Morphology of Microtubules Grown in Agarose Gels: Effect of Diffusion and Confinement**

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The notion that microtubules (MTs) might serve as active biomolecular nanostructures that can assemble into hierarchical functional networks and systems has held for some time a special allure for bio-nanotechnology researchers. The controlled assembly of active MT-based networks and systems offer great promise in numerous technologies. In recent years there has been an emerging interest in building MT arrays or networks to serve as tracks for kinesin-functionalized micrometer-size cargo such as microcrystals or polymer beads.[1–5] Due to the difficulties encountered in the assembly of such nanoscale tracks, MTs have also been used to serve as transport platforms in kinesin-coated microfluidic channels.[6,7] In addition, MTs have been shown to effectively serve as templates for spatially selective metallization paths.[8] However, the development of effective molecular systems based on MTs is impeded by competition between the natural mixing caused by both diffusion and directional motor-protein transport. Overcoming this mixing problem can be achieved by building MT tracks in media such as gels that retard molecular diffusion. MTs are self-assembling, dynamic, tubular structures composed of polymerized tubulin proteins with nanometer size diameters and large aspect ratios.[9] In the presence of the small molecule guanosine triphosphate (GTP), tubulin monomers form GTP-bound tubulin heterodimers (Tu-GTP). Under suitable conditions, these heterodimers will self-assemble into the MT polymer. MT ends are polarized in that each one exhibits unique and specific biochemical moieties. Dynamic instability is an intrinsic property of MTs.[10] For tubulin concentrations above a critical concentration (Cc), tubulin dimers polymerize into MTs whereas below Cc, MTs depolymerize. Near Cc, MTs undergo apparently random periods of assembly and disassembly. During mitosis, this rapid elongation and shrinkage of MT ends tethered to the kinetochore generate force on the chromosomes.[11] In the presence of MT associated proteins (MAPs), MTs also exhibit different organization.[12] from dynamic spindles and self-organized acentrosomal networks[13] to parallel arrays in axonemes.

To date, the vast majority of work involving in vitro growth of MT networks has dealt with bulk-phase aqueous solutions of tubulin. So far, only two-dimensional MT networks have been grown from such aqueous solutions, using patterned solid substrates with control of both position and polarity. For instance, selectively patterned centrosomes on solid substrates have been used as templates for the directed polymerization of microtubules.[14] We have recently reported the controlled nucleation and growth of microtubules from arrays of gold pads on a hydrophilic oxidised silicon wafer functionalized with γ-tubulin, a natural nucleating agent for microtubule growth.[15] In contrast to two-dimensional networks, the design, engineering and construction of three-dimensional networks of MTs requires MT growth in three-dimensional scaffolds such as gels. There are few reports of experimental studies on the impact of the competition between tubulin diffusion and assembly reaction on MT growth in high viscosity media containing gelling agents.[16] We have shown recently diffusion plays a critical role in the collective MT dynamics,[17] where modeling and simulations support the importance of interplay between reaction and diffusion on the growth dynamics and morphology of MTs. The geometry of the environment in which MTs grow not only impacts the MT network morphology by affecting diffusion but also does by creating a crowded environment. Extensive theoretical studies of the effects of crowding by additions of inert macromolecules or confinement on assembly of protein structures shown that the chemical potential of the solute protein can be drastically modified leading to an enhancement of nucleation and association.[18,19] In addition to the impact on thermodynamics properties, crowding may affect the kinetics of protein assembly.[20] When assembly is chemical reaction limited crowding is expected to increase association. For diffusion limited assembly crowding decreases the transport of available proteins to the assembling structure. Furthermore prediction of formation of rod-like protein aggregates in crowded or confined environments supports a tendency toward alignment and even bundling.[21]

MT reaction dynamics are generally simulated in the context of a molecular cap model.[22–30] Numerous models have been developed to simulate the collective oscillations of microtubules[31–35] but the majority of existing models are based on mean-field approaches and have assumed homogeneity of the tubulin heterodimer concentration. However, while studies have shown that tubulin diffusion is important for pattern
Existing models of MT dynamics with diffusion have been limited to two-dimensional phenomenological representation of MT dynamics and diffusion.

In this paper, we report on the morphology of MTs grown in a crowded environment in the form of high viscosity fluids containing agarose and in confined environments in the case of solid agarose gels. We have chosen agarose because: a) it is chemically and electrically neutral, b) it forms a porous gel network, and c) by varying its concentration, it is possible to influence the diffusion coefficient of molecules in high viscosity solutions and gels. We show experimentally that as the agarose concentration increases, the morphology of the MTs evolves from numerous short, straight MTs to longer MTs to fewer long bundles of MTs that are curved or contorted. The same behavior is also observed in a three-dimensional reaction-diffusion model of MT dynamics where the diffusion coefficient of free tubulin is varied. The modeling also provides an explanation as to the observed bundling and contorting of MTs due to the confinement of MTs in the porous structure of the gel.

Ten MT-agarose samples (MTs polymerized in agarose containing solutions or gel) were prepared with agarose concentrations of 1%, 0.75%, 0.50%, 0.25%, 0.10%, 0.08%, 0.05%, 0.035%, 0.02%, and 0% (control) with a fixed tubulin concentration of 0.33 mg ml⁻¹. The concentrations above 0.25% do not contain MTs (with agarose concentrations of 2.5, 1.5, 1, 0.75, and 0.50%) were also studied by FESEM.

The physical structure of the agarose gel is composed of an arrangement of long filaments with diameters between 12 to 18 nm, forming a cross-linked three-dimensional network. According to the analysis by FESEM of the gel samples over fractured surfaces, the porosity of the gel was characterized by large pores separated by a denser network of agarose filaments. The large pores exhibit diameters in the order of hundreds of nanometers, whereas the denser network has small pores with diameters no bigger than a few tens of nanometers.

In Figure 1a we attempted to capture a two-dimensional representation of this three-dimensional structure. We observed a notable change in the structure of the five different agarose gel samples as the agarose concentration was increased. The sample with the lowest concentration showed a more open structure compared to those of higher concentration where a more compact structure was present (Fig. 1b and d). Also, we note that as the concentration of agarose was decreased, the size of the large pores appeared to increase, but the denser network remained the same (Fig. 1c and e).

Figure 2 illustrates the morphology of MTs grown in viscous fluids comprised of various concentrations of agarose. Because these samples (0.25% agarose and below) were difficult to analyze due to the high density and three-dimensional arrangement of MTs in the solution, the images in Figure 2 were obtained from samples that were carefully smeared on a microscope slide in order to isolate MTs and measure their lengths. The inevitable mechanical introduced by smearing may have several effects on the MTs in our samples namely breakage of MTs or separate MTs from bundles. We note qualitatively that as the agarose concentration increases the average length of the observed MTs increases, supporting the fact that MTs had indeed lengthened. Furthermore we have verified that fluorescence images of MTs grown in the viscous media do not show MT bundling. In Figure 3 we report a quantitatively analysis of this effect of agarose content on MT’s length which shows unambiguously that increase crowding leads to assembly of longer MTs.

In Figure 4 we show the morphology of MTs in solid gels. For those samples with higher concentration of agarose, the MTs appear as thicker filaments which correspond to bundles of MTs. The average length of the thick filaments was quantified by the measurement of their projected lengths. At these
high agarose concentrations, the filaments exhibit lengths exceeding 30 \(\mu\)m and many of them were curved or contorted (Fig. 4).

Additional insight into the nature of the long, thick and contorted MT filaments was provided by the FESEM characterization of high concentration gels containing MTs. Figure 5a and b show that what appeared as a thick filament in the fluorescent microscopy images are indeed bundles of individual MTs.

Figure 2. Fluorescence microscopy images (100 X) of samples smeared on a microscope slide. These samples are in liquid solution with a very low agarose concentration. a) 0.0 wt %, b) 0.02 wt %, c) 0.035 wt %, d) 0.05 wt %, and e) 0.08 wt % agarose.

Figure 3. Effect of agarose content on MT's length at very low agarose concentrations (liquid solutions). Void circles represent different batches. Inset: Maximum lengths found in each sample.

Figure 4. Fluorescence microscopy (100 X) of MTs grown in solid gel a) 0.5 wt %, b) 0.75 wt %, c) 0.75 wt %, and d) 1 wt % agarose.

Figure 5. FESEM images of MT-gel samples. Surface images of: a) MT bundle at high magnification (0.75 % agarose), b) MT bundle (arrow) at the surface (0.75 % agarose). Fractured surface of a sample showing: c) MT bundle at high magnification (arrow pointing a single MT) (0.5 % agarose), d) same MT bundle at lower magnification, e) single MT at high magnification (0.5 % agarose), and f) same MT at lower magnification. Accelerating voltage was 5 kV.
In Figure 5a we show a bundle over the surface of a sample at 0.75 % agarose. In 5c and d a bundle emerging from a fractured surface of a sample at 0.5 % agarose is clearly visible. In both cases bundles have diameters very similar to diameters of the larger pores of the gel structure, approximately 200 to 300 nm. The bundle contains several tens of MTs that are shorter than the lengths of the bundles themselves. In Figure 5e and f we show a single MT in a fractured surface of a sample at 0.5 % agarose. In this sample we found few single MTs (as confirmed by diameters of 24–25 nm), but bundles were more common.

For the purpose of understanding the experimental observations, we have extended the two-dimensional reaction-diffusion model of MT growth introduced by Glade et al. to three dimensions. We believe that this model can be used to shed light on our experimental observations if not quantitatively, qualitatively, for the following reasons. Although we have grown MTs in the presence of taxol, it has been shown that taxol inhibits neither the binding of GTP nor its hydrolysis. Furthermore taxol depresses the critical concentration by changing the energetic of the binding of tubulin in MTs. In addition, Janosi et al. have found their GTP cap model in agreement with experiments of MTs grown in the presence of taxol. Based on these observations we assume that the GTP cap model used by Glade et al retains some level of validity.

The reaction and diffusion space of the model is a three-dimensional grid, organized as a cube (30 μm per side) with periodic boundary conditions applied in all directions. We used a hydrolyzing cap model, in which the growth of MTs is driven by the Tu-GTP concentration at its ends. Upon growth, the length of the Tu-GTP cap is increased. As the simulation steps forward in time, the Tu-GTP along the length of the microtubule is hydrolyzed into Tu-GDP at a constant rate. In the event that the cap becomes too short, the MT rapidly disassembles into solution, releasing Tu-GDP which can be regenerated in the solution back to Tu-GTP, due to an excess of free GTP and then contribute again to MT growth. To account for the effect of taxol on the growth of MTs we have modified the rate of reaction used by Glade in the following manner. Glades employed Hill’s equation for this rate. This equation describes a sigmoidal step function with a parameter \( \lambda_{\text{growth}} \) that correspond to the point of inflection of the function taken as the concentration of tubulin at which MT growth explodes. Glades takes this concentration at 2 mg ml\(^{-1}\). Since taxol depresses the critical concentration for MT growth we used a significantly smaller value of \( \lambda_{\text{growth}} = 0.1 \text{ mg ml}^{-1} \).

The model tracks the concentration of Tu-GTP and Tu-GDP at all points on the grid. We assume that the Tu-GTP and Tu-GDP diffuse independently of each other throughout the reaction space because their concentrations are far below the saturation point. We employ a finite difference scheme to solve the diffusion equation on a three-dimensional square grid with grid spacing of 0.5 μm. The diffusion equation and reaction kinetics are solved numerically at time intervals of 0.005 s. MTs may nucleate at any grid point with random orientation. The nucleation probability is chosen to be linearly dependent on the concentration of Tu-GTP in the vicinity of the nucleation site (we have tested a quadratic function of concentration which did not change qualitatively the results reported below). If conditions are optimal, there is a 1 % chance that a tube will nucleate at a given site during that cycle. The details of the linear dependence seem to have little effect on the self-organizational morphology of the system, as a 0.2 % nucleation probability factor produced results similar to the 1 % case. The 0.2 % probability factor per time step was arbitrarily selected because the time step was very small (1/150). MTs are not allowed to change direction once growth is initiated. Any MT that fails to grow may be replaced by another nucleus with some other orientation. In this way, microtubules will form preferentially along orientations conducive to their growth if given sufficient time, regardless of their initial orientations. For our calculations, a Tu-GTP concentration of 0.3 mg ml\(^{-1}\) was used because it closely resembles the concentrations used in our experiments.

In one test, we varied the diffusion coefficient of tubulin, equivalently simulating varying concentrations of agarose in the solution (Fig. 6). With fast diffusion, at the end of the calculation (polymerization time of 10 min) the simulated systems were essentially composed of short MTs (length ca. 1 μm) and few long MTs. We observed that a slower diffusion coefficient (i.e., higher agarose concentration) results in an increase in the number of long, stable MTs. These results are qualitatively in agreement with our experimental observations.
tions. Some quantitative discrepancies exist between the model and our experiments, but we believe that they arise from the choice of some parameters for the reaction kinetics which may not match exactly the actual chemistry of our tubulin solutions.

Even based on these calculations, we can not conclusively state that diffusion is the only factor that controls the length of the MTs growing in the crowded environment, indeed we have shown that the lengths of MTs may be controlled by the ratio of reaction rate of assembly to the diffusion coefficient. In such a case as discussed by Minton crowding that have shown that the lengths of MTs may be controlled by the state that diffusion is the only factor that controls the length of the MTs growing in the crowded environment, indeed we have shown that the lengths of MTs may be controlled by the ratio of reaction rate of assembly to the diffusion coefficient. In such a case as discussed by Minton crowding that leads to higher association reaction rates will also produce longer MTs.

In the second simulation, we constrained the direction of growth of the MTs to evaluate the possibility that the agarose structure of the solid gel influences MTs’ morphology. Limiting the MT growth by imposing a no-growth condition outside the pre-defined pathway enables us to simulate growth in solid agarose gels where MTs growth may be limited to the large pores of the gel (Fig. 1). The calculation shows that although the initial orientation of MT nuclei is random, the MTs that grow are those that align with the predetermined pathway (Fig. 7). The computer model does not allow bundling (i.e., MT-MT interaction) but it is apparent that the confinement of MTs inside the gel pore would favor MT-MT interactions leading to bundling. Therefore this observation combined with our experimental data agrees with Madden and Herzfeld.

The present work demonstrates that the morphology of MTs grown in gels is directly dependent on the tubulin diffusion coefficient. We have shown experimentally that as the gel density increases, i.e., tubulin diffusion coefficient decreases, the system evolves from an aqueous solution containing numerous short, straight single MTs to a distribution of a few, but long bundles of MTs. At high gel concentrations, the MT bundles are also contorted. MT bundling appears to be caused by MT confinement in the porous structure of the gel. This is supported by FESEM images indicating that the MT bundles have a diameter similar to that of the largest pores of the gel. MT bundling resulting from pore confinement may be due to MT electrostatic and/or steric interaction assisted also by the presence of taxol, which has been reported to facilitate the formation of bundles.

There are two differing explanations for this contorting effect. The first and simplest explanation could be attributed to directed growth along the large pores of the gel. A second explanation may be found in the capacity of a MT to exert a force on neighboring MTs during the process of polymerization producing compression stresses along the longitudinal axis of the bundles, leading to buckling.

A three-dimensional reaction-diffusion model of MT dynamics, where the diffusion coefficient of free tubulin was varied, produced MT length distributions in good qualitative accord with the experimental results. Application of this model to the growth of MTs in a confined pore-like space showed that although the initial orientation of MT nuclei may have been random, MT growth aligned along the predetermined pathway. These results support our speculation about the bundling of MTs due to confinement by the gel.

Agarose gel provides an excellent environment for growth of arrays of MTs in solid or semi-solids environments. Agarose gels do not interfere chemically with the MT polymerization process, although they do physically interact with the samples by modifying the diffusion coefficients of the reacting species and possibly constraining MT growth. In addition to providing physical support for MT arrays, agarose gels could be used to promote altered MT morphologies such as the anisotropic MT growth in an electric field.

**Experimental**

A low gelling temperature agarose [ultra pure Agarose low melting point, gelling temperature (2 wt %) 26–30°C, remelting temperature (2 wt %) ≤ 65°C from GibCobrl Life technologies] was mixed with PEM80 buffer (80 mM PIPES, 0.5 mM EGTA, 4 mM MgCl2) at a pH of 6.9, and boiled to form a clear solution. We prepared solutions with agarose concentrations of 1%, 0.75%, 0.5%, 0.25%, 0.1%, 0.08%, 0.065%, 0.05%, 0.035%, 0.02%, and a sample containing no agarose (0%) was used as a control.

The agarose-buffer solutions were cooled down to 32°C and maintained at this temperature in a liquid state. To this, guanosine 5' triphosphate (GTP) and Paclitaxel (taxol) from Sigma were then added and the samples vortexed, the final concentration of GTP and Paclitaxel were 0.208 mM and 0.016 mM respectively. Fluorescent rhodamine tubulin (Cytoskeleton cat. #T331M) was added and mixed; the final concentration of tubulin was 0.33 mg mL−1. A small amount of this mix (5–10 μl) was put into several small cuvettes (3 mm in diameter and 1 mm thick with an optically transparent bottom). The sam-

![Figure 7](image-url)
samples were subsequently cooled down to 4 °C during 10 min in order to form a solid gel. The low temperature treatment also served to disassemble any tubulin that may have polymerized during the mixing process (the absence of polymerized tubulin was verified by fluorescence microscopy). Then the cuvettes filled with tubulin-containing agarose gels were placed into an incubator at 37 °C for 30 min to support MT polymerization. Finally the samples were stored at room temperature prior to their characterization by fluorescence optical microscopy. Also we prepared several specimens for FESEM analysis. MT-gel samples were fixed with a solution of 0.05 M PIPES buffer pH 7.4 and 3 % of glutaraldehyde during three hours. The samples were then desiccated by means of critical CO2 dry point, and finally a platinum coating was applied to the surface of the samples by sputtering. Control gel samples which do not contain MTs were prepared using the same agarose and PEMS80 buffer used before, but no tubulin. GTP or taxol were added. The same process of gelation was followed as well. The pure gel sample preparation for FESEM analysis was done in a way identical to that of the MT-gel samples.

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