

Expression and Purification of N-terminally Acetylated microtubule binding protein Tau

An undergraduate project presented

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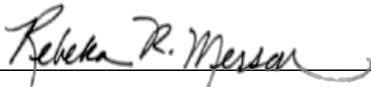
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**Expression and Purification of N-terminally Acetylated microtubule binding
protein Tau**

By Abigail Fleurima

**A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
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ABSTRACT

The microtubule-associated protein Tau (MAPT) plays a critical role in many neurodegenerative diseases. Tau functions to stabilize microtubule structures that are essential for transport within the neuron, and transport disruption leads to loss of neuronal function. Tau binding is regulated by phosphorylation with the help of kinases that add phosphate groups which block microtubule binding sites, and phosphatases that remove phosphate groups and expose the microtubule-binding regions. Tau can be found in hyper-phosphorylated states, which causes Tau to self-assemble into aggregates and prevent microtubule binding. It's clear that post-translational modifications (PTMs) of Tau play a key role in the dysregulation of neuronal function due to abnormal conformational changes, however not all PTMs are as thoroughly studied as phosphorylation. N-terminal acetylation is the most common PTM of all proteins and Tau is predicted to be a target. N-terminal acetylation is a co-translational process that is catalyzed by N-terminal acetyltransferases, which adds an acetyl group to the N-terminus of Tau, thus neutralizing the positive charge on the N-terminus. Commonly, studies endogenously express Tau in a prokaryotic system that lack PTMs. Utilizing a co-expression system in *E. coli* allows for the purification of Tau with a modified N-terminus. Purifying modified Tau with Fast Protein Liquid Chromatography (FPLC) will allow us to probe the structural and functional effects of N-terminal acetylation by assessing changes in Tau's aggregation and microtubule binding affinity and polymerization.

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INTRODUCTION:

I. Neurodegenerative Diseases

Neurodegenerative diseases are characterized by neuronal atrophy related to abnormal protein dynamics due to an array of interrelated factors (Yamamoto et al. 2000; Auluck et al. 2002; McKee et al. 2009; Grimaldi et al. 2018; Bandyopadhyay et al. 2007). Some major factors may include oxidative stress, mitochondrial dysfunction, disruption in the proteostasis network, and genetics. Neurodegenerative diseases are characterized based on the unique combinations of these factors, which can be interrelated where one causes the other (Jellinger, 2010).

Oxidative stress refers to the redox imbalance in cells and organisms, where the concentration of reactive oxygen species (ROS) is increased and not balanced by antioxidant defense in order to maintain redox homeostasis. This causes a disturbance in cellular metabolism and regulation of cellular constituents, especially proteins (Breitenbach and Eckl, 2015). The nervous system is especially sensitive and susceptible to oxidative stress because it makes up approximately 20% of the body's basal O₂ consumption (Halliwell, 2006). This large amount of consumption is due to the large amount of ATP needed to propagate action potentials from neuron to neuron and overall neurosecretion (Halliwell, 2006). ROS are primarily produced from escaping electrons of the respiratory chain reacting with oxygen in the inner membrane of mitochondria due to mutations in mitochondrial DNA, metal-iron-associated Fenton reactions, lipid peroxidation, and nitric oxide induced protein nitrosylation in neurons (Jellinger, 2010). These ROS have shown to non-selectively oxidize proteins and therefore mediate protein misfolding and protein radicals that can cause a cascade of events such as dysregulation of calcium homeostasis, reactive atrogliosis both observed in neurodegeneration (Jellinger, 2010). Additively, cells that cannot correct their oxidative imbalance enter apoptosis with rapid cell death adding to oxidative stress's contribution to neuronal degeneration (Jellinger, 2010)

The mitochondria is both an important producer and target of ROS. High levels of oxidants can induce mitochondrial permeability transition and uncouple oxidative phosphorylation with catastrophic effects on mitochondrial energetics and metabolism, meaning

lowered production of ATP, that contribute to cytotoxicity via apoptosis (Jellinger, 2010). However, mitochondrial dysfunction relates to abnormal protein folding because decreased ATP production has shown to increase the production of amyloid β peptides that make up amyloid protein plaques (Jellinger, 2010). These amyloid β peptides can easily enter the mitochondria due to the increased permeability of its membrane and therefore induce the production of free radicals, decrease cytochrome oxidase activity and inhibit ATP generation (Jellinger, 2010). So in a clever system, mitochondrial dysfunction may increase abnormal protein dynamics but also abnormally folded proteins interact with the mitochondria and further fuel its dysfunction.

Disruption of the proteostasis network is probably one of the most important factors pertaining to abnormal protein misfolding because protein formation is regulated by this network (Jellinger, 2010). The network's regulation responsibility involves strict control of the production, folding, abundance, and subcellular localization of proteins, as well as maintenance of protein conformation (Klaips et al. 2018). The first branch of the network, synthesis, is comprised of 279 components, the second branch, folding which is often coupled with transport and assembly, is comprised of 332 components, the third branch, degradation, is comprised of both the ubiquitin-proteasome and autophagy system to make a total of 1388 components (Klaips et al. 2018). Molecular chaperones play a major role in this network as they act as liaisons between the network branches and prevent the misfolding of disease-causing proteins (Klaips et al. 2018). The folding process is inherently error prone due to the large number of possible conformations a polypeptide can adopt (Jellinger, 2010). Therefore, when the network's ability to cope with the production of misfolded protein decreases it leads to the quick accumulation of these proteins that attract to each other and form lesions and aggregates that become toxic to the cell (Jellinger, 2010). The decreased ability of this network is normally caused by aging which results in the gain of mutations in genes that express the disease-causing protein and faulty molecular chaperones (Klaips et al. 2018; Jellinger, 2010)

Some of the neurodegenerative diseases related to abnormal protein dynamics include Parkinson's disease (PD), Huntington's disease, Chronic traumatic Encephalopathy (CTE), Alzheimer's disease (AD), and Tauopathies. Parkinson's disease (PD) is considered the second most common neurodegenerative disorder (Auluck et al. 2002). PD normally affects people in later years of life in multiple systems (Sveinbjornsdottir, 2016). PD is diagnosed clinically, based

on the presentation of symptoms, specifically the first motor symptom (Sveinbjornsdottir, 2016). Symptoms exhibited can be motor or non motor, where non motor symptoms appear before motor ones starting as early as 10 years prior (Sveinbjornsdottir, 2016). Motor symptoms can be slowness of voluntary movements, muscular rigidity, resting tremors, and posture instability. Non-motor symptoms can be lack of emotional involvement and interest, excessive daytime sleepiness, sleep problems, and constipation (Sveinbjornsdottir, 2016).

A. Parkinson's Disease

PD is primarily the result of damaged extrapyramidal neurons due to ubiquitous α -synuclein filamentous inclusions in the form of Lewy bodies, though it can affect other types of regional neurons (Auluck et al. 2002). Aggregation of α -synuclein is said to be caused by a missense mutation at position 53 (A53T) in its gene and gives rise to cytotoxicity generally through mitochondrial dysfunction, endoplasmic reticulum stress, proteasome system dysfunction, and inflammatory responses in microglia (Auluck et al. 2002). Since PD is caused by a cascade of factors and affects multiple systems of the body there is no treatment available to simply stop the progression of the disease (Sveinbjornsdottir, 2016). However, treatment with dopaminergic drugs are used to relieve symptoms and correct motor disturbance (Sveinbjornsdottir, 2016). There are surgical treatments available, like deep brain electrical stimulation, but it is only used for a select group of patients that do not respond to drug therapy (Sveinbjornsdottir, 2016).

B. Huntington's Disease

Huntington's disease (HD) is an autosomal dominant inherited disorder that starts showing at mid-age and death follows no more than 10 to 20 years after onset at middle age (Yamamoto A. et al. 2000). This is a rare disorder that is prevalent in only 10 out of 100,000 people in the Caucasian population (Roos, 2010). Symptoms that characterize Huntington's are normally involuntary motor changes and movements (Roos, 2010). Dystonia, which is the first motor signs of Huntington's, ticks, "drunk" walking, dysarthria, and dysphagia are some of the other motor signs (Roos, 2010). Behavioral and psychiatric symptoms present early on before motor symptoms; The most frequent sign is depression, but others are apathy, inactivity, low self

esteem, and feelings of guilt or anxiety (Roos, 2010). Diagnosing HD is based on the presentation of clinical symptoms described previously and the individual having a parent with HD; It is essentially to take a family history of a person with symptoms, because with it diagnosis is not very difficult (Roos, 2010).

Huntington's disease is caused by the lack of communication between cortical pyramidal neurons and striatal medium spiny neurons (Yamamoto et al. 2000). The lack of communication results from the expansion of glutamine repeats in the N-terminal region of the huntingtin protein which promotes its aggregation due to a mutation in the huntingtin gene (HTT) (Yamamoto et al. 2000).

There is no cure for HD but there are therapeutics available for treating the symptoms to increase one's quality of life (Roos, 2010). The most targeted set of symptoms to treat are the motor ones, dopamine receptor blocking or depleting agents are used to treat them (Ross, 2010). The prognosis of individuals with HD normally worsen with increased lack of motor control and ability, eventually death occurs because of complete physical dependence or suicide (Roos, 2010).

C. Chronic Traumatic Encephalopathy

Chronic Traumatic Encephalopathy (CTE) is a neurodegenerative disease prevalent in athletes playing contact sports like football and in military veterans who were in combat settings (Fesharaki-Zadeh, 2019). The disease is characterized in 3 areas; The first area is behavioral, including aggression, depression, apathy, impulsivity, and delusions; The second area is cognitive, where diminished attention and concentration, memory deficits, executive functioning deficits, and dementia are exhibited (Fesharaki-Zadeh, 2019). Motor is the third and last domain which consists of dysarthria, gait abnormalities, incoordination, and tremors (Fesharaki-Zadeh, 2019). Based on these different symptoms from the different domains there are 4 diagnostic subtypes defined, definite, probable, possible, and improbable CTE that can be determined based on the worsening and frequency of the symptoms (Fesharaki-Zadeh, 2019). Unfortunately, conclusive diagnosis of CTE is only accomplished with neuropathological examination of brain tissue (Mckee et al. 2015). CTE results from frontal and temporal lobe deterioration, thinning of the hypothalamic floor, shrinkage of the mammillary bodies, pallor of the substantia nigra,

enlargement of ventricle, and reduction of brain mass resulting from variable amyloid- β deposits, TDP-43 positive inclusions, and neurites (McKee et al., 2009). However, the most important cause of pathology is localized neuronal and glial accumulation of phosphorylated Tau (p-Tau) that start out as neurofibrillary tangles (NFT) before forming plaques and aggregates (McKee et al. 2009).

This disease has always been known to be associated with boxing and football due to the damage these sports cause with repetitive trauma to the brain; This trauma is what causes the accumulation of p-Tau that result in neurological deterioration (McKee et al. 2009). Though there is no set treatment for CTE, it's been said that applying traumatic brain injury (TBI) treatment in a time sensitive manner may provide a viable option for those that suffer from it, as well as implementation of preventative measures for athletes and military personnel (Fesharaki-Zadeh, 2019).

D. Alzheimer's Disease

Alzheimer's Disease's (AD) is most prevalent in the elderly, and accounts for 60-70% of all dementia cases (Grimaldi et al. 2018). Symptoms of AD are the same ones exhibited in those with dementia (Grimaldi et al. 2018). Diagnosis is currently based on cognitive assessments of patients presenting symptomatic features of cognitive and behavior changes, biomarker testing of cerebrospinal fluid, and MRI images of the brain (Grimaldi et al. 2018 and Hane et al. 2017). AD's hallmark is cytoskeletal alterations stemming from the formation of abnormal Tau protein structures in a few neuronal types in the CNS. These abnormal formations include the accumulation of amyloid β plaques extracellularly and hyperphosphorylated Tau intracellularly (Grimaldi et al. 2018). Their precursors are amyloid proteins and neurofibrillary tangles (Grimaldi A. et al. 2018). Neuronal loss for AD individuals begins 10-15 years prior to any cognitive impairment (Grimaldi et al., 2018). In terms of cognitive impairment that may ensue, those include behavioral and cognitive deficits (Grimaldi et al. 2018).

There isn't an effective treatment to slow or prevent AD, a lot of the treatments are tested to modify the progression of the disease targets, the accumulation of plaques; However, based on the failure of these treatments in trials its assumed that this route of treatment alone isn't effective (Hane et al. 2017). Amyloid β proteins are the precursors for the plaques that form, therefore

target treatments that inhibit β and α secretase essentially prevent this precursor protein's production, and in combination with other forms of treatment it can improve cognitive assessment and the presence of biomarkers. In turn, immunotherapy is another form of treatment. It utilizes antibodies targeted for disease causing Tau and certain conformations of amyloid- β to prevent the formation of aggregates and plaques and increase cognitive assessments (Hane et al. 2017). There are also non-pharmaceutical treatments that involve cognitive training for reading and speed that have shown to improve cognitive and functional abilities (Hanes et al. 2017). The main goal of treatment is essentially preventative because once these amyloid- β plaques and Tau aggregates cause neuronal degeneration, that neuronal loss cannot be regained again (Hanes et al. 2017). Thus treatment focuses on reducing the plaques and aggregates before they become neurotoxic (Hanes et al. 2017).

E. General Tauopathies

Tauopathies are a range of incurable and progressive age-associated neurodegenerative diseases (Coughlin and Irwin, 2017). Individuals with a tauopathy normally exhibit a range of symptoms that can be cognitive and/or motor which come in the form of memory or movement impairment (Coughlin and Irwin, 2017). Tauopathies are clinically and pathologically diverse, which makes it hard to diagnose; Therefore to definitely diagnose the specific tauopathy a person suffered from, an autopsy is needed to confirm clinical anatomical features (Coughlin and Irwin, 2017). These range of diseases are characterized by the aggregation of varying Tau isoforms that appear as filaments initially. These filaments are each tau molecule's adaptation of a β -conformation; The more they aggregate the more β -sheets of Tau stacks together in neurons and glia (Bandyopadhyay B. et al. 2007). A keystone tauopathy we know of is AD, however it also stretches to affect other neurodegenerative diseases such as PD and CTE that was previously mentioned Bandyopadhyay B. et al. 2007).

As already said tauopathies are incurable but continued research has shown promising results from treatment trials. One treatment technique, microtubule stabilizing agents, aimed to restore the loss of Tau's microtubule stabilization in tauopathies; Initial studies have been promising but have concerning downfalls like the central nervous system side effects that result from extensive exposure to stabilizing agents, like paclitaxel (Coughlin and Irwin, 2017). Tau

immunotherapy has become another area of interest in terms of therapeutics; Immunizing mouse models of different types of tau fragments has shown to improve safety and efficacy of reducing tau pathology (Coughlin and Irwin, 2017).

II. Tau Structure and Function

Tau is a protein that is heavily related to microtubules in neurons. Microtubules have very dynamic regions that play architectural roles in neurons and allow them to achieve and maintain exaggerated shapes, which is important for a neuron's communication to different parts of the body (Dent and Baas, 2014). Not only do they serve an architectural role, but they also serve a transportation role; Microtubules act as highways where molecular motor proteins convey cargo (Dent and Baas, 2014). Tau fits into the functional picture of microtubules because it is a microtubule associated protein that stabilizes and promotes microtubule polymerization in neurons (Drubin and Kirschner, 1986). Tau stabilizes microtubules by preventing depolymerization by slowing the rate of transition to the disassembling state down (Drubin and Kirschner, 1986). Primarily, to make a protein, the transcription of the Tau gene is essential. Tau is transcribed from the MAPT gene that's located on chromosome 17 in humans (Adams et al. 2010). Just like any other mRNA transcript, Tau mRNA must undergo processing before it is a translatable transcript. More specifically, alternative splicing is a processing technique that makes different variants of Tau. The nuclear RNA of Tau contains six different isoforms, due to alternative splicing; These isoforms are characterized based on whether they lack or contain exons 2,3, and 10 (Adams et al. 2010). Respectively, besides the other 3 microtubule-binding regions that are nascent to Tau, exon 10 codes for a microtubule-binding region that is similar to the other 3 (Adams et al. 2010). Therefore, Tau isoforms that contain exon 10 have 4 microtubule-binding regions (4R), and those that don't contain 3 microtubule-binding regions (3R) (Adams et al. 2010). Due to the alternative splicing process of Tau mRNA, proteins produced from different isoforms differ in primary structure because they contain different exons.

A. Primary Structure

The primary structure of the Tau protein is divided into an N-terminal and C-terminal region (Adams et al., 2010). There are 11 conserved residues in Tau that have been seen to be important to its role; M11, A152, K174, S214, T231, R279, K280, C322, N368, S409, and D421 (Avila et al. 2016). When a truncated Tau protein starts with methionine-11, it results in a change in the tertiary structure of Tau (Carmel et al. 1996). When lysine-174 is acetylated it results in the production of a toxic protein (Min et al. 2015). Serine-214's phosphorylation has a positive effect on the microtubule binding affinity of Tau (Scott et al. 1993). Threonine-231's dephosphorylation can potentially make the protein toxic because of its spatial effect on 2 proline residues next to it (Illenberger et al., 1998). Arginine-279's deamination could facilitate the self-aggregation of Tau (Dan et al. 2013). Loss of Lysine-280 and oxidation of cysteine-322 both encourage the formation of Tau filaments (Gorsky et al. 2016). Cleavage of Asparagine-368 results in toxic fragmentation (Zhang et al. 2014). Serine-409 has an affinity for the Rho kinase, which increases likelihood of Tau's phosphorylation; Phosphorylation decreases the likelihood of degradation and results in Tau's accumulation (Amano et al. 2003). Lastly, the truncation of aspartic-421 aids in Tau aggregation and toxicity (Basurto-Islas et al. 2016).

Tau is primarily expressed in neurons (Melo et al. 2016). The expression of Tau is strictly regulated because its different isoforms interact with microtubules in different ways (Panda et al. 2003). Since Tau lack hydrophobic residues, its characterized as a intrinsically disordered protein; Combining this with the fact that it contains microtubule-binding region repeats, it has the ability to bind to different heterodimers of tubulin at the microtubule lattice (Melo et al. 2016).

III. Post Translational Modification

Once a protein is translated and even before it folds into its proper conformation, it normally undergoes post-translational modifications. These chemical modifications can be reversible or irreversible additions of chemical groups, lipids, carbohydrates, even residues or polypeptide chains (Uversky, 2013). Just like splicing, PTMs represent a way of diversifying the different types of polypeptides that can arise from one mRNA transcript of a particular gene (Uversky, 2013). Reversible PTMs normally act as switches for proteins, turning it on so it

carries out its function and turning it off so it changes conformation and is unable to function; The addition or removal of a particular chemical group can play either role (Uversky, 2013). Though PTMs can occur at any stage in a protein's life, the time when a PTM is carried out determines what role it plays for the protein (Uversky, 2013). When it is carried out prior to the final conformation of a protein, its role is to alter the folding efficiency and stability of the protein and therefore determining where it will reside in the cell (Uversky, 2013). If the PTM is carried out after the protein has folded and has a determined location in the cell, its role is to activate or inactivate the function it's supposed to carry out (Uversky, 2013).

A. Phosphorylation

The function of Tau, which is the stabilization of microtubules of neurons through binding, is driven by the post translational modification (PTM) of its higher order structure. Notably, Tau kinases have 4 phosphorylation sites, Ser262, Ser293, Ser324, or Ser356 , all located in all four microtubule binding repeats; And when these sites gain a phosphate group it decreases Tau's binding affinity to microtubules (Drewes et al. 1995). There are three general groups of kinases: proline-directed serine/threonine-protein kinases, non-proline-directed serine/threonine-protein kinases, and protein kinases specific for tyrosine residues (Paudel et al. 1993; Dillon et al. 2020; Lee et al. 1998). The purpose of kinase phosphorylation correlates to Tau's role in stabilizing axonal transport. Tau binding to tubulin interferes with the axonal transport of motor proteins; and when certain motor proteins encounter Tau on a microtubule they fall off (Dixit et al. 2008). Therefore, phosphorylation ensures that tau out of the way of motor protein traveling on microtubules, preventing them from falling off (Alonso et al. 2004)

The regulatory function of the phosphorylation PTM not only requires kinase proteins, but also protein phosphatase. Protein phosphatase 2A accounts for the majority of the phosphatase activity for Tau (Gong et al. 2000). Through research it's been seen that not only can phosphatase 2A (PP2A) dephosphorylate phosphorylation sites, but it can also restore the ability of misfolded Tau protein to bind to microtubules (Wang et al. 2007).

Nonetheless, phosphorylation can become a dysregulated process resulting in hyperphosphorylation because of the downregulation of PP2A (Tsujiro et al. 2005). It was discovered that hyperphosphorylated Tau is a running cause for Alzheimer's diseased (AD)

brains; The normal brain contains Tau that has 2-3 moles of phosphate per mole of Tau, while an AD brain has 4-fold that amount (Kopke et al. 1993). Hyperphosphorylated Tau loses its function because it loses its affinity to microtubules while simultaneously promoting self-polymerization (Alonso et al. 2018). It's said that hyperphosphorylation must occur before aggregation starts because positively charged microtubule-binding domains, which inhibit self-assembly, acquire a negative charge when they are phosphorylated; This gives hyperphosphorylated negatively charged Tau an attraction to positively charge regions of other Tau molecules (Alonso et al. 2018).

B. Glycosylation

Another type of post translational modification that affects Tau is glycosylation which works in concert with phosphorylation, specifically hyperphosphorylation. Glycosylation involves the addition of a sugar normally to the asparagine residues of Tau (N-glycosylation) (Liu et al. 2002). Carbohydrates being polar entities and essentially negatively charged, has the potential to greatly affect the conformational dynamic of Tau and it shows. It has been found that the glycosylation of Tau occurs prior to hyperphosphorylation in Tau, therefore playing a role in its facilitation (Liu et al. 2002). This is because protein kinases have shown to have a greater affinity for glycosylated Tau than normal Tau (Liu et al., 2002). However, there are two distinct types of glycosylation that occurs in Tau; N-linked glycosylation which adds monosaccharides to phosphorylated Tau specifically, and O-linked N-acetyl- D- glucosamine that specifically adds sugars to normal Tau (Liu et al. 2002).

The normal process of N-linked glycosylation begins in the endoplasmic reticulum where a sugar is transferred to a polypeptide with the help of an oligosaccharyltransferase (Zielinska et al. 2012) . O-linked N-acetyl-D-glucosamine occurs with the help of a β -O-linked N-acetylglucosaminidase that is normally localized in the cytoplasm and has a similar dynamic to phosphorylation (Wells et al. 2001).

C. Ubiquitination

Ubiquitination is a protein modification that works to mark an unfolded or misfolded protein which then gets eliminated by a ATP-driven ubiquitin proteasomal system (UPS)

(Petrucci et al. 2004). CHIP (carboxyl terminus of Hsc-70 interacting protein) directly ubiquitinates Tau in the presence of a E2 conjugase (Petrucci et al. 2004). However, based on immunoreactivity experiments of AD and Pick's neurons, Tau lesions were found to be positive for ubiquitin, therefore correlating ubiquitination to the self aggregation of Tau, instead of its degradation (Petrucci et al. 2004)

D. N-terminal Acetylation

The least studied but most common post translational modification of proteins is N-terminal acetylation which occurs co-translationally with the protein (Arnesen et al. 2009). N-terminal acetylation involves the transfer of an acetyl group from acetyl-coA to an alanine residue of the N-terminal region of the protein where it becomes covalently bound (Arnesen et al. 2009). The protein enzyme responsible for this modification is called Nat A, it acts on an array of proteins with N-termini containing serine, alanine, threonine, glycine, cysteine, or valine in humans (Arnesen et al. 2009). However, it has been shown that the specific acetylation of lysine residue K280 both weakens Tau's ability to bind and stabilize microtubules as well as enhances Tau aggregation (Cohen et al. 2011). When positively charged lysine residues of the N-terminal region of Tau is acetylated, it neutralizes that charge and the resulting acetylated Tau will act similar to heparin and promote aggregation (Kamah et al. 2014). A Tau protein has a positively charged N-terminal region and negatively charged C-terminal region; So the acetylated species that only carries a negatively charged domain will have an electrostatic attraction to other positively charged N-terminal domains of unacetylated Tau proteins, therefore causing the generation of neurofibrillary tangles and aggregates (Rosenberg et al. 2008; Kamah et al. 2014). Not only does acetylation promote aggregation, but it also prevents the degradation of misfolded and aggregated Tau species by preventing the ubiquitination and subsequent stabilization of Tau by the UPS (Min et al. 2010).

On the other hand, N-terminal acetylation of Tau works in concert with other modifications such as truncation. Truncation of either the C-terminal or both regions of Tau result in the gain of a toxic function, self-aggregation. Even so, through experimentation, acetylation of truncated species of Tau at the Gln-124 residue have shown to bind and stabilize microtubules more than unacetylated species (Derisbourg et al. 2015).

IV. Aggregation

A. Higher-Ordered Structures

Though Tau is intrinsically disordered, lacking tertiary structure, it has the capability of forming quaternary structures like dimers, oligomers, and large polymers that are characterized as pathogenic Tau conformations through interactions with its microtubule binding domains. These interactions are made possible due to the cysteine residues that are present in the microtubule binding domain of the protein (Soeda et al. 2015). The biophysics and biochemistry research of Montejo de Garcini showed that tau filaments and fragments assemble in an anti-parallel arrangement using the microtubule-binding domains (1986). However, when the domains are not accessible due to it being bound to a microtubule, tau proteins still find a way to interact and form dimers using their N-terminal regions (Montejo de Garcini. 1986).

Before Tau forms quaternary structures it is hypothesized that there is a transitional mechanism that occurs prior, liquid:liquid phase separation. Liquid:liquid phase separation (LLPS) is the formation of membrane-less organelles that separates and compartmentalizes biological constituents, like proteins and reactions, which is essential for cells (Kanaan et al.,2020). LLPS of Tau has shown to be influenced by the electrostatic interactions of its domains, its post translational modifications, and interactions with tubulin (Kanaan et al. 2020). This suggests that Tau may have additional functions to stabilizing and promoting tubulin polymerization because it forms these membrane-less organelles, suggesting that maybe their aggregates do as well (Kanaan et al. 2020).

In vitro studies that form LLPS Tau using wild-type Tau and mutants Tau that represents pathogenic modifications of Tau at physiological concentrations show that disease-like modifications actually increase and enhance LLPS in vitro (Kanaan et al. 2020). When LLPS mutant Tau is incubated for prolonged periods of time it reduces the movement of soluble Tau in the droplets and increases the seeding and formation of specific pathogenic conformations Tau that are toxic prior to the generation of Tau filaments (Kanaan et al. 2020).

B. Formation

Structurally, what drives the formation of aggregates are 2 six residue segments: VQIINK at the start of repeat 2 and VQIVYK at the start of repeat 3 of the microtubule binding domain (Seidler et al. 2017). Through electron diffraction, atomic resolutions of fibrillary structures revealed that VQIINK segments form tighter steric zipper interfaces than the VQIVYK and adhere more to other Tau peptides than the VQIVYK as well (Seidler et al. 2017). Further evidence supports this adhesion because engineered Tau constructs only containing either segments showed that VQIINK constructs aggregate more rapidly than the wild type or VQIVYK constructs that produced a mixture of Tau species; Monomers, oligomers, and fibers (Seidler et al. 2017). Physiologically, when the zipper interfaces adhere to other Tau proteins it creates a Tau fibril containing a core composed of repeats 3 and 4 of the MTBD (Seidler et al. 2017). Majority of the core contained VQIVYK segments while the VQIINK segments coated fibril (Seidler et al. 2017). Based on the seeding experiments, VQIINK inhibitors were more effective at preventing seeding than VQIVYK inhibitors (Seidler et al. 2017). This led to the conclusion that both segments have separate specific roles that helps drive aggregation (Seidler et al. 2017). VQIINK zipper-interfaces, which coats fibrils, serves to be more accessible to Tau monomers promoting seeding, while VQIVYK zipper interfaces, which forms the core, serves as a solvent-excluded scaffolding clustering the fibrils together into a more compact shape, basically driving and stabilizing monomeric and quaternary structure formation (Seidler et al. 2017).

C. Description of Aggregated Tau Structure

The lowest-energy structures that form the varied possibilities of globular Tau structures have secondary structural elements; 11% helical and 21% antiparallel β structures (Popov et al. 2019). The overall globular structures are considered compact containing 4 difference subdomains that have a tetrahedral arrangement (Popov et al. 2019). The core of the structure contains multiple β structure elements (Popov et al. 2019). This core was able to form due to re-orientation of hydrogen bonds from intra-protein to inter-protein cross- β bonding, which results in the rearrangements of the N- and C-terminal and intermediate domains in a aggregated globular Tau structure (Popov et al. 2019).

D. Role of Oligomers

Tau oligomeric species are not only the source of cytotoxicity, as opposed to neurofilaments, but they represent the transient structure between monomers and larger higher order aggregates of Tau (Lasagna-Reeves et al. 2012). This is exemplified when the relevance of protein modification and aggregation is investigated (Lasagna-Reeves et al. 2012). Phosphorylation of specific threonine residues of Tau filaments and fibrils have led to their oligomerization (Lasagna-Reeves et al. 2012). Overall, the accumulation of oligomeric Tau results in behavioral deficits and neuronal loss preceding any formation of neurofibrillary tangles (Lasagna-Reeves et al. 2012).

E. Neurotoxicity of Aggregates

Intracellular aggregation of Tau is toxic due to its presence in neurons or glia when cognitive decline and neurodegeneration occurs (Bandyopadhyay et al. 2007). Based on co-immunoprecipitation of soluble Tau/tubulin and fibrillary Tau/tubulin, only soluble Tau coprecipitates with tubulin; This shows that Tau aggregation directly affects Tau function by preventing Tau from binding to tubulin (Bandyopadhyay et al. 2007). With loss of tubulin stability, microtubule cytoskeleton structures of neurons can't properly carry out functions like transportation of certain molecules along axons, mechanical support, and organize the cytoplasm. Intracellular Tau aggregates decrease cellular viability; In the presence of a nuclear stain ToPro3, a dead cell indicator, Tau aggregated cells prove to be the only cells that stain compared to cells with soluble Tau (Bandyopadhyay et al. 2007).

Besides the obvious effect on neuronal axons and transport, aggregates exhibit their toxicity by the harmful effects it can have on the genome of a neuron when present in the nucleus (Niewiadomska et al. 2021). Tau contains a DNA binding domain and can form protein-DNA complexes. When dysfunctional or pathogenic Tau enters the nucleus, it may disrupt chromatin organization leading to cell cycle re-entry and dysregulation of gene expression which will bring about more faulty protein (Niewiadomska et al. 2021). With genetic defects ensuing, it will eventually lead to the degradation of the Ubiquitin proteasomal system and further accumulation of aggregated misfolded Tau proteins (Niewiadomska et al. 2021). Additionally, Tau oligomers

can interact with the mitochondria directly and is internalized in its membrane, inducing changes in its adhesion (Niewiadomska et al. 2021). This causes energy impairments in neurons which is essential in axonal terminals and synapses (Niewiadomska et al. 2021).

Aggregates of Tau are not spatially limited species, considering their varied size and shape. Apart of the toxicity of these aggregates is its ability to propagate to other cells (Kfoury et al.,2012). Cell-Cell propagation of aggregates is facilitated by the uptake of Tau fibrils from the extracellular space; Tau fibrils are perfect size to swiftly pass through an impermeable cell membrane and enter a cell (Kfoury et al. 2012). Since Tau fibrils are the precursors to the formation of oligomeric and larger Tau aggregates, everytime a fibril enters a cell a aggregation inducer is present in a cell that expresses Tau. Because these pathogenic Tau species can spread from neuron to neuron it can cause widespread neuronal death and degeneration which drives the progression of neurodegenerative diseases.

V. Relevance of Studying the Effects of Tau's N-terminal Acetylation

This project addresses what effects N-terminal acetylation has on Tau structurally and functionally, based on possible changes in aggregation and microtubule binding affinity. Previous research has shown that N-terminal acetylation does affect Tau's propensity to form aggregates and therefore affect its function (Derisbourg et al. 2015; Fanni et al. 2019). However there has not been much research addressing the specific structural effects N-terminal acetylation has that leads it to the formation of a new conformation that leads to possible aggregation and effects on Tau function.

The hypothesis of this project is that N-terminal acetylation changes the conformation of Tau in a way that it promotes aggregation and decreases function.

To test this hypothesis different experimental techniques will be used such as NMR spectroscopy; It will assess the structural changes of the unacetylated versus acetylated species of tau based on the changes in the magnetic spin properties of the atomic nuclei that are bonded or in close range to each other due to protein folding (Howard et al.1998). Another spectroscopy

technique, Circular dichroism (CD), will provide a visual of the secondary composition of unacetylated and acetylated species of tau, and whether more helices or β sheets form as a result of acetylation; considering the globular structure of Tau contains transient secondary elements (Micsonai et al. 2015). These structural analysis will be done on Tau 1-100 protein fragments, which represents a snapshot of the N-terminal region of Tau alone, and Full Length Tau representing the entirety of the protein acetylated and unacetylated. A protein aggregation assay will be used to evaluate the propensity of Full length Tau to form aggregates, with and without N-terminal modification.

The different species of Tau aggregates can then be visualized using Native Gel electrophoresis. This electrophoresis technique allows for a protein to remain in its natural nascent structural state as it travels through a polyacrylamide gel without denaturing compared to a normal SDS-PAGE technique (Nowakowski et al. 2014). Native Gel electrophoresis will visualize the aggregates that naturally form when Tau is acetylated versus unacetylated, and whether or not aggregates are soluble depending on how the differing samples run through the gel. To correlate structure with function, experimental assays such as microtubule binding and tubulin polymerization assays will assess the effect these structural changes of acetylation has on Tau's primary function (Huda et al. 2017; Sun et al. 2012). All of these experimental techniques will tie in together to tell a story about the effect N-terminal acetylation has on Tau as specific as the neighboring residue interaction changes, to secondary composition, to the formation of aggregates, and lastly function.

MATERIALS AND METHODS:

Recombinant DNA

HB45: Nat A DNA in Pet15 Vector

HB75: Tau 1-100 DNA in PET22 vectors with C-terminal His-tag and Ampicillin cassette

HB76: Full length Tau DNA in PET22 vectors with C-terminal His-tag and Ampicillin cassette

Cloning Process

Primers were designed for Tau 1-100 and full length Tau DNA constructs that include designated Nde1 and Xho1 cut sites that correlate with the same cut sites in the PET22 vector. With the use of RT-qPCR, primers were added to the ends of Tau 1-100 and Full Length Tau constructs in a manner where Nde1 cut site was appended to the beginning of the construct and Xho1 cut site to the end of it, while simultaneously amplifying the number of Tau construct copies. The PCR reaction consisted of 5 μ L of OneTaq 10x buffer, 2 μ L of dNTP, 2 μ L of the engineered forward primer, 2 μ L of the reverse primer, 38 μ L of water, 0.5 μ L of Q5 polymerase, and 0.5 μ L of either full length Tau construct and Tau 1-100. The RT-qPCR software settings were 3 minutes and 30 seconds at 96 °C, 30 seconds at 55 °C, and 72 °C for 3 minutes.

PCR products of Tau 1-100 and full length Tau DNA were transformed with Top 10 *E. coli* cells, cells competent for DNA replication. Transformed cells were grown on ampicillin luria broth agar plates. Colonies from transformation plates were grown up into 3 mL cultures made with 3 mL of liquid Luria broth and 3 μ L of ampicillin antibiotic in order to increase the amount of usable DNA for the ligation and digest screen process. A Thermo Fisher Scientific DNA miniprep kit was used to extract Tau 1-100 and Full Length DNA from Top 10 cells (ThermoFisher, 2021).

To clone the constructs into the Pet22 vector, both Tau 1-100 and full length Tau constructs and Pet22 vectors were digested. 1 μ L of both Xho1 and Nde1 enzymes each, 5 μ L of 10x Cutsmart Buffer, 15 μ L of the designated construct or vector, and 28 μ L of water was mixed together, and incubated at 37 °C for 45 minutes to an hour. Tau 1-100 and full length Tau digests were ligated with digested Pet22 vectors at the appropriate Xho1 and Nde1 cut sites. To determine the ratios of construct to pet22 vector, calculations were made using NEBcalculator. The most successful being 7:1 for full length Tau and 5:1 for Tau 1-100. The ligation solution for full length Tau's most successful ligation into the pet22 vector contained: 1 μ L of digested vector, 3 μ L of digested full length Tau construct, 2 μ L of ligation buffer, 13.5 μ L of water, and 0.5 μ L of ligase buffer. The ligation solution for Tau 1-100 was: 1 μ L of digested vector, 2 μ L of ligation buffer, 0.5 μ L of ligase buffer, 1 μ L of digested Tau 1-100 construct, and 15.5 μ L of water. Both solutions were incubated at room temperature for several hours. Ligation mixtures were then transformed into Top 10 cells, where the cells had the plasmids extracted using the Thermo Fisher Scientific DNA

miniprep kit, digested, and visualized by a 0.8% Agarose DNA Gel. When Tau 1-100 and full length Tau successful cloning into Pet22 vectors was confirmed by the agarose gel, stocks of transformed Top 10 cells of successful ratios were made and stored in the -80 °C freezer.

Single and Double Transformation

For a single transformation, HB75 or HB76 were transformed with DE3 *E. coli* cells, cells competent for protein expression. 50 µL of competent cells and 2 µL of either construct was mixed together for a total of 52 uL. If it is a ligation transformation, 10 µL of incubated ligation mixture is mixed with 50 µL of competent cells (Top 10) for a total of 60 uL. The mixture is left on ice for 30 minutes and heat shocked at 37 °C for 90 seconds. Transformed heat shocked cells were diluted with 250 µL of luria broth, and the dilution was shaken for approximately 60 minutes at 37 °C, 200 µL of the dilution was spread on LB ampicillin agar plates. The plates were incubated at 37 °C overnight or at room temperature for 48 hours.

For a double transformation, HB75 or HB75, and HB45 were transformed with DE3 cells. In this case, 50 µL of competent cells was mixed with 2 µL of either Tau constructs and 2 µL of the HB45 construct, for a total of 54 uL. The same procedure from the single transformation is followed for the double transformation, except a double antibiotic LB agar plate is used.

Induction Test

To confirm that transformed DE3 cells can produce the desired Tau protein, colonies from single or double transformations were grown up in 3 mL of luria broth and 3 µL of ampicillin overnight. 1 mL of the overnight was diluted with 4 mL of luria broth and 4 µL of ampicillin and allowed to grow for approximately an hour. Once the O.D. reached between 0.2 A and 0.4 A, 5 µL of IPTG was added to the cultures and allowed to incubate for a 1 hr or 24 hr induction. Induced cells were visualized using a 15% SDS-PAGE. A previous student's work has shown that Tau is 80% acetylated when double transformed and induced DE3 cells are analyzed by mass spectroscopy.

Harvesting Large Scale Inductions

From a 3L to 4L overnight induction for both the single and double transformations of either Tau constructs, 6 250 mL centrifuge tubes are filled with the overnight induction and spun at 10,000 RPM for 10 mins in a Sorvall centrifuge. The supernatant was removed leaving the pellet and any remaining overnight was poured into the centrifuge tubes and spun again. It takes a total of 3 spins to pellet all the bacteria from a 4L induction. Pellets were washed with 75 mL of water using a spatula and 25 ml pipet. Resuspension fluid from all 6 tubes were pelleted into 2 250 mL centrifuge tubes. The supernatant was removed and the pellets were resuspended in 25 mL of water each. The resuspension fluid was transferred to 2 50 ml conical tubes and spun at 10,000 rpm for approximately 20 minutes. The supernatant was removed and the pellets were stored at -20 C.

His-tag Purification

To purify expressed His-tagged protein out of frozen pellets, a FPLC (Fast Protein Liquid Chromatography) technique was used. Lysis buffer containing 50 mL of Buffer A (50 mM NaCl 1M Tris pH 9.0), 1 protease inhibitor tablet, and 250 μ L of PMSF (0.04 mg/ μ L) was used to resuspend 2 frozen pellets, 25 mL for each pellet. Each resuspended pellet was sonicated at 80% amplitude, with 15 second pulse and 15 second rest for a total of 1 minute and 30 seconds. After sonication, centrifuge tubes containing sonicated resuspension were boiled for about 15 minutes in a water bath erlenmeyer flask until the temperature reached about 75-85 C. Once complete, both tubes were immediately placed on ice for 15 minutes, then centrifuged in the Sorvall at 18,000 rpm for 45 minutes at 4 C. The supernatant from both tubes was carefully removed. If there was an excess of white precipitate in the boiled lysate supernatant, then the supernatant was filtered through a 20 micron filter on a luer lock syringe into a clean 50 mL conical tube. Before using the FPLC, the UNICORN start software was booted and the His-Tag purification protocol uploaded. The sample volume was adjusted to about 1-2 mL less than the total amount of boiled lysate. The sample inlet was placed in the 50 ml conical tube of lysate. 3 conical tubes was used to collect the flow through, wash, and elution. The output inlet had to manually moved into each conical tube throughout each stage of the chromatography purification once the FPLC was started. On the elution stage, the UV was monitored in order to gage when to move the output inlet into the elution conical tube. When the UV started to peak 2-3 units that's when the inlet

was moved to the elution tube, and once the UV returned to the baseline, the output inlet was placed in the waste beaker to prevent dilution of the pure protein collected.

TCA Precipitation

The eluate was transferred to a 15 ml conical tube and diluted with lysis buffer to a volume of 10 mL and put on ice. 4.5 grams of ammonium sulfate was added gradually into the 15 ml conical tube as it rotated in a 4 °C fridge. It was then left to rotate overnight. The next day, 1 mL of the sample was aliquoted into 12-14 microcentrifuge tubes each. The aliquots were spun at 13,000 rpm for 60 minutes at 4 °C. The supernatant from each tube was removed and aliquots were dried for 30 minutes at 95 °C and stored at -20 °C.

Real Time-PCR Aggregation Experiment

Solution preparation for this assay included making: Aggregation buffer (1 M Tris pH 7.4, 4 M NaCl, 292 mM EDTA), 100 mM DTT, 3 mM Thioflavin T, 5 uM Heparin. Control and Tau protein preparation involved making BSA (4mg/ml in aggregation buffer), and solubilizing Tau protein pellets in 1 ml of 50 mM Tris pH 8. Using a 96 well plate, each well contained a 200 µL reaction: 100 µL of protein, control BSA or Tau, 2 µL of Thioflavin T, 2 µL of DTT, 20 µL of Heparin, and 76 µL of aggregation buffer. The overall assay took 20 minutes total while being held at 25 °C with fluorescence readings of 454 nm using the Real Time-PCR.

Dialysis Aggregation Experiment

All the eluent sample for an FPLC run was pipetted into a dialysis tubing bag, sealed on both ends, and then placed in an Erlenmeyer flask of 0.1M phosphate buffer pH. 7.0. The dialysis bag was spun with a stir magnet for 1 hour. Every hour, the phosphate buffer in the flask was exchanged with fresh buffer. After about 4 hours, the dialysis bag was left to stir overnight.

The next day, a sample of the white visible aggregates from the dialysis bag were placed on a slide and visualized under a confocal microscope.

Tubulin Polymerization Assay

Solution preparation for this assay was all made on ice and included making: G-PEM buffer (80 mM PIPES pH 6.9, 2 mM MgCl₂, 0.5 mM EGTA, 1 mM GTP, 5% glycerol), 100 uM of positive control paclitaxel, overnight solubilization and dilution of acetylated and unacetylated Tau protein to a 10X concentration, 10 uM to 40 uM, and 4 mg of tubulin solubilized in 1 mL of G-PEM. Using a 96 well plate, each well contained a 110 µL reaction: blank (110 µL of general tubulin buffer), negative control (10 µL of general tubulin buffer and 100 µL of tubulin), positive control (10 µL of paclitaxel and 100 µL of tubulin), Tau (10 µL of 10x Tau and 100 µL of tubulin), and acetylated Tau (10 µL of 10x A-Tau and 100 µL of tubulin). All well solutions were added and incubated at 37 °C, except for the tubulin until the 96 well plate was placed in the plate reader. Using a Biosystems plate reader, measurement kinetics were set to 60 cycles consisting of 1 reading every 30 seconds, absorbance wavelength was set at 340 nm, and it was set to shake once at the start of the reaction for 5 seconds.

RESULTS:

Cloning of Tau Constructs into Pet22 Vectors

Our initial goal was to utilize affinity chromatography to purify the Tau protein. This required Tau constructs with an appropriate affinity tag and we chose the commonly used His tag found in the vector pET22b (Fig. 1A). Tau sequences were amplified by PCR utilizing primers with Xho1 and Nde1 cut sites(Fig. 1B). We produced two constructs; the entire Tau gene (FL-Tau) and the first 100 amino acids (Tau 100). Both PCR amplified fragments were digested with the restriction enzymes Xho1 and Nde1, as was the Pet22 vector to produce complementing sticky ends. Next, in order to ensure proper ligation of PCR fragments into the vector, different ratios of digested vector, PCR product, and ligase were transformed into competent *E. coli* cells. To ensure proper insertion, resulting colonies had their plasmids purified and were subjected to restriction digest with Xho1 and Nde1. Plasmids with a correct insert for Tau100 would produce

a digested fragment around 500bp (Fig. 1C) and FL-Tau would produce a fragment around 1000bp (Fig. 1D), Taken together, these results suggest we have successfully cloned both Tau constructs into a vector that will add a C-termini His-tag to protein.

In order to ensure the ligation, or cloning, of PCR constructs into the vectors, a ligation mixture of digested constructs and vector, and ligase was transformed with *E. coli* cells and digested to screen for the Pet22 vector and Tau 1-100 construct. Digest screens of colonies from transformations showed that a specific ratio of construct and vector is required in order for the Tau constructs to ligate into the Pet22 vectors. For Tau 1-100 a ratio of 5:1 presented the best results in the screen; This is the only ratio that showed the most colony growth, and lanes that presented the correct base pair length for both the Pet22 vector and Tau 1-100 construct when visualized by an agarose gel (Fig. 1C). Out of the 8 colonies that were selected and screened, the 5:1 ratio represented half of those colonies (Fig. 1C). Out of the 4 colonies, 2 of them showed the correct base pair for the vector and construct; A red arrow points out the correct Tau 1-100 fragment for context (Fig. 1C).

For the full length Tau construct, a ratio of 7:1 is the only ligation ratio in the transformation because the previous full length Tau construct that was used before this nickel column purification approach, was cloned using that same ligation ratio. With this, out of the 8 colonies screened, 3 of those colonies had the appropriate vector and full length construct base pair (Fig. 1D). This means not only was there a high number of colonies to screen on the agar plate, demonstrating significant growth, but close to 40 percent of those colonies contained the full length Tau construct in the Pet22 vector; A red arrow points out the correct full length Tau fragment for context (Fig. 1D). Overall, the specific ratio needed to clone Tau 1-100 into a Pet22 vector was 5:1 (HB75), and for the full length Tau construct it was a standard 7:1 (HB76).

Transformation of Newly Cloned Tau constructs (HB75 & HB76)

Once the Tau constructs were successfully cloned into the Pet22 vector with a C-termini His-tag (HB75 and HB76), its transformation and induction capability needed to be tested in order for it to be possible to make large amounts of His-tagged Tau protein with transformed *E. coli*. The single transformations that used HB75 and HB76 plasmids alone showed significant

amounts of growth on ampicillin luria broth agar plates (Fig. 2A and B). The double transformations that used HB75 or HB76 plasmids with the HB45 plasmids, showed less growth on the luria broth ampicillin and chloramphenicol agar plates (Fig. 2C and D). These results can be explained by the decreased probability of bacterium uptaking not only 1 but 2 different plasmids. In general, transforming *E. coli* with newly cloned Tau constructs is possible, and therefore promising for the intentions of making His-tagged Tau protein for the nickel column purification.

Induction Test of Transformed *Escherichia coli*

Transformed *E. coli* cells with HB75 and HB76 underwent a small scale 5 mL induction to test their inducibility. Tau's molecular weight ranges from 45-110 kDa and we know Tau 1-100 is an extremely small protein (Fischer and Baas, 2020). As demonstrated by the red arrow it was deduced that a 1 hr and 24 hr induction with IPTG shows the presence of full length Tau at approximately 70 kDa and Tau 1-100 at approximately 11 kDa (Fig 3). Conclusively this insight showed us that when large scale inductions take place at volumes like 3 to 4 L, inductions should be held for a duration of 24 hours in order for full length Tau and Tau 1-100 to be expressed and accumulate in transformed cells.

Large Scale Induction and Purification of His-tagged Tau

With transformation and inducing capability established, large scale inductions of both Tau constructs were carried out to start the production process of acetylated and unacetylated Tau protein. Tentatively, Tau protein expressed with the C-termini His-tag will undergo a series of steps that are crucial to separate Tau from any of the cellular components that aided in its expression. The specific combination of steps from sonication to elution provide cleaner and cleaner samples of pure Tau as it progresses through each step, with the cleanest being the elution step (Fig. 4A and D). The purpose of the sonication step is to physically open up the cells in order to access the cytoplasm where the expressed tagged Tau protein is. Though sonication

provides access to the Tau protein it doesn't necessarily separate the protein from the crude components of *E. coli*.

The hallmarks of this purification are the boil, elution, and centrifuge spins between each step up until nickel column purification. The boiling step takes advantage of the intrinsically disordered structure of Tau. One would expect that when a protein is boiled it should denature and lose its shape, however because soluble Tau endogenously doesn't have a structure or shape, it will remain unaffected when boiled, and majority of the other proteins of *E. coli* will denature out of solution. With the centrifuge spin, those proteins will separate from the supernatant of mostly soluble Tau and therefore represent the first progressive step towards obtaining pure Tau. Looking at both SDS-PAGEs of the Tau constructs' purifications, the clear difference in purity is evident comparing the sonication and boil lanes (Fig. 4A and D). The only prominent protein bands present in lysate samples after boil and centrifugation carry molecular weights of around 70 kDA and less than 11 kDA (Fig. 4A and D). Those molecular weights validate and confirm the deduced masses of Full length Tau and Tau 1-100 in the induction test (Fig 3).

Comparatively, looking at the elution lanes of both constructs, there is an increased lack of faint protein bands surrounding the even more prominent approximate 11 kDA and 70 kDA protein bands (Fig. 4A and D). This suggests that between the boil step and the elution step, the majority of the proteins that actually attached to the nickel column were the His-tagged Tau proteins, which results in higher pure protein output (Fig. 4A and D).

Focusing more on how much pure protein output results from using this nickel column purification technique, induction volume has shown to be of major significance. Inducing cultures transformed with HB75 for 24 hours at higher volumes, provides higher output of pure protein. This is shown by the difference in integrated elution peaks of a 3 L induction nickel column chromatograph compared to the 2 L induction chromatograph during high %B elution (Fig. 4B and C). The 3 L Tau 1-100 induction has an integrated peak that was 3.19 units greater than the 2 L induction (Fig. 4B and C). The same trend applies for the full length Tau, 3 L induction's integrated elution peak was 3.15 units greater than the 2 L induction (Fig. 4E and F). Since integration represents the retention of these elution peaks in volume, higher integration means higher volumes of pure protein collected after elution.

Tubulin Polymerization Experiment

We next wanted to assess the function of the purified acetylated and unacetylated Tau. To achieve this, we utilized a tubulin polymerization assay. Briefly, we monitored tubulin as it polymerized into microtubules, since polymerized microtubules will absorb light at 340nm. Paclitaxel is a known tubulin polymerizing agent, and we were successful in observing tubulin polymerization changes in terms of O.D. and rate overtime when polymerization is actively occurring over a 30 minute time frame (Fig. 5C and D). Tau will also induce tubulin polymerization and our initial characterization shows that there is a slight increase in the rate of polymerization over control (Fig. 5C). Interestingly, when comparing the performance of acetylated and unacetylated Tau exclusively, acetylated Tau showed to increase the rate of tubulin polymerization more than unacetylated Tau, therefore promoting tubulin polymerization (Fig. 5C). Though this comparison is not statistically significant it is enough to be an adequate preliminary result

While there was an increase in tubulin polymerization, the response to the purified Tau is lower than expected. A number of inferences can be inferred from these results: there is not enough soluble Tau present, Tau protein in the soluble sample fragmented during solubilization preparations, or the Tau protein aggregated before it was added to the wells. We resolved a sample of the solubilized Tau by SDS-PAGE and based on the visualization there were protein bands of molecular weights lower than 70 kDa present in the samples used in the tubulin polymerization (Fig. 5E). This validates that fragmentation and therefore low concentrations of Tau are the most plausible explanations for the lack of change in tubulin polymerization when tubulin is in the presence of our pure Tau samples (Fig. 5E). Overall, these polymerization results show that the assay does work, however, it does need some modifications in terms of Tau sample preparation.

Aggregation Experiments

We then wanted to see if our purified Tau could be used to observe aggregate formation in vitro. Using the purified Tau, we performed preliminary aggregation experiments to evaluate the aggregation behavior of Tau. We utilized a dye, Thioflavin S, that specifically binds and fluoresces when it binds to aggregated Tau fibers. We then observed changes in fluorescence over time to assess aggregation rate. We predicted that aggregation of purified Tau could be followed over an hourly time scale at a microgram concentration. Initially we utilized heparin to induce aggregation and found that aggregation occurred over a shorter time scale (Fig. 5A). We next asked if aggregation would take place without heparin to assess Tau's innate ability to form aggregates. Visible aggregation occurred over after 24 hours without the need of an aggregation inducer at milligram concentrations of acetylated Tau protein which was not seen in the control protein (BSA) (Fig 5B.) In all, initially it can be deduced that the Tau protein, whether acetylated or unacetylated has a high propensity to aggregate in the presence and without an aggregation inducer like heparin.

DISCUSSION:

Relevance of the Single and Double Transformation Technique using a Prokaryotic System

Bacteria don't perform post translational modifications on their proteins as often as eukaryotic organisms do; They are essentially only carried out when it's needed for the bacteria's adaptation to an environment (Macek et al. 2019). Majority of the modifications actually carried out are so minute that they are difficult to see any effect by structural or functional analysis (Macek et al. 2019). This only speaks to the innovation behind using a prokaryotic system to produce large scale amounts of the Tau protein with and without N-terminal acetylation, and this all starts with the transformational capability of a prokaryotic system like *E. coli*. A laboratory DNA transformation of *E coli* depends on chance. Where it really counts is at 2 important points in the transformation procedure, the heat shock step and the growth of transformed cells on agar plates. It's been assumed that using competent cells that allow for the absorption of a desired

plasmid through the plasma membrane with the help of Ca^{2+} ions and adding a heat shock step ensures that the DNA is taken up into the cytosol of the bacterium (Rahimzadeh et al. 2016).

But to ensure that competent cells transform successfully and can hold onto the plasmid over generations of progeny, they have to be grown on agar plates that contain the antibiotic that the desired plasmid has an encoded resistance for. Successfully transformed bacteria have to be able to successfully fulfill two of these requirements to be culturable and therefore inducible to make large quantities of acetylated and unacetylated Tau protein, which is to be used in different assays. What was described was the chances of a successful single transformation, therefore the successful artificial double plasmid transformation of *E. coli* requires double the amount of successful fulfillment of the 2 requirements. Other protocols have shown that the success frequency of a single plasmid transformation is 4-6 fold greater than the frequency of a double plasmid transformation (Weston et al. 1979). This can explain the low numbers of colonies found in the double transformation plates compared to the single transformation (Fig. 2).

Importance of Induction Method

Knowing that single and double transformations are possible, the next big accomplishment was the induction step. This is probably one of the most crucial steps in the whole process of protein production because this defines how much pure protein we were going to get in the end based on the nickel column purification method, think of it as the “rate-limiting step”. Through previous trials of varying induction volumes we concluded that high induction volumes yield higher pure protein yield. The chromatograph integrated elution peaks showed that the higher the integrated value, the higher the volume of protein coming out of the inlet during the elution step (Fig. 4). There have been many protocols published that have proven that high density cell cultures, referring to high O.D., provide high yield protein (Sivashanmugam, 2009). However, there are many challenges that come about with using high density cell cultures and those are: the structural features of the gene sequence being expressed, the stability and translational efficiency of mRNA, protein folding, degradation of protein by host proteases, and codon usage toxicity of the protein to the host (Sivashanmugam, 2009).

Now the challenges that specifically pertain to full length Tau and Tau 1-100 is protein folding and degradation of the protein by the host proteases. If full length Tau were to be made by every cell in a high cell density culture then there will be an increased accumulation of Tau in the culture. Considering how intrinsically disordered Tau is, and purification issues that arose due to spontaneous aggregation when concentrating high outputs of Tau after elution using dialysis or a concentrator tube, one can safely assume that Tau would form aggregates and precipitate when expressed in a high density culture before it were to undergo purification. This could therefore activate the proteases in the host cells that would degrade Tau and get rid of the aggregates. As for Tau 1-100, because it is technically only a piece of the Tau protein being expressed, the cell may recognize it as a mistranslated protein fragment and activate the proteases causing its degradation. Therefore to counteract the high O.D. effects of induction, higher volumes of culture are induced overnight; It proves that these higher volumes provide higher yields of protein, based on integrated elution peak values (Fig. 4).

Purification Technique

Proteins expressed in induced cultures need to be purified out in order for it to be useful and provide clean results for structural and functional analysis. One of the main structural analysis techniques that would have been used to study unacetylated and acetylated Tau is NMR analysis. NMR spectroscopy (Nuclear Magnetic Resonance spectroscopy) has played a major role in monitoring the formation of distinct aspects of protein structure; It fulfills this role through its ability to analyze the distributions of hydrogen and deuterium in readily changing sites in proteins (Dobson et al. 1998). The quality of the structures produced by NMR is comparable to that of x-ray crystallography; However, structural quality is dependent on the extent and quality of the data that can be obtained, which depends on the purity of the protein sample (Montelione et al. 2000). This is why the type of purification used to purify Tau is critical and important, because it determines what the results of assays and analysis will look like. Due to the design of the DNA constructs induced in *E. coli* bacterial cultures, the Tau protein expressed has a His-tag on the C-termini. This affinity tag has a very high affinity for Nickel, which is what the column used in the chromatography step is made of. Passing lysates of the affinity tagged Tau protein over the column ensures that when a high salt buffer is used to

dislocate material attached to it, the only thing that elutes off of the column is the Tau protein. It's shown that using this affinity-tag purification technique to purify protein out of crude samples is highly efficient; Specifically using the His-tag technique provides good yields even when using an inexpensive antibody purification practice (Lichty et al. 2005).

One of the major concerns we had with the affinity-tag technique is that once the pure protein is extracted from the bacterial culture, we were not sure what the His-tag's effect would be on the protein's functionality and structural conformation. Assays performed on a similar intrinsically disordered protein, FUS with the His-tag, showed some differences in results with the tag versus no tag at all (Bock et al. 2020). Liquid-liquid phase separation experiments using tagged and untagged FUS showed that the His-tag enhanced the propensity of the protein to phase separate, acetylated or unacetylated (Bock et al, 2020). Withal, attempts were made to cleave off the His-tag using a TEV protease enzyme, since the Tau-tagged protein carries a TEV epitope between the C-termini and the His-tag. However, commercially available TEV protease is not at a concentration that it would be effective at cleaving off the tags on all the proteins in our highly concentrated eluent. Alternative efforts turned to producing TEV protease in the lab, however the TEV protease plasmid on hand was not inducible in transformed DE3 *E. coli* cells.

On the concentrating end, eluent concentration was successful. High salt elutions of Tau-tagged protein was TCA precipitated, which allowed for the aliquotation of protein into large quantities of individual microcentrifuge tubes, which on average reached about 0.5-1 mg/ml of protein for each tube.

Experiments

Using pure Tau from the nickel column purification, assays were still carried out for preliminary results. The first aggregation assay was an unintentional method of forming precipitates of aggregated Tau based on concentrating high salt eluent of Tau using dialysis. The less salt and imidazole present in the dialysis bag results in closer interactions between Tau-tagged proteins. Based on the globular structure of Tau, the prion nature of the protein allows for more and more scaffolds to form between Tau proteins as well as the formation of larger aggregates the longer it dialyzes (Fig. 5B).

Using the tubulin polymerization assay to look at the hypothesis from the functional perspective, initial results were not significant. However, there is a fold increase difference in the rate of polymerization between the acetylated Tau and unacetylated Tau protein. Acetylation seems to promote the tubulin polymerization ability of Tau. It is safe to assume there is a possibility that significant results were not attained because of the concentration of Tau being used. Through consistent probing of TCA precipitated Tau protein, aliquots did not contain the same amount of protein. The concentrations ranged from 12-40 uM, this could alter results from trial to trial when new aliquots of protein are being used for each trial assay. Furthermore, based on the SDS PAGE of the Tau samples used for the assay, Tau isn't the only protein present in these samples (Fig. 5E). Proteins of lower molecular weights are in the samples, and those protein bands could represent fragments of Tau resulting from mistreatment of solubilized samples. This theory can potentially be supported because visualization of another solubilized TCA precipitated sample showed distinct amounts of smearing that could be degradation (Fig. 4D). Those lower kda protein bands can also represent proteins from the crude lysate sample that was not purified from the Tau protein using the Nickel column in the purification.

Future directions

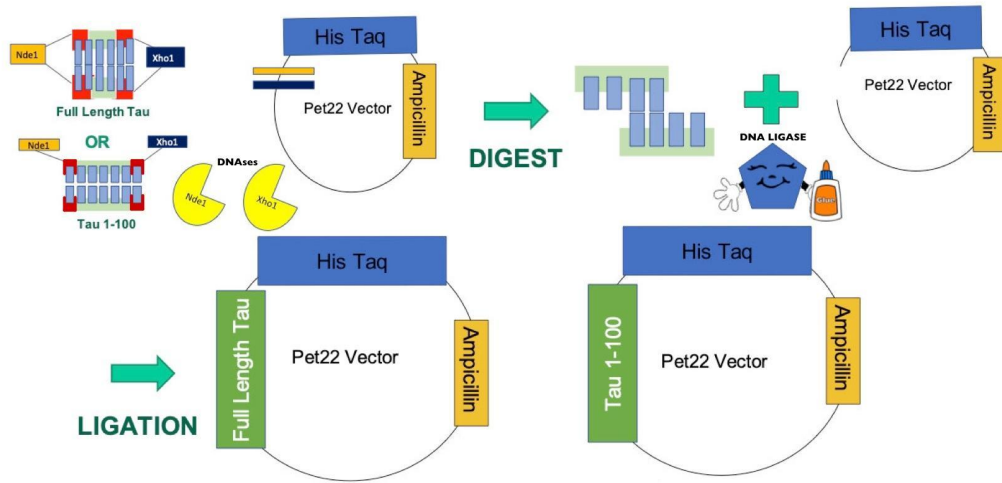
The purification methodology used to obtain pure Tau needs to be modified to bring about consistent and clean samples of protein that can be used in the polymerization assay and future experiments. Modifications include doing 2 runs of the chromatography purification step; The first run will be done using the nickel column and the second run will be done using an ion-exchange Column. The His-tag on the Tau protein contains positively charged histidine residues that will bind to the ion-exchange column differently than the other remnant proteins in the sample that may have had some affinity to the nickel column. So when proteins elute off of the Ion-exchange column they will do so at different times based on their difference in charge and therefore affinity (Nakatani et al. 2012). These proteins will be detected through the UV detector. Fractioning samples during the elution step from different UV peaks at different time points, and visualizing those samples by SDS-PAGE can feasibly display a certain UV fraction that only contains the acetylated or unacetylated Tau protein band. Then based on the SDS-PAGE,

identifying the time point where the Tau protein alone elutes off of the Ion-exchange column could provide cleaner and highly concentrated samples of pure Tau.

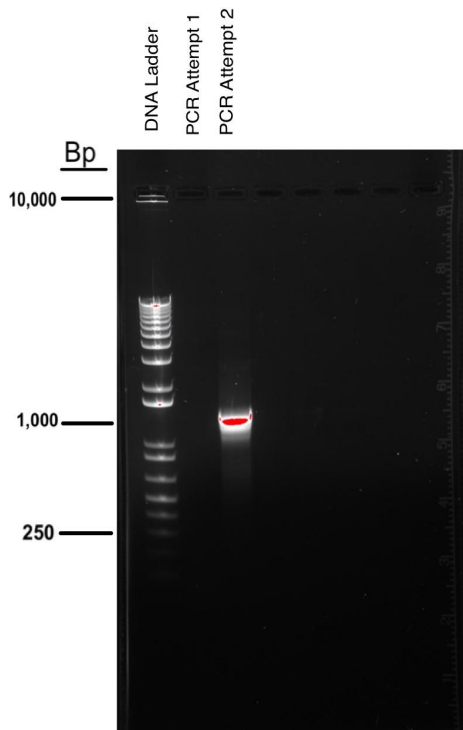
Continuing to troubleshoot the His-tag removal step using a TEV protease is significantly necessary for our pure Tau 1-100 protein, rather than full length Tau. Tau 1-100's purpose is primarily for the structural analysis of the N-terminal region of the Tau protein, so with a positively charged tag attached to such a small protein it could drastically affect the NMR and CD analysis that could potentially be done. These types of analysis are primarily based on the chemical properties and charges of residues neighboring each other (Howard et al. 1998). In terms of the full length Tau, using the TEV protease on this protein is not entirely necessary, because the His-tag is not near the microtubule binding domain which is what Tau uses to carry out its functions and aggregation.

Majority of the research done over the course of 4 years focused on perfecting a purification technique that can be used to provide the possibility for both the structural and functional analysis of Tau. Though there are some modifications and further investigation needed, some of the functional assays that can be done in the future are tubulin binding assays and aggregation assays. These two assays are a key addition to the polymerization experiment performed because one can decipher the effects of N-terminal acetylation on Tau's affinity to tubulin and its polymerization when you compare assay results. Further analysis of aggregates using an aggregation assay can also show the effect of acetylation. On that same note, using aggregates that may form from the aggregation assay in the polymerization and binding assays can provide key information on how acetylated and unacetylated aggregates affect tubulin polymerization and stabilization. Structural analysis like NMR and CD of both the FL Tau and especially Tau 1-100, could reveal a new concept on a mechanism behind the dynamics of aggregates with the added bonus on how N-terminal acetylation affects that mechanism. Overall, this is all physiologically relevant because the dynamic of Tau aggregates is what drives Tauopathies. Therefore finding out how N-terminal acetylation affects Tau's aggregation, tubulin binding, and tubulin polymerization through structural and functional assays can potentially lead to another therapeutic treatment that can be used to treat a large class of neurodegenerative diseases.

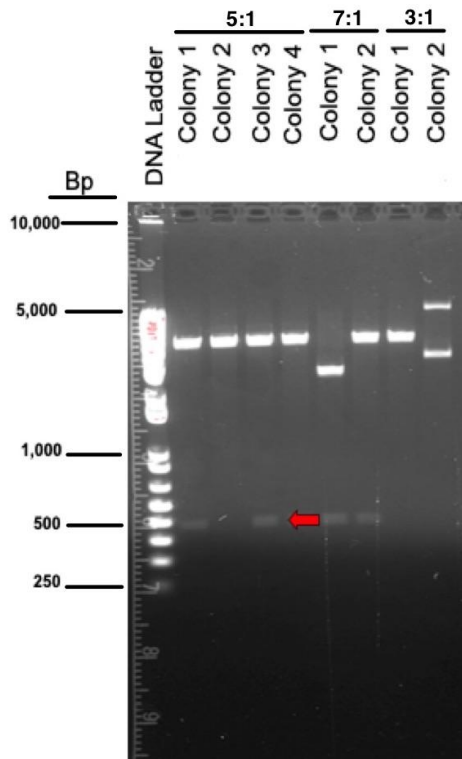
FIGURES:



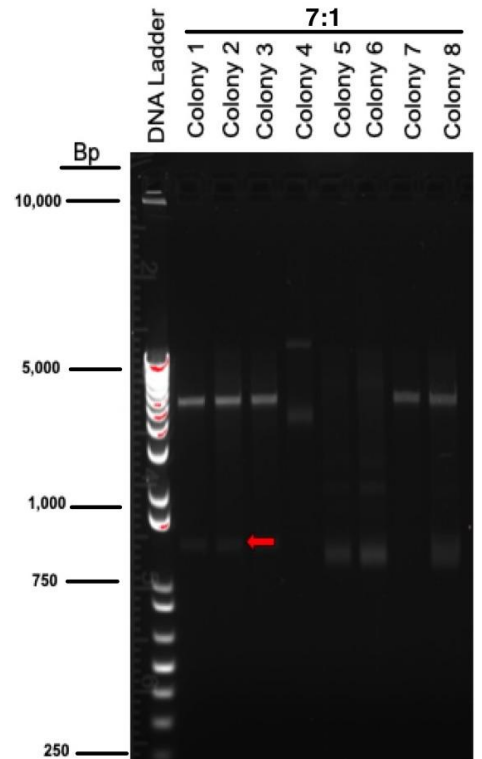
A.



B.

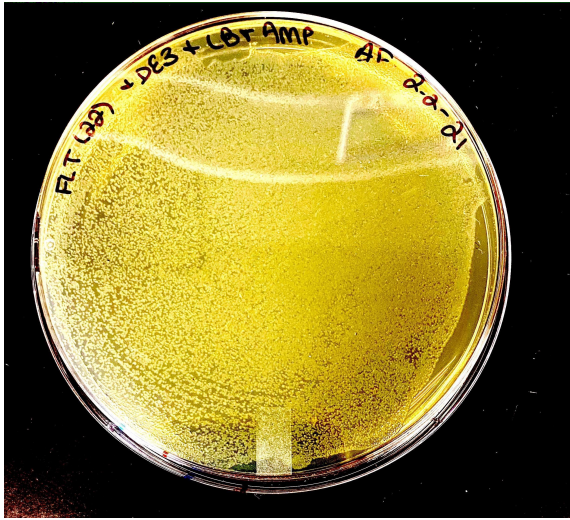


C.

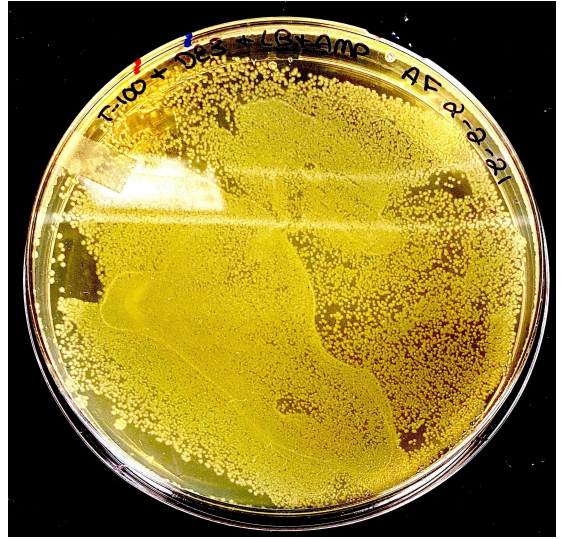


D.

Figure 1. A. Schematic of the Cloning Process for Full Length Tau and Tau-100 into Pet22 vectors. B. PCR Construct Screen of Full Length Tau Construct. To ensure that the PCR performed was successful a DNA Agarose Gel was used to visualize the presence of the desired Full Length Tau constructs with the designated forward and reverse primers with Xho1 and Nde1 cut sites. **C. Digest Screen of Tau 1-100 Clones of Varying Ligation Ratios (Construct to Vector).** Selected colonies from the 5:1, 7:1, and 5:1 ligation transformations were grown up into 3 ml cultures. The DNA from each of the cultures was extracted and the DNA plasmids were digested to check for the correct insert construct, Tau 1-100. The digests were ran through an agarose gel in 1X TAE Buffer, and visualized by UV. **D. Digest Screen of Full Length Tau Clones for a 5:1 Ligation Ratio.** Selected colonies from the 5:1 ligation transformations were grown up into 3 ml cultures. Same procedure from Figure 1B was performed.



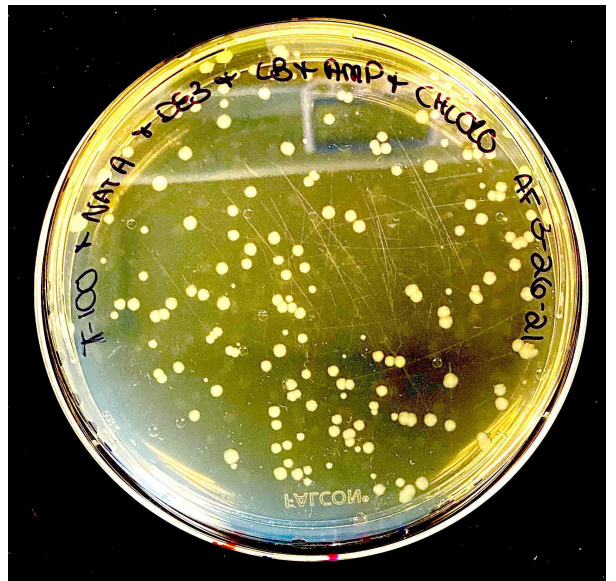
A.



B.



D.



E.

Figure 2. Single and Double Transformation in Ecoli Prokaryotic System.

A. Single Transformation with Full Length Tau . Transformation was done using DE3 competent *E. coli* cells and Full-length Tau PET22 His-tag vectors (FL-T PET22) that encoded a ampicillin cassette for plasmid selection under conditions described in the Materials and Methods section. Transformed cells were plated on Luria Broth (LB) Agar plates that contained ampicillin.

B. Single Transformation with Tau 1-100. Transformation was done using DE3 competent *E. coli* cells and Tau 1-100 PET22 His-tag vectors (T-100 PET22) that encoded an ampicillin cassette for plasmid selection under conditions described in Materials and Methods. Transformed cells were plated on LB Agar plates that contained ampicillin.

C. Double Transformation with Full Length Tau and Nat A complex. Transformation was done using DE3 competent *E. coli* cells and FL-T PET22 and a Nat A complex construct (HB45) that encoded a chloramphenicol cassette for plasmid selection under conditions described in the Materials and Methods.

D. Double Transformation with Tau 1-100 and Nat A complex. Transformation was done using DE3 competent *E. coli* cells and T-100 PET22 and HB45 that encoded a chloramphenicol cassette for plasmid selection under conditions described in the Materials and Methods.

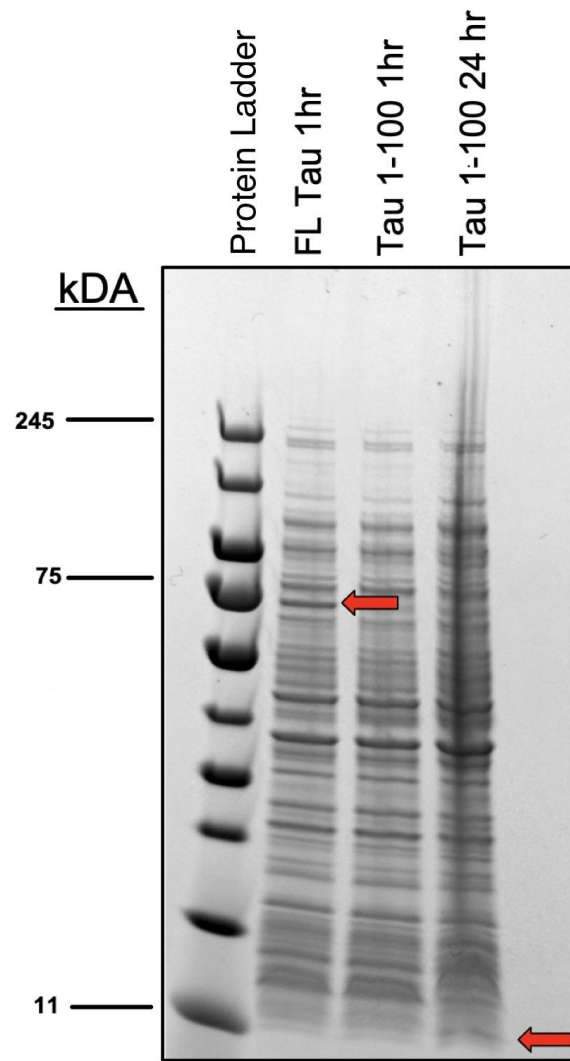
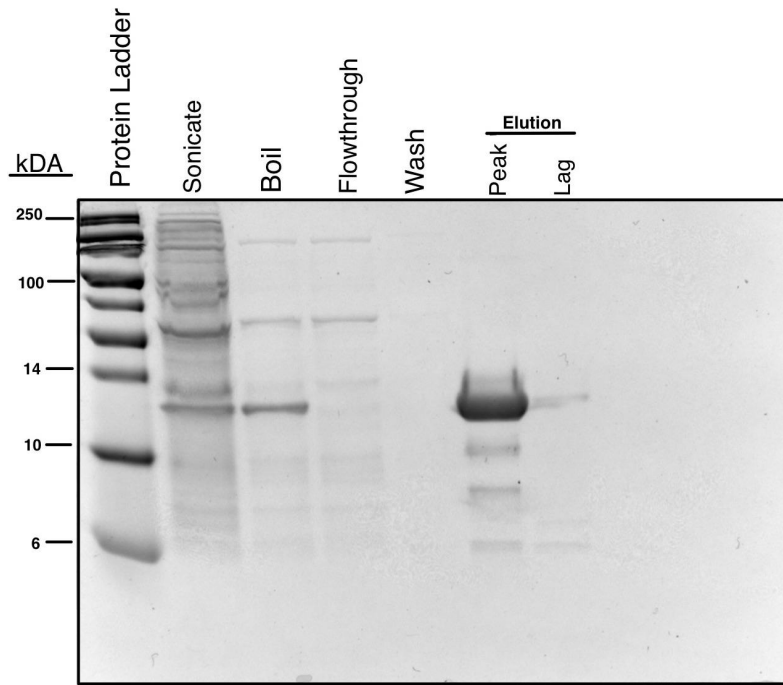
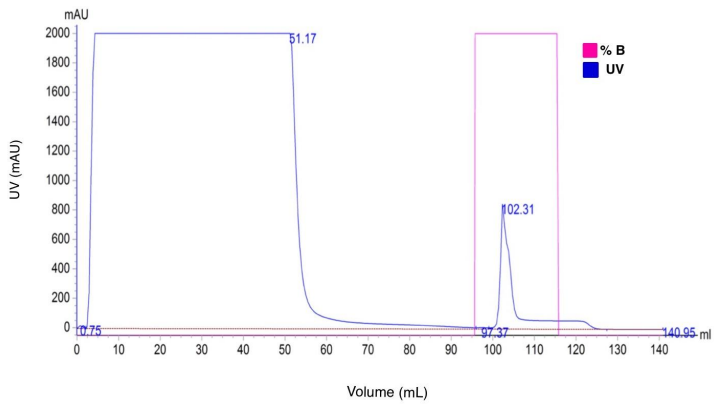


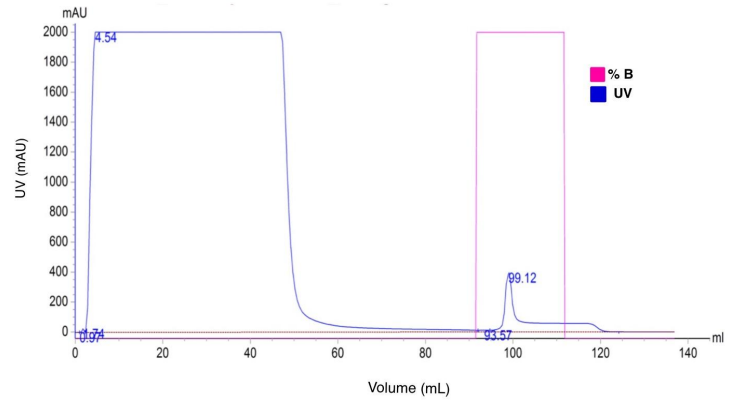
Figure 3. Induction of DE3 Cells Transformed with FL-T and T-100 Constructs. 2 aliquots of 5 ml cultures of Full Length Tau and Tau-100 were induced with IPTG for a 1 hr induction period. A third 5 ml culture of Tau-100 was induced with IPTG for a 24 hr induction period. 1 ml of each culture was lysed by boiling, ran through a 4-20% Coomassie blue SDS-PAGE, and visualized by Coomassie Blue.



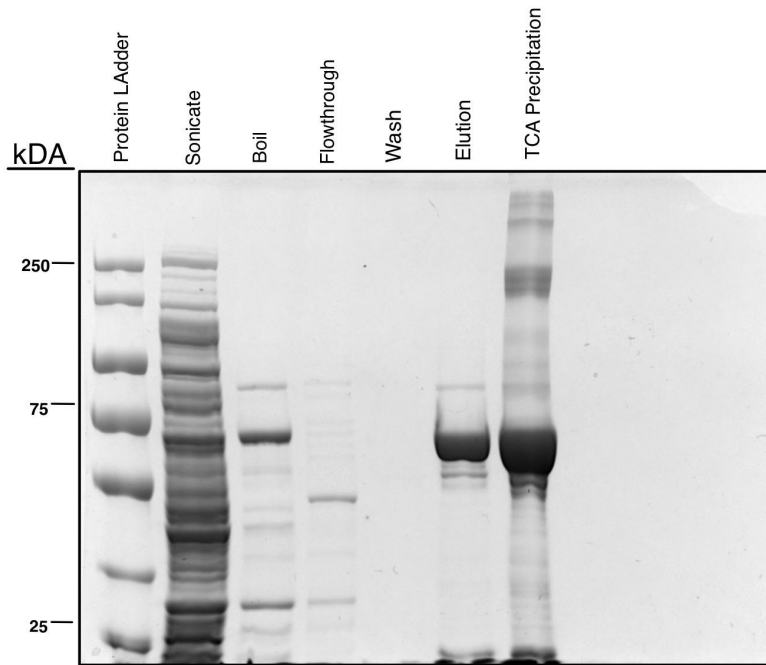
A.



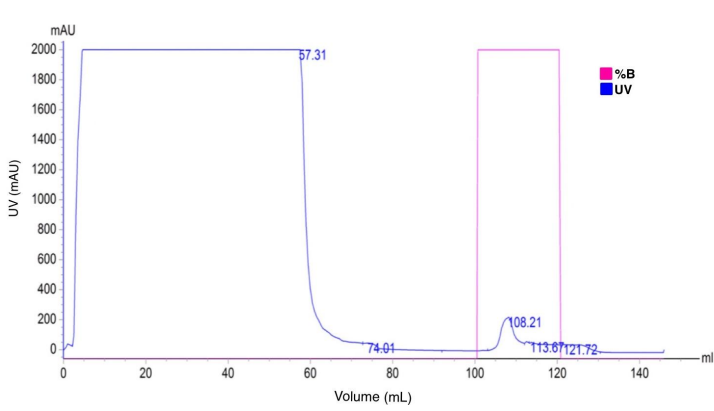
B.



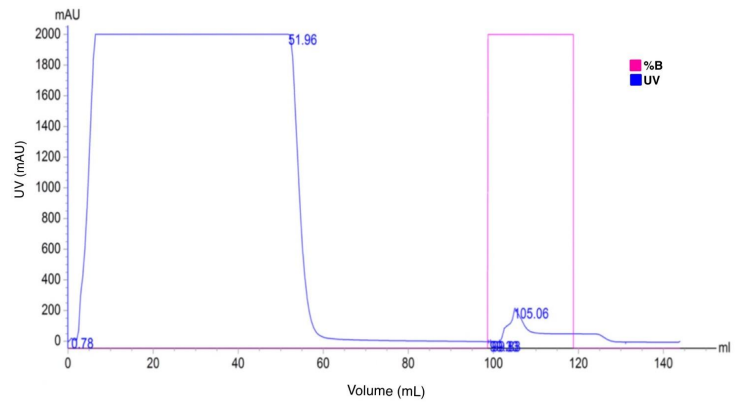
C.



D.

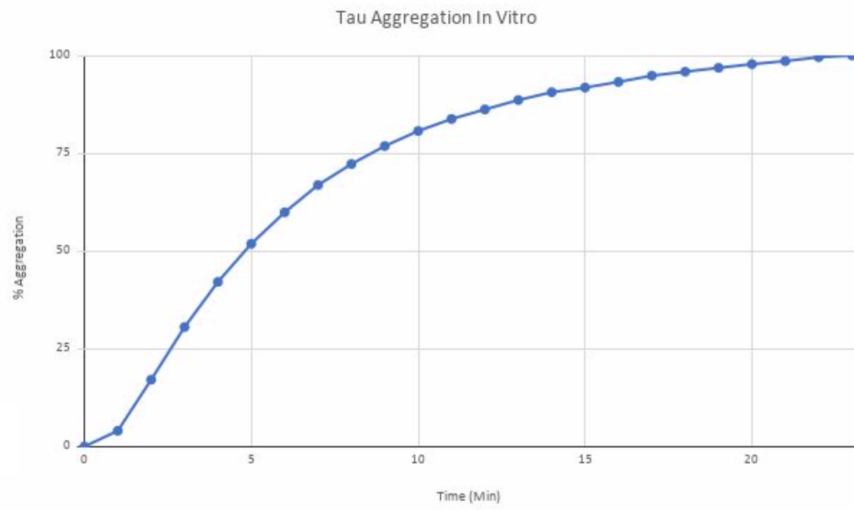


E.

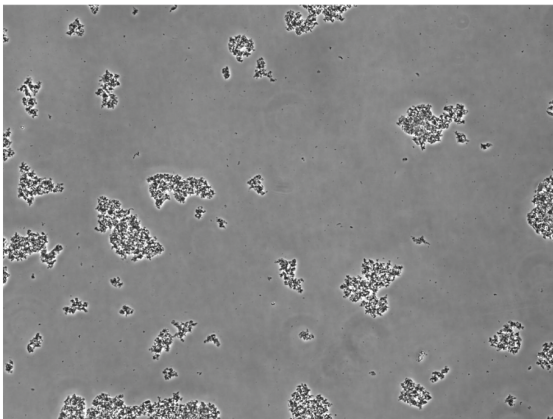


F.

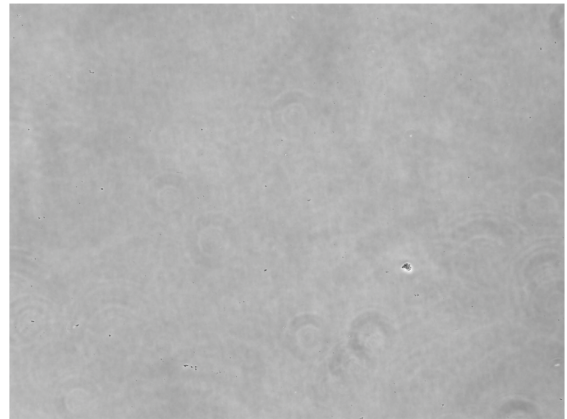
Figure 4. Purification Scheme with FPLC His-Column Method: Tau 1-100 and FL Tau Purification with 3L Induction and Chromatograph comparison of Varying Induction Volumes. A. 3L Purification of Tau 1-100. 15% Coomassie blue SDS-PAGE was used to visualize each step of the purification. 20 uL samples of the 3L induction of acetylated Tau-100, lysed product, boiled product. 20 uL samples from each step of the Fast Protein Liquid Chromatography (FPLC) run was taken; Flowthrough, wash, and elution were sampled. **B. Chromatograph of 3L Tau 1-100 Nickel Column Purification.** The chromatograph of a 3L culture of induced Tau-100 demonstrates the nickel column purification process which includes washing approx 50 ml of acetylated Tau-100 protein of impurities with low salt Buffer A (50 mM Tris pH 9.0 50 mM NaCl) once injected into the column, and eluting the pure protein off of the column with a high salt Buffer B (50 mM Tris pH 9.0 50 mM NaCl). The eluted protein passes through a UV detector, a chromatograph containing UV peaks of material passing through the detector were integrated. **C. Chromatograph of 2L Tau 1-100 Nickel Column Purification.** The chromatograph of a 2L culture of induced Tau-100 that underwent the same nickel column purification as in B. **D. 2L Purification of FL Tau.** An 8% Coomassie blue SDS-PAGE was used to visualize each step of the purification of a 3L. Sample prep was the same. **E. Chromatograph of 3L FL Tau Nickel Column Purification.** The chromatograph of a 3L culture of induced full length Tau whose boiled lysates underwent nickel column purification using the ACORN FPLC, same as B. **F. Chromatograph of 2L of FL Tau Nickel Column Purification.** The chromatograph of a 2L culture of induced full length Tau whose boiled lysates underwent nickel column purification using the ACORN FPLC, same as B.



A.



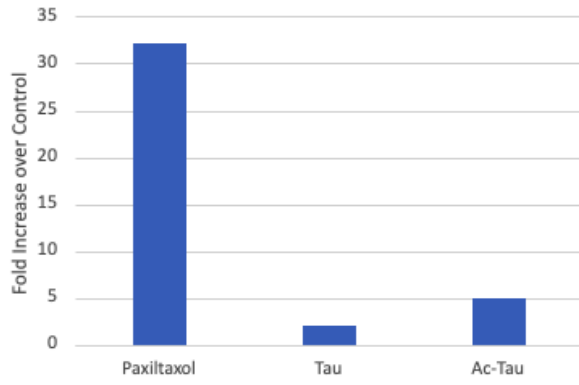
Acetylated Tau



Bovine Serum Albumin

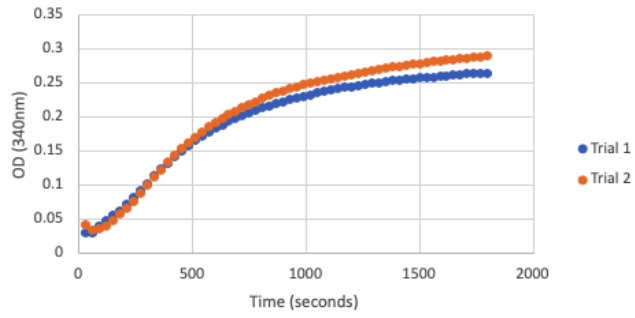
B.

Tubulin Polymerization Rates

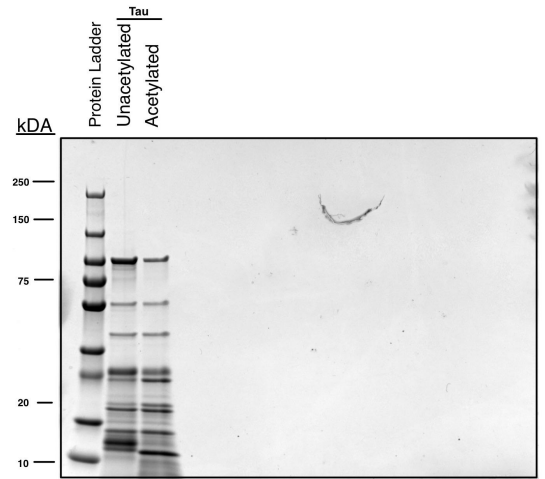


C.

Tubulin Polymerization with Pacitaxil



D.



E.

Figure 5. Tau Aggregation and Tubulin Polymerization Preliminary Experiments. (A). 96 well plates contained full length Tau, heparin, Thioflavin T, DTT, and aggregation buffer bringing the reaction volume for each well to 200 ul. Using Real-Time PCR the aggregation of Tau was observed over the course of 0-1 hr. **(B).** Microscopy images of acetylated Tau aggregates were obtained by using a dialysis bag stirring in 50 mM phosphate buffer that contained eluted acetylated Tau. The formation of visible aggregates was observed over the course of 72 hours. Bovine Serum Albumin served as a negative control. **C. Tubulin Polymerization Assay.** Using a 96 well plate and Biosystems plate reader, each well reaction contained 110 uL reactions. For the positive control, a tubulin polymerization promoter paclitaxel was used. For the protein samples, Tau and acetylated Tau was solubilized overnight in G-PEM buffer. The overall assay took 30 minutes with readings every 30 seconds at 340 nm. **D. Line Graph of Positive Control in Tubulin Polymerization Assay.** Change in O.D. for the positive control wells in the tubulin polymerization assays 1 and 2 recorded over a 30 minute period. **E. Tubulin Polymerization Protein Sample Gel.** Visualization of overnight solubilized Tau and acetylated Tau samples using 4-20% Coomassie Blue SDS-PAGE.

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