is a disease with a heavy burden on the person suffering from the disease, the healthcare system, the caring next of kin and society as a whole from personal suffering to lost income [113].

AD pathology is characterized by accumulation of extracellular plaques formed by the A β peptide [114] and intraneuronal deposits of neurofibrillary tangles consisting of different isoforms of tau [59]. It is not always clear what causes the neurodegeneration, but the accumulation of amyloid plaques and neurofibrillary tangles appears early, before any symptoms have started to show. Immuno-reactivity against abnormally phosphorylated tau can be detected even in children [115]. The authors state that in this case AD can be viewed as a disease with a very slow progress that extends into old age, instead of an age-dependent disorder [115, 116].

There are several different hypotheses on what causes the disease. The most widely acknowledged hypothesis is the amyloid cascade hypothesis. Aggregation of A β is the cause of cell loss and aggregation of hyper phosphorylated tau [117]. This is supported by mutations in or flanking the A β peptide in A β PP or mutations in proteins involved in the A β PP metabolism, which cause aggressive forms of AD. At the same time mutations decreasing A β production and aggregation are protective against AD [118]. Several clinical trials are running with the A β

plaques as the therapeutic target. There are several other hypotheses regarding the cause of Alzheimer's disease: aggregation of tau, inflammation, microbial infections etc.

The spreading of A β and tau in the brain has been mapped and divided into different phases and stages (see Figure 12):

Different stages of A β plaques: Stage A: initial deposits in basal portions of isocortex; Stage B: deposits in virtually all isocortical association area, hippocampal formation mildly involved; Stage C: Deposits in entire isocortex [86].

Different stages of NFT: Stage I-II: Alterations in a single layer of transentorhinal region; Stage III-IV: severe involvement of entorhinal and transentorhinal layer; Stage V-VI: isocortical destruction [86]. Stage I-II corresponds to pre-clinical AD, III-IV mild cognitive impairment and stage V-VI corresponds to fully developed Alzheimer's disease [86].

Figure 12. Showing general progression of A β plaques (left) and tau inclusions (right) in AD. Top row corresponding to Stage A (A β plaques left) and Stage I-II (tau inclusions right). Middle row corresponding to Stage B (A β plaques left) and Stage III-IV (tau inclusions right). Bottom row corresponding to Stage C (A β plaques left) and Stage V-VI (tau inclusions right). Image redrawn from [119].

Biochemical differences of AD, CBD, PSP

It is difficult to discriminate between different tauopathies. On a biochemical level this can be done because western blots will show differences between tauopathies. AD will show three major bands due to being characterized by NFTs of both 3R and 4R tau (60, 64 and 68 kDa). CBD and PSP will show two major bands at 64 and 68 kDa due to being 4R tauopathies. CBD and PSP can be discriminated between due to different bands after proteolytic cleavage indicating different structures of the filaments. A 33 kDa fragment dominates in PSP and a double band at 37 kDa dominates in CBD [120]. This has been further backed up by more recent studies indicating different fibrillary morphology between AD, CBD, and PSP by proteolytic digests and electron microscopy of filaments [121].

AIM

It is evident that amyloidogenic proteins can manifest as amyloid fibrils in several different structures, polymorphs, albeit generated from the same amino acid sequence. The overall objective of this thesis has been to use biophysical methods to characterize fibrillated proteins associated with neurodegenerative diseases, with an emphasis on tau and A β . By applying both established and novel approaches to the problem we can gain knowledge of how to discriminate between the different structures from different proteins and different polymorphs of fibrils from the same protein.

High quality monomeric protein is a prerequisite for the detailed studies of amyloid polymorphism *in vivo*. Hence, establishing high-quality production of recombinant proteins for usage in further biophysical measurements under native conditions is of the utmost importance for the project.

Conformation sensitive fluorescent amyloid probes are important and versatile tools for detecting and discriminating amyloids *in vitro* and *in vivo*. A large library of fluorescent probes from different classes would increase the resolution of detail in fluorescence readouts by adding new possibilities to find binding modalities that are unique for one or a few of the existing amyloid polymorphs.

Most of the amyloidogenic proteins associated with neurodegeneration present disease associated point mutations. By addressing the mutated sequence with biophysical characterization we can learn more about how these mutations can interfere with the native function of the protein, affect the rate of amyloid formation, alter the amyloid structure and ultimately give a deeper insight into what dictates the formation of disease-causing amyloid structure. One such mutant is MAP-tau G273R, which is possibly linked to FTLD.

Tau aggregation is involved in a number of different diseases termed tauopathies. Different tauopathies are dominated by different splicing variants of tau. The recent development of structural determination using cryo-EM has revealed that there are also differences in amyloid structure between the different patient samples. By utilizing the recombinant tau in a seeding assay and applying our library of conformation sensitive probes, we aim to characterize polymorphs of aggregated tau from brain-derived material by: seeding propensity, super structure and fluorescent read out, with the ultimate goal of fingerprinting amyloid polymorphs *in vitro* and *in vivo*.

METHODS

Biochemical methods

Protein expression

A more detailed description of purification of the A β peptide and tau protein can be found in Paper I [122].

In this work *E. coli* has been used to produce recombinant protein for the *in vitro* measurements. *E. coli* was transformed with plasmids using the same general system of induction: Plasmid where the gene of interest is inserted downstream of the lac operator and the T7 promoter. Gene expression is induced by isopropyl β -D-1-thiogalactopyranoside (IPTG). IPTG activates the protein production in the cell by binding to the lac repressor, which is bound to the operator sequence in the DNA inhibiting transcription, the repressor releases from the repressor sequence and RNA polymerase can initiate transcription of the gene.

The tau constructs

Tau is a soluble intrinsically disordered protein and can be expressed in BL21 cells. Paper I describes the protocol used for tau production in Paper III and IV. Tau used in Paper II was purified according to [123]. The latter construct does not contain any modifications to the tau construct. Tau contains cysteine residues (0N3R C322 and 0N4R C291, C322). To prevent disulfide bond formations during fibrillation, reducing agents such dithiotreitol (DTT) and β -mercaptoethanol needed to be added to the reactions. However, these agents oxidize quickly at neutral pH. Hence, in kinetic runs stretching over several days the chemical environment would change during the time of the experiment. Replacing cysteine residues with serine avoids the risk of studding tau with different disulfide crosslinking patterns during different time points in the experiments. Hence, in this work a pseudo wild type (PWT) variant of 0N3R and 0N4R has been used. In the PWT variants all the afore mentioned cysteines were replaced by serine. For purification purposes a hexa his-tag with a linker is added in the N- terminal.

The plasmid containing the A β construct was a kind gift from Sara Snogerup Linse. The construct contains an additional start codon methionine in the N-terminal of the peptide sequence [124].

Protein purification

The plasmid was transformed into *E. coli* by electroporation. For tau, BL21/DE3 cells were used as tau is expressed as a soluble protein. BL21 pLysS cells were used for A β which is expressed in inclusion bodies. These cells were inoculated to 2X LB-media grown to an appropriate OD and expression induced by IPTG. Expression was allowed for 4 h and cells were harvested by centrifugation.

Tau: Harvested cells were lysed by sonication followed by boiling and centrifugation. Most of the undesired proteins precipitated during the boiling step while tau, being natively disordered remain in solution. The cleared supernatant was further purified as described below.

A β : The desired A β formed inclusion bodies in the pLysS cells. The harvested cells were sonicated, centrifuged and the pellet resuspended in buffer. This sonication, centrifugation and

resuspension was repeated 3 times. The resulting washed inclusion bodies were dissolved in urea.

Liquid Chromatography

Once a cleared lysate is achieved, there are several different forms of liquid chromatography techniques suitable for different areas of protein science.

Affinity chromatography (His tag)

Affinity chromatography is a purification based on specific interactions between the target molecule and a binding target. In the case of tau purification, the N-terminally added hexa his-tag and its affinity towards metal ions was used. The sample is applied to a column with metal ions attached by a chelator to the stationary phase. Non-interacting molecules are washed out with buffer and interacting molecules can be eluted by imidazole groups in the elution buffer. Imidazole competes to bind to the metal ions. Affinity chromatography was used to purify all the tau constructs used in Paper III and IV.

Ion exchange chromatography

Ion exchange chromatography is a separation method based on interactions between molecules with opposite charge. In this case a stationary phase with charged molecules, cation exchange and anion exchange for stationary phases with anions or cations respectively, is used. These interact with charged molecules in the solution while non-interacting molecules can be eluted. Molecules that interact with the stationary phase can be eluted by increasing the ionic strength of the buffer or changing the pH. pH changes result in changes in the charge of the molecule and/or stationary phase resulting in lost affinity. Ion exchange chromatography was used to purify the A β and tau construct used in Paper II.

Size exclusion chromatography

Size exclusion chromatography (SEC) is a technique where molecules are separated according to their hydrodynamic radius. Size exclusion columns are built by having porous materials, eg polymers or agarose, as a stationary phase and flowing a buffer of choice as the mobile phase through the porous material. Molecules too large to enter the pores will only have access to the volume outside the pores particles, and smaller molecules will have access to the volume in the porous particles and the void volume. Due to various sizes of the pores within the same column, the molecules solved in the mobile phase will have access to different volumes depending on their size. This results in the column separation limit between the largest molecule with complete access to the pores to the smallest molecule with no access to the pores.

The larger molecules will be eluted with the void volume, smaller molecules will elute at the total volume. SEC is a crucial purification step to guarantee high-quality homogeneous protein preparation. This final step of purification ensures that no preformed aggregates or truncated variants will participate in the reaction and affect the assembly of monomers, the morphology of fibrils, inhibit the fibrillation or add errors to monomer concentration determination. Size exclusion chromatography was used as the final purification step for all constructs used in Paper II, III and IV.

***In vitro* fibril formation**

In vitro fibril formation, or fibrillation, of monomers will start as soon as the protein/peptide is in an environment with the appropriate chemical composition and temperature at a concentration interval allowing nucleation and fibrillation. Therefore it is important to keep the time for sample preparations after SEC down to a minimum to be able to capture early events

in the measurements. Protein/peptide concentration after SEC was determined spectrophotometrically by absorption at 280 nm. Technical replicates were achieved by mixing protein/peptide with the appropriate probe, inducer and buffer to the intended concentration to form a master mix for aliquoting. For seeding experiments, seeds were aliquoted to the wells first and the master mix added to the respective seed.

Seeding

Recombinant seeds used for fibrillation assays were taken from fibrillation experiments done under the same conditions and no sample preparation was done.

Fresh frozen brain material from patients diagnosed postmortem with CBD or PSP as well as samples from patients with pathological aging (Braak stage 2) who were diagnosed with other dementias were used to investigate the seeding and amyloid polymorph templating propensity. Seeds from patient derived brain material were analyzed for total tau and phosphorylated tau content using electrochemical luminescence (ECL). The purified filaments were added at 1-3% to the monomeric Tau protein at the start of the fibrillation assay to promote the formation of fibrillar morphology resembling that of the seed.

Biophysical characterization

Fluorescence

Luminescence is when a substance relaxes from an electronically excited state to its ground state by the emission of a photon. This can be divided into two categories:

1. Phosphorescence: which is a slow relaxation process due to the same spin of the electron in the excited orbital as the electron in the ground state orbital, making this transition forbidden.
2. Fluorescence: is faster than phosphorescence due to opposite spins of the electron in the excited state compared to the electron in the ground state orbital making this transition allowed. In this thesis the focus is on conjugated organic molecules and their characteristic fluorescence in interactions with fibrillated proteins.

For a more elaborate introduction to fluorescence see reference [125].

Molecules can be excited by the absorption of a photon. The molecule can return to its ground state by the emission of a photon. The emitted photon is usually of a longer wavelength (lower energy) than the excitation photon. The lower energy of the emitted photon is due to energy losses or stabilization of the excited state which can have several mechanisms. This difference in wavelength between excitation and emission is usually referred to as a Stokes shift. Absorption is a fast event on the femto (10^{-15}) second scale. This makes the absorption spectra less sensitive to solvent effects because vibrational relaxation or rearrangements of atom nuclei and molecules do not happen within this time frame (Figure 13).

Figure 13: A: Jablonski diagram showing general scheme of absorption and emission. Black arrow pointing upwards represents the absorption of a photon. Yellow squiggly arrows represent vibrational relaxation. Black arrows pointing downwards represent fluorescence. B, and C: normalized emission spectra of ThT and p-FTAA respectively. Red trace probe bound to A β fibril, Blue trace free probe in glycerol (high viscosity) and green trace free probe in PBS (low viscosity). D: general illustration of spectral shifts of ThT in more and less polar solvents, due to for example reorientation of solvent dipoles in more polar samples. E: general illustration of spectral shifts of p-FTAA due to restrictions in conformations. Listed to the right are other factors that can result in spectral shifts of the emission.

Fluorophores are excited from the ground singlet state (S_0) to the first singlet state (S_1) or the higher electronic states (S_2 , S_3 ,...). If they are excited to a higher singlet state the internal conversion (IC) relaxation is fast, usually on the scale of picoseconds (10^{-12}) to the first singlet state. The fluorophore is often excited to higher excited vibrational level within the first singlet state and this excess in vibrational energy is quickly lost to the solvent, ranging from 10s to 100s of picoseconds compared to fluorescence, which is in the order of nanoseconds (10^{-9}). This loss of vibrational energy is the reason for the Stokes shift. Emission of photons is a random event and the fluorescent lifetime is only the average time the fluorophore spends in the excited state. This is an important parameter because this is the time the probe has to interact with or diffuse across the environment, determining what information can be extracted from the emission spectra.

The dipole moment of a molecule in the excited state can be more pronounced and/or have a different orientation than the dipole in the ground state. If the molecule is excited in a polar environment this will lead to energy losses due to reorientation of the dipoles and molecules in the surrounding environment, resulting in a more redshifted (lower energy) spectrum of the emission compared to a nonpolar environment.

Molecules with an electron donating group and electron accepting group can undergo internal charge transfer (ICT) when in the excited state. When excited, the molecule can have a transfer of electron from the donor to the acceptor, creating a charge separation across the excited molecule. In a polar solvent, this excited ICT state could be the conformation with the lowest energy, compared to the same molecule in a non-polar environment, where the locally excited state is the most favored in terms of energy. There are cases where the excited molecule needs to undergo rotations or “twists” of groups in order to perform the charge transfer; this is referred to as twisted internal charge transfer TICT. TICT states can be strongly emissive or almost

completely quenched depending on the molecule and environment [126]. Quantum yield explains how many of the excited molecules actually emit a photon after excitation. The higher the quantum yield, the higher the brightness of the probe. The quantum yield is the ratio of number of photons emitted to number of photons absorbed.

Electrochemical luminescence (ECL)

Electro chemical luminescence is a technique where photons are emitted from a chemical reaction maintained by an electric current. In this case we have used instruments and kits from Mesoscale discovery (MSD). Detection is enabled when a ruthenium complex (in MSD called SULFO-TAG) is covalently bonded to the detection antibody and emits photons at 620 nm when reduced by tripropylamine (TPA) through a radical mechanism [127]. An electric current is generated at the bottom of the plate by having a dielectric barrier between the periphery anodes and the center carbon cathode that acts as a support which can adsorb capture molecules. This cycle can be repeated by the oxidation of the ruthenium complex. This is done in a 96 well plate format, where there are options for analyzing more than one analyte in the same well. Generally, a capture antibody is attached on the carbon electrode surface of the plate, which binds to the analyte. A detection antibody with a SULFO-TAG can then bind to the analyte.

The assay used in Paper III is of the sandwich type, where two analytes can be measured in the same well by having different capture antibodies in separated areas. One capture antibody has the specific epitope of phosphorylated threonine 231 and a second total tau capture antibody. The detection is then mediated by a SULFO-TAG total tau antibody. To be able to calculate concentrations, a standard curve is included in the measurements.

In Paper IV an ECL assay made in-house was used. Taxol stabilized microtubules or F-actin were adsorbed to the carbon cathode acting as capture molecules. Tau was added to allow for binding to the capture molecules followed by a primary antibody against tau and a secondary antibody towards the primary with a SULFO-TAG. A standard was generated by letting different concentrations of tau bind to the carbon surface.

Fluorescent ligands

Most proteins do not absorb or emit light in the visible region. Hence, they are not visible to the naked eye. The aromatic amino acids in protein are not always useful as intrinsic probes for amyloid formation. Therefore there is a need to use extrinsic ligands with light absorbing or emitting properties that can bind to amyloid fibrils and report on their presence in a sample. There is a need for specific interaction characteristics to be able to monitor the species of interest. Fibrils, once formed, can have several potential binding pockets for ligands (Figure 14).

These binding pockets will have different characteristics depending on which amino acids are presented in the binding pocket, what protein is forming the fibril, the conformation of the fibril and how the fibrils are bundled. As a result, the binding probes will experience different chemical environments and have different conformations depending on the interactions in every unique binding pocket. Binding pockets will also be ligand specific, so different probes might not bind to the same structural motif structures of different ligands.

Figure 14: Hypothetical formation of different binding sites when assembling the same β -strand motif in different orientations. The beta strand used is A β 1-42 PDB ID: 5oqv [128].

Thioflavin T (ThT)

ThT has been extensively used in *in vitro* fibrillation assays since it was shown to have a linear relationship between amount of amyloid and fluorescence signal [129]. The low emission intensity of ThT in a water solution is thought to be due to non-radiative relaxation through twisted internal charge transfer (TICT) like a molecular rotor [130]. The torsional angle between the benzotiazole and the aminobenzene with the lowest energy in the ground state is thought to be 39°, and the conformation with the lowest energy in the excited state is thought to be 90° due to internal charge transfer. When in a highly viscous sample the changes in angle and reorientation of solvent molecules are slow and relaxation to the ground state can occur through emission of a photon. But in a less viscous sample, the excited molecule can twist to the excited state's most favorable angle and solvent reorientation can occur, resulting in non-radiative relaxation [131].

Luminescent conjugated oligothiophenes (LCOs)

LCOs show shifted excitation and emission peaks when bound to amyloids. LCOs have been extensively used and characterized. Their ability to specifically stain amyloid both *in vitro*, *in vivo* and *ex vivo* has been shown. LCOs also show characteristic spectral shifts between different types of fibrils due to the thiophene backbone being restricted to certain conformations, depending on the conformations of the fibrillar structure. One of the most used LCOs is p-FTAA [132-134].

Other ligand classes

In Paper II and III several probes from classes other than LCOs were used, as characterized previously (for examples see Figure 15). These, have been shown to maintain the Congo red binding site without competing for the PiB site and bind to A β and tau fibrils from *in vivo* and *in vitro* sources.

The styrylbenzothiadiazoles previously described and characterized were used to stain A β plaques and neurofibrillary tangles or map heterogeneity in A β plaques due to spectral shifts or

preference towards coronas or core of the plaque [135, 136]. Bi-thiophene-vinyl-benzothiazoles (bTVBTs) showed selective detection of tau pathology in brain sections from AD brains [137].

Figure 15: Fluorescent amyloid ligands used in this thesis; styrylbenzene: DF-9, styrylbenzothiazole ligands: BTD-1-14. Congo red analogs: X-34, NSB and BTDSB, LCOs: p-FTAA and q-FTAA-CN, Bi-thiophene-vinyl-benzothiazoles (bTVBTs): bTVBT-3 and bTVBT-4.

Fluorescence measurements

The basic principle for any fluorescence measurement is that a light source is needed. A lamp or laser can be used. The light is guided through filter(s) or a monochromator to generate light of a certain specified wavelength. The resulting wavelength of light should correspond to a wavelength where the fluorescent probe will absorb light and be transferred to its excited state. The filtered or monochromatic light is guided to the sample, which is excited and emits light. This is then guided through a new set of filter(s) or monochromators and guided to the detector. Several different setups exist with the ability to use techniques based on fluorescence.

Monitoring of *in vitro* fibrillation kinetics

Monitoring of *in vitro* fibrillation kinetics was conducted in 96 well plate format, using a plate reader with shaking before measurements at 37°C. Shaking time and measurement intervals differ depending on which protein is being monitored. As amyloid ligand ThT and/or p-FTAA was used at a 2 µM or 300 nM concentration respectively. Both ligands were excited with a wavelength of 440 nm and emission spectra were acquired from 460 nm to ≥ 650 nm. The reason for monitoring emission spectra instead of single wavelength is to be able to analyze the shape of the emission profile. Both ligands will show emission intensity increases when bound to amorphous aggregates, but will have characteristic shifted peaks when bound to amyloid aggregates (ThT = 482 nm when bound to fibrils and over 500 nm when free in PBS buffer p-FTAA = 510 nm when bound to fibrils and 540 nm in PBS buffer).

Figure 16. Example of fibrillation kinetics monitored by fluorescence from amyloid ligand. Black curve shows fitted data using Equation 1 [138]. Gray trace experimental data of emission intensity. Dotted line (right) shows estimated $T_{1/2}$ from equation, dotted line (left) shows calculated lag time (lag time = $T_{1/2} - 2\tau$).

$$\text{Equation 1: } Y = y_i + m_i t + \frac{y_f + m_f t}{1 + e^{-\frac{(t - T_{1/2})}{\tau}}}$$

Equation 1. Y = emission intensity, y_i and y_f are the intercept of initial and final baseline, m_i and m_f are the slope of initial and final baselines. $T_{1/2}$ is time needed to reach halfway through the elongation phase and τ is the elongation time constant. For a general example see Figure 16.

For samples labeled with ThT or p-FTAA, emission intensity at 480 nm and 510 nm respectively, was plotted against time. Fibrillation kinetics were defined as $T_{1/2}$, which is the time required to reach half of the maximum emission intensity. In Paper IV $T_{1/2}$ was estimated using equation 1. When bound to mature fibrils, p-FTAA shows a characteristic double peak at 510 nm and 545 nm. One alternative way of determining fibrillation kinetics using p-FTAA is to plot ratios between the 510 nm and 545 nm peaks, as the 545 nm peak dominates when the probe is free in solution or bound to amorphous aggregates, but the 510 nm peak dominates when bound to mature amyloid fibrils.

Characterization of *in vitro* formed fibrils

In Paper III a variety of fluorescent ligands are used to characterize tau fibrils seeded with filaments derived from patients. Which were diagnosed with different tauopathies. Measurements were performed on 3 μM fibrils with 300 nM fluorescent ligand. Wavelengths where: ex 376: em 406-576, ex 440: em 470-640 and ex 500: em 530-700 nm. Emission intensity and emission shift were used to identify differences in ligand binding, interpreted as differences and similarities in morphology between fibrils.

Fluorescence Microscopy

In fluorescence microscopes a light from a light source is guided through a filter to select the wanted excitation wavelength and is then guided by mirrors to illuminate the sample. The emitted light is guided through the objective and lenses to the detector through an emission filter, which removes light of unwanted wavelengths. This is used to obtain data revealing structures on a microscopic level of tissue, cells, subcellular structures, distribution of other microscopic structures. The filters used to select excitation or emission wavelengths can be of varying kinds. Band pass filters are filters selecting light of a certain wavelength within a given span. Short pass filters cut out all wavelengths longer than a specified value and long pass filters cut out all wavelengths shorter than a specified value.

Epifluorescence microscope

Hyperspectral imaging (long pass filters vs short pass filters). By using long pass filters for the emission light to eliminate reflected light from the excitation beam and a diffraction source before the detector, it is possible to generate a fluorescent spectrum from the image. The term hyperspectral imaging means obtaining a spectrum in every pixel of the image.

Laser scanning confocal microscope

In confocal microscopy excitation of the sample is achieved by focusing a laser excitation beam in small points moving across the selected area. The emitted light is then guided to the detector by passing a pinhole aperture placed at a focal point in the light path. By having the pinhole aperture at a focal point emitted light which is not in focus does not reach the detector. This results in improved resolution of the image.

Fluorescence spectroscopy

Fluorescence spectroscopy can be performed in single samples in a cuvette or on multiple samples in multi-well plates. The advantage of the plate reader system is the high throughput of samples that can be measured in a short time.

Monochromator system (Tecan)

A monochromator based system was used for all *in vitro* fibrillation kinetic experiments (Papers I, II, III, and IV). The light source provides excitation light which is transmitted to a grating through an entry slit. On the grating, the light is diffracted into its optical spectrum. Light of the intended wavelength, determined by the angle of the grating, is transmitted to a second grating through the next set of slits. The second grating further diffracts the light, which passes through a third slit and is guided to the sample. The purpose of having two monochromators is to reduce stray light. The same setup is then used for the emission light, which is guided through the same double monochromator setup to the detector.

Filter based system (Clariostar)

Was used in ligand screen Paper III. The light is guided through a set of two linear variable filters (LVF) which together remove light above and below the intended wavelength. The monochromatic light is then reflected with a dichroic mirror to excite the sample. The emitted light is passed through the dichroic mirror to a second set of filters, preventing light of unwanted wavelengths from reaching the detector. LVF filters are filters whose spectral properties vary linearly from one end of the filter to the other. By placing two filters in parallel, both with opposing ends to each other, it is possible to separate light of distinct wavelengths and adjust the bandwidth and wavelength according to the user's requests.

Transmission electron microscopy (TEM)

When an object is so small that it is no longer able to scatter visible light because the wavelength of the incoming light is larger than the object of interest, we turn to electron microscopy. For an introduction to electron microscopy, see [139]. In transmission electron microscopy, a beam of electrons is transmitted through the sample. Electrons from the beam can be scattered or absorbed by the object analyzed, and the electrons transmitted can be detected. This generates a negative image which can be magnified through a system of magnetic lenses generated by electric currents rather than the glass lenses used in conventional light microscopy.

The sample is thin to allow the transmission of electrons. The higher the electron density of the sample, the more electrons will be scattered or absorbed, which gives higher contrast to the image. Proteins consist mostly of relatively light atoms (C, N, O, H) which give poor contrast. Therefore there is a need to increase the contrast by adding heavy atoms. In protein chemistry this is generally done by staining the samples with heavy atom salt solutions e.g. uranyl acetate, after adhering the protein of interest to the carbon coated copper meshed grid used for microscopy.

The images acquired can be analyzed using image analysis software such as ImageJ. This technique provides the opportunity to study super structure motifs of fibrils. Investigating bundling, thickness, twist or “pitch”, and the length of the fibril. Amorphous aggregates and oligomers present in the sample can also be imaged.

Isothermal titration calorimetry (ITC)

ITC is a technique based on measuring the heat taken up or evolved from a reaction, depending on whether the reaction is endothermic or exothermic respectively. This is achieved by having two cells under constant temperature. One cell is the reference cell containing the buffer and the second cell the sample containing the buffer and the molecule of interest, with the interaction partner titrated to the sample cell. Measurements are performed by comparing the amount of energy required to keep the two cells at the same temperature per second. By knowing the volume and concentration of the molecule of interest and the titrant, it is possible to determine: Enthalpy changes (ΔH), binding affinity (K_a), and binding stoichiometry (n).

SUMMARY OF PAPERS

Paper I

Working with amyloid proteins offers a number of challenges when it comes to acquiring enough material in the form that the scientist wishes to investigate. Therefore we need to be able to express and purify high quality protein in well-defined conformational states. Working with aggregation prone proteins offers several challenges and there are multiple pitfalls. Purification protocols can be complicated and all the details on how to handle these proteins are not always clear. Here we present three protocols for expression and purification of proteins associated with neurodegenerative disease. These proteins are routinely used in our group and are the foundation of this thesis. We describe the process in detail and give general tips and suggestions on how to avoid some pitfalls, as well as highlighting some parameters to take into consideration for successful aggregation studies.

Amyloid beta (A β 1-42) is one of the prime culprits in Alzheimer's disease. The peptide is very aggregation prone and can be toxic for the host cell when expressed recombinantly. Several crucial steps need to be considered when handling A β during the purification process. Stringent management of the protein is necessary to control at which state in the aggregation process the measurements are performed. Therefore protocols for expressing A β need to handle the toxicity of A β . The purification protocol needs to enable close tracking of monomer production. The fibrillation process needs to be probed by a distinct marker for monitoring the start and progress of aggregation.

Aggregated tau can be found in the brain during many different brain disorders. Due to its involvement in several neurodegenerative diseases tau is highly interesting to study *in vitro*. Tau is phosphorylated *in vivo*, whereas recombinantly expressed tau lacks these post translational modifications. Non-phosphorylated tau molecules are not prone to form amyloid aggregates unless an inducer is added. Several native disulfide bonds also interfere with the fibrillation process. This issue needs to be controlled via reducing agents or by replacing the cysteine residues with amino acids that do not form intramolecular covalent bonds. Our protocol allows for purification of high-quality monomeric tau. The unphosphorylated tau protein can be turned into amyloid-like fibrils under various conditions. This process can be followed by ThT assay and the resulting fibrillar structures can be imaged using electron microscopy.

The prion protein has been linked to several disease in humans [140] and in a large number of mammals [141]. The prion protein (PrP) can be purified using different purification protocols and several fibrillation protocols involve aggregation under strongly denaturing conditions. This raises problems if interactions between the prion protein and other folded proteins are to be studied. In order to be able to study aggregation of the prion protein together with other natively folded proteins, it is necessary to have a fibrillation protocol under conditions allowing proteins to be folded. PrP *in vivo* under normal healthy conditions has a well-organized, tightly folded α -helical structure. This also underlines the relevance of studying the misfolding process of PrP using natively folded, monomeric PrP as the starting material. In this protocol PrP is harvested from *E. Coli* under denaturing conditions and refolded stepwise to its native structure. The final product, natively folded PrP, is eluted from a size exclusion column. The native monomeric protein is then used for the assays of choice.

Conclusions

We have adapted robust protocols for purification of A β , tau and the prion protein. With no or minor changes, we can purify different mutants and isoforms of the proteins. These protocols also allow for fibrillation under native conditions.

Paper II

X-34 is a highly fluorescent analog to the well described amyloid dye Congo red. It is used in the amyloid research field for detection of amyloid both *in vivo* and *ex vivo*.

X-34 was first described by Styren et al [142]. Little has been studied when it comes to replacing the central benzene ring of X-34. Here we demonstrate that when this motif is substituted by different heterocyclic motifs, it still retains its amyloid binding properties and generates new fluorescence properties. Four probes were synthesized and compared against the established amyloid ligands X-34 (parent compound) and ThT (Figure 17).

Figure 17: Structures of the amyloid ligands; A: Congo red, B: X-34, C: BTDSB, D: TSB, E: QSB and F: NSB.

Photophysical characterization of the probes was done both on the probes free in solution and their interactions with A β and tau fibrils. NSB showed a similar absorption peak to X-34, assigned to the π - π^* transitions. TSB showed a 35 nm redshift. This is ascribed to enhanced planarity and conjugation length. QSB and BTDSB had two absorption peaks where the blue peak is assigned to π - π^* transitions and the red peak to charge transfer transitions, where blue and red are relative to the absorption peak for X-34. When emission spectra for the probes in PBS buffer were collected, NSB showed a broad peak and emission peak near X-34. TSB has a blue shifted emission and QSB and BTDSB have red shifted peaks compared to X-34.

When bound to fibrils of recombinant A β 1-42, all ligands showed a red shift (10–20 nm) in their absorption spectra, presumably due to the planarization of the ligand when bound to fibrils. When mixed with A β monomers and oligomers, the fluorescence was only moderately increased, indicating weak interactions (Table 2). TSB showed a weak emission intensity, which increased when binding to A β or tau fibrils (1.9 and 1.7 respectively). QSB and BTDSB both showed the same interesting feature: high intensity increases when bound to A β fibrils (67 and 46 respectively), but when bound to tau fibrils the emission intensity fold increases were reduced to 10 and 13. All the probes had lower K_d values than ThT as monitored by saturation

binding assays based on fluorescence. The corresponding binding affinity towards A β and tau fibrils is in order of decreasing affinity from highest TSB, BTDSB, QSB, NSB, X-34 and lowest ThT. It was shown that the new probes competed with ^3H -X-34 but not ^3H -PiB when binding to A β fibrils. EC₅₀ values in the competition assays when competing against ^3H -X-34 were as follows: TSB 16 nM; QSB 120 nM; BTDSB 140 nM and NSB 750 nM. X-34 competing with ^3H -X-34 was 80 nM, revealing that the thiophene group which is an electron donating group, decreases the EC₅₀ but the ligand QSB with an electron withdrawing group and the more electron withdrawing BTDSB showed an increase of EC₅₀ values, as did NSB with the naphthalene ring. The same competition experiments on tau fibrils showed lower EC₅₀ values for all probes (Table 2 in Paper II).

Table 2: The absorption and emission maxima of ligands free in PBS or bound to A β 1-42 fibrils.

	In PBS		A β 1-42	
Ligand	λ_{max} Abs (nm)	λ_{max} Em (nm)	λ_{max} Abs (nm)	λ_{max} Em (nm)
ThT	413	494	413	483
X-34	364	490	384	448
NSB	365	505	380	446
TSB	399	475	409	481
QSB	333, 436	571	448	577
BTDSB	330, 472	620	489	622

Fluorescence lifetime experiments were also done on the probes either free in solution or bound to A β 1-42 fibrils in PBS. Accurate lifetimes for ThT, QSB and BTDSB free in solution were not measurable due to instrument limitations. All probes showed increased fluorescent lifetimes when bound to fibrils compared to free in solution indicating interactions with the fibrils.

The ability for the probes to stain A β plaques and neurofibrillary tangles in Alzheimer's disease brain sections was also tested. All probes were able to specifically stain A β plaques and all but QSB were able to stain neurofibrillary tangles. QSB's ability to stain tau fibrils formed *in vitro* but not *in vivo* might be attributed to the different morphology and to the post translational modifications that occur in the brain.

Conclusions

We showed that the central benzene ring of X-34 can be replaced with heterocyclic motifs and still retain its X-34 binding site without competing with the PiB binding site on recombinant A β 1-42 fibrils. The ability to specifically stain neurofibrillary tangles and amyloid plaques was also retained, except for QSB which was plaque specific. These new ligands and the possibility to keep developing probes with new optical properties will expand the number of available ligands for different amyloid aggregates.

Paper III

Is there a disease specific tau aggregate polymorph? We addressed this question using a method for kinetic studies of recombinant tau protein replication of brain derived fibrils *in vitro*, which were further investigated with transmission electron microscopy and a panel of fluorescent amyloid ligands generating an amyloid ligand pattern code (AP-code).

Microtubule associated protein tau can be linked to several neurodegenerative diseases. There are six different isoforms of tau in the adult brain and different isoforms of the protein can be found in aggregates in different diseases. 3R/4R mixture in Alzheimer's disease, 3R tau in Pick's disease and 4R tau in cortical basal degeneration (CBD) and progressive supranuclear palsy (PSP). It is also thought that different polymorphs of fibrils can be linked to individual strains of disease resembling that of the prion protein [30, 36, 119, 143].

For these fibrillation experiments recombinant 0N3R and 0N4R pseudo wild type tau [122] was used with and without the fibrillation inducer heparin. Without heparin, these tau constructs are not capable of spontaneously forming fibrils *in vitro*. Seeds were isolated from five patient brains. We quantified total tau content and the relative phospho-tau (Thr-231) content between the samples. A seeding assay was used to determine the seeding activity of the samples. End-point aliquots were analyzed using transmission electron microscopy (TEM). To further distinguish the fibrils, an assay with structurally different fluorescent amyloid ligands was used.

The concentration determination of the tau content in the seeds showed that the samples contained 2 000 – 6 000 ng/mL total tau and phospho-tau content varied up to three-fold between the samples. No trend of tau concentration based on clinical diagnosis was found.

From the kinetic seeding assay using 0N4R PWT as a substrate without heparin, it was clear that samples PSP-1, PSP-2, CBD-1 were the most active seeds PA-1 was less active, while PSP-3 did not show any seeding activity in this assay. TEM showed large fibril assemblies for PSP-1, PSP-2 and CBD-1. Fibrils were absent in PSP-3 and PA-1. The AP assay showed that PSP-1 and CBD-1 were the most overlapping samples and they seemed to most closely resemble PA-1. PSP-2 and PSP-3 showed a generally weaker signal, with PSP-2 showing a clear preference for probes containing a thiophene backbone. PSP-3 showed preference for BTB-probes.

0N4R was further used as a substrate, this time with heparin generating spontaneous fibrillation of non-seeded tau. Here all samples except PSP-3 showed clear seeding activity by considerably shortening the fibrillation time. In TEM images, all samples contained large amounts of fibrils. Overall, the AP-code assay results were different from the AP-code assay where 0N4R without heparin was used. PSP-1 and CBD-1 still had similar patterns, as did PA-1 but with a clear preference for ligands with a thiophene backbone. PSP-2 differed from PSP-3 by having lower intensities for BTB probes, but similar intensities when interacting with Congo red analogs.

For the 0N3R seeding activity assay without heparin, PSP-1 and CBD-1 were moderately active, with incomplete conversion within the replicates. PSP-2, PSP-3 and PA-1 showed no seeding activity. TEM revealed fibrils in the PSP-1 and CBD-1 samples and fibrils but in low amounts in the PA-1 sample. The AP assay showed clear differences from AP assays where 0N4R tau was used. PSP-1 showed a unique preference for BTB probes, DF-9 and bTVBT-3 and bTVBT-4. CBD-1, PA-1 PSP-2 were similar overall and PSP-3 showed the weakest signal.

When running 0N3R seeding assay in the presence of heparin, PSP-1, PA-1 and CBD-1 showed seeding activity, and PSP-3 was moderately active, as indicated by collecting the $T_{1/2}$ distribution and increasing conversion fidelity. One interesting feature was that PSP-2 appeared to slow down the fibrillation reaction. TEM analysis revealed large amounts of fibrils in PSP-1, PSP-3, CBD-1 and PA-1. Seeding with PSP-2 showed large amounts of fibrils clustered in a web-like network. In the AP assay PSP-1, CBD-1 and PA-1 showed very similar patterns. PSP-2 was more reactive against BTD-2, BTD-3 and BTD-14, and less reactive against DF-9, bTVBT-3 and 4. PSP-3 was generally weaker. Overall recombinant seeded 0N3R tau deviated from the brain derived seeded variants.

Conclusions

All together, fibrillation kinetics TEM and AP-code reveal distinct differences between seeds derived from different brains. There are clear differences depending on what substrate is being used in the assay (0N3R or 0N4R) and whether or not the fibrillation inducer heparin is present. Our finding that PSP-2 seed delayed the onset of fibril formation shows that kinetic data alone might not be sufficient. The use of TEM was critical due to it being revealed to be a more sensitive detection method than p-FTAA alone in some instances. The AP-code appears to differentiate between different polymorphs. Our main finding is that each patient derived seed behaves differently, indicating different polymorphs in each individual.

Paper IV

Several functions of microtubule associated protein tau (tau) are known. Beyond its native functions, stabilizing microtubules and crosslinking microtubules and F-actin etc. [56, 58], it also forms aggregates in several neurodegenerative diseases. Neurodegenerative diseases with involvement of aggregated tau are generally termed tauopathies. The mechanisms involved in tauopathies are not fully understood and it is not clear if disease is due to loss of function or gain-of-toxic function. It is also complicated to establish a correct diagnosis for the different tauopathies as well as for the neurodegenerative diseases as a whole.

The G273R mutant is possibly associated with the neurodegenerative disease Frontotemporal lobe dementia [144]. We have studied samples from the second patient ever to be found with this rare mutation in a genetic screen.

The tau protein is subjected to alternative splicing at three exons of the protein generating six variants. Two exons in the N- terminus and one in the C terminus of the tau protein. These can be present in different numbers due to varied splicing of the tau gene. The N-terminal regions are termed N for short and the C-terminal repeats are termed R. N appears in zero, one or two copies, 0N 1N or 2N for short, whereas in the C-terminal the second repeat can be spliced (exon 10), generating 3R or 4R tau. A total of six isoforms are found in the adult brain. The G273R mutant is located in the first of the C-terminal repeats. The mutant is located within a microtubule binding site and close to an F-actin binding site. It is also located near or in the fibril core of aggregated tau. Hence it is interesting to investigate it from a protein chemistry point of view and look for changes in either function (binding to the cytoskeletal proteins) or aggregation propensity, as well as fibril morphology. These results can then be put in the context of the phenotype of the patient.

The G273R mutant was found in one case as part of a large scale study through exome sequencing of cases labeled as CBD across brain banks in Europe, North America and Australia. Tissue from the anterior frontal cortex, temporal cortex, parietal cortex, hippocampus, striatum and midbrain were stained with hematoxylin and eosin and tau immunohistochemistry. The main histological findings resemble patterns consistent with those observed in sporadic progressive supranuclear palsy (PSP), neurofibrillary tangles and tufted astrocytes. On the other hand, several features typical of corticobasal degeneration (CBD), such as astrocytic plaques and ballooned neurons, were also present.

For the *in vitro* experiments we used recombinant expressed and purified 0N4R, 0N4R G273R, 0N3R and 0N3R G273R. All experiments were performed with monomeric tau as the starting material. We performed fibrillation kinetic experiments where heparin was used as a fibril inducer and seeds were acquired from aggregation experiments performed under identical conditions. The kinetics were monitored by the fluorescent dye p-FTAA and $T_{1/2}$ was defined as the time it takes for the emission intensity to reach half of its maximum intensity (see Figure 2 and Figure S4 in Paper IV). 4R tau showed the fastest aggregation kinetics. When the reaction was seeded, it was clear that the reaction followed a sequence dependent manner. 4R was the most potent seed for any of the 4R variants and the G273R variant was more potent for seeding the G273R variant compared to the wild type seed. Transmission electron microscopy was used to image the resulting fibrils at end point. The images were analyzed by manually measuring the fibril widths using ImageJ. The fibrils of 0N4R G273R were thinner than 0N4R PWT while the relationship was reversed for the 0N3R variants, G273R formed thicker fibrils than the PWT. When observing the 0N4R variants, it was clear that there were two different variants of fibrils, interpreted as paired helical filaments (PHF) and straight filaments (SF) [40]. Both the PHF and SF were thinner for the mutant compared to the corresponding filament type for the

wild type. The binding of the tau variants to taxol stabilized microtubules and F-actin was assessed using an ECL assay. The assay revealed that 0N4R G273R had an enhanced binding to taxol stabilized microtubules and 0N4R PWT binds better than 0N3R PWT. 0N4R PWT has a lower EC₅₀ value when binding to F-Actin than the 0N4R G273R variant.

Conclusions

Our results from biophysical measurements reveal distinct properties of the G273R mutant that separate the mutant from its wild-type counterpart. Our data show a reduced lag phase of fibril formation for the G273R mutant compared to its wild type counterpart and a sequence similarity dependent manner to seed the formation of 4R fibrils. This mutation also alters the morphology of the fibrils formed, indicated by different fibrillary widths between the wild type and the G273R mutant. The G273R variant also showed different binding to taxol stabilized microtubules and F-actin with an enhanced binding to microtubules and a diminished binding to F-actin. Taken together, our biophysical data are compatible with the mutation G273R causing a 4R tauopathy.

CONCLUDING REMARKS

Robust protocols have been established for the expression and purification of recombinant amyloidogenic proteins. The use of well-defined recombinant proteins and robust fibrillation protocols are the foundation of this thesis. These protocols can be utilized to purify a variety of isoforms and mutations of the intended proteins.

We have contributed to expanding the library of conformation sensitive amyloid probes by development and characterization of X-34 analogs with the ability to detect A β fibrils formed *in vitro* and *ex vivo*. We demonstrate that minute changes in the chemical structure of the molecule influence how and to which amyloid polymorph they bind. One of our ligands was A β plaque specific and showed no staining of neurofibrillary tau tangles and one ligand showed a clear preference for A β fibrils and plaques but also weak staining of tau tangles. We also found that two probes were able to bind to recombinant tau fibrils and neurofibrillary tangles as well as recombinant A β fibrils and A β plaques. The various probes also showed different optical properties, paving the way for new experiments using combination of dyes.

We addressed the question of the effect of the disease associated point mutation G273R by employing our recombinant tau purification and fibrillation assays. Seeding experiments reveal the potency of self-seeding (mutant seed in mutant substrate and wild type seed in wild type substrate) in the background of 4R. When using 3R as substrate the outcome was different and seeding was not efficient. Ultrastructure analysis revealed differences in fibril width between the mutant and wild type fibrils. Alterations in the binding to cytoskeletal proteins were also shown. Altogether, this could lead to the altered function of monomeric tau, leading to differences in accessible monomer or an altered conformation being more aggregation prone. Our biophysical data rationalize the identification of the G273R mutant causing a 4R tauopathy.

Again, seed brain material from 4R tau pathologies showed selectivity towards seeding 4R recombinant tau. We were able to fingerprint amyloid polymorphs using our novel AP-code assay. Different patients appear to carry different polymorphs of tau fibrils. This is possibly linked to disease phenotype.

FUTURE PERSPECTIVES

There is a large gap in the understanding of the importance of amyloid polymorphs. Several questions remain unanswered. So far there is no clear knowledge regarding more or less toxic polymorphs. Which polymorphs are the most aggregation prone? What determines which polymorphs spread from cell to cell, propagating the disease? What dictates which anatomical route a certain disease follows? An understanding of these questions could lead to more focused research aimed at finding relevant diagnostics and perhaps relevant treatments.

The continuous development of the AP-code assay could perhaps help answer these questions. This assay could be further developed by finding optimal conditions for the propagation of disease relevant morphologies. Also one should study microscopy sections of the same individuals using the same ligands, screening through more ligands to find those relevant for discrimination between disease related/generating morphologies. One attractive future development would be to use seeds derived from cerebrospinal fluid (CSF) in the assay, mapping species found in CSF, discriminating between morphologies capable of generating disease, and perhaps ultimately predicting which disease the subject is at risk of developing, depending on the AP-code. A similar method has been developed by Saijo, et. al, specifically for 3R tauopathies or 4R tauopathies from CSF [145, 146]. But this method lacks the accuracy to discriminate between morphologies and give more or less of a digital readout for 3R or 4R pathology.

Further investigation of disease related mutants of tau is required. Over 100 putative disease mutations of tau are known. For these experiments, accessible animal models such as *Drosophila melanogaster* could be used to investigate different aspects of diseases. Here one can investigate the toxicity of tau mutants by expression in different cell types, to investigate which cell types are more sensitive or resistant to misfolded proteins. Similar experiments have previously been done with A β expressing flies, revealing selective toxicity in neurons [147]. Such experiments allow for characterization of aggregates and their relevance for human disease. There is also a need for further investigations into disease mechanisms on the effects of tau interactions with cytoskeletal proteins and disease phenotype on a sub cellular level. This is doable using reporter lines developed for this purpose [148].

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Linköping Studies in Science and Technology Dissertation No 2022, 2019
Linköping University
SE-581 83 Linköping, Sweden

www.liu.se