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# **STRUCTURAL FLEXIBILITY OF TAU IN ITS INTERACTION WITH MICROTUBULES AS VIEWED**

## **BY SITE-DIRECTED SPIN LABELLING EPR SPECTROSCOPY**

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## ABSTRACT

Tau is a microtubule associated protein that belongs to the Intrinsically Disordered Proteins (IDPs) family. IDPs or Intrinsically Disordered Regions (IDRs) play key roles in protein interaction networks and their dysfunctions are often related to severe diseases.<sup>1</sup> Defined by their lack of stable secondary and tertiary structures in physiological conditions while being functional, these proteins use their inherent structural flexibility to adapt to and interact with various binding partners.<sup>2</sup> Knowledge on the structural dynamics of IDPs and their different conformers are crucial to finely decipher fundamental biological processes controlled by mechanisms such as conformational adaptations or switches, induced-fit or conformational selection events. Different mechanisms of binding have been proposed: among them, the so-called « folding-upon-binding » in which the IDP adopts a certain conformation upon interacting with a partner protein, or the formation of a « fuzzy » complex in which the IDP partly keeps its dynamical character at the surface of its partner.<sup>3</sup> The dynamical nature and physico-chemical properties of unbound as well as bound IDPs, makes this class of proteins particularly difficult to characterize by classical bio-structural techniques and require specific approaches for the fine description of their inherent dynamics.

Among other techniques, Site-Directed Spin Labelling combined with Electron Paramagnetic Resonance (SDSL-EPR) spectroscopy has gained much interest in this last decade for the study of IDPs.<sup>4-7</sup> SDSL-EPR consists in grafting a paramagnetic label (mainly a nitroxide radical) at selected site(s) of the macromolecule under interest followed by its observation using and/or combining different EPR strategies.<sup>8,9</sup> These nitroxide spin labels detected by continuous wave (cw) EPR spectroscopy are used as perfect reporters or “spy spins” of their local environment, being able to reveal structural transitions, folding/unfolding events... Another approach is based on the measurement of inter-label distance distribution in the [1.5-8.0] nm range using pulsed dipolar EPR experiments, such as Double Electron-Electron Resonance (DEER) spectroscopy or DEER.<sup>10</sup> The technique is then particularly well suited to study the behaviour of Tau in its interaction with its physiological partner: microtubules (MTs). In this chapter we provide a detailed experimental protocol for the labelling of Tau protein and

its EPR study while interacting with preformed (Paclitaxel-stabilized) MTs, or using Tau as MTs inducer. We show how the choice of nitroxide label can be crucial to obtain functional information on Tau/tubuline complexes.

**KEY WORDS:** Tau protein, Tubuline dimers, microtubules, Site-directed Spin labelling, nitroxide radical, spin labels, Electron Paramagnetic Resonance, intrinsically disordered proteins, fuzzy complex.

## 1. INTRODUCTION

Tau is an IDP that physiologically regulates assembly, dynamic behaviour and spatial organization of microtubules (MTs). Tau belongs to the family of MTs associated proteins (MAPs). Among MAPs, Tau is probably the most studied protein because of its implication in a group of neurodegenerative diseases called tauopathies, including Alzheimer's disease. These diseases are associated to Tau aggregation into intra-neuronal deposits<sup>11</sup> and to loss of MTs/Tau binding leading to neurodegeneration. Even if the MTs binding (MTB) region of Tau has been determined,<sup>12-15</sup> numerous studies tried to understand where and how Tau binds to MTs, the model of Tau:MTs interaction still remaining a topic of debate. Some studies suggested that the MTB region is located inside the MTs, in the vicinity of the Paclitaxel site localized on  $\beta$ -tubulin in the inner MTs surface.<sup>16,17</sup> Others proposed that Tau binds exclusively to the outside surface of MTs, not only along protofilaments but also across.<sup>18</sup> In the last few years, the hypothesis of longitudinal binding of Tau on the external surface of MTs seemed to be reinforced.<sup>8,9</sup> Recently, the combination of cryo-EM at near-atomic resolution and of Rosetta modelling generated models of the Tau/MTs interaction, highlighted the crucial residues of Tau MTB domain directly implicated in the interaction.<sup>21</sup> In the same year, we used Site-Directed Spin Labelling combined with Electron Paramagnetic Resonance (SDSL-EPR) to study the binding mode of the longest isoform of Tau, to Paclitaxel-stabilized MTs and to tubulin dimers, with Tau used as the sole inducer of MTs formation in the latter case.<sup>22</sup>

Since the pioneering works of the group of Hubbell,<sup>5</sup> SDSL-EPR has emerged as a valuable tool for studying protein dynamics such as structural transitions in a wide range of proteins (soluble as well as membrane proteins, the size of the protein not being a limitation for the technique). The strategy of SDSL-EPR involves the insertion of a paramagnetic label at a selected site of a protein and its observation by EPR spectroscopy (for reviews see<sup>4,8,23-25</sup>). The paramagnetic label is usually a stable nitroxide reagent, the most commonly used being the MTSL spin label (1-oxyl-2,2,5,5-tetramethyl- $\delta$ 3-pyrroline-3-methyl methane thiosulfonate) which forms a disulfide bond with the sulfhydryl group of a cysteine residue (Fig. 1A). Depending on the case, other nitroxide spin labels targeting cysteine

residues such as maleimido Proxyl can also be chosen (Fig. 1A).<sup>8</sup> The common approach consists in the analysis of the continuous wave (CW) EPR spectral shape reflecting the nitroxide motion reflecting its microenvironment. This approach is particularly well suited to monitor conformational changes induced by protein-protein or protein-ligand interactions, since such interactions modify the mobility of the label located at the interface, and consequently, the EPR spectrum of the spin-labelled protein.<sup>5,26–28</sup> Moreover, it has been shown to be very well adapted to study induced folding events within intrinsically disordered proteins (IDPs).<sup>4,29–32</sup> A second approach consists in measuring distances between two spin probes, through spin-spin interaction analysis detected by Double Electron Electron Resonance (DEER) sequence requiring pulsed EPR technology.<sup>33–35</sup> This technique goes beyond the scope of this chapter but has been successfully applied on Tau by other groups.<sup>36,37</sup>

This chapter provides a guideline to perform SDSL-EPR spectroscopy on the longest isoform of Tau protein using CW-EPR. As the longest isoform of Tau (441 aa) contains two natural cysteines (C291 and C322), located in the MTB region, we targeted them for spin labelling (Fig. 1B) using either MTSL or Proxyl (Fig. 1A). We identified a thiol-disulfide exchange between Tau and tubulin or Paclitaxel-stabilized MTs. Depending on the MTs formation conditions, differences in the thiol-disulfide exchange kinetics allowed us to propose the localization of two binding regions of Tau on tubulin: a first one involving the Cys347 of  $\alpha$ -tubulin subunit and a second one the Cys131 in the 3j6f structure (Cys 129 in the amino acid sequence) of  $\beta$ -tubulin subunit. The protocol described here is applicable to various protein samples and intends to describe the best practices in spin labelling. How to graft efficiently spin labels on cysteine residues? How to record and analyse EPR spectra? And finally, which advantage do we get by using several labels?

### **Site-directed spin-labelling (SDSL)**

Classically, nitroxides spin labels are functionalized to react specifically with cysteine residues. Most often, a cysteine residue is introduced at the desired position *via* site-directed mutagenesis on a “cys-less” mutant, unless one natural cysteine is already present in the region of interest. Here, no mutagenesis was necessary because the longest isoform of Tau contained two natural cysteine

residues at positions 291 and 322 located in the MBD. The presence of the label usually does not affect the protein properties thanks to its rather small size, which is comparable to that of a tryptophane residue (7 Å).<sup>38</sup> However, control experiments are needed to check that the presence of the label does not modify the behaviour of the system under study. For Tau, functionality experiments were done using turbidimetry (Fig. 2) to check the ability of Tau to induce MTs formation before and after labelling of Tau either with MTSL or Proxyl, referred to as Tau<sup>MTSL</sup> or Tau<sup>Proxyl</sup> in the following text.

A critical point for achieving high labelling yields is the reduction of cysteine residues prior to incubation with the nitroxide reagent. Indeed, cysteine residues may be involved in disulfide bridges. Incubation of Tau with TCEP, is carried out as the first step and a gel filtration onto a desalting column is then used to remove excess of reducing agent. Once the reductant has been removed, it is recommended to immediately proceed to spin labelling to avoid re-oxidation of cysteines. In our experience, incubating the protein with the label under an argon flow gave rise to higher labelling yields, although successful spin labelling has also been reported in the absence of argon.<sup>39</sup> Quantification of the labelling yield is achieved by calculating the  $C_{Spin}$  to  $C_{Protein}$  ratio, where  $C_{Spin}$  is measured after spin integration of the absorption spectrum of labelled protein and  $C_{Protein}$  is the protein concentration as described in section 3.4.2 (Fig. 3A).

### **CW-EPR spectroscopy of nitroxide labels grafted to proteins**

Nitroxide radicals are anisotropic paramagnetic centres characterized by the interaction between an electronic spin magnetic moment ( $S = \frac{1}{2}$ ) and a nuclear spin magnetic moment ( $I = 1$ ) arising from the magnetism of the <sup>14</sup>N nucleus located in the vicinity of the unpaired electron. As a consequence, the typical EPR signal of such radical is a 3-line spectrum observed at room temperature. The anisotropy of both  $g$  tensor (Zeeman interaction) and more importantly  $A$  tensor (hyperfine interaction) makes the EPR spectrum critically dependent on the radical mobility (Fig. 3B). When nitroxide spin labels are allowed to tumble rapidly, magnetic interactions ( $g$  and  $A$ -tensors) are completely averaged (Fig. 3B, spectrum a), this is the case for a free (non-grafted) radical in solution.

As the motion becomes progressively slower (after grafting for example), the magnetic anisotropy is no longer totally averaged and this results in a differential broadening of lines in the spectrum, while line positions remain constant (Fig. 3B, spectrum b). This is the so-called fast motional regime. By further reducing the mobility of the radical, the averaging of tensor components becomes less and less efficient, leading to shape distortions of the EPR spectrum corresponding to the intermediate motional regime (Fig. 3B, spectrum c). Finally, the slow motional regime is reached when the full effects of the anisotropy of  $g$  and  $A$ -tensors are observed, this situation corresponding to the spectrum observed in frozen solution of nitroxide radical (Fig. 3B, spectrum d).<sup>8</sup>

### **EPR spectral shape analysis of labelled Tau**

EPR spectra of labelled Tau were analysed using two methods: spectral simulation for Tau<sup>Proxyl</sup> and semi-quantitative analysis for Tau<sup>MTSL</sup>, both alone or in presence of tubuline or MTs.

#### *(1) EPR spectra analysis of labelled Tau<sup>Proxyl</sup>*

EPR spectral simulation allowed decomposing the spectrum into different components and extracting, for each of them, the relative proportion and a dynamic parameter, namely, the rotational correlation time  $\tau_c$ .<sup>5,40,41</sup> The presence of several spectral components might arise from 1) different mobilities of the label or 2) several protein conformations. Simulations were performed using SimLabel,<sup>40</sup> a MATLAB Graphical User Interface (GUI) that uses some functions of the EasySpin toolbox<sup>40,41</sup> and is dedicated to multicomponent simulations of EPR spectra from SDSL-EPR experiments. EPR spectrum of Tau<sup>Proxyl</sup> alone was typical of a label grafted on a disordered region, where both the mobility of the label and the high flexibility of the side-chain contributed to the narrowing of the lines. The spectrum was simulated using a single component with a rotational correlation time  $\tau_c$  of  $0.24 \pm 0.02$  ns (Fig. 4). The single  $\tau_c$  value showed that the two labelled cysteine (C291 and C322) environments were not discernible.

#### *(2) EPR spectra analysis of Tau<sup>Proxyl</sup> with tubulin dimers and Paclitaxel-stabilized MTs*



In the case of this study, two conditions of interacting partners were studied. The first was the interaction of Tau<sup>Proxyl</sup> in presence of  $\alpha\beta$ -tubulin (it has to be freshly prepared prior to use, see section 3.1.1), when Tau was directly used as an inducer of MTs formation, which represents the physiological condition. The second condition was the interaction of Tau<sup>Proxyl</sup> with Paclitaxel-stabilized MTs.

Slight broadening of the EPR spectral shapes were observed when Tau<sup>Proxyl</sup> was mixed with its partner (Fig. 4A). Two components were required for the simulation of the spectrum of Tau<sup>Proxyl</sup> when used as MTs inducer, with  $\tau_c$  values of  $0.24 \pm 0.02$  and  $0.76 \pm 0.02$  ns accounting for 19 and 81% respectively (Fig 4B). The first component corresponds to Tau<sup>Proxyl</sup> alone in solution, *i.e.* not bound to tubulin. The second component has a  $\tau_c$  value which is slightly larger than the first one, but is still in the rapid regime of mobility. In presence of Paclitaxel-stabilized MTs, the EPR spectrum was also simulated using two components with  $\tau_c$  of  $0.24 \pm 0.02$  ns and  $1.19 \pm 0.02$  ns accounting for 55 and 45% respectively (Fig. 4B). Again, the first component corresponds to a proportion of unbound Tau<sup>Proxyl</sup>. In both cases, the slight changes observed showed that Tau remains in a high mobility regime in its bound form. This observation is in agreement with the character of fuzzy complex of Tau:MTs in which the IDP keeps a high flexibility in the bound state as already described in the literature.<sup>42</sup>

### *(3) EPR spectra analysis of Tau<sup>MTSL</sup> alone and with tubulin dimers and Paclitaxel-stabilized MTs*

When the MTSL spin label was used, we unexpectedly observed the release of the label after interaction with the partner protein (tubulin dimers or Paclitaxel-stabilized MTs) as described in Fig. 1A. To follow the kinetics of label release, we used a semi-quantitative analysis of the EPR spectra based on the measurement of the ratio between two lines.<sup>43,44</sup> In the fast motional regime of mobility, the parameter of choice is the ratio between the peak-to-peak amplitude  $h$  of the lateral lines at high field  $h(-1)$  over the one of the central line  $h(0)$ . The values -1, 0, +1 come from the different spin states of the nitroxide <sup>14</sup>N nucleus  $M_I = -1, 0, +1$ , (Fig. 5A). An increase of this parameter indicates an increase of the mobility of the probe.

The  $h(-1)/h(0)$  ratio for Tau<sup>MTSL</sup> alone in solution had a value of 0.48, this value being stable with time (Fig. 5B). Tau<sup>MTSL</sup> was then mixed with tubulin in a 1:2 molar ratio at 37 °C in order to form MTs. The EPR spectrum of Tau<sup>MTSL</sup> evolved with time until reaching the spectral shape of free nitroxide spin molecule after approximately 25 min (Fig. 5B). Plotting the ratio  $h(-1)/h(0)$  as a function of time was performed in order to measure the label release kinetics, followed by data fitting with an mono-exponential curve giving a  $k$  value of  $0.23 \text{ min}^{-1} \pm 0.01 \text{ min}^{-1}$ . The same experiment was performed by mixing Tau<sup>MTSL</sup> with Paclitaxel-stabilized MTs at 37 °C. As previously, the release of the label was observed, but with a slower kinetics with a  $k$  value of  $0.054 \pm 0.002 \text{ min}^{-1}$  (Fig. 5B). Following the  $h(-1)/h(0)$  ratio measurement over time allowed us to find out the breakage of the disulfide bond between the sulfur atoms of the Tau C291 and C322 cysteines and the label (*i.e.* label release) that implied the necessary involvement of cysteines in the partner protein (tubulin), since the Tau-SS-tubulin complex was not observed by western-blotting in non-reducing conditions.<sup>22</sup> Differences of label release kinetics for the two conditions and comparison of the 3D reconstructions of MTs with (PDB: 3j6g) and without Paclitaxel (PDB: 3j6f)<sup>45</sup> allowed us to propose a Tau longitudinal binding model involving two sites (1 and 2) on tubulin associated with the two natural cysteines of Tau. Tau R2 repeat (more precisely C291) binds to the  $\alpha$ -subunit of tubulin at site 1 involving the region of the most reactive cysteine known in tubulin  $\alpha$ -C347, whereas Tau R3 repeat (C322) binds to the  $\beta$ -subunit involving  $\beta$ -C131 and  $\beta$ -C129 in the 3j6f structure (Cys 129 and Cys 127 in the amino acid sequence) (Fig. 6).

## 2. MATERIALS

All solutions were prepared using ultrapure milliQ® water and analytical grade reagents. All reagents were stored and prepared at room temperature (unless otherwise indicated). Diligently followed all waste disposal regulations.

### 2.1. Spin labelling

#### 2.1.1. Reagents and solutions

1. Lyophilized wild type Tau protein (441 aa) purified as described in De Bessa *et al.*<sup>46</sup>
2. 20 mM sodium phosphate buffer, pH 6.5.
3. 1.5 mL and 0.5 mL Eppendorfs.
4. Tris(2-carboxyethyl)phosphine (TCEP) (Sigma-Aldrich).
5. Spin labels: 1-oxyl-2,2,5,5-tetramethyl- $\delta^3$ -pyrroline-3-methylmethane-thiosulfonate (MTSL) (Toronto Research Chemicals Inc.) or 3-Maleimido proxyl (Proxyl) (Sigma Aldrich).

#### 2.1.2. *Special equipment*

1. PD-10 Desalting Columns (GE Healthcare).
2. Argon gas.
3. UV-vis absorption spectrophotometer and quartz cuvettes.

## 2.2. **Microtubule formation**

#### 2.2.1. *Reagents and solutions*

1. 1 M magnesium chloride ( $\text{MgCl}_2$ ) commercial aqueous solution.
2. 400 mM phosphate buffer stock (1 L): mix 27.6 g of  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  with 28.39 g anhydrous  $\text{Na}_2\text{HPO}_4$  and add ultrapure water up to 1 L in a graduated flask.
3. 20 mM phosphate buffer pH 6.5 (1L): put 50 mL of the 400 mM phosphate buffer in a graduated flask. pH must be at 6.95 at 25 °C. Adjust the pH to 6.5 by adding HCl and adjust volume to 1 L.
4. 0.1 M of guanosine triphosphate sodium salt hydrate (GTP): weigh 0.0523 g GTP in a microtube and add 1 mL of ultrapure water. Check the concentration by measuring the OD at 256 nm with a molecular extinction coefficient  $\epsilon_{256 \text{ nm}} = 13,700 \text{ M}^{-1}\text{cm}^{-1}$ . Make aliquots of 100  $\mu\text{L}$  and store at  $-20 \text{ }^\circ\text{C}$  (see **Note 1**).

5. 1 M of Tris(2-CarboxyEthyl)Phosphine (TCEP, Sigma-Aldrich): weigh 1.43 g in a 5 mL plastic tube and add 4.5 mL ultrapure water. Use a Vortex to strongly mix the solution during few minutes (see **Note 2**). Make 500  $\mu$ L aliquots and store them at  $-20^{\circ}\text{C}$ .
6. G25 Preparation: Sephadex G-25 medium was purchased from GE Healthcare (Uppsala, Sweden). Pack the Sephadex G25 beads (pre-incubated in ultrapure water overnight) in a column of 25 cm high and 1 cm diameter (25 cm  $\times$  1 cm) (see **Note 3**).
7. Guanidine-HCl 6 M: weigh 11.46 g of guanidine-HCl and adjust volume up to 20 mL with water (see **Note 4**).
8. Non-Assembly Condition Buffer: 20 mM phosphate buffer pH 6.5, 0.1 mM GTP, 1 mM TCEP. In a 500 mL glass beaker, add 25 mL of the 400 mM phosphate buffer to 450 mL of ultrapure water. Adjust pH to 6.5 with HCl. Transfer the buffer in a 500 mL graduated flask and complete volume to 500 mL. Add 0.1 mM GTP and 1 mM TCEP freshly just before used.
9. Paclitaxel: Paclitaxel was purchased from Alexis, and used without any purification.  $10^{-3}$  M Paclitaxel: weigh 0.8 mg of paclitaxel in 1 mL in dimethyl sulfoxide (DMSO). Its concentration is measured spectrophotometrically using the value of epsilon  $\epsilon_{273\text{nm}}=1,700 \text{ M}^{-1} \text{ cm}^{-1}$  in ethanol.

### 2.2.2. *Special equipment*

1. Gravity column (1cm\*25cm) with peristaltic pump, and tubes to collect the fraction.
2. UV-vis absorption spectrophotometer and quartz cuvettes.
3. Thermostated bath or incubator or room thermostated at  $37^{\circ}\text{C}$ .

## 2.3. *Electronic Microscopy*

### 2.3.1. *Reagents and solutions*

1. Uranyl acetate 2%: weigh 0.02 g uranyl acetate and add 10 mL of ultrapure water. Mix with vortex and check carefully for the dissolution of crystals (see **Note 5**). Pass the solution through a 20  $\mu\text{m}$  Millipore filter with a syringe.
2. 200 mesh carbon-coated formvar films on copper grids.

#### *2.3.2. Special equipment*

1. Transmission electron microscope

#### **2.4. EPR spectroscopy**

1. X-band (9.9 GHz) BRUKER Elexsys 500 spectrometer
2. Bruker Elexsys Super High Sensitivity resonator
3. Bruker variable temperature unit with liquid N<sub>2</sub> (ER4131VT)
4. Quartz capillary of 1.0-1.1 mm inner diameter corresponding to a sample volume of 30  $\mu\text{L}$ -40  $\mu\text{L}$ .
5. 100  $\mu\text{L}$  syringe (SGE or Hamilton) equipped with a 20 cm-long needle.

### **3. METHODS**

#### **3.1 Tau and Tubulin purifications**

Tau and Tubulin were prepared as described in the previous edition in chapter 4 untitled "Tau interaction with Tubulin and Microtubules : from purified proteins to cells".<sup>46</sup>

##### *3.1.1. Tubulin preparation*

Because tubulin was stored in a buffer containing 1 M of sucrose,<sup>46</sup> it was necessary to remove it by size exclusion chromatography to avoid any biased effect due to its presence. Tubulin was prepared (see **Note 6**) in the appropriate buffer (assembly or non-assembly condition) on gel filtration Sephadex G25 resin on a gravity column (1 cm x 25cm).

1. Equilibrate the column with 40mL of cold appropriate buffer, supplemented in GTP 0.1 mM at a flow rate of 1mL/min.
2. Rapidly unfreeze the tubulin sample that was stored in liquid nitrogen. When the buffer is removed (without completely drying the resin), inject tubulin solution (see **Note 7**) on the top.
3. When all the protein has entered into the resin, add buffer and start elution at a flow rate of 1mL/min. Collect 500  $\mu$ L (or approximately 13 drops) fractions and store them on ice.
4. Tubulin concentration is determined spectrophotometrically at 275 nm. Dilute 20  $\mu$ L of elution buffer in 500  $\mu$ L of 6M Guanidine-HCl and make the blank. Put 20 $\mu$ L of each tube presenting a meniscus in 500  $\mu$ L and measure the OD (see **Note 8**).
5. Determine the concentration using the Lambert-Beer law with a molecular extinction coefficient of  $\epsilon_{275 \text{ nm}} = 1.09 \text{ mL.mg}^{-1}.\text{cm}^{-1}$  taking into account the dilution factor (here 26). Mix the most concentrated fractions in one tube and measure the concentration in triplicate as described above. Dilute it at the desired concentration.

### *3.1.2. Tau preparation*

After Tau purification, the protein was lyophilized and stored at  $-20^{\circ}\text{C}$ .<sup>46</sup> Before use, Tau was dissolved in the desired buffer, centrifuged at 3,000g during 10 min and its concentration was determined by recording a UV-visible absorption spectrum from 240 to 400 nm. After correction of the light diffusion,<sup>46</sup> the concentration of Tau was obtained using the maximal absorbance at 280 nm, the extinction coefficient being  $\epsilon_{280\text{nm}} = 7700 \text{ M}^{-1}.\text{cm}^{-1}$ .

### **3.2. Site-directed spin-labelling (SDSL)**

1. Add a small quantity of buffer to a lyophilised and purified Tau in order to keep a high solution concentration (typical concentration range of 100-200  $\mu$ M). Add a 50-molar excess of TCEP to the solution and incubate for 30 min on ice.
2. Equilibrate the PD-10 desalting column with 30 mL of 20 mM phosphate buffer pH 6.5.

3. Add the mixture to the column and let it enter the packed bed. Add equilibration buffer to adjust the volume up to 1 mL and let it enter the packed bed completely. Discard the flow through.
4. Use Eppendorf (1.5 mL) tubes for fraction collection. Elute with 500  $\mu$ L of equilibration buffer and collect the eluate. Repeat 4 times. This constitutes the dead volume that should not contain any protein (check anyway that this is the case by absorption measurement at 280 nm or EPR).
5. Use Eppendorf (0.5 mL) tubes for fraction collection. Elute with 250  $\mu$ L of equilibration buffer and collect the eluate. Repeat 8 times. 8 fractions of 250  $\mu$ L each are thus collected. Keep these fractions in ice.
6. Check each fraction for protein concentration by measuring the absorption at 280 nm as described in **3.1.2** section.<sup>21</sup> Pool the fractions containing the protein (typical final volume of 1 mL).
7. Add the MTSL or Proxyl spin label to the pool of reduced protein in a molar excess of 10. Keep the mixture under a flow of Argon in an ice bath, in the obscurity and under gentle stirring for 1 hour. The labelled proteins will be designed hereafter as Tau<sup>MTSL</sup> or Tau<sup>Proxyl</sup>. Be careful not to dry too much the solution, by always keeping a minimum of volume of buffer.
8. Equilibrate a second PD-10 desalting column with approximately 30 mL of 20 mM sodium phosphate pH 6.5 buffer so as to remove the excess of label (MTSL or Proxyl).
9. Use Eppendorf (1.5 mL) tubes for fraction collection. Elute four fractions of 500  $\mu$ L each using the equilibration buffer.
10. Use Eppendorf (0.5 mL) tubes for fraction collection. Elute 12 fractions of 250  $\mu$ L each (numbered from 1 to 12) using the equilibration buffer and keep them in an ice bath.
11. Check every fraction : 1/ for protein concentration by recording an UV-visible absorption spectrum and 2/ for spin concentration by recording an EPR spectrum at room temperature

on approximately 50  $\mu\text{L}$  of solution injected in a quartz capillary (see below for details) (for an example of a typical elution profile see Habchi *et al.*<sup>6</sup>).

12. Pool the fractions containing the labelled protein and giving rise to a sufficient EPR signal (usually fractions 2 to 6, see **Note 9**) and concentrate it up to a high final concentration (100-150  $\mu\text{M}$ ) by flushing Ar gas (see **Note 10**). Make sure no bubbles are present in the protein solution. Check the concentrated sample for protein and spin concentration in order to determine the labelling yield (see section **3.4** for details on EPR and spin concentration calculation). Concentrations of labelled proteins in the range of 20  $\mu\text{M}$ -100 $\mu\text{M}$  are required to obtain EPR spectra with a good signal to noise ratio in typically 90 s.

### **3.3. Preparation of microtubules**

MTs were formed by either adding Paclitaxel, an anticancer agent that stabilizes them, or by adding Tau protein to tubulin dimers. In the first case, we monitored the interaction of Tau over time on preformed and Paclitaxel-stabilized MTs at room temperature and in the second case, we monitored the interaction of Tau with tubulin dimers during the polymerization process at 37°C.

#### *3.3.1. Tau induced MTs*

Purified tubulin was prepared using size exclusion chromatography in 20 mM phosphate buffer pH 6.5, GTP 0.1 mM as described in **3.1.1** section. For the functionality tests, the experiment was followed by measuring the turbidimetry time course at 350 nm at 37 °C (Fig. 2). At this wavelength, the absorbance was proportional to the amount of microtubules formed.<sup>47</sup> For functionality experiments, the different steps are described below:

1. Switch on the spectrophotometer with thermostated water bath or with a Peltier temperature control device, set the temperature to 37°C and choose the wavelength to 350 nm.
2. Before starting the time-driven microtubule formation, make the blank with buffer alone or on air.



3. Inject 200  $\mu\text{L}$  of tubulin at 7  $\mu\text{M}$  in the cuvette and insert it in the thermostated spectrophotometer at 37°C. Measure the absorbance time course at 350 nm during 30 min or more. After the first 5 min, add 80  $\mu\text{L}$  of different labelled Tau concentration (for example 80  $\mu\text{L}$  of labelled Tau at 15  $\mu\text{M}$  for a final concentration of 4.3  $\mu\text{M}$ ). The signal should increase just after adding labelled Tau indicating the formation of MTs (Fig. 2).

### *3.3.2. Paclitaxel-stabilized MTs*

Tubulin was prepared as described in section **3.1.1** using 20 mM phosphate buffer pH 6.5, GTP 0.1 mM, 8 mM  $\text{MgCl}_2$ . 40  $\mu\text{M}$  of tubulin (total volume between 100 and 300  $\mu\text{L}$ ) was incubated 20 min at 37°C with 60  $\mu\text{M}$  of Paclitaxel. The solution was turbid due to MTs formation.

### *3.3.3. Co-sedimentation assay on Tau<sup>MTSL</sup> and Tau<sup>Proxyl</sup>*

To ensure that the labelling of Tau with either MTSL or Proxyl did not modify the propensity of Tau to interact with MTs, co-sedimentation assays were performed. Unbound and bound Tau to Paclitaxel-stabilized MTs were separated by centrifugation through a 60 % glycerol cushion to avoid any non-specific binding.

1. Prepare the glycerol cushion by adding 6 mL of glycerol to 4 mL of 20 mM phosphate buffer pH 6.5, GTP 0.1 mM and warm it at 37°C.
2. Form Paclitaxel stabilized microtubule as described in the **3.4.1** section using 10  $\mu\text{M}$  of tubulin and 15  $\mu\text{M}$  of Paclitaxel (see **Note 11**).
3. Mix 150  $\mu\text{L}$  of Paclitaxel-stabilized MTs at 10  $\mu\text{M}$  with 150  $\mu\text{L}$  of various concentration of labelled Tau (the stock concentration should be twice higher than the desired final concentration)
4. Put carefully (without mixing) 200  $\mu\text{L}$  of a mix of Paclitaxel-stabilized MTs/labelled Tau on 200  $\mu\text{L}$  glycerol 60% cushion in pre-warmed (at 37 °C) ultracentrifuge tubes and centrifuge at 88 000 g for 20 min at 37°C in a pre-warmed rotor.

5. Collect supernatant (200  $\mu$ L) containing free tubulin and labelled Tau and mix it with 40  $\mu$ L of 5X SDS-PAGE loading buffer. Turn the tubes containing the pellets upside down to remove the rest of the supernatant.
6. Wash the pellets three times with 200  $\mu$ L of warm buffer (37°C)
7. Dissolve the pellet in 200  $\mu$ L of cold buffer to depolymerize microtubules. A manual action may be necessary to correctly suspend pellets. Add 40  $\mu$ L of 5X SDS-PAGE loading buffer to the 200  $\mu$ L dissolved pellets.
8. Heat samples during 10 min at 95°C.
9. Load both 10  $\mu$ L of pellets and supernatants on the 12%Bis-Tris precast SDS gel.

The protein content was quantified by measuring the band intensity using the image J software.

#### *3.3.4. Transmission electronic microscopy*

Even if there was an increase of turbidimetry, it was important to check the structure of the MTs formed from tubulin in presence of labelled Tau. At the end of the Paclitaxel or Tau induced microtubules formation experiment, collect 10  $\mu$ L of the sample (see **note 12**).

1. On a parafilm, put a 200-mesh carbon-coated formvar films on copper grids.
2. Put 3  $\mu$ L of the 10  $\mu$ L sample on the grid and incubate during 15 seconds.
4. Eliminate the excess of sample by taking the grid with tweezers and absorb sample with filter paper (apply the grid perpendicularly).
5. For staining, add 3  $\mu$ L of uranyl acetate on the grid during 30 seconds.
6. Eliminate the excess of acetate as in step 4.
7. Keep the grids in a dry area during 24h.
8. Observe the grids with a transmission electron microscope.

### 3.4. EPR spectroscopy

#### 3.4.1. Optimizing parameters of acquisition

Analysis of the EPR spectral shape recorded at room temperature gives access to the nitroxide spin probe mobility (Fig. 3A).<sup>8</sup> It is thus crucial to record EPR spectra without distorting the EPR spectral shape and with an optimized signal to noise ratio to reduce the measurement time. In practice, the parameters used for all spectra of labelled Tau at room temperature are: microwave power  $P = 10$  mW, modulation field (amplitude  $MA = 1$  G, frequency  $\nu = 100$  kHz), field sweep = 150 G, sweep time = 81.92 s, conversion time = 80 ms. If necessary, accumulation can be done to obtain a spectrum with a convenient signal to noise ratio.

#### 3.4.2. Labelling yield quantification

1. Record the EPR spectrum of a standard solution of known concentration (ideally a 100 $\mu$ M stable nitroxide radical solution) and measure the double integration of the EPR signal  $I_{std}$  (Fig. 3B). A baseline correction after the first integration is highly recommended.
2. Record the EPR spectrum of the labelled protein (Tau<sup>MTSL</sup> or Tau<sup>Proxyl</sup>) of unknown concentration (see section 3.1.2) and measure the double integration of the EPR signal  $I$ . In practice, it is better to use the same tube and the same volume than for the standard EPR tube so the EPR cavity is equally filled in. In the same way, a baseline correction after the first integration is highly recommended.
3. Spin concentration  $C_{spin}$  is obtained by calculating:

$$C_{spin} = C_{std} \frac{I}{I_{std}} \frac{MA_{std}}{MA} \sqrt{\frac{P_{std}}{P} \frac{NA_{std}}{NA}}$$

where  $I$  is the result of the double integration of the signal of the labelled protein (Fig. 3A),  $MA$  the modulation amplitude of the magnetic field,  $P$  the microwave power, and  $NA$  the number of accumulations. Note that the other experimental parameters are supposed to be equal for

the two spectra. The subscript *std* corresponds to the standard solution. Make sure that the field resolution of the spectrum is taken into account in the calculation of *I* (check in the user's manual of the spectrometer).

4. Divide the spin concentration by the protein concentration measured as described in section **3.1.2** to obtain the labelling yield. As full-length Tau has two natural cysteines, the labelling yield typically ranged from 150 to 200%, reflecting the overall good accessibility of cysteine residues.

### 3.4.3. Spectral shape analyses

#### (1) EPR spectral shape analysis of Tau<sup>Proxyl</sup>

1. Record the spectrum of Tau<sup>Proxyl</sup> (40-100  $\mu$ M) in the absence of any partner at 37°C.
2. Record the spectrum of Tau<sup>Proxyl</sup> (40-100  $\mu$ M) in the presence of tubulin dimers or Paclitaxel-stabilized MTs (see sections **3.1.1** and **3.1.2**) at 37°C. Make sure the spectral shape of the spectra did not change with time by recording EPR spectra after typically two hours.
3. Analyse the spectrum by spectral simulation using SimLabel.<sup>40,41</sup>

#### (2) EPR spectral shape analysis of Tau<sup>MTSL</sup>

1. Record the spectrum of Tau<sup>MTSL</sup> (40-100  $\mu$ M) in the absence of any partner at 37°C.
2. Record a 2D experiment (magnetic field, time) of Tau<sup>MTSL</sup> (40-100  $\mu$ M) in the presence of tubulin dimers or Paclitaxel-stabilized MTs (see sections **3.1.1** and **3.1.2**) at 37°C to follow the spectral shape modification associated to the label release. The delay between 2 spectra is 150 ms to ensure the magnetic field flyback. 20 to 100 spectra are recorded per kinetics.
3. Extract *h(-1)* and *h(0)* amplitudes over time thanks to *kinetics.m* (see **note 13**) for both experiments of Tau<sup>MTSL</sup> in the presence of tubulin dimers or Paclitaxel-stabilized MTs.

4. Plot the ratio  $h(-1)/h(0)$  as a function of time using *easyplot.m* (see **note 13**). Fit the kinetics data using the single-exponential curve  $y = y_0 + (A - y_0) * (1 - \exp(-kt))$  (red curves, Fig.5B) where  $y_0$  is the  $h(-1)/h(0)$  value at  $t = 0$  min,  $A$  is the maximum value of  $h(-1)/h(0)$  and  $k$  is the kinetic constant (in  $\text{min}^{-1}$ ) of the label release.
5. Compare the  $k$  values obtained for the two different conditions.

#### 4. NOTES

1. GTP should be freshly added to buffer to avoid hydrolysis of GTP into GDP, which is inactive for MTs formation.
2. TCEP powder is very difficult to dissolve at concentration of 1 M. TCEP is preferred to DTT to reduce disulfide bonds because it is more stable. TCEP is a useful reductant over a much wider pH range (1.5– 8.5 (8)) than DTT.<sup>48</sup> It is used to maintain Tau in its monomeric form and have no effect on MTs formation.
3. Add a pinch of azide to prevent bacteria or fungus growth. After use, unpack resin, wash ten times with ultrapure water (it can be used ten times). Wear gloves and protective clothing and work in a fume cupboard when weighing.
4. In the case of guanidine-HCl, the volume increases when it is dissolved and the reaction is endothermic. Do not put a volume of water larger than the half of the total volume.
5. This solution must be filtered just before use to remove crystals or possible pollutants which perturb coloration. To use uranyl acetate, safety conditions imply using gloves and work in a fume cupboard when weighing.
6. Tubulin is sensitive to temperature. All preparation steps must be performed at 4 °C in a cold room or using an ice box with cold buffer.
7. If you put 150  $\mu\text{L}$  of tubulin solution at 670  $\mu\text{M}$  (10 mg of protein), you must recuperate 6–7 mg into four tubes of approximately 35  $\mu\text{M}$  corresponding to the 2/3 of the amount of protein initially put on the top of the column. If you need a higher concentration of protein, increase both the volume and the tubulin solution concentration which is put on the top of the column.
8. If tubulin concentration is too high, use only 10 or 5  $\mu\text{L}$  of tubulin in 500  $\mu\text{L}$  of Guanidine-HCl and change the dilution factor to accurately determine the concentration.
9. It can happen that some free label or biradical contaminates the fractions containing the labelled protein. The resulting EPR spectrum will then be a superimposition of the signals arising from the labelled protein and the free radical or biradical. If this is the case, the contaminated fractions are discarded and not pooled with the other protein containing fractions. For more sensitive systems, one

could go further by performing the whole labelling experiment (reduction and addition of the label) into a glove box when using MTSL, avoiding formation of  $S = 1$  disulfide biradicals coming from the condensation of two MTSL labels (this reaction is not possible when using the Proxyl label).

10. In the case of Tau protein, concentrators were not used because it triggers a high loss of protein. Instead, we chose to concentrate the solution by flushing gas, in our case, Argon gas.

11. In a more general way, it is necessary to add a concentration of Paclitaxel 1.5 times higher than that of the tubulin to form MTs.

12. To maintain stable MTs, grids preparation was made in a thermostated room at 37°C. At room temperature, rings of tubulin might form.

13. GUI developed by Emilien Etienne (freely available at <https://bip.cnrs.fr/epr-facility/software-and-scripts/>) using MATLAB and some functions of the EasySpin toolbox.<sup>41</sup>

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## Figures captions

**Figure 1.** Labelling reactions and schematic representation of Tau sequence. (A) Labelling reactions of cysteines with MTSL and Proxyl spin labels, and MTSL-probe derivative release after interaction with tubuline. (B) Hatching boxes represent exons 1 and 2 in the N-terminal domain and exon 10 in the MTB domain differencing the Tau isoforms by alternative splicing of its mRNA. PR1 and PR2 boxes are proline rich regions. R1, R2, R3, R4 are the imperfect MT-binding repeats. Arrows indicate the 291 and 322 positions of the natural cysteines.

**Figure 2.** Turbidimetry time course at 350 nm to monitor the Microtubule formation at 37 °C. (A) 5  $\mu$ M of tubulin alone (a) or in presence of 5  $\mu$ M of Tau in phosphate buffer 20 mM, GTP 0.1 mM, pH 6.5, 1 mM TCEP (b) or 5  $\mu$ M of Tau<sup>MTSL</sup> (c) or 5  $\mu$ M Tau<sup>Proxyl</sup> (d) in phosphate buffer 20 mM, GTP 0.1 mM, pH 6.5. (B) 20  $\mu$ M of tubuline alone (f) and 5  $\mu$ M Tau<sup>Proxyl</sup> (e). The Microtubule formation is induced by adding the different Tau samples (arrow). Electronic micrographs of the Microtubules formed in each condition are also represented.

**Figure 3.** (A) EPR spectrum of a nitroxide radical grafted on a protein and its absorption form after first integration. The filled blue surface represents the integrated intensity that is proportional to spin concentration. (B) EPR spectral shape modifications as a function of the mobility of the spin label described by its rotational correlation time  $\tau_c$ . The spectra have been simulated using SimLabel<sup>40,41</sup> for different values of  $\tau_c$ : 0.01 ns (spectrum a), 1 ns (spectrum b), 10 ns (spectrum c) and 100 ns (spectrum d).

**Figure 4.** (A) EPR spectra (black) of Tau<sup>Proxyl</sup> alone (10  $\mu$ M), in presence of 20  $\mu$ M tubulin and in presence of 20  $\mu$ M Paclitaxel-stabilized MTs superimposed with the simulated spectra (red) using SimLabel.<sup>40,41</sup> (B) Values of  $\tau_c$  with proportions (%) of each component for Tau<sup>Proxyl</sup> alone, in presence of 20  $\mu$ M tubulin and in presence of 20  $\mu$ M Paclitaxel-stabilized MTs. The higher is the  $\tau_c$  the more restricted is the mobility of the spin label. Buffer: phosphate buffer 20 mM pH 6.5, GTP 0.1 mM. Temperature: 37 °C.

**Figure 5.** (A) EPR spectrum of Tau<sup>MTSL</sup> alone and the illustration of  $h(+1)$ ,  $h(0)$  and  $h(-1)$  peak-to-peak amplitudes of each line. (B)  $h(-1)/h(0)$  ratio as a function of time of a 1:2 molar ratio of Tau<sup>MTSL</sup>: tubulin (■), of a 1:2 molar ratio Tau<sup>MTSL</sup>: Paclitaxel-stabilized MTs (o) and of Tau<sup>MTSL</sup> alone (▼). Data were fitted using the single-exponential curve  $y = y_0 + (A - y_0) \cdot (1 - \exp(-kt))$  (red curves). Tau<sup>MTSL</sup> concentration was 10  $\mu$ M. Buffer: phosphate buffer 20 mM pH 6.5, GTP 0.1 mM. Temperature: 37 °C.

**Figure 6.** Model of Tau:tubulin interaction involving the Tau R2 repeat (C291) with the  $\alpha$ -subunit of tubulin at Site 1 involving the region of  $\alpha$ -C347 and the Tau R3 repeat (C322) with the  $\beta$ -subunit of

tubulin at Site 2 involving  $\beta$ -C131 and  $\beta$ -C129 in the 3j6f structure (Cys 129 and Cys 127 in the amino acid sequence).<sup>22</sup>