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Protein Semisynthesis Provides Access to Tau Disease-Associated Post-translational Modifications (PTMs) and Paves the Way to Deciphering the Tau PTM Code in Health and Diseased States

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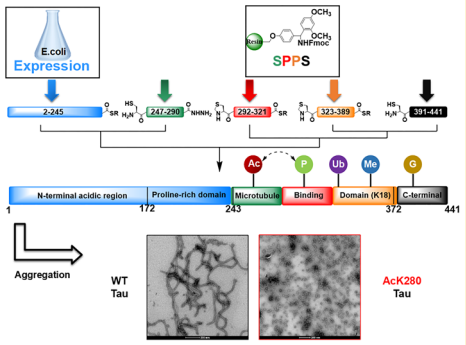
Supporting Information

ABSTRACT: The microtubule-associated protein Tau plays a central role in neurodegeneration and is a leading therapeutic target for the treatment of Alzheimer's disease (AD). Several lines of evidence suggest that post-translational modifications (PTMs) regulate the function(s) of Tau, including its subcellular localization, clearance, aggregation, toxicity, and pathological spreading. However, the lack of tools and methodologies that allow site-specific introduction of PTMs in Tau have limited our ability to dissect the role of PTMs in regulating Tau functions in health and disease. To facilitate deciphering the Tau PTM code, we have developed, for the first time, semisynthetic strategies that allow for the site-specific introduction of single or multiple physiological or disease-associated PTMs that occur within residues 246−441 of Tau, which includes the microtubule-binding domain (MTBD). As a proof of concept, we produced unmodified Tau and three Tau variants with single or multiple disease-associated PTMs that were not previously accessible as homogeneously modified proteins, AcK280, pY310, and pS396/pS404. We then focused on investigating the effect of acetylation at lysine 280 (AcK280) on the structure, aggregation, and microtubule binding properties of Tau. Our results show that site-specific acetylation at K280 significantly enhances the aggregation rate of Tau and impairs microtubule assembly. Surprisingly, compared with unmodified Tau, which forms long and flexible filaments, AcK280 Tau forms predominantly globular oligomers and short fibrils (<200 nm) that exhibit a reduced propensity to assemble into long filaments. These findings are consistent with the increased aggregation propensity and pathogenicity of this mutant in animal models of AD and suggest that acetylation at this residue might enhance the seeding capacity or formation of toxic Tau species *in vivo*. Beyond acetylation and phosphorylation, the development of this semisynthetic strategy provides new opportunities to investigate other types of Tau PTMs and to study the cross-talk between PTMs that occurs within residues 246−441, which were previously inaccessible, thereby paving the way to deciphering the Tau PTM code in health and disease.



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INTRODUCTION

Increasing evidence suggests that the microtubule (MT)-associated protein Tau plays a causative role in the pathogenesis of Alzheimer's disease (AD) and several other neurodegenerative diseases that are collectively known as Tauopathies. Tau accumulation in the form of hyperphosphorylated paired helical filaments (PHFs), neurofibrillary tangles (NFTs), or other aggregated states in the brain represent key pathological features that are commonly used to diagnose[1] and monitor the progression of AD[2,3] and Tauopathies.[4,5] Although the mechanisms by which Tau contributes to the pathogenesis of these diseases remain unknown, increasing evidence from neuropathology research, brain imaging, and animal models suggest that Tau dysfunction, misfolding, and aggregation play central roles in the cascade of events that precede neurodegeneration, cognitive deficit, and eventually death.[6]

Several physiological functions have been attributed to Tau, including its role in (1) assembly, stabilization, and dynamics of microtubules,[7] (2) regulation of axonal transport and axonal outgrowth,[8] (3) organization of the cytoskeleton network, and (4) regulation of synaptic function and plasticity.[9,10] The high solubility, chemical diversity (six major isoforms), and conformational flexibility of Tau combined with its ability to undergo a broad range of reversible post-translational modifications (PTMs)[11] contribute to expanding the Tau proteoform and could thus explain the ability of Tau to perform a wide variety of physiological functions.

PTMs represent important mechanisms for regulating protein function, subcellular localization, the protein interactomes, and protein turnover in health and disease.[12−14] Tau is known to undergo several PTMs including ubiquitination, methylation, polyamination, prolyl-isomerization, acetylation, glycation, glycosylation, nitration, and truncation. However, most studies on Tau PTMs have focused on Tau phosphor-

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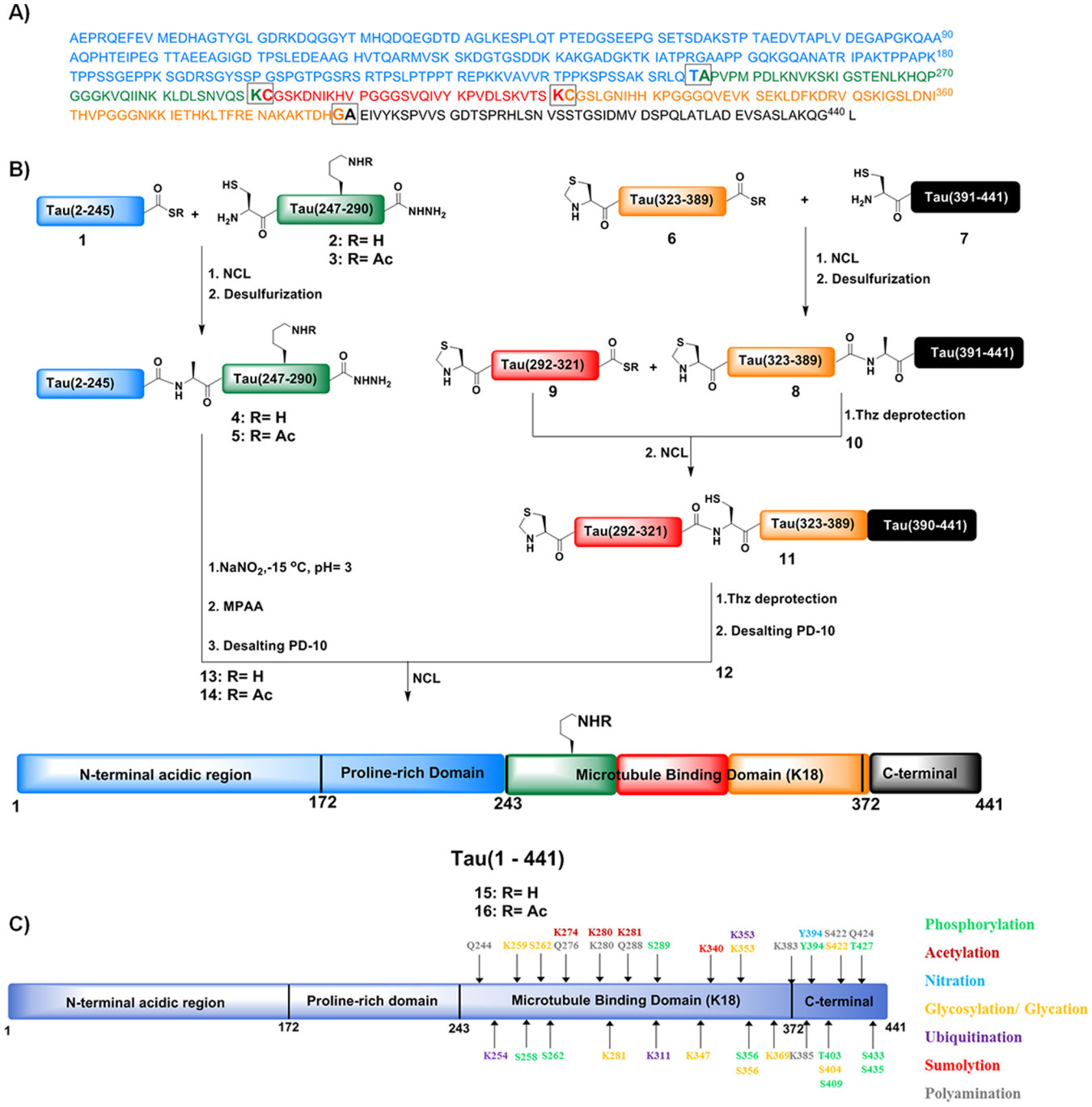
Scheme 1. Semisynthetic Strategy of Wild-Type (WT) and K280 Acetylated Tau[a]



[a](A) The sequence of full-length Tau highlighting the ligation sites. (B) Scheme showing the assembly of the five fragments: the recombinant fragment (blue) and the synthetic fragments (green, red, orange and black), where PTMs could be introduced. (C) Tau disease-associated PTMs at the microtubule binding and C-terminal domains that are covered by that current strategy.

ylation for several reasons: (1) pathological Tau in AD and other Tauopathies is hyperphosphorylated; (2) Tau hyperphosphorylation significantly disrupts its binding to tubulin, thereby leading to microtubule destruction;[15,16] (3) phosphorylation represents the most common and frequent Tau PTM. At least 45 out of the possible 85 serine (S) and threonine (T) residues have been shown to undergo phosphorylation in PHF Tau;[17] (4) several kinases and phosphatases that regulate Tau phosphorylation have been identified compared to a very limited number of enzymes that regulate other PTMs, such as acetylation, ubiquitination, and glycosylation; and (5) the use of phosphomimetics (the substitution of serine (S) or threonine (T) to aspartic acid (D) or glutamic acid (E)) has provided an acceptable alternative approach to bona fide Tau phosphorylation *in vivo* and *in vitro* when the kinases targeting specific sites are not known. The clustering of the different PTMs within short sequence motifs and functional domains suggests that Tau function and dysfunction could be regulated by a complex interplay between different PTMs. Therefore, our ability to decipher the Tau PTM code and utilize it effectively

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to develop novel diagnostic and therapeutic strategies for Tauopathies will require new methodologies for the site-specific introduction of single or multiple PTMs into a Tau sequence to determine how these modifications alter its structure, function, and cellular properties.

To address similar challenges with other proteins, native chemical ligation (NCL)[18] and expressed protein ligation (EPL)[19] strategies have been widely utilized to produce highly homogeneous, site-specific, post-translationally modified proteins, including proteins that have been implicated in neurodegenerative diseases, such as α-synuclein, the prion protein, and N-terminal huntingtin fragments.[20−25] Tau is approximately four times larger than these neuronal proteins and contains two native cysteines, which must be preserved because of their important role in Tau function and aggregation. Recent attempts to achieve the semisynthesis of Tau have focused primarily on PTMs that occur within the last 50 C-terminal amino acids. Hackenberger and co-workers successfully introduced site-specific phosphorylation[26] and O-GlcNAcylation[27] at the C-terminus of Tau using an EPL approach. Their strategy relies on assembling the expressed N-terminal Tau fragment (1−389)-SR with the synthetic C-terminal Tau fragment (A390C-441), which bears an alanine (A) to cysteine (C) mutation at position 390 to allow NCL. In addition to the desired PTM (e.g., phosphorylation), biotin was also introduced at lysine438 (K438) to facilitate the purification of the ligation product. Subsequent studies from the same group reported a photocleavable version of biotin that can be removed after purification.[28] Despite this important achievement, this strategy suffers from several limitations and drawbacks. First, the mutation of A390 to C at the ligation site results in the introduction of a non-native cysteine residue that might form intermolecular or intramolecular disulfide bridges with other native C residues (C291 and C321) or with itself, which could alter the structure and aggregation properties of the protein.[29−31] Second, this strategy provided access to only PTMs within the C-terminal part of the protein (the last 50 amino acids), whereas PTMs in other regions of the protein, especially those within the microtubule-binding domain (MTBD), which is implicated in regulating Tau aggregation and functional properties,[6,32] remained inaccessible.

To address these limitations, we sought to develop a semisynthetic strategy that would provide access to PTMs within the MTBD and allow for the investigation of PTMs cross-talk in the MTBD and the C-terminus of the protein. Here, we describe an efficient modular semisynthetic strategy that enables the site-specific introduction of single or multiple PTMs within residues 246−441 of the Tau protein for the first time. This strategy preserves the native sequence of Tau, including the cysteine residues, which have recently been shown to play critical roles in the autoacetylation and autoproteolytic processing of Tau.[33,34]

At the outset, we demonstrated the semisynthesis of unmodified full-length Tau and showed that it exhibits a similar structure and functional activity to that of recombinant Tau prepared in *E. coli*. Next, we employed our semisynthetic strategy to produce full-length Tau site-specifically acetylated (at Lysine 280(K280)), monophosphorylated (at Tyrosine(Y310)), and diphosphorylated (at S396 and S404). In the current study, we chose to focus on Tau acetylation because of the increasing evidence that implicates Lysine acetylation within the MTBD as an important mechanism for regulating its pathogenic properties and physiological functions.[35−37] Tau acetylation is increased in human brains at the early and moderate Braak stages, even before the formation and accumulation of NFTs, thus supporting the idea that Tau acetylation is an early event in disease pathology.[35,38] Tau acetylation in AD and other Tauopathies, including corticobasal degeneration (CBD), progressive supranuclear palsy (PSP), and FTDP-17 familial dementia occurs primarily in the MTBD at K280.[39] Although site-specific acetylation at K280 has not been possible, the acetylation-mimicking mutation at K280 was shown to disrupt Tau−MT binding and increase Tau fibrillization *in vitro* and is associated with distinct pathological features in AD.[36] The overexpression of Tau mutants that mimic the acetylation at K271/K281 (K → glutamine (Q)) was also shown to disrupt the cytoskeletal sorting machinery, thus leading to the mislocalization of Tau in the axon initial segment (AIS). Mimicking acetylation at K274/K281 in a transgenic mouse model of AD was shown to promote cognitive decline and a disruption of synaptic plasticity by reducing postsynaptic kidney/brain (KIBRA) protein, a memory-associated protein, and by disrupting activity-dependent actin dynamics and AMPA receptor trafficking and insertion.[37] In addition to its role in promoting Tau aggregation and Tauopathies, K280 acetylation also plays a critical role in Tau-mediated neurodegeneration, cognitive deficits, and memory loss and was recently shown to disrupt synaptic plasticity.[37] Acetylation has also been reported to occur at lysine residues other than K280, and acetylation at some of these lysine residues (e.g., at K274 and K281) are also elevated in AD brains and are associated with cognitive decline in AD.[37,40] Although we focused our proof of concept studies on this particular PTM, the semisynthetic strategy outlined herein can be used to introduce acetylation or other PTMs at any putative sites in the MTBD or the C-terminal domain of Tau.

■ RESULTS

Our strategy for the semisynthesis of the longest isoform of Tau (2N4R) exploits the two native cysteine (C^{291} and C^{322}) residues in the Tau sequence and is based on sequential NCL involving 5 fragments: a recombinantly expressed fragment comprising residues 2−245 with a C-terminal thioester (**1**) and four synthetic fragments (**2**, **6**, **7**, **9**) comprising residues 246−441 (Scheme 1). The synthetic segment of Tau covers almost half of the protein sequence and contains the MTBD (also called K18) and the C-terminal part of the protein, thus allowing for the site-specific introduction of the majority of disease-associated PTMs.[11,41,42] The selection of the ligation sites was based on our desire to preserve the native sequence of the protein, including the cysteine residues. Therefore, the native chemical ligation sites were chosen at K290−C291, K321−C322, T245−A246 and G389−A390 (Scheme 1A), where the native alanine was mutated temporarily to cysteine and then converted back to the native alanine after NCL by a desulfurization reaction.[43,44] The N-terminal fragment of Tau (2−245) was expressed in *E. coli* as a fusion with Mxe-His6 at the C-terminus and then spliced and purified to afford the desired thioester, Tau (2−245)-SR (**1**), with a yield of 4.7 mg/L of bacterial culture (Figure S1). The synthetic Tau fragment (A246C−K290)-$NHNH_2$ (**2**) was prepared via Fmoc-solid phase peptide synthesis (Fmoc-SPPS) using Rink amide resin functionalized with 3-(Fmoc-amino)-4-(methylamino) benzoic acid (Fmoc-MeDbz). After peptide elongation, the peptide was converted to an activated *N*-acyl-*N*′-methylurea (MeNbz) as described by Dawson and co-workers,[45,46] followed by

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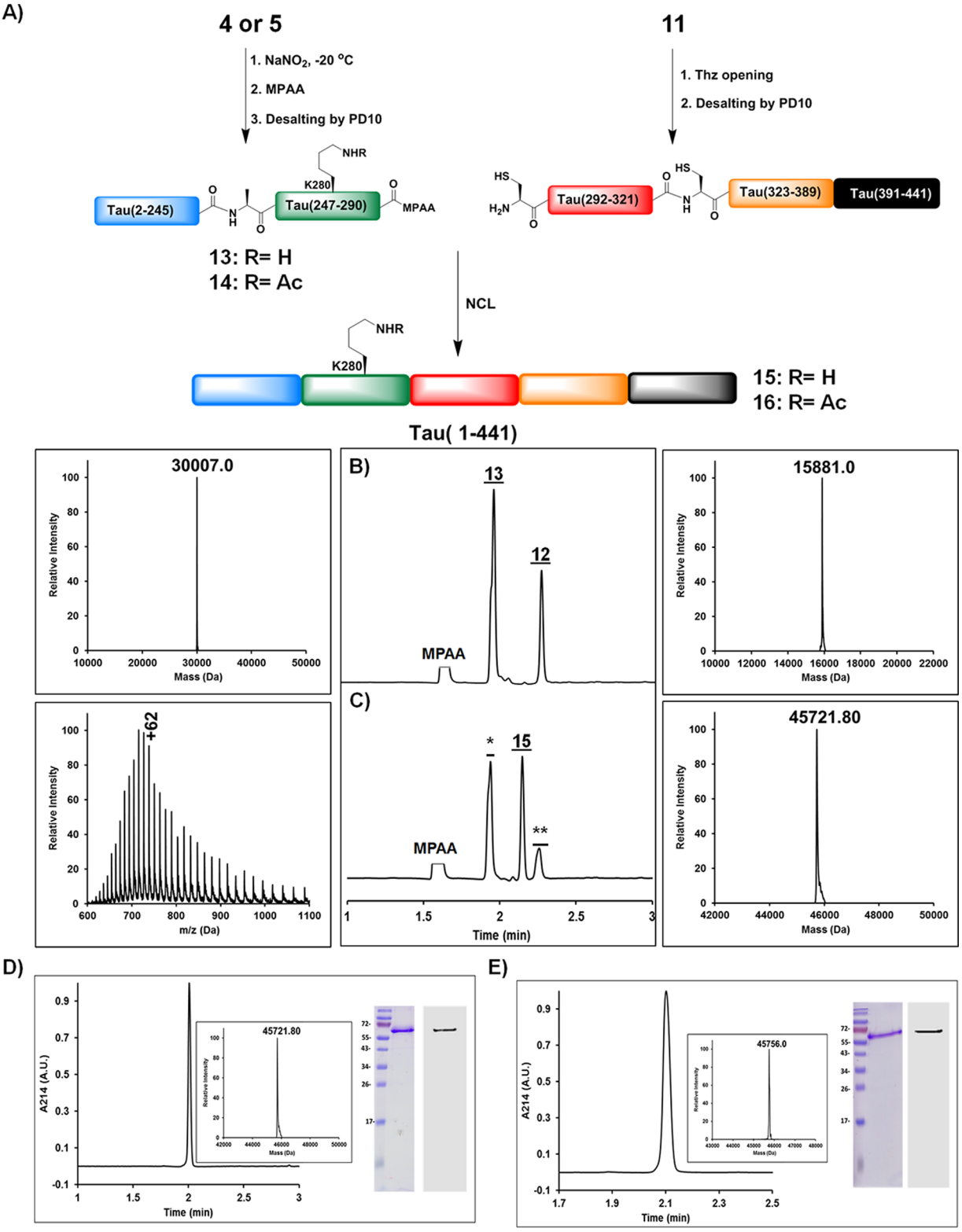

Figure 1. Semisynthesis and characterization of the full-length Tau. (A) Schematic representation of the last ligation step involving the ligation of the N- and C-terminal parts of Tau. (B, C) Analytical UPLC traces/(ESI-MS) of the ligation reaction between Tau (2−290)-MPAA (**13**) (observed mass of 30 007 Da) and Tau (291−441) (**12**) (observed mass of 15 881 Da), at 0 h (B) and after 6.5 h (C). Peak 15 corresponds to the ligation product (**15**) (observed mass of 45 722 Da; calculated 45 719 Da). Peak * is the hydrolyzed thioester, while peak ** corresponds to the remaining traces of the starting material. (D) Characterization of the purified WT Tau by analytical UPLC, ESI-MS, SDS-PAGE, and immunoblotting with antibody that specifically recognize Tau (anti-Tau 13 antibody [B11E8] (ab19030), from Abcam). (E) Characterization of the purified AcK280 Tau (**16**) by analytical UPLC, ESI-MS (observed mass, 45 756 Da; calculated mass 45 760 Da), SDS-PAGE, and immunoblotting with antibody that specifically recognize AcK280 Tau (anti-Tau antibody (acetyl-K280), AS-56077, from Anaspec).

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hydrazinolysis to obtain the C-terminal hydrazide, which can be converted selectively to a thioester (Figure S2). The hydrazide chemistry, developed by the Ramage and Liu groups,[47,48] was chosen to avoid cyclization and peptide polymerization and to allow for the use of the convergent chemical approach, which provides many advantages over the linear approach[49−52] during the final assembly of the protein.

The synthetic peptides Tau Thz-(292−321)-SR (**9**) and Tau Thz-(323−389)-SR (**6**) were prepared using the same resin with the MeDbz linker at the C-terminus and thiazolidine (Thz) at the N-terminus (Figures S8 and S12). The last Tau fragment (A390C-441) (**7**) was prepared using standard Fmoc protocols using preloaded Wang resin with leucine (L) as the first amino acid (Figure S9). Notably, the synthesis of the longest fragment (**6**) with 68 amino acids was not straightforward and required optimization of the SPPS reaction conditions (see the Supporting Information).

Having all Tau building blocks in hands, we focused on assembling these building blocks and the preparation of full-length Tau. First, we focused on assembling the N-terminal part of the protein using EPL between the expressed fragment thioester (**1**) and peptide (**2**), which carries a C-terminal hydrazide (Scheme 1B). Fragments (**1**) and (**2**) were dissolved in 8 M urea, and then 50 mM tris(2-carboxyethyl)phosphine (TCEP) was added as a reducing agent. To apply the one-pot ligation−desulfurization approach, we used the alkyl thiol trifluoroethanethiol (TFET), which was recently shown to have similar reactivity to 4-mercaptophenylacetic acid (MPAA), as a thiol additive.[53] The desulfurization reaction was accompanied by a side product resulting from an adduct addition of +43 Da, which increased significantly under the desulfurization conditions. All attempts to eliminate the addition of the +43 adduct failed. We reasoned that this adduct was formed from the carbamylation of an amino group caused by isocyanic acid (O═C═NH), which was derived from urea. Consistent with this hypothesis, the use of 6 M guanidine hydrochloride (GdnHCl) and 100 mM lysine as a ligation buffer prevented the carbamylation reaction and generated the desired ligation product Tau (2−290)-$NHNH_2$ (**4**) with high purity and 41% overall yield for the two steps (Figure S4 and S5).

Next, we focused on the assembly of the C-terminal part of the protein by ligating the fragments (**6**), (**7**), and (**9**). First, the C-terminal peptide (**7**) was ligated with the peptide thioester (**6**) bearing Thz at the N-terminus using the ligation buffer (i.e., 8 M urea and 50 mM TCEP at pH 7.0) in the presence of 2% (v/v) TFET as a thiol additive. The ligation was completed after 1 h, and the resulting crude peptide (**8a**) was subjected to the metal-free desulfurization conditions described by Danishefsky and colleagues[44] (Figure S10). After completion of the desulfurization reaction, the peptide was purified using RP-HPLC to afford Tau Thz-(323−441) (**8**) in 30% overall yield (Figure S10 and S11). Subsequently, the Thz of peptide **8** was deprotected by treatment with 0.1 M methoxyamine for 2 h at pH 4.0 to produce **10** with the desired N-terminal cysteine. After pH adjustment to 7.0, peptide thioester **9** was added to peptide **10**, to afford Tau Thz-(291−441) (**11**) in 16% yield (Scheme 1B and Figure S13).

Next, we focused on assembling the full-length protein by ligating the N-terminal part of the protein spanning residues 2−290-$NHNH_2$ (**4**) and the C-terminal part spanning residues 291−441 (**11**) (Figure 1A). Each peptide was dissolved separately in the ligation buffer (pH 3−4). Peptide **11** was treated for 2 h with 0.1 M methoxyamine to unmask the N-terminal C, and peptide **4** was oxidized to the corresponding acyl azide using sodium nitrite at −15 °C, followed by the addition of TFET to generate Tau (2−290)-TFET. Finally, peptide **4** was added to peptide **12**, and the pH of the reaction was adjusted to 7.0 by the careful addition of 1 M NaOH. After 1 h, we observed complete hydrolysis of the thioester without any traces of the ligation product. Initially, we reasoned that this reaction could have been caused by the high reactivity of the thioester TFET under oxidizing conditions. However, the same extent of hydrolysis was observed using the MPAA instead of the TFET. Then, we explored the possibility that the oxidizing agent might have increased the susceptibility of thioester to hydrolysis. Therefore, after the oxidation treatment of hydrazide and addition of MPAA, the oxidizing agent was removed by passing the Tau (2−290)-MPAA (**13**) through a PD-10 desalting column (Figure 1A and Figure S15). Moreover, peptide **11** was also purified using a PD-10 column after methoxyamine treatment to generate peptide **12** (Figure 1A and Figure S14). Finally, the lyophilized powders were dissolved in ligation buffer, which was adjusted to pH 6.9. Following these optimized conditions, the hydrolysis side-reaction was suppressed, and the ligation was completed after 6.5 h at 30 °C to afford the native form of the full-length Tau (**15**) on a milligram scale (Figure 1B−D). The purity of the protein was confirmed by mass spectrometry, UPLC, SDS-PAGE gel electrophoresis, and Western blot (WB) (Figure 1).

To verify that the semisynthetic Tau adopted the correct conformation and was functional, we assessed and compared its secondary structure and tubulin polymerization activity[54] to those of recombinant Tau produced from *E. coli* (see Supporting Information and Figure S18). As shown in Figure 2, both semisynthetic and recombinant Tau exhibit similar propensities to promote MT polymerization and identical circular dichroism (CD) spectra that are consistent with a predominantly disordered conformation.

After successfully performing the semisynthesis of wild-type (WT) Tau, we then focused our efforts on the semisynthesis of Tau acetylated at K280. Although, several enzymes that regulate Tau acetylation (P300 acetyltransferase, CREB-binding protein, acetyl-CoA substrate) and deacetylation (sirtuin 1 (SIRT1) and histone deacetylase 6 (HDAC6)) have been identified, site-specific acetylation is not yet possible. *In vitro* acetylation by the acetyltransferase CBP increases Tau aggregation and impairs MT assembly,[36] whereas acetylation of Tau by p300 (the homologue of CBP) inhibits Tau fibril formation.[55,56] The discrepancy observed when using different acetyltransferases might be caused by the differences in the efficiency or specificity of the acetylation induced by these enzymes, which are known to acetylate Tau at multiple residues (Figure S28). These findings underscore the critical importance of developing methodologies that allow for site-specific acetylation at single or multiple lysine residues. To achieve this goal, we proceeded with the semisynthesis of AcK280-Tau. The Tau synthetic fragment (A246C-290, AcK280)-$NHNH_2$ (**3**) was prepared using the same strategy as that used for the peptide (**2**), and monoacetylated Tau at K280 was produced using the semisynthetic strategy used to produce unmodified Tau (see Figure 1E, Supporting Information, and Figures S3, S6, S7, S16, and S17).

To demonstrate the strength of our semisynthetic approach and the flexibility it offers in terms of generating Tau with various PTMs at different sites within the residues 246−441, we applied it to generate mono- and diphosphorylated Tau at

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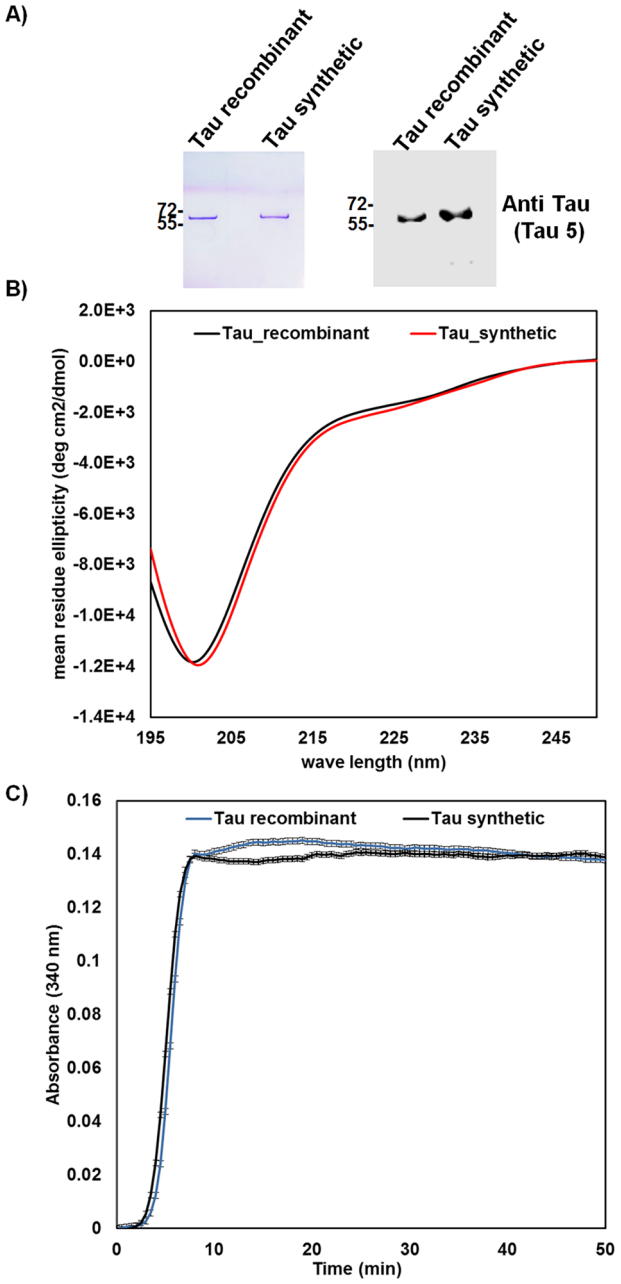

Figure 2. Characterization of the wild-type (WT) semisynthetic Tau compared with recombinant Tau produced in *E. coli*. (A) SDS-PAGE analysis of WT recombinant and synthetic Tau (left) as well as a Western blot using the Tau specific antibody (Tau Monoclonal Antibody (TAU-5), MA5-12808, ThermoFisher) (right). (B) Circular dichroism spectra of the WT recombinant (5 μM, black) and synthetic (5 μM, red) Tau. (C) Tubulin polymerization assay in the presence of WT recombinant Tau or synthetic Tau.

tyrosine 310 (pY310) or serine 396 and serine 404 (pS396, pS404), respectively (Figure 3, Supporting Information, and Figures S20−S26). We chose these modifications for the following reasons: (1) they occur on different segments of the protein; (2) phosphorylation at these residues has been associated with different stages of AD,[57−60] play a role in abnormal Tau processing, and alter the microtubule and aggregation properties of Tau;[61] and (3) there are currently no methods that allow site-specific introduction of these modifications in the full-length Tau. The c-Abl kinase phosphorylates Tau at tyrosine 310, but it also efficiently phosphorylates other tyrosine residues in the N- (Y18, Y29, and Y197) and C-terminus of the protein (Y394). Similarly, several enzymes (e.g., GSK-3b, cdk5, TPK-II, CK1) have been shown to phosphorylate Tau at both S396 and S404, but with very limited specificity or efficiency.[61−65] Indeed, all of the kinases known to phosphorylate Tau tend to phosphorylate multiple serine/threonine residues throughout the sequence of the protein.[65] As shown in Figure 3, our semisynthetic method enables sites specific introduction of these modifications and production of these proteins in highly pure forms. The purity was assessed by SDS-PAGE, UPLC, and mass spectroscopy and further checked by Western blot analysis using pan-specific and PTM-specific Tau antibodies (Figure S30).

To determine the effect of K280 acetylation on the kinetics of Tau fibril formation, *in vitro* fibrillization studies were conducted by incubating unmodified and AcK280 Tau (5 μM) in the presence of heparin (1.25 μM) at 37 °C without shaking in phosphate buffer (pH 7.4), and fibril formation was followed by monitoring changes in the fluorescence intensity of thioflavin S (ThS). We observed that the acetylation of Tau at K280 (AcK280 Tau) significantly enhanced the rate of Tau fibril formation (3-fold) compared with that for unmodified recombinant (Figure 4a and Table S1) and semisynthetic Tau (Figure S27). As expected, in the absence of heparin, AcK280 Tau did not form fibrils. These results are consistent with those obtained in previous studies in which acetylation with the enzyme CBP (which acetylates Tau at multiple sites (see also Figure S25), including K280) was shown to enhance Tau aggregation.[36]

Analysis of the aggregates structure by transmission electron microscopy (TEM) showed that the WT Tau formed flexible fibrils with a broad length distribution 50−1000 nm (average length of 369.27 ± 15.91 nm and average diameter of 19.94 ± 0.51 nm). Interestingly, AcK280 Tau formed predominantly a mixture of oligomers (∼80−90%) with an average diameter of 26.30 ± 0.29 nm and short fibrils with an average length of 154.24 ± 7.23 nm and average diameter of 24.70 ± 0.53 nm (Figure 4B,C). Consistent with the size and distribution of the Tau aggregates observed in the TEM data, the aggregation of both proteins assessed via a sedimentation assay showed a significant accumulation of unmodified Tau fibrils in the insoluble fraction, whereas the AcK280 Tau aggregates (oligomers and short fibrils) remained predominantly in the soluble fractions (Figure S28). These findings suggest that acetylation at K280 promotes Tau oligomerization and favors the formation of short fibrils.

The majority of the published studies on the acetylation of Tau at K280 and other residues have relied primarily on the use of acetylation mimetic K → Q to dissect the role of acetylation in regulating Tau aggregation, function(s), and toxicity.[36,37,40,56,66−69] In the absence of site-specifically acetylated forms of Tau, it has not been possible to determine whether this mutation indeed reproduces all aspects of acetylation. Toward addressing this knowledge gap, we sought to compare the aggregation kinetics and aggregates morphology of acetylated Tau AcK280 and corresponding acetylation mimetic K280Q Tau, produced and purified from *E. coli* as described in the Supporting Information (Figure S19). As shown in Figure 3A, both AcK280 Tau and Tau K280Q proteins show similar kinetic trends of fibril formation and exhibit faster aggregation compared to the WT protein (See also Table S1). Interestingly, the K280Q aggregates exhibited higher ThS values. Further-

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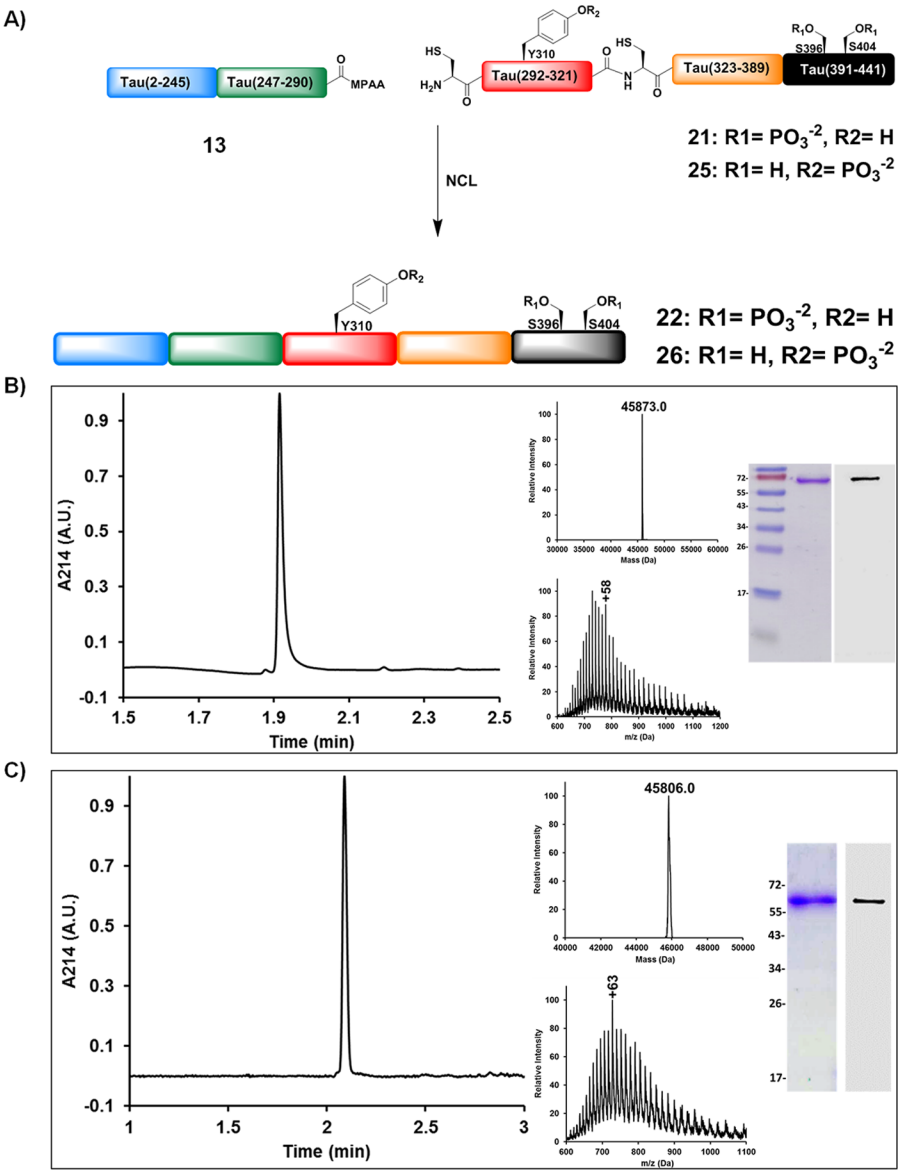

Figure 3. Semisynthesis and characterization of the mono- (pY310) and diphosphorylated (pS396/pS404) Tau. (A) Schematic representation of the last ligation step. (B) Characterization of the purified pS396, pS404 Tau (**22**) by analytical UPLC, ESI-MS (observed mass, 45 873 Da; calculated mass 45 878 Da), SDS-PAGE, and immunoblotting with antibody that specifically recognize Tau phosphorylated at Ser396 and Ser404 (PHF-1, from Peter Davies (Albert Einstein College of Medicine)). (C) Characterization of the purified pY310 Tau (**26**) by analytical UPLC, ESI-MS (observed mass, 45 806 Da; calculated mass 45 799 Da), SDS-PAGE, and immunoblotting with antibody that specifically recognize Tau phosphorylated at Tyr310 (pY310, generated by Eurogentec S.A. (Belgium)).

more, analysis of the aggregate structures by TEM revealed that the K280Q mutant forms predominantly short fibrils with an average length of 198.17 ± 7.19 nm and average diameter of 24.03 ± 0.37 nm, compared to AcK280, which exhibit an average length of 154.24 ± 7.23 nm. However, unlike AcK280, no significant amounts of oligomers were found to accumulate in the K280Q samples (Figure 4B,C). These findings demonstrate that the acetylation mimetic mutation seems to reproduce many of the effects of acetylation on Tau fibrillization but does not reproduce fully the effect of acetylation on Tau oligomerization.

One of the critical functions of Tau proteins is the stabilization and assembly of MTs. Previous studies have shown that acetylation by CBP of K18 at several sites prevents the ability of K18 and Tau (to a lesser extent) to promote MT assembly.[36] Another study showed that acetylation-mimicking mutations at K280 (K280Q) reduced the rate of MT assembly by 50%.[68] To assess the effect of the authentic acetylation at K280 on the regulation of MT assembly, tubulin polymerization experiments were performed in the presence of either WT or AcK280 Tau proteins at 37 °C. MT assembly was followed by a measurement of the absorbance at 340 nm as described previously.[70,71] As expected, acetylation at K280 of Tau slightly impairs the ability of tubulin to form MTs, which is consistent with a previous report[36] (Figure 5, and Table S2). This observation might be explained by the ability of acetylation to neutralize the positive charge of lysine, which could potentially abrogate Tau−MT binding.

■ DISCUSSION

The MT-binding protein Tau is one of the most heavily post-translationally modified proteins in the brain. Both under

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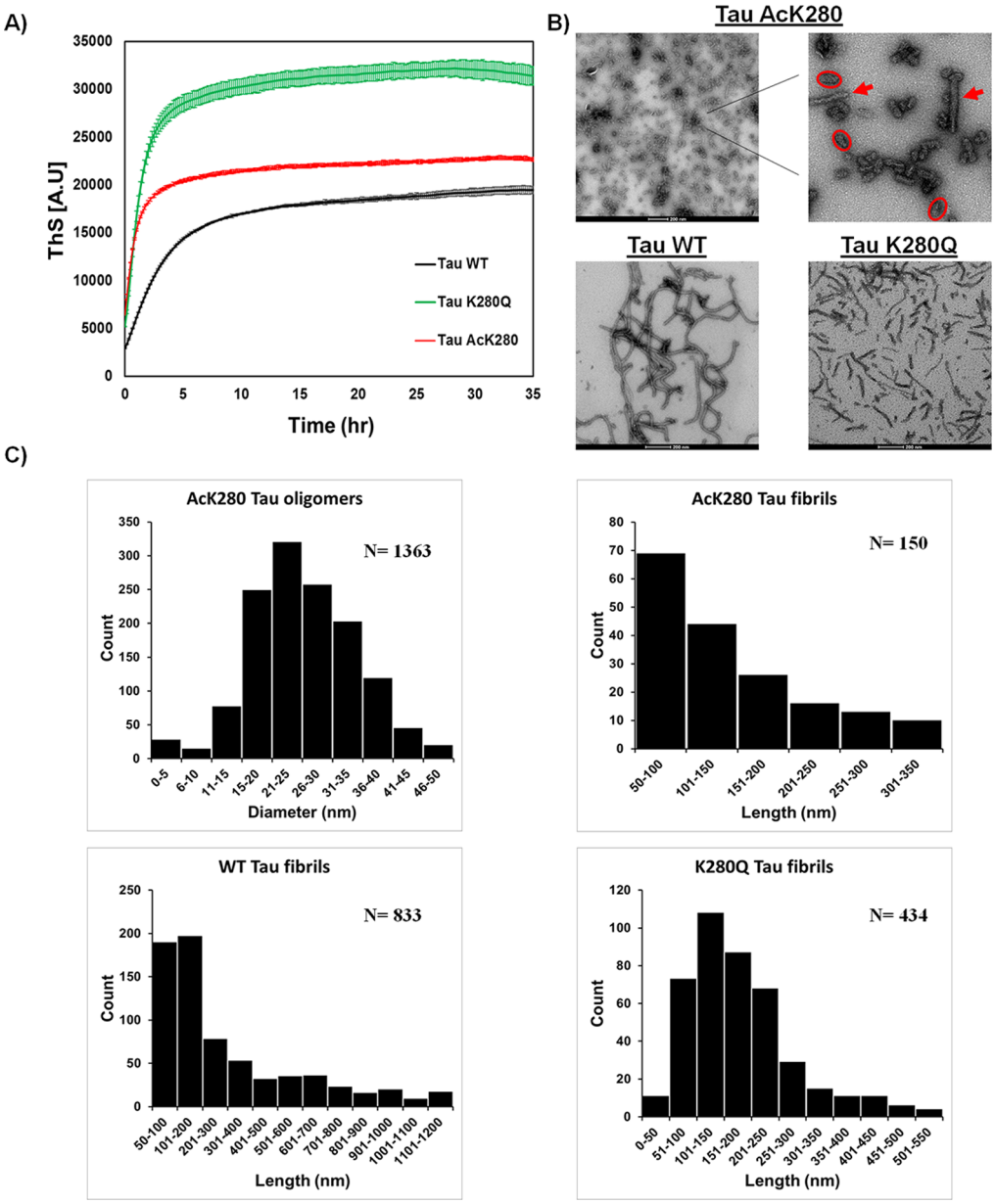

Figure 4. Comparison of the aggregation kinetics of AcK280 Tau, WT Tau, and K280Q Tau. (A) Thioflavin S fluorescence assay of the samples measured at 490 nm (mean ± SEM, $n = 3$). (B) TEM images of WT, AcK280, and K280Q Tau after 48 h of the aggregation. AcK280 Tau oligomers and fibrils are marked with circles and arrows, respectively (scale bars = 200 nm). (C) Quantification of the oligomer size and fibril length distribution. Since AcK280 Tau forms aggregates that were enriched in both globular oligomers and short fibrils, the two species were quantified separately, (top left) oligomers with an average diameter of 26.30 ± 0.29 nm and (top right) short fibrils with an average length of 154.24 ± 7.23 nm. Length measurements of WT Tau aggregates (bottom left, with an average length of 369.27 ± 15.91 nm), and K280Q Tau (bottom right, with an average length of 198.17 ± 7.19 nm).

physiological conditions and in pathological aggregates (e.g., PHFs), Tau has been shown to undergo several types of PTMs, including phosphorylation, acetylation, nitration, glycosylation, and ubiquitination, often at multiple sites and within the same molecule.[11,72] The distribution of PTMs throughout the sequence of the protein and the enrichment of PTMs in regions that have been implicated in regulating Tau functions and aggregation, combined with the observation of several PTMs co-occurring on the same Tau molecule, suggest that Tau functions are modulated by a complex regulatory network that involves cross-talk between different types of PTMs. However, the complexity and diversity of Tau PTM patterns and the lack of methodologies that allow for the site-specific introduction of these PTMs has hampered systematic studies aimed at deciphering the Tau PTM code in health and disease. Together, these challenges and limitations have limited ability to assess and exploit the potential of Tau PTMs in developing novel diagnostics and therapeutic strategies for the treatment of AD and other Tauopathies.

Herein, we described, for the first time, an efficient method for the semisynthesis of Tau, and this allows the introduction of single or multiple site-specific modifications within the MTBD or the C-terminal domain of the protein or both. This method preserves the native sequence of Tau, including the cysteine residues, and addresses many of the limitations and challenges experienced in previous attempts to achieve the semisynthesis

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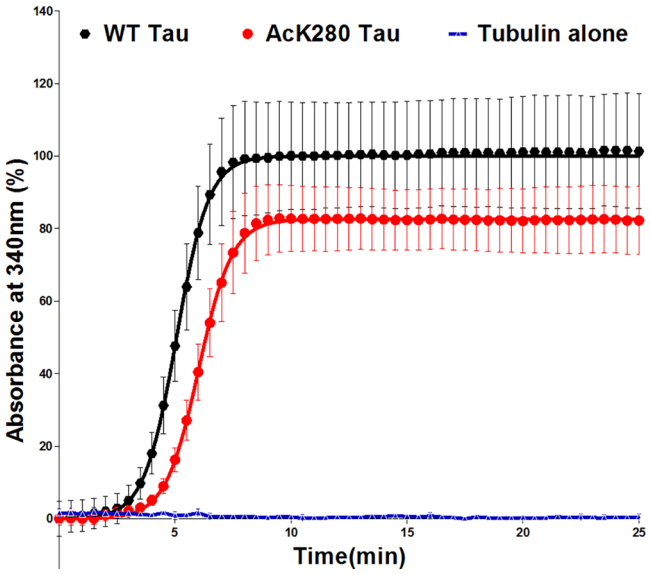

Figure 5. Tubulin polymerization in the presence of WT or AcK280 Tau. Light scattering assays of Tau proteins show a slight inhibition of tubulin polymerization when using Tau acetylated at K280. The assay was conducted by mixing Tau proteins (15 μM) and tubulin (3 mg/mL) in tubulin buffer at 37 °C, and the reaction was assessed by monitoring the absorbance every 30 s at 340 nm. Error bars indicate the standard error of the mean (SEM) from n = 3 independent replicates.

of Tau. As a proof of concept, we demonstrated the semisynthesis of unmodified full-length Tau and showed that it exhibits a similar structure and functional properties to that of recombinant Tau prepared in *E. coli*. Next, we employed our semisynthetic strategy to prepare full-length Tau that was specifically acetylated at the K280 site and to investigate the effect of this modification on the structure, aggregation, and microtubule binding properties of Tau. To demonstrate the utility and the flexibility of our semisynthetic strategy, we produced site-specifically mono- (pY310) and diphosphorylated (pS396/pS404) Tau, thus showing that our semisynthetic strategy enables the introduction of different types of PTMs in different parts of the Tau sequence. We choose to focus here on Tau acetylation because of the increasing evidence of lysine acetylation within the MTBD as an important mechanism for regulating both its pathogenic properties and physiological functions.[73,74]

Although several studies have reported the effect of lysine acetylation on Tau, including acetylation at K280, most of these studies relied on the use of acetylation "mimicking" mutations (K → Q/R) or the use of acetyltransferases, which are not specific and acetylate Tau at multiple lysine residues (see the Supporting Information and Figure S29). Therefore, our work represents the first attempt to elucidate the role of site-specific acetylation at K280 and paves the way for elucidating the role and relative contribution of various disease-associated Tau acetylation events in modulating the aggregation, toxicity, and cell-to-cell spreading of Tau. Our findings show that acetylation at K280 significantly enhances the aggregation kinetics of Tau, impairs MT binding, and favors the formation of predominantly ThS positive oligomers and short fibrils, thereby suggesting that acetylation plays a critical role in the pathogenesis of AD and other Tauopathies. In the case of acetylation at K280, this could be through stabilizing and promoting the accumulation of predominantly toxic oligomers and short Tau fibrils, which could result in the presence of a larger number of fibril seeds, thus favoring the increased aggregation and possibly the increased cell-to-cell propagation of Tau aggregates. Previous studies have shown that the levels of Tau acetylated at K280 are increased in AD brains and that acetylation occurs prior to NFT formation in human brains.[35,38,39] Together, these observations and our findings suggest that therapeutic approaches targeting AcK280 Tau oligomers and fibrils represent a viable strategy for preventing the initiation of Tau aggregation or blocking the spreading of Tau aggregates in the brain in AD and other Tauopathies.

Despite the fact that the R1−R2 motif, which contains K280 has a high affinity for MTs, our results show only moderate inhibition of Tau MT polymerization. This is consistent with previous findings using nonselective lysine acetylation strategies.[36,68] Thus, several modifications including phosphorylation, acetylation, and other PTMs, likely regulate different aspects of Tau interactions with MTs. It is also plausible that for certain PTMs such acetylation might represent early events that lead to the partial disassociation of Tau from MTs and facilitate its modification, especially within the MTBD, by other enzymes.

Interestingly, the acetylation of Tau within the KXGS motifs, K259, K290, K321, or K353, was found to be decreased in AD brains, and this decrease was associated with increased Tau phosphorylation in both AD brains and transgenic models of AD. Acetylation at these residues inhibits Tau aggregation,[56] suggesting a potential protective role. These findings underscore the critical importance of developing methodologies that can enable elucidation of the relative contribution of each acetylation site and the role of the cross-talk between the different acetylation sites and other competing PTMs in regulating these functional and pathogenic properties of Tau. For example, acetylation has been suggested to play a key role in the initiation of Tau pathology by competing with ubiquitination, thereby slowing Tau degradation and promoting its accumulation, phosphorylation, and aggregation.[35]

Our results on comparing the effect of the acetylation mimetic mutation K → Q and authentic acetylation show that the mimetic approach provides a reasonable strategy to investigate the effect of selective acetylation at this residue. However, this mutation did not reproduce the effect of acetylation on the oligomerization of Tau, highlighting the importance of performing direct comparison studies, which is now possible using our semisynthetic methods, to improve the design of *in vivo* studies and interpretation of cellular and *in vivo* results obtained using PTM mimetics.

Finally, our semisynthetic strategy and the ability to generate homogeneous site-specific acetylation of different forms of Tau provide unique opportunities to elucidate the mechanisms by which Tau acetylation influences the aggregation, clearance, and MT-binding properties of Tau *in vitro* as well as synaptic plasticity and pathological spreading in cell culture and animal models of AD. Beyond acetylation and phosphorylation, the development of this semisynthetic strategy opens new opportunities for investigating other types of PTMs that occur within the previously inaccessible residues 246−441, thus paving the way to deciphering the Tau PTM code in health and disease. The current strategy provides access to all disease-associated acetylation or ubiquitination sites within residues 246-441 and allows for the investigation of the cross-talk between different Tau PTMs for the first time. Current efforts

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are underway to extend these strategies to enable site-specific modifications within the N-terminal domain (residues 2–245), with the ultimate goal of developing protein synthetic strategies that will allow for the introduction of single or multiple PTMs throughout the Tau protein sequence. These advances will enable us for the first time to systematically investigate the role of PTMs in regulating Tau misfolding, aggregation, toxicity, and the spreading of Tau pathology in AD and related Tauopathies. The ability to reproduce the diversity of the Tau proteoforms with high purity presents unique opportunities to advance biomarker discovery, and to develop more accurate assays to quantify total Tau, and assess the levels of specific Tau PTMs or changes in the Tau proteoforms during the progression of AD and Tauopathies.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.8b02668.

Expression of Tau (2-245)-SR (**1**), synthesis of Tau (A246C-K290)-$NHNH_2$ (**2**) and Tau (A246C-K290, AcK280)-$NHNH_2$ (**3**), preparation of Tau (2-290)-$NHNH_2$ (**4**), preparation of Tau (2-290, AcK280)-$NHNH_2$ (**5**), synthesis of Tau (C322Thz-389)-SR (**6**), synthesis of Tau (A390C-441) (**7**), preparation of Tau (C322Thz-441) (**8**), synthesis of Tau (C291Thz-321)-SR (**9**), preparation of Tau (C291Thz-441) (**11**), Thz opening of Tau (C291Thz-441) (**12**), switching Tau (2-290)-$NHNH_2$ (**4**) to Tau(2-290)-MPAA (**13**), ligation of Tau(2-290)-MPAA (**13**) with Tau(291-441) (**12**), ligation of Tau(2-290, AcK280)-MPAA (**14**) with Tau(291-441) (**12**), expression and purification of Tau (2-441), expression and purification of Tau (2-441, K280Q), preparation of the full length Tau (2-441, pS396, pS404) (**22**), synthesis of Tau (A390C-441, pS396, pS404) (**17**), preparation of Tau (C322Thz-441, pS396, pS404) (**8**), preparation of Tau (C291Thz-441, pS396, pS404) (**20**), ligation of Tau(2-290)-MPAA (**13**) with Tau(291-441, pS396, pS404) (**20**), preparation of the full length Tau (2-441, pY310) (**26**), synthesis of Tau (C291Thz-321, pY310)-SR (**23**), preparation of Tau (C291Thz-441, pY310) (**24**), ligation of Tau(2-290)-MPAA (**13**) with Tau(291-441, pY310) (**24**), biophysical and biochemical analysis, circular dichroism (CD) measurements, in vitro aggregation studies, sedimentation assay, length measurements of aggregates, tubulin polymerization assay, Western blot analysis, amino acid analysis (AAA), transmission electron microscopy (TEM), in vitro acetylation, supplemental results, aggregation kinetics of the semisynthetic WT Tau and AcK280 Tau, sedimentation assay of the WT and AcK280 Tau using ultracentrifugation, summary of the aggregation kinetic parameters, summary of the MTs polymerization kinetic parameters, in vitro acetylation of the WT Tau by p300 and CREB, and Western blot analysis of the semisynthetic Tau variants (PDF)

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Notes

The authors declare no competing financial interest.

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