Seeded Propagation of Tau Fibrils

Paul David Dinkel
University of Denver

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SEEDED PROPAGATION OF TAU FIBRILS

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Doctor of Philosophy

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by

Paul D. Dinkel

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Advisor: Dr. Martin Margittai
Abstract

In various neurodegenerative diseases, including Alzheimer’s disease, progressive supranuclear palsy, Pick’s disease, and corticobasal degeneration, the deposition of fibrils composed of misfolded tau protein is observed. Recent evidence suggests that tau fibrils transfer between cells and spread throughout the brain, underscoring the significance of fibril propagation.

Six tau isoforms exist in the adult human brain that can be grouped into 4-repeat (4R) tau and 3-repeat (3R) tau based on the presence or absence of the second of four microtubule binding repeats. We demonstrate in vitro that seeded fibril growth, a prerequisite for the spreading of the tau pathology, is crucially dependent on the isoform composition of individual seeds. Seeds of 3R tau and 3R/4R tau recruit both types of isoforms. Seeds of 4R tau recruit 4R tau, but not 3R tau, establishing an asymmetric barrier. Conformational templating of 4R tau onto 3R tau seeds eliminates this barrier, giving rise to a new type of tau fibril.

Tau fibrils formed in vitro routinely utilize polyanionic molecules as cofactors to stimulate nucleation. A broad set of negatively charged cofactors, including nucleic acids, polypeptides, and glycosaminoglycans were applied to induce fibril assembly. Utilizing electron paramagnetic resonance (EPR) spectroscopy, we found that the core structure of the fibril is conserved, regardless of cofactor used. Additionally, we assessed whether a cofactor provides a role in tau aggregation beyond inducing the initial
nucleation events, and observed that the presence of a cofactor is needed for fibril propagation to be sustained. The cofactor-fibril interaction was investigated, revealing that cofactors are bound to the fibril and that the basis of the interaction is electrostatic. Cofactor binding is dynamic, as introduction of an alternative cofactor was shown to result in exchange with the bound cofactor.

Protein misfolding cyclic amplification (PMCA) is a tool used for the detection of dilute concentrations of prion fibrils. We have successfully applied this concept towards the amplification of tau fibrils for the first time and demonstrated that fibrils can be amplified, even when diluted by several orders of magnitude.

These findings provide basic mechanistic insights into the seeding, propagation, and diversification of tau fibrils. Importantly, we demonstrate that cofactors are not only of consequence for inducing nucleation events, but decorate the fibril and provide a critical role in fibril propagation.
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<tr>
<td>4R tau</td>
<td>4-repeat tau</td>
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<tr>
<td>3R tau</td>
<td>3-repeat tau</td>
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<tr>
<td>Aβ</td>
<td>beta-amyloid protein</td>
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<tr>
<td>Acrylodan</td>
<td>6-Acryloyl-2-Dimethylaminonaphthalene</td>
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<tr>
<td>Cf</td>
<td>Cofactor</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>EM</td>
<td>Electron microscopy</td>
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<tr>
<td>EPR</td>
<td>Electron paramagnetic resonance</td>
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<tr>
<td>FTDP-17</td>
<td>Frontotemporal dementia and Parkinsonism linked to chromosome 17</td>
</tr>
<tr>
<td>HEPES</td>
<td>(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, sodium salt</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo bases</td>
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<tr>
<td>MTSL</td>
<td>[1-oxy-2,2,5,5-tetramethyl-pyrroline-3-methyl]methanethiosulfonate</td>
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<td>NFTs</td>
<td>Neurofibrillary tangles</td>
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<td>PIPES</td>
<td>piperazine-N,N’-bis(2-ethanesulfonic acid)</td>
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<tr>
<td>PMCA</td>
<td>Protein misfolding cyclic amplification</td>
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<tr>
<td>Poly(A)</td>
<td>Polyadenylic acid</td>
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<tr>
<td>Poly(AU)</td>
<td>Polyadenylic acid-polyuridylic acid double stranded RNA</td>
</tr>
<tr>
<td>PolyGlu</td>
<td>Polyglutamic acid</td>
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<tr>
<td>Poly(U)</td>
<td>Polyuridylic acid</td>
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<td>Abbreviation</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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<td>SDS PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
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<tr>
<td>ThT</td>
<td>Thioflavin T</td>
</tr>
<tr>
<td>Tris</td>
<td>2-Amino-2-hydroxymethyl-propane-1,3-diol</td>
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<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
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Chapter 1: Introduction

1.1 Protein misfolding and amyloids

1.1.1 Misfolding and disease

The 3-dimensional structure of a protein has been shown to be governed by its primary structure \((1, 2)\). While linear strands of most proteins fold into highly structured native states, there are also proteins whose native states are devoid of structure in large regions or the entirety of the protein and are considered to be intrinsically disordered \((3)\). The association of disordered proteins with various neurodegenerative diseases has been well documented, with aggregated forms of the natively disordered protein found accumulated in the form of fibrillar aggregates \((4, 5)\). Among these disordered proteins is the microtubule-associated protein tau \((6, 7)\) that is found deposited as misfolded aggregates in Alzheimer’s disease brain. The appearance of these aggregates has instigated an effort to better understand the structure of aggregates as well as the mechanisms responsible for their initiation and growth.

Protein aggregation is the result of a protein misfolding to a conformation other than its native soluble state. For folded proteins it has been suggested that the main event leading to aggregate formation is the destabilization of the native state, which results in an unfolded intermediate that is then more prone to aggregation \((8, 9)\). As intrinsically disordered proteins already lack secondary or tertiary structure, the process of unfolding
is not required and instead the already unfolded protein can switch more directly to the conformation of the aggregate.

Protein misfolding is a common event that can have deleterious consequences. Within the cell there exist corrective mechanisms to ameliorate the effects of misfolded proteins, namely the recognition and degradation of the potentially harmful conformation through the proteosome (10) or the intervention by the chaperone system that allows a misfolded conformation to fold properly (11, 12). Misfolded proteins that are not remedied can pose serious risks to the cell and provide the basis of any one of a number of diseases referred to as protein conformational disorders (13). How a misfolded conformation can induce a harmful effect is not entirely understood and may vary according to disease and disorder. A general paradigm is that misfolded proteins can contribute to the disease pathogenesis by providing a toxic gain of activity (i.e. the misfolded protein is toxic) or that the harmful effect stems from a protein losing its natural function as a consequence of misfolding. For misfolded conformations that lead to intermolecular associations and aggregation, it is possible that the protein aggregates produce the toxic gain or loss of function that is witnessed in disease (4, 13). Although the causative link of these aggregates to the progression of disease is not conclusive, the appearance of these protein aggregates has warranted extensive investigation nevertheless.

1.1.2 Amyloid structure of aggregates in disease

The deposition of aggregates containing misfolded proteins is observed in numerous neurodegenerative diseases other than Alzheimer’s disease, and includes Parkinson’s disease, and Huntington’s disease, Amyotrophic lateral sclerosis, as well as
the transmissible spongiform encephalopathies (14). Common to all these protein aggregates is a conserved amyloid structure (14) and a fibrillar morphology (15, 16). Structurally characteristic of amyloid fibrils is a conserved cross β-structure, in which fibrils are composed of stacked protein strands hydrogen bonded as β-sheets and aligned perpendicular to the long axis of the fibril (17, 18). Additionally, different amyloids share a general property used for their structural identification, namely the ability to bind and enhance the fluorescence of dyes such as thioflavin T and thioflavin S (19). As to the specific composition of amyloids, the protein involved varies considerably depending on disease. In Alzheimer’s disease, proteins of tau and amyloid-beta (Aβ) are observed as amyloid deposits (20), while in Parkinson’s disease and the spongiform encephalopathies, amyloids are composed of α-synuclein and prion proteins, respectively (14). Despite the potential to form a conserved structure, the proteins found to assemble into amyloids have no general sequence homology, nor any overall resemblance in native structure (14, 21). The general paradigm of amyloid aggregation was that the occurrence was rare and was only specific to a small group of proteins (around 20) that were found to assemble into such structures observed in disease. However, the SH3 domain of phosphatidylinositol 3-kinase, and acylphosphatase—proteins whose aggregation is not observed in disease—were shown to be capable of forming amyloid aggregates under non-physiological conditions (22, 23). Furthermore, myoglobin, a well-structured globular protein that lacks β-sheets in its native state, was induced to form fibrillar aggregates under specific conditions (24). It is believed that almost any protein possesses the capacity to form amyloid fibrils, given the right environmental conditions (23).
1.1.3 Model of amyloid formation

The pathway that describes the conversion of the monomeric protein or polypeptide species into their assembled amyloid counterparts has long been considered crucial for the therapeutic intervention of amyloid diseases. Despite decades of research, however, a comprehensive and generally accepted mechanism that describes the step-wise assembly of amyloid fibrils has remained elusive (25, 26). The course by which intra and intermolecular associations occur towards the polymerization of fibrils proceeds via a nucleation dependant mechanism (27, 28). Observations of the kinetics of a polymerization reaction reveals a long lag phase where little or no assembly is shown to occur, followed by a dramatic acceleration and then gradual plateau of amyloid growth (Figure 1.1). The events related to the lag phase are not fully understood, but are defined by the time required for nucleation to occur. Nucleation of fibrils during the lag phase can be divided into two general processes described as primary or secondary nucleation (29, 30). Primary nucleation involves the conversion of monomer into nuclei that are competent for fibril growth, while secondary nucleation can be attributed to fibril breakage that leads to additional nucleation competent species (more fibril ends) (29-31).

Explanations for the events preceding the formation of competent nuclei range from conformational rearrangements of monomers and the intermolecular assemblies of monomers into oligomeric species (25-27). In the case of oligomer formation it is thought that the successive assembly of monomers occurs until nuclei are formed that are capable of adding monomers to form a fibril. Once the nuclei are formed, the addition of monomers will proceed to elongate fibrils, presumably by the addition of monomer at the ends of the fibrils. As the lag phase leading up to the elongation phase is dependent on
the slow formation of nuclei (29), the introduction of competent nuclei would be expected to bypass the nucleation event and lead directly to the elongation of the fibril. This phenomenon is observed when preformed fibrils are added to monomers as seeds, resulting in the abolishment of the lag phase and the direct induction of fibril growth (27, 28).

**Figure 1.1: Aggregation kinetics of amyloids.** Observed in the aggregation of amyloids is a lag phase which is dependent on the time required for nuclei to form. Nucleation is a prerequisite for fibril growth, and can be circumvented by the addition of fibril seeds.
1.2 Tau and disease

1.2.1 Tau aggregates are found in disease

Alzheimer’s disease is a form of dementia that is estimated to affect up to 24 million people worldwide (32). In Alzheimer’s disease there are two fundamental pathological hallmarks that are observed in the diseased brain. Lesions of aggregated material are found both extracellularly as neuritic plaques and intracellularly as neurofibrillary tangles (NFTs) (20, 33). The chief component of neuritic plaques was identified as amyloid aggregates composed of A\(\beta\) protein (34, 35). Studies focusing on the intracellular NFTs revealed an abundance of fibrils composed of paired helical filaments that were capable of being stained with congo red dye, which like A\(\beta\) suggested an amyloid structure (36). The proteinaceous composition of these NFTs began to be elucidated when it was shown that antibodies to tau were also selective to neurofibrillary tangles (37). Furthermore, antibodies to fibrils are selective to tau and vice versa, suggesting that the fibrils in NFTs contain tau (38, 39). The finding that the protein purified from NFT derived fibrils shared the amino acid sequences found in tau confirmed that the fibrils are in fact composed of tau (40). Importantly, the prevalence of lesions composed of tau is not specific to just Alzheimer’s disease, as tau lesions are found in other neurodegenerative diseases and disorders as well. Combined there are more than 20 diseases besides Alzheimer’s where tau lesions are central to the pathology, termed tauopathies, and include progressive supranuclear palsy, Pick’s disease, coricobasal degeneration, and Down’s syndrome (41, 42).

The protein composition of fibrils has long been known, but what role the fibrils play in disease is not well understood. For instance, in what manner the fibrils are
responsible for the progression of disease has been a matter of debate. Alternative perspectives of the impact of tau fibrils even speculate that the fibrils could provide a protective role by recruiting and therefore reducing species that are toxic to the cell (43).

While the specific role of tau species in disease is not known, the contribution of tau towards disease has been verified by genetic data. In frontotemporal dementia and Parkinsonism linked to chromosome 17 (FTDP-17), mutations in the tau gene have been implicated to disease suggesting a hereditary basis. Additionally, tau gene mutations have been linked to an increase in risk of developing other tauopathies including Pick’s disease and progressive supranuclear palsy (41). Combined, these cases linked to tau gene mutations indicate that tau dysfunction can be attributed to disease. Evidence that links tau fibrils to disease includes the observations that the distribution of NFTs is not random, but can be differentiated into six successive stages known as Braak stages. Also, the advanced Braak stages observed occur with concomitant cases of dementia (44). Further evidence that indicates a relationship between tau fibrils and disease include the observation that the severity of dementia caused by Alzheimer’s disease correlates with the amount of NFTs present (45). These findings have helped to shed light on the disease pathology and suggest that tau fibrils contribute to disease progression.

1.2.2 Tau fibril propagation

Tau fibrils have been associated with the development of disease, and therefore it is important to better understand the mechanisms behind the misfolding of monomeric tau into fibrils. The aggregation of monomeric tau into fibrils was suggested to occur through a nucleated assembly mechanism. With the addition of fibrils formed in vitro or extracted from Alzheimer’s disease brain, monomeric tau can be recruited onto fibril
seeds (46). In another study it was shown that monomeric tau elongated on fibril seeds and adopted the conformation of the seed in a templated mechanism (47). Earlier paradigms regarding the tendency of tau to misfold and form fibrillar aggregates proposed the event to occur autonomously in cells and therefore the effect that fibrils could have on neighboring cells was not thought to be of great importance (48). With recent findings it has been proposed that tau fibril formation may not occur independently within each cell, but that fibrils having nucleated in one cell could propagate and spread to neighboring cells. The ability of fibrils to recruit monomer when introduced to cells was shown by Nonaka et al. when fibrils transfected into cells overexpressing tau resulted in the aggregation of the endogenous monomer (49). Additionally, Frost et al. demonstrated that tau aggregates are capable of being taken up by cells; that intracellular aggregates can transfer between co-cultured cells; and that once cells are transfected with tau aggregates, the misfolded state is propagated in the endogenous tau population (50). In other studies it was shown that cells are capable of internalizing fibrils and that the endogenous tau is recruited as aggregates resembling NFTs (51, 52). When characterizing the tau species that are internalized by cells it was revealed that small aggregates and short fibrils (10-100 µm) are taken up while monomer and larger fibrils are not (53).

Propagation of tau fibrils and the misfolded tau pathology has also been demonstrated in the brains of mice. When insoluble extract containing fibrils from the mouse brain was injected into healthy mouse brain, the healthy mouse’s brain developed a fibrillar tau pathology that spread from the site of injection (54). Similarly, when brain extracts from humans who had died from various tauopathies was injected into mouse
brain, the formation of tau lesions was induced (55). In other mouse models, pathogenic misfolded tau originating in the entorhinal cortex has been shown to spread to neighboring brain regions resulting in the aggregation of the local monomer (56, 57).

From these findings it is clear that the propagation of tau fibrils is not limited to the cell or brain region of origination, but instead tau fibrils are found to spread intercellularly via undefined mechanisms. From cell and animal models it has been well established that misfolded tau in the form of fibrils can exert profound effects when introduced, as the misfolded state of the fibril can be passed onto the endogenous tau population.

1.2.3 Structure of fibrils

Investigation of the structural features of tau aggregates reveals a commonality to amyloid structures, as tau fibrils also possess an ordered cross-β structure. The spacing between the stacked strands perpendicular to the long axis of the fibril occurs every 4.7 Å (58) (Figure 1.2A). Analysis of tau fibrils (Figure 1.2B) by electron microscopy and atomic force microscopy reveal tau fibril structure is actually diverse, with variations observed with respect to fibril width and twist periodicity (59). While atomic resolution structures have yet to be elucidated, studies using in vitro formed fibrils indicate that the β strands of the fibril are arranged parallel to one another with the same residues in different strands stacking in-register (60-62) (Figure 1.2C). The arrangement for how the β-sheets of a fibril pack with respect to one another has been difficult to determine and only recently have experiments been implemented that are capable of providing
distance constraints within a strand. From these distance measurements the first models have been created that provide structural insights into how the β-sheets pack within fibrils (63).

Figure 1.2: **Structure of tau fibrils.** (A) Stacked β-sheets of tau strands are arranged perpendicular to the long axis of the fibril, with 4.7 Å spacing between strands. (B) Electron microscopy image of tau aggregates reveal fibrillar morphology akin to other amyloids. (C) Strands within a β-sheet are arranged parallel with side chains (yellow circles) stacked in-register.
1.2.4 Tau function and properties

The primary function of tau was first elucidated when it was observed that tau was found associated with microtubules, and furthermore that the polymerization of tubulin into microtubules was reliant on this associated protein (64). Later it was revealed that tau provided a role not only for initiating formation, but also for the growth of microtubules through elongation (65). Tau has been shown to bind and support microtubules (66, 67), while also modulating microtubule dynamics, both accelerating the rate of polymerization and inhibiting depolymerization (68).

In determining what region of tau is responsible for the binding of microtubules it was demonstrated that it is actually a relatively small portion of the protein that associates. This binding region consists of semi conserved sequence repeats of around 30 amino acids located towards the carboxyl terminus of the protein (69-71). Using constructs composed of these microtubule binding repeats, it was shown that the repeats alone are capable of aiding tubulin assembly into microtubules (72). Interestingly, the core of the fibrils is located within the microtubule binding repeats (60, 62, 73), with the remaining majority of the protein remaining unstructured and providing a peripheral “fuzzy coat” (74, 75).

The complexity of the study of tau protein is amplified in that there are 6 isoforms expressed in the adult human brain (76). Tau can have either 3 or 4 microtubule binding repeats depending on alternative splicing of exon 10 in the tau gene (77) (the individual microtubule binding repeat sequences along with the largest full tau sequence, htau40, are depicted in Appendix A). Towards the N-terminus of tau there exist one, two, or no insert regions, and between the N-terminal inserts and microtubule binding repeats is a
proline rich region (70). The differences in the number of N-terminal inserts and microtubule binding repeats present within tau are the basis for the variations between the tau 6 isoforms (Figure 1.3).

Figure 1.3: Tau isoforms. Six isoforms are produced in the adult human brain. Variations occur in the N and C termini of the protein. Either 0, 1 or 2 N-terminal inserts can be included (gray hatched), while differences in C-terminus arise from the inclusion of either 3 or 4 microtubule binding repeats (shaded gray). Tau can be described based on the number of microtubule binding repeats as either 3R or 4R tau.

Additional variations in tau also occur due to post-translational modifications including phosphorylation, glycosylation, and proteolysis (78, 79). The phosphorylation of tau is believed to be a key event in the progression of Alzheimer’s disease. In diseased Alzheimer’s brain hyperphosphorylation has been shown to occur at 45 possible sites compared to only 10 sites in normal control brains (80, 81). Additionally, tau is
found phosphorylated in disease brain 3-4 times the extent than tau in normal brain (82). The effects of hyperphosphorylation are not fully understood, but have been shown to cause a decrease in microtubule affinity and a reduced capacity to promote microtubule assembly (83, 84). It has been suggested that when tau is detached from microtubules due to hyperphosphorylation, aggregation is more probable due to the increased tau concentration in the cytosol, which ultimately leads to the formation of fibrils found in disease. This hypothesis is supported by experiments that have shown that hyperphosphorylated tau extracted from Alzheimer’s disease brain is capable of aggregating at low concentrations within a short time-period (85). Also, hyperphosphorylation of tau has been found to occur prior to the earliest detection of any tau aggregation in disease (86). Despite this evidence it has not been substantiated whether phosphorylation is necessary for tau aggregation in disease. Additionally, hyperphosphorylation of tau has even been shown to inhibit aggregation of tau, suggesting a protective role from aggregation (87). Contrary to the proposed opinion that hyperphosphorylation leads to the fibrillization observed in disease, evidence has been shown by recombinant protein that phosphorylation is not a prerequisite for tau aggregation (88, 89). While the phosphorylation of tau is a hallmark of Alzheimer’s disease, the hyperphosphorylation events that have been linked to Alzheimer’s also have been shown to occur during normal embryonic development, suggesting that by itself phosphorylation does not result in aggregation and disease progression (90).

### 1.2.5 In vitro tau fibrils and polyanionic cofactors

The deposition of misfolded tau into ordered fibrillar aggregates is observed in disease, but can also be recapitulated in vitro, as recombinant tau monomer has been
shown to assemble into fibrils (89, 91, 92). The commonality between fibrils found in disease and those formed in vitro is reflected by comparable structural properties. Specifically, fibrils derived from disease and fibrils formed in vitro have both been shown to have a common cross-β structure with 4.7 Å spacing between strands (58). Also, the morphological appearance of in vitro fibrils is indistinguishable from fibrils found in disease (93). While recombinant tau is capable of forming fibrils consistently under relatively simple conditions, as compared to conditions within a cell, an interesting stimulant utilized for the process to occur is the addition of negatively charged cofactors, including the glycosaminoglycan heparin (94). The manner by which a cofactor facilitates the transition of monomeric tau to highly structured aggregates is not fully understood. One general observation is that the cofactor greatly enhances the nucleation process needed for fibril growth to occur. Speculated mechanisms describing how a cofactor might induce aggregation include neutralizing the repulsive electrostatic interactions within tau that inhibit aggregation (95), and binding to multiple tau monomers to increase local concentrations that lead to aggregation (96). Interestingly, hyperphosphorylated tau extracted from Alzheimer’s disease brain is capable of assembling into fibrillar aggregates without the addition of a polyanionic cofactor (85). One explanation for the effect observed from the hyperphosphorylation of tau is that the negative phosphates introduced neutralize the basic side-chains within the microtubule binding region and stimulate aggregation (84).

Polyanionic cofactors bind to tau monomer (97-99) and cofactors can induce secondary structure formation (98) in a normally unstructured monomer. With this in mind, it is possible that the cofactor binding to the monomer allows the monomer to
adopt a conformation that is more amenable to nucleation and growth of fibrils. Considerations beyond the effects on nucleation, is whether the cofactor has any role in the growth or sustainability of the fibril once nucleation has occurred. How the cofactor interacts with the mature fibrils has been difficult to determine, and reports are conflicting on whether the cofactor binds and is therefore included within the fibril (98, 100-102). Questions like these are important for providing a more comprehensive understanding of tau fibrils, as the role of the cofactor could have significant influences on how these fibrils propagate, as well as modulate the conformations of fibrils produced.

When considering the possible roles a cofactor may have on tau fibrils, it is important to recognize the various types of molecules shown to be capable of facilitating aggregation. Sulfated glycosaminoglycans such as heparin (Figure 1.4A), heparan sulfate, and dextran sulfate have been shown to induce fibril formation in vitro (92, 103). Importantly, observations that uncharged glycosaminoglycans do not stimulate fibril formation, and that increases in glycosaminoglycans sulfonation results in increases in fibril formation indicate that the ability of a cofactor to aid in fibril aggregation is dependent on the negative charges of the molecules (103). Incubating tau with other biological polymers such as nucleic acids (104) and polyglutamate (Figure 1.4B) (105) has also been shown to lead to the formation of fibrils. As these polymers contain negatively charged functional groups that are not sulfates (i.e phosphodiesters and carboxylates), the requirements for polymers that could act as cofactors for fibril formation would be expected to be less discriminating and simply require the presence of multiple negative charges. Furthermore, functional cofactors are not limited to negatively charged polymers, as arachidonic acid, phosphatidylserine, and alkyl sulfate
and sulfonate detergents, also induce tau aggregation (106, 107). Interestingly, in these cases the facilitation of aggregation appears to proceed through the formation of micelles and liposomes (107).
Figure 1.4: Heparin and polyglutamate cofactors. (A) Heparin sulfate derived from porcine intestinal mucosa consists of a major disaccharide unit that is homogenously sulfonated and a minor unit that is heterogeneously sulfonated (108). (B) Polyglutamate peptide with varying lengths of glutamate units.
1.3 Description of research

In the work described here, the misfolding of tau monomer into fibrils was investigated. Previously, our group had shown that fibrils can form that are compositionally distinct based upon the number of microtubule binding repeats present in tau. Fibrils composed of 4R tau, 3R tau, or mixed 3R/4R tau all have been shown to have a parallel, in-register arrangement of their β-strands (62). However, whether fibrils are conformationally distinct according to composition was unknown. In these studies we have compared the seeded growth of both 3R and 4R tau upon addition of the different fibril types and observed variations in seeding properties that can be attributed to differences in conformation. In order to assess the seeding properties, we developed an intrinsic assay that utilizes the fluorophore acrylodan as a reporter. From this developed assay we were able to specifically observe the recruitment of tau monomer when fibril seeds were added.

The assembly of fibrils is dependent on the addition of a polyanionic cofactor to aid in the initial nucleation event. Using a diverse set of polyanionic cofactors including nucleic acids and polyglutamate we induced fibril formation and characterized the resulting fibrils by EPR spectroscopy as well as by their seeding properties. Furthermore, the effect of a cofactor beyond the nucleation events was investigated, revealing that cofactors are not only needed for the initiation of nucleation events, but also facilitate the growth, as determined from seeding experiments. When studying the fibril-cofactor interaction we found that the cofactor is incorporated with the fibrils, but can be exchanged when another cofactor is introduced.
In the prion field the amplification of undetectable quantities of prion amyloids is accomplished using a protein misfolding cyclic amplification (PMCA) method (109). As described herein we have applied this amplification method for the first time to tau fibrils. With our applied method we have demonstrated that fibrils diluted several orders of magnitude can be amplified through seeded assembly to levels that are then detectable. This method is expected to be used for the amplification and study of fibrils derived from diseased brain, and could eventually be applied for diagnostic purposes.
Chapter 2: Methods

2.1 Tau constructs

Htau40 and htau23 were cloned in pET-28b plasmids (62). Natural cysteines (in the second and third repeats), two in htau40 and one in htau23, were replaced with serines using site-directed mutagenesis with the QuikChange method from Stratagene/Agilent Technologies. The tau constructs with the natural cysteines replaced are described as “cysteineless” tau. In order to generate constructs containing the microtubule binding repeat regions and excluding the N and C termini, fragments of K18 and K19 (110) were created and cloned into pET-28b plasmids at XhoI and NcoI sites. The introduction of unnatural cysteines to which labels could be attached was accomplished using site-directed mutagenesis. All sequences were verified by DNA sequencing.

2.2 Transformation of tau DNA

Approximately 1 µg of tau DNA (gene in pET-28b plasmids) was added to 20 µl BL21-DE3 bacterial cells (Agilent) in 14 ml polypropylene tubes (BD Biosciences cat. # 352059) and incubated at 4 °C for 30 minutes. Tubes were then submerged in a 42 °C bath for 50 seconds and immediately placed on ice for 2 minutes. 800 µl of NZY+ (10 g/L NZ-amine, 12.5 mM MgCl₂, 12.5 mM MgSO₄, 20 mM glucose) was added to sample. Bacteria was then placed in a shaking incubator at 37 °C for 40 minutes and plated on an agar plate.
2.3 Expression and purification of tau

Tau was expressed in BL21(DE3) competent cells (Agilent). Overnight cultures were prepared by adding a bacterial colony or applying bacteria from a glycerol stock to LB Miller solution and allowing bacteria to grow for 15 hr at 37 °C. Bacteria from overnight cultures (15ml) was added to 1.5 Liters of LB miller (Difco) and cells were grown at 37 °C until the OD$_{600}$ reached 0.8-1.0, after which isopropyl β-D-1-thiogalactopyranoside was added for a final concentration of 1 mM. Cells were further incubated for 3 ½ hours and pelleted at 3,000 g for 15 minutes. Bacterial pellets were resuspended in 500 mM NaCl, 20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), 5 mM ethylenediaminetetraacetic acid (EDTA), 50 mM 2-mercaptoethanol, pH 6.5 buffer and stored at -80 °C until later purification. Samples were heated to 80 °C for 20 minutes, cooled for 5 minutes at 4 °C, lysed by sonication, and centrifuged at 15,000 g for 30 minutes. To the supernatants, a 55% w/v addition of ammonium sulfate was added. Samples were allowed to incubate for 1 hour with gentle rocking, after which samples were centrifuged at 15,000 g for 10 minutes. Pellets were taken up in dH$_2$O with 4 mM dithiothreitol (DTT), sonicated for 40 seconds, syringe filtered, and applied to a Mono S 10/100 GL cation exchange column (GE Healthcare) in 50 mM NaCl, 20 mM PIPES, 500 nM EDTA, pH 6.5 buffer. Tau was eluted with a 50 mM-1 M NaCl gradient and fractions were analyzed by SDS PAGE. Enriched fractions were further purified with a Superdex 200 size exclusion column in buffered solution (100 mM NaCl, 20 mM 2-Amino-2-hydroxymethyl-propane-1,3-diol (Tris), 500 nM EDTA, pH 7.4). Purified tau was pooled and precipitated overnight at 4°C with an added 4-fold volumetric excess of
acetone for constructs K18 and K19 or a 1-fold volumetric addition of methanol for constructs htau23 and htau40, with 5 mM DTT. Precipitated tau was sedimented at 11,000 g for 20 minutes, washed in acetone (K18 and K19) with 2 mM DTT or methanol (htau23 and htau40) with 2 mM DTT and stored at -80°C.

2.4 Acrylodan labeling of tau

Precipitated tau (approximately 5 mg) was dissolved in 8 M guanidine hydrochloride. Acrylodan (Invitrogen) was conjugated to a single introduced cysteine (Figure 2.1) by adding dye in a 10 fold molar excess and incubating in the dark for 1 hr. Unbound acrylodan and guanidine hydrochloride was removed by passing sample through a PD-10 desalting column (GE Healthcare) with 100 mM NaCl, 10 mM (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, sodium salt (HEPES), pH 7.4 buffer. Non-labeled, cysteineless tau was treated identically, with the exception that no acrylodan label was added. Protein concentration of eluted tau was determined by the BCA method (Pierce).

![Acrylodan](image)

**Figure 2.1:** Acrylodan labeling of tau. Mutated cysteine 310 (unless otherwise stated) of tau was conjugated to fluorophore acrylodan.
2.5 Fibril seed preparation

Fibrils were prepared by incubating 25 µM K18 or K19 (or mixture of 12.5 µM K18 and 12.5 µM K19 in the case of mixed fibrils) for 3 days under stirring conditions with the presence of 50 µM heparin (average molecular mass of 5,000 Da, Celsus, Cincinnati, OH). Fibrils were cooled on ice for 15 minutes and 500 µL was sonicated with a microtip for 20 seconds using a Fisher Scientific Sonifier (150 Series).

2.6 Seeded assay and fluorescence detection

Monomeric tau was present at a concentration of 10 µM for K18 and K19, and 20 µM for htau40 and htau23. Monomer consisted of molar fractions of 98% cysteineless tau and 2% acrylodan labeled tau. Acrylodan label was conjugated to a single cysteine positioned at amino acid 310, unless otherwise noted. All reactions were carried out in 100 mM NaCl, 10 mM HEPES, pH 7.4 buffer. Reaction kinetics were initiated with a 2-fold molar addition of heparin and a 3% molar addition of fibril seeds. Temperature was maintained at 37 °C with a solid state Pelletier element. Fluorescence scans were obtained using a Fluorolog 3 system (Horriba, Jobin) fluorometer, with the excitation occurring at 360 nm and the corresponding emission obtained at 400 to 600 nm. Excitation and emission slitwidths were set at 5 nm.

For reactions that involve multiple steps of seeding, tau monomer and seed concentrations were provided at 25 µM and 8% per mol respectively at each step. These concentrations were increased to enhance the rate of the reactions. Reactions were allowed to progress for 1 hr. at 37 °C, and each reaction was sonicated to provide seeds for the subsequent reaction. In the final seeding step of the multi-step procedure 3%
seeds were used with monomer concentrations at 10 µM for K18 and K19 and 20 µM for htau40 and htau23.

2.7 Sedimentation of tau fibrils

After the completion of fibril growth from the seeded reactions, protein aggregates were sedimented for 40 min at > 100,000 g. The pellets were taken up in equal volumes of gel loading buffer and applied onto a 15% SDS-PAGE gel. Proteins were stained with Coomassie Blue and quantified by densitometry.

2.8 Thioflavin T Seeding Assay

Fibril formation was monitored by the change in ThT fluorescence (3) upon ThT binding to tau fibrils. Reactions were carried out in a HEPES buffer (100 mM NaCl, 10 mM HEPES, pH 7.4) at 37°C, with 5 µM ThT. Tau (10µM K18 or K19) was pre-incubated for 5 min at 37°C, whereupon reactions were initiated with 3 mol % of respective seeds. All reactions contained 2-fold molar excess of heparin (average MW = 5000) to monomeric tau. Fluorescence was measured using a Fluorolog 3 spectrofluorometer (Horiba Jobin Yvon), with the excitation wavelength set at 440 nm and the emission wavelength set at 480 nm. The excitation and emission slit widths were 5 nm.

2.9 Recruitment of K19 monomer with ΔK280 K18 seeds

Fibril seeds were formed combining 25 µM tau monomer with 50 µM heparin in buffer (100 mM NaCl, 10mM HEPES, pH 7.4), and allowing reaction to incubate at 25 °C under stirring conditions for 3 days. Fibrils were fragmented by sonicating 500 µL of sample for 20 seconds with a microtip sonicator. For sedimentation experiments a 10%
molar addition of K18 WT or ΔK280 K18 seeds was added to 10 µM K19 WT monomer in buffer with 20 µM heparin, and incubated for 3 hours at 37 °C. Samples were centrifuged at > 100,000 g for 30 minutes at 10 °C. Pellets were resuspended with 40 µL 1X SDS PAGE sample buffer and aggregated tau was assessed by SDS PAGE.

2.10 Electron microscopy

250-mesh carbon coated copper grids were placed on 10 µL drops of the incubated sample for 40 s, 30 s on 10 µL 2% uranyl acetate, and air-dried on filter paper. Samples were imaged with a Philips/FEI Tecnai-12 electron transmission microscope equipped with a Gatan CCD camera.

2.11 Spin-labeling of tau monomer

Purified mutants of K18 and K19 tau containing single cysteines were labeled as shown in (Figure 2.2). Precipitated tau pellets were dissolved in 200-400 µL of 8 M guanidine hydrochloride. An approximate 10-fold molar excess of [1-oxy-2,2,5,5-tetramethyl-pyrroline-3-methyl]methanethiosulfonate (MTSL) spin label (Toronto Research Chemicals, Downsview, Canada) was added to tau and allowed to incubate for at least 1 hr. Tau samples were then purified with a PD-10 column (GE Healthcare) to remove denaturant and unreacted spin label. Concentrations of tau monomers were determined using the BCA method (Thermo Scientific).
2.12 Fibril formation and EPR measurement

Spin-labeled tau was incubated with respective cofactor at room temperature for 3 days under stirring conditions. Fibrils were formed in buffered solution (100 mM NaCl, 10 mM HEPES, pH 7.4) with poly(A) RNA (Sigma P9403) at 70 µg/ml and 35 µM tau. Poly(A) RNA size distribution was determined by agarose gel electrophoresis to be 0.2-2.0 kb. Additional reactions were carried out with poly(U) (Sigma P9528), double stranded poly(AU) (Sigma P1537), transfer RNA (Sigma R8759), and RNA from baker’s yeast (Sigma R650) with concentration ranges of 140-200 µg/ml RNA and 35-50 µM tau. Fibrils were centrifuged at > 100,000 g for 30 min., washed with buffer, and centrifuged for 10 minutes at > 100,000 g. Sedimented fibrils were transferred into capillary tubes with a 0.60 mm inner diameter x 0.84 mm outer diameter (VitroCom CV6084-100). Samples were measured with a Bruker EMX spectrometer equipped with an ER 4119HS resonator. Spectra were obtained with a scan width of 150 G and a microwave power of 12 mW, and normalized according to total number of spins as compared with a double integration. The EPR signal for residual spin-labeled tau monomer, < 0.5%, was subtracted from all spectra.
2.13 Size distribution of RNA

The size distribution of poly(A) RNA was determined using agarose gel electrophoresis. A 1% w/v agarose gel was cast containing 1 µg/ml of ethidium bromide and 600 µg of RNA was loaded. The size range of the RNA was determined using a RiboRuler High Range RNA Ladder (Thermo).

2.14 Kinetics of seeded reactions using RNA polyA

Seeded aggregation kinetics were monitored as previously described using the intrinsic acrylodan based assay. Fibrils were formed for seeded reactions by combining 25 µM tau (K18 or K19) and 50 µg/ml of poly(A) RNA (Sigma P9403) in buffered solution (100 mM NaCl, 10 mM HEPES, pH 7.4) and allowing reactions to proceed for 3 days under stirring conditions. Reactions were chilled on ice for 15 minutes and sonicated for 20 seconds to induce breakage of fibrils. Fragmented fibrils were used as “seeds” to initiate aggregation of monomeric tau. Total monomer concentrations for K18 and K19 were 10 µM, with 98% of monomer containing cysteineless tau (cysteines replaced with serines) and 2% acrylodan labeled tau (acrylodan covalently linked to a cysteine at amino acid 310). Reaction kinetics were initiated with the addition of 20 µg/ml poly(A) RNA and a 10 % (seed to monomer) molar addition of fibril seeds. Kinetics were measured at 37 °C using a Fluorolog 3 fluorometer (Horiba, Jobin). The excitation wavelength was 360 nm and the emission spectra were scanned from 400-600 nm with a 5 nm excitation and emission slitwidth.
2.15 Successive seeding reactions

Multistep seeding reactions were designed with and without the addition of poly(A) RNA as a cofactor. Initial reactions were prepared combining 25 µM K18 WT or K19 WT, 125 µg/ml RNA, and a 10% molar addition of fibril seeds. Seeded reactions proceeded overnight, whereupon 500 µL was removed, sonicated on ice and used for the seeding of the subsequent reaction. Each additional reaction (25 µM tau monomer) was seeded with a 10% molar addition of seeds and was incubated overnight with and without the addition of 125 µg/ml RNA. Fibrils for each reaction were sedimented at >100,000 g for 30 min., pellets were washed with buffer (100 mM NaCl, 10 mM HEPES, pH 7.4) and centrifuged for an additional 10 min. Pellets were taken up in sample buffer and analyzed by SDS PAGE.

2.16 RNA binding to tau and exchange with heparin

Tau fibrils of either K18 WT or K19 WT were formed with the addition of 25 µM monomeric tau, 50 µg/ml poly(A) RNA, and 10% (molar) fibril seeds in buffer (100 mM NaCl, 10 mM HEPES, pH 7.4). Seeded reactions were inverted 3 times and incubated for 2hr at 37 °C. Resulting fibrils were sedimented for 30 min. at >100,000 g. Pellets were dissolved in 60µL 2% w/v SDS and the total volume was brought up to 1250 µL with buffer. To supernatant samples 2% SDS was added and total volume brought to 1250 µL with buffer. RNA contained in pellets and supernatants was determined using a Cary 100 Bio UV-Visible spectrophotometer.

For heparin exchange experiments fibrils were formed as above mentioned and heparin (average molecular weight 5000, Celsus, Cincinnati, OH) was added for a final
concentration of 50 µM. Samples were inverted 5 times and incubated further overnight at 37 °C. Fibrils were sedimented, resuspended, and analyzed by UV-Vis as before. Minor absorbance due to tau protein was subtracted from all spectra.

2.17 Fluorescent heparin binds to fibrils

Fibrils of K18 and K19 were prepared by adding a 10% (molar) of respective seeds to 10 µM monomer and 50 µg/ml RNA poly(A) in 100 mM NaCl, 10 mM HEPES, pH 7.4 buffer. Reactions proceeded for 2 hr at 37 °C. Fluorescent heparin (Invitrogen H7482) was dissolved in PCR certified H₂O (Teknova W3340) and centrifuged at >100,000 g for 10 min and any insoluble material removed. Fluorescent heparin (average molecular weight 18,000) was added to fibrils for a final concentration of 15 µM. Samples were inverted 5 times and incubated overnight at 37 °C. Samples were sedimented at 100,000 g for 30 minutes and the supernatants removed from fibril pellets. Pellets were dissolved with 60 µL 2% SDS. A corresponding concentration of SDS was provided for supernatant samples. Both pellets and supernatants were brought up to the same volume with reaction buffer and samples were analyzed by UV-vis spectroscopy. Absorbance of fluorescent heparin was determined in both pellet and supernatant at 490 nm. RNA present was determined by absorbance at 260 nm with minor contributions from fluorescent heparin being subtracted.

2.18 Heparin binding to fibrils by anisotropy

Binding of fluorescein-conjugated heparin (average molecular weight 18,000, Invitrogen H7482) was monitored using a Fluorlog 3 fluorometer (Horriba, Jobin) with an excitation wavelength of 480 nm and a measured emission of 516 nm. Integration
times were set to 0.1 sec. and the excitation and emission slit widths were set to 4 nm and 8 nm, respectively. Fluorescent heparin alone was measured for 5 min in buffered solution (100 mM NaCl, 10 mM HEPES, pH 7.4). Tau fibrils (25 μM tau, 50 μg/ml poly(A) RNA) were formed as aforementioned and added to heparin for final concentrations of 6 μM tau fibrils (total tau) and 250 nM heparin.

2.19 Aggregation of tau fibrils at high temperature and subsequent cooling

Aggregation reactions were carried out in HEPES buffer (10 mM HEPES, pH 7.4, 0.1 M NaCl or 2.0 M NaCl). Total tau monomer concentrations were 10 μM, with a molar ratio of 98% cysteine-free (cysteines replaced with serines) and 2% acrylodan-labeled tau (position 310 replaced with cysteine and labeled). Heparin (Celsus, Cincinnati, OH) when present was 20 μM. Stirring was utilized in all reactions to promote aggregation. The progression of fibrillization was monitored using a Fluorolog 3 fluorometer (Horriba, Jobin). All reactions were allowed to proceed for 80 min at 343 K. Temperature was adjusted using a solid state Pelletier element. Upon cooling from 343 K to 275 K, samples were allowed to equilibrate and were then measured. Excitation of samples occurred at 360 nm with a 5 nm excitation and emission slit width.

2.20 Inhibition of heparin-mediated fibril growth by high salt

Tau fibrils were prepared by incubating 25 μM K18 or K19 with 50 μM heparin (10 mM HEPES, pH 7.4) for 3 days at 25 °C under stirring conditions. Fibrils were chilled on ice for 10 min and sonicated for 20 seconds to induce breakage. Monomeric K18 and K19 (10 μM; 98% cysteine free tau and 2% acrylodan labeled at position 310) were seeded with a 3% molar addition of sonicated fibrils composed of their respective
construct. Reaction conditions were maintained at 10 mM HEPES, pH 7.4 in the presence of low (0.1 M NaCl) or high (1 M NaCl) salt. Heparin was added in all reactions at 20 µM. Assembly of tau monomers on fibril seeds was monitored by observing the shift in fluorescence maximum of acrylodan label. Excitation of label occurred at 360 nm with 5 nm excitation and emission slit widths.

2.21 PMCA of tau fibrils

All reactions were carried out in Nunclon (237105) 96-well plates (Thermo Scientific) and covered with BioDot Microplate sealing tape (dot scientific #T393). Reactions containing tau monomer were carried out with 10 µM htau40 WT (cysteineless), 40 µM of heparin (Celsus, average MW = 5000), and 5 µM thioflavin T (Sigma T3516) in buffer (100 mM NaCl, 10 mM HEPES, pH 7.4). Fibril seeds were prepared by incubating 1.4 ml of 25 µM K18 WT (or K19 WT) for 3 days or htau40 WT for 6 days in buffer with the presence of 50 µM heparin under stirring conditions at room temperature. For seed dilution experiments, seeds were diluted in buffer with the presence of 40 µM heparin. Fibril seeds (not initially sonicated) were added to reactions prior to incubation or PMCA sequence and are present according to the molar percentage of seed per monomeric tau. After reactions were covered with sealant tape, plates were covered with respective cover, and secured with sealed transfer pipettes to prevent plates from sinking in sonicator bath (see Appendix C). Plates were placed in a water filled microplate horn (Q Sonica #431MPX), coupled to an ultrasonic processor (Q Sonica Q700). Temperature of bath was regulated at 37 °C with a recirculating chiller (Q Sonica #4900). PMCA was programmed to undergo cycles of PMCA with 5 second pulses at
5% amplitude, followed by 30 minutes of incubation at 37 °C (for one cycle). After completion of PMCA cycles, plates were centrifuged at 1,650 g for 2 minutes at room temperature to remove liquid accumulated on cover. Samples were analyzed by fluorescence using a Tecan Infinite M1000 microplate reader. Spectra of samples were obtained using a 440 nm excitation wavelength and scanning the emission from 450 nm – 530 nm with 5 nm excitation and emission bandwidths. Quantification of tau aggregation was determined by thioflavin T emission at 480 nm. For sedimentation experiments same samples were pooled and 1.3 ml was centrifuged for 30 minutes at > 100,000 g at 10 °C. Pellets were washed with 1 ml of buffer and dissolved in 75 µL of 1X SDS PAGE sample buffer and run on SDS PAGE.
3.1 Development of fluorescence assay to monitor 3R and 4R tau seeding

As there are 6 different isoforms of tau present—which include either 3 or 4 microtubule binding repeats—the composition of tau fibrils with respect to isoform can vary. Previously, our group studied tau fibril structure and determined that 3 compositionally distinct fibrils can be formed, which include fibrils composed of 3R tau, 4R tau, and fibrils that have both 3R and 4R tau integrated into the same fibril (62). In order to better understand how microtubule binding repeats could affect fibril propagation, we wanted to devise a methodology that would allow us to monitor the recruitment of monomeric tau as it assembles onto fibrils. To observe this monomer recruitment we formed fibrils of tau using heparin and applied ultrasonic perturbation to shear the fibrils into smaller fragments that could then be used to seed the aggregation of monomeric tau. Constructs K18 and K19 were used that contained the region responsible for forming the core of the fibril and included either 4 or 3 microtubule binding repeats, respectively, while omitting the C and N termini (Figure 3.1). The ability of these constructs to form fibrils had previously been demonstrated (96). To monitor the recruitment of monomer onto seeds we developed a fluorescent-based assay that utilizes the environment sensitive dye acrylodan (111). Acrylodan was cross-linked to a cysteine introduced at amino acid 310 in the third repeat. As the third microtubule binding repeat is within the core of the fibrils (60, 62) it was expected that the acrylodan reporter at this
position would be buried in the hydrophobic region of the fibril which would result in a blue-shift in the fluorescence emission. In all reactions acrylodan labeled tau was mixed with unlabeled cysteineless tau in a 1:50 molar ratio. Sonicated fibril seeds (Figures 3.2A,B) were added to monomer, and recruitment of monomer was monitored by observing the acrylodan fluorescence maximum. Before the introduction of seeds, the fluorescence of the acrylodan attached to monomeric tau (both K18 and K19) was observed to have a maximum of 523 nm, indicative of a site that is exposed to a polar environment (112). This accessibility to the surrounding aqueous environment is not surprising as tau monomer has been shown to be intrinsically disordered (6, 7). Upon a 3% molar addition of K19 seeds to K19 monomer, the acrylodan emission was drastically blue-shifted over time, demonstrating the aggregation of the monomer (Figure 3.2C). The shift in the reciprocal of the \( \lambda_{\text{max}} \) was plotted as a function of time and revealed the elongation kinetics of K19 fibrils (Figure 3.2D). Likewise, K18 seeds added to K18 monomer produced a similar shift in emission that indicated the aggregation of the monomer (Figure 3.2E,F). Importantly, without the addition of seeds no change in emission was observed. This is expected as tau aggregation is characterized by a lag-phase that reflects the time needed for nucleation to occur (46). The concentration of seed used was shown to modulate the seeding efficiency for both K18 and K19 seeding reactions, as increasing the seed concentration from 2% to 8% resulted in accelerated seeding kinetics (Figure 3.3A,B). These combined results demonstrate the recruitment of monomer by fibrils composed of the same protein. Furthermore, we have
demonstrated that our acrylodan assay is capable of monitoring the aggregation of monomer as it is recruited onto fibrils composed of the same protein.

In collaboration with Dr. Ayisha Siddiqua, tau seeding experiments were conducted and irrespective of researcher, identical seeding properties for 3R and 4R tau recruitment were determined (as will be discussed), indicating the robust nature in how these fibrils propagate according to variations in the microtubule binding repeat region.

**Figure 3.1: Truncated constructs K18 and K19.** Constructs K18 and K19 are derived from the microtubule binding repeat region of full-length tau. 4R tau is represented by construct K18 and 3R tau by construct K19.
**Figure 3.2: Intrinsic tau seeding assay.** Electron microscopy images of fibrils of K19 (A) and K19 (B) that were sheared by ultrasonication and used as seeds onto which tau monomer was grown. (C) Addition of 3% K19 seeds to a mixture of monomers of 98% K19 and 2% K19_310A1 (total concentration: 10 μM) results in a shift in the emission maximum from 523 nm to 465 nm. Excitation: 360 nm, Emission: 400 nm – 600 nm. (D) The inverse wavelength of the emission maximum plotted as a function of time reveals the progression of K19-seeded aggregation. In the absence of seeds no aggregation is observed. (E) When K18 seeds (3%) are added to a mixture of K18 monomers (98% K18, 2% K18_310A1) a similar spectral shift as for K19-seeded reactions is observed. (F) The inverse wavelength plotted as a function of time reveals the progression of K18-seeded aggregation. In the absence of seeds no changes are observed. Values represent mean ± s.d. (n = 3 experiments).
3.2 Cross-seeding of 3R and 4R tau

Tau constructs K18 and K19 differ by the presence or absence of the second microtubule binding repeat (Figure 3.1). In order to determine whether this variation in sequence would affect the conformation of fibrils, as determined by seeding properties, we carried out cross-seeding experiments that assessed the capability of 3R to grow on 4R fibril seeds and 4R to grow on 3R fibril seeds. Reactions were performed as previously described by introducing a 50 molar excess of monomeric unlabeled cysteine-free tau to acrylodan labeled tau and adding the respective fibril seeds. While K18 monomers were capable of elongating onto K18 fibrils, K19 monomers were not (Figure 3.4A). The aggregation of self-seeding K18 fibrils was assessed by electron microscopy (EM), which confirmed the growth of long fibrillar aggregates (Figure 3.4B) that were
distinguishable from the short K18 fibril seeds (Figure 3.2B). EM images taken from attempts to grow K19 on K18 fibrils did not reveal any fibrils (Figure 3.4C), concurring with the kinetic data that K19 does not grow on K18 seeds. As K18 was not capable of seeding K19, we anticipated that likewise, K19 fibrils would be unable to cross-seed K18 monomer. Surprisingly, K19 seeds introduced to K18 monomer resulted in the aggregation of monomer (Figure 3.4D). Notably, the growth of K19 monomer onto K19 seeds was more efficient than K18 on K19 seeds. The aggregation of both K18 and K19 monomer was confirmed by EM (Figure 3.4E,F).
Figure 3.4: Asymmetric barrier for K18 and K19 cross-seeding. (A) Aggregation of K18 monomers (98% K18, 2% K18_310A1) and K19 monomers (98% K19, 2% K19_310A1) in the presence of 3% K18 seeds. (B) Electron micrograph of K18 fibrils seeded with K18. (C) Electron micrograph of K19 grown onto K18 seeds. (D) Aggregation of K19 monomers (98% K19, 2% K19_310A1) and K18 monomers (98% K18, 2% K18_310A1) in the presence of 3% K19 seeds. (E) Electron micrograph of K18 grown onto K19 seeds. (F) Electron micrograph of K19 grown onto K19 seeds. Protein concentrations were 10 μM. All values represent mean (s.d. (n = 3 experiments).

By cross-seeding K18 and K19 we observed a barrier that prevents 3R tau from growing onto 4R tau fibrils. To further test this barrier we added K18 seeds to K19 monomer at concentrations 3 and 5 times those first used (Figure 3.5A). Importantly, even at elevated seed concentrations K19 does not grow on K18. To ensure that the inability of K19 to grow on K18 fibrils does not originate from acrylodan fluorophore
being attached at amino acid 310, we introduced the fluorophore at positions 317 and 322 and assessed the ability of K19 to grow on K18 fibrils (Figure 3.5B). As before K19 does not grow on K18 fibrils, which indicates that the barrier observed is not a consequence of labeling the 310 amino acid position. Thus, our acrylodan assay reveals an asymmetry in the cross-seeding of 3R and 4R tau, which allows fibrils of 3R tau to seed 4R tau, but prevents 4R tau fibrils from seeding 3R tau.

![Figure 3.5: Asymmetric barrier for K18 and K19 cross-seeding.](image)

**Figure 3.5: Asymmetric barrier for K18 and K19 cross-seeding.** (A) A robust barrier prevents growth of K19 monomers onto K18 seeds. Circles = 9% seeds, diamonds = 15% seeds. Growth of K18 monomers onto 3% K18 seeds is shown for comparison (triangles). In all samples acrylodan is attached to position 310. (B) K19 aggregation monitored through acrylodan-labeled constructs K19_317A1 (circles) and K19_322A1 (squares) indicates that regardless of which position is labeled, K19 does not grow onto K18 seeds. Growth of K18 onto K18 seeds (triangles) is depicted for comparison. In all cases 3% seeds are added to 10 μM monomers.

### 3.3 Cross-seeding analysis by sedimentation

Using our intrinsically based fluorescent assay as well as electron microscopy we were able to observe an asymmetric barrier between 3R and 4R tau. In order to further validate our findings and provide an additional determination of the degree of
aggregation we employed an assay that would measure the insoluble aggregates obtained through seeded reactions. After monomer was allowed to aggregate with the addition of seed, reactions were given a high speed spin to sediment any resulting fibrils formed. Pelleted fibrils were solubilized and applied on SDS PAGE gels, and the density of the resulting bands was quantified. The fibrils recovered from these sedimentation experiments is in agreement with the results of the acrylodan kinetic assay. K19 monomer was shown to be recruited by K19 fibrils, but not by K18 fibrils (Figure 3.6A). Alternatively, K18 monomer aggregated both in the presence of K18 and K19 seeds, with K18 seeds resulting in considerably more aggregation, consistent with acrylodan kinetics data (Figure 3.6B).

Figure 3.6: Sedimentation of K18 and K19 fibrils. (A) Sedimented fibrils of K19 were analyzed by 15% SDS PAGE (upper panel) and quantified by densitometry (lower panel). (B) Sedimented fibrils of K18 were analyzed by SDS PAGE (upper gel) and quantified by densitometry (lower panel). Protein concentrations were 10 μM. All values represent mean ± s.d. (n = 3 experiments).
3.4 Thioflavin kinetics

The kinetics of cross-seeding 3R and 4R tau was determined using an intrinsic fluorescent based assay. Traditional amyloid determination assays typically utilize extrinsic fluorescent dyes such as thioflavin T (113). Thioflavin T unbound has excitation and emission maxima of 350 and 438 nm, respectively. Upon binding to amyloid fibrils the fluorescence of the dye substantially increases with a concomitant shift in the excitation and emission to 450 and 482 nm, respectively (114). The mechanism responsible for the spectral shifts and increased fluorescence is not fully understood, but appears to occur through the binding of the fluorophore, whose long axis runs parallel to the length of the fibril (113, 115-117). The practicality of using thioflavin T to determine amyloid abundance is well documented. When amyloids of the same protein are assessed under the same designated conditions, the fluorescence intensity of the binding dye is proportional to amount of amyloid available (105, 113, 114, 117). In order to verify the kinetics demonstrating an asymmetric barrier between 3R and 4R tau, we employed thioflavin T as an extrinsic reporter. The kinetics were similar to what was observed using the acrylodan assay, as K18 was able to grow on both K18 and K19 seeds, but K19 only grew on K19 seeds (Figure 3.7A,B). These results further confirm the asymmetric barrier observed in the previous experiments.
Figure 3.7: Thioflavin kinetics of 3R and 4R cross-seeding. (A) Seeded fibril growth monitored with Thioflavin T (5 μM). Changes in fluorescence intensity indicate that K18 monomers grow onto K18 seeds, but that K19 monomers do not. (B) ThT fluorescence reveals growth of K19 monomers onto K19 seeds and reduced growth of K18 monomers onto K19 seeds. In all thioflavin experiments, 3% seeds are added to 10 μM monomers. All values represent mean ± s.d. (n = 3 experiments).

3.5 Seeding full-length tau

All our previous seeding experiments between 3R and 4R tau used truncated constructs K18 and K19 as monomers to be recruited. Although these constructs contain the region that forms the core of the fibril, we wanted to determine whether there were any differences in the seeding properties if full-length tau was recruited. Utilizing monomeric full-length tau of htau40 that contains all 4 microtubule binding repeats and htau23 that only contains 3 repeats (missing the second repeat) we repeated our acrylodan kinetic experiments. Cysteine-free full-length tau was again mixed with a 50-fold excess to acrylodan labeled tau at position 310. Monomer was added to fibril seeds composed of either K18 or K19 and the kinetics observed as before. When K18 seeds were added to htau40 monomer, htau40 was efficiently recruited as evidenced by the aggregation.
kinetics. While an immediate shift in the emission maximum did occur, htau23 did not appear to grow efficiently on K18 seeds (Figure 3.8A). The aggregation of htau40 into fibrils was confirmed by EM (Figure 3.8B). No fibrils were observed when htau23 was used as monomer when EM images were taken (Figure 3.8C). Next, K19 fibrils were used to seed full-length tau monomer. In both htau40 and htau23, monomer was recruited (Figure 3.8D), reaffirming the asymmetric barrier between 3R and 4R tau observed for K18 and K19. To ensure that aggregation occurred for both htau23 and htau40 EM images were taken after the reaction and confirmed the growth of fibrils for both types of reactions (Figure 3.8E,F).

In our kinetic experiments we demonstrate that full-length tau is capable of being seeded by the addition of K18 or K19 fibrils and that the barriers are the same as for the truncated versions. As in reactions where K18 and K19 were grown on fibril seeds, full-length tau is recruited onto the fibril seeds, thus elongating the fibril. To demonstrate that the aggregation of htau40 and htau23 does not originate due to nucleation events occurring within the time-window monitored, we prepared reactions of full-length tau where no seeds were added. Without the addition of seeds, full-length monomer does not aggregate (Figure 3.9), substantiating that the growth of full-length (Figure 3.8) occurs due to recruitment by fibril seeds and not due to nucleation.
Figure 3.8: Templated growth of htau23 and htau40. (A) Aggregation of htau23 monomers (98% htau23, 2% htau23_310A1) in the presence of 8% K19 seeds or 8% K18 seeds. (B) Electron micrograph of K19-seeded reaction. (C) Electron micrograph of K18-seeded reaction. (D) Aggregation of htau40 monomers (98% htau40, 2% htau40_310A1) seeded by addition of 8% K18 seeds or 8% K19 seeds. (E) Electron micrograph of K18-seeded reaction. (F) Electron micrograph of K19-seeded reaction. All values represent mean ± s.d. (n = 3 experiments)
Figure 3.9: Full-length tau monomer does not aggregate without seeds. (A) Kinetics of htau23 without the addition of seeds. (B) Kinetics of htau40 without the addition of seeds. All values represent mean ± s.d. (n = 3 experiments)

As observed in our acrylodan assay, the addition of K18 seeds to htau23 monomer resulted in an immediate shift in the emission maximum (Figure 3.8A). This shift was minor in comparison to htau40 growing on K18 seeds. However, in order to determine whether this shift was the result of aggregation of htau23 we performed sedimentation experiments at the end of the reaction. When htau40 was seeded with K18 a substantial amount of insoluble fibrils were recovered (Figure 3.10A), whereas K18 seeding htau23 resulted in little or no fibrils (Figure 3.10B). Alternatively, when K19 seeded hau40 and htau23 monomer, both full-length proteins were found to have aggregated (Figure 3.10A,B). These basic findings are in agreement with our kinetic and electron microscopy data that indicate an asymmetric barrier allows the cross-seeding of 3R fibrils to recruit 4R tau, but prevents 4R fibrils from recruiting 3R tau. Therefore, it is evident that the seeding properties remain unchanged, regardless of using full-length tau monomer, or monomer composed of solely the microtubule binding repeats.
3.6 Seeding with coassembled fibrils of 3R and 4R tau

Previously, it was shown that 3R and 4R tau are capable of assembling into the same fibril [45]. We formed mixed fibrils of K18 and K19 and assessed the seeding properties. With similar kinetics both K18 and K19 were recruited (Figure 3.11A). Fibrils were confirmed by EM at the end of the reaction (Figure 3.11B,C). These results indicated that fibrils of mixed 3R/4R tau recruit both 3R and 4R tau monomer, and do so with comparable kinetics.
Figure 3.11: Seeds of K18/K19 effectively template growth of K18 and K19. (A) The inverse wavelength plotted as a function of time reveals the progression of K19 and K18 aggregation. Values represent mean ± s.d. (n = 3 experiments). (B, C) Electron micrographs of K18 and K19 fibrils produced through K18/K19-seeded reactions, respectively.

3.7 Cross-seeding 4R on 3R seeds creates new type of 4R tau fibrils

Tau aggregation results in the intermolecular stacking of strands in a parallel and in-register manner; therefore, assembly of fibrils occurs by a template-assisted mechanism in which the assembling monomer would assume the conformation of the fibril (60). As we have observed that 4R tau is capable of being recruited onto 3R tau fibril seeds, the question arose as to whether the resulting elongated fibrils of 4R tau would then be able to seed 3R tau. When growing 4R tau on 3R tau fibrils it would be anticipated that the 4R tau monomer would adopt the conformation of the 3R tau seeds. As such, the resulting 4R tau fibrils would be expected to now be capable of recruiting 3R tau monomer. In order to test our hypothesis, we designed a 3 step seeding experiment. In the first step, K18 was grown on seeds of K19. In the second step K18 (on K19 seeds) fibrils were enriched by carrying out 3 successive seeding and elongation cycles. In the third step the enriched K18 fibrils were used to seed K19 monomers
As was hypothesized, the K18 fibrils that were templated on K19 seeds were capable of seeding K19 monomer (Figure 3.12B, top trace). The effect of the K19 seeds that were used to seed K18 in the first step can be discounted as the concentration of K19 seeds was drastically diluted through the enrichment steps undertaken in the second step of the experiment. The concentration of K19 seeds was diluted by 65,000 fold to bring K19 seeds from a concentration of $2.00 \times 10^{-6}$ M to $3.1 \times 10^{-11}$ M. At this concentration K19 seeds do not accelerate aggregation, and therefore the growth of K19 monomer in the final step cannot be attributed to any K19 seeds carried over during the reaction sequence. In order to verify the aggregation of K19 monomer into fibrils, EM images were taken at the end of the reaction (Figure 3.12C). Importantly, when the multistep seeding procedure was carried out with K18 seeds being used initially (instead of K19 seeds), K19 was not capable of being recruited in the final step (Figure 3.12B, bottom trace). To ensure that the templating experiment would provide the same result had full-length tau been used, htau23 monomer was used in the final step and shown to be recruited (Figure 3.13A, top trace). EM was used to confirm the presence of fibrils (Figure 3.13B). Again, when K18 seeds were used in the initial seeding step no growth of 3R tau monomer was observed (Figure 3.13A, bottom trace). The above described observations are important as they demonstrate that the same protein, such as K18, can assemble into different fibril conformations. The differences in the fibrils produced is dependent on the initial template the monomer is offered. Furthermore, our observations indicate that the seeding properties are propagated over multiple generations of fibril elongation.
Figure 3.12: Tau fibril diversification. (A) Experimental design of multicycle seeding reactions. (B) Aggregation of K19 monomers (98% K19, 2% K19_310A1) in the presence of 3% seeds (last step in A) monitored as a change in inverse emission wavelength over time (top trace). When instead of K19 seeds K18 seeds were present in the initial seeding step, no aggregation occurred (bottom trace). Values represent mean ± s.d. (n = 3 experiments). (C) Electron micrograph representing the end point of the top trace in (B).
Figure 3.13: Tau fibril diversification with full-length tau. A multi-cycle seeding procedure as outlined in Fig. 4A is used with the only difference that seeds (8 mol %) in final reaction are added to htau23 (20 μM) instead of K19. (A) Aggregation of this reaction (98% htau23, 2% htau23_310A1) is monitored as a change in inverse emission wavelength over time (red). When initial seeds in this five-step procedure are K18 and not K19 fibrils, aggregation does not occur (black trace). Values represent mean ± s.d. (n = 3 experiments). (B) Electron micrograph representing the end point of the red trace in A.

3.8 Mutations can affect recruitment of tau monomer by fibril seeds

Our results indicate that 4R tau can assemble into at least two conformations, one of which is capable of recruiting 3R tau. Frost et al has shown that conformational differences in fibrils can occur due to mutations in tau. Specifically, fibrils composed of wild-type tau are conformationally distinct from fibrils containing the disease mutant ΔK280 (47). As we have demonstrated that conformational differences in fibrils can dictate the recruitment of monomer, we wondered whether fibrils formed from tau with the ΔK280 mutation, which are conformationally distinct from wild-type tau fibrils, might recruit monomer differently. To determine this we formed fibrils with ΔK280 K18 tau and assessed whether K19 monomer could be recruited. When a 10% molar addition
of ΔK280 K18 seeds was added to K19 monomer and incubated we found that the K19 monomer was recruited by the seeds, as determined by sedimentation experiments (Figure 3.14A). K19 monomer without the addition of seeds did not sediment, and with the addition of K18 seeds only a marginal amount of K19 was observed in the pellet. Importantly, the ability of K18 ΔK280 to seed K19 is not due to K18 ΔK280 fibrils aggregating more efficiently that K18 WT, as the sedimanted seeds of K18 WT and K18 ΔK280 seeds were found to be of comparable density. Triplicate analysis of sedimentation experiments confirmed the ability of ΔK280 K18 seeds to recruit K19 monomer (Figure 3.14B). Growth of K19 on K18 ΔK280 seeds is furthermore supported by EM images taken after a K18 ΔK280 seeded reaction, revealing an abundance of long fibrils that can be clearly distinguished from the short and fragmented ΔK280 K18 fibril seeds (Figure 3.14C,D). Additionally, we applied our acrylodan assay to assess whether K19 was recruited by ΔK280 seeds. When ΔK280 K18 seeds were added to acrylodan labeled K19 monomer, the acrylodan emission was blue-shifted by approximately 50 nm (Figure 3.14 E), indicating a local environment of increased hydrophobicity that would suggest aggregation had occurred. From these results we demonstrate that a single mutation (in this case a deletion) can alter seeding properties of tau. The effects of mutations on the recruitment of monomer are further being investigated by Virginia Meyer.
Figure 3.14: ΔK280 K18 fibrils recruit K19 monomer. (A) Recruitment of K19 WT by ΔK280 K18 seeds. K19 WT (10µM) was grown on 10% seeds of ΔK280 K18 or K18 WT fibrils with 20 µM heparin. After 3 hours of incubation aggregates were sedimented, dissolved in 2% w/v SDS (to dissociate fibrils), and analyzed by SDS PAGE and (B) quantified according to band density of aggregated K19 monomer. Values represent the mean ± SEM (n = 3). (C) EM image of K19 WT (10 µM) seeded with 10% ΔK280 WT seeds after 3 hours of incubation at 37 °C. (D) EM image of 25 µM or sonicated ΔK280 seeds. Bars = 400 nm. (E) ΔK280 K18 seeds (10%) were added to acrylodan labeled K19 monomer (amino acid 310). Emission is shown before addition and after addition with 2 hours incubation at 37 °C. Excitation = 360 nm.
Chapter 4: Tau cofactors bind and sustain fibril propagation

4.1 Fibril structure determination by EPR spectroscopy

In previous experiments the structural core of tau fibrils had been elucidated by spin-labeling single-cysteines introduced throughout the microtubule binding repeats and measuring the labeled fibrils by EPR spectroscopy (60, 62). In these studies heparin was utilized to initiate the aggregation of the fibrils. As a cofactor such as heparin stimulates aggregation of tau by inducing nucleation, we hypothesized that the nucleation of tau with different cofactors could lead to variations in fibril conformations. Previously, Kampers et al. had shown that RNA was capable of functioning as a cofactor to induce fibril formation (104). We hypothesized that fibrils formed by RNA could have a different structural core than fibrils formed by heparin. In order to compare the structure of fibrils formed with RNA to those with heparin we carried out EPR experiments.

Tau monomer that is spin-labeled with MTSL gives a spectrum with 3 sharp lines that is indicative of high mobility (60) and is in line with tau monomer’s disordered structure. The 3 lines observed are expected for an MTSL labeled sample. Hyperfine splitting gives rise to lines that can be determined based on the equation 2nI + 1, where n = the number of equivalent nuclei and I = nuclear spin. For a radical such as MTSL, nuclei of $^{14}$N give n = 1 and I = 1, and therefore 3 lines are observed (118). Tau fibrils formed with heparin, however, reveal a broad single-line spectrum, indicative of a structured core composed of parallel, in-register β-strands (60). Stacking of labels from
different tau molecules allows spin-exchange, leading to the loss of hyperfine structure \((60, 119)\). Here, we formed spin-labeled tau fibrils with polyadenylic acid \((\text{poly}(A))\) RNA and analyzed the fibrils by continuous wave EPR. Fibrils of K18 or K19 were labeled at 6 different positions within the third microtubule binding repeat. As was observed for fibrils formed with heparin, poly(A) RNA produced fibrils with single-line spectra (Figure 4.1), indicating a parallel, in-register arrangement of \(\beta\)-strands at the third repeat. These findings reveal that even when using an alternative cofactor such as poly(A), the third repeat of tau is highly structured. Next, we assessed whether the ability to form ordered aggregates of tau was limited to RNA, or whether we could also use DNA. Using polydeoxyadenylic acid \((\text{poly}(dA))\) we formed fibrils with spin-labeled tau as before and measured the fibril samples. As was seen for RNA, all 6 sites measured produced single-line spectra for K18 and K19 (Figure 4.2). Using poly(A) and poly(dA) we showed that purine-based nucleic acids were capable of producing highly ordered tau aggregates. In order to determine whether pyrimidine-based nucleic acids could also form fibrils with a highly structured third repeat we applied polyuridylic acid \((\text{poly}(U))\) to spin-labeled tau (position 310) and again observed single-line spectra for both K18 and K19 (Figure 4.3A). Furthermore, we characterized the fibrils formed using double-stranded polyuridylic and polyadenylic acids \((\text{poly}(AU))\), transfer RNA extracted from yeast \((\text{tRNA})\), and total RNA from yeast. All of these RNA species produced fibrils with single-line spectra (Figure 4.3A). As we had demonstrated that a glycosaminoglycan (heparin) and a broad class of nucleic acids effectively promoted highly ordered aggregation of tau, we next tested whether polyglutamate \((\text{poly}(Glu))\) would also induce
the formation of highly ordered fibrils. The application of polyglutamate had previously been shown to promote fibril assembly (105). As was shown for heparin and nucleic acids, polyglutamate produces fibrils from spin-labeled monomer that provided single-line spectra (Figure 4.3B). Combined, these data reveal that the parallel, in-register structure of tau fibrils is conserved regardless of cofactor used.

**Figure 4.1: Spectra of spin-labeled tau fibrils using poly(A) RNA.** Polyadenylic acid poly(A) was used to induce the aggregation of spin-labeled monomer into fibrils. Spin-labels were attached at 6 different sites (conjugated to introduced cysteines). Fibrils were analyzed by EPR and single-line spectra were found to occur at each of the 6 positions within the third microtubule binding repeat for fibrils composed of both K18 and K19 tau.
Figure 4.2: DNA produce ordered fibrils. Polydeoxyadenylic acid poly(dA) was used to induced the aggregation of spin-labeled monomer into fibrils. Spin-labels were attached at 6 different (conjugated to introduced cysteines). Fibrils were analyzed by EPR and single-line spectra were found to occur at each of the 6 positions within the third microtubule binding repeat for fibrils composed of both K18 and K19 tau.
Figure 4.3: Nucleic acids and polyglutamate produce ordered fibrils. (A) Polyuridylic acid (poly(U)), double-stranded polyuridylic and polyadenylic acids, transfer RNA (tRNA) and total RNA extracted from yeast and (B), polyglutamate (polyGlu) produce fibrils that are highly ordered as indicated from single-line spectra obtained from spin-labeled tau.
4.2 Seeding properties of tau using RNA as a cofactor

Previously when the seeding properties of tau fibrils were studied, we had utilized heparin as a cofactor to initiate nucleation (120). Our findings showed that an asymmetric barrier exists between fibrils of 3R and 4R tau (see Chapter 3), and that conformational variability can exist in fibrils composed of the same isoform. Although, parallel in-registry is preserved, fibrils formed in the presence of different cofactors could have different conformations. We next wanted to test whether cofactors affect seeding properties of tau fibrils, as differences in the seeding properties would indicate changes in fibril conformation. Using poly(A) RNA as a cofactor we formed fibrils of K18 or K19 and assessed these fibrils’ competency to recruit K18 and K19 monomer. When using K18 fibrils as seeds we observed the efficient recruitment of K18 monomer, but not K19 monomer (Figure 4.4A). When applying K19 fibrils to monomer, the recruitment of both K18 and K19 monomer was observed (Figure 4.4B). However, the cross-seeding of K18 with K19 seeds was far less efficient than K19 seeding K19. From these results we see that the asymmetric barrier between 3R and 4R tau is recapitulated when applying nucleic acids for aggregation. Importantly, when K18 and K19 are incubated with poly(A) RNA, but with no seeds, aggregation does not occur (Figure 4.5), indicating that RNA alone was not responsible for tau aggregation observed in the seeding experiments. The seeding experiments presented here indicate the robust nature of the seeding properties of tau, as the asymmetric barrier was observed even when using an alternative cofactor.
Figure 4.4: Seeding properties of 4R and 3R tau using poly(A) RNA. Seeding of K18 and K19 monomers with a 10% molar addition of (A) K18 seeds or (B) K19 seeds. Total monomer concentrations were 10 µM (98% WT, 2% acrylodan labeled_310A1), with 20 µg/ml of poly(A). All values represent mean ± s.d. (n = 3 experiments).
Figure 4.5: Aggregation does not proceed without addition of seeds. Acrylodan assay of the kinetics of K18 and K19 (10 µM) fibril formation with the addition of poly(A) RNA, but without seeds. All values represent mean ± s.d. (n = 3 experiments).

4.3 Size distribution of poly(A) RNA

Based on our EPR measurements and seeded reactions poly(A) RNA is capable of producing tau fibrils. What sizes of poly(A) were used to facilitate tau aggregation was unknown. As we utilized poly(A) extensively in previous and subsequent experiments, we wanted to characterize the size distribution present in the poly(A) sample. Poly(A) RNA purchased from Sigma had previously been shown to contain broad length distributions of 0.2-6.0 kb (121). To determine the size distribution of the poly(A) RNA used for our experiments we performed agarose gel electrophoresis with ethidium bromide staining. From the agarose gel we found that the predominant distribution of poly(A) RNA ranged from 0.2-2.0 kb (Figure 4.6). This indicates that the poly(A) RNA species was not a defined size, but instead was composed of a relatively broad length range.
4.4 Cofactors sustain growth of tau fibrils

The aggregation of tau monomer into fibrils has been shown to proceed through a nucleation-dependant mechanism (46). From our seeding experiments we have also shown that preformed fibrils added to monomer abolish the lag-phase attributed to nucleation, which also supports a nucleation-dependent mechanism for fibril formation. It was previously suggested that cofactors are only needed for the initiation of nucleation, and are not required for the subsequent elongation of the fibril (102). If this model is
valid then once the nucleation event is initiated by the cofactor, the presence of the cofactor should no longer be necessary for the fibril growth through elongation. To test whether the cofactor was needed for the propagation of the fibrils we created an experiment where multiple seeding steps were carried out (Figure 4.7A). The sequence of the experiment consisted of five successive seeded reactions (cycles 1-5), whereby fibril seeds from the previous reaction were used to seed the aggregation reaction. For our cofactor we again utilized poly(A) RNA. In one set of reaction cycles poly(A) RNA was included (+cf), while in another set of reaction cycles poly(A) RNA was only included in the first reaction (-cf). At the end of each reaction cycle the fibrils were sedimented and run on an SDS PAGE gel. For reactions using either K18 or K19 the addition of poly(A) RNA in each of the five reaction cycles resulted in the continued propagation of fibrils (Figure 4.7B, left panel). When the poly(A) RNA was omitted after the first reaction cycle the fibril propagation was reduced and eventually no fibrils were found to have sedimented (Figure 4.7B, right panel). These results clearly demonstrate that cofactor was needed for the fibril propagation to be sustained.
Figure 4.7: Cofactor sustains propagation of tau fibrils. (A) Multi-step reaction sequence for the propagation of tau fibrils. Tau fibrils are propagated by the successive seeding of fibrils from one reaction to another. In all reaction cycles tau monomer is present (25 µM) and the fibrils from the previous reaction are used as seeds to recruit available monomer. In one reaction sequence, (+cf), poly(A) RNA cofactor is provided in all 5 reaction cycles, whereas in another reaction sequence, (-cf), poly(A) cofactor is only provided in the first reaction cycle. (B) SDS PAGE gels of sedimented fibrils from the experiment using either K18 or K19. The sedimented fibrils from each reaction cycle are shown with poly(A) RNA in all reactions (left panel), or with poly(A) RNA only included in the initial reaction (right panel).

In the multi-step seeding experiment we observed a strong dependence of the propagation of the fibrils with the addition of cofactor. In previous kinetic experiments using heparin as a cofactor (120), and the above described seeding experiments using poly(A) RNA as a cofactor (Figure 4.4), we had always included the cofactor with
monomer, to which seeds were added. Based on the results from the multi-step seeding experiment (Figure 4.7), we would expect that when conducting a seeded reaction that there would be a significant difference in the seeding efficiency depending on whether a cofactor was included. Using poly(A) RNA as a cofactor we carried out seeding experiments with and without the addition of cofactor (Figure 4.8). Our kinetics data reflected the multi-step seeding experiment, showing for both K18 and K19 that without the addition of cofactor the fibril propagation is dramatically reduced. Furthermore, as nucleation of fibrils was shown not to occur in the time-period observed (Figure 4.5), the growth induced by the addition of seeds can be fully attributed to the elongation of the fibril seeds and not due to the formation of new fibrils through nucleation.

Figure 4.8: Cofactor is needed for elongation of fibrils. Acrylodan kinetics of seeded aggregation of K18 (A), or K19 (B) on respective seeds (10 % by mole). Reactions were carried out with (20 µg/ml) or without the addition of poly(A) RNA. All values represent mean ± s.d. (n = 3 experiments).
4.5 Tau-cofactor interaction is electrostatic

By observing the effect of the cofactor on seeding efficiency we had observed a strong dependence of fibril propagation on the presence of cofactor. It could be expected that as the core of the fibrils contain a largely basic region, that a polyanionic cofactor would facilitate fibril growth through electrostatic interactions with the fibril core. To demonstrate that interactions between two species occur via electrostatics, the application of high salt can be used (122, 123). When binding occurs through oppositely charged surfaces the conditions of high ionic strength through the introduction of high salt is seen to be capable of abolishing these electrostatic interactions. If the negatively charged cofactor facilitated fibril growth through oppositely charged electrostatic interactions with tau monomer or fibril, then it would be expected that here also elevating the ionic strength would reduce the fibril assembly. We observed the self-seeding kinetics of K18 and K19 fibrils (K18 fibrils seeding K18 and K19 fibrils seeding K19) in the presence of low (0.1 M NaCl) and high (0.5 or 1.0 M NaCl) salt. When using poly(A) RNA as a cofactor low salt conditions allowed for the seeded growth of K18 and K19 fibrils, whereas when the concentration of salt was elevated the fibril growth was abolished (Figures 4.9A,B). To see whether the inhibitory effect of high salt was also reflected when using a different cofactor we carried out the experiments using heparin instead. As was observed for poly(A) RNA the introduction of high salt inhibited the fibril growth (Figure 4.9C,D). These experiments indicate that the cofactor interacts with tau during the elongation of the fibril and that the interactions are electrostatic.
Figure 4.9: High salt concentrations inhibit fibril elongation. Acrylodan kinetics of seeded-aggregation with conditions of low and high salt. (A) Growth of K18 monomer on K18 seeds with 20 µg/ml RNA, and (B) growth of K19 monomer on K19 seeds with 20 µg/ml RNA. (C) K18 self-seeded growth with 20 µM heparin (D) K19 self-seeded growth with 20 µM heparin. All tau concentrations were 10 µM. All values represent mean ± s.d. (n = 3 experiments).

4.6 Cold dissociation of tau fibrils

Computational studies with our collaborators have indicated that at high temperature (340 K) β-sheet-strand probability reaches a maximum (124). The temperatures used for our aggregation reactions ranged from 25 °C to 37 °C. In all of our experiments thus far we have provided cofactor for the nucleation of fibrils. Whether
fibrils could be formed at higher temperatures conducive to β-sheet-strand probability (340 K) without the presence of a cofactor was not known. Using our acrylodan assay we monitored the fibril formation with and without the addition of heparin at 343 K. With the addition of heparin the aggregation was rapid, while without any cofactor the reaction proceeded slowly (Figure 4.10A). At the end of the reactions sample was mounted on EM grids. EM images revealed fibrils formed with and without heparin. However, fibrils formed without heparin appeared to have disintegrated (Figure 4.10B). We reasoned that this disintegration could have originated from the temperature change as the fibrils cooled during the mounting procedure carried out at room temperature. To test the temperature effects on tau fibril integrity we monitored changes in the emission spectra. The acrylodan fluorescence blue-shifted during amyloid formation at 343K with and without heparin (although to a lesser extent) (Figure 4.10C, D). After heating, the K18 solution was cooled to 275 K. When heparin was added, cooling to 275 K resulted in an additional blue-shift in the acrylodan fluorescence, which could be attributed to a further maturation of the fibrils (Figure 4.10C). However, when K18 fibrils without heparin were cooled to 275 K the main peak of acrylodan fluorescence red-shifted back to the original monomeric position (Figure 4.10D). Meanwhile, the shoulder region between 400 and 450 nm increased, possibly indicating an increase in soluble oligomers. These experiments indicate that K18 can form fibrils without heparin at high temperatures, and when cooled the fibrils cold dissociate. Fibrils formed with cofactor do not cold-dissociate suggesting that the heparin provides a stabilization role in preventing dissociation.
Figure 4.10: Heparin locks the tau protein K18 fibril and prevents its reversion. (A) Kinetics of fibril formation at 343 K in the presence (blue) and absence (red) of heparin, as determined by the shift of the emission maximum of acrylodan labeled tau. Values represent mean ± s.d. (n = 3 experiments). (B) EM images of fibrils formed at 343 K in the presence (left panel) and absence (right panels) of heparin. Two images are shown for fibrils formed without heparin. Bar = 200 nm. (C and D) Representative spectra of aggregation reactions in the presence and absence of heparin, respectively. Spectra of monomeric tau (green), after aggregation of fibrils at 343 K (blue), and when cooled to 275 K (purple). Protein concentration = 10 µM. NaCl concentration = 0.1 M. The data indicate that heparin prevents tau fibrils from dissociating in the cold.
4.7 Cofactor binds to fibrils

The cold dissociation experiments indicated that the introduction of a cofactor prevents fibril dissociation when cooled. If the cofactor provides a stabilizing role it would be likely that the cofactor binds to the fibril. To assess cofactor binding to fibrils we utilized poly(A) RNA as a cofactor for fibril formation. After forming the fibrils with the RNA the insoluble fibrils were sedimented and pelleted fibrils were separated from supernatants. The pellets were dissolved in 2% w/v SDS (to dissociate fibrils) and adjusted to the same volume as the supernatant with buffer and 2% w/v SDS. As RNA absorbs strongly in the UV region, we utilized UV-Vis spectroscopy to determine whether RNA was associated with the fibrils (pellet) or whether it did not bind (supernatant). Absorbance contributions due to tau protein were negligible, as we utilized truncated constructs that contained only 1 tyrosine and no tryptophan residues. Spectra for the analysis of RNA bound to K18 revealed that a majority of the RNA was contained in the pellet, indicating that RNA binds to tau fibrils (Figure 4.11A). The total RNA concentration used for these reactions was also analyzed and was found to be approximately equal to the sum of the RNA in the pellet and supernatant. Triplicates confirmed the binding of RNA to K18 fibrils (Figure 4.11B). For K19 fibrils we also observed that a majority of the RNA provided is bound to the fibrils (Figure 4.11C,D). These findings indicate that the cofactor-tau interaction is not limited to monomer-tau during nucleation, but instead the cofactor binds and is a constituent of the fibrils.
Figure 4.11: RNA binds to tau fibrils. Tau fibrils were prepared incubating 25 µM tau with 50 µg/ml poly(A) RNA. Sedimented samples were analyzed for poly(A) RNA contained in the pellet and supernatant. The pellet was dissolved and fibrils dissociated using 2% w/v SDS. Spectra for RNA contained in pellet and supernatant, and total RNA used, for (A) K18 fibrils and (B) K19 fibrils. Absorbance of RNA for triplicate samples for K18 (B) and K19 (D) fibrils. Values for (B) and (D) represent mean ± s.d. (n = 3 experiments).
4.8 Cofactors can exchange on the fibril

4.8.1 Cofactor exchange by sedimentation analysis

In the case of poly(A) RNA we have shown that the cofactor binds to the fibril. We next wanted to determine whether once bound, if a cofactor was capable of being removed through the addition and exchange of another cofactor, or whether the cofactor binding was more permanent. To determine this we formed fibrils with poly(A) RNA and added an excess of heparin (50 µM) to see whether the heparin was capable of exchanging with the RNA. When analyzed by UV-Vis we found that the heparin had substantially removed the poly(A) RNA, as the majority of the RNA was now found in the supernatant. The poly(A) RNA removal was observed for fibrils of either K18 (Figure 4.12A) or K19 (Figure 4.12D). We next wanted to determine whether the removal of RNA through the addition of heparin had any effect on the integrity of the fibril. If heparin were removing RNA, possibly through replacement, it is possible that structural disturbances or general change in aggregate morphology would be observed. By EM we analyzed fibrils of K18 before (Figure 4.12B) and after (Figure 4.12C) the addition of heparin and found that the integrity of the tau aggregates was still upheld, with no changes in the fibrillar morphology detected. The same structural adherence was also observed for fibrils of K19 with and without the addition of heparin (Figure 4.12 E,F).
Figure 4.12: Removal of RNA by the addition of heparin. The removal of RNA was determined after the addition of heparin for K18 (A) and K19 (B) fibrils. Poly(A) RNA found in the supernatant was analyzed by UV absorbance at 260 nm. All values in represent mean ± s.d. (n = 3 experiments). EM images were taken of sample before and after the addition of heparin. For K18 fibrils, (B), before and (C) after heparin addition. For K19 fibrils, (E) before, and (F) after addition of heparin. Bar = 400 nm.

The addition of heparin to fibrils formed with poly(A) RNA resulted in the dissociation of RNA from the fibrils. It could be assumed that the heparin removal occurred via the exchange of heparin bound to the fibril. However, when heparin was added to remove the RNA (Figure 4.12), it was not clear whether the heparin was binding and taking the place of the RNA. To test whether heparin exchanges with RNA on the
fibril we utilized heparin conjugated with fluorescein. When fluorescent heparin (15 µM) was added to fibrils formed with RNA, the RNA was shown again to be removed (Figure 4.13A,B). The fluorescent heparin was also accounted for in the pellet and supernatant samples, with the majority of the heparin found to be in the pellet (Figure 4.13C,D). For both K18 and K19 fibrils the addition of heparin therefore resulted in the removal of the RNA from the fibrils and the binding of the heparin. From these sedimentation experiments it is clear that heparin added to the fibrils exchanges with the RNA.
Figure 4.13: Exchange of RNA for heparin. Fluorescein-conjugated heparin was added to poly(A) RNA/tau fibrils. RNA in the pellet and supernatant after addition of heparin was assessed by Abs 260 nm, for (A) K18 and (B) K19 fibrils. After exchange heparin bound to fibrils was determined by heparin found in the pellet and supernatant (Abs at 490 nm) for (C) K18 and (D) K19 fibrils. All values represent mean ± s.d. (n = 3 experiments).

4.8.2 Cofactor exchange by anisotropy analysis

To determine the binding of heparin by an alternative assay we devised a fluorescence anisotropy experiment. The binding of heparin in solution to large fibrils would be expected to reduce the mobility of the heparin, which could be observed as a decrease in the rate of tumbling, resulting in an increase in the fluorescence anisotropy.
When the heparin fluorophore is excited with vertically polarized light the anisotropy $\langle r \rangle$ can be determined by measuring the intensities of the vertical ($I_{VV}$) and horizontal ($I_{VH}$) emissions of the fluorophore and applying the equation

$$\langle r \rangle = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}}$$

(1)

Polarization biases introduced by the instrument—for example due to biases in monochromators to transmit vertical versus horizontal emissions—can be accounted for by applying the $G$ factor (125-127), or grating factor. The determination of the G factor requires the excitation of the fluorophore with horizontally polarized light and the measurement of the resulting vertical ($I_{HV}$) and horizontal ($I_{HH}$) emissions. The G factor is described as

$$G = \frac{I_{HV}}{I_{HH}}$$

(2)

Applying $G$ factor to anisotropy gives

$$\langle r \rangle = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}}$$

(3)

We measured the anisotropy of fluorescent heparin for three-hundred seconds and added poly(A) RNA/tau fibrils and observed the change in anisotropy. Both fibrils of K18 and K19 caused large increases in the anisotropy (Figure 4.14A,B) indicating the binding of the heparin to the RNA fibrils. In studies focusing on the interaction between tau and cofactors, it was shown that cofactors bind to tau monomer (97, 98). To ensure that the
large increases in anisotropy observed when adding fibrils to heparin were not caused by binding to tau monomers contained in the fibril mixture, we also performed experiments where tau monomer alone was added to fluorescent heparin. Conservatively, we added the same concentration of tau monomer that our fibrils contained. When monomer was added to K18 fibrils an increase in the anisotropy occurred (Figure 4.14C), but was marginal compared to adding fibrils to heparin. When adding the same concentration of K19 monomer to the heparin the anisotropy increased (Figure 4.14D), but was significantly less than the anisotropy change when K18 monomer was introduced.

Together our results show that cofactors are incorporated into tau fibrils. Furthermore, the binding of a cofactor to the fibril is not a static event, as we have shown that cofactor exchange on the fibril can occur. These findings are important as they indicate that the cofactor is not only important for nucleation and growth, but is also a component of the fibril.
Figure 4.14: Heparin binds to tau fibrils as shown by fluorescence anisotropy. The fluorescence anisotropy of fluorescein-conjugated heparin was monitored before and after the addition of tau fibrils. (A) Addition of K18 poly(A) RNA fibrils after 300 seconds. (B) Addition of K19 poly(A) RNA fibrils after 300 seconds. In control experiments tau monomers were added to heparin, (C) K18 and (D) K19. Final concentrations of heparin and tau fibrils were 250 nM and 6 µM, respectively. All values represent mean ± s.d. (n = 3 experiments).
Chapter Five: Amplification of tau fibrils using PMCA

5.1 Seeding efficiency is dependent on seed concentration

From our seeding experiments we have shown that we can rapidly propagate tau fibrils when providing tau monomer and cofactor for recruitment. The efficiency of monomer recruitment is dependent on the concentration of fibril seeds introduced. When seeding K18 and K19 monomer with respective fibrils, we observed an acceleration in the reaction kinetics when increasing the concentration of fibril seeds (Figure 3.3). In order to further assess the efficiency of aggregation we applied a broad set of seed concentrations and monitored the recruitment of monomer by thioflavin T fluorescence. Htau40 monomer was seeded with non-sonicated fibril seeds of K18 WT, with molar seed concentrations ranging from 0.1% to 8% seed (Figure 5.1). After the addition of seeds the reactions were sonicated for 5 seconds (in bath) and after 6 hours of incubation the efficiency of the reactions was determined. Our data indicate that the efficiency of aggregation drastically decreases with decreasing seed concentration. After accounting for fluorescence intensity contributions of introduced seeds we observed that the seeding capacity was limited at 0.1% seeds. At 0.1% seeds the aggregation of monomer was nearly indistinguishable from samples without seeds (monomer only controls).
Figure 5.1: Seeding of tau monomer with varying seed concentrations. Various concentrations of K18 WT seeds were added to recruit monomeric tau. Htau40 WT monomer (10 µM) was incubated with seeds and 5 µM thioflavin T (ThT) for 6 hr. Fluorescence emission was measured at 480 nm, with an excitation of 440 nm. Heparin was included in all reactions at concentrations of 40 µM, except for samples of “ThT only” (Thioflavin T only). All values represent mean ± SEM (n = 4 experiments).

5.2 Fibril amplification concept

We have shown that small amounts of fibril seeds added to monomeric tau are capable of recruiting the monomer into the fibril. However, when seed concentrations are too low the seeding capacity is lost and the conversion of monomer to fibril does not
occur within the time period-frame of the experiment. In the prion field a protein misfolding cyclic amplification (PMCA) assay that amplifies minute concentrations of misfolded prion amyloid has been developed (109). The basis for the assay includes the alternation of fibril growth and breakage cycles that are applied to amplify undetectable concentrations of fibrils. As we have also witnessed a limitation in the seeding capacity at a lowered seed concentration (0.1%) for tau fibrils, we asked whether the principles of PMCA could be applied to amplify tau fibrils previously undetectable. We designed an experimental setup to amplify tau fibrils that utilizes a similar approach as is used for prion amplification. The sequence includes the introduction of minute concentrations of tau fibrils as seeds to monomer, and the subsequent sonication and incubation to amplify the fibrils. The sonication is applied to break the fibrils and create additional fibril ends, onto which monomeric tau can grow. The prolonged incubation phase allows for the time needed for the elongation of the fibrils to occur through the recruitment of monomer. As a result of multiple cycles of breakage and elongation (Figure 5.2), an initially low and undetectable fibril seed concentration can be amplified through propagation to a fibril concentration that is then detectable.
Figure 5.2: PMCA applied to tau fibrils. Tau fibril seeds (blue arrows) are introduced to tau monomer. Initial fibrils are propagating by undergoing alternating cycles of growth (incubation) and breakage (applied sonication). At the end of multiple cycles of PMCA, an initially low concentration of fibril seeds has been amplified.
5.3 Fibril amplification of tau fibrils

In collaboration with Virginia Meyer, the PMCA concept was applied to tau fibrils. To test the PMCA concept on the amplification of tau fibrils we added K18 fibril seeds (initially unsonicated) at a concentration of 0.1% to htau40 WT monomer. This concentration was chosen because a seeded reaction after 6 hours was shown to result in a minimal amount of monomer aggregation (Figure 5.1). As before, we used the thioflavin T assay for the determination of fibrillar aggregation. The seeded reactions underwent 12 cycles of PMCA, with a 5 second sonication pulse, followed by a 30 minute incubation time, giving a total elapsed time of 6 hours. At 4 cycles, 8 cycles and 12 cycles samples were measured by fluorescence (Figure 5.3A), which showed that with successive cycles the aggregation of monomer increased. Importantly, to rule out the occurrence of monomer aggregation through nucleation, we included a monomer control that contained no added seeds (monomeric tau was ensured by applying chemical denaturant) and was carried out through the 12 cycles of PMCA. As this monomer control did not aggregate, it is evident that the aggregation enhancement imparted by PMCA cycles is due to the amplification of the initial fibril seed added. Additionally, thioflavin T alone after 12 cycles of PMCA did not result in an increased fluorescence intensity (Figure 5.3A).

To compare seeded reactions with PMCA reactions we sonicated the seeded reaction for 5 seconds and measured the growth after 6 hours of incubation—the same incubation time elapsed after 12 cycles of PMCA. As was expected a single pulse of sonication was not sufficient to effectively promote the aggregation of the monomer
(Figure 5.3B). The proposed mechanism of propagation by which PMCA amplifies a dilute concentration of fibril seed is suggested to occur not due to the total time the reaction undergoes sonication, but due to the periodic pulses of sonication (breakage) that are spaced between steps incubation (growth). To test whether the periodicity of sonication was important for fibril amplification we applied an initial sonication pulse of 60 seconds (total sonication time for 12 cycles of PMCA) to the seeded reaction and allowed the reaction to incubate for 6 hours. As was expected the reaction did not result in a substantial amount of aggregation of the monomer (Figure 5.3B), in agreement with the idea that PMCA functions to promote fibril amplification due to applied cycles of sonication and incubation. After the reactions were completed identical samples were pooled, sedimented and analyzed by SDS PAGE to verify the findings observed using the thioflavin T assay. As was observed using the thioflavin T fluorescence assay, seeded reactions undergoing 12 cycles of PMCA resulted in the aggregation of the monomer. Control samples, to which no seeds were added, did not result in the aggregation of monomer after 12 PMCA cycles. When applying 5 second and 60 second pulses to seeded samples and incubating for 6 hours, there was a minimal amount of aggregated monomer, which is consistent with the marginal fluorescence increases observed with the thioflavin T assay. Together, the thioflavin T fluorescence assay and fibril sedimentation experiments reveal the efficacy of applying PMCA to amplify tau fibrils.
**Figure 5.3: PMCA applied to tau fibrils.** Htau40 WT (10 µM) underwent PMCA with the addition of 0.1% K18 WT seeds with 40 µM heparin. (A) ThT fluorescence of htau40 WT with no seeds (12 PMCA cycles) and 4, 8, and 12 PMCA cycles with the addition of 0.1% seeds. ThT was present in all reactions at 5 µM. (B) Samples were sonicated continuously for 5 seconds (duration of 1 sonication step of PMCA) and 60 seconds (duration of combined 12 cycles of PMCA sonication), and then incubated for 6 hours. Samples undergoing 12 cycles, with and without seed addition are shown for comparison. (C) SDS PAGE of sedimented fibrils at the end of 12 PMCA cycles (or 6 hours). All values represent mean ± SEM (n = 8 experiments).
5.4 Sensitivity of PMCA

We had demonstrated the efficacy for PMCA applied to tau fibrils when using 0.1% K18 fibrils as seeds and performing 12 cycles of PMCA. Next we wanted to assess the sensitivity of PMCA to determine whether seeds further diluted could be amplified. We seeded htau40 WT monomer with fibril seeds of K18 ranging from 0.1% to 0.00001% of monomer tau concentration and applied 40 cycles of PMCA. Interestingly, at all the seed concentrations tested we observed amplification, with decreasing amplification occurring with each seed dilution. Notably, the control for no seeds added did not result in any aggregation after 40 PMCA cycles. As was expected, ThT only and 0.01% seeds (seeds only) also did not result in elevated fluorescence (Figure 5.4A). When observing PMCA reactions substantial variability is observed between identical samples (Figure 5.5). Emission spectra of thioflavin T indicate that the variability in amplification can arise, sometimes deviating due to a single reaction (e.g. see reaction in well B1). It is possible that this variability originates due to an uneven force distribution in the bath. If PMCA were to be applied as an assay for the accurate determination of tau fibrils, the variability would need to be accounted for when determining the number of trials chosen. In summary, the results indicate that tau fibrils can be amplified, even when diluted by several orders of magnitude.
**Figure 5.4: Amplifying K18 fibrils at dilute seed concentrations.** Reactions underwent 40 cycles of PMCA (or 20 hours of incubation), and aggregation efficiency was determined by thioflavin fluorescence (thioflavin T present in reactions at 5 µM). (A) Varying initial seed concentrations of K18 fibrils from 0.1 to 0.00001% per mole of monomer (10 µM). Control reactions were performed with 10 µM htau40 WT monomer and underwent 40 PMCA cycles without added seeds (no seeds). (B) Samples with added heparin (40 µM) and no added heparin were compared for effect of cofactor. The influence of periodic sonicating was determined by applying 5 seconds and 200 seconds of sonication (1 cycle and 40 cycles of PMCA, respectively) and incubating samples for a total duration equivalent to of 40 cycles of PMCA (20 hours). All values represent mean ± SEM (n = 8 experiments).
Previously we had demonstrated a requirement of a cofactor for fibril propagation when carrying out seeded reactions. In all the PMCA experiments thus far the cofactor heparin was included in the reactions. As fibril breakage and elongation are the basis for PMCA we would expect that the omission of cofactor from the reactions would inhibit PMCA by reducing the efficiency of the elongation phase. When comparing PMCA reactions using the same seed concentration, but with and without added heparin (40 µM), we observed a substantial decrease in the amplification for reactions without added heparin (Figure 5.4B). It should be noted that when introducing the seeds a low concentration of heparin (1.6 µM) is carried over to the reaction, which would account for the minor amplification observed for the “no heparin reaction”. Again, we tested whether the amplification of the fibrils was occurring in part due to the periodic incubation cycles and not solely due to the incubation phase. When sonicating for one cycle (5 seconds) there was only slight aggregation observed. When sonicating for the total duration of 40 PMCA cycles (200 seconds) at the beginning of the reaction there was no substantial aggregation observed, further indicating that PMCA occurs due to the periodic breakage of fibrils in between incubation phases. Our results reiterate the dependence of fibril elongation on the presence of a cofactor and validate that the mechanism of PMCA is based on cycles of combined breakage and growth of fibrils.
Figure 5.5: Variability of PMCA. Variability of ThT spectra after 40 cycles of PMCA using K18 fibrils as seeds and htau40 WT as recruited monomer. Spectra were obtained in a 96-well plate (n = 8). A1-A8 = 0.1% K18 seeds; B1-B8 = 0.01% seeds; C1-C8 = 0.001% seeds; D1-D8 = 0.0001% seeds; E1-E8 = 0.00001% seeds; G1-G8 = no seeds; A11-H11 = 0.01% seeds (no heparin); A12-H12 = 0.01% seeds only. Excitation = 440 nm, Emission = 450nm -530 nm.

5.5 Seeding with full-length seeds

In all of our seeding reactions thus far we used K18 fibrils (unfractured) as initial seeds and propagated the fibrils by recruiting htau40 WT monomer. In order to determine whether fibrils of htau40 WT could be propagated when htau40 WT fibrils were used as seeds we performed 40 cycles of PMCA with concentrations of 0.1% to 0.00001% htau40 WT seeds. As was seen when using K18 seeds, htau40 seeds could be effectively propagated, even to some extent when using the lowest seed concentration
tested (0.00001%) (Figure 5.6A). Also, we again assessed the impact of omitting cofactor, and found that without added heparin the reaction efficiency was significantly reduced (Figure 5.6B). Furthermore, the importance of applying sonication periodically to break the fibrils was again demonstrated as reactions with sonication applied only at the beginning of the reaction did not result in effective amplification (Figure 5.6B). These results indicate that like fibrils composed of K18 tau, htau40 fibrils are capable of being amplified.

Next, we asked whether 3R tau could be amplified by PMCA. Using htau23 as monomer and htau23 fibrils (unfractured) as seeds we assessed the amplification sensitivity (Figure 5.7A). After 40 PMCA cycles we observed amplification compared to that contained monomer but no seeds; however the seed concentration amplified was limited to 0.001%. The addition of cofactor was again shown to enhance amplification, as reactions with no added cofactor were amplified to a significantly lower extent (Figure 5.7B). Thus it is clear that fibrils composed of truncated tau, as well as fibrils of full-length tau (either htau40 or htau23), are capable of being amplified.
Figure 5.6: Amplifying htau40 fibrils. Reactions underwent 40 cycles of PMCA (or 20 hours of incubation) with 10µM htau40 WT monomer, and aggregation efficiency was determined by thioflavin fluorescence (thioflavin T present in reactions at 5 µM). (A) Varying initial seed concentrations of K18 fibrils from 0.1 to 0.00001% per mole of monomer (10 µM). Control reactions with 10 µM htau40 WT monomer underwent 40 PMCA cycles without added seeds (no seeds). (B) Samples with added heparin (40 µM) and no added heparin were compared for effect of cofactor. Influence of periodic sonication was determined when applying 5 seconds and 200 seconds of sonication (1 cycle and 40 cycles of PMCA, respectively) and incubating samples for total duration of 40 cycles of PMCA (20 hours). All values represent mean ± SEM (n = 8 experiments).
Figure 5.7: Amplifying htau23 fibrils. Reactions underwent 40 cycles of PMCA (or 20 hours of incubation) with 10 µM htau23 WT monomer, and aggregation efficiency was determined by thioflavin fluorescence (thioflavin T present in reactions at 5 µM). (A) Varying initial seed concentrations of K19 fibrils from 0.1 to 0.00001% per mole of monomer (10 µM). Control reactions with 10 µM htau23 WT monomer underwent 40 PMCA cycles without added seeds (no seeds). (B) Samples with added heparin (40 µM) and no added heparin were compared for effect of cofactor. Influence of periodic sonicating was determined when applying 5 seconds and 200 seconds of sonication (1 cycle and 40 cycles of PMCA, respectively) and incubating samples for total duration of 40 cycles of PMCA (20 hours). All values represent mean ± SEM (n = 8 experiments).
Chapter 6: Discussion

6.1 Seeding of 3R and 4R tau

There have been three distinct types of fibrils identified based on the composition of 3R and 4R tau (3R fibrils, 4R fibrils, and 3R/4R fibrils). Studies have revealed that the core of these fibrils is composed of the microtubule binding repeats, and that β-strands of the fibril are all arranged parallel with their side-chains in-register (60-62). While the core of the fibrils and the β-sheet stacking are conserved within compositionally distinct fibrils, structural variations could occur based on differences in the packing of β-sheets within the fibril. Such variations in the β-sheet packing would therefore impart conformational differences among fibrils, and would be expected to alter biophysical properties. The results of our seeding experiments have shown that key differences exist between the identified fibril types, and indicate that the fibrils are conformationally distinct. Our findings show that 3R tau fibrils, as well as mixed 3R/4R fibrils, seed both 3R and 4R tau. Fibrils of 4R tau, however, seed 4R tau, but not 3R tau indicating an asymmetric barrier exists between 3R and 4R tau. When 4R tau was first seeded with 3R tau fibrils, 3R tau was then capable of being seeded by 4R tau fibrils, indicating that a new conformation of 4R tau fibrils had been produced and further instilling the idea that fibril propagation proceeds by a templated mechanism. Based on seeding properties, we have demonstrated the occurrence of four conformationally distinct fibrils—which
include 3R fibrils, 3R/4R fibrils, and two conformations of 4R fibrils (one that seeds 3R tau and one that does not).

In our seeding experiments we have utilized truncated constructs K18 and K19 that contain the microtubule binding repeat region and omit the N and C termini. These truncated versions of tau are reflective of the protease resistant core region of fibrils found in Alzheimer’s disease. Similar to constructs K18 and K19, this protease resistant core of Alzheimer’s disease fibrils is composed of around 100 amino acids, and includes most of the microtubule binding repeat region (73). Additionally, the truncation of tau through proteolysis is observed in fibrils found in disease (128, 129) and has been linked as a precursor towards the aggregation of tau into NFTs (130)—despite other findings disputing this requirement (131). Furthermore, tau that has been truncated at the C and N termini beyond the microtubule repeat region has been shown to assemble into fibrils more quickly and efficiently than full-length tau (129), and is capable of nucleating the aggregation of full-length tau (132). Our findings indicate that fibrils composed of truncated constructs K18 and K19 effectively seed both truncated and full-length tau. As the seeding properties observed were the same whether truncated or full-length constructs were used, it would be expected that the region of the protein outside the fibril core may not have any marked contributions to the fibril conformation. This would be in line with the flanking regions forming an intrinsically disordered coat (74, 75).

The diversification of amyloid fibrils is well documented in prions, and is reflected by strain variations in both mammals and yeast (133-136). The basis of distinct prion strains is considered to originate from conformational differences between fibrils
When monomeric protein is assembled onto the prion fibril, the conformation of the fibril is adopted in a templated manner through seeded aggregation (137, 138). Proteins that contain sequence variation, either between species or due to polymorphisms within a species, may also be recruited by prions if common permissible conformations exist (139). In cases where sequence variation between fibril and monomer prevents a monomer from being recruited, transmission of the misfolded prion protein is prevented and a barrier between divergent sequences exists (138). Transmission barriers in prions have been observed between species and can be introduced by minor mutations (137). The incompatibility of cross-seeded aggregation between sequences with minor differences is not limited to prions but includes other amyloids as well. Amyloids formed by sequences of the OsmB membrane associated protein were shown to be capable of seeding monomer of the same sequence, but not monomers with subtle sequence variations (140). Fibrils of α-synuclein are capable of seeding the growth of monomeric α-synuclein, but are not capable of seeding β- and γ-synuclein, despite having a substantial sequence homology (141). Additionally, barriers between lysosome fibrils have also been demonstrated between species (142). In our experiments that cross-seed 3R tau with 4R tau we demonstrate the existence of a barrier similar to what has been observed for other amyloids. The barrier was proven to be robust, as even a 5-fold addition (15%) of 4R fibril seeds did not allow for the recruitment of 3R tau monomer. To verify the seeding properties between 3R and 4R tau we utilized several experimental approaches. From our kinetics experiments, using the intrinsic reporter acrylodan and the extrinsic fluorophore thioflavin T, as well as by electron microscopy and fibril
sedimentation experiments we found that 3R tau does not assemble on 4R tau fibril seeds. The seeding properties we observe in the in vitro experiments are also in agreement with cellular studies that focused on the aggregation induced by fibril seeds. When 4R tau fibril seeds were transfected into cells overexpressing 4R tau, the monomer was observed to aggregate. However, transfected 4R tau fibrils into cells overexpressing 3R tau did not result in the aggregation of the endogenous tau (49). Despite containing a majority of the 4-repeat sequence, the lack of 30 amino acids leads to an incompatibility for 3R tau recruitment by 4R tau fibrils. Interestingly, we observed the ability of 4R tau to be competent for the recruitment by 3R fibril seeds. This is in agreement with Clavaguera et al. who showed that brain extracts from Pick’s disease—which contain aggregated deposits of predominantly 3R tau—were able to induce the formation of 4R tau in transgenic mice (55). The cross-seeding asymmetry that we demonstrate is not the first observed between tau fibrils. When studying the cross-seeding of fibrils composed of disease mutant P301L tau and wild-type tau, it was shown that fibrils of P301L are competent to seed P301L but not wild-type monomer. Alternatively, fibrils composed of wild-type tau seed both wild-type and P301L tau, establishing an asymmetric barrier (143).

Elongation of amyloid fibrils occurs through the subsequent addition of soluble protein. The conformational influence that the fibril seed has on the conversion of monomer has been demonstrated in prions and reveals that the seed conformation is imprinted onto the assembling monomer. The robustness of the templating nature was tested when distinct prion strains were propagated through multiple steps, with the
resulting fibrils maintaining the strain identity of the initial seed (144). Conformational variation is implicit in prion amyloids and is manifested in the prion seeding properties. Prions composed of the same protein sequence can have different conformations, which may diverge in the ability to recruit a given sequence of monomer (145). When cross-seeding prions of different species, variations in sequence can prevent adoption of the misfolded conformer from one species to another. For instance, an asymmetric seeding barrier was observed between mouse and Syrian hamster prions that allowed the hamster fibrils to seed mouse, but prevented mouse fibrils from seeding hamster. When mouse monomer was seeded with hamster fibrils the resulting mouse fibrils were then capable of seeding mouse monomer (138). The circumvention of the transmission barrier between mouse fibrils and hamster monomer through the templating of mouse monomer on hamster fibrils resulted in the emergence of a new conformation of mouse fibril. From the seeded growth, mouse monomer was found to assume the conformation of the hamster fibrils (137).

We had hypothesized that the same principles governing the propagation of prion conformation could be applied to tau fibrils. In our experiments we analyzed the seeding properties of 3R and 4R tau and observed an asymmetric barrier, similar to what was shown to exist between mouse and hamster prions. This observation provided the opportunity to assess the parallels between tau and prion fibrils with respect to a common conformational templating mechanism. When growing 4R tau monomer on 3R tau fibrils, the 4R tau assumed the 3R tau fibril conformation and the resulting fibrils permitted the recruitment of 3R tau monomer. Importantly, when multiple steps of
propagation were performed to remove the initial 3R tau seed, the 3R tau seeding properties were maintained within the 4R tau fibrils as indicated by the ability of the templated 4R tau fibrils to seed 3R tau. This finding was important as it indicates that tau and prion fibrils propagate via a common mechanism despite sharing no similarity in sequence. In view of our findings it would be interesting to determine whether the barrier observed preventing disease mutant P301L from seeding wild-type tau could be bypassed when templating P301L mutant monomer on wild-type tau seeds.

The adaptation of the 4R tau monomer to the 3R tau fibril conformation indicates that the same sequence of tau is capable of assuming different conformations that possess variable seeding properties (i.e. one conformation that seeds 3R tau monomer and one that does not). The ability of a tau sequence to assume variable conformations was also demonstrated by Frost et al., who observed that 4R tau grown on distinct fibril seeds produces different conformations (47). The conformational diversity observed in tau fibrils is reminiscent of the strain phenomenon observed in prions.

From our in vitro experiments we have identified at least four conformationally distinct fibrils based on the corresponding seeding properties (Figure 6.1). Electron paramagnetic resonance spectroscopy studies that measure intramolecular distances within a fibril have indicated that the conformational basis of fibrils is even more complex, and that within a fibril sample multiple conformations can exist (63). The effects of mutations, cofactors, chaperones, and post-translational modifications such as phosphorylation could also influence the conformational variability of tau fibrils. In our experiments we have demonstrated the ability of tau fibrils to seed monomeric tau,
propagate, and diversify into different conformations. We also identified the existence of conformational barriers that impart significant consequences on isoform recruitment. As we have demonstrated that fibril seeds can act to provide nuclei to recruit monomeric tau, it is therefore expected that the initial nucleation events leading to aggregation would have a profound effect on the subsequent fibril formation. Nuclei that contain both 3R and 4R tau, as was previously demonstrated (62), could be relevant to Alzheimer’s disease, where all 6 isoforms are found to deposit (146). Nucleation events of 4R tau could be reflective of what is observed in tauopathies like progressive supranuclear palsy, argyrophillic grain disease, and corticobasal degeneration, where 4R tau is found to deposit (41). Based on our findings the preferential deposition of 3R tau in Pick’s disease could not be explained by a barrier that prevents 4R tau from growing on 3R tau fibrils, as we have demonstrated that 3R tau nuclei do seed 4R tau. However, one explanation for the lack of 4R tau in Pick’s disease could be that another 3R tau fibril conformation exists that does not seed 4R tau. Alternatively, it is possible that the inefficient growth of 4R tau on 3R tau fibrils, as we observe, could leave the fibrils susceptible to cellular clearance. Our findings presented here have provided basic insights into the molecular mechanisms responsible for tau fibril propagation. Attributes of prions such as strain diversity, transmission barriers, and strain emergence are clearly inherent in tau fibrils, suggesting that similar phenomena might occur in tauopathies.
Figure 6.1: Templated fibril growth of tau. Monomers of 3R and 4R tau (top) can only grow onto the ends of 3R (square), 3R/4R (oval), and 4R fibrils (triangle and hexagon) if their sequence is compatible with the conformation of the seed (arrows). Structural incompatibility gives rise to a seeding barrier (crossed out arrow). Two conformations are possible for 4R fibrils based on the capacity to seed 3R tau. The conformations observed could be reflective of conformations in disease that would create preferences for isoform deposition. PID = Pick’s disease, AD = Alzheimer’s disease, PSP = progressive supranuclear palsy, CBD = corticobasal degeneration, AGD = argyrophilic grain disease.

6.2 Tau cofactors bind and sustain fibril propagation

6.2.1 Fibril core is retained with diverse cofactors

The structural core of in vitro formed tau fibrils has been well characterized when utilizing the cofactor heparin to induce fibril formation (60-62, 147). We have further analyzed the effects of alternative cofactors on the structure of tau. Using the single-stranded poly(A) RNA nucleic acid as a cofactor, we formed aggregates with tau spin-
labeled in the third repeat, and observed single-line spectra by EPR at six different sites. Single-line spectra were also observed at sites labeled in the third repeat when using heparin \((60, 62)\). The single-line spectra indicate a close proximity among the spin-labels in the fibril. Importantly, the distance constraint implicit by a single-line spectrum narrows the structure of the fibril core to stacked \(\beta\)-sheets that are parallel, with side-chains positioned in-register.

From our EPR analysis we observed that tau aggregates formed from poly(A) RNA are highly-ordered and contain a structural core containing the third repeat that is also found to exist in heparin induced fibrils. The ability to promote parallel, in-register fibrils was also observed for other cofactors such as single-stranded pyrimidine RNA, double-stranded RNA, DNA, tRNA, RNA extracted from yeast, and polyglutamate. We demonstrate that a diverse set of nucleic acids and peptide cofactors all produce fibrils with a structured third repeat, suggesting that the third repeat invariability remains structured. This structural arrangement is therefore consistently conserved within tau fibrils and does not appear to vary according to the cofactor used. The herein described secondary structure is not limited to tau fibrils, as parallel, in-register \(\beta\)-sheet arrangements have also been found to be the structural basis of other amyloids \((148-150)\).

**6.2.2 Seeding properties conserved using RNA as cofactor**

When using heparin as a cofactor to form fibrils we had previously determined the seeding properties of tau and observed an asymmetric barrier between 3R and 4R tau \((120)\). As the cofactor initiates the formation of fibrils through nucleation, we had speculated that alternative cofactors could produce different conformations of fibrils that
might be reflected in variations in the seeding properties of 3R and 4R tau. The seeding properties of tau using poly(A) RNA as a cofactor did not deviate from experiments using heparin. The consistency of the seeding properties of tau, even when using a different class of cofactor, suggests a robust nature of tau propagation according to isoform. While the seeding properties remained unchanged when using a different cofactor, we cannot exclude that poly(A) RNA produces fibrils with conformations different from fibrils formed in the presence of heparin. It still remains possible that utilizing alternative cofactors such as RNA could result in conformational variations that are manifested in differences in β-sheet packing.

The method for determining the seeding properties utilized our developed acrylodan conjugated assay. The study of tau aggregation in the presence of nucleic acid cofactors is hampered by the inability to utilize the routinely employed thioflavin assay. Thioflavin T has been shown to bind to nucleic acids and produce enhancements in fluorescence (151) that could be mistaken for the detection of amyloids. The use of the acrylodan assay in these experiments further demonstrates its utility for measuring tau aggregation.

6.2.3 Cofactor promotes elongation of fibril

Cofactors function to facilitate tau aggregation by promoting nucleation. In our seeding experiments we introduced sonicated pre-formed fibril seeds that allow the nucleation phase of aggregation to be bypassed. The introduction of seeds provides the nuclei needed for aggregation and leads to the recruitment of monomer to elongate the fibril. When studying the in vitro aggregation of tau, cofactors can be employed to
induce fibril assembly. However, it is difficult to differentiate fibril growth phases of nucleation and elongation. As seeding experiments result in the elongation of the fibril, we were able to monitor fibril growth and determine whether the presence of a cofactor contributed a role in elongation. From our multi-step seeding experiment we observed that the propagation of fibril seeds was sustained through successive seeded reactions only when a cofactor was provided. This result suggests that the cofactor not only initiates the nucleation of tau aggregation, but once nucleated, also facilitates the elongation of the fibril. Our kinetic experiments monitoring the seeded aggregation of tau also support this conclusion, as the addition of both seed and cofactor promoted the assembly of tau fibrils, while the addition of only the seed did not. As incubating the same concentrations of tau and RNA within the same time-period did not result in aggregation we can eliminate the possibility that nucleation was occurring within the time period and confirm that the cofactor significantly promotes the elongation of the fibril. Our findings suggest that a “nucleation only” role for cofactors as proposed previously (102) can be excluded.

Our results are in agreement with Zhu et al. who found that increasing the concentration of heparin (up to a 1 heparin to 1 tau molar ratio), leads to an acceleration in the growth phase of the aggregation kinetics. Consisted with our findings, this observation suggested that heparin facilitates fibril elongation (99). The effect of a cofactor on tau fibrils also parallels the cofactor contribution to prion amyloids. When using RNA as a cofactor, Deleault et al. showed that the successive seeding of prion fibrils is sustained through the addition of cofactor, but lost when the cofactor is removed.
While our experiments indicate that a cofactor promotes fibril elongation and sustains aggregation, it has been shown that fibrils can be formed with high concentrations of tau or at high temperatures without the presence of a cofactor \((89, 124)\). Fibrils formed without cofactor could be expected to have conformations that deviate from fibrils formed in the presence of cofactor. It is possible that fibril conformations nucleated without a cofactor may experience little or no reliance on a cofactor for the elongation of the fibril.

### 6.2.4 Cofactor stabilizes fibrils through binding

At an elevated temperature we demonstrated that fibrils could be formed with and without the addition of cofactor \((124)\). A ~20 nm blueshift in the acrylodan emission maximum after 80 minute incubation indicated protein aggregation. The presence of fibrilar structures was confirmed by EM imaging. When cooled, however, the fibrils formed without cofactor were found to dissociate, while the fibrils formed with added cofactor did not. This observation indicated that the cofactor provides a role in stabilizing the fibril, preventing reversion to the initial monomeric state. If the cofactor stabilizes the fibril then it could be expected that the cofactor would bind as an accessory component. We formed fibrils with RNA as a cofactor and carried out sedimentation experiments to determine whether the RNA co-sedimented with the insoluble fibrils. We observed that the majority of the RNA was found bound to the fibrils. The interaction between the tau fibrils and the cofactor has been previously investigated, however, reports deviate on whether the cofactor binds and is therefore part of the fibril \((100-102)\). Importantly, our results demonstrating the binding of cofactor to tau fibrils are in
agreement with observations of aggregated tau found in disease. In NFTs found in Alzheimer’s disease, the abundance of glycosaminoglycans is well documented (152-156), and glycosaminoglycans have furthermore been shown to be localized on the fibrils (155, 156). The use of nucleic acids as cofactors, as we have utilized here, is also comparable to potential cofactors that would be found available in the cell. The inclusion of RNA in NFTs has been shown to occur, which again suggests the occurrence of cofactor binding to fibrils in vivo (157). The RNA found in NFTs was not characterized according to type (tRNA, mRNA, etc), but based on our findings we demonstrate an indiscriminant requirement for nucleic acids in the facilitation of tau aggregation. As we have shown that poly(A), poly(U), poly(dA), poly(AU), tRNA, and RNA extracted from yeast all are capable of forming highly ordered aggregates, the potential cofactors in the cell are likely to be based more on availability than on specific structure.

The observation that a broad class of polyanionic molecules facilitates the aggregation of the highly basic region of tau suggests that the binding event between tau and cofactor occurs through electrostatic interactions. In reactions where tau was nucleated with the presence of a cofactor the application of elevated salt inhibited aggregation (105, 158). If the electrostatic interactions occurring between tau and cofactor were limited to the nucleation phase, then elevated salt should not affect the elongation of the fibril. When treating seeded reactions with elevated salt we observed the abolishment of fibril growth suggesting that the cofactor-tau interactions are not limited to nucleation events, but are also important for fibril elongation. The prevention of fibril elongation is even expected, since we have shown that cofactors bind to fibrils.
Disrupting the electrostatic interactions by introducing high salt would therefore inhibit binding and prevent aggregation as was observed.

6.2.5 Cofactor exchange on the fibril

When using RNA as a cofactor to induce tau aggregation we have observed the incorporation of the cofactor into the fibrils. As we had used truncated constructs containing the binding repeats, the binding event is likely to occur at or near the fibril core and not at the unstructured N- and C-termini. We had asked whether the introduction of another cofactor could exchange the cofactor originally used for induction. When heparin was incubated with fibrils with bound RNA, the co-sedimentation of RNA with the insoluble aggregates no longer occurs, indicating the removal of the RNA from the fibrils had taken place. The assumption that the RNA removal occurred through the exchange of heparin was verified when fluorescein conjugated heparin was used as the exchanging cofactor. The dye-labeled heparin was found to cause the release of the RNA from the fibril and was found to be associated with the sedimented aggregates. Also, the increase in the fluorescence anisotropy of the fluorescent heparin was observed with the addition of RNA induced fibrils, further confirming the effect of cofactor exchange.

The observation that cofactors can exchange on the fibril is important, as it indicates that the binding of the cofactor to the fibril does not occur at a buried site, but instead binds at a region that would be accessible for cofactor exchange. The cofactor therefore would be expected to bind on the surface of the fibril. Based on tau fibril structure, the orientation of tau strands in the fibril would require side-chains to be
stacked on top of each other. As the repeat region of tau fibrils is highly basic, the resulting fibril structure would require positively charged side-chains to be positioned in close proximity. Specifically, in repeats two and three, where tau is highly structured, there are five and four lysines present, respectively, that would introduce charge repulsions between parallel stacked β-strands. The binding of the polyanionic cofactor could neutralize these repulsions and stabilize the fibril. It would seem probable that the cofactor-tau binding occurs with the length of the cofactor polymer stretched along the long fibril axis, similar to what has been observed for DNA bound to α-synuclein fibrils (159). In this manner the cofactor could act as a scaffold to bridge and neutralize the positive repulsions between multiple strands of tau. As was shown by the replacement of RNA by heparin, cofactors are not bound statically, but are dynamic and can exchange on the fibril (Figure 6.2). The binding of cofactor to tau could have significant implications for the fibril properties. Namely, the decoration of fibrils by varying cofactors could affect the fibril surface and impart the ability to transfer between cells. Proteoglycans have been shown to be distributed on the cell surface (160) and could be a means by which tau fibrils could transfer into cells. Based on heparin exchange with RNA on the fibril, we have shown that tau fibrils can bind glycosaminoglycans through an exchange mechanism. As proteoglycans have been shown to bind cationic ligands such as polypeptides and provide a role of endocystosis (161), it is possible that the same internalization mechanisms may be applied to tau fibrils. In fact, cell surface heparan sulfate proteoglycans have recently been shown to facilitate the cellular binding and internalization of tau fibrils (162). These findings further indicate the importance
inherent in the tau-cofactor association. It is possible that cofactors bound to tau fibrils could exchange for proteoglycans on the cell surface, providing a mechanism for the internalization of tau fibrils into the cell, which ultimately could account for the spreading of the tau pathology.

Figure 6.2: Cofactor binding and exchange on the fibril. The envisioned mode of cofactor binding is that a cofactor polymer such as poly(A) RNA could bind along the fibril and neutralize repulsive positive charges introduced from side-chain proximity between strands. The introduction of a cofactor such as heparin can result in the exchange of the bound cofactor.
6.3 Tau fibril amplification by PMCA

From our in vitro experiments we have demonstrated that we can transcend the limitations of low seed concentrations when carrying out seeded reactions of tau. For the first time we have applied the principles of PMCA used to amplify prions towards the amplification of tau fibrils. As shown by the thioflavin T assay, and verified by sedimentation of fibrils, we have successfully propagated fibrils whose concentration was shown previously to be too dilute for efficient seeded amplification. Next, we assessed the sensitivity of seed dilution and found that we could amplify a concentration of seed diluted $10^4$ fold of what was found to be insufficient to recruit monomer effectively in a single sonication cycle seeded reaction (0.1% seed). At this concentration (0.00001% seeds) tau in the fibrils is present at $10^8$ molecules (5 X $10^8$ molecules per mL), while the total tau monomer (10 µM) consists of $10^{15}$ molecules. Whether we had used seeds of truncated (K18) or full-length (hta40) tau to amplify, similar sensitivity was observed. Htau40 WT was the recruited monomer in all of the experiments. As truncated tau nucleates to form aggregates in a shorter time period than full-length tau (163, 164), we would not have been able to apply as many PMCA cycles before the monomer only (no seeds) negative controls would have begun to aggregate. Therefore, by using htau40 monomer to be recruited we were able to perform more cycles (40 cycles), and meanwhile avoid spontaneous nucleation. Since the monomer only (no seeds) was never shown to aggregate during the 40 cycles, we can be confident that the fibril growth observed through the addition of seeds is not due in part to monomer nucleation, but solely to the elongation and propagation of the fibril seeds. Induced aggregation of tau
monomers is best promoted under conditions of low salt (0.1M NaCl) (158) and is aided by oxidizing conditions when native cysteines are present (96). Additionally, full-length tau may take days to aggregate at low µM concentrations (4-50 µM) at 37 °C (96). Attention to the applied conditions is therefore important when considering the prevention of spontaneous nucleation of monomer.

In the PMCA reactions carried out, the cofactor heparin was added. When cofactor was not included we found that the amplification efficiency was drastically reduced, regardless of whether K18 or htau40 were used as seeds. This finding was expected as we had previously shown that the addition of a cofactor was needed for the fibril propagation to be sustained. Interestingly, when PMCA is applied to prion amyloids it has been shown that the fibril amplification is also dependent on the presence of a polyanionic cofactor (121, 165), further suggesting common requirements needed for growth of both tau and prion fibrils.

The incubation and breakage (sonication) steps are believed to be the basis of amplification when PMCA is applied to prions. In fact, PMCA applied to prions without the incubation steps (166) or without the sonication steps (109, 166, 167) results in a loss of amplification. Similarly, we found that a reaction, in which the fibrils were only sonicated 1 cycle (5 seconds) and incubated for 20 hours were not substantially amplified. Reactions in which all the sonication steps were carried out consecutively (40 cycles, 200 seconds) and incubated for 20 hours also did not result in considerable amplification. This observation indicates the need for sonication steps to be
spaced between the incubation steps, in order to allow time for fibril elongation to occur before the next breakage step is induced.

Our findings presented here indicate that PMCA can be applied to the amplification of dilute quantities of tau fibrils. The function of this assay could ultimately be applied towards the amplification of tau fibrils derived from biological samples. When applied to prions, PMCA has been used to quantitatively determine the concentration of prions within a sample. Using animal models the PMCA assay has demonstrated a heightened sensitivity that allows for the detection of dilute prion concentrations in sources that include brain, blood, and urine (168, 169). Similarly it is possible that PMCA could provide a diagnostic tool for the presence of tau fibrils found in comparable tissue and fluid samples. We have taken the first important steps to optimize an assay and implement PMCA for the amplification of tau fibrils. The utility of PMCA applied to tau fibrils is expected to support fundamental studies of fibrils derived from \textit{in vivo} sources.

6.4 Future work

The described studies were performed using recombinant tau protein that was not phosphorylated. As phosphorylation of tau is associated with disease, further studies are needed to elucidate whether phosphorylation of tau is critical for the phenomena observed. For example, it is not known whether the seeding properties of tau fibrils are influenced by tau phosphorylation. We observed that fibril propagation is dependent on the presence of a cofactor. As phosphorylation assists in the aggregation of tau (85), it will be important to determine whether phosphorylated tau requires cofactors for template growth.
Finally, we have developed an assay for the amplification of tau fibrils. Further optimization is needed to enhance its sensitivity and to adopt it for use in conjunction with *in vivo* derived material.
References


103. M. Hasegawa, R. A. Crowther, R. Jakes, M. Goedert, Alzheimer-like changes in microtubule-associated protein Tau induced by sulfated glycosaminoglycans


Appendix A: Tau constructs

Repeat Region of tau

MTBR Region

MAEPRQEFVE MEDHAGTYGL GDRKDGQGYT MHQDGQEDTD AGLKESPLQT PTEHDGSEEPG 60
SETSDKSTP TAEDVTAPLV DEGAPGKQA QAPHHTEIPEG TTAEEAGIGD TPSLEDDEAAG 120
HVTQARMVSK SKDGTGSDDK KAKGADGTKK IATPRGAAPP GQKGQANATR IPAKTPPAPK 180
TPPSSGEPPK SGDRGYYSSP GSPGTGSSRS RTPSLPTPPT REPKKVAVVRR TPPKSPSSAK 240
SRLQTAPVPM PDGBKVKSKIS GSTEMLKQHP GGGKQVIINV KLDLSNVQSG CGSKEDNIKHY 300
EGGGVQIVY KPVDSDKVTS KCGSLGNIHH KPGGGQVEVK SEKLDKFDVR QSKIGSDLNI 360
THVPGGGNKK IETHKLTFRE NAKAKTDHGAEIVYKSPVVS GDTSRPHLSN VSSTGSIDMV 420
DSPQLATLAD EVSASLAKQG L

QTAPVPM PDGBKVKSKIS GSTEMLKQHP GGGKQVIINV KLDLSNVQSG CGSKEDNIKHY Repeat 1
QVIINKKLDLSNVQSGSKGSKDNIKHVPGGGQ Repeat 2
QIVYKPVDSKVTSCGSLGNIHHKPGGGQ Repeat 3
VEVKSEKLDKFDVRQSGSKIGSDLNI THVPGGGN Repeat 4

*cysteines found in repeat 1 and 2
Htau40
MAEPRQEFEV MEDHAGTYGL GDRKDQGGYT MHQDQEGDTD AGLKESPLQT PTEDGSEEPP 60
SETSDAKSTP TAEDVTAFLV DEGAPGQQAQ AQPHTIEPEG TTAEAGIGD TPSLEDEAAG 120
HVTQARMVSK SKDGTGSDDK KAKGADGKTQ IATPRGAAPP GQKQGQANATR IPAKTPPAK 180
TPPSSGEPPK SGDRSGYSSP GSPGTGPSRS RTPSLPTPPT REPKKVAVV TPPSPSSAK 240
SRLQTAPVPM PDLKNVKSKI GSTENLKHQP GGGKVQINK KLDSLNVQSK CGSKDNIKHV 300
PGGGSVQIVV KPVDLSKVTS KCGSLGNIHH KPGGQVEVKS SEKLDKDRVQSKIGSDLNI 360
THVPGGNKK IETHKLTFFRE NAKAKTDHGAE IVYKSPVVVS GDTSRPHLSNV SSTGSIDM 420
DSPAQLATLAD EVSASLAKQG L 441

Estimated pI = 8.24
M.W = 45850

Htau23
MAEPRQEFEV MEDHAGTYGL GDRKDQGGYT MHQDQEGDTD AGLKAEAGI GDTPSEDEA 1
AGHTQARMV SKSDKTGSDDK KAKGADGKTQB TATPRGAAPP GQKQGQANATR IPAKTPPA 2
PKTTPSSGEPPK SGDRSGYSSP GSPGTGPSRS RTPSLPTPPT REPKKVAVV TPPSPSSAK 8
AKSRLQTAPV PMPDLNKVS KIGSTENLKHQP GGGKVQINK KLDSLNVQSK CGSKDNIKHV 16
HKPGGQVEV KSEGDKFDRQ VQSKIGSDLN ITHVPGGNKKI EETHKLTRN ENAKAKTDHG 24
AEIVYKSPVV SGDTSPHLSNV SSTGSIDM VDSPQLATLAD EVSASLAKQG L 32

Estimated pI = 9.38
M.W = 36760
K18 Construct

MQTAPVPMPD LKNVSKIGS TENLKHQP GGKVQIINKKL DLSNVQSKCG SKDNikhVP GGSVQIVYKV PVDLSKVTSCG SLGNIHHHP GGGQVEVKSE KLDFKDRVQS KIGSLDNITH VPGGGNKKE

Estimated pI = 9.7
M.W = 13813

K19 Construct

MQTAPVPMPD LKNVSKIGS TENLKHQP GGKVQIVYKPV DLSKVTSCG SLGNIHHHP GGQVEVKSE KLDFKDRVQS KIGSLDNITH VPGGGNKKE

Estimated pI = 9.67
M.W = 10567
Appendix B: Tau purification

Elution program and gradient profile

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<table>
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<td>Eluate fraction size (ml)</td>
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<td>Linear gradient</td>
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<tr>
<td>Target concentration buffer B (%B)</td>
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Length of gradient (column volumes) 11.0

Linear gradient for cation exchange elution of tau. Slope of increasing buffer B versus ml of eluate.
Htau40 WT cationic exchange elution profile

Cationic exchange elution chromatograph for htau40 WT. Absorbance at 280 nm shown in blue with the linear gradient shown in green. Eluate fractions (3ml) shown at the bottom in red.
Htau40 WT gel filtration elution profile

Gel filtration elution chromatograph of Htau40 WT. Htau40 WT purified over Superdex 200 medium packed column
Appendix C: PMCA setup

Bath sonicator horn used for applied cycles of PMCA
PMCA reactions in 96-well plate with sample cover.
96-well plate with cover and plate lid. Two transfer pipette bulbs were sealed and fastened to plate to allow for plate to float in bath sonicator.
Covered plate was allowed to float in bath sonicator while undergoing PMCA.
Appendix D: Publications


P.D. Dinkel, R. Mazzarino, N. Matin, M. Margittai, Cofactors Sustain Seeded Filament Growth (Manuscript in Preparation)