Angewandte Chemie
Eine Zeitschrift der Gesellschaft Deutscher Chemiker

Supporting Information

Single Posttranslational Modifications in the Central Repeat Domains of Tau4 Impact its Aggregation and Tubulin Binding

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Supporting Information

Water was purified, using a Milli-Q®Integral Water Purification System, and used for all experiments. AcOH, EtOH, Toluene (all from Sigma Aldrich, Vienna, Austria), ACN, DCM, DMF, Et_2O, *i*PrOH, MeOH and TFA (all from VWR, Vienna, Austria) were purchased in HPLC or peptide synthesis grade. All amino acids, HATU, HBTU, DIEA, DIC, DMS, 2,6-lutidine, MESNa, MPA, MPAA, OxymaPure, piperidine, TCEP, thioanisole, TIS and trityl chloride resin were obtained from commercial sources (Bachem AG, Bubendorf, Switzerland; Iris Biotech, Marktredwitz, Germany; Merck, Darmstadt, Germany; Novabiochem, Nottingham, UK; Sigma Aldrich, Vienna, Austria and VWR, Vienna, Austria).

All chemicals used for molecular biology experiments, especially buffer preparation, and aggregation assays were purchased from Sigma Aldrich (Vienna, Austria) and VWR (Vienna, Austria). Materials for cloning (restriction enzymes, ligases, vectors, etc.) and protein expression were obtained from Agilent Technologies (Vienna, Austria), Promega (Mannheim, Germany) and New England Biolabs (Frankfurt, Germany). All constructs were sequenced at Eurofin Genomics AT (Vienna, Austria). The PCR purification kit was purchased from Qiagen (Hilden, Germany) and the Gene JET Plasmid Miniprep Kit from Thermo Fisher Scientific (Vienna, Austria). The protein standard Amersham Low Molecular Weight (LMW) Calibration Kit for SDS Electrophoresis (GE Healthcare, Vienna, Austria) and equipment (multi-casting chamber, etc.) from Biorad (Vienna, Austria) were used for SDS-PAGE experiments. 96-well black untreated polystyrene plates for fluorescence detection and Adhesive plate seals from Greiner Bio-One GmbH (Kremsmuenster, Austria) were used for aggregation assays. The 5 ml His-Trap Ni-NTA column from GE Healthcare (Vienna, Austria) and chitin beads from New England Biolabs (Frankfurt, Germany) were used for affinity purification. The protein concentrations of the aliquots used in aggregation assays were determined using the Pierce BCA Protein Assay Kit from Thermo Scientific (Vienna, Austria).

HPLC buffers: Buffer A (H_2O + 0.05% TFA) and buffer B (ACN + 0.05% TFA) were used on the Waters Auto Purification HPLC/MS system. On the Dionex Ultimate 3000 instrument for analytical HPLC, buffer A (H_2O + 0.1% TFA) and buffer B (ACN + 0.08% TFA) were used.

SDS-PAGE: All gels were run under conditions described by Laemmli using 15% separating gels [1]. Sample loading was carried out in 2x SDS-loading buffer (0.5 M Tris-HCl, 6% (w/v) SDS, 35% (vol%) glycerol, 3.5% (vol%) β-mercaptoethanol and 0.05% (w/v) bromophenol blue, pH 6.8). For gel staining and de-staining, a Coomassie solution (0.1% (w/v) Coomassie R250, 10% (vol%) acetic acid and 45% (vol%) methanol in water) and de-staining solution (10% (vol%) acetic acid and 40% (vol%) methanol in water) were used. For silver staining the following buffers were used: fixation solution (see de-staining solution), incubation solution (50 ml aq. solution with 15 ml EtOH, 3.4 g NaOAc and 0.1 g sodium thiosulfate), staining solution (50 ml aq. solution with 0.05 g $AgNO_3$ and 30 µl 36% formaldehyde), developing solution (50 ml aq. solution with 1 g Na_2CO_3 and 15 µl 36% formaldehyde), and stop solution (50 ml aq. solution with 0.04 M EDTA at pH 8 and 10% v/v glycerol).

Protein expression: 2YT medium (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl) containing antibiotics as required (ampicillin: 100 mg/l, chloramphenicol: 30 mg/l, kanamycin: 30 mg/l) was used for all expressions. All media were autoclaved at 121°C for 30 min.

Protein purification: Tris buffers used for TauN affinity purification are salt-Tris buffer (50 mM Tris-HCl and 500 mM NaCl, pH 7.5) and no-salt-Tris buffer (50 mM Tris-HCl, pH 7.5). The Tris buffers used for TauC are imidazole-containing Tris buffer A (30 mM Tris-HCl, 0.3 M NaCl and 10 mM Imidazole), Im-Tris buffer B (30 mM Tris-HCl, 0.3 M NaCl and 500 mM Imidazole) and TEV-cut buffer (50 mM Tris-HCl, 100 mM NaCl, 0.5 mM EDTA and 1 mM DTT). The MES-buffer (50 mM MES·H_2O and 5 mM DTT, pH 6.9) was used for fl Tau4 variants.

SPPS: Trityl chloride resin was derivatized with the SEA-linker and loaded with the first amino acid according to Ollivier *et al*. [1]. SPPS was performed manually or on the Liberty Blue Automated Microwave Peptide Synthesizer (CEM GmbH, Kamp-Lintfort, Germany). Each peptide was synthesized on a 0.05 mmol scale (except for TauMid with CML294: 0.05 mmol resin; 0.1 mmol scale on the synthesizer) using Fmoc-protected amino acids with following standard side protecting groups: Asn(Trt), Asp(O*t*Bu), Gln(Trt), His(Trt), Lys(Boc), Ser(*t*Bu), Thr(*t*Bu) and Tyr(*t*Bu). In addition, the pseudo-proline dipeptides Fmoc-Leu-Ser($\psi^{Me,Me}$pro)-OH and Fmoc-Gly-Ser($\psi^{Me,Me}$pro)-OH were used at suitable positions to disrupt secondary structure formation and to improve yields [3]. Ninhydrin tests [4] were carried out if required in the manual synthesis, especially before and after the coupling of the Fmoc-Ser(PO(OBzl)OH)-OH building block. During manual synthesis, Fmoc protecting groups were removed with 20% piperidine (vol%) in DMF for 3 and 7 min each. Amino acids (2.5 eq.) were activated with HBTU (2.38 eq.) in DMF and then coupled in the presence of DIEA (5 eq.) for 30 min at rt. For double couplings, this activation and coupling step was repeated. During automated synthesis, amino acids (5 eq.) were activated with DIC (5 eq.) and then coupled in the presence of OxymaPure® (5 eq.) under standard microwave conditions at 70 and 90°C. Histidine and dipeptides were coupled at lower temperatures. Finally, after finishing the peptide sequence, the resin was washed (DMF, DCM and MeOH) and dried under reduced pressure. MTT removal was achieved by swelling the resin for 1 h in DCM and by subsequent treatment with a solution of TFA/TIS/DCM (1/1/98, vol%). After 1-2 min of shaking, the yellowish cleavage solution was removed. This procedure was repeated (20 x) or until the yellowish color disappeared. The resin was washed with DCM and DMF and swollen in DMF for 1 h.

Alkylation of the lysine side chain with BrAcOtBu was achieved by washing the resin with DIEA/DMF (10/90, vol%, 2 x 1 min) and then with DMF. BrAcO*t*Bu (0.8 eq., calculated for 0.05 mmol scale and 100% yield) and DIEA (1.8 eq., calculated for 0.05 mmol scale and 100% yield) in DMF (~1.5 ml) were added to the resin at 0°C and the mixture was shaken o/n at 4°C. The resin was washed (DMF, DCM and MeOH) and dried under reduced pressure.

The Fmoc-Ser(PO(OBzl)OH)-OH building block was coupled manually after standard Fmoc deprotection as described before. Fmoc-Ser(PO(OBzl)OH)-OH (2.5 eq.) was activated with HATU (2.38 eq.) in DMF and then coupled in the presence of DIEA (5 eq.) for 60 min at rt. For double coupling, this activation and coupling step was repeated. All following amino acids of the peptide sequence were coupled manually.

All peptides were globally deprotected and cleaved from the resin using the standard SEA-cleavage mixture (TFA/TIS/H_2O/DMS/thioanisole: 90/2.5/2.5/2.5/2.5, vol%, 10 ml/g resin) for 3 h at rt [5]. The crude peptides were precipitated by addition of three volumes of cold Et_2O and

then centrifuged. The pellet was washed twice with cold Et_2O, dissolved in H_2O/ACN (50/50, vol%) and lyophilized.

All peptides were purified by RP-HPLC using (semi-)preparative C4 columns (Kromasil 300-10-C4, 21.2 x 250 mm and 10 x 250 mm with 10 µm particle size) on a Waters Auto Purification HPLC/MS system. The gradient from 5 to 30% buffer B in buffer A in 40 min was used. For final analysis, an analytical HPLC was performed on the Dionex System using a C4 column (Kromasil 300-5-C4, 4.6 x 150 mm with 5 µm particle size). The standard gradient from 5 to 65% buffer B in 30 min at a flow of 1 ml/min was used. In addition, a mass spectrum was recorded via direct injection using a maXis classic instrument (Bruker) or a Waters Auto Purification HPLC/MS system operating in the positive mode. The use of the SEA linker gives rise to an equilibrium between a C-terminal amide and thioester linkage on each of the TauM peptides. The resulting isomers split into two peaks with the same mass on HPLC chromatograms. We carefully collected only the major peak for each TauM, which were subsequently lyophilized. Re-analyzing TauM segments on the HPLC indicates that the equilibrium is re-established (see Figures S3-S5, S7).

Expressed Protein Ligation (EPL)/Native Chemical Ligation (NCL): First, the synthetic peptide TauM, containing a SEA-thioester at the C-terminus, and TauC were ligated. After purification, removal of the cysteine protecting group and another purification, the ligation product Tau291-441, bearing now again an N-terminal cysteine, was ligated to the segment TauN.

For the first ligation of the segments, a SEA ligation buffer was prepared according to the conditions described in the literature [5]. Gdn.HCl (6 mmol), MPAA (0.2 mmol) and TCEP (0.2 mmol) were dissolved in 0.1 M sodium phosphate buffer (1 ml, pH 7.5) and the pH was adjusted to 7.5 using NaOH (5 M aqueous solution). The resulting ligation buffer was degassed by purging with Argon (10-15 min), aliquoted (100 µL) and stored at -80°C. Before use, the buffer was again freshly degassed with Argon. For each ligation, the TauM peptide (4 mM) and the TauC segment (2 mM) were dissolved in ligation buffer and shaken for 48 h at 37°C and 600 rpm under inert atmosphere. The ligation was monitored by SDS-PAGE and NanoHPLC/MS. For this, an aliquot (0.4-0.5 µl) of the ligation mixture was diluted with 50 µl H_2O, 1 µl was used for HPLC/MS.

For the second ligation, a ligation buffer was prepared containing less TCEP. Gdn.HCl (6 mmol), MPAA (0.2 mmol) and TCEP (0.1 mmol) were dissolved in 0.1 M sodium phosphate buffer (1 ml, pH 7.5) and the pH was adjusted to 7.5 using NaOH (5 M aqueous solution). The resulting ligation buffer was degassed by purging with Argon (10-15 min), aliquoted (100 µL) and stored at -80°C. Before use, the buffer was again freshly degassed with Argon. For each ligation, the Tau291-441 segment (2 mM) and the TauN segment (2 mM) were dissolved in ligation buffer and shaken for 4 h at 37°C and 600 rpm under inert atmosphere. The ligation was monitored by SDS-PAGE and/or HPLC/MS.

For purification, the ligation mixtures were acidified with 10% aqueous TFA (1 µl/ µl reaction mixture) and extracted with Et_2O to remove MPAA before injection. Purification was performed by peak collection using a C4 column (Kromasil 300-5-C4, 4.6 x 150 mm with 5 µm particle size or a new Kromasil C4, 4.6 x 250 mm) on the Dionex system. The gradient from 5 to 40% buffer B in buffer A in 35 min was used and maximum loading on the column was 1 mg of peptide/protein ligation mix.

The Acm protecting group was removed as described by Wang *et al*. [6]. Following the first ligation, the purified product Acm-Tau291-441 (~1 mg/100 µl buffer final concentration) was dissolved in degassed AcOH/H_2O (85/15, vol%). AgOAc (1 mg/100 µl buffer) was added and the mixture was stirred for 1 h at rt under inert atmosphere. The reaction was stopped by addition of degassed Gdn-buffer (6 M Gdn.HCl, 1 M DTT, 1 µl/ µl reaction mixture) and the mixture was stirred for another 30 min at rt, followed by centrifugation. The supernatant was then directly purified by HPLC on the Dionex system using a C4 column (Kromasil 300-5-C4, 4.6 x 150 mm with 5 µm particle size or a Kromasil C4, 4.6 x 250 mm) and a short gradient (from 5 to 30% buffer B in 5 min, then from 30 to 40% buffer B in 10 min). The reaction progress was followed by HPLC/MS. For this, an aliquot (1 µl) was treated as described above for stopping the reaction.

TCA precipitation: 40% TCA (w/v, 15 µl) were added to the ligation sample (50 µl). After mixing, the samples were precipitated for 15 min on ice and centrifuged. The supernatant was discarded and the pellet resuspended in 200 µl EtOH/H_2O (90/10, vol%) and again centrifuged. The pellet was dried and dissolved in water (5 µl) and 2x SDS-loading buffer (5 µl).

TauN: For the generation of TauN-thioester, the Tau4(1-290)-sequence was cloned into a pTWIN1 vector encoding an *Mxe* intein-CBD and ampicillin resistance. For large-scale expression, 2YT medium (3 x 2 l) containing ampicillin was inoculated with o/n culture (3 x 200 ml 2YT medium, inoculated with glycerin stock and then incubated for 16-18 h) until a starting OD_{600} of 0.1 was obtained. After cell growth for 2-2.5 h and an OD_{600} of 0.6-0.8, the expression was induced with 1 mM IPTG for 5 h. The expression was monitored by regular OD_{600} measurements and samples for SDS-PAGE were taken. Cells were harvested by centrifugation for 20 min at 15900 g at 4°C. The supernatant was discarded and the remaining pellets were re-suspended in 200 mL 1x PBS-buffer and again centrifuged for 30 min at 4255 g and 4°C. The washing buffer was discarded and the pellets stored at -80°C. Before lysis at 1.9 kbar and 10°C, the defrosted pellets were re-suspended in 50 ml salt-Tris buffer containing 200 µM PMSF (diluted in 100 µl EtOH before addition). The lysis mixture was centrifuged at 48,384 g for 30 min at 4°C. The expressed protein construct was in the supernatant, which was directly used for affinity purification and the pellet was discarded. Chitin beads, stored in 20% aq. EtOH, were washed (1 x H_2O and 3 x salt-Tris buffer). The supernatant after cell lysis was added and the beads were incubated for 2 h on a rotator at 4°C. The supernatant was removed and the chitin beads were washed (1 x no-salt-Tris buffer, 2 x salt-Tris buffer and 1 x no-salt-Tris buffer). To cleave the TauN segment as a thioester, 200 mM MESNa in no-salt-buffer (~1 column volume) were added and the suspension was incubated on a rotator at 4°C. Samples for SDS-PAGE were taken after 24h and 48h. The cleavage was stopped after 48 h by collecting the supernatant and the chitin beads were washed with fresh 200 mM MESNa in no-salt-buffer (~1 column volume). The combined supernatants containing TauN thioester, but also traces of uncleaved TauN-*Mxe* intein-CBD and intein, were filtrated (pore size ≤0.22 µm) and stored at -80°C. Further purification was achieved using a Varian ProStar RP-HPLC System with a preparative C4 column (Kromasil 300-10-C4, 10 x 250 mm with 10 µm particle size). The gradient, including 20 min desalting, from 5 to 30% buffer B in 5 min and 30 to 60% buffer B in 30 min at a flow of 10 ml/min was used. For final analysis of the pooled, lyophilized fractions containing the desired product, an analytical HPLC was performed on the Dionex System using a C4 column (Kromasil 300-5-C4, 4.6 x 150 mm with 5 µm particle size). The

standard gradient from 5 to 65% buffer B in 30 min at a flow of 1 ml/min was used. In addition, a mass spectrum was obtained via direct injection using a Waters Auto Purification HPLC/MS system in the positive mode.

TauC: For the generation of the TauC segment bearing an N-terminal-cysteine, the Tau4(322-441)-sequence was cloned into a pET28a vector encoding a His_6–tag with a TEV-cleavage site and kanamycin resistance. For large-scale expression, 2YT medium (3 x 2 l) containing kanamycin was inoculated with o/n culture (3 x 200 ml 2YT medium, inoculated with glycerin stock and then incubated for 16-18 h). After cell growth for ca. 2 h and an OD_{600} of 0.6-0.8, the expression was induced with 1 mM IPTG for 5 h. The expression was monitored by regular OD_{600} measurements and samples for SDS-PAGE were taken. Cells were harvested by centrifugation for 20 min at 15900 g and 4°C. The supernatant was discarded and the remaining pellets were re-suspended in 600 ml medium and again centrifuged for 20 min at 11899 g and 4°C. The washing medium was discarded and the pellets stored at -80°C. Before lysis at 1.9 kbar and 10°C, the defrosted pellets were re-suspended in 80 ml Im-Tris buffer A. The lysis mixture was centrifuged at 48384 g for 30 min at 4°C. The expressed protein construct was in the supernatant, which was directly used for affinity purification and the pellet was discarded. After centrifugation of the cell lysis suspension, the protein concentration of the supernatant was measured to determine the maximum volume that can be loaded on the column. The supernatant was then directly loaded onto a Ni-NTA column that was equilibrated with Im-Tris buffer A at rt. The flow through was collected and the column was washed with Im-Tris buffer A until a constant UV trace was recorded. The gradient from 0 to 100% Im-Tris buffer B in 60 min and a flow of 1ml/min was used. The fractions containing the H_6-TEV-TauC were determined using SDS-PAGE, combined and concentrated. The protein concentration was determined and TEV-protease (dilution 1:10) was added. The mixture was dialyzed against TEV-cut buffer o/n at 4°C. The protein mix after the TEV-cut was diluted 2 x with 6 M Gdn.HCl (pH 4.5) and directly injected into a Varian ProStar RP-HPLC System equipped with a semi-preparative C4 column (Grace Vydac 214TP510, 10x250 mm with 5 µm particle size). The gradient, including 20 min desalting, from 5 to 45% buffer B in 40 min at a flow of 3 ml/min was used. For final analysis of the pooled, lyophilized fractions containing the desired product, an analytical HPLC was performed on the Dionex System using a C4 column (Kromasil 300-5-C4, 4.6 x 150 mm with 5 µm particle size). The standard gradient from 5 to 65% buffer B in 30 min at a flow of 1 ml/min was used. In addition, a mass spectrum was obtained via direct injection using a Waters Auto Purification HPLC/MS system in the positive mode.

Full length Tau4: The gene encoding full-length (fl) Tau4 was obtained in a pET29b vector encoding a kanamycin resistance. The expression and heat treatment steps were performed according to a published procedure [7]. For large-scale expression, 2YT medium (3 x 2 l) containing kanamycin was inoculated with o/n culture (3 x 200 ml 2YT medium, inoculated with glycerin stock and then incubated for 16-18 h) until a starting OD_{600} of 0.1 was obtained. After cell growth for 2-2.5 h and an OD_{600} of 0.6-0.8, the expression was induced with 1 mM IPTG for 4 h. The expression was monitored by regular OD_{600} measurements and samples for SDS-PAGE were taken. Cells were harvested by centrifugation for 20 min at 15,900 g and 4°C. The supernatant was discarded and the remaining pellets were re-suspended in 70 ml 1x PBS-buffer and again centrifuged for 30 min at 4255 g and 4°C. The washing buffer was discarded and the pellets stored at -80°C. The defrosted pellets were re-suspended in 50 ml MES-buffer and then lysed at 1.9 kbar and 10°C. The lysis mixture was centrifuged at 48,384 g for 30 min at 4°C. The expressed protein construct was in the supernatant, which was directly used for pre-purification and the pellet was discarded. The supernatant was stirred at 95°C.

After 20 min, the mixture (containing white precipitate corresponding to undesired products) was centrifuged for 45 min at 75,600 g and 4°C. The remaining supernatant was filtered (pore size ≤0.22 µm) and samples for SDS-PAGE and HPLC analysis were taken. Following heat treatment and filtration, the supernatant was directly injected into a Varian ProStar RP-HPLC System equipped with a preparative C4 column (Grace Vydac 214TP1022, 22 x 250 mm with 10 µm particle size or a Kromasil 300-10-C4, 10 x 250 mm with 10 µm particle size). The gradient, including 20 min desalting, from 5 to 30% buffer B in 5 min and 30 to 60% buffer B in 30 min at a flow of 10 ml/min, was used. The purity of the fractions of wt fl tau4 was not sufficient for aggregation assays, so another purification with a semi-preparative C4 column (Grace Vydac 214TP510, 10x250 mm with 5 µm particle size) on a Waters Auto Purification HPLC/MS system was performed. The gradient, from 5 to 20% buffer B in 5 min and 20 to 50% buffer B in 30 min at a flow of 10 ml/min was used. For final analysis of the pooled, lyophilized fractions containing the desired product, an analytical HPLC was performed on the Dionex System using a C4 column (Kromasil 300-5-C4, 4.6 x 150 mm with 5 µm particle size). The standard gradient from 5 to 65% buffer B in 30 min at a flow of 1 ml/min was used. In addition, a mass spectrum was obtained via direct injection using a Waters Auto Purification HPLC/MS system in positive ion mode.

Aggregation assays: The procedure for aggregation assays was modified from Nanavaty *et al*. [8]. chapter 6 in (Smet-Nocca, C., ed. *Tau Protein - Methods and Protocols*. 1 ed. Methods in Molecular Biology, ed. S.S.B.M.N. York. 2017, Humana Press) using ThT dye fluorescence. All buffers were prepared, filtered (pore size ≤0.22 µm) and stored in aliquots at -20 or 4°C. The following buffers were used: 5x aggregation buffer (50 mM HEPES with 500 mM NaCl at pH 7.4 and 5 mM DTT), 2 mM octadecyl sulfate (dissolved in 50% (v/v) aqueous *i*PrOH) and ThT stocks (100 µM in H_2O). For the aggregation assays, 1x aggregation buffer (freshly mixed with DTT aliquots) with 50 µM ODS was freshly prepared from the stock solutions and the tau-4 variants were dissolved (final concentration: 2.4-2.6 µM). 90 µl of each mixture were pipetted into a well plate containing glass pearls for improved mixing and 10 µl of the ThT stock solution were added. The plate was sealed with an adhesive foil and put into the microplate-reader device. Aggregation was monitored at wavelengths λ_{ex}=440 nm and λ_{em}=490 nm and at 37°C under medium shaking for 24 h. A mixture of 1x aggregation buffer, already containing ODS (90 µl), and ThT stock solution (10 µl), was used as a blank. To assess the amount of soluble Tau after aggregation, samples were collected from wells and centrifuged at 200,000 x g at 4°C for 20 min (Optima ultracentrifugation system, Beckman Coulter Inc.). Supernatants were injected into a Dionex HPLC system equipped with a C4 column (Kromasil 300-5-C4, 4.6 x 150 mm with 5 µm particle size) running a gradient from 5 to 65% solvent B in 30 min at a flow rate of 1 ml/min (see Figure S16).

Tubulin binding/polymerization assay: 15 µM concentrations of all Tau4 variants in TP buffer (80 mM PIPES pH 6.9, 2 mM $MgCl_2$ and 0.5 mM EGTA) were mixed with tubulin (3 mg/ml) in the presence of 1 mM GTP at 37°C. [9-11] Absorbance (scattering) was measured at 340 nm over 30 min and normalized to 1 for the highest absorption. Protein concentration and homogeneity was checked by SDS-PAGE (see Figure S15).

Scanning Electron Microscopy (SEM): Samples from aggregation assays (1 µl each) were directly applied to a Thermanox™ coverslip (Thermo scientific), wisk-washed on the slides and air-dried. The coverslips were then placed onto sample holders and sputter coated with gold in high vacuum (Bal-Tec SCD 005) and measured.

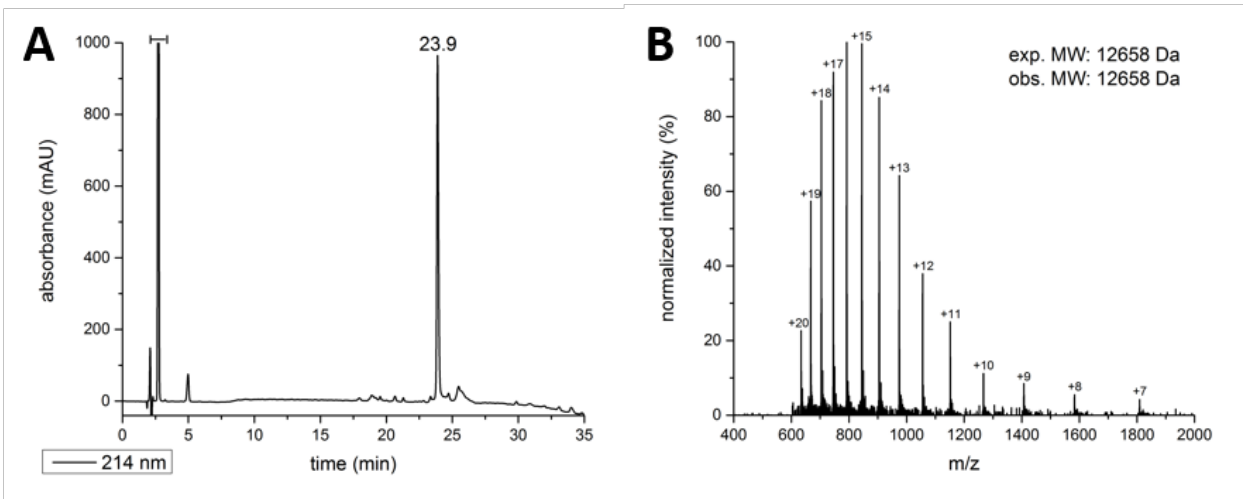

Figure S1: Characterization of the purified TauC segment; A: analytical RP C4 HPLC chromatogram; B: ESI-MS spectrum; exp. MW: 12,658 Da; obs. MW: 12,658 Da.

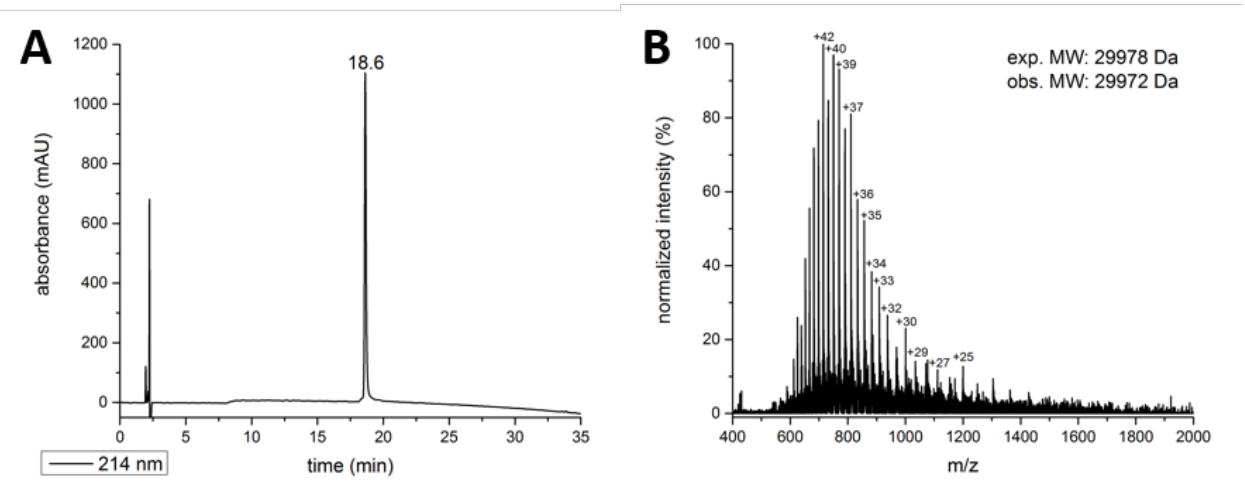

Figure S2: Characterization of the purified TauN segment; A: analytical RP C4 HPLC chromatogram; B: ESI-MS spectrum; exp. MW: 29,978 Da; obs. MW: 29,972 Da.

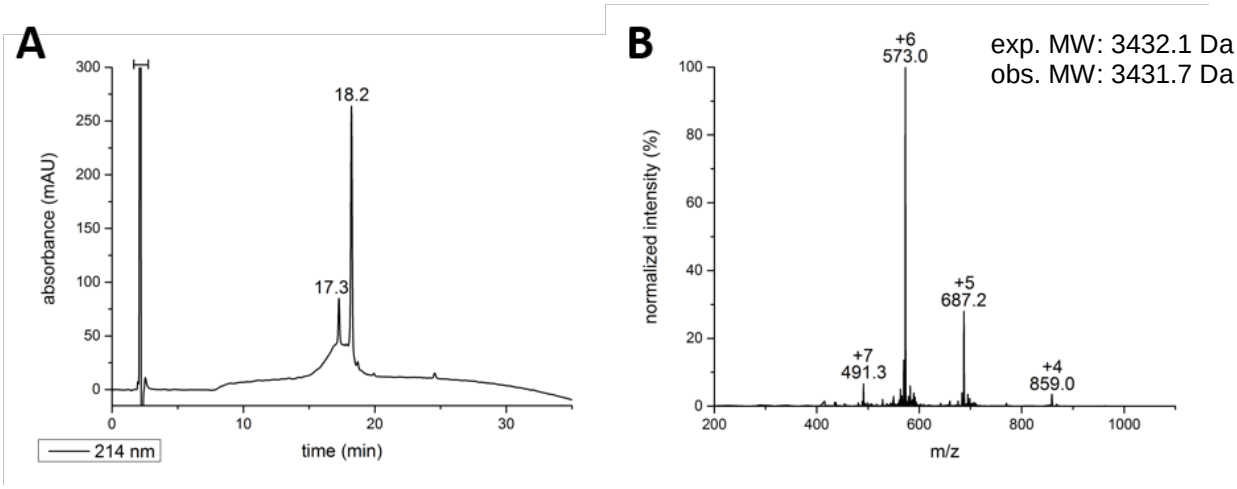

Figure S3: Characterization of unmodified Acm-TauM-SEA peptide; A: analytical RP C4 HPLC chromatogram; B: ESI-MS spectrum; exp. MW: 3432.1 Da; obs. MW: 3431.7 Da.

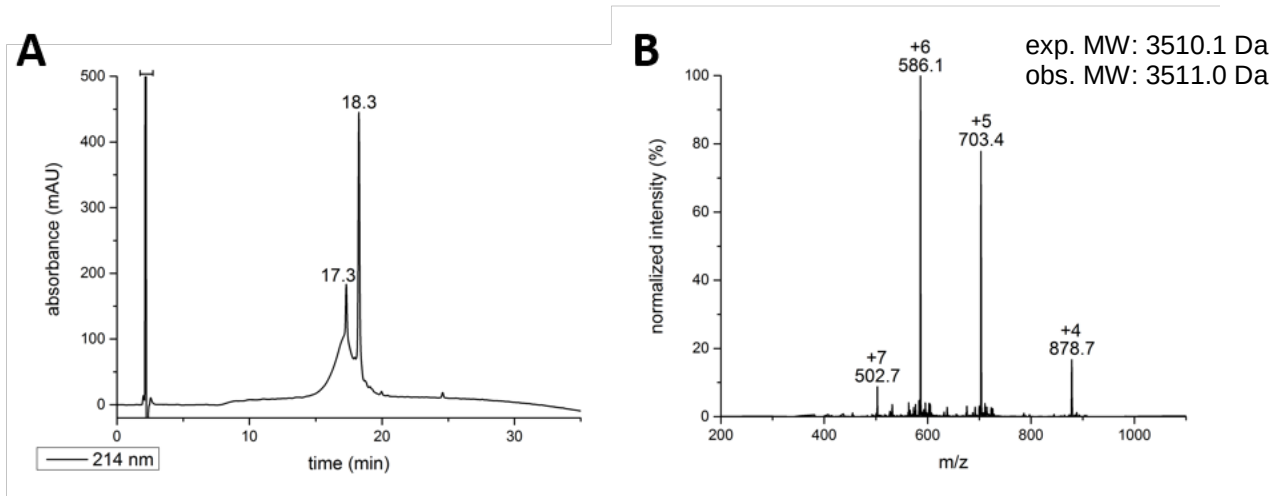

Figure S4: Characterization of Acm-TauM-SEA – pS293 peptide; A: analytical RP C4 HPLC chromatogram; B: ESI-MS spectrum; exp. MW: 3510.1 Da; obs. MW: 3511.0 Da.

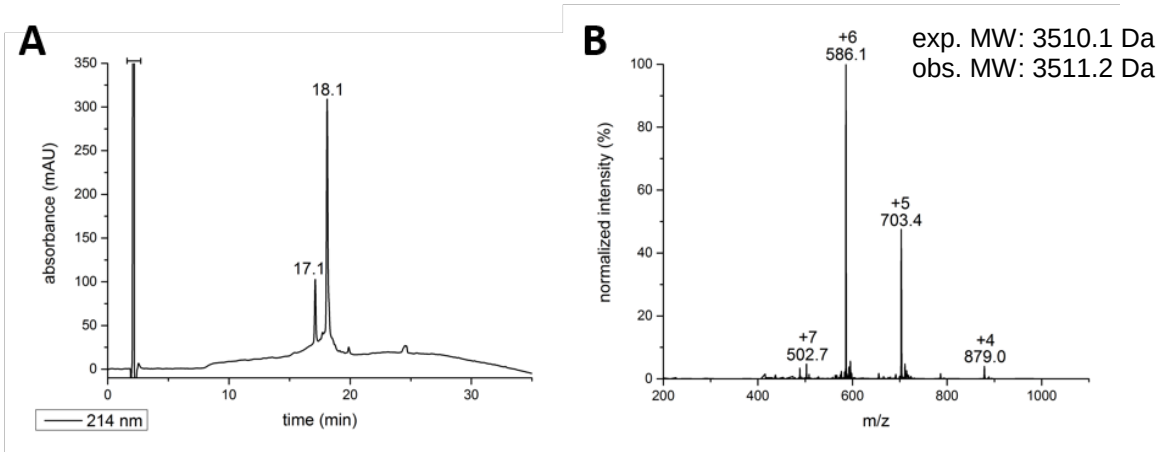

Figure S5: Characterization of Acm-TauM-SEA – pS305 peptide; A: analytical RP C4 HPLC chromatogram; B: ESI-MS spectrum; exp. MW: 3510.1 Da; obs. MW: 3511.2 Da.

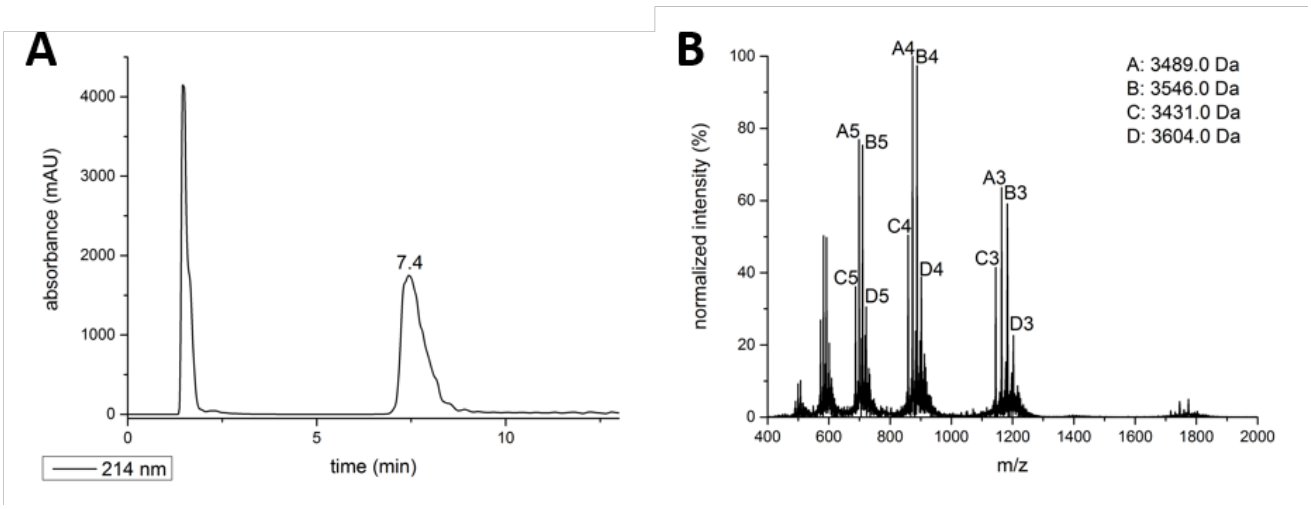

Figure S6: Test-cleavage after alkylation; crude Acm-TauM-SEA – CML294 peptide; A: analytical RP C4 HPLC chromatogram; B: ESI-MS spectrum; observed masses: A: 3489.0, B: 3546.0 Da, C: 3431.0 Da and D: 3604.0 Da.

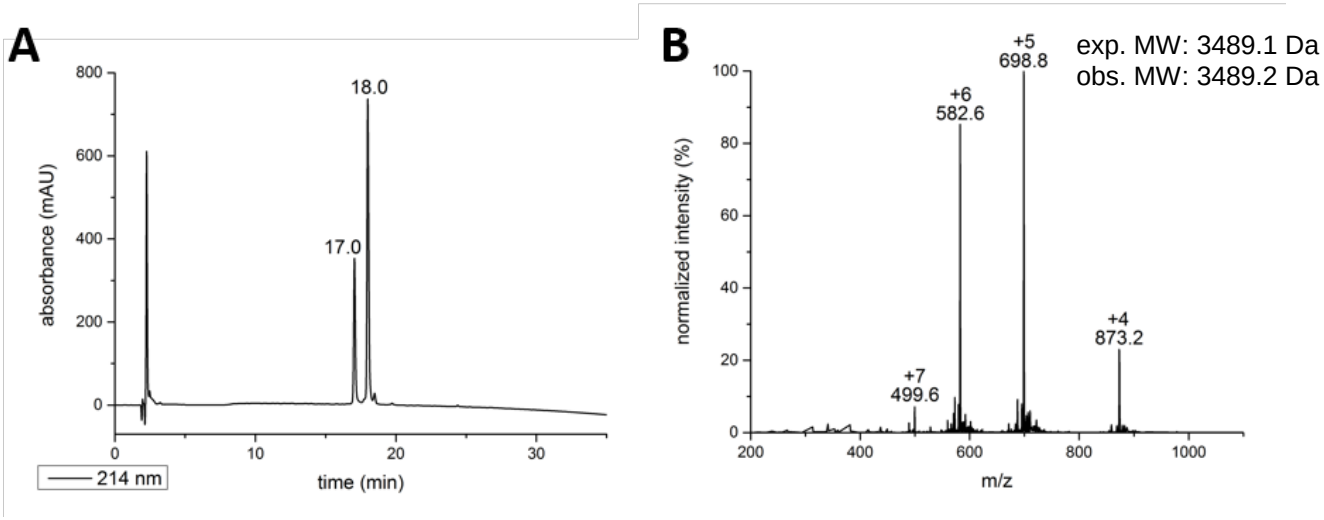

Figure S7: Characterization of Acm-TauM-SEA – CML294 peptide; A: analytical RP C4 HPLC chromatogram; B: ESI-MS spectrum; exp. MW: 3489.1 Da; obs. MW: 3489.2 Da.

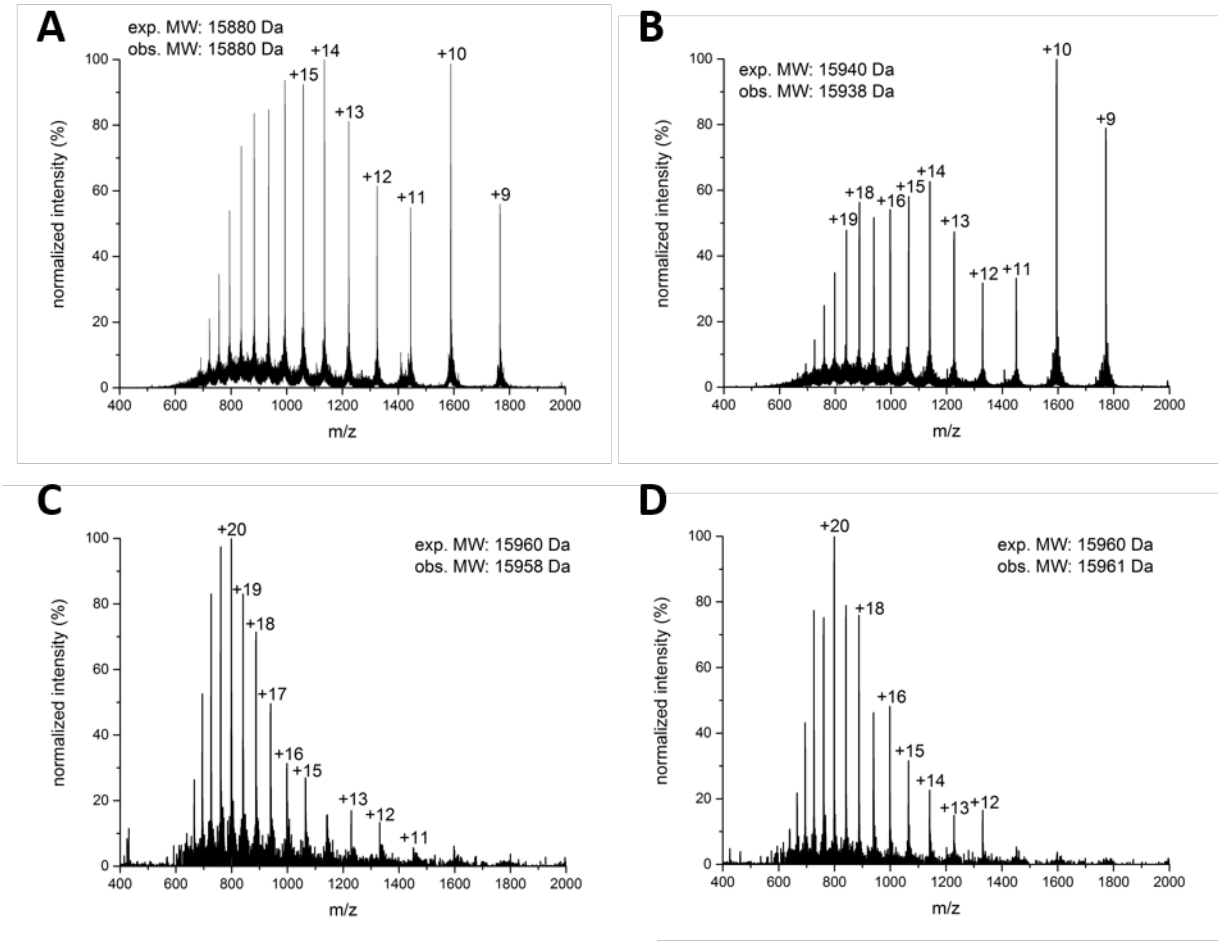

Figure S8: Mass spectra of Tau291-441 variants; A: unmodified Tau291-441 (exp. MW: 15,882 Da; obs. MW: 15,880 Da); B: Tau291-441 [CML294] (exp. MW: 15,940 Da; obs. MW: 15,938 Da); C: Tau291-441 [pS293] (exp. MW: 15,960 Da; obs. MW: 15,958 Da); D: Tau291-441 [pS305] (exp. MW: 15,960 Da; obs. MW: 15,961 Da).

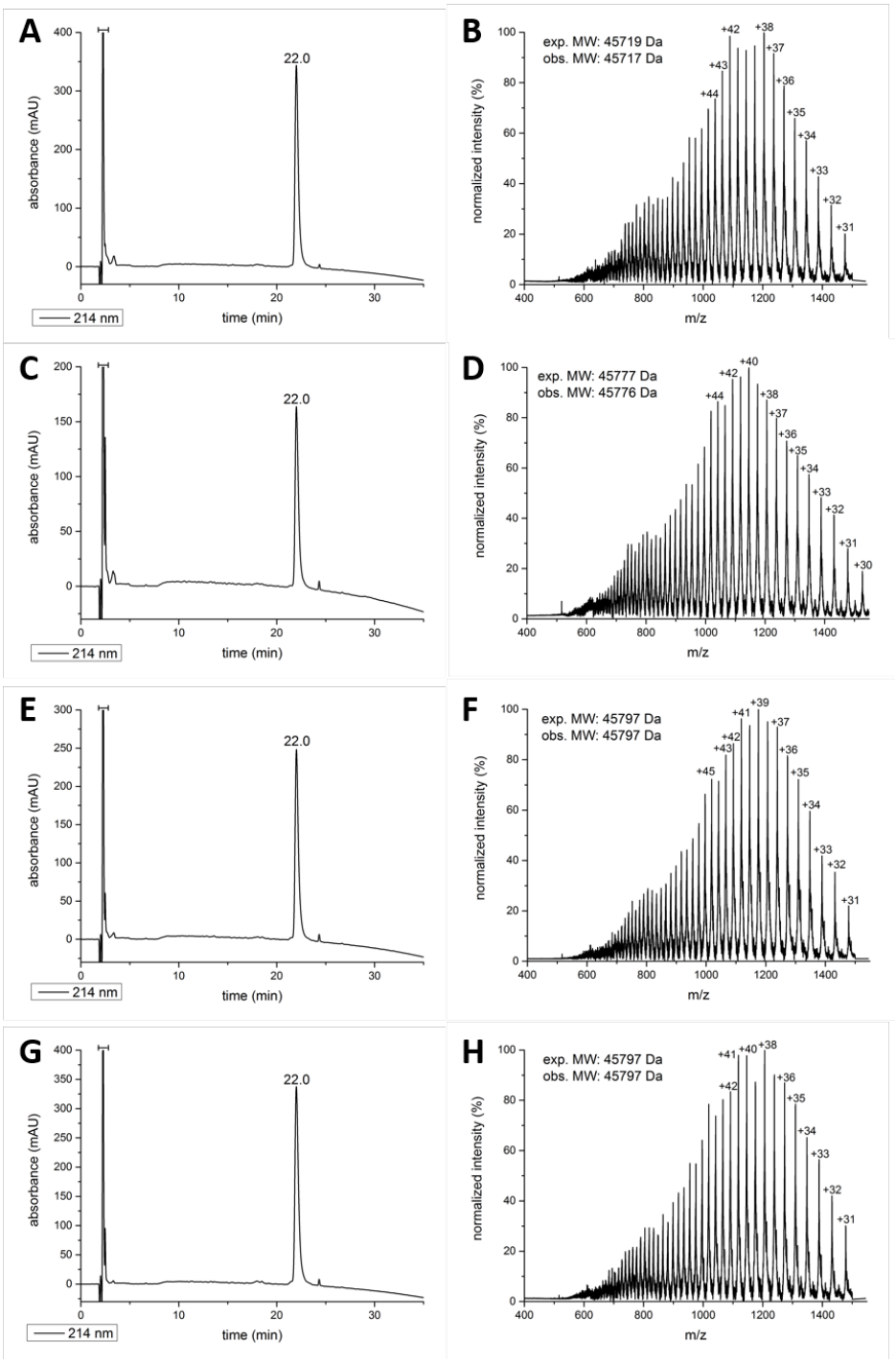

Figure S9: Characterization of all semisynthetic fl Tau4 variants; all mass spectra baselines corrected using OriginPro; **A**: analytical RP C4 HPLC chromatogram of unmodified fl Tau4; **B**: mass spectrum of unmodified fl Tau4 (exp. MW: 45,719 Da; obs. MW: 45,717 Da); **C**: analytical RP C4 HPLC chromatogram of fl Tau4 [CML294]; **D**: mass spectrum of fl Tau4 [CML294] (exp. MW: 45,777 Da; obs. MW: 45,776 Da); **E**: analytical RP C4 HPLC chromatogram of fl Tau4 [pS293]; **F**: mass spectrum of fl Tau4 [pS293] (exp. MW: 45,797 Da; obs. MW: 45,797 Da); **G**: analytical RP C4 HPLC chromatogram of fl Tau4 [pS305]; **H**: mass spectrum of fl Tau4 [pS305] (exp. MW: 45,797 Da; obs. MW: 45,797 Da).

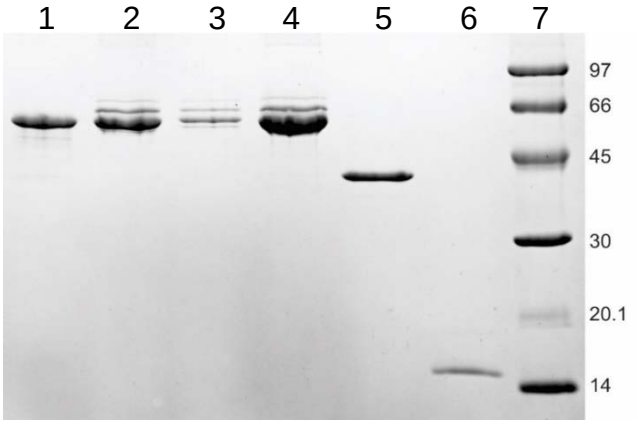

Figure S10: SDS-PAGE analysis of full length Tau4 variants generated with Thz-protected TauM. The additional band above the strong band at ~60 kDa contains Tau4 with an additional insertion of TauM due to undesired premature Thz conversion into cysteine (1. recomb. wt fl Tau4; 2. fl Tau4 [CML294]; 3. fl Tau4 [pS293]; 4. fl Tau4 [pS305]; 5. TauN; 6. TauC; 7. LMW marker). This conversion is most likely caused by the presence of bis(2-sulfanylethyl)amine (released from TauM with a C-terminal SEA group during ligation) over the long reaction time of 48 h. The ability of the SEA group to form a thiazolidine has previously been used in ligation reactions [12]. No such deprotection of Thz was observed during the assembly of Tau4 via a different strategy employing the same ligation site [13], however no bis(2-sulfanylethyl)amine was present during this reaction.

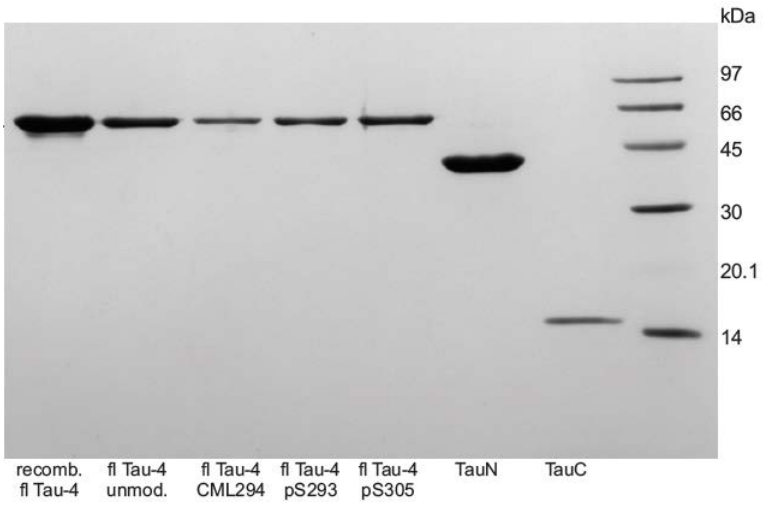

Figure S11: SDS-PAGE analysis of all semisynthetic fl Tau4 variants prepared with Acm-protected TauM, recombinant wt fl Tau4 and recombinantly produced Tau segments TauN and TauC.

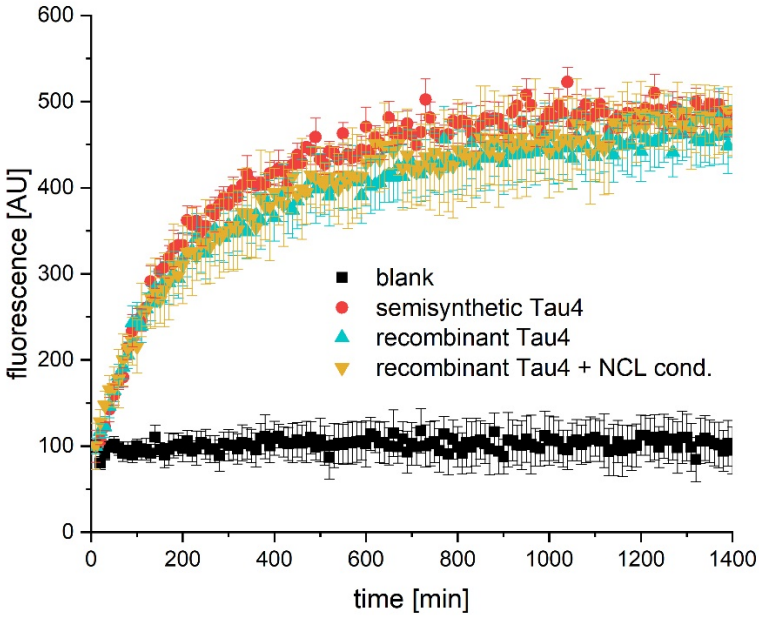

Figure S12: Aggregation behavior of recombinant Tau4, recombinant Tau4 after treatment with ligation buffer (NCL cond.) and semisynthetic Tau4 .

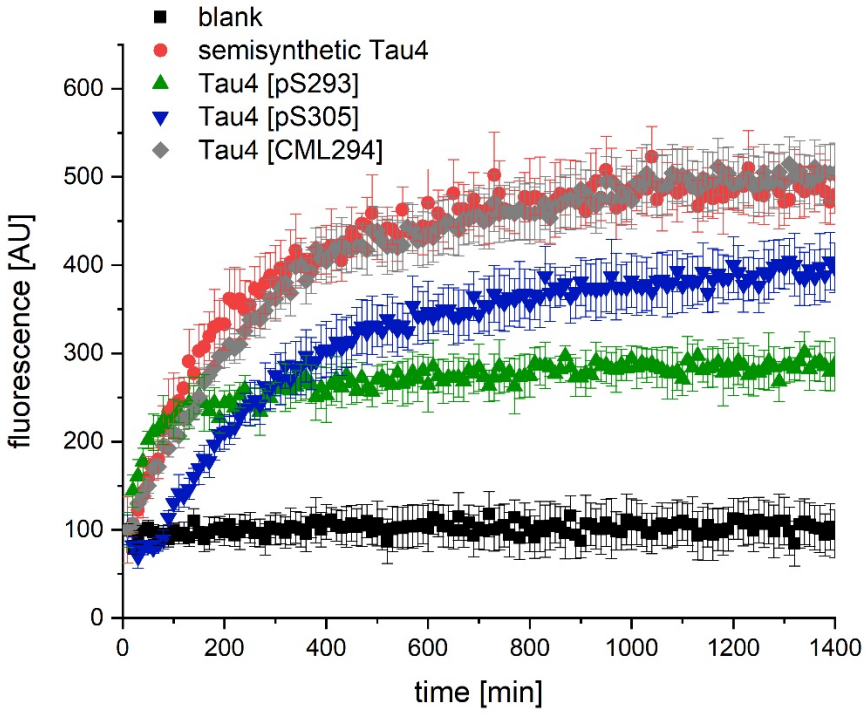

Figure S13: Aggregation behavior of all modified semisynthetic tau4 variants (Tau4 [pS293], Tau4 [pS305] and Tau4 [CML294]) compared to recombinant Tau4 with error bars.

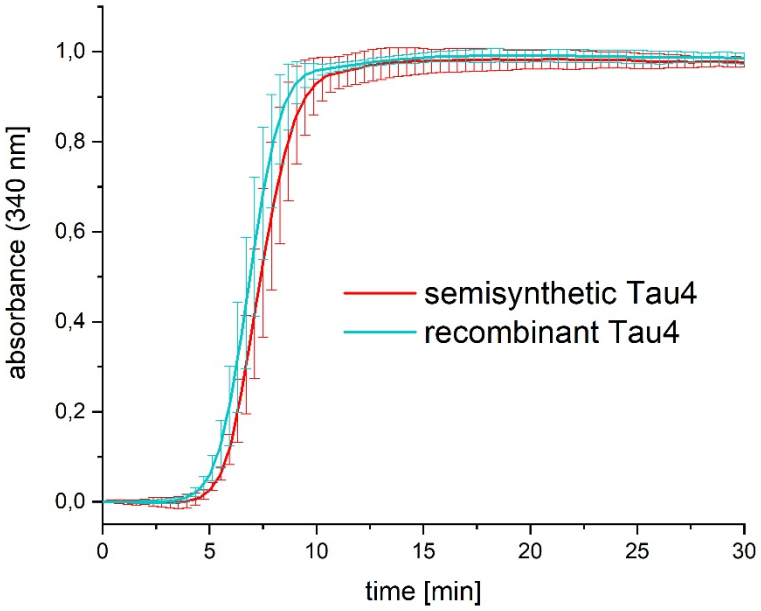

Figure S14: Tubulin binding of semisynthetic fl Tau4 and recombinantly produced fl Tau4. No significant difference in the scattering signal of polymerized tubulin is observed

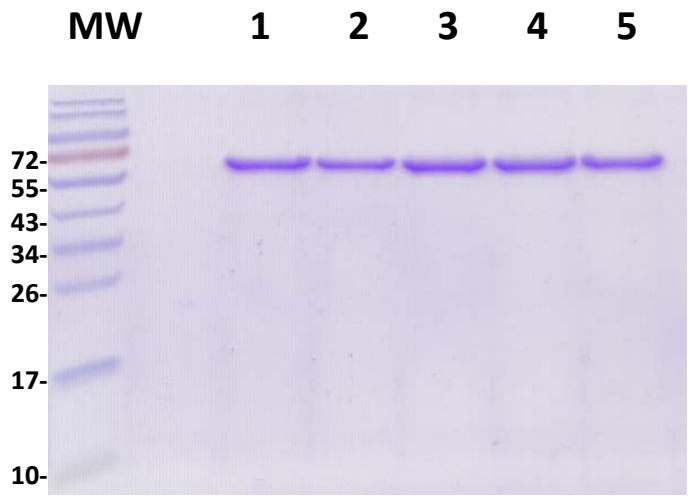

Figure S15: SDS-PAGE of Tau4 samples used to confirm homogeneity and equal concentrations for MT binding assays. 1. Recombinant wt Tau4; 2. Semisynthetic wt Tau4; 3. Tau4 [p293]; 4. Tau4 [p305]; 5. Tau4 [CML294].

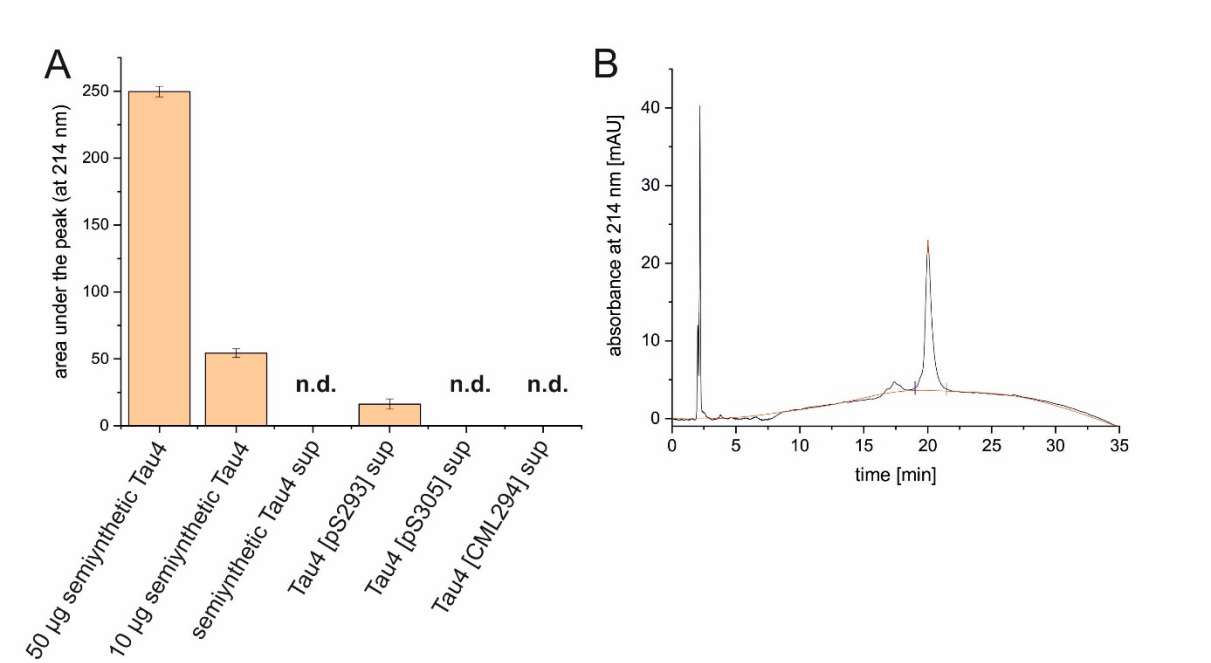

Figure S16: Detection of soluble Tau4 variants in supernatants of aggregation assays. **A:** Area analysis. Calibration with 50 and 10 µg of semisynthetic Tau4. Only for Tau4 [pS293] soluble protein was detected. The peak area corresponds to 3 µg (~30% of total protein used in aggregation assay). **B:** RP-HPLC chromatogram of Tau4 [pS293] supernatant after aggregation. The red line indicates the baseline used for integration of the peak at 20 min. **n.d.** = not detectable

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