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Tubulin Conformation and Anaesthetic Interaction - An Experimental Study

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Abstract

The current endeavour of neuroscience and philosophy is to discover the code of consciousness or rather how the material brain produces our in Material sense of awareness. Some scientists believe that consciousness is something distinct from the physical brain and body, as consciousness continues to exist even when the brain is not functioning. But from a scientific viewpoint, consciousness is a function of the brain. Since the brain is a material entity, consciousness is subject to the study of science. The human brain is a complex mass of tissue endowed with extraordinary capabilities. Microtubules turn out to be a common target of neurotransmitter action and play a significant role in learning and memory. Memory and consciousness are interrelated, thus, microtubules could be the link between these two phenomena. Microtubules are cylindrical hexagonal lattice polymers of the protein tubulin, comprising 15% of total brain protein. Microtubules regulate synapses and are suggested to process information via interactive bit-like states of tubulin. Microtubules are very dynamic polymers whose assembly and disassembly is determined by whether their heterodimer tubulin subunits are in a straight or curved conformation. Curvature is introduced by bending at the interfaces between monomers. This is because GTP hydrolysis promotes bends in protofilaments. However, while GDP-bound protofilaments are still associated together as a microtubule or 2-D sheet, the contacts between neighbouring subunits constrain them to remain in a straight form. The resulting tension is proposed to store conformational energy that is released during depolymerisation. Also, the mechanism by which anaesthetics prevent consciousness remains largely unknown because the mechanism by which brain physiology produces consciousness is unexplained. Tubulins have other smaller non-polar regions that contain pi electron-rich indole rings separated by only about 2 nm. Penrose-Hameroff Orchestrated Objective Reduction (Orch-OR) Theory proposes that these electrons are close enough to become quantum entangled. They suppose that quantum-superposed states develop in tubulins, remain coherent and recruit more superposed tubulins until a mass-time-energy threshold, related to quantum gravity, is reached called as the 'bing' moment. This paper characterizes the conformation of tubulin in presence of anesthetics.

Keywords: Tubulin; Anaesthetic interaction; Microtubules

Introduction

Consciousness can be defined in a number of ways. Many eminent researchers believe that consciousness continues even when the brain is not functioning. In other words, consciousness is something separate from the physical brain and body. Also from a scientific viewpoint, consciousness is a function of the brain. Since the brain is a material entity, consciousness is subject to the study of science. The human brain is a complex mass of tissue endowed with extraordinary capabilities. The brain is the control centre for the central nervous system in higher animals. Now a day's many eminent scientist and researchers are involved in decoding the mystery of consciousness. They are mapping brain of Franciscan nuns, Buddhist monks and many others during prayer to explain spirituality or consciousness. Now the question is can a powerful brain imaging technology explain consciousness which is immaterial in nature. According to theory of consciousness given by Penrose and Hameroff, consciousness emerges from the brain and focus particularly on complex computation at synapses that allow communication between neurons. Neurons possess a highly organized arrangement of microtubules due to a high number of specialized non-motor microtubule associated proteins (MAPs) with nerve cells. MAP 1A, MAP 1B, MAP2 and Tau are localized within axons and dendrites of neurons. It has been suggested that the different MT organizations within axons and dendrites may play a role in neuronal signalling. Evidences indicate that links exist between MT activity and cognitive functions by showing that during the period when the formation of synapses and visual learning occur at their highest rates the visual cortex of the brain produces massive amounts of tubulin [1]. Recently the link between microtubules and cognition has been illustrated by studies involving patients with Alzheimer's disease [2]. It has been shown that Alzheimer's compromises axonal transport indicating a link to MTs since MTs are responsible for the transport of material from the soma to the axon terminal. it has been suggested that MTs can provide a non-selective mechanism for general anaesthetics. Tabony explained the analogy between the way ants and microtubules self-organize. Like ant colonies leave behind the chemical trails (pheromones) and communicate, under appropriate conditions, neighboring microtubules or microtubule population may communicate indirectly with one another by a stigmergic process involving the chemical trails (free tubulin) that they themselves produce. Thus, this raises the intriguing questions as to what extent microtubules like ants and other social insects spontaneously develop very high-level behavior extending up to what is called 'swarm intelligence' [3]. Microtubules also undergo a conformational change when the beta-tubulin bound GTP hydrolyses resulting in a 27° shift between the original centre to centre line joining the alpha, beta tubulin monomers and the new configuration centre line and during this it releases approximately 0.42 eV of energy per molecule. Vibrations are expected to be generated in microtubules from the MHz to GHz region. It is also suggested that consciousness derives from quantum vibration in microtubules. As consciousness cannot be

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directly observed, measured or verified, we can only measure neural correlates of consciousness. Also we need to know what are those biomolecules which are involved in maintaining a state of heightened concentration/or attention during meditation.

In this paper, efforts have been made to understand the nature of secondary structure of tubulin heterodimer and also the interaction between microtubules and propofol (an anesthetic) with the help of circular dichroism spectroscopy and time resolved fluorescence spectroscopy. We have also carried out kinetics studies with the help of Eon spectrophotometer to optimize the reaction conditions.

Materials and Methods

Tubulin (#TL238), taxol (#TXD01), GTP (#BST06) and General Tubulin Buffer (#BST01) (Microtubule/Tubulin Biochem kit Cat#BK015) are supplied by Cytoskeleton Inc. Denver, CO. USA.

Preparation of buffer T

GTP stock solution (100 mM) is added to General Tubulin Buffer (80 mM PIPES pH 6.9, 2 mM $MgCl_2$, 0.5 mM EGTA) at a final concentration of 1 mM GTP. The buffer T will be stable for 2-4 hours on ice.

Fluorescent reporter buffer

Fluorescent reporter buffer contains 80 mM Piperazine-N,N'-bis{2-ethanesulfonic acid} seuisodium salt; 2.0 mM Magnesium chloride; 0.5 mM Ethylene glycol-bis (b-amino-ethyl ether) N,N,N',N'-tetra-acetic acid, pH 6.9, 10 μM DAPI (4',6-diamidino-2-phenyindole).

Tubulin Reconstitution

1 mg of lyophilized tubulin is resuspended in 1 ml of buffer T at 0-4°C

(final tubulin concentration is 1 mg/mL). The reconstituted tubulin solution is not stable and needs to be used soon after its preparation.

Propofol (2,6-di-isopropylphenol) (Anesthetic) is supplied by MP Biomedicals, Mumbai. Three different concentrations of Propofol (0.5, 1.0 and 100 mM) were prepared for the study.

Throughout the experiments, all solutions were prepared in MilliQ water. pH measurements were carried out using EUTECH instruments-pH 510.

Kinetics study of polymerization of bovine brain tubulin in presence of propofol

Polymerization of Bovine brain tubulin was studied with spectrophotometric technique. The kinetics of microtubule assembly (bovine brain tubulin) was monitored by Eon Spectrophotometer (Biotek) at 350 nm for 1-2 hours with the time interval of 1 minute at 37°C. Polymerization of tubulin protein is carried out using standard protocol [4-8].

The effect of Propofol on polymerization of tubulin was also studied. Chemically, propofol is 2,6-di-isopropylphenol. It has 95-99% protein binding affinity. Its half-life is 30-60 minutes. Propofol has been proposed to have several mechanisms of action, both through potentiation of GABA receptor activity, thereby slowing the channel-closing time, and also acting as a sodium channel blocker. EEG research upon those undergoing general anesthesia with propofol finds that it causes a prominent reduction in the brain's information integration capacity at gamma wave band frequencies. So the effect of different concentrations of propofol (0.5, 1.0 and 100 mM) on polymerization of tubulin in presence of guanosine tri-phosphate (GTP) was also monitored. As Tubulin proteins do not polymerize into microtubules in the presence of zinc ions and guanosine di-phosphate (GDP), an attempt was also made to understand the exact mechanism of propofol binding to tubulin in presence of zinc ions and guanosine di-phosphate using different concentrations of propofol (0.5, 1.0 and 100 mM).

Circular Dichroism (CD) Measurements

The isothermal studies of Tubulin by CD measurements were carried out with Chirascan, a polarimeter of Applied Photophysics equipped with a Quantum Northwest-TC125, a Peltier-type temperature controller. The instrument was calibrated with d-10-camphorsulfonic acid. All the isothermal CD measurements were made at 25°C. Spectra were collected with 20 nm/min scan speed, 0.1 nm data pitch, and a response time of 2 s. Each spectrum was the average of 10 scans. The Far-UV CD spectra (200–260 nm) were taken at protein concentrations of 0.889 mg/ml in a cell of 0.1 cm path length. All spectra were smoothed by the Savitzky-Golay method with 25 convolution width. CD values (θ) in mdeg, were obtained from the instrument readings.

TRFS measurements

Excited-state lifetime measurements were performed using a time-correlated single photon counting (TCSPC) spectrometer (Edinburgh FLS920). For our experiments a LASER having its central wavelength at 375 nm was used as the source for exciting the DAPI present in fluorescent buffer. Emission was subsequently collected at 440 nm through a single monochromator with a 5 nm bandpass over a total time range (TAC) of 100 ns for all samples. Emission polarizer was set at 55.4 degree magic angle to exclude rotational anisotropy lifetime decay to simple decay life time data. Emission decays were fit with appropriate instrument response functions (IRF) collected using a scattering solution. The FWHM (full width at half-maximum) of the IRFs collected was typically in the range of ~120 ps.

Results and Discussion

The kinetics of microtubule assembly (bovine brain tubulin) was monitored by Eon Spectrophotometer (Biotek) at 350 nm for 1-2 hours with the time interval of 1 minute at 37°C.

Polymerization of bovine brain tubulin was carried out with and without guanosine triphosphate (GTP) (Figure 1). Bovine brain tubulin in presence of GTP showed different polymerization- depolymerization behaviour when compared to that without GTP (Figure 2). In presence of GTP, the microtubule polymerization was found to be stable to some extent and less dynamic. In absence of GTP, the microtubule was found to be less stable. This also showed that GTP, not only enhances the rate of polymerization, but is essentially required for polymerization. The effect of taxol was also seen on polymerization of microtubule in presence of GTP and glycerol buffer. It shows that microtubules are stabilized in presence of taxol (Figure 3).

The effect of different concentrations of propofol (0.5, 1.0 and 100 mM) was also seen on polymerization of tubulin in presence of 1 mM GTP and glycerol buffer (Figures 4-9). Figure 1 is the control for Figures 4-6. It was seen that propofol affected polymerization or self organization behaviour with all concentrations of propofol. With 0.5 mM propofol, polymerization was affected upto 10 minutes, with 1 mM Propofol, the rate of polymerization was affected upto 30 minutes and with 100 mM propofol, polymerization was affected upto 60 minutes.

When the effect of propofol was seen on tubulin polymerization in the presence of GDP and zinc ions, it was observed that polymerization

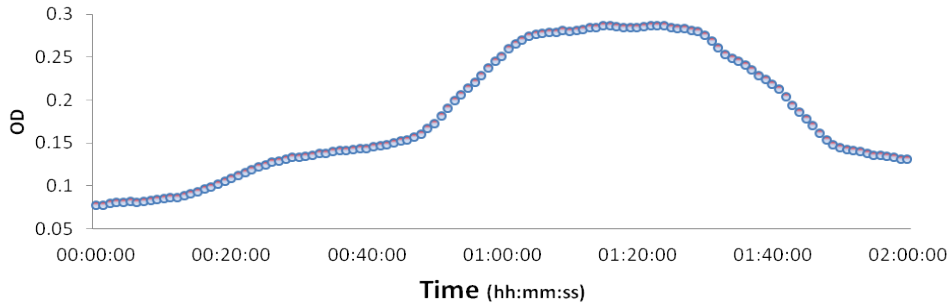

Figure 1: Graph showing kinetics of bovine brain tubulin with 1 mM GTP and glycerol buffer.

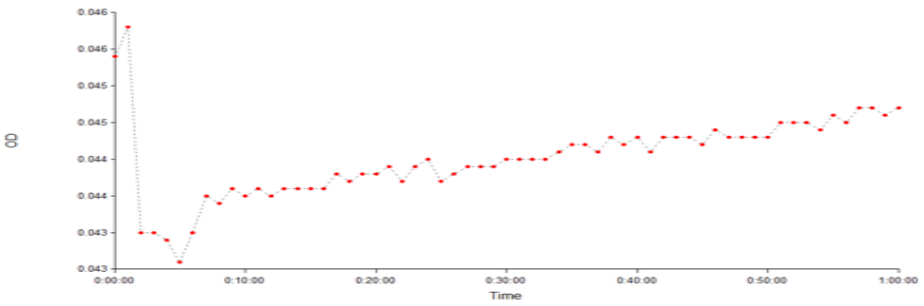

Figure 2: Graph showing kinetics of bovine brain tubulin in presence of glycerol buffer and in absence of GTP.

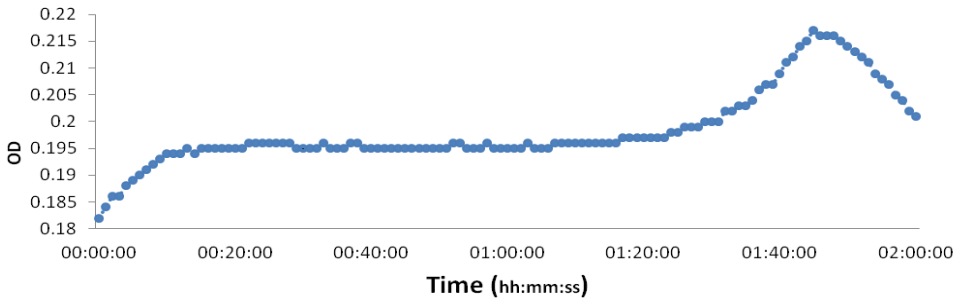

Figure 3: Effect of 2 mM taxol on polymerization of bovine brain tubulin in presence of 1 mM GTP and glycerol buffer.

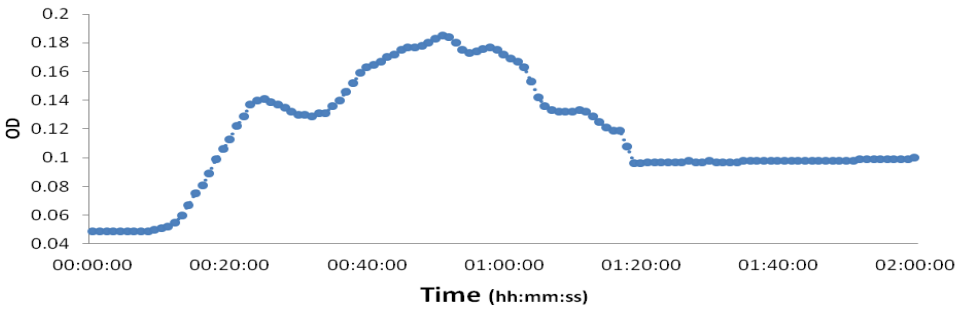

Figure 4: Effect of 0.5 mM Propofol on polymerization of bovine brain tubulin in presence of 1 mM GTP and glycerol buffer. (Figure 3.2 is the control for figures 3.5, 3.6 and 3.7).

was strongly affected with all three concentrations of propofol and the same trend was observed with all three sets of experiments.

With low concentration of propofol (0.5 mM), the polymerization or self-organizational behaviour of tubulin was affected upto 10 minutes (Figure 4). From this, it is inferred that the effect of propofol diminishes after 10 minutes with 0.5 mM Propofol as its half-life is 30-60 minutes. But with a little higher concentration of propofol (1 mM), self-organizational behaviour was affected upto 30 minutes (Figure 5). When polymerization of tubulin was studied with 100 mM propofol, the self-organizational behaviour of microtubule was strongly affected upto one hour (Figure 6). From above observations, it is inferred

that propofol (anesthetic) binds to hydrophobic pockets of tubulin via weak Vander waals london dispersive forces [9]. Propofol's effect is time- and dose-dependent, and can be reversed when propofol is removed. For further experiments, we have chosen propofol's dose to be 100 mM [10-20].

Efforts have also been made to determine the conformation of tubulin protein by circular dichroism spectroscopy. CD is a spectroscopy technique widely used for the evaluation of the conformation and stability of proteins. Circular dichroism is a method that would account for the helix content in a polypeptide based on the presence of alpha helix, beta sheet and random coil. Two deep minima near 208 nm and 222 nm are characteristic of α helical structure in aqueous solution due to π-π^* and n-π^* transitions, respectively and β-sheet is having negative at 218 nm (π-π^*) (Figures 10 and 11). Figure 10 clearly reflects characteristics of (α + β) type of structure. There is a weak n-π^* transition centered around 210 nm and an intense π-π^* transitions about 190 nm [20-25].

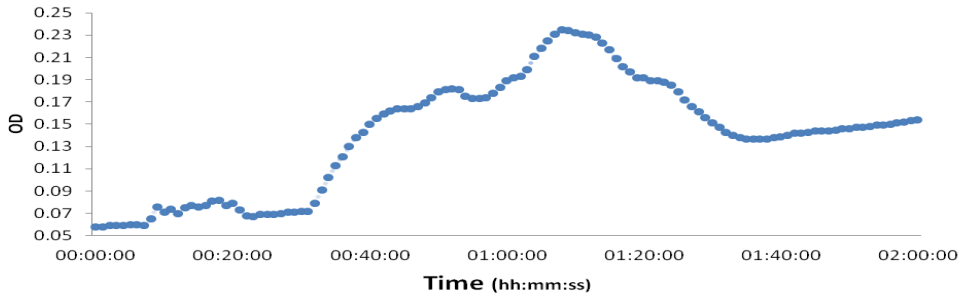

Figure 5: Effect of 1 mM propofol on polymerization of bovine brain tubulin in presence of 1 mM GTP and glycerol buffer.

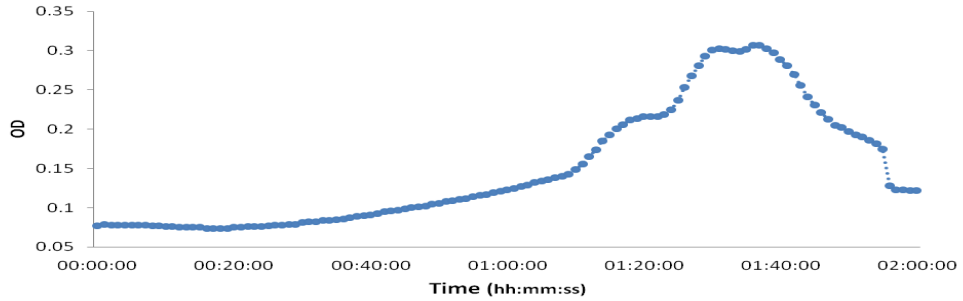

Figure 6: Effect of 100 mM propofol on polymerization of tubulin protein in presence of 1 mM GTP and glycerol buffer.

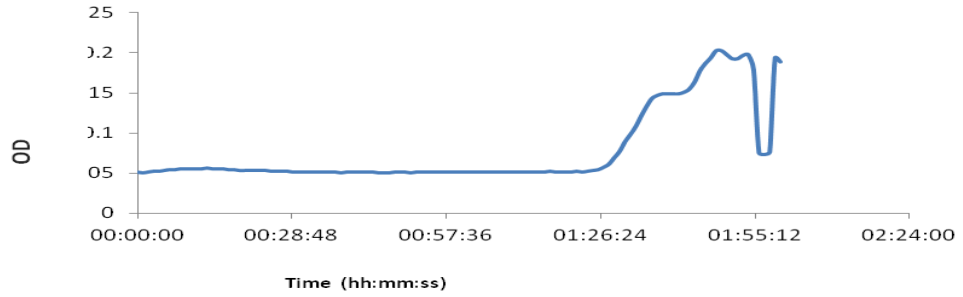

Figure 7: Effect of 1 mM Propofol on polymerization of bovine brain tubulin in presence of guanosine di-phosphate (GDP), Zn^{2+} and glycerol buffer.

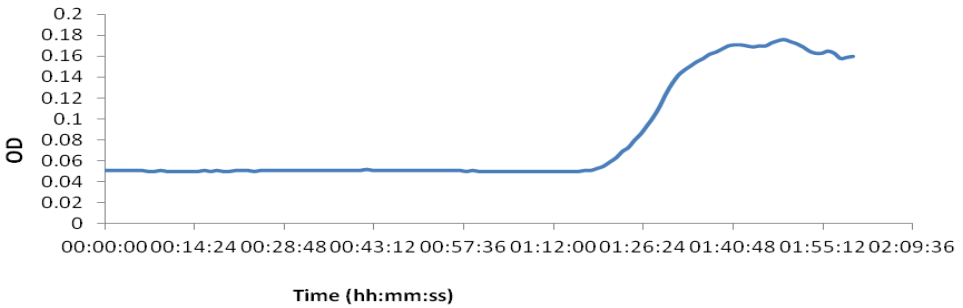

Figure 8: Effect of 0.5 mM Propofol on polymerization of bovine brain tubulin in presence of GDP, Zn^{2+} and glycerol buffer.

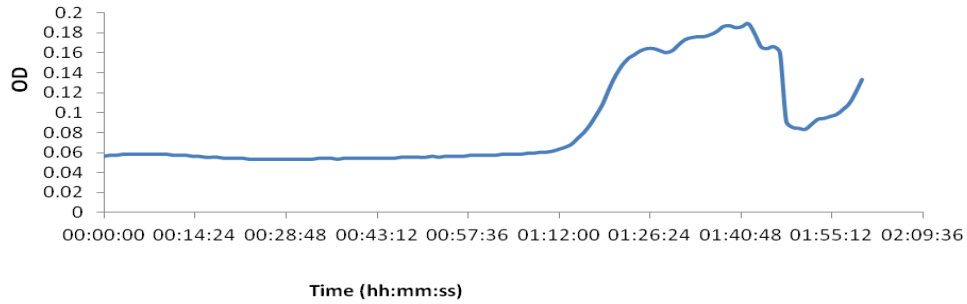

Figure 9: Effect of 100 mM Propofol on polymerization of bovine brain tubulin in presence of GDP, Zn^{2+} and glycerol buffer.

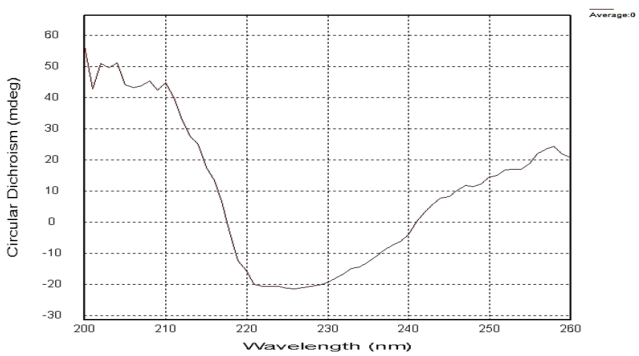

Figure 10: Circular dichroism spectra of bovine brain tubulin with 1 mM guanosine tri-phosphate and glycerol buffer.

Figure 11: Circular dichroism spectra of tubulin in presence of guanosine tri-phosphate and propofol.

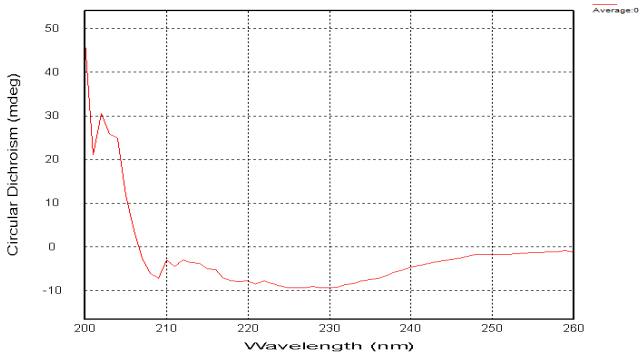

Figure 12: Circular Dichroism spectra of bovine brain tubulin in presence of zinc chloride and propofol.

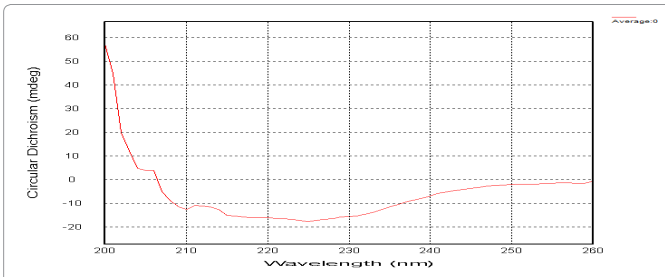

Figure 14: TRFS plot of tubulin in presence of fluorescent tubulin glycerol buffer and guanosine tri-phosphate.

As zinc chloride was added to tubulin protein, a notable rearrangement of spectrum occurred with lowering in major minima as well as slight changes in the shape of spectra. A lowering in negative value of ellipticity at 208 nm and 222 nm indicated a sign of α-helical reduction due to intramolecular H-bonding rearrangement. After the addition of propofol to the tubulin in presence of zinc chloride (Figures 12 and 13), there is complete collapse of structure, the nature of the CD spectrum has completely changed suggesting major changes in its overall conformation [26-30].

For further confirmation of results, we have also carried out time resolved fluorescence study of tubulin in presence of anesthetic. Fluorescence lifetime is a very sensitive parameter for analyzing the excited-state interactions and the local environment present around the fluoropore. In this study, we have used DAPI as fluoropore. DAPI (4',6-Diamidino-2-phenylindole), a polycationic fluorescent reagent, binding site in tubulin is located at the interface of both subunits (alpha- and beta- tubulin). Its binding site is different from that of colchicine, vinblastine, or taxol, does not interfere greatly with microtubule polymerization. The fluorescence decay(s) of DAPI (4',6-diamidino-2-phenyindole) in buffer could be well described by a biexponential fit throughout, irrespective of the different environment used. DAPI (4',6-diamidino-2-phenyindole) itself shows complicated excited-state photo physics even in buffer and hence here we have used the average fluorescence lifetime to monitor the proximity of DAPI (4',6-diamidino-2-phenyindole) to the tubulin and drug molecules. The lifetimes of DAPI (4',6-diamidino-2-phenyindole) in the buffer and tubulin (Figure 14) are much longer than that observed in buffer alone (Figure 10). From the graph, it is hypothesized that when DAPI binds to tubulin, the tubulin heterodimers acquire such a conformational state that DAPI does not lose its energy to its neighbouring molecule. DAPI (4',6-diamidino-2-phenyindole) lifetime in buffer is understandably short because of the close proximity of the of DAPI (4',6-diamidino-2-phenyindole) to glycerol and other neighbouring buffer molecules, resulting in a significant amount of energy transfer from of DAPI (4',6-diamidino-2-phenyindole) to the glycerol and other neighbouring

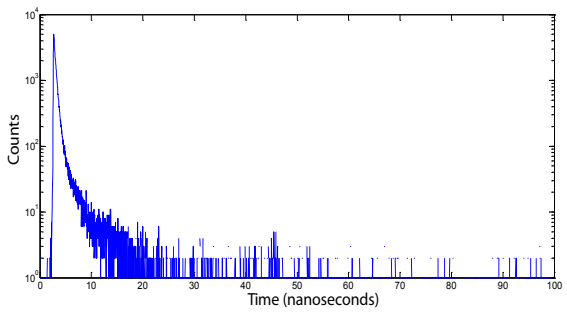

Figure 15: TFRS plot of control (Fluorescent tubulin buffer).

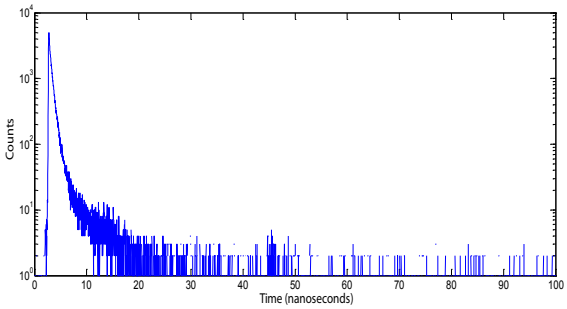

Figure 16: TRFS plot of tubulin in presence of Fluorescent Tubulin Glycerol Buffer, Guanosine Tri-Phosphate and propofol.

buffer molecule (Figure 15). However, the same in the vicinity of tubulin protein molecule and the lifetime undergoes a gradual increase as the tubulin changes the local environment of the DAPI (4', 6-diamidino-2-phenyindole) (Figure 14). Also, this trend is quite expected on the basis of the fact that relaxation (increase) in the confining dimensions should bring about a change in state resulting in a decrease in energy transfer (and hence increased lifetime). When the DAPI (4', 6-diamidino-2-phenyindole) is in neighbor of tubulin and propofol in the buffer then there is no change in the average life time data of DAPI (4', 6-diamidino-2-phenyindole). Infact the lifetime data of DAPI (4', 6-diamidino-2-phenyindole) in this condition (Figure 16) matches as that of DAPI (4', 6-diamidino-2-phenyindole) in buffer alone. No change in lifetime with a change in neighboring condition signifies that the DAPI (4', 6-diamidino-2-phenyindole) probably does not bind to tubulin heterodimer and it remains free in the reaction condition. Also, it is understood that there is preferential binding of propofol to tubulin binding site than that of DAPI. Therefore, DAPI transfers energy to its neighbouring molecules [31-34].

Conclusion

Kinetics studies show that propofol strongly affects polymerization of tubulin or self-organization of microtubules. It is hypothesized that propofol's effect is time- and dose- dependent, and can be reversed when propofol is removed. Microtubule population in presence of anesthetic propofol is not capable of carrying out collective action. Microtubules do not self-organise by a reaction-diffusion process in presence of propofol and do not communicate indirectly with each other. Self-organizing patterns suggesting the potential for MTs to process information do not form in the presence of anesthetic. Circular dichroism of tubulin in presence of propofol suggests major changes in its overall conformation. It is inferred that binding of anesthetics to tubulin protein causes an alteration in secondary structure. TRFS study further supports the change in secondary structure of tubulin protein when it binds with anesthetic (propofol).

We are not sure that assembly/polymerization of microtubule is the best measure of microtubule activity relevant to consciousness. Also we have not considered conditions ideal for quantum brain structures relevant to consciousness. But our future endeavor would be to determine the conformation of tubulin under conditions ideal for quantum brain structures relevant to consciousness. In future we may discover quantum effects in microtubules responsible for information processing.

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