Title: Paclitaxel suppresses Tau-mediated microtubule bundling in a concentration-dependent manner

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Abstract

Background: Microtubules (MTs) are protein nanotubes comprised of straight protofilaments (PFs), head to tail assemblies of $\alpha\beta$ -tubulin heterodimers. Previously, it was shown that Tau, a microtubule-associated protein (MAP) localized to neuronal axons, regulates the average number of PFs in microtubules with increasing inner radius $\langle R_{in}^{MT} \rangle$ observed for increasing Tau/tubulin-dimer molar ratio Φ_{Tau} at paclitaxel/tubulin-dimer molar ratio $\Lambda_{Ptxl}=1/1$.

Methods: We report a synchrotron SAXS and TEM study of the phase behavior of microtubules as a function of varying concentrations of paclitaxel ($1/32 \leq \Lambda_{Ptxl} \leq 1/4$) and Tau (human isoform 3RS, $0 \leq \Phi_{3RS} \leq 1/2$) at room temperature.

Results: Tau and paclitaxel have opposing regulatory effects on microtubule bundling architectures and microtubule diameter. Surprisingly and in contrast to previous results at $\Lambda_{Ptxl}=1/1$ where microtubule bundles are absent, in the lower paclitaxel concentration regime ($\Lambda_{Ptxl} \leq 1/4$), we observe both microtubule doublets and triplets with increasing Tau. Furthermore, increasing paclitaxel concentration (up to $\Lambda_{Ptxl} = 1/1$) slightly decreased the average microtubule diameter (by ~ 1 PF) while increasing Tau concentration (up to $\Phi_{3RS} = 1/2$) significantly increased the diameter (by $\sim 2-3$ PFs).

Conclusions: The suppression of Tau-mediated microtubule bundling with increasing paclitaxel is consistent with paclitaxel seeding more, but shorter, microtubules by rapidly exhausting tubulin available for polymerization. Microtubule bundles require the aggregate Tau-Tau attractions along the microtubule length to overcome individual microtubule thermal energies disrupting bundles.

General Significance: Investigating MAP-mediated interactions between microtubules (as it relates to *in vivo* behavior) requires the elimination or minimization of paclitaxel.

1. Introduction

Microtubules (MTs) are a key component of the eukaryotic cytoskeleton and are involved in a wide variety of cellular functions (e.g. intracellular trafficking, cell motility, chromosome segregation) [1]. Microtubules are made up of straight protofilaments (PFs), polarized head-to-tail assemblies of globular $\alpha\beta$ -tubulin heterodimers, which dynamically assemble into hollow nanotubes (diameter ≈ 25 nm) that are stabilized by lateral PF-PF interactions (Fig. 1A). However, microtubules can switch between growth and shrinkage phases in a process known as dynamic instability [2], wherein tubulin polymerization into microtubules is periodically interrupted by rapid depolymerization (“catastrophe”). Catastrophe occurs when GTP-bound tubulin at the polymerizing end of microtubules (the microtubule GTP cap) is fully hydrolyzed, causing protofilaments to peel off as highly bent GDP-tubulin oligomers and depolymerizing until a new microtubule GTP cap is formed, restarting (rescuing) the polymerization process [2,3]. The disassembly of microtubules is especially notable in cold (4°C) and with the addition of calcium ions [4].

Microtubule dynamic instability would have made many experiments intractable were it not for the discovery that paclitaxel (known popularly as its chemotherapeutic formulation Taxol) promoted and stabilized microtubules against cold- and calcium ion-induced microtubule depolymerization [5]. It was later shown that at near-stoichiometric paclitaxel concentrations (paclitaxel/tubulin-dimer molar ratio $\Lambda_{\text{Ptxl}}=1/1$) microtubule dynamic instability was almost completely suppressed [6–8] by kinetically trapping protofilaments, made up of GDP-tubulin, into a straight conformation [9–11]. Although the use of paclitaxel did alter microtubule structure by decreasing the PF number (at $\Lambda_{\text{Ptxl}}=1/1$) [12], minor changes to the microtubule structure were outweighed by the robustness of microtubules for experiments, especially at room temperature ($\approx 25^\circ\text{C}$). Paclitaxel-stabilized microtubules served as an effective platform to investigate microtubule-dependent motor proteins (such as kinesin [13]) and microtubule-associated proteins (MAPs), which serve to regulate microtubule polymerization/depolymerization.

One particular MAP, Tau, is localized to the axon in mature neurons and has been heavily investigated due to its key role in neuronal development as well as the fact that aberrant Tau behavior has been implicated in many neurodegenerative diseases (“tauopathies”) including Alzheimer’s, FTDP-17, progressive supranuclear palsy, Pick’s, and more recently, chronic traumatic encephalopathy [14–16]. Difficulty in understanding Tau structure and function is due, in large part, to its intrinsic disorder; Tau does not form a secondary structure in solution. As a result, Tau structure and function is often described sequentially from the N- to C- terminus: the “projection domain” (PD, which projects off the microtubule surface due to low microtubule affinity), the proline-rich region (PRR, intermediate microtubule affinity), the microtubule-binding region (MTBR, high microtubule affinity), and a carboxyl-terminal tail (CTT, low microtubule affinity) (Fig. 1B,C). While even truncated Tau domains have also been shown to be disordered [17], the lack of structure in solution does not preclude induced structures of Tau domains on the microtubule surface. Recently, the Tau MTBR was shown to adopt a hairpin

conformation on microtubules [18] and the projection domain of Tau was demonstrated to undergo a conformational mushroom-to-brush transition as a function of increasing Tau concentration on the microtubule surface [19]. Furthermore, the concentration where the Tau mushroom to Tau brush transition occurs is consistent with Tau being in an extended state in the mushroom phase.

Fig. 1. A schematic of a microtubule (A) and the shortest wild type isoform of Tau, 3RS (B). (C) Charge (averaged over 10 residues) versus amino acid residue number for 3RS Tau. Data were obtained from the NCBI protein databank (NP_058525.1 for 3RS). Starting at the amino-terminus (N), the N-terminal tail (NT) is made up of the projection domain (PD, yellow background, 92 AAs) and proline-rich region (PRR, green background, 94 AAs), followed by microtubule-binding region (MTBR, blue background, 93 AAs), and ending at the carboxyl-terminus (C) with the C-terminal tail (CT, pink background 73 AAs).

While experiments suggest that microtubule surfaces promote conformational changes to Tau upon binding, conversely, Tau affects microtubule assembly dynamics and structure (much like paclitaxel). Tau has been shown to suppress dynamic instability in paclitaxel-free microtubules as a function of Tau-concentration [20], with Tau/tubulin-dimer ratio $\Phi_{\text{Tau}} = 1/1$ nearly eliminating microtubule catastrophe [21]. Additionally, increasing concentrations of Tau were found to increase the ensemble-averaged PF number (from $\langle N_{\text{PF}} \rangle \approx 13$ at $\Phi_{\text{Tau}} = 0$ to $\langle N_{\text{PF}} \rangle \approx 14$ at $\Phi_{\text{Tau}} \geq 1/5$) of paclitaxel-stabilized microtubules (at $\Lambda_{\text{ptxl}}=1/1$) [22]. Thus, although Tau and paclitaxel both suppress dynamic instability, they modulate the diameter of microtubules by

different amounts and in different directions (i.e. Tau can increase PF number by 2-3 [19,22], while microtubules at $\Lambda_{\text{Ptxl}}=1/1$ decreases PF number by 1 [12]).

Importantly, although it was widely assumed that paclitaxel-stabilized microtubules were fully reflective of *in vivo* microtubules, this was never well established. This is especially important with respect to cell free investigations of the interaction of Tau with microtubules, where many studies utilized paclitaxel-stabilized microtubules [18,19,22–24]. Interestingly, cell free experiments indicated that Tau does not induce bundling of paclitaxel-stabilized microtubules at $\Lambda_{\text{Ptxl}}=1/1$ [22,23]. In marked contrast, groundbreaking *in vivo* electron microscopy studies of the fine structure of neuronal axons (where Tau is localized) revealed that the axon initial segment (AIS) contained small string-like microtubule bundles with wide microtubule wall-to-wall spacing [25]. Moreover, seminal studies of cells overexpressing transfected Tau cDNA in non-neuronal cells concluded that Tau induced hexagonally-ordered microtubule bundles resulting in neurite-like processes [26,27]. Thus, there is a conflict between the cell free experiments utilizing paclitaxel-stabilized microtubules and *in vivo* biological observations.

Here, in an attempt to understand the opposing and distinct regulatory mechanisms of both Tau and paclitaxel we report the phase diagram of microtubules as a function of both increasing concentration of one of the human isoforms of Tau (3RS, with $0 \leq \Phi_{3\text{RS}} \leq 1/2$) and increasing paclitaxel ($1/32 \leq \Lambda_{\text{Ptxl}} \leq 1/4$) through synchrotron small-angle X-ray scattering and transmission electron microscopy. We find that 3RS Tau increases $\langle R_{\text{in}}^{\text{MT}} \rangle$ (equivalently, $\langle N_{\text{PF}} \rangle$) as a function of increasing $\Phi_{3\text{RS}}$, while increasing paclitaxel concentration (Λ_{Ptxl}) serves to decrease $\langle R_{\text{in}}^{\text{MT}} \rangle$. Furthermore, at low Λ_{Ptxl} and high $\Phi_{3\text{RS}}$ microtubule doublets and triplets were observed (coexisting with singular microtubules), indicating that paclitaxel serves to suppress the Tau-mediated microtubule bundling.

Our results are consistent with the hypothesis that paclitaxel acts as a seed-promoting molecule in facilitating microtubule polymerization. This seeding hypothesis is borne out in experiments which show that paclitaxel-stabilized MTs have an average length of 2 μm [28] versus an average length of 20 μm for Tau-stabilized MTs without paclitaxel [29]. Tubulin polymerization is expected to lead to more, but shorter, MTs as the concentration of paclitaxel increases. Thus, Tau-mediated MT bundling should occur at low enough paclitaxel concentration (i.e. with resultant longer microtubules) where the sum of Tau-Tau attractions (on opposing microtubules) along the entire microtubule length overcomes the thermal kinetic energy of each microtubule (on the order of a few $k_{\text{B}}T$).

2. Materials and Methods

2.1 Purification of Tau and Tubulin

Tubulin was purified from bovine brain, as previously described [30]. Briefly, crude brain extract was subjected to three cycles of microtubule polymerization/depolymerization, with resultant MAP-rich microtubules separated into MAPs and tubulin through a phosphocellulose anionic exchange column. Purified tubulin was suspended in PEM50 (50 mM PIPES at pH 6.8, 1 mM

MgSO₄, and 1 mM EGTA) with protein concentration between 7 and 12 mg/mL, as measured by a Bradford assay using BSA as a standard. Tubulin was drop-frozen in liquid nitrogen and stored at -70° C freezer until use.

Tau was expressed and purified, as previously described [31]. Briefly, Tau was expressed in BL21(DE3) competent cells (Life Technologies, Carlsbad, CA) carrying the 3RS-Tau encoding plasmid. After incubation in auto-induction media (10g of tryptone, 5g of yeast extract, 0.5g of dextrose, 2g of α -D-lactose and 5 mL of glycerol per liter of 25 mM Na₂HPO₄, 25 mM KH₂PO₄, 50 mM NH₄Cl, 5 mM Na₂SO₄ in DI water) for 24 hours, cells were collected via centrifugation. Collected pellets were resuspended in BRB80 (80 mM PIPES at pH 6.8, 1 mM EGTA, 1 mM MgSO₄), lysed via sonication, then subsequently boiled and centrifuged again. Resulting supernatant (with Tau) was run through a phosphocellulose anionic exchange column, with Tau binding to the phosphocellulose and eluted with increasing concentration of NaCl in BRB80. Fractions with Tau were buffer exchanged into pure BRB80 via Amicon Ultra-15 Centrifugal Units with MWCO=10,000 (EMD Millipore, Darmstadt, Germany). Tau was then loaded into a HiTrap hydrophobic interaction chromatography column (GE Healthcare Life Sciences, Pittsburgh, PA), eluted with decreasing concentration of (NH₄)₂SO₄ in BRB80. Tau was then concentrated and buffer exchanged with pure BRB80 through successive centrifugation cycles using Amicon Centrifugal Units. The concentration of each Tau stock was determined by SDS-PAGE comparison with a Tau mass standard (originally measured via amino acid analysis).

2.2 Microtubule Assembly with Paclitaxel and Tau

Microtubules were assembled from 50 μ M tubulin in PEM50 buffer in the presence of 1 mM GTP and 5 wt% glycerol for 20 min at 35 °C, and then stabilized by desired paclitaxel/tubulin molar ratios ranging from $1/32 \leq \Lambda_{\text{Ptxl}} \leq 1/1$. Tau in BRB80 was then mixed with the assembled MTs at desired 3RS Tau/tubulin-dimer molar ratios ranging from $0 \leq \Phi_{3\text{RS}} \leq 1/2$. All measurements were taken or samples set within 3 hours from assembly to preclude microtubule end-to-end annealing (the vast majority of annealing occurs within the first several seconds of polymerization [32] with subsequent stable microtubule length over the course of five hours [6]).

2.3 Synchrotron Small Angle X-ray Scattering (SAXS)

Samples of paclitaxel-stabilized microtubules with Tau were centrifuged at 16,000g for 30 minutes and loaded in 1.5 mm quartz capillaries. Synchrotron SAXS of samples was performed at the Stanford Synchrotron Radiation Laboratory (SSRL) beam-line BL 4-2 at 9 keV ($\lambda=1.3776$ Å) with a Si(111) monochromator. Scattering data was collected via a 2D area detector (MarCCD, Mar USA). Sample-to-detector distance was set to be 2.5 m and calibrated with a silver behenate reference sample. Scans were repeated for different heights along each sample to confirm data consistency; data analysis was performed for one scan per sample.

2.3.1 Model Fitting of SAXS Data

SAXS data was analyzed, as described [22]. Briefly, after azimuthally-averaging SAXS intensity data, the averaged intensity data was background subtracted by fitting background to a

polynomial that passed through the scattering minima of SAXS data. Background-subtracted data were fit to the square of the form factor corresponding to a model hollow cylinder powder averaged over all orientations in q -space:

$$|F_{\text{MT}}|^2 \propto \left[\frac{\sin(q_z L/2)}{q_z} \left[(r_{\text{in}} + \delta) J_1(q_{\perp}(R_{\text{in}} + \delta)) - r_{\text{in}} J_1(q_{\perp} R_{\text{in}}) \right] \right]^2$$

With wall thickness δ (49 Å, an input parameter [12]), and average microtubule length L (2 μm, an input parameter [33]), yielding the ensemble-averaged microtubule inner radius $\langle R_{\text{in}}^{\text{MT}} \rangle$. $\langle R_{\text{in}}^{\text{MT}} \rangle$ is linearly proportional to the ensemble-averaged protofilament number in cross section, $\langle N_{\text{PF}} \rangle$. Error bars for $\langle R_{\text{in}}^{\text{MT}} \rangle$ result from fits.

2.4 Transmission Electron Microscopy (TEM)

TEM was performed on a JEM-3011HR (JEOL) electron microscope in the National NanoFab Center (NNFC) at KAIST with the electron beam set at 300 kV. The microtubule samples of 0.1 mg/mL were loaded on formvar coated copper grids (Ted Pella), followed by staining with 1 wt% uranyl acetate.

3. Results

3.1 Increasing paclitaxel concentration decreases the average MT protofilament number

The azimuthally-averaged synchrotron SAXS data of microtubules present scattering consistent with microtubules in an orientationally-ordered nematic state (with no higher-order structures, i.e. microtubule bundles) [22], with increasing amounts of paclitaxel ($\Lambda_{\text{ptxl}} = 1/32, 1/16, 1/8, 1/4, 1/1$) clearly showing an increase in scattering wave vector q of the peak position of the form factor, indicative of a decrease in the radial size of microtubules (Fig. 2A). To quantitatively assess this effect, azimuthally-averaged data were background-subtracted (Fig. 2B) and fit (Fig. 2B, fit results as colored solid lines) to a powder-averaged hollow cylinder form factor with radially constant electron density (Fig. 2B, inset) of defined wall thickness δ . In previous measurements [19,22], the change of the ensemble-averaged microtubule inner radius $\langle R_{\text{in}}^{\text{MT}} \rangle$ (with no change in wall thickness) corresponded to a change in the average number of protofilaments $\langle N_{\text{PF}} \rangle$. Thus, from the fits of the data the ensemble-averaged microtubule inner radius $\langle R_{\text{in}}^{\text{MT}} \rangle$ (equivalently, $\langle N_{\text{PF}} \rangle$) was extracted as a function of Λ_{ptxl} , showing a clear monotonic decrease in both average inner radius (and average protofilament number) as a function of increasing paclitaxel (Fig. 2C), from $\Lambda_{\text{ptxl}}=1/32$ yielding $\langle R_{\text{in}}^{\text{MT}} \rangle = 89.6 \text{ Å}$ ($\langle N_{\text{PF}} \rangle \approx 14.5$) to $\Lambda_{\text{ptxl}}=1/1$ yielding $\langle R_{\text{in}}^{\text{MT}} \rangle = 77.9 \text{ Å}$ ($\langle N_{\text{PF}} \rangle \approx 13$) (Fig. 2D). The non-integer values of $\langle N_{\text{PF}} \rangle$ imply a distribution of PF numbers in microtubule cross section. The change in $\langle N_{\text{PF}} \rangle$ is consistent with the previous discovery even of the active exchange of protofilaments between microtubules and a “reservoir” of free tubulin [22].

Fig. 2. (A) Azimuthally-averaged SAXS data of microtubules as a function of decreasing paclitaxel concentration (Λ_{ptxl}) show a clear shift in form factor of microtubule to lower q . (B) Background-subtracted SAXS data were fit (colored lines) to a hollow cylinder form factor with constant radial electron density (see inset). (C) Plotting the extracted ensemble-averaged microtubule inner radius $\langle R_{\text{in}}^{\text{MT}} \rangle$ (equivalently, $\langle N_{\text{PF}} \rangle$) decreasing as a function of increasing Λ_{ptxl} , suggesting that paclitaxel modulates PF-PF interactions in such a way to decrease the number of protofilaments in cross section. Error bars are extracted from fits of background-subtracted azimuthally-averaged data. (D) A schematic of paclitaxel binding to the inner microtubule lumen and affecting the average protofilament number of microtubules. All data were obtained at room temperature and taken 3 hours after preparing the reaction mixture, precluding microtubule end-to-end annealing.

3.2 At all initial paclitaxel concentrations, increasing Tau increases the average MT protofilament number

With increasing paclitaxel concentration decreasing the average microtubule protofilament number and increasing Tau concentration known to increase the average microtubule protofilament number (at $\Lambda_{\text{ptxl}} = 1/1$) [22], the competitive effect of 3RS Tau and paclitaxel in affecting the average microtubule protofilament number was investigated. Microtubules were assembled at varying Λ_{ptxl} with subsequent paclitaxel-stabilized microtubules mixed with varying amounts of 3RS Tau, with Tau “coating” microtubules [34]. At representative paclitaxel concentrations ($\Lambda_{\text{ptxl}} = 1/4, 1/8, \text{ and } 1/32$), azimuthally-averaged SAXS

data reveal a noticeable shift in the microtubule form factor peaks to lower q as a function of the amount of 3RS Tau added (Φ_{3RS}) to the reaction mixture (Fig. 3, A-C), indicative of the increase in microtubule cross-sectional size. As before, azimuthally-averaged data were background subtracted (Fig. 3, D-F) and fit to a powder-averaged form factor for a hollow cylinder, (Fig. 3, D-F, fit results as colored solid lines).

Fig. 3. (A-C) Azimuthally-averaged SAXS data of increasing Φ_{3RS} at $\Lambda_{ptxl} = 1/4$ (A), $\Lambda_{ptxl} = 1/8$ (B) and $1/32$ (C) show a clear shift in peak position as a function of increasing Φ_{3RS} , indicative of change in microtubule radius. (D-F) Background-subtracted SAXS data with resultant fit (colored lines). (G) Fit results of background-subtracted SAXS data at various Λ_{ptxl} (1/1, 1/4, 1/8, and 1/32) for increasing Φ_{3RS} yields the ensemble-averaged microtubule inner radius $\langle R_{in}^{MT} \rangle$ (equivalently, $\langle N_{PF} \rangle$), which demonstrates the increase in protofilament number with the addition of Tau, although paclitaxel competes to decrease the protofilament number. Error bars are extracted from fits of background-subtracted azimuthally-averaged data. The hatched area represents data unable to be fit by form factor alone due to scattering from microtubule bundles. (H) Schematic of the competition of paclitaxel and Tau in affecting the microtubule protofilament number. All data were obtained at room temperature and taken 3 hours after preparing the reaction mixture, precluding end-to-end microtubule annealing.

Plotting $\langle R_{in}^{MT} \rangle$ as a function of Φ_{3RS} for various Λ_{ptxl} (Fig. 3G) reveal a monotonic increase in average microtubule inner radius (and thus, average PF number) with increasing addition of 3RS Tau, at all paclitaxel concentrations. However, the increase is modulated by the initial conditions set by Λ_{ptxl} . For example, for stoichiometric paclitaxel concentrations ($\Lambda_{ptxl} = 1/1$) at $\Phi_{3RS} = 0$ the $\langle N_{PF} \rangle \approx 13$, while at $\Phi_{3RS} = 1/6$ the $\langle N_{PF} \rangle$ increases to ≈ 14 (adapted from [22]). However, at sub-stoichiometric paclitaxel concentrations ($\Lambda_{ptxl} = 1/4$), with no added 3RS Tau ($\Phi_{3RS} = 0$) the $\langle N_{PF} \rangle \approx 14$ but with some added 3RS Tau ($\Phi_{3RS} = 1/6$) the $\langle N_{PF} \rangle$ increases to ≈ 14.5 . The behavior indicates an opposing regulatory effect between Tau and paclitaxel in affecting the curvature of microtubules, most likely by influencing the active exchange of PFs (Fig. 3H), resulting in microtubules with as little as 13 PFs to as much as 15 PFs in our experimental range of Φ_{3RS} and Λ_{ptxl} . Indeed, the finding that Tau binds longitudinally along the outer ridges of microtubule protofilaments [34,35] lends credence to the idea that Tau modulates conformation of protofilaments leading to a change in microtubule curvature.

Interestingly, at lower paclitaxel concentrations ($\Lambda_{ptxl} \leq 1/4$) and high 3RS Tau concentration ($\Phi_{3RS} \geq 1/5$) (gray-hatched area, Fig. 3G), the powder-averaged hollow cylinder form factor is insufficient to fit the resultant azimuthally-averaged data, with the resulting SAXS showing a broad correlation peak [36], indicative of the onset of microtubule bundles (discussed below).

3.3 With increasing Tau and decreasing paclitaxel concentrations, microtubule bundles emerge

SAXS of reaction mixtures at high paclitaxel concentrations ($\Lambda_{ptxl} = 1/1$) but varying Tau concentration ($1/2 \geq \Phi_{3RS} \geq 1/10$) result in scattering data consistent with microtubule form factor with no higher-order structure (Fig. 4A, bottom 3 profiles). However, a lower paclitaxel concentration ($\Lambda_{ptxl} = 1/8$) over the same Tau concentration range presents scattering consistent

with microtubules in a higher-order structure (i.e. a microtubule bundle) at high Tau concentration ($\Phi_{3RS} = 1/2$), with weak correlation peaks evident (Fig. 4A, middle 3 profiles, correlation peaks noted with arrows for scattering of reaction mixture at $\Phi_{3RS} = 1/2$). This phase transition of non-bundled microtubules to bundled microtubules is clearly evident (via scattering) with increasing Tau and decreasing paclitaxel. Indeed, at a lower concentration of paclitaxel ($\Lambda_{Ptxl} = 1/32$), correlation peaks are clearly evident at $\Phi_{3RS} = 1/2$ with scattering inconsistent with form factor alone (i.e. non-bundled microtubules) at $\Phi_{3RS} = 1/6$ (Fig. 4A, top 3 profiles, correlation peaks noted with arrows). This observation of microtubule bundles at low paclitaxel concentrations ($\Lambda_{Ptxl} \leq 1/4$) and high Tau concentrations ($\Phi_{3RS} \geq 1/5$) suggests an opposing effect in mediating inter-microtubule interactions as a function of paclitaxel and Tau, requiring a map of phase behavior as a function of Λ_{Ptxl} and Φ_{3RS} (Fig. 4B).

Fig. 4. (A) Azimuthally-averaged SAXS data reveals the emergence of correlation peaks at low paclitaxel and high Tau concentrations, indicative of higher-order structures (bundles) of microtubules induced by Tau. (B) A phase map of the emergence of microtubule bundles as a function of Λ_{Ptxl} and Φ_{3RS} by SAXS data, with bundles emerging at the shade blue area. (C-E) TEM independently confirms microtubule bundles, with microtubule bundles coexisting with singular microtubules. (F-H) TEM also shows a lack of microtubule bundles at high paclitaxel and/or low Tau

concentrations. All data were obtained at room temperature; all SAXS measurements were taken 3 hours after preparing the reaction mixture and all EM measurements were prepared after approximately 4 hours after preparing the reaction mixture, precluding microtubule end-to-end annealing.

The phase diagram of the higher order structure of microtubules reveals the emergence of microtubule bundles with increasing Tau and decreasing paclitaxel concentration (specifically, with microtubule bundles emerging at $\Phi_{3RS} \geq 1/6$ for $\Lambda_{Ptxl} = 1/32$, $\Phi_{3RS} \geq 1/4$ for $\Lambda_{Ptxl} = 1/8$, and $\Phi_{3RS} \geq 1/2$ for $\Lambda_{Ptxl} = 1/4$). TEM independently confirms small microtubule bundles (MT doublets and triplets) co-existing with non-bundled microtubules occurring at reaction mixtures at $\Phi_{3RS} = 1/4$ for $\Lambda_{Ptxl} = 1/32$ (Fig. 4C), $\Phi_{3RS} = 1/6$ for $\Lambda_{Ptxl} = 1/8$ (Fig. 4D), and $\Phi_{3RS} = 1/2$ for $\Lambda_{Ptxl} = 1/4$ (Fig. 4E), with only non-bundled microtubules occurring elsewhere (Fig. 4F-H).

4. Discussion

The opposing regulatory roles of Tau and paclitaxel in increasing and decreasing, respectively, the diameter of microtubules is consistent with previous studies showing a decrease in microtubule protofilament number upon paclitaxel stabilization [12] and an increase in protofilament number of paclitaxel-stabilized microtubules upon the addition of Tau [19,22]. Indeed, change in the curvature of microtubules is a recurring theme with other molecules. Cationic membranes (consisting of mixtures of neutral and charged lipids) coating microtubules increase the average microtubule diameter (decreasing microtubule curvature) as a function of increasing membrane charge density [37,38]. Multivalent cations have been observed to invert the axial curvature of microtubule protofilaments into inverted tubulin tubules consisting of a helical assembly of PF rings [10]. The changes induced in microtubule structure by such a diverse array of molecules and ions strongly indicate there are probably other biologically relevant proteins and ions that do so as well.

The finding that Tau-mediated microtubule bundling is suppressed at stoichiometric paclitaxel concentrations ($\Lambda_{Ptxl} = 1/1$) [22] and our finding in this paper that shows a small region in the Φ_{3RS} - Λ_{Ptxl} phase diagram where microtubule bundles form (Fig. 4B, at low Λ_{Ptxl} and high Φ_{3RS}) at room temperature, could quite possibly explain why earlier cell-free reconstitutions of paclitaxel-stabilized microtubules have never bundled in the presence of Tau [23], as opposed to TEM data of string-like microtubule bundles found in the axon initial segment [25] and hexagonally-ordered microtubules in neurite-like processes of non-neuronal cells overexpressing transfected Tau cDNA [26].

The emergence of microtubule bundles with increasing Tau and decreasing paclitaxel suggest the bundling mechanism may lie in how Tau and paclitaxel differentially regulate microtubule structure. Along with the aforementioned regulation of the microtubule protofilament number distribution ($\langle N_{PF} \rangle$, Fig. 3G), it is also known that Tau-stabilized microtubules are, on average, much longer ($\approx 20 \mu\text{m}$ [29]) than paclitaxel-stabilized microtubules ($\approx 2 \mu\text{m}$ [28]). As both lengths are much shorter than reported microtubule

persistence lengths [39–41], both Tau-stabilized and paclitaxel-stabilized microtubules can be essentially treated as stiff rods of differing lengths. While the average thermal kinetic energy of stiff rods of differing lengths is the same (of order of a few $k_B T$ by the equipartition theorem), stabilization of those rods into bundles would require a commensurate interaction between rods greater than the thermal kinetic energy of individual rods. Because the interaction between rigid rods is length dependent (i.e. resulting from the aggregate of individual Tau-Tau interactions over the entire length of opposing microtubules), bundling itself would also be length dependent.

Although increasing paclitaxel favors the polymerization of tubulin into microtubules, it is entirely possible that high or stoichiometric quantities of paclitaxel favor microtubule seeds over increasing polymerization of existing microtubules (i.e. more, shorter microtubules over less, longer microtubules) by rapidly depleting the supply of tubulin available for microtubule assembly. In this manner, decreasing paclitaxel concentration should increase the length of the average microtubule, which allows for microtubule-length dependent mechanisms, like Tau-mediated microtubule bundling, to occur.

5. Conclusions

Although it is well appreciated that both Tau and paclitaxel enhance tubulin assembly and suppress microtubule dynamic instability in a concentration dependent manner [20,28], through SAXS and TEM we demonstrate the opposing regulatory roles of Tau and paclitaxel in affecting microtubule structure (i.e. average PF number) and inter-microtubule interactions. The immediate impact of this work would be to suggest that in order to more closely replicate microtubule geometries found *in vivo* it may be necessary to minimize paclitaxel use, despite the obvious benefits in experimental tractability.

The emergence of microtubule bundles as a function of increasing Tau and decreasing paclitaxel may shed light on the less-understood domains of Tau. The conservation of the repeat motifs in the microtubule-binding region (MTBR) of Tau suggest a very specific role; combined with the evidence that the MTBR specifically binds to microtubules [34,35,42] strongly indicates the MTBR regulates individual microtubule structure and assembly. However, the role of the projection domain and proline-rich region are less clear. Hirokawa and associates concluded that the Tau projection domain determined inter-microtubule spacings in hexagonally-ordered microtubule arrays in neurite-like processes of non-neuronal cells transfected with Tau cDNA [26]. With Tau being highly overexpressed, this study could not determine if the hexagonal arrays resulted from Tau-mediated attractions or was a repulsive lattice under confinement. Our study shows that the conclusion [23] that Tau acted as a purely repulsive “spacer” of paclitaxel-stabilized microtubules cannot be generalized to paclitaxel-free microtubules.

While this study focused on paclitaxel-stabilized microtubules at room temperature ($\approx 25^\circ$ C), future studies involving microtubules at physiological temperatures ($\approx 37^\circ$ C) may yield additional interesting results. As the “straight” conformation of protofilaments is favorable at 37° C (with the “bent” conformation increasingly favorable as temperature drops) [43,44], it would be reasonable to speculate that the phase diagram of non-bundled microtubules and microtubule

bundles as a function of paclitaxel and Tau concentration would be altered at physiological temperatures ($\approx 37^\circ\text{C}$).

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