Reversible and Irreversible Modulation of Tubulin Self-Assembly by Intense Nanosecond Pulsed Electric Fields

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Tubulin self-assembly into microtubules is a fascinating natural phenomenon. Its importance is not just crucial for functional and structural biological processes, but it also serves as an inspiration for synthetic nanomaterial innovations. The modulation of the tubulin self-assembly process without introducing additional chemical inhibitors/promoters or stabilizers has remained an elusive process. This work reports a versatile and vigorous strategy for controlling tubulin self-assembly by nanosecond electropulses (nsEPs). The polymerization assessed by turbidimetry is dependent on nsEPs dosage. The kinetics of microtubules formation is tightly linked to the nsEPs effects on structural properties of tubulin, and tubulin-solvent interface, assessed by autofluorescence, and the zeta potential. Moreover, the overall size of tubulin assessed by dynamic light scattering is affected as well. Additionally, atomic force microscopy imaging reveals the formation of different assemblies reflecting applied nsEPs. It is suggested that changes in C-terminal modification states alter tubulin polymerization-dependent conformations. Although the assembled tubulin preserve their integral structure, they might exhibit a broad range of new properties important for their functions. Thus, these transient conformation changes of tubulin and their collective properties can result in new applications.

Tubulin heterodimers (α/β-tubulin) are the building blocks that assemble into tubular filaments and form a highly complex microtubule (MT) structure (Figure 1A). The MT network is an exquisite superstructure that is important for many cellular functions such as cell division and movement, intracellular transport, and cell signaling.[1] MTs are intensively studied due to their critical role in cellular processes, neurodegenerative diseases, and because they are valuable targets for cancer treatment.[2] Moreover, due to the highly regulated assembly of tubulin, MTs structures have an effective monodisperse nanoscale diameter with a tunable length. Additionally, MTs diverse amino acid composition imparts rich chemical functionality. Therefore, MTs are attractive structures as templates for the growth of nanoscale materials assembly, and organization of nanoparticles.[3–5] The basis of the above-mentioned functions of MTs is their capability to switch between states of assembly and disassembly (i.e., dynamic instability). MT dynamics is dependent on the hydrolysis of guanosine triphosphate (GTP) and helps to remodel the MTs network, and produce forces during the cell cycle. Cells rely on MT-associated proteins (MAPs) to selectively target specific tubulin conformations to regulate MT dynamics, and mechanical forces can also influence MT dynamics by altering the balance of tubulin conformations.[6] However, even in the absence of MAPs, MTs can display self-organizing properties in vitro, such as striped structures in spectrophotometer cuvettes.[7,8]

Whereas cells rely on a complex system of intracellular factors to modulate MTs dynamics,[9] a chemical approach was followed in the majority of previous works to inhibit/stabilize or promote the normal MT dynamics in biological applications. Although tubulin-binding agents (TBA) are important components in the treatment of many cancers, the potential dose-dependent toxic side effects, as well as development of resistance, can limit TBA clinical applications.[10] An essential drawback is the irreversibility of the tubulin polymerization once TBA were used. In nanomaterial applications, the development of a wide range of hybrid or composite nanomaterials, such as semiconductor nanocrystals, focused mainly on organization of nanoparticles at the individual filament level. Even though the ability to re polymerize was retained, there are practical
limitations to use such natural tubulin-nanomaterials composites for a scalable technological application.\cite{5,11} Although artificial MTs were developed, an artificial dynamic instability has not been achieved yet. Despite the varied applications, the recent studies confirmed that the conformation state of tubulin is the key factor toward the control of MTs dynamics into delerate, multidimensional and functional assemblies.\cite{12,13} Therefore, a new approach has to be developed to act on tubulin subunits conformation in a controllable fashion.

A perspective modulation strategy to overcome the above-mentioned limitations is looming from the extraordinary electrical properties of tubulin dimers,\cite{14,15} and suggests efficient targeting of tubulin with electromagnetic fields.\cite{16} Recent studies showed that intense nanosecond pulsed electric field affected cellular fibrous structures such as actin filaments and MTs.\cite{17} Additionally, it has been recently shown that MTs can be disrupted with nanosecond pulsed electric field independently from Ca\textsuperscript{2+} release.\cite{18}

While these studies were performed in complex cellular systems, knowledge about the mechanisms of action of nanosecond pulsed electric field on MTs in vitro is so far very limited. Herein, we used a bottom-up approach to act on tubulin subunits conformation in a controllable manner.

We used highly purified porcine brain tubulin shown in lane 3 (Figure 1B) (purity >95\% on Coomassie Blue stained polyacrylamide gel after 1D-PAGE; preparation of tubulin from fresh porcine brain obtained from a local slaughterhouse is described in Section S1 Supporting Information). The ability of tubulin to self-assemble was verified for different concentrations, and the critical concentration was 4.5 mg mL\textsuperscript{-1}. Therefore, in all our experiments, we used a tubulin concentration of 5 mg mL\textsuperscript{-1}.

Isolated tubulin was stored in BRB80 buffer (80 \times 10\textsuperscript{-3} m PIPES pH 6.9, 1 \times 10\textsuperscript{-3} m MgCl\textsubscript{2}, 1 \times 10\textsuperscript{-3} m EGTA) at \textdegree C. Thawed tubulin at 4 \textdegree C on ice was transferred to 2 mm electroporation cuvettes and placed between high voltage electrodes. The electric field across the parallel electrodes was 20 kV cm\textsuperscript{-1} (calculated as \(E = V/d\), where \(V\) is the voltage peak and \(d\) is the gap distance between electrodes; 2 mm). The applied voltage pulse shape is shown in Figure S1 (Supporting Information). The pulse width was 11 ns, the frequency of repetition was 1 Hz, and number of applied pulses was variable parameter. Immediately after pulsing, tubulin was characterized or polymerized at 37 \textdegree C in BRB80 buffer supplemented with 1 \times 10\textsuperscript{-3} m GTP and 3.0 m glycerol.

The both \(\alpha\) and \(\beta\)-tubulin subunits contain multiple tryptophan, and tyrosine amino acid residues (Figure S2, Supporting Information). These two amino acids are both fluorescent, and can be preferentially excited at 275 and 295 nm wavelength for tyrosine and tryptophan, respectively.\cite{19} The tryptophan and the tyrosine autofluorescence emission is often used to assess conformational changes due to their high sensitivity to the local environment. Autofluorescence from those aromatic residues can be used to get information about the structure, folding.
and binding interactions. Important information about molecular structure is carried by the two main parameters of autofluorescence spectrum; autofluorescence intensity and the wavelength of the intensity peak.

Tryptophan autofluorescence intensity ($\lambda_{ex} = 295$ nm) of nsEPs-treated tubulin (100–800 pulses) measured just before polymerization at 37 °C was similar to the untreated tubulin, except for tubulin treated with 200 pulses when a significant increase was observed (Figure 2A, solid bars, autofluorescence axis). After depolymerization of MTs at 4 °C, the autofluorescence intensity of treated tubulin with 100 and 200 pulses was similar to that of untreated tubulin. In contrast, the tubulin polymerization at 37 °C was similar to the untreated tubulin, except for tubulin treated with 200 pulses when a significant increase was observed (Figure 2A, solid bars, autofluorescence axis). After depolymerization of MTs at 4 °C, the autofluorescence intensity of treated tubulin with 100 and 200 pulses was similar to that of untreated tubulin. In contrast, the tubulin

Figure 2. Structural characterization of untreated and nsEPs-treated tubulin. A) Effect of nsEPs on autofluorescence intensity and peak wavelength of tubulin's tryptophans. B) Effect of nsEPs on autofluorescence and wavelength peak for tubulin's tyrosines. Tubulin samples were treated with various number of nsEPs (1 Hz pulses at 37 °C). Continuous lines depict data for tubulin before polymerization; dashed lines depict data for tubulin submitted to polymerization to MTs that were depolymerized by cold. C) Molecular structure of tubulin heterodimer including C-terminus, tryptophans, and tyrosines locations. D) Immunostaining of tubulin samples separated by 2D-PAGE with anti-α-tubulin antibody. 1) Untreated tubulin, 2) tubulin treated with 200 pulses before polymerization, 3) tubulin treated with 200 pulses, then polymerized to MT and depolymerized by cold, 4) tubulin treated with 800 pulses before polymerization, and 5) tubulin treated with 800 pulses, then polymerized to MT and depolymerized by cold. Major, intensively stained spots represent positions of α-tubulin; minor, faintly stained more acidic spots denote positions of β-tubulin. The pI scale is shown along the bottom of the picture. E) Effect of 1 Hz pulses at 37 °C in BRB4 buffer on zeta potential of tubulin immediately before polymerization (continuous lines) and after depolymerization of formed MTs (dashed lines). F) The scheme to summarize the effect of temperature and nsEPs on tubulin size. G) Effect of pulses at 22 and 37 °C in BRB4 buffer on the hydrodynamic radius intensity distribution of tubulin dimers before polymerization determined by DLS. H) Average hydrodynamic radius of at least three independent measurements at 22 and 37 °C and the difference between them.
treated with 400 and 800 pulses showed a decrease in autofluorescence intensity (Figure 2A, dashed bars, autofluorescence axis). The peak wavelength around 330 nm is typical of tryptophans located in protein body not having contact with water solvent.[21] The wavelength shift of the peak toward the blue region was also observed in untreated tubulin or tubulin treated with 100, 200, and 800 pulses (Figure 2A, wavelength axis). The tyrosine autofluorescence intensity (λem = 275 nm) was not affected by nsEPs compared to untreated tubulin before polymerization, except for tubulin treated with 200 pulses where a significant increase was observed. However, after depolymerization, the autofluorescence intensity was much lower for 400 and 800 pulses (Figure 2B, autofluorescence axis). The peak emission wavelength around 324 nm of untreated tubulin was not affected by nsEPs before polymerization or after depolymerization (Figure 2B, wavelength axis). Therefore, data from both tryptophan and tyrosine autofluorescence suggest that the tubulin conformation state is changed by nsEPs in a dose-dependent manner. Before polymerization, the structure of tubulin seems more compact (stable) as suggested from the invariance or increase of autofluorescence intensity of treated tubulin compared to untreated tubulin as shown in the full spectrum of autofluorescence (Figure S3, Supporting Information). Moreover, the shift of the peak wavelength toward the blue region reflects disequilibrium of electrostatic forces for the tubulin treated with more than 200 pulses.[22,23] After depolymerization, the initial conformation was re-established only for untreated and nsEPs-treated tubulin up to 200 pulses. In contrast, a new state of electrostatic forces equilibrium was established in tubulin structure. Likely, this new electrostatic equilibrium reflects changes in the flexible C-terminal tail that contains fluorescent amino acid residues. Moreover, the other tryptophans and tyrosines in tubulin body are in close proximity to the tail region (Figure 2C).

After cold depolymerization, there was a significant structural change for 400 and 800 pulses, which might reflect not only conformational changes but also a tubulin damage. Therefore, we checked the preservation of the tubulin structure. We performed immunoblotting with antibody TU-01,[24] reacting with all brain α-tubulin isotypes,[25] on untreated or nsEPs-treated tubulin samples separated by 2D-PAGE.[26] Additionally, we measured the zeta potential to assess any changes in the effective electric charge of tubulin. Immunoblotting analysis of untreated tubulin (Figure 2D-1) or tubulin treated with 200 (Figure 2D-2) or 800 pulses (Figure 2D-4) before polymerization did not reveal degradation of α-tubulin. The same held true in the case of tubulin samples that were treated with 200 (Figure 2D-3) or 800 pulses (Figure 2D-5), polymerized to MTs and then depolymerized by cold.

The overall charge of tubulin dimers is negative.[27] This negative charge is mainly located in highly flexible C-terminal tail regions on both tubulin subunits.[28] We measured the zeta potential of tubulin (untreated and nsEPs-treated) diluted in BRB4 buffer (4 × 10⁻³ M PIPES pH 7.0, 0.05 × 10⁻³ M EGTA, and 0.05 × 10⁻³ M MgCl₂) at 37 °C to minimize the ionic condensation effect on tubulin surface. The zeta potential of native tubulin (~29.39 ± 1.77 mV) was similar to that reported in literature (~27.1 ± 3.3 mV).[29] Upon increasing the number of pulses, the zeta potential of tubulin decreased significantly in the case of tubulin treated with 400 and 800 pulses before polymerization to ~21.38 ± 1.04 and ~20.2 6 ± 0.87 mV, respectively. In contrast, the zeta potential of tubulin measured after cold depolymerization was not affected significantly (Figure 2E). The configuration of the protein affects the effective protein charge.[30] Hence, modifying the configuration of the flexible tubulin C-terminal tail might explain changes observed in zeta potential. In this respect, the zeta potential results corroborate our findings from autofluorescence data suggesting that the tubulin conformation is affected by nsEPs. This effect is reversible, as the surface charge was re-established after cold depolymerization of MTs in a manner that maintain the tubulin stable in the new solvent interface arrangement. Since the C-terminal tail of tubulin is flexible and charged, we hypothesize that the overall size of tubulin can be affected by C-terminal tail configuration. We, therefore, measured tubulin hydrodynamic radius.

The hydrodynamic radius (R̅) of tubulin was measured by dynamic light scattering (DLS). Herein, the measurements were done at two different temperatures (22 and 37 °C) in BRB4 buffer (Figure 2G). The R̅ values of untreated tubulin at 22 °C are consistent with the tubulin size values in literature,[31,32] as our measurements resulted in the R̅ of 3.348 ± 0.192 nm. However, at 37 °C the R̅ increased to 5.066 ± 0.335 nm. The radius of tubulin treated with nsEPs at 22 °C increased up to 3.749 ± 0.174 nm when compared to native tubulin. This increase was, however, not statistically significant. In contrast, at 37 °C, the radius of treated tubulin with nsEPs decreased to 4.898 ± 0.138 when compared to untreated tubulin. These results, on the one hand indicate that during polymerization tubulin undergoes structural changes due to temperature-dependent size increase. The ~1.73 nm R̅ difference in the case of untreated tubulin at 22 and 37 °C was substantial. This difference might be caused by more elongated C-termini tails, which can extend few nm from tubulin body at higher temperature.[15] On the other hand, the nsEPs-treated tubulin showed less R̅ variation at both temperatures. Smaller variation of the response could be due to different response of the nonelongated versus elongated configuration of the C-termini at 37 versus 22 °C (Figure 2H). The results from DLS thus support our results from autofluorescence, and zeta-potential analysis. In the latter case, the conformational changes due to nsEPs are likely initiated at the C-terminal level. The scheme (Figure 2F) summarizes the effect of temperature and nsEPs on tubulin size.

The polymerization of tubulin at 37 °C in the presence of 3 M glycerol and 1 × 10⁻³ M GTP was monitored by turbidimetry measuring absorbance at 350 nm. The polymerization of untreated tubulin shows a typical curve of MTs formation following three phases (nucleation, rapid formation, and stable length).[33] The effect of nsEPs on the tubulin polymerization is shown in (Figure 3A left-hand side curves). The treated tubulin showed an nsEPs-dependent decrease in the MTs formation. Additionally, the lag phase was also affected. The assembled MTs were thereafter depolymerized by cooling at 4 °C for 90 min to insure a complete disassembly into tubulin subunits. After cold depolymerization, the tubulin samples
were again heated on to 37 °C. Surprisingly, tubulin samples treated with 100 and 200 pulses repolymerized to the same extent as the untreated sample (reversible polymerization). The tubulin treated with 400 and 800 pulses could polymerize, but not to the same extent as untreated tubulin (irreversible polymerization) (Figure 3A right-hand side curves). These data suggest that the structural changes in tubulin subunits generated by nsEPs affect tubulin polymerization capability as it was quantified in term of absorbance decrease up to 26% for 800 pulses (Figure 3A-table). Additionally, we measured the autofluorescence during self-assembly (Figure S4, Supporting Information). Fluorescence spectroscopy revealed that autofluorescence intensity was decreasing during polymerization, and was affected by nsEPs. As the added GTP has a fluorescence quenching ability, the decrease of the autofluorescence of native tubulin reflects the binding process. The nsEPs decreased the autofluorescence intensity even more. Therefore, the conformational changes and polarity in the vicinity of the fluorescent amino acids (tryptophan and tyrosine) reflect a reversible or irreversible alteration of the binding sites.

Figure 3. Characterization of assembled untreated and nsEPs-treated tubulin. A) Turbidimetry assays of untreated and nsEPs treated tubulins. Effect of nsEPs on MTs polymerization (left) and repolymerization (right), solid thick lines are the calculated average shaded solid lines (forming symmetric envelope) are depicting standard deviation data, circles are maximum absorbance values used to calculate the percentage of the relative absorbance changes due to pulses (inset table). B) Atomic force microscopy images and height of structures formed from untreated tubulin. C) Atomic force microscopy images and height of structures formed from nsEPs-treated tubulin with 200 pulses. D) Atomic force microscopy images and height of structures formed from nsEPs-treated tubulin with 200 pulses. E) Scheme of the corresponding structures formed from untreated and nsEPs-treated tubulin.
C-terminal tails are known to affect MTs length dynamics, and are major sites of tubulin post-translational modification, which regulates binding interactions.[39] On the other hand, the fluorescence quenching reflects the alterations in binding sites on the tubulin body. We suggest that nsEPs affect C-terminal tails configuration that is followed by conformational changes at the tubulin body. Depending on the nsEPs dose, the tubulin's binding sites on the tubulin body are affected either reversibly or irreversibly. To investigate the effect of nsEPs on the structures polymerized from the treated tubulin, we performed atomic force microscopy (AFM) imaging.

Using atomic force microscopy, we investigated the nature of the structures formed by untreated and nsEPs-treated tubulin. The untreated tubulin formed typical structures of MTs (Figure 3B) with different diameters varying between ≈10 and ≈25 nm. This diameter range suggests a tubular structure or collapsed MTs (Figure 3E). 200 pulses generated structures with diameters ranging from ≈4 to ≈10 nm (Figure 3C). This reflects a collapsed or an open-up structures (Figure 3E). In contrast, at 800 pulses, the diameter was less than ≈7 nm. This might reflect an open-up structure (Figure 1D). Moreover, a high density of unpolymerized tubulin/oligomers was detected on the mica surface.

In conclusion, we show that tubulin building blocks of MTs are sensitized by nsEPs to engineer their function. We also show that nsEPs induced conformational changes and electrostatic forces disequilibrium. In this regard, we suggest that the C-terminal tails are affected at first by nsEPs followed by a modification of the tubulin's binding interactions sites. Moreover, we show that the ability to polymerize is linked to the induced structural modifications. Recovery of polymerization-competent tubulin conformations depends on nsEPs dosage. AFM revealed that different structural configurations could be generated after application of nsEPs. Therefore, this work reports a versatile and vigorous strategy for controlling tubulin polymerization by tuning of nanosecond pulsed electric field parameters. Although the assembled tubulin preserved its integral structure, it may exhibit a broad range of new characteristics important for its functions and potential applications. These perspective applications will be a direct consequence of the transient conformation changes of tubulin and their collective property while interacting within the cell environment or with nanostructures. For instance, one could envisage using nsEPs to steer the self-assembly of tubulin to engineer nanostructured templates for the creation of 3D to-be-metalized electrical interconnects where only biochemical control was possible so far.[36] Steering of the self-assembly would be possible both in quantitative and qualitative manner: controlling the self-assembly rate and generating nanostructures of a different nanoscopic profile.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords

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