Gravitational Symmetry Breaking Leads to a Polar Liquid Crystal Phase of Microtubules *In Vitro*

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Abstract. Recent space-flight experiments performed by Tabony's team provided further evidence that a microgravity environment strongly affects the spatio-temporal organization of microtubule assemblies. Characteristic time and length scales were found that govern the organization of oriented bundles under Earth's gravitational field (GF). No such organization has been observed in a microgravity environment. This paper discusses physical mechanisms resulting in pattern formation under gravity and its disappearance in microgravity. The subtle interplay between chemical kinetics, diffusion, gravitational drift, thermal fluctuations, electrostatic interactions and liquid crystalline characteristics provides a plausible scenario.

Key words: microtubules, microgravity, tubulin, assembly, pattern formation

Microtubules (MTs) are the key protein filaments of the cytoskeleton in eukaryotic cells [1]. In their assembly behavior, growing phases are stochastically interrupted by catastrophic depolymerization events (CDE) caused by so-called dynamical instability [2]. So far, only sporadic efforts have been made to understand the influence of electrical [3], gravitational [4], magnetic [5] and electromagnetic [6] fields on MT dynamics.

Symmetry breaking is a powerful concept relevant in various fields including developmental biology where spatial symmetry breaking elucidates pattern formation in tissue development [7]. Transitions mediated by chemically reactive substances leading to symmetry breaking phenomena are crucial in the establishment of physico-chemical self-organization in biology [7, 8].

Many components of living cells [9] such as cell membranes, DNA in chromosomes, and proteins in muscle and in connective tissues (collagens), exhibit liquid crystal (LQ) properties. Present physical considerations demonstrate that under GF, *in vitro* MTs assemblies behave as typical polar LQs.

Tabony's team, in a series of experiments (Tabony's experiments) demonstrated a significant role of GF in the formation of spatio-temporal patterns by assemblies

of MTs *in vitro* [10–13]. In these experiments, purified tubulin (TB) was polymerized by warming the sample from 7–37 °C in an appropriate solution. MTs were assembled in rectangular spectrometer cells with dimensions $40 \times 10 \times 1$ mm. The MT assembly in Tabony's experiments exhibits a gradual and progressive self-organization lasting approximately 12 h and eventually reaching equilibrium macroscopic patterns of both MT concentration and orientation. The most striking result obtained when the largest dimension (40 mm) is parallel to the GF shows a pattern of horizontal stripes of MTs. When the smallest dimension (1 mm) is parallel to the GF, circular vortex-like morphologies appear. Therefore, the sample's orientation with respect to the GF crucially and irreversibly affects MT self-assembly if the process is unperturbed for at least the first 6 min. Once this critical period has passed, the morphology of self-organization that subsequently develops is independent of sample orientation.

Under microgravity conditions (2×10^{-4} g) [13], specimens which underwent the self-assembly process for at least the first 10 min at microgravity, show no specific pattern formation. A second set of samples (control) taken into space was subjected to an equivalent of 1 g obtained by centrifugation and showed striped or circular patterns of self-organization in complete analogy to the results obtained on Earth. Importantly, GF was pointed out [10–13] as a trigger for spatial self-organization under conditions close to a bifurcation point for these non-linear systems.

In the experiments with the largest dimension (40 mm) oriented parallel to the GF (1 g), after the initial period of 6 min and over the next period of 3 h the development of larger scale patterns covering the area of 10×40 mm was observed (Figure 1a). The pattern seen is that of parallel bundles of MTs forming what we call “curtains” that are respectively at angles of 45° and 135° to the horizontal axis, x -axis in Figure 1a, and at a right angle to each other.

The next stage of self-organization was observed between 3 and 12 h from the start of Tabony's experiments; horizontal stripes of MT bundles with an approximate width of 0.5 mm appeared. In addition to the above periodicity, periodic patterns with characteristic dimensions of 3 mm, 100 μ m, 20 μ m, 5 μ m and 1 μ m could be discerned as shown in Figure 3 of [13]. This structured pattern is illustrated in (Figure 1b). It was also reported [13] that the average length of MTs was approximately $L = 5 \mu$ m.

In a dilute solution of TB (at 10 mg/ml), the TB–TB interactions at an average distance can be neglected, being smaller than their thermal energy $k_B T$, at room temperature. In such a system, TB density fluctuations in a volume element exhibit a Poisson distribution reflecting a thermally-induced disorder. Increasing T above 7 °C raises the TB–TB binding rate according to the Arrhenius law. Consequently, TB forms cylindrical polymers – MTs – inducing TB concentration gradients. Due to simultaneous depolymerization, GDP-TB that is not assembly competent is released into solution. This form of the TB dimer can be thought of as promoting uniformity of distribution since it does not bind to other dimers prior to rephosphorylation.

Figure 1. (a) Approximately 20 min after the onset of assembly, when the dimension of 40 mm is parallel to g during the first 5 min, the “curtains” of highly oriented MTs develop. On the left half are MTs with an angle of 45° , and on the right half MTs with an angle of 135° , with respect to the horizontal x -axis. The angle between bundles in the middle is 90° . (b) The characteristic periodicities occurring within a single stripe in experimental samples exposed to gravitation of g during the first 10 min, developed after 12 h.

Diffusion plays an important role in MT-assembly for centrosome-nucleated MTs that are less than $5 \mu\text{m}$ long [14]. Furthermore, MTs have been shown capable of mutual interactions via TB concentration gradients if they are separated by a distance less than a characteristic diffusion growth length defined by $l_d = DV_g^{-1}$,

where D is the TB diffusion constant and V_g is the MT growth rate. For TB from sea urchin eggs, $D = 5.9 \times 10^{-12} \text{ m}^2/\text{s}$ and $V_g = 7 \text{ } \mu\text{m}/\text{min}$, giving $l_d = 50 \text{ } \mu\text{m}$. Since the average separation between MTs in TE is on the order of $0.3 \text{ } \mu\text{m}$, the condition for TB gradient-mediated MT interactions is safely met.

Another possible mode of MT movement and reorganization in solution is via treadmilling in which the $+$ end grows at the same rate that the $-$ end shrinks. The *in vitro* MT treadmilling rate reaches $4.2 \text{ } \mu\text{m}/\text{min}$ [15], which would give a treadmilling distance over 6 min of $25 \text{ } \mu\text{m}$ that is close to one of the periodicities shown in (Figure 1b). For the reported time of 12 h, treadmilling could reach the 3 mm limit, in agreement with the greatest periodicity in TE [12].

Both non-linear chemical kinetics and symmetry breaking disturb the otherwise Poissonian nature of the TB spatial distribution. In addition to the anisotropy of the two ends of a MT, there is an asymmetry of the growing and shrinking processes. While growth is gradual, shrinking can be a CDE when a lateral GTP cap at the $+$ end is lost. We proposed earlier a solitonic mechanism as a possible trigger for CDEs in MT dynamics [16–18]. The crucial point is that a single CDE can result from a disassembly of thousands of TB dimers [19]. However, the thus depolymerized TBs remain correlated spatially via a short-range order associated with non-Poissonian behavior.

Thus, in addition to diffusion, another mechanism playing the role of symmetry breaking is drift induced by GF. This avalanche-like correlated cluster (ACC) will over time spread through the available volume of exploration (Figure 2). The scenario for the spatio-temporal MT self-organization, at the onset of Tabony's experiments, strongly relies on the interplay of CDE and ACC dynamics. In this connection, we estimate the Peclet number characterizing the sedimentation of denser clusters in a solution [20], being defined as the ratio of the cluster's gravitational and thermal energy.¹ For a single TB dimer with a mass of $m_{\text{TB}} = 1.66 \times 10^{-22} \text{ kg}$ and a diameter of roughly $d_{\text{TB}} = 6 \text{ nm}$, the Peclet number is $\approx 10^{-8}$ indicating that the gravitational drift of a single TB dimer is negligible. However, this is vastly different for an ACC of TB dimers released back into solution following many correlated CDEs. Assuming that the diameter of an average ACC is on the order of the smallest periodicity in the striped configuration observed in Tabony's experiments, $d_{\text{ACC}} = 1 \text{ } \mu\text{m}$, we estimate the mass of an ACC as $m_{\text{ACC}} = 10^{-16} \text{ kg}$. At $T = 310 \text{ K}$, the Peclet number is $P_e = 0.1$. Since a predominantly diffusive behavior occurs for $P_e \ll 1$, it follows that drift-led sedimentation becomes a competitive process in the case of ACC.

For TBs to be recycled after a catastrophic depolymerization event, GDP-TB must be converted to GTP-TB (since only GTP-TB is assembly competent). It is expected that an ACC sweeping through solution facilitates GDP rephosphorylation. Such recycled ACC showers encounter nucleation centers so that new MTs grow predominantly in the direction of gravitational drift (Figure 2). The TB dimer distribution around a single growing MT is oriented in the z -direction based on [21].²

Figure 2. (a) Avalanche-like correlated cluster (ACC) of TB created by CDE falls toward nucleation centers. (b) Showered by rephosphorylated TB, nucleation centers have grown in highly oriented parallel MT's. This brings about the onset of LQ phase.

Without ACC effects, it follows [21] that the concentration gradient of TB around a growing MT decays to the background level within ≈ 50 nm of the MT tip. With ACC effects, however, the correlated TB mobility is comparable to the MT growth rate V_g and the TB concentration rate increases dramatically, yielding a spatially correlated MT growth.

The correlation length for this reaction-diffusion-drift effect is $l_{\text{cor}} = [\frac{D(1+P_e)}{K_{\text{CDE}}}]^{1/2} = 23 \mu\text{m}$, where K_{CDE} is the CDE rate taken to be 0.012 s^{-1} [14], $P_e = 0.1$ and $D = 5.9 \times 10^{-12} \text{ m}^2/\text{s}$. This value is also close to the observed periodicity of $20 \mu\text{m}$. Approaching the bifurcation point this correlation effect becomes macroscopic, giving rise to a globally self-organized state with associated domains (“curtains”) of highly oriented MTs (Figure 1). This is a collective phenomenon which cannot be explained by a single chemical reaction and its rate. Crucially, the coupling of a local chemical process (CDE) with transport processes (diffusion and drift due to GF) results in large deviations from the equilibrium TB density distribution further magnified non-linearly via chemical kinetics. In the absence of coupling between volume elements, these density fluctuations are thermalized. Furthermore, impeding chemical kinetics, as shown in Tabony’s experiments [11] with taxol stabilization of MTs, leads to homogenization of TB concentration.

Thus, both physical and chemical aspects of the process are needed to create spatial and temporal self-organization due to their comparable characteristic time scales. The characteristic time scale for the TB spread by diffusion and ACC effects through length L in the z -direction is $\tau_{\text{tr}} = L^2[D(1+P_e)]^{-1}$. When spatial coherence is established over a macroscopic distance it requires a substantial amount of time due to slow diffusion and gravitational drift. Taking the characteristic length scale from Tabony’s experiments to be $L = 0.5 \text{ mm}$, together with D and P_e used before, yields $\tau_{\text{tr}} = 4.1 \times 10^4 \text{ s} \approx 11 \text{ h } 24 \text{ min}$. This is very close to the time required for the formation of the striped patterns in Tabony’s experiments [12], reported to be 12 h (Figure 1).

We earlier conjectured [16,17] that single MTs possess ferroelectric and piezoelectric properties. The successful X-ray crystallography determination of the atomic-resolution structure of TB [22] enabled molecular dynamics simulations that lend additional support to some of these predictions. It was reported [23] that a large negative charge is distributed ($\approx 20e$ per TB monomer) on the surface of MTs with visible asymmetry between the $+$ and $-$ ends. The TB’s permanent dipole moment was calculated as approximately 1800 debye [24], almost perpendicular to the protofilament axis. Chiral ferroelectric features of TB dimers within polymerized MTs underlied a LQ model [18] for the non-linear dynamics of a single MT leading to the above mentioned solitonic trigger mechanism for ACC.

However, the net charge on MT’s is mostly screened by counterions in physiological solutions. The parameters relevant for electrostatic interactions between MT cylinders are:

- The Bjerrum length l_B , which is relevant for MT tips. Although as many as $20e$ charges can reside on each TB monomer in vacuum, a small fraction of this number pertains to the exposed end since most are located on the outer surface of an MT. Estimating conservatively that two elementary charges per TB are present on each bottom tip and assuming that only 20% of this charge is unscreened and

gives an effective charge per tip to be approximately $q_{\text{eff}} = 5e$, and the Bjerrum length $l_B = q_{\text{eff}}^2 (4\pi \epsilon_0 \epsilon k_B T)^{-1}$, with $\epsilon = 80$ for water, at room temperature, to be roughly $l_B = 20$ nm. This is comparable to the diameter of the MT cylinder $d = 25$ nm, and indicates that two MTs separated by l_B measured tip to tip can be electrostatically correlated, leading to the LQ phase of MT bundles.

- The screening effects of positive ions are reflected by the Manning parameter $\xi = \frac{l_B}{b}$, where b^{-1} is the linear density of condensed counter-charges along MTs. The effective linear charge density is $\tilde{q}_{\text{eff}} = \frac{q'}{L} = (Z\xi)^{-1}$ where Z is the counterion valence. For DNA, if q_{eff} goes below 10% of the unscreened value, then electrostatic repulsion between two chains switches to attraction [25].

Due to correlated fluctuations of counterions, the forces between MT cylinders should become attractive at high screening and are additive with respect to cylinder–cylinder attraction [26]. The effective free energy³ due to the screening charge fluctuations calculated for two MT bundles separated by a distance R with the angle of θ consists of three terms: two repulsive terms, due to monopole–monopole and dipole–dipole interactions, and one attractive van der Waals term expressed for smaller R . The attractive term is only angle-dependent through $(2\cos^2\theta + 1)$. The free energy minimization with respect to θ leads to two values, $\theta = 0$ and $\theta = \pi/2$. This corresponds to parallel orientations of MT bundles within each half of the “curtains” (Figure 1a) and the angle $\theta = \pi/2$ the boundary of the two halves.

Furthermore, even long neutral cylinders tend to align parallel to each other when their density exceeds a critical number [27], namely $\nu_{\text{cr}} \sim L^{-2}d^{-1}$ (for MTs, $L = 5 \mu\text{m}$ and $d = 25$ nm, $\nu_{\text{cr}}^{\text{MT}} \sim 1.5 \times 10^{18} \text{ m}^{-3}$). In Tabony’s experiments, the TB concentration is 10 mg/cm^3 , roughly 70% of which is assembled into MTs. Thus, $\nu_{\text{TE}}^{\text{MT}} = 3 \times 10^{18} \text{ m}^{-3} > \nu_{\text{cr}}^{\text{MT}}$, satisfying the conditions for the onset of a LQ phase. Consequently the LQ features dominate the pattern formation after the bifurcation point is reached under gravitational ACC effects. Thus the free energy thermodynamics of this polar LQ together with a high viscosity of 50 Pa s [13] prevents the MT sedimentation within the “curtain” pattern.

LQ features of Tabony’s experimental system also exert influence on the alignment of MTs within the surface of the cell. A cylindrical MT can either be perpendicular, parallel or obliquely oriented to the glass surface of the cell. For long MT cylinders ($L \gg d$) in Tabony’s experiments, condensing many MT ends at the surface carries a high entropy cost and is therefore unlikely to occur. This is consistent with the experimental fact that MTs are aligned parallel to the cell wall corresponding to the largest area $2 \times (40 \times 10) \text{ mm}^2$ and pointing toward the smallest surface area. Moreover, due to an oblique alignment of MTs with the smaller surface areas ($\theta = \pi/4$) a further reduction in the number of MTs in contact is accomplished. Since the position of a MT end with respect to the wall is determined up to one Bjerrum length $l_B \approx d = 20 \mu\text{m}$, this significantly reduces the configurational

entropy of one MT in contact by $\Delta S_{\text{conf}} = k_B \ln \left(\frac{L}{a} \right) \approx 5.3k_B$. This is why MTs in Tabony's experiments are not vertically oriented as expected from gravitational ACC effects responsible for the oriented growth of MTs. This orientation forming a close-packed layer of MTs perpendicular to the surface would create a new surface a distance L into the bulk, at which location the next layer of MTs must be properly arranged, further reducing the entropy. Oblique orientations induce the splay effect, especially near the corners of the experimental cell; gaps between MTs open up, into which other MTs can penetrate, restoring the random distribution of MTs ends. However, in the bulk, the highly parallel oriented MTs still have to "solve" the cooperative entropy reduction problem.

The rotational relaxation of a single cylinder of fixed length is hindered by its disordered neighbors when cylinder concentrations exceed $\nu^* = L^{-3}$. With $L = 5\mu\text{m}$, one obtains $\nu^* = 10^{16} \text{ m}^{-3}$, which is much more dilute than in Tabony's experiments. This is why in the low gravitational Tabony's experiments no pattern formation appears.

Disorderly grown MTs are prevented from establishing LQ order. Conversely, if the system already possesses highly parallel ordering, as in the case of gravitationally directed MTs within "curtains", then the entropy-frustrated state should be relaxed by the spread of slow rotational waves [28] starting from the walls and lasting approximately 12 h [12]. These rotations are facilitated by existing dynamical instability of MTs but are less dramatic than at the moment of chemical instability, 6 min after the onset of the reaction. The most likely contribution to this thermodynamic stabilization comes from treadmilling processes. The newly formed stripes significantly increase the entropy due to splay deformations (Figure 1b).

Implications of the microgravity experiments and their explanation extend to cell behavior which was seen to be affected by microgravity in another space flight experiment [29]. As recently reported [30], in climbing plants competing for light energy, MTs create a twist obliquely oriented toward the cell's long axis in the root. This is suggestive that symmetry breaking by the GF, together with structural changes within α -TB subunits related to the rigidity of MTs, is the mechanism responsible. Patterns in Figures 3 and 4 of [30] clearly reveal the LQ phase of MTs discussed in the present paper.

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Notes

1. The Peclet number is defined as $P_e = (1 - \rho/\rho_0)mgd(k_B T)^{-1}$, where m is the cluster mass, g the constant of gravity, d the diameter of the cluster, ρ the density of solution, ρ_0 the density of the cluster and $k_B T$ the thermal energy.
2. If the direction of the z -axis coincides with $\vec{g}$, the equilibrium TB concentration $C(r, t)$ around a single growing MT is described by:

$$D\nabla^2 C + \mu g \frac{\partial C}{\partial z} - V_g \frac{\partial C}{\partial z} = 0$$

where $\mu = mD(1 - \rho/\rho_0)(k_B T)^{-1}$ is the TB mobility within ACC. Using cylindrical symmetry and $\zeta = \sqrt{(r^2 + z^2)}$, the solution of the above equation is

$$C(r, z) = C_\infty - \frac{W}{4\pi D\zeta} \exp\left[-\frac{V_g - \mu g}{2D}(\zeta - z)\right]$$

where C_∞ is the asymptotic TB concentration as $\zeta \rightarrow \infty$, and $W = 4\pi\zeta^2 D(\frac{\partial C}{\partial \zeta})_{\zeta=\zeta_0}$, with ζ_0 being the radius of the growing MT tip.

3. The free energy per unit volume attributed to the counterion charge fluctuations at MTs in solution calculated for two bundles separated by a distance R and containing N cylinders/m² of the area normal to the MT's axis is:

$$F_{\text{vol}} = N^2 k_B T \left\{ \alpha \frac{1}{R^2} + R\kappa^4 \ln(2\kappa R) - \frac{1}{R^3} [\beta_1 + \beta_2(2 \cos^2 \theta + 1)] \right\}$$

where θ is the angle between the orientations of the two bundles, $\kappa^{-1} = (\frac{\epsilon_0 \epsilon k_B}{4\pi n_c e^2})^{1/2}$ is the screening length with n_c denoting the average concentration of the condensed counterions along MTs and ϵ the dielectric constant of the solution. The effects of the apparent dielectric anisotropy of MTs are incorporated through remaining model parameters α , β_1 and β_2 .

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