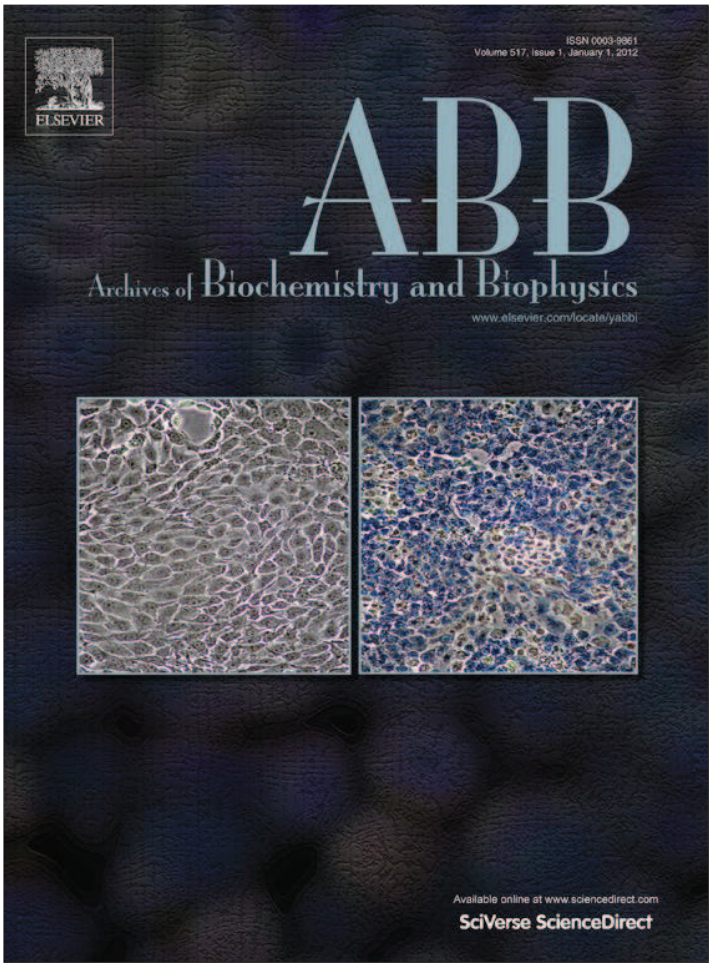

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Spin probe analysis of microtubules structure and formation

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ABSTRACT

Microtubules (MTs) control cell replication, material transport and motion in eukaryotic cells, but MT role in several pathologies is still unknown. These functions are related to the MT physico-chemical properties and MT formation mode starting from tubulin molecules. This study describes a new method, based on the computer aided analysis of the electron paramagnetic resonance (EPR) spectra of selected spin probes to obtain structural and dynamical information on tubulins and MTs and the kinetics of MTs formation promoted by guanosine-5′-triphosphate (GTP). It was found that tubulin and MTs avoid radical quenching caused by ethylene glycol tetraacetic acid (EGTA). MT formation showed different kinetics as a function of tubulin concentration. At 5 mg/mL of tubulin, MTs were formed in 8 min. These results are also useful for getting information on MT–drug interactions.

Introduction

Microtubules (MTs)[1] are protein polymers that characterize the cytoskeleton of all eukaryotic cells and which are essential for controlling various cellular functions such as transport of materials inside the cell, the movement of organelles or cytoplasmic vesicles and cell division [1].

MTs are extremely important in the process of mitosis, during which the duplicated chromosomes of a cell are separated into two identical sets before cleavage into two daughter cells. Their importance in mitosis and cell division makes MTs an important target for anticancer drugs. In addition to this important function, their involvement in learning and memory was also demonstrated. The MTs shape and unique architecture in the neurons are responsible for the regulation of neuronal morphology through changes in synaptic structure and transport of essential components for synaptic function. MTs are capable of enormous computational complexity because of the numerous post-translational modifications of its constituent tubulin proteins [2–4].

The structural components of MTs are tubulins, heterodimeric proteins of 110 kDa, formed by association of two homologous subunits known as α and β-tubulin, encoded by separate genes. The structure of α- and β-tubulin dimer is shown in Fig. 1.

Although the association between the two subunits is due solely to non-covalent interactions, the term "tubulin" refers to heterodimer α,β which is usually considered as a unit [5].

The tubulins make MTs dynamic structures that allows the cell to perform many functions through a process of polymerization/depolymerization.

Tubulin polymerization depends on various physical and chemical factors. Under normal conditions, tubulin assembly depends on GTP binding. As shown in Fig. 1, each tubulin dimer can bind two GTP molecules, one at a non-exchangeable site on the α monomer, another at an exchangeable site on the β monomer at which GTP can hydrolyze to guanosine-diphosphate (GDP), imparting energy and conformational flexing to the underlying tubulin. During assembly of the MTs, the exposed tubulin binds GTP and forms a temporary cap that prevents disassembly. On the other hand, GTP will undergo hydrolysis within a brief time if not covered by another tubulin. Thus, MTs, which continue to grow and add GTP tubulin, are stable. However, if the assembly is stalled, the terminal GTP undergoes hydrolysis and triggers rapid disassembly (called 'MT catastrophes').

The dynamic nature makes MTs sensitive to several pharmacological agents, i.e. some classes of anticancer agents that are able to destroy or stabilize their structure [6–10]. The understanding of MT formation process, structure and properties is important for

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[1] *Abbreviations used:* EPR, electron paramagnetic resonance; MT, microtubules; CAT8, 4-octyl-dimethylammonium-2,2,6,6-tetramethyl-piperidine-1oxyl bromide; Tempo, 2,2,6,6-tetramethyl-piperidine-1oxyl; Tempol, 4 hydroxy-2,2,6,6-tetramethyl-piperidine-1oxyl; CAT1, 4-trimethyl ammonium-2,2,6,6-tetramethyl-piperidine-1oxyl bromide; DC, doxylcholestane; 5DSA, 5 doxylstearic acid; GTP, guanosine-5′-triphosphate; GDP, guanosine-diphosphate; EGTA, ethylene glycol tetraacetic acid; PIPES, 1,4-piperazinediethanesulfonic acid.

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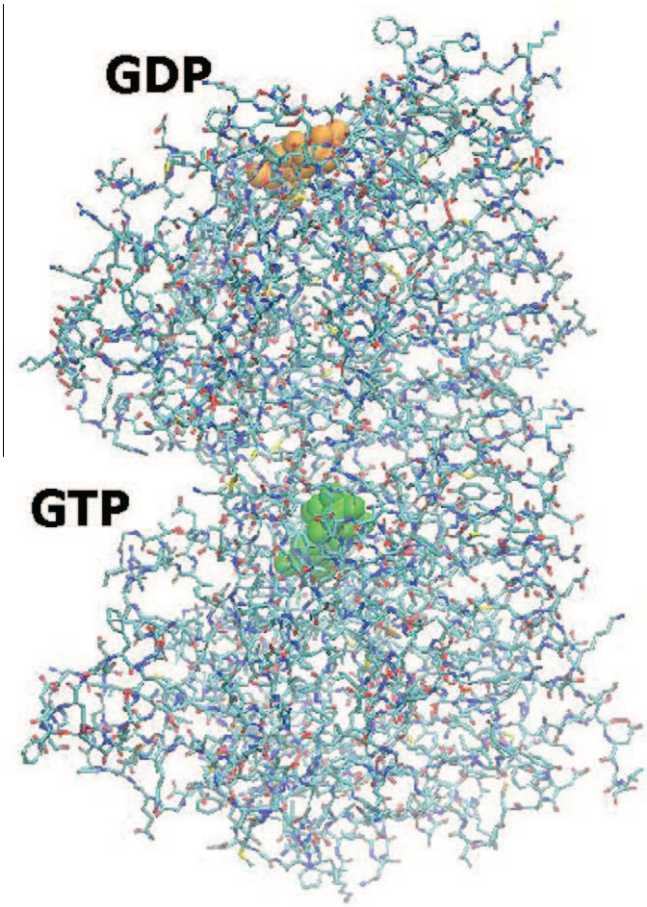

Fig. 1. α- and β-tubulin dimer after GTP hydrolysis. α-tubulin contains the non-hydrolyzable GTP (in green) while β-tubulin contains the hydrolyzed GTP in GDP (orange). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

characterizing the MT binding sites and interaction modes towards drugs and various biomolecules, as the so-called MT associated proteins (MAPs), which also work as protecting agents for MT depolymerization [11–13].

In the last decade, many theories and papers have been published concerning the biophysical role of MTs, including the hypothesis of MTs implication in coherent quantum states in the brain evolving in some form of energy and information transfer [14]. The most discussed theory on quantum effects involving MTs has been proposed by Hameroff and Penrose that published the OrchOR Model in 1996 and revisited it in 2011 [15,16]. Pathologic implications of MTs network have been also hypothesized by Cocchi and coworkers [17].

The relevance of MTs in biological and biomedical fields requires a deeper understanding of the formation mechanism and of the processes involving MTs. In this contest, electron paramagnetic resonance (EPR) has revealed to be a very fruitful technique in providing structural and dynamical information on biomolecular structures and their mutual interactions [18–23]. Both spin probes and spin labels may be used to gain information about the biosystem. Spin labelling has a lot of limitations and contraindications, mainly due to (a) the perturbative effect that the label may play because it permanently modifies the structure and the properties of the molecule to which it is attached; (b) the location of the label in a given position, which therefore only monitors the environmental modifications at that site and prevents site action, if the site is the active one. Nonetheless, previous studies by our group demonstrated the usefulness of selected spin probes to analyze similar complex systems [18–20], demonstrating the validity of the spin-probe method to provide specific and unique information about the system itself, which are mainly significant in a comparative way. Furthermore, a spin probe may be selected to mimic a biochemical structure or biomolecule which is relevant in the system under study, or is known to interact with the studied system.

In this paper we describe the EPR spin probe analysis of tubulin both in absence and presence of GTP as polymerization promoter. The kinetics of MTs polymerization was also followed by EPR. We are confident that these results make it possible to extend the study to more complex systems, where the MTs interact with drugs.

Materials and methods

EPR probes and their conditions of use

2,2,6,6-Tetramethyl-piperidine-1oxyl (Tempo), 4 hydroxy-Tempo (Tempol), 4-trimethyl ammonium Tempo bromide (CAT1), doxylcholestane (DC) and 5 doxylstearic acid (5DSA) were purchased from Sigma–Aldrich and used as received. 4-Octyl-dimethylammonium Tempo bromide (CAT8) was kindly provided by Dr. X. Lei, Columbia University, New York. The hydrophilic probes were directly solved in double distilled (Millipore) water both in absence and presence of the other ingredients, at a starting concentration of 1 mM (dilution experiments were first tried, as described thereafter). The solutions were stable for days if stored at 4 °C, but a blank spectrum was always performed before using them to verify the reproducibility. Conversely, the hydrophobic probes were first solved in chloroform (Sigma) at initial concentration of 1 mM. The portion of the solution (eventually diluted) containing the needed amount of probe for each experiment was then transferred into a vial, the solvent evaporated and the water solution, containing the other ingredients needed for the experiment, eventually added and left equilibrating under stirring for 1 h before using it in the EPR experiment.

EPR experiments by using the commercial tubulin polymerization assay kit

The tubulin polymerization assay kit was purchased from Cytoskeleton company and contains pure Porcine tubulin (>99%), the "general tubulin buffer" (80 mM PIPES – pH 6.9; 2 mM $MgCl_2$; 0.5 mM EGTA) and guanosine-5′-triphosphate (GTP). A stock solution of GTP (100 mM) is added to the general tubuline buffer to obtain a final concentration of 1 mM. This solution is used within 4 h and stored at 0 °C. Each of the different probes was added to this solution before addition of the tubulin and blank spectra were runned. Then, tubulin was added at different concentrations (from 1 to 10 mg/mL) and the EPR experiments were repeated in the same experimental conditions.

EPR experiments by using the different ingredients of the polymerization assay kit

PIPES, $MgCl_2$ and EGTA were purchased from Sigma–Aldrich and used as received. A Millipore water solution of each of these ingredients and each of the probes (0.1 mM) were prepared and tested by EPR. Similarly, tubulin and GTP (from the tubulin polymerization assay kit) were separately tested by EPR with the probes at the concentrations used in the experiments using the commercial tubulin polymerization assay kit. Similarly, the different mixtures of the various ingredients were tested by EPR.

Kinetics of formation of MTs

The kinetics of formation of MTs was monitored by using Millipore water solutions of CAT8 at concentration of 0.1 mM containing 80 mM PIPES (pH 6.9), and 2 mM $MgCl_2$. A first experiment was performed by adding tubulin at two concentrations (1 and 5 mg/mL) and following the spectral variations over time. Then, the same experiment was repeated in the same experimental conditions by also adding GTP to promote tubulin polymerization. The spectra were recorded over time at a selected temperature (see thereafter).

Method

EPR spectra were recorded by means of a EMX-Bruker spectrometer operating at X band (9.5 GHz) and interfaced with a PC (software from Bruker for handling and recording the EPR spectra). The temperature was controlled with a Bruker ST3000 variable-temperature assembly cooled with liquid nitrogen. The reproducibility was verified by repeating each experiment at least three different times.

Optimization of the experimental conditions to analyze MT formation

Selection of the spin probe

Several different spin probes were used to select the one which better reports on the structural variations and interactions occurring in tubulin and MT solutions.

Among the commercially available hydrophobic probes, DC and 5DSA showed a non negligible solubility in tubulin solutions, but the spectra were poorly informative. Among the hydrophilic probes, Tempo, Tempol and CAT1 were soluble in tubulin and MT solutions, but showed limited spectral variations in the different experimental conditions we tried. Conversely, CAT8 showed interesting spectral variations by changing the experimental conditions for the tubulin and MT solutions, as expected on the basis of previous studies using this probe [19]. Therefore, CAT8 was selected as a probe for studying MT formation. Anyway, it is important to verify the absence of perturbations played by the probe on the system under study, and in case perturbations occur, it is critical to know the extent of these perturbations, and identify, or, at least, propose the interacting site of the probe if it is mimicking a drug or a biomolecule. Consequently, the concentration of CAT8 was changed in different systems before starting with the EPR analysis to verify if different amounts of the probe affect the EPR response. We verified that concentrations of the probe $\leqslant$ 1 mM did not show any significant spectral differences; therefore these concentrations could be trusted for experiments where CAT8 is used to probe the molecular interactions in solution.

Selection of the tubulin concentration

In the usual "general tubulin buffer" (80 mM PIPES – pH 6.9; 2 mM di $MgCl_2$; 0,5 mM EGTA), both in the absence and in the presence of GTP (1 mM), and in the absence of tubulin, the EPR intensity (calculated by double integration of the EPR signal) of CAT8 (1 mM) almost goes to zero (Figure S1(a): Supplementary Data). However, if tubulin is added to the buffer solution at a concentration of 1 mg/mL, the EPR intensity is largely recovered. As shown in Figure S1(a), the EPR spectrum of CAT8 in the general tubulin buffer solution in the presence of 1 mg/mL of tubulin at 298 K is the usual three-lines spectrum of CAT8 in free conditions in solution; the three-lines spectrum arises from the hyperfine coupling of the electron spin with the nitrogen nuclear spin ($I = 1$, No. of lines = $2I + 1$). Therefore, if the strong decrease in intensity is due to a "radical quenching" effect of the buffer, the intensity recover is by itself a demonstration of the interactions of tubulin with the probe and/or the radical quencher in solution. Therefore the tubulin extracts the probe from this deleterious buffer component.

Interestingly, by adding increasing concentrations of tubulin, the intensity remains constantly high for concentrations between 1 and 5 mg/mL (Figure S1(b): Supplementary Data). At higher concentrations, the intensity decreases, probably due to phase separation of the polymerized tubulin. Therefore, concentrations of tubulin of 1 and 5 mg/mL were used.

Selection of the temperature conditions

As already found for other biological systems studied by EPR analysis [18–22], the room temperature spectra were poorly informative, because the probe is fast moving and fast exchanging at the protein/bulk interphase and the fast motion spectrum of the probe in water solution is dominating and almost only contributing to the EPR signal at room temperature. The way to solve such a problem is to decrease the temperature, that causes the decrease in the exchange rate and consequently allows to monitor by EPR the structural modifications of the probe environment. However, it is not convenient to decrease the temperature progressively, because molecular structure and intermolecular interactions may change with respect to those occurring at room or physiological temperature. We found suitable to the analysis to put the samples directly in the EPR cavity at a temperature which shows slow motion conditions in the EPR time scale, that is, a rotational mobility, measured by the correlation time for the rotational diffusion motion, higher than 1 ns. The temperature of 255 K demonstrated to well characterize the interactions occurring in solution. Several other temperatures were tried. In most cases, higher temperatures gave rise to fast motion spectra with no spectral modifications among the various samples (therefore not shown), whereas lower temperatures gave rise to phase separation in the system. To analyze the kinetics of MT formation, the samples were taken at subsequent times from the mixture that was left equilibrating at 310 K and directly inserted into the EPR cavity thermostatized at 255 K. We already verified in other studies the reliability of this method, as proved by integrating the EPR analysis with other techniques [19,20,22].

Blank spectra and selection of the buffer ingredients

The EPR experimental spectra at 255 K obtained for the CAT8 probe in the absence of tubulin in water and after progressive addition of each of the general tubulin buffer ingredients and, finally, of GTP are shown in Figure S2 (Supplementary Data).

The EPR spectra were computed (red lines in Figure S2) using the well established procedure by Budil and Freed [24]. The computation allowed us to extract the following main magnetic and mobility parameters: (a) the g_{ii} main components of the tensor for the coupling between the electron spin and the magnetic field (if not differently specified, these parameters were assumed equal to 2.009, 2.006, 2.003, as already used in previous studies with the same probe) [18]; (b) the A_{ii} main components of the tensor for the hyperfine coupling between the unpaired electron spin and the nitrogen nuclear spin ($I_N = 1$) (the increase of these components corresponds to an increase in the environmental polarity of the probes). The accuracy in the A_{ii} parameters in the spectral computation was ±0.01 G. For simplicity, we assumed $A_{xx} = A_{yy} = 6$ G and therefore only A_{zz} was considered a variable parameter. But, if the fitting between experimental and computed spectra was not obtained with those constant A_{xx} and A_{yy} values, it became convenient for getting information about the polarity variation, to compare the average A_N value, given by $\langle A_N \rangle = (A_{xx} + A_{yy} + A_{zz})/3$; (c) the correlation time for the rotational diffusion motion, τ (the increase of this parameter means an increased strength of interaction of the probe with its environment). We assumed either a Brownian or a Jump rotational diffusional motion, with $\tau = 1/(6D)$

Table 1
Polarity (A_{zz} and $\langle A \rangle$) and mobility (τ) parameters extracted from the computation of the EPR spectra at $T = 255$ K of "blank" samples (without tubulin), and samples containing tubulin at concentrations of 1 and 5 mg/mL.

Sample	A_{zz}/G	$\langle A \rangle/G$	τ/ns
Blank: water	38.40	16.80	0.15
Blank: $MgCl_2$	38.40	16.80	0.15
Blank: PIPES	38.45	16.81	0.24
Blank: EGTA	Intensity 0		
Blank: PIPES + $MgCl_2$	38.22	16.75	1.83
Blank: PIPES + $MgCl_2$ + EGTA	Intensity 0		
Blank: PIPES + $MgCl_2$ + GTP	38.47	16.82	1.62
Blank:PIPES + $MgCl_2$ + EGTA + GTP	Intensity 0		
PIPES + $MgCl_2$ + EGTA + GTP tubulin 1 mg/ml	38.50	16.83	1.85
PIPES + $MgCl_2$ + EGTA + GTP tubulin 5 mg/ml	39.00	17.00	2.35
PIPES + $MgCl_2$ tubulin 1 mg/ml	37.70	16.80	7.08
PIPES + $MgCl_2$ tubulin 5 mg/ml	38.70	16.90	5.25
PIPES + $MgCl_2$ + GTP tubulin 1 mg/ml	38.00	16.90	6.45
PIPES + $MgCl_2$ + GTP tubulin 5 mg/ml; $t > 8$ min	38.50	17.30	6.50
PIPES + $MgCl_2$ + GTP tubulin 5 mg/ml; $t = 0$ min slow component more 'fluid' component	38.50	17.30	6.20
	38.00	16.65	2.65

and $\tau = 1/D$, respectively, where D is the diffusion coefficient. The accuracy of this parameter in the spectral computation was ±0.01 ns.

It is interesting to describe the information gained from the main parameters obtained from the computations of the "blank-samples" spectra, which are reported in Table 1, together with the main parameters obtained for all samples in the present study.

The environmental polarity of the probe (measured by $\langle A \rangle$) slightly increases by adding PIPES, but then the addition of $MgCl_2$ decreases both the polarity and the mobility (measured by τ). This result may be related to two synergistic effects: (a) the repulsion played by the salt on the hydrophobic (lipidic) part of the probe which better associates with the low polar moiety of PIPES; (b) the PIPES-Mg^{2+} interaction/complexation which also neutralizes the PIPES charges and favours the interactions of the probe with the complex itself. The addition of GTP turns back to a slight increase in polarity and mobility because GTP interacts with the buffer components and with their adducts in solution and, therefore it competes with CAT8 for these interactions. In any case, on the basis of the EPR analysis, CAT8 interactions are weak, mainly dipole–dipole or ion–dipole with a synergistic effect of hydrophobic interactions played by the CAT8 chain.

The parameters obtained by also adding EGTA are not reported in Table 1, because the intensity of the EPR spectra was almost zero. This demonstrated that EGTA has a "quenching" effect towards CAT8. However, EGTA is not able to work as an antioxidant, that is, to reduce CAT8 to the parent amino derivative. Conversely, the chemical structure of EGTA well justifies the formation of chelate complexes in which EGTA is able to bind two (or more) CAT8 radicals due to a synergic effect of hydrophylic interactions (between the CAT group and the ═N—COOH groups) and hydrophobic interactions (among the C8 chains of the radicals and the etheric chain of EGTA); the radicals in dimeric forms are EPR silent due to strong spin–spin exchange interactions. So, EGTA works as a radical scavenger or radical quencher by forming chelate complexes with CAT8. As described above, tubulin is able to avoid such an effect by impeding CAT8-EGTA interactions, thus recovering the EPR intensity. In any case, it resulted interesting to compare the MT formation in the absence and in the presence of EGTA in the buffer, as it will be described in the Results and discussion section.

Results and discussion

Analysis of MT formation by using the tubulin polymerization assay kit

The tubulin polymerization assay kit contains the general tubulin buffer, in which, as described above, the antioxidant effect of

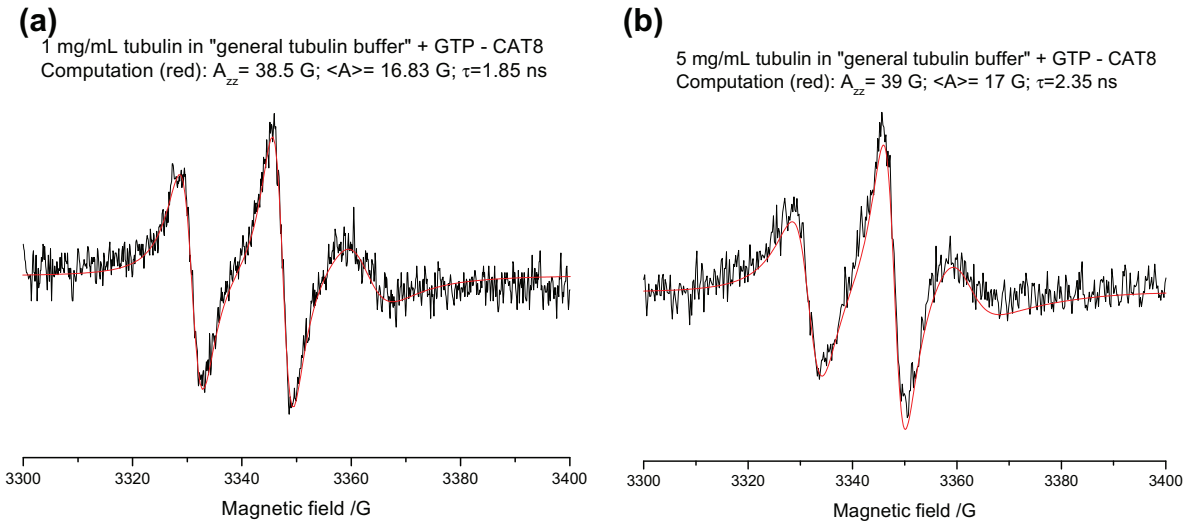

Fig. 2. Experimental and computed spectra of CAT8 obtained for the general tubulin buffer + GTP in the presence of 1 mg/mL (a) and 5 mg/mL (b) of tubulin. The main parameters of computation are also reported in the figure.

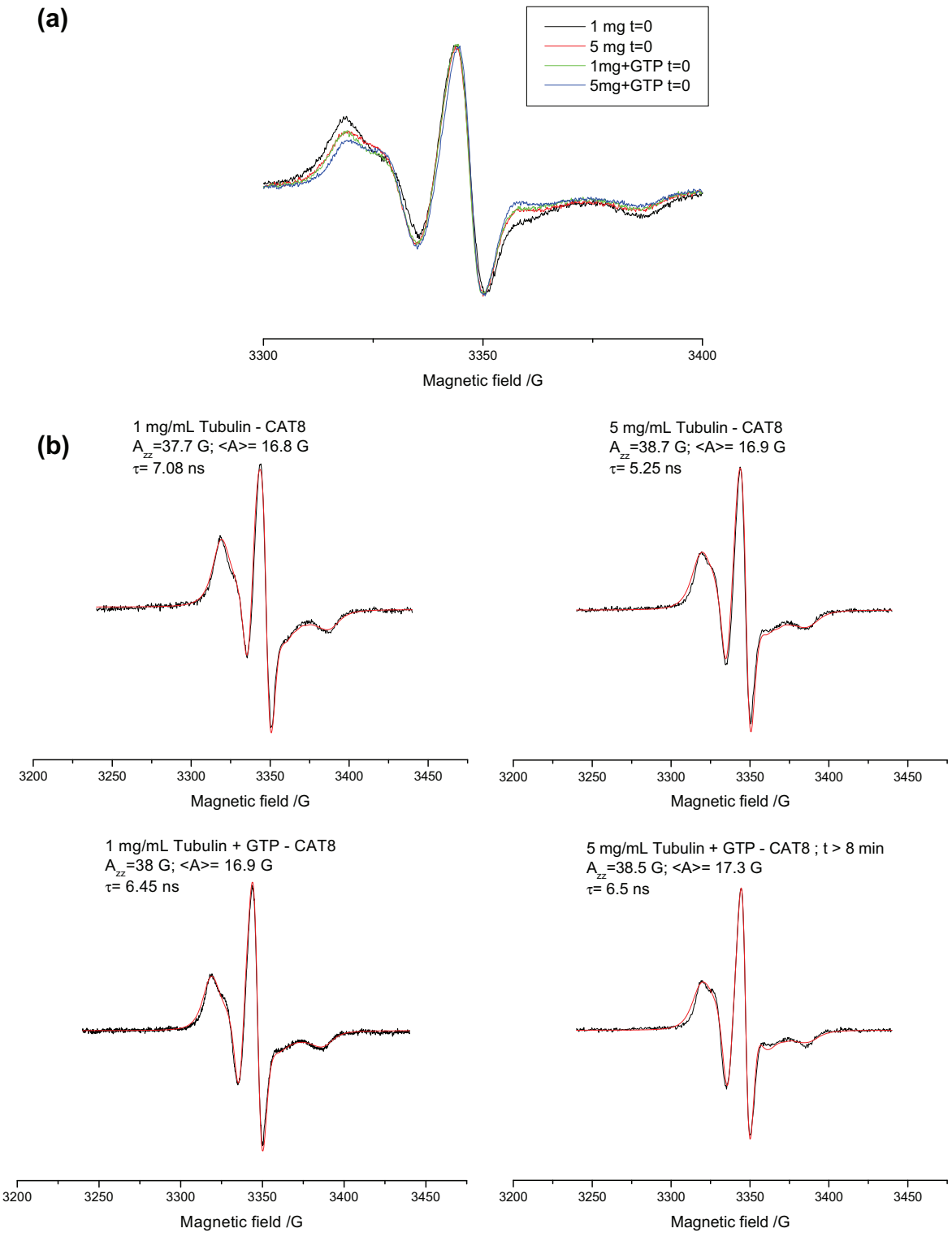

Fig. 3. (a) Comparison among the experimental EPR spectra of CAT8 at 255 K obtained in the four different experimental conditions (PIPES and $MgCl_2$ buffer, with and without GTP, with 1 or 5 mg/mL of tubulin); (b) experimental and computed spectra of CAT8 in PIPES and $MgCl_2$ buffer in the presence of 1 mg/mL or 5 mg/mL of tubulin, and in the absence and presence of GTP. The main parameters of computation are also reported in the figure.

EGTA degrades the radical preventing the EPR analysis. The addition of GTP does not modify the situation. However, addition of tubulin recovers the EPR signal, whose computer aided analysis provides some interesting structural and dynamical information on the newly formed MTs. Fig. 2 shows the experimental and computed spectra of CAT8 obtained for the general tubulin buffer + GTP in the presence of 1 mg/mL (a) and 5 mg/mL (b) of tubulin (T = 255 K).

The main parameters obtained from computation are shown in Fig. 2 and also listed in Table 1. From these spectra and parameters it is clear that CAT8 probe monitors the formation of MTs by showing an increase in environmental polarity and a decrease in

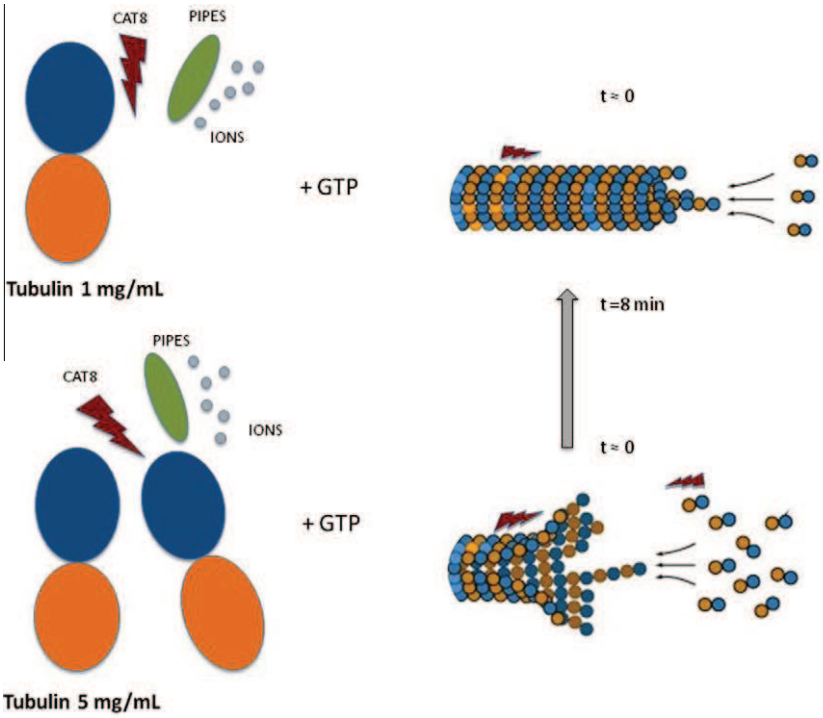

Fig. 4. Scheme showing the proposed interactions occurring in the tubulin solutions (PIPES and $MgCl_2$ buffer) at two concentrations (1 and 5 mg/mL) in the absence and presence of GTP, as inferred by the EPR computer aided analysis of CAT8 spectra.

mobility with respect to those measured for the "blank" system only containing PIPES + $MgCl_2$ + GTP. Therefore the probe transfers from the buffer solution to the MT surface, thus following the structural and mobility variations occurring in the system when the MTs are formed. Furthermore, the polarity and mobility variations as a function of tubulin concentration provide a direct measure of the MTs formed in solution. However, since the general tubulin buffer also contains EGTA which "quenches" the spin probe, we could not compare the EPR parameters of the mixtures of PIPES + $MgCl_2$ + EGTA + GTP in the absence and in the presence of MTs and eventually follow the MT formation from tubulin due to GTP addition. Also, we need a more accurate comparison of spectra in different experimental conditions in the presence of tubulin to eventually identify the CAT8-tubulin binding sites with the purpose of using this spin probe to mimic drug interactions. For these reasons, we repeated the EPR experiments by adding tubulin to the buffer only containing PIPES + $MgCl_2$, as described in the next section.

Analysis of tubulin solutions in the absence and presence of GTP: kinetics of MT formation

The buffer only containing PIPES and $MgCl_2$ allowed us to perform a comparative analysis of the kinetics over time in the absence and in the presence of GTP (which promotes polymerization of tubulin) by adding tubulin at two concentrations, that is, 1 and 5 mg/mL. Fig. 3(a) shows the experimental EPR spectra of CAT8 at 255 K obtained in the four different experimental conditions (with and without GTP, with 1 or 5 mg/mL of tubulin). The samples were immediately inserted in the EPR cavity at 255 K after addition of tubulin: this time is considered $t = 0$. The time for recording the spectra was always the same: 30 s for the temperature equilibration and 10 s for the magnetic field scan. From the simple visual inspection of the spectra it resulted clear that the spin probe well differentiate among the four different systems.

The computation of the spectra, shown in Fig. 3(b), provides deeper information, by means of the parameters extracted from computation and reported in both Fig. 3(b) and Table 1.

First, the $\langle A \rangle$ parameters indicate that an increase in tubulin concentration leads to an increase in polarity, as already found when EGTA is present in the buffer; however, in the absence of GTP the mobility also increases (decrease of τ) by increasing tubulin concentration. This effect seems strange because an increase in concentration usually determines an increase in viscosity which corresponds to a decrease in probe mobility; but, this increase in probe mobility may be rationalized considering that tubulin at the higher concentration perturbs the CAT8 binding mode and location.

This effect is sketched in Fig. 4. This is nicely in line with what we see by adding GTP. Indeed, the presence of GTP increases the polarity, but, regarding mobility, the trends are the opposite at the two tubulin concentrations: at 1 mg/mL, the mobility increases from the absence ($\tau = 7.08$ ns) to the presence ($\tau = 6.45$ ns) of GTP; conversely, at 5 mg/mL of tubulin, the mobility decreases from the absence ($\tau = 5.27$ ns) to the presence ($\tau = 6.20$ ns) of GTP. Therefore, at 1 mg/mL the formation of MTs after GTP addition determines a partial mobilization of the probe, whereas, at 5 mg/mL, the starting location of CAT8 is in a more mobile situation, and, therefore, the formation of MTs due to GTP provokes a relative immobilization of the probe. However, the situation at 5 mg/mL is more interesting, as described below and sketched in Fig. 4.

The computation of the spectra in the presence of GTP at $t = 0$–8 min and [tubulin] = 5 mg/mL was more complicated because the spectra were constituted by two superimposed signals arising from not exchangeable probes (in the EPR time scale) in two different environments. Usually, subtraction of experimental spectra, which contain the signals at different relative intensities allows to extract the two signals, calculate their relative intensities by double integration, and compute each of them.

However, one of the component, which is the only one at $t > 8$ min, is changing over time from $t = 0$ to $t = 8$ min. Therefore,

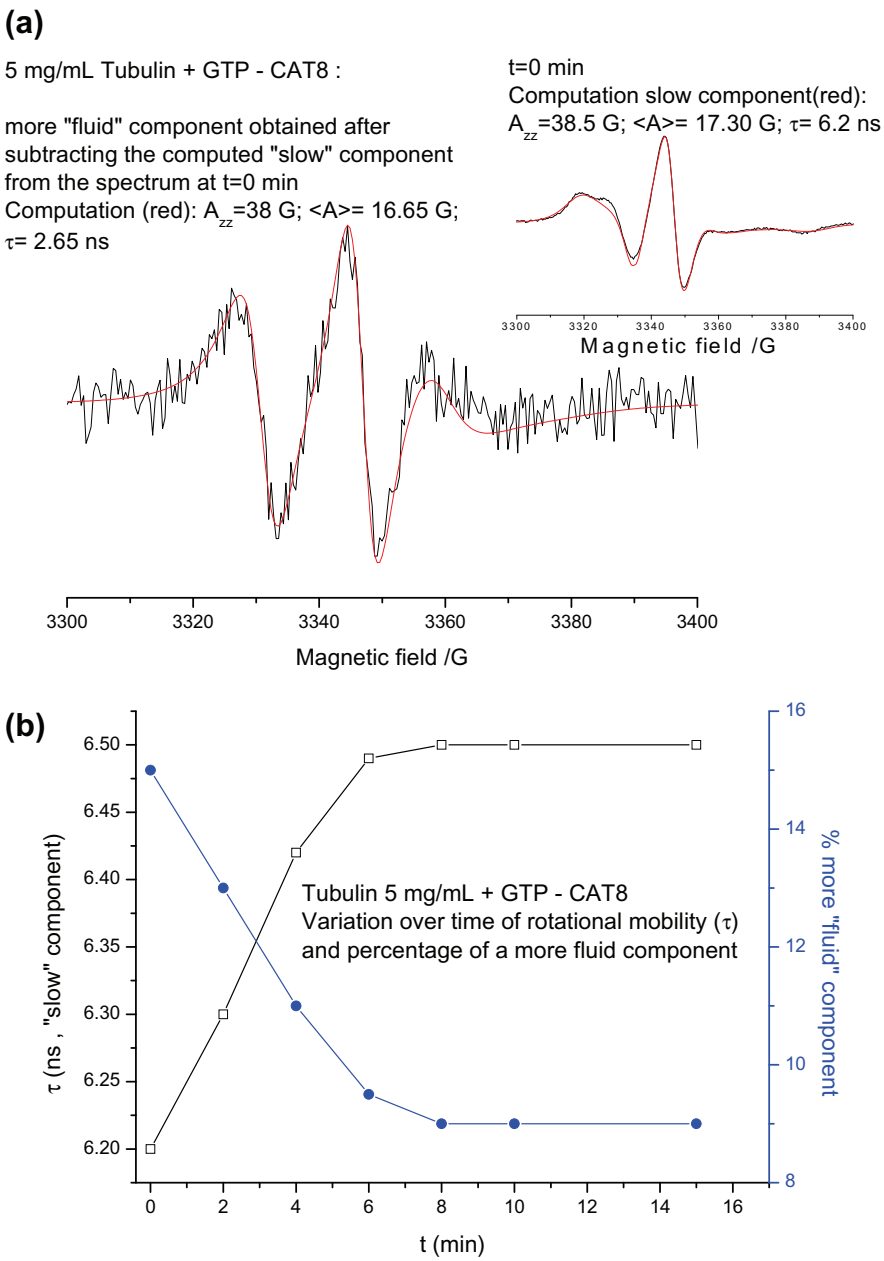

Fig. 5. (a) Computation of the "faster" component obtained by subtracting the computed "slow" component from the experimental spectrum of CAT8 in PIPES + $MgCl_2$, in the presence of GTP and (tubulin) = 5 mg/mL at $t = 0$ and $T = 255$ K. The main parameters used for computation are shown in the figure; (b) variation over time of the correlation time for the rotational diffusion motion (measuring the microviscosity) of the "slow" component, and of the relative percentage of the more "fluid" component: these two components constitute the spectrum of CAT8 in PIPES + $MgCl_2$, in the presence of GTP and (tubulin) = 5 mg/mL at $T = 255$ K.

first, we computed the main component at $t = 0$ (insert in Fig. 5a); then, this computed component was subtracted from the $t = 0$ spectrum to extract the second component, which was computed as shown in Fig. 5a. The first main component was named "slow", because the correlation time is characteristic of slow motion probes, due to their interactions with MT surface (Fig. 4). The second further component, on the basis of the parameters obtained from computation (Table 1), is relatively more mobile (corresponding to a more "fluid" environment, showing $\tau = 2.65$ ns) with respect to the "slow" main component (showing $\tau = 6.20$ ns). Interestingly, in the time range from $t = 0$ min to $t = 8$ min two spectral modifications occurred: (1) the mobility of the "slow" component decreased, and (2) the relative percentage of the more "fluid" component decreased. Both these trends are displayed in Fig. 5b. We interpreted these results on the basis of the formation of MTs whose kinetics becomes measurable by EPR in these experimental conditions. This means that the tubulin molecules at a higher local concentration slow down the polymerization process. Therefore the formation of MTs at 5 mg/mL of tubulin may be followed by EPR because all radicals show a progressive quenching in their mobility while MTs are assembled, and the portion of radicals in a relatively more fluid condition is also progressively trapped into the newly formed MTs. After 8 min, the spectrum for the sample at 5 mg/mL becomes similar to the one found for 1 mg/mL at $t = 0$ compared to a negligible presence of the more fluid component. However, the mobility is lower and the polarity is higher for the 5 mg/mL sample with respect to the 1 mg/mL one, in agreement with the results found by using the general tubulin buffer. The proposed pathway of MTs formation for the two tubulin concentrations is sketched in Fig. 4.

A further concern about the effect of EGTA may be discussed by comparing the results for MT solutions obtained by using the general tubulin buffer (containing EGTA) and the buffer without EGTA (Table 1, and Figs. 2 and 3, respectively). It resulted that EGTA significantly increases CAT8 mobility which means that the probe poorly interacts with MTs. This information is not useless, if we consider that this probe mimics well the behaviour of several drug or biologically interesting molecules, for the presence of hydrophilic, hydrophobic and charged groups. In this respect, it is relevant to eventually identify the interacting sites of CAT8 at the tubulin surface. The main advantage of CAT8-tubulin interaction is its dynamic and therefore not perturbative interaction (for this reason it is necessary to perform the EPR measurements at 255 K to slow down the exchange of the spin probe between the binding site and the bulk solution). Therefore the binding mode is of the ion–dipole + hydrophobic interactions type. Tubulin structure is well known [25], and, on the basis of this structure, the binding sites for drugs and biomolecules structurally similar to CAT8 may be identified. The tubulin dimer is known to bind one nonexchangeable and one exchangeable GTP. The binding and hydrolysis of exchangeable GTP regulates microtubule assembly. Three other important binding domains have been identified [26], namely the taxanes (paclitaxel, docetaxel), vinca alkaloids (vincristine, vinblastine, vinorelbine) and podophyllotoxins/colchicines. Taxol binds to the polymerized dimer, but in the lateral contact site. Vinblastine binds at the polar, opposite side of β-tubulin at the plus-end interface, adjacent to the hydrolysable GTP site, whereas colchicine binds at the inter-dimeric interface between the α–β-tubulin dimers. Furthermore binding of vinblastine induces a conformational change in tubulin in connection with tubulin self-association [26]. CAT8 is an amphiphilic molecule with a small ring as a polar head, such kind of structure could bind to one of each already known binding sites, however, on the basis of the EPR results, we assume it binds at the plus-end interface nearby the GTP interacting sites and therefore in a critical position for monitoring the structural–dynamical modification of tubulin when it is polymerizing.

Conclusions

This study reports about the polymerization of tubulin to form MTs promoted by GTP, studied by means of a computer aided EPR analysis of a selected spin probe, termed CAT8.

First, to optimize the experimental conditions needed to obtain structural and dynamical information on MT solution in the general tubulin buffer, the different ingredients of the buffer were separately added to the probe solution. EGTA worked as radical scavenger, but tubulin in the concentration range between 1 and 5 mg/mL prevented the radical scavenging effect of EGTA. The probe localized at the tubulin or MT/water interphases forming ion–dipole bonds with polar sites with a synergistic effect of hydrophobic interactions due to the presence of the C8 chain.

MT formation follows different kinetics as a function of tubulin concentration. At low tubulin concentration (1 mg/mL), MTs are immediately formed after GTP addition and the probe more strongly interacts with tubulins than with MTs. At 5 mg/mL of tubulin, MTs are formed in 8 min after GTP addition because the mobility progressively decreases, and, also, a fraction of more mobile probes almost disappears in that time.

The results obtained in this work are useful for understanding physico-chemical properties of tubulin and MT buffer solutions and provides a method to follow the formation of these aggregates in different experimental conditions. Furthermore, these results provide a set of physical measurements on samples of MT and tubulin in the presence of a spin probe which well mimics the drugs and biomolecules of medical and pharmacological interest which are already known to interact with MTs. The possibility to investigate and therefore clarify the role of MTs in drug curing efficacy is really important in pharmacological and medical studies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.abb.2012.04.002.

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