A validated method to prepare stable tau oligomers

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Abstract

There is growing evidence that tau oligomers are a major pathological species in a number of tauopathies including Alzheimer's disease. However, it is still unclear what exact mechanisms underlie tau oligomer-mediated dysfunction. Studies of tau oligomers *in vitro* are limited by the high propensity for aggregation and consequent changes in aggregation state of the produced tau samples over time. In this protocol, we provide a step-by-step description of a validated method for producing stable and structurally characterised oligomers of tau that can be used in biochemical, cellular and animal model studies to evaluate mechanisms of action of tau in tauopathies.

Keywords: tau, oligomer, protein expression and purification, tauopathies, Alzheimer's disease, stable oligomer

1. Introduction

Tau is a highly soluble microtubule-associated protein, encoded by the microtubule associated protein tau (*MAPT*) gene, which acts within neurons to modify microtubule stability and to maintain cellular and axonal morphology [1–4]. Tau also plays key roles in regulating axonal transport [5–8], and synaptic plasticity [9,10]. As a monomer, tau is intrinsically disordered and lacks significant secondary structure [11–13]. Under physiological conditions, the phosphorylation level of tau is maintained by the balanced actions of kinases and phosphatases. If this balance is interrupted, abnormally phosphorylated tau dissociates from microtubules, altering their stability [4] and leaving free hyperphosphorylated tau protein in a soluble form. These monomers are initially believed not to be aggregation-prone [14] and require monomer-monomer interactions via disulphide bonding, hexapeptide motif

interactions or both to drive conformational changes to a new structured, aggregation-competent conformation that is rich in beta-sheets [13,15,16]. The subsequent aggregation cascade leads initially to the formation of small soluble oligomers that can aggregate further into fibrils and finally to neurofibrillary tangles (NFTs) [17,18].

The intracellular deposition of NFTs is a defining pathological feature of a group of neurodegenerative diseases, collectively termed tauopathies. In many tauopathies, it was thought that NFTs were the key causative pathological agent as their presence correlated well with disease progression. However, recent evidence suggest that soluble, low molecular weight oligomers have a range of harmful effects on neurons, sometimes in the absence of NFTs, establishing tau oligomers as a highly toxic aggregate species in these diseases [19,20,20–23]. In some tauopathies with specific disease-associated mutations in the *MAPT* gene, NFTs are rare whilst oligomers are the major aggregation products, further suggesting that oligomers may be more fundamental to the disease process than the larger aggregates [24,25]. Tau oligomers of different conformations, seeding capacities and ultrastructural properties have been prepared from recombinant sources [26–30] or isolated from tauopathy brains [19,20,31,32]. However, it is unclear if these properties are features that define specific tauopathies. A principal aim in tauopathy research is therefore to understand the structural and molecular properties of tau oligomers and how these characteristics may differ between diseases.

In vitro studies using recombinant tau have greatly improved the understanding of tau function in pathological processes. For instance, recombinant forms of tau encoding different pathological mutations show varying structural and biochemical properties that are reminiscent of the divergent disease characteristics observed in humans [33,34]. Such differences in tau aggregate conformations are supported by high-resolution structural data from cryo-electron microscopy studies [35–37]. However, biochemical studies are often limited by their inability to correlate observed functional effects to specific aggregate conformations of tau, due to its strong aggregation propensity. Moreover, the use of chemicals such as

heparin to induce tau aggregation *in vitro* precludes application of the resulting aggregates in specific experimental contexts, for example in animal model systems, since heparin is an anticoagulant. These challenges can lead to wide experimental variation and could thus mask tau-mediated effects. It is therefore vital to have a method that can produce preparations of tau oligomers that can be structurally characterised and tested experimentally, with confidence that the aggregation state is stable, without using inducing agents that may cause unintended physiological effects.

Our protocol describes an inducer-free method for producing stable oligomers of full-length tau-441 and its microtubule-binding fragment (tau-K18; amino acids 244-372) [23,34,38]. The method involves the labelling of a single-cysteine residue with maleimide derivatives, either the green fluorophore Alexa Fluor 488-C5-maleimide or the non-fluorophore *N*-ethyl maleimide, in reducing conditions. The advantages of this method are two-fold. Firstly, it generates fluorescently labelled oligomers of tau, which allow for real-time studies into the diversity of aggregate conformers, cell-to-cell spread, intracellular seeding and for electrophysiological and localisation effects within single neurons [23,34,38]. Secondly, our method allows *in vitro* experiments to be carried out with continuity across samples - as the bound label prevents further aggregation and maintains low molecular weight oligomers [23,34,38]. The method therefore permits the study of the action of soluble small tau oligomers that can be structurally characterised and correlated with observations. It is important to know which of the many forms of tau along the aggregation cascade are involved in driving toxicity in tauopathies, in order to properly target treatments.

2. Materials

Unless otherwise stated, all chemicals were obtained from Sigma Aldrich.

2.1 Preparation of recombinant tau protein

2.1.1 Tau Expression

1. *Escherichia coli* BL21 (DE3) carrying pProEX plasmids coding for wild-type full-length tau-441 (Uniprot ID: P10636-8) or the tau-K18 fragment with cleavable N-terminal 6xHis and FLAG tags to enable purification of the expressed protein (see Note 4.1 and Figure 1).
2. 15 ml Luria-Bertani broth, also known as Lysogeny broth. Mix 10 g tryptone, 10 g NaCl and 5 g yeast extract in 950 ml. Adjust pH to 7.0 with 5 N NaOH and adjust the final volume to 1 L. Autoclave for 20 min to sterilise.
3. Ampicillin. Prepare a 10 ml of 100 mg/ml stock by dissolving 1g sodium ampicillin in 1L double distilled water (ddH_2O). Sterilise by passing through ddH_2O-cleaned 0.22 µm sterile filter.
4. Chloramphenicol: prepare 10 mg/ml stock by dissolving 0.34 g in 10 ml of 100 % ethanol.
5. Shaking incubator suitable for use at 37 °C, 180 rpm
6. Isopropyl β-D-1 thiogalactopyranoside (IPTG): prepare 1M stock by dissolving 2.38 g in 10 ml ddH_2O. *Cover bottle with aluminium foil since this chemical is light-sensitive; store at 4* °C
7. Spectrophotometer
8. 500 ml centrifuge tubes
9. 100 % methanol
10. Trigene
11. Sterile water

12. Weighing balance (for balancing centrifuge tubes)
13. Centrifuge: 10 minutes at 4 °C at 9800 xg
14. Stock solution of 0.5 M NaH_2PO_4: 30 g in 500 ml ddH_2O.
15. 0.5M stock solution of Na_2HP0_4: 35.5 g in 500 ml ddH_2O.
16. 0.5M sodium phosphate buffer pH 7.4 or 7.0: transfer 200 ml NaH_2PO_4 to a clean bottle and add dibasic until you reach pH 7 or 7.4 respectively

2.1.2 Tau purification

1. Water bath at 100 °C
2. Protease inhibitor cocktail tablet (Thermofisher Scientific, #A32963; 1 tablet per 50 ml lysate)
3. DNAse I (Roche, #10104159001)
4. 5 ml of 1X BugBuster® protein extraction reagent (Merck, #70584)
5. Probe sonicator
6. Centrifuge at 4 °C, at 48000 g
7. 0.2 µm filter (Sartorius Minisart Plus Syringe filters, #10730792 or similar)
8. Sodium phosphate buffer (500 mM pH 7) and (500 mM pH 7.4):
9. 1 M imidazole: mix 68 g with 1L ddH_2O
10. 5 M NaCl: 292 g in 1L ddH_2O
11. 10 mM $NiCl_2$/NaAc solution:
    - 40 ml 100 mM $NiCl_2$, 200 ml 100 mM NaAC, 160 ml ddH_20, adjust to pH 4 with HCl/NaOH
12. Immobilised metal affinity chromatography (IMAC) using Ni-NTA column (Econo-Pac, Biorad)
13. 70 % ethanol.
14. Chelating sepharose resin (GE Healthcare, UK, #GE17-0575-01)
15. Buffer A: 50 mM sodium phosphate buffer pH 7.0, 500 mM NaCl, 10 mM imidazole

16. Buffer B: 50 mM sodium phosphate buffer pH 7.0, 500 mM NaCl, 25mM imidazole
17. Buffer C: 50 mM sodium phosphate buffer pH 7.0, 500 mM NaCl, 500 mM imidazole
18. Clean the column with ddH_2O and then with 70 % ethanol

2.2 Confirmation of purification

2.2.1 Detection using SDS-PAGE

1. Cast 6 % gels and allow to set. *See note 4.1*

   1X loading buffer (25 mM Tris HCl pH 6.8, 25 % v/v glycerol, 2% SDS, 0.01 % w/v bromophenol blue. Diluent = ddH_2O). Add 262.5 μl of 1X loading buffer to 37.5 μl beta mercaptoethanol (βME) immediately before use.
2. Running buffer made (200 ml running buffer (25 mM Tris, 190 mM glycine, 0.1% SDS) + 1800 ml distilled water)
3. 15 µl protein + 5 µl loading buffer for each sample
4. PCR machine: 95 degrees for 5 mins
5. Biorad Mini-PROTEAN Tetra system (BioRad Laboratories, California, USA)
6. Protein ladder (#P7712 or #P7712S from New England BioLabs, size range = 11–245 kDa)
7. Instant Blue (Expedeon, #ISB1L)
8. SynGene G-Box imaging system.
9. AGFA Curix 60 processor (Agfa Healthcare, Greenville, SC, USA)

2.2.2 Concentrating the tau protein

1. 10 mM sodium phosphate buffer pH 7.4: Dilute 10ml of the 500mM stock (see recipe above) in 500ml ddH_2O.
2. Slide-A-Lyzer™ MINI Dialysis devices (10K MWCO; Thermo Scientific, #69570)
3. Centrifuge: 2600 xg x 10 mins x 4 degrees
4. Gel filling tips

2.2.3 Bicinchoninic acid (BCA) assay for protein quantification

1. Bicinchoninic acid assay kit (G-Biosciences, #786-570)
2. For instructions on how to produce the standard/reference curve, see *note 4.3*
3. Water bath at 37 °C
4. Spectrophotometer (absorbance at 562 nm)

2.3 Preparation of labelled tau protein

1. Freshly-purified monomeric tau
2. Tris(2-carboxyethyl)phosphine (TCEP; Sigma Aldrich, #C4706)
3. 10 mM sodium phosphate buffer pH 7.4
4. Alexa Fluor 488-C5-maleimide (#A10254, Molecular Probes) or *N*-ethyl maleimide (Thermo Scientific, #23030)
5. Dry ice
6. 100 % ethanol
7. Dialysis buffer (50 mM Tris HCl pH 7.5, 100 mM NaCl)
8. Slide-A-Lyzer™ MINI Dialysis device (10 K MWCO; Thermo Scientific) with perforated reservoirs
9. 2-L container

10. SDS-PAGE gels, see *note 4.4*
11. Ultraviolet light exposure for detection. *Note that when working with UV, caution must be taken to avoid exposure, i.e., with the use of a face shield and UV resistant gloves.*

2.4 Characterisation of the tau oligomers

2.4.1 Transmission electron microscopy (TEM)

1. Formvar/carbon-coated 300-mesh copper grids (#S162, Agar Scientific, UK)
2. ELMO glow discharge system from Cordouan Technologies
3. Labelled or unlabelled purified tau preparations
4. Filter paper
5. 5 μl of 2 % uranyl acetate
6. JEOL-2100F transmission electron microscope

2.4.2 Circular Dichroism

1. 10 μM samples of labelled and dialysed tau (diluted in sodium phosphate buffer pH 7.4)
2. Jasco J-815 CD spectropolarimeter (conditions: 1 mm path length cell, response time: 1 s, data pitch: 0.1 nm, scan speed: 100 nm/min, wavelength: 190 nm to 280 nm, high-tension voltage: ≤ 550 V)

2.4.2 Dot blots

1. Amersham Hybond electrochemiluminescence nitrocellulose membrane (Sigma, #GERPN3032D)

2. 10 % non-fat milk in phosphate buffered saline (PBS) containing 0.05% Tween (50 ml TBS, 250 μl tween, 5 g of milk powder)
3. 10% Tris buffered saline (TBS)-Tween
4. Primary antibody (total tau [K9JA; #A0024] from Dako or anti-tau oligomer antibody [T22, #ABN454 from Merck]) dissolved in 10% TBS-Tween solution
5. Secondary antibody (anti-rabbit immunoglobulin G; 31450, Thermofisher)
6. Electrochemiluminescent detection kit (Sigma, #GERPN2232)
7. X-ray film (Super RX, Fuji Medical X-ray Film)
8. AGFA Curix 60 processor (Agfa Healthcare, Greenville, SC, USA).
9. ImageJ software https://imagej.nih.gov/ij/
10. Prism software (GraphPad Inc., CA, USA)

2.4.3 Thioflavin T (ThT) aggregation kinetic assay

1. 10 mM sodium phosphate buffer pH 7.4
2. Thioflavin T (ThT)
3. Heparin (6000 molecular weight)
4. Purified tau (0.16 mg/ml)
5. FLUOTRAC™ 96-well plate (Greiner Bio-One #655077)
6. ThT fluorescence at 440 nm excitation and 510 nm emission in a CLARIOstar microplate reader (BMG Lab Tech).
7. Prism software (GraphPad Inc., CA, USA)

2.4.4 Atomic force microscopy

1. Purified tau
2. Heparin (6000 molecular weight)
3. Freshly-cleaved mica surface (Agar Scientific, #G250-3)

4. ddH_2O.
5. MFP3D AFM instrument (Asylum Research, UK)
6. AFM conditions: scan mode = AC Air Topography, scan rate of 1 Hz, set point of 590.49 mV, an integral gain of 3, x- and y- offsets of 0, points and gains of 256 and drive amplitude of 129.06 inches
7. Igor Pro software
8. ImageJ software https://imagej.nih.gov/ij/

2.4.5 Western Blot

1. Precast 4-20 % Tris-glycine gels (NuSep)
2. Loading buffer (262.5 μl loading buffer [see recipe above] + 37.5 μl βME
3. Protein ladder (#P7712 or #P7712S, New England BioLabs, size range = 11 – 245 kDa)
4. Precision Plus standard (#161-0374, Biorad; size range = 10 – 250 kDa)
5. Biorad Mini-PROTEAN Tetra system (BioRad Laboratories, California, USA)
6. Instant Blue (Coomassie-based stain, Expedeon, Cambridge)
7. SynGene G-Box imaging system
8. MagicMark^{TM} XP Western standard (#LC5602, Invitrogen)
9. Running buffer x10 stock (25 mM Tris, 190 mM glycine, 0.1% SDS)
10. Diluent = ddH_2O
11. Transfer buffer: 25 mM Tris, 190mM glycine, 2 % methanol
12. Amersham Hybond Electrochemiluminescence Nitrocellulose membrane
13. 5 % w/v non-fat dried milk in TBS-Tween
14. Primary antibody in 10% TBS-Tween
15. Secondary antibody in 10% TBS-Tween
16. Amersham Electrochemiluminescence detection reagents

17. X-ray film (Fuji Medical X-ray Film Super RX or CL-XPosure Film)
18. AGFA Curix 60 processor (Agfa Healthcare, Greenville, SC, USA).
19. ImageQuant™ LAS4000 (GE Healthcare).
20. ImageJ software https://imagej.nih.gov/ij/
21. Prism software (GraphPad Inc., CA, USA)

Methods

3.1 Preparation of recombinant tau protein

3.1.1 Tau Expression

1. Generate or source *Escherichia coli* BL21 (DE3) carrying pProEX plasmids coding for wild-type full-length tau-441 (Uniprot ID: P10636-8) or its microtubule binding fragment (amino acids 244-372, also known as tau-K18; Fig. 1) with N-terminal 6xHis and FLAG tags (see *Notes 4.1)*
2. Cysteine modifications (C291A/C322A/I260C) are introduced by site directed mutagenesis to prevent potential functional interference of the maleimide label (see *note 4.2)*
3. Inoculate single colonies of BL21(DE3)* Rosetta cells (described in 3.1.1.1) into Luria-Bertani broth (15 ml) supplemented with ampicillin (100 μg/ml) and chloramphenicol (35 μg/ml)
4. Incubate at 37 °C and 180 rpm overnight. A turbid (cloudy) appearance shows successful culture growth
5. Add overnight culture to 750 ml LB broth with ampicillin (100 μg/ml) and place the new flask in the shaking incubator (37 °C at 180 rpm) for 90 minutes
6. Measure the optical density (OD_{600}) using a spectrophotometer.

Transfer 1 ml reference media (fresh Luria-Bertani broth) into a cuvette and use this to blank the spectrophotometer. Transfer 1 ml of the large culture in a fresh cuvette and measure its OD_{600}.

OD_{600} should be around 0.6 – 0.7. If not, incubate for longer in the shaking incubator and measure every 15-30 minutes until it reaches $OD_{600} = 0.6$

7. Add 0.5 mM IPTG to induce tau expression and return the culture to the incubator for 1 hour.
8. Before using centrifuge bottles, rinse with 100 % methanol and allow to dry.
9. Add samples to methanol-rinsed 500 ml centrifuge tubes and balance with sterile water.
10. Centrifuge for 10 minutes at 4 °C at 9800 xg
11. Remove the supernatant and discard.
12. Wash pellet twice with 10 mM sodium phosphate buffer pH 7.4
13. Resuspend pellet in 10 mM sodium phosphate buffer pH 7.4. Store samples at -20 °C until use.
14. After use, wash centrifuge tubes with Trigene and allow to dry for the next user (this is very important if the centrifuge tubes are shared by multiple users for different cultures)

3.1.2 Tau purification

1. Defrost the expressed tau cultures on ice and then boil at 100 °C for 10 mins
2. Add a protease inhibitor cocktail tablet (1 tablet/~ 50 ml lysate), DNAse I and 5 ml of 1X BugBuster® protein extraction reagent and leave to dissociate for 1 hour at room temperature
3. Sonicate the culture at 70% power for 1 minute
4. Centrifuge at 4 °C, at 48000 g for 15 min
5. Decant the supernatant containing the soluble fraction (crude extract), filter through a 0.2 µm filter and purify as described below.

6. Samples are purified by IMAC technology using a Ni-NTA column
7. Wash the column with 70% ethanol
8. Pack chelating sepharose resin (GEHealthcare, UK) into to the column and allow to set
9. Charge the resin with 10 mM $NiCl_2$/NaOAc pH 4.0
10. Equilibrate with two column volumes of buffer A
11. Add the crude extract
12. Collect 1ml of the flow through – this is the unbound protein fractions
13. Wash through the column once with two column volumes of buffer A, and then twice with 2 column volumes of buffer B (increased imidazole concentration for washing; 25 mM)
14. Right before using buffer C, add DTT (700mg DDT to 500 ml)
15. Wash once with buffer C (+ DTT) to elute the purified protein from the column. Collect the flow-through into 1ml collection tubes and retain all fractions.
16. Store the eluted fractions at -20 °C until use.

3.2 Confirmation of purification and selecting high-yield fractions for downstream applications

3.2.1 Detection using SDS-PAGE

1. Analyse the collected 1ml fractions of eluted protein on six percent hand-cast non-denaturing SDS-PAGE gels (see *note 4.4*) to identify those with the highest yields.
2. Cast 6 % gels and allow to set (see *note 4.4)*
3. Defrost samples on ice
4. Add βME to the loading buffer (262.5 µl loading buffer + 37.5 µl βME)
5. Prepare a working dilution of the running buffer (200 ml of 10x running buffer + 1800 ml distilled water)

6. Add 15 μl protein fraction of interest to 5 μl loading buffer in a 0.5 ml Eppendorf tube
7. Heat the tubes in the PCR machine (or water bath) to 95 °C for 5 min
8. Assemble the gel in the gel tank, ensuring it does not leak. Fill with running buffer to the instructed level
9. Load 12 μl of the loading mixture into each well. Load the ladder into your preferred ladder lane (2 μl)
10. Run the gel

    150 volts for 15 mins – should see it start to separate

    175 volts for ~ 1 hour, wait for it to run close to the bottom

11. Stain the gels with Instant Blue (Expedeon, #ISB1L) for 1 hour at room temperature
12. Image the gel to identify for fractions that contain high protein yields. Pool these fractions together for concentrating.
13. If break-down products are detected by SDS-PAGE, polish the purified tau by size exclusion chromatography following published protocols [**37**].

3.2.2 Concentrating the high-yield purified tau protein fractions

1. Add 1 ml 10 mM sodium phosphate buffer pH 7.4 to each of the Slide-A-Lyzer^{TM} MINI Dialysis devices (10K MWCO; Thermo Scientific, #69570)
2. Centrifuge at 2600 xg, 10 mins, 4 °C to rinse and equilibrate the filter device with washing buffer.
3. Decant the liquid and pipette out the extra buffer from the top and the bottom. Use gel-loading tips to remove leftover buffer from the top part of the device in order to avoid damaging the filter membrane.

4. Add defrosted samples to the tube in the filter device and centrifuge for 30 minutes, 2600 xg, 4 °C until ~20 % of the starting volume is left.
5. Prepare the BCA assay reagents according to the manufacturer's instructions
6. For how to produce the standard curve see *note 4.3*.
5. Add 50 μl of the concentrated sample to 1 ml of BCA copper solution
6. Leave for 30 minutes at 37 °C in a water bath
7. Use the spectrophotometer (wavelength = 562nm) to measure the protein concentration, with 1x PBS as a control
8. Where needed, repeat the dialysis process on the remaining liquid further with additional spins until all samples have reached 1 mg/ml

3.3 Oligomerisation and labelling of tau protein

1. Perform the labelling process at 4 °C. It is important to perform the entire labelling and oligomerisation process in cold conditions (see Note 4.5).
2. Adjust the purified tau samples to 1mg/ml. Add 5X molar excess of TCEP to reduce the protein down to monomers. Incubate the reaction mixture for 1 hour. *TCEP is preferred over the commonly used reducing agent DTT because TCEP has a longer half-life.*
3. Add 4X molar excess of the label chemical Alexa-Fluor-maleimide or *N*-ethyl maleimide diluted in sodium phosphate buffer pH 7.4 and allow to reform oligomers by incubating at 4 °C overnight.
4. Prepare unlabelled control samples identically except that use equal volume of 10 mM sodium phosphate buffer pH 7.4 in place of the maleimide label
5. Snap-freeze samples on dry ice and 100 % ethanol before being stored at -80 °C overnight

6. Remove free fluorophore and reducing agent by extensive dialysis against dialysis buffer (50 mM Tris HCl pH 7.5, 100 mM NaCl) in a Slide-A-Lyzer™ MINI Dialysis device (10 K MWCO; Thermo Scientific)
7. To ensure efficient dialysis, the reservoir should be perforated and placed in a container large enough to accommodate a 2 L volume of buffer.
8. Sample is added into the top of the dialysis device and the buffer fills the container surrounding. Dialysis buffer is added and should come to just above the level of the sample
9. The buffer needs to be changed every 2 hours for 10 hours
10. Samples are then recovered and stored at - 80 ˚C until use
11. Labelling can be confirmed with non-denaturing sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) followed by ultraviolet light exposure.
12. Labelling efficiency is measured spectrophotometrically using Beer's law and the molar extinction coefficient of 72,000/cm/M for Alexa-Fluor-maleimide
13. The oligomerisation process is illustrated in Figure 2. Characterisation of the prepared stable oligomers by gel electrophoresis is shown in Figure 3, and Figure 4 shows structural characterisation using transition electron microscopy. Figure 5 demonstrates the importance of performing the oligomerisation process in cold conditions compared with at room temperature.

3.4 Characterisation of the tau oligomers

3.4.1 Transmission electron microscopy

1. TEM Formvar/carbon-coated 300-mesh copper grids, shiny side up (#S162, Agar scientific, UK) are glow-discharged using the ELMO system from Cordouan Technologies for 1 min

2. Pipette 5 μl of labelled or unlabelled tau preparations onto each grid and allowed to bind for 1 min
3. Remove excess samples with a strip of filter paper
4. Add 5 μl of 2% uranyl acetate for 1 min
5. Remove excess stain with a strip of filter paper
6. Grids are now ready to be imaged. They can be stored in the Grid Box until use.
7. Samples can be imaged using a JEOL-2100F transmission electron microscope

3.4.2 Circular Dichroism

1. 10 μM samples of the purified labelled and dialysed tau (diluted in 10 mM sodium phosphate buffer pH 7.4 buffer) can be analysed using a Jasco J-815 CD spectropolarimeter. The conditions are as follows: a 1 mm path length cell, response time was 1 s with a data pitch of 0.1 nm and the scan speed was 100 nm/min
2. Collect 32 spectra accumulations for each sample at wavelengths ranging from 190 nm to 280 nm. Take the average of these measurements.
3. The high-tension voltage should be ≤ 550 V throughout.
4. For each CD assay, conduct three independent experiments using different batches of protein preparations.

3.4.3 ThT kinetic assay

1. Dilute samples to 0.2 mg/ml (11 μM) in 10 mM Na_2PO_4 pH 7.4
2. Seed samples at 100 μl per well (heparin-free aggregation studies) of a FLUOTRAC™ 96-well plate (Greiner Bio-One #655077).
3. In heparin-induced aggregation studies, 10 μl each of ThT (20 μM final concentration) and heparin (0.15 mg/ml = 25 μM final concentration) is added to 80 μl tau 0.16 mg/ml (8.85 μM; final concentration) per well of a FLUOTRAC™ 96-well plate (Greiner Bio-One #655077).

4. Tau aggregation is monitored for up to 40 h by measuring ThT fluorescence at 440 nm excitation and 510 nm emission in a CLARIOstar microplate reader (BMG Lab Tech)
5. Sensitivity is set to 20 % to prevent signal saturation
6. Fluorescence readings are taken with the top optic setting every 5 min after 200 rpm shaking for 30 s.
7. Data analysis can be done with Prism 7 software (GraphPad Inc.)

3.4.4 Atomic force microscopy

1. To closely monitor the structural transformation of tau during aggregation, 0.7 mg/ml (38.5 µM) tau is mixed with 0.75 mg/ml (125 µM) heparin and incubated at 37 ˚C.
2. Samples can be taken at pre-determined time points, snap-frozen and stored at -80 ˚C until use
3. Tau protein samples are adsorbed onto freshly-cleaved mica surface (Agar Scientific, #G250-3) for 15 min
4. Unbound protein is removed by washing with excess ddH_2O.
5. Random positions on the mica surface are chosen for the collection of 1 µm 2 height images using a cantilever
6. AFM conditions: scan mode = AC Air Topography, scan rate of 1 Hz, set point of 590.49 mV, an integral gain of 3, x- and y- offsets of 0, points and gains of 256 and drive amplitude of 129.06 inches
7. Scan using MFP3D AFM instrument (Asylum Research, UK)
8. Data processing is done using the “Flatten” function of the Igor Pro 6.37 software
9. Sample sizes are measured using Image J as previously described [**41**]

3.4.5 Dot blots

1. Spot 2 µl aliquots of purified tau samples onto a piece of nitrocellulose membrane and air dried for 15 mins
2. Block the membrane with 10% non-fat milk in phosphate buffered saline (PBS) containing 0.05% Tween (50 ml TBS, 250 µl tween, 5 grams milk) for 30 min at room temperature
3. Wash subsequently five times with 10% Tris buffered saline (TBS)-Tween. Incubate for 2 h with primary antibody (total tau [K9JA] or T22) in 10% TBS-Tween
4. Wash five times with 10% TBS-Tween Treated with secondary antibody (anti-rabbit immunoglobulin G) for 2 h in 10% TBS-Tween

   All antibodies should be used at 1:1000 dilutions in the PBS-Tween buffer
5. Wash five times with 10% TBS-Tween
6. Visualise dot blot bands by adding electrochemiluminescent detection reagents, exposing to X-ray film for 2 min and then developing the blots in an AGFA Curix 60 processor
7. Analyse blot intensities using ImageJ and GraphPad Prism 7 software.

3.4.6 Western blot

1. Separate protein samples on precast 4-20 % NuSep Tris-glycine gels or standard handcast 15 % Tris-glycine gels with (reducing gels) or without (nonreducing gels) βME and 5 min heating at 95 ˚C
2. Analyse samples against protein ladder for 35 min at 200 V in a Biorad Mini-PROTEAN Tetra system
3. Stain the gels with Instant Blue for 1 h at RT with no washing steps required
4. Image gels using a SynGene G-Box imaging system

5. Perform western blotting to identify tau-positive bands, following the instructions below
6. Use MagicMark™ XP Western standard as a loading marker
7. Transfer gels overnight at 4 ˚C or 2 hr at room temperature onto Amersham Hybond electrochemiluminescence nitrocellulose membrane
8. Block for 15 min (in 5 % w/v non-fat dried milk in TBS-Tween)
9. Incubate for 2 h incubation with the primary antibody
10. Remove unbound antibody by 5 x 5 min washes with 10 % TBSTween in ddH_2O
11. Incubate for 2 h at RT with the secondary antibody
12. Remove unbound antibody by 5 x 5 min washes with 10 % TBSTween in ddH_2O
13. Antibody detection is then be performed using the Amersham electrochemiluminescence detection reagents according to the manufacturer's instruction and bands visualised by exposure to X-ray film and developed in an AGFA Curix 60 processor
14. Western blot detection is performed using the ImageQuant™ LAS4000 biomolecular imaging system Densitometry analysis of protein bands can be performed using the ImageJ software, and the data statistically analysed with Prism 6 (GraphPad Inc., CA, USA)

Notes

4.1 Introducing the MAPT genetic construct into the pProEx plasmid

1. Add 5 ng of plasmid DNA to 50 µl competent BL21(DE3)*pRosetta cells
2. Incubate on ice for 30 min.
3. Heat-shock cells at 42 ˚C for 30 s

4. Incubate for 2 min on ice.
5. Add 950 µl LB broth and incubate at 37 ˚C for 1 hour.
6. Plate 40 µl of the mixture on LB agar supplemented with ampicillin (100 µg/ml) and chloramphenicol (35 µg/ml) and incubate overnight at 37 ˚C

4.2 Cysteine changes

Cysteine modifications are introduced to allow labelling of the protein outside the microtubule-binding core to avoid introducing steric hindrance. The purpose of the cysteine modifications is to have a single cysteine residue located outside the microtubulebinding region that can be specifically labeled by a fluorophore without potentially interfering with the protein's functions; this approach has been widely used and shown to have no apparent detrimental effects [23,34,39–41]. The cysteine modifications were generated using site directed mutagenesis (Q5® SDM kit) using the primers below:

4.3 Producing a BCA standard curve

1. A linear plot is generated by monitoring the 562 nm absorbance of serial dilutions of manufacturer-provided bovine serum albumin (BSA) as the standard protein.
2. Prepare nine 1:2 serial dilutions of a stock 2 mg/ml BSA and thoroughly mix
3. Add 50 µl aliquots to 1 ml working solution (recipe: 10 ml BCA reagent + 200 µl copper solution).
4. The absorbance of each mixture should be measured with an Ultrospec 2100 spectrophotometer after 30 min incubation at 37 ˚C.
5. To identify the absolute protein concentration per dilution, the absorbance value for the working solution alone (the last tube in the dilution series with no protein present) is deducted from all collected absorbance values.

6. The normalised absorbance readings are subsequently plotted against protein concentration to generate a standard curve, which is used to estimate the concentration of purified proteins used in this work.
   Concentrations in mg/ml can be converted to molarity using each protein's molar mass.

4.4 Making Gels for SDS-PAGE

1. Resolving gel solution (6 %): 5.2 ml H_20_2 ml bis-acrylamide (Sigma Aldrich #A3574), 2.6 ml 1.5 M Tris/HCl pH 8.8, 100 μl 10 % SDS (Sigma Aldrich #L3771), 100 μl 10 % APS (Sigma Aldrich #A3678), 10 μl TEMED (Sigma Aldrich #T-7024)
2. Stacking gel solution: 2.975 ml H_20, 0.67 ml bis-acrylamide, 1.25 ml 0.5M Tris/HCl pH 6.8, 50 μl 10% SDS, 50 μl 10% ammonium persulfate (APS; made fresh), 5 μl tetramethylethylenediamine (TEMED)
3. APS and TEMED are added **immediately before use.**
4. Assemble the gel holders and ensure that they are water tight
5. Add the resolving gel mixture to the gel holders. Fill to around 3/4 full and then top with 70% ethanol to flatten. Leave for 30 mins
6. Remove the ethanol, then add the stacking gel and teeth. Fill from the sides to ensure no gaps
7. Leave for half an hour to set.
8. Load the gel or store at 4 ˚C (wrapped in cling film until use)

4.5 Conditions for tau labelling and oligomerisation

1. It is important to treat the starting purified tau material with TCEP to provide a uniform population of monomers for downstream oligomersation (see Figure 3). This step is necessary partly because tau can undergo slow aggregation processes even when stored at -80 ˚C for long durations [42].

2. Perform the labelling and oligomerisation process in cold conditions (at 4 ˚C) to ensure the generation of pure populations of oligomers. the We have noted that doing so at room temperature generates a mix of oligomers and larger aggregates including fibrils (Figure 5). This agrees with previous publications showing that maleimide labelling of tau is more efficient at 4 ˚C [43,44], and that labelling at room temperature or 37 C leads to fibril contamination of oligomers [39,41].

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Figure legends

Figure 1: Plasmids for bacterial expression of tau. pProEX plasmids coding for (A) full-length tau-441, Uniprot ID: P10636-8), and (B) tau-K18 (amino acids 244-372 of full length tau-441).

Figure 2: Schematic illustration of the method of tau oligomer stabilization described in this protocol. The method involves first treating highly-purified monomers with the reducing agent TCEP to monomerise apparent oligomers. Monomers are then labelled in cold conditions with either maleimide derivative (Alexa-Fluor-488-maleimide or N-ethlyl maleimide). Excess maleimide derivatives are removed by dialysis. Labelled oligomers can then be characterized using an array of biochemical and biophysical tools, including non-denaturing SDS-PAGE, dot/western blotting and visualisation by transmission electron microscopy.

Figure 3: Characterisation of tau-K18 oligomers by protein gel electrophoresis. The non-reducing SDS-PAGE gels (samples analysed without heat or beta mercaptoethanol treatment) in (A) show tau-K18 oligomers prepared following the method described in this Chapter. (B) shows aggregates prepared without initial treatment with the reducing agent

TCEP, highlighting the importance of this step. Figure taken from [38] in accordance with CC.BY 4.0 open access license.

Figure 4: Characterisation of full-length tau and tau-K18 oligomers using transmission electron microscopy. (A) Buffer-only negative control, (B) Full-length tau-441 oligomers, and (C) Tau-K18 oligomers. Insets refer to oligomers observed at higher magnification. Scale bars = 200 nm for main figures and 20 nm for insets. Panels A and C taken from [23] and panel B from [34] in accordance with CC.BY 4.0 open access license.

Figure 5: Importance of labelling tau oligomers in cold conditions. (A) Maleimide labelling of tau-K18 at room temperature produces a mix of globular oligomers and fibrils. (B) Unlabelled tau aggregates into oligomer, protofibrils and fibrils. Scale bars = 100 nm. Figure taken from [34] in accordance with CC.BY 4.0 open access license.