Spinodal decomposition and the emergence of dissipative transient periodic spatio-temporal patterns in acentrosomal microtubule multitudes of different morphology

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We have studied a spontaneous self-organization dynamics in a closed, dissipative (in terms of guansine 5′-triphosphate energy dissipation), reaction-diffusion system of acentrosomal microtubules (those nucleated and organized in the absence of a microtubule-organizing centre) multitude constituted of straight and curved acentrosomal microtubules, in highly crowded conditions, *in vitro*. Our data give experimental evidence that cross-diffusion in conjunction with excluded volume is the underlying mechanism on basis of which acentrosomal microtubule multitudes of different morphologies (straight and curved) undergo a spatial-temporal demix. Demix is constituted of a bifurcation process, manifested as a slow isothermal spinodal decomposition, and a dissipative process of transient periodic spatio-temporal pattern formation. While spinodal decomposition is an energy independent process, transient periodic spatio-temporal pattern formation is accompanied by energy dissipative process. Accordingly, we have determined that the critical threshold for slow, isothermal spinodal decomposition is 1.0 ± 0.05 mg/ml of microtubule protein concentration. We also found that periodic spacing of transient periodic spatio-temporal patterns was, in the overall, increasing versus time. For illustration, we found that a periodic spacing of the same pattern was 0.375 ± 0.036 mm, at 36 °C, at 155th min, while it was 0.540 ± 0.041 mm at 31 °C, and at 275th min after microtubule assembly started. The lifetime of transient periodic spatio-temporal patterns spans from half an hour to two hours approximately. The emergence of conditions of macroscopic symmetry breaking (that occur due to cross-diffusion in conjunction with excluded volume) may have more general but critical importance in morphological pattern development in complex, dissipative, but open cellular systems. © *2013 AIP Publishing LLC*.
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Periodic patterns transiently accompany slow isothermal spinodal decomposition in a closed and dissipative acentrosomal microtubules' reaction-diffusion system constituted by multitude of straight and curved microtubules. Indeed, at highly crowded conditions, i.e., above the bifurcation threshold, cross-diffusion in conjunction with excluded volume is the key driving force of these processes. This finding strongly supports the recent predictions that a variety of transient periodic spatio-temporal structures may emerge in reactive-diffusion systems due to cross-diffusion in conjunction with excluded volume where "the spatial distribution of one species may affect the motion of other species."[1] Acentrosomal microtubules play a critical role in cellular signalling, which is accompanied by cellular morphological changes such as those occurring in dendritic spines during memory formation and learning.[2–5] The emergent conditions of symmetry breaking may have quite general but critical importance in morphological pattern development and signal processing in complex, dissipative, and open biological systems. These emergent conditions occur due to a cross-diffusion in conjunction with excluded volume and apart from energy dissipation, which enable transient periodic pattern formation in acentrosomal microtubules multitudes, in reaction-diffusion closed system *in vitro*. This work confirms as revealed in other related studies that microtubules have great non-linear dynamic capacity to form and reform an

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abundance of different spatio-temporal patterns, which enables them to process and encode biological signals during cell division and tumorigenesis. Furthermore, this study may be helpful in understanding the fundamental nature of the mechanisms of these phenomena.

I. INTRODUCTION

This study pursues to better understand the fundamental mechanism(s) of microtubule self-organization phenomena *in vitro*. Therefore, it is directly relevant to understanding of the self-organizational phenomena of *acentrosomal* microtubules *in vivo*. In order to facilitate the interpretation of the results obtained in this work, we will first outline the basic physico-chemical (thermodynamics) points regarding acentrosomal microtubules *in vitro*. We will then briefly describe the biological relevance of acentrosomal microtubules.

A. The basic physico-chemical (thermodynamics) characteristics of acentrosomal microtubules *in vitro*

1. *Experimental microtubule's system* in vitro *is closed system*

All in vitro experiments were performed with microtubules and their precursors in cuvette at a given temperature. After the necessary components (see Materials and Methods) were placed in the experimental cuvette, nothing else was added nor it was taken out. The heat was constantly supplied to the system in order to hold the system at a given working temperature of 36 °C (unless differently indicated). Therefore, the system is thermodynamically closed system.

2. *Microtubule growth and self-organization: Consumption and dissipation of energy, irreversible hydrolysis of guansine 5′-triphosphate (GTP), and dynamic instability*

The growth of a single microtubule, and a spatio-temporal self-organization of a microtubules multitude, is considered to be a non-equilibrium and a dissipative system.[6–12] The microtubule growth and self-organization of its spatio-temporal structures *in vitro* requires continual consumption and dissipation of energy, which comes from organic phosphate contained in reservoir of GTP. Therefore, in order to initiate and maintain microtubule growth, and its spatio-temporal self-organization, *in vitro*, it is necessary to add an appropriate amount of GTP in the experimental cuvette at the beginning of the experiment.

$\alpha\beta$-tubulin (100kDa) bound by GTP or GDP (further GTP-tubulin and GDP-tubulin) are basic building blocks of a microtubule wall. However, the microtubule self-assembly proceeds *only* after the addition of $\alpha\beta$-tubulin-GTP to each tip of both microtubule ends. The GTP-tubulin is considered to be the activator, while GDP-tubulin is considered to be the inhibitor of the microtubule assembly. GTP-tubulin addition includes its association/dissociation. The addition is accompanied by somewhat lagging irreversible hydrolysis of GTP bound at exchangeable site (E) at β-tubulin subunit. After GTP hydrolysis, GDP-tubulin remains within microtubule lattice, while inorganic phosphate (P_i) is released into solution. It is considered that one part of the free energy, obtained by GTP-hydrolysis, is dissipated as free energy accompanied with (P_i) release, while the other part is stored (in a form of GDP-tubulin) in microtubule wall lattice as a mechanical strain.[9,13] Intermediate part of a microtubule wall built by GDP-tubulin is less stable than terminal part, which is prevalently built by GTP-tubulin.[8] Terminal part, which has stabilized the whole microtubule structure, is called GTP-cap.[14–17] The energy of the mechanical strain powers fast microtubule disassembling during dynamic instability.[18,19] What is dynamic instability? Dynamic instability is the key and the most peculiar feature of microtubule growth discovered in the mid 1980s,[17,20] and recent detailed elaboration can be found elsewhere.[21] Dynamic instability consists of stochastic and abrupt changes in microtubule length. This instability includes a switch (catastrophe) between a phase of stable growth to fast depolymerisation, and a reverse switch (rescue), due to which the growth is resumed. It is due to the dynamic instability (i.e., due to irreversible GTP-hydrolysis as primary cause) that microtubule growth is a non-equilibrium phenomena accompanied by the dissipation of energy and should be described by an appropriate non-linear concept.[13,22,23] Dynamic instability is tightly related to irreversible GTP-hydrolysis. It is considered that after hydrolysis, part of microtubule wall, which is prevalently built by GDP-tubulin, is less stable and may lead, under some conditions, to microtubule fast depolymerisation. For example, once hydrolysis reaches the tip of microtubule, rapid depolymerisation may start. This depolymerisation may destroy the whole microtubule or may be arrested due to rescue and the growth may resume. Therefore, due to dynamic instability, and regardless of the bulk critical concentration within the multitude of growing microtubules, there are always a certain number of microtubules that shrink.

The ability of dynamic instability to enable microtubule to disassembly and reassembly during self-organization has a far reaching biological consequences. For example, during self-organization and due to disassembly and reassembly, the microtubules can change its length and orientation in very fine readjustments.[24]

For the purpose of this work, let us point out that due to dynamic instability, curved microtubule can be transformed into straight ones, and vice versa. In addition, local fluctuations of both GTP-tubulin and GDP-tubulin and consequently local displacement and reorientation of curved and straight microtubules may occur due to dynamic instability as the first cause.[25–28]

3. *Reaction-diffusion system, excluded volume, and local fluctuations*

It is considered that reaction-diffusion processes play pivotal role in microtubule growth, as well as in spontaneous spatio-temporal self-organization of microtubule multitudes.[28–31]

As an activator GTP-tubulin promotes microtubule growth, while GDP-tubulin as an inhibitor inhibits microtubule growth. Growing end of microtubule may deplete GTP-tubulin from its frontal zone, while shrinking end may leave

the zone behind with increased concentration of GDP-tubulin. Thus, initially homogenous solution becomes inhomogeneous asymmetrically, due to GTP-tubulin and GDP-tubulin concentration fluctuations. These fluctuations are direct consequences of a microtubule assembly dynamics and the intrinsic asymmetry of a microtubule body. These fluctuations are massively enforced by dynamic instability. Since microtubule length is at the scale of microns, it is expected that local fluctuations, with their asymmetry, occur at the same scale. It is important to point out that the fluctuations are athermal and have a local character, i.e., they are not caused by any gradient.[27] It is obvious that inhomogeneity of microtubule assembly solution emerges endowed with certain spatial symmetry qualities. On the other hand, asymmetry in spatial distribution of GTP-tubulin and GDP-tubulin may lead directly to the local diffusion of GTP-tubulin and GDP-tubulin. Now, let us imagine that each microtubule is surrounded by its neighbouring microtubules. It is obvious that neighbouring microtubules will mutually affect each other due to creation of the pattern of local concentration fluctuations of GTP-tubulin and GDP-tubulin and its local diffusion. For example, if the growing tip of a neighbouring microtubule hits a zone depleted of GTP-tubulin by other microtubule(s), or it hits a zone with increased GDP-tubulin concentration (due to depolymerisation of other microtubule(s)), it will stop to grow, and likely proceed to disassembly. The spatial configuration of local fluctuations may induce directional changes in growing microtubule.[28] Thus, one can say that neighbouring microtubules may "communicate" through the changes of local concentration, i.e., through the local diffusion of GTP-tubulin and GDP-tubulin. Here, we may recognise phenomena known as "cross diffusion" found in a quite different chemical systems.[1] The magnitude of these effects will increase with the number of microtubules in a solution. Furthermore, the effect of excluded volume will become more prominent as neighbouring microtubules get closer to each other. The effect of excluded volume will also be enhanced significantly with the presence of microtuble-associated proteins (MAPs).

Numerous studies of spontaneous self-organization phenomena have shown that excluded volume is a critical parameter, which drives the self-organization process in the systems constituted by the rod-like particles.[10,32–35] Since the microtubule is long hollow cylinder (microns) with an outer diameter of 25 nm, it is reasonable to expect that the effect of the excluded volume is significant.

On the other hand, it has been argued that microtubule form dissipative structures that self-organise over macroscopic distances exclusively by a combination of reaction and diffusion.[12,24,28,31,36]

However, it is likely that both factors, reaction–diffusion as well as excluded volume, act synergistically during microtubule self-organization. In addition, MAPs are structural part of microtubule cylinder in biological systems. They are densely distributed across microtubule surface, and they protrude from the outer surface of microtubule wall for about 15 nm in a solution. Thus, they effectively increase microtubule diameter of up to about 55 nm. MAPs play a dual role as spacers between the neighbouring microtubules, but also as joiners.[37] Due to MAPs, the neighbouring microtubules cannot be stack to each other, and yet cannot escape either because of MAPs-MAPs strong electro-static interactions.[38] They are thus adjoined at a distance and form a spatial network. Obviously, the effect of excluded volume is significant in microtubule system, in particular in those with MAPs. *In vitro* preparations, MAPs may or may not be present. In our system, MAPs are present. Thus, in interpretation of some of the results, this has to be taken into account due to the effect of excluded volume.

4. Some relevant findings in this work

It was predicted recently that significant cross-diffusion phenomena can occur between two different species in the reaction-diffusion systems where excluded volume plays an important role, which may induce a spatio-temporal symmetry pattern formation.[1,39] Apropos of this prediction, our data may give experimental evidence that cross-diffusion in conjunction with excluded volume is the underlying mechanism on the basis of which a demix of microtubules with different morphology (straight and curved) may occur.

We found that demix is constituted by two distinct processes: a very long and a short one. The long demix starts at a very early stage of microtubule assembly (at about 2–3 min from the initiation of microtubule assembly) and it proceeds, as far as we observed, up to 12 h after microtubule assembly commenced. Since the chemical energy of GTP is already consumed at a very late stage of this long running demix indicates that this demix is not energy dependent. Furthermore, at an intermediate interval (approximately between 90th and 305th min after microtubule assembly commenced), the new type, that is, the short running process of demix is observed. The short running demix is manifested by the formation of periodic spatio-temporal patterns. These patterns have transient character (their duration is between 30 min to 2.0 h). Their duration coincides with a period of energy dissipation obtained by GTP hydrolysis.

At this stage, we speculate that each, short and long running, demix components are driven by cross-diffusion in conjunction with excluded volume. Short running demix forms a transient periodic patterns and depends on the energy obtained from GTP hydrolysis. This, in turn, is responsible for the formation of local concentration pattern of GTP-tubulin and GDP-tubulin that, in final instance, leads to a microtubule local displacement and a microtubules transient periodic pattern formation.[28]

In conclusion, this work shows, perhaps for the first time, that two spontaneous processes of self-organization of microtubules occur in the closed system of acentrosomal microtubules of different morphology. We have identified the first process as a long isothermal demix of straight and curved microtubules. This demix, although accompanied by cross-diffusion (i.e., local fluctuations and excluded volume effects), is not accompanied by dynamic instability (i.e., it is not accompanied by energy consumption). We recognized this long demix process as an isothermal slow spinodal decomposition.[40] We identified the second process as a spontaneous process of the spatio-temporal transient periodic

microtubule pattern formation, which is accompanied by dynamic instability, energy dissipation, and cross diffusion. These two processes may coincide for the certain period of time.

B. Biological relevance of acentrosolmal microtubules

Microtubules are one of the three major components of the cytoskeleton of eucaryotic cells. Within a cell, they appear as centrosomal microtubules (those apparently nucleated and spatio-temporally organized by microtubules organizing a center (MTOC)), and acentrosomal (non-centrosomal) microtubules (those nucleated and organized in the absence of a MTOC).[41–43] Acentrosomal microtubules are present in a substantial number in a variety of cell types from yeast, through plant to mammalian cells.[44] Acentrosomal microtubules prevalently appear as spatio-temporally ordered structures, including microtubule bundles, arrays, and networks, in a variety of cells including neuronal cells, epithelial cells, and muscle cells.[41,45,46] It was found that if negative (slow) end of microtubule is blocked, then acentrosomal (free) microtubule may exist in a solution or in a cytoplasm, so that it may undergo a variety of self organizing processes.[41,47] Furthermore, it was proposed that acentrosomal microtubules spatio-temporal behavior is ruled by the self-organization as the leading principle in conjunction with specialized proteins including MAPs.[41] In regard to acentrosomal microtubules spatio-temporal nucleation and self-organization, recent experimental data have indicated that specialized proteins, such as gama-tubulin,[48–50] MAPs (non-motor) proteins,[41,51–56] motor proteins,[57–61] including the proteins from pericentriolar materials (PCM),[48] play critical role in acentrosomal microtubules self-organization, rather than conventional MTOC. The spatio-temporal microtubules control has been previously thought in terms of conventional MTOCs. However, the phenomena of acentrosomal microtubules clearly indicates that the microtubules spatio-temporal self-organization is more complex than previously thought, so that conventional concept of microtubule organizing centers has to be re-evaluated in a sense of systems biology.[48]

It is evident from the gross relevant experimental data that, because of their great capacity to undergo spatio-temporal self-organization, the acentrosomal microtubules are quite commensurable to mediation of cellular signaling processes. This is truly the reason why nature places them in neural cells, where memory formation and learning are taking central place. For a long period of time, it has been staunchly argued that acentrosomal microtubules are endowed with a physical capacity to process and encode biological signals, i.e., information for memory formation and learning process.[62–73] Recent experimental evidence shows that morphological changes in dendritic spine, which are major sites of excitatory synaptic input, are directly responsible for memory and learning.[5,74,75] On the other hand, it has been shown that acentrosomal microtubules may change dendritic spine morphology and synaptic plasticity (i.e., they can influence memory formation and learning).[2–4,76] In terms of self-organization, in the rest of text, we will consider microtubules in solution *in vitro* as acentrosomal microtubules, thus, unless stated otherwise, the term "microtubules" relates to the phrase "acentrosomal microtubules."

Regarding acentrosomal microtubules' aspect, the basic condition for the cellular signaling proceeding is that an appropriate multitude of microtubules act as a collective, cooperatively, or even synergistically. Acentrosomal microtubules have the capacity to fulfill this condition, since they are endowed by great ability to be self-organized at the right time, and at the right locations. *In vitro*, at macroscopic scale, acentrosomal microtubule multitudes may undergo self-organization, which may be extended at the scale of solution volume at disposal.[6,10,77,78] As per fundamental biological importance of self-organization phenomena, it is important to mention that it has been already recognized that the self-organization phenomena, which, as a rule of thumb, occur in far from thermodynamic equilibrium conditions, may be an emergence stage for autonomous pattern formation.[79,80] Therefore, understanding the self-organizational mechanisms of how acentrosomal microtubules can form an abundance of temporal (periodic) dissipative structures is a prerequisite for understanding the intracellular patterns formation.[81] From practical point of view, this is a critical step in understanding fundamental biological processes in which dynamics of microtubules spatio-temporal patterning is a driving force, such as cellular division, and its pathological counterpart—tumorigenesis.

II. MATERIALS AND METHODS

Microtubule protein (MTP) was prepared using bovine brain as described elsewhere.[82] Unless stated otherwise, all experiments were performed under standard solution conditions, with microtubules reconstituted in Mes buffer, containing (in mM): 100 Mes, 1 EGTA, 0.5 $MgCl_2$, 1.0 GTP, and 6.6 pH. Microtubules were reconstituted by different concentrations of MTP. The spectra of MTP concentrations used in our experiments were (in mg/ml): 0.5, 1.0, 2,0, 3.0, 5.0, and 10.0. MTP refers to 95% of $\alpha\beta$-tubulin + 5% MAPs.[82] For all experiments, microtubule assembly was initiated in temperature controlled spectrophotometric couvette (in further text just couvette) by adding 1 mM GTP to the 0.5 ml of MTP solution, at 36 °C, unless stated otherwise. In all experiments (light transmittance (turbidity) and birefringence), couvette (0.2 cm × 1.0 cm) filled with MTP solution was held vertically in forward scattering geometry, between the source of light and detector. Importantly, neither external flow conditions nor temperature gradient was imposed on the sample solution.

Microtubule morphology was assessed by analytical Philips CM12 transmission electron microscope (EM) at the Australian Key Centre for Microscopy and Microanalysis, University of Sydney.

A. The spatial extent of microtubule self-organized and non-self-organized pattern

We have used the birefringence phenomena to visualise the extent of self-organized (anisotropic) and non-self-organized (isotropic) pattern of microtubules in a microtubule solution at macroscopic scale (across the whole sample). The

dynamics of the microtubule spatio-temporal pattern was followed by birefringence obtained from the sample by placing the temperature-controlled couvette between the two cross-polarizers.[6] Due to birefringence, white and black regions of the sample represent, unless stated otherwise, self-organized and non-self-organized microtubules regions, respectively. The birefringence patterns were photographed using Kodak TMX 100 film and a Nikon single lens reflex camera fitted with a Micronikkor 60 mm objective, using an aperture of f2.8 and an exposure of 2 s.

B. The critical threshold

To determine the critical threshold of MTP at which onset of ordered microtubule phase occurs, the light scattering experiments were done in transmittance mode in forward scattering configuration, which assumes that sample couvette is held between source of light and detector. When 1 mM GTP was added at 36 °C into the MTP solution in couvette, microtubules began to assemble. The microtubules strongly scatter the light at 350 nm with intensity proportional to the number of the microtubules.[83] In a forward scattering geometry, the detector recorded transmitted and eventually forwarded scattered light. In an MTP solution, the absorption of light is negligible at 350 nm. The light scattered by $\alpha\beta$-tubulin dimmers and MAPs is negligible in comparison to the light scattered by microtubules. Thus, prior to initiation of microtubule assembly, the forward positioned detector will detect maximal light intensity. As long as the number of microtubules increases, they will scatter more light (aside), so less light will strike the forward positioned detector. At lower microtubule concentrations, i.e., when microtubule protein concentration is <1 mg/ml, microtubules scatter the light predominantly in a ballistic fashion and the decrease of the intensity of detected light as a function of the MTP concentration is single-exponential.[83] Hence, the logarithm of decreasing transmittance is a *linear* function of the MTP concentration.

At a higher MTP concentration, the number of the microtubules is increased and multiple light scattering may occur. Hence, the forward positioned detector will detect proportionally less light. However, it is important to bear in mind that if anisotropic (ordered) phase is present in solution, then significant *additional forward scattered* light intensity will occur.[84] The *forward scattered intensity* will contribute to the total intensity of transmitted intensity recorded by the forward positioned detector. As we demonstrated elsewhere, although the ordered phase increases in the sample due to an increased number of microtubules (e.g., due to higher MTP concentration), this effect becomes prominent.[6] Hence, it is expected that MTP concentration dependence of forward detected light intensity (transmittance + forward scattered by ordered phase) has nonlinear form. The point at which nonlinearity appears represents onset of an ordered microtubule phase in the sample.

C. The morphology of microtubule self-organized and non-self-organized phase at microscopic scale

We used electron microscopy (EM) technique to observe microscopic details, in terms of individual microtubules, of a macroscopically self-organized and non-self-organized phase observed in birefringence experiments. For that purpose, specimens were taken from each phase separately from birefringence samples. In order to view microtubules by EM, the specimens were prepared according to Langford's procedure, which includes fixation and staining with 2% uranyl acetate on 200 mesh grids.[85] Specimens were left overnight at room temperature before microscopic examination. EM images were obtained using a Gatan multiscan CCD camera with an exposure time of 1.51 s.

III. RESULTS

We are here interested in the mechanism(s) of macroscopic microtubule self-organized spatio-temporal patterns in the closed system, *in vitro*, constituted of self-organized multitude of straight and multitude of non-self-organized curved microtubules.

A. Referential measurements

Formation of microtubules spatio-temporal structures is tightly related to microtubules morphology and kinetics of their assembly from $\alpha\beta$-tubulin. In this section, we have designed measurements to obtain referential data in regard to the system in order to facilitate the interpretations of the subsequent and final experimental results of spatio-temporal microtubules patterns formation. Kinetics of microtubule assembly was observed by measuring turbidity using typical MTP concentrations [mg/ml] (0.5; 1.0; 2.0; 5.0; and 10.0) (Figs. 1(A-a)–1(A-c)). Microtubules morphological study was performed using electron-microscopy technique (Figs. 1(B) and 1(C)).

The height of the stationary state of microtubule assembly and the time in which it was reached were proportional to the MTP concentration (Fig. 1(A-c)). However, the stationary state ($\pm 0.5\%$), observed by turbidity measurements, was reached within the approximately 30 min for all MTP concentrations used in our experiments. The kinetics have shown more prominent biphasic character in higher MTP concentrations. Typical biphasic kinetic is shown for the case of MTP concentration of 5.0 mg/ml in Figs. 1(A-a) and 1(A-b). As we have shown elsewhere, the first phase in biphasic kinetic graphs corresponds purely to the microtubule assembly, while the second phase corresponds to the formation of microtubules ordered phase (Buljan *et al.*, 2009). From these data, we can see that ordered phase may start to develop (see turning point in Fig. 1(A-b)) very early (within 2–3 min) after the microtubule assembly has commenced. Once the microtubules assembly reaches the stationary state, the system is constituted of multitude of self-organized straight and non-self-organized curved microtubules. At MTP concentrations higher than 2.0 mg/ml, straight microtubules are primarily aligned in domains, while curved microtubules are irregularly entangled (Figs. 1(B-a) and 1(B-b)). The average length of aligned microtubles is shorter than those out of domains.

It is obvious that some solution factors may affect microtubule morphology in terms of straight and curved, but it is little known which solution factors act in which way.

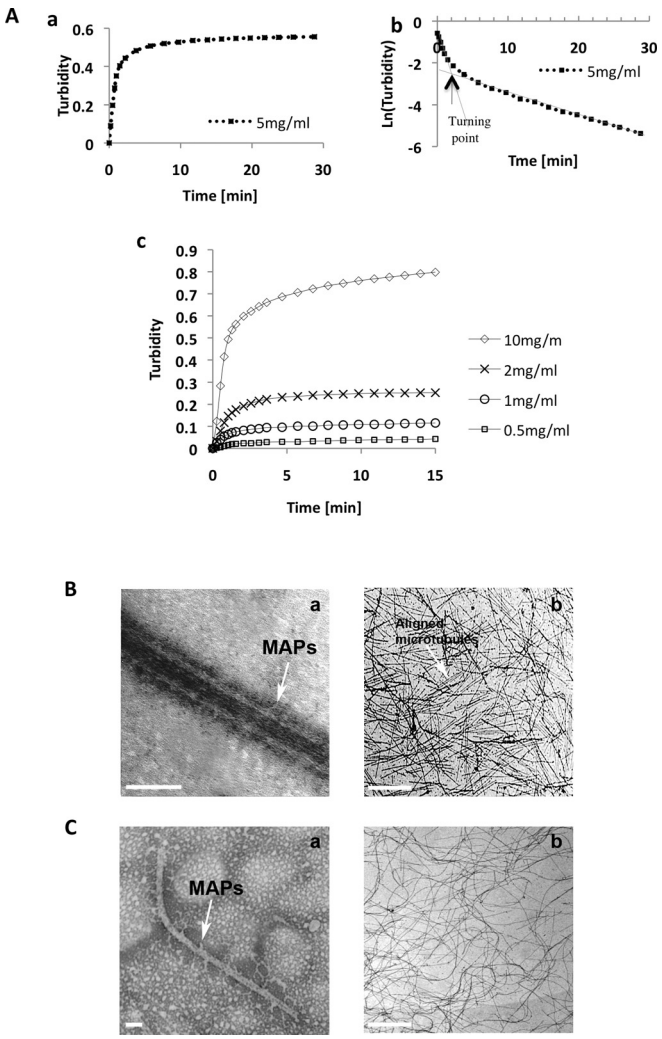


FIG. 1. Microtubule assembly kinetic, morphology, and spontaneous ordering. A. (a) Time-dependent microtubule assembly at MTP concentration of 5 mg/ml, measured by turbidity. (b) When the time dependence of logarithm of turbidity was graphed, it reveals biphasic character. Biphasic character is more pronounced at higher protein concentration. B. Straight microtubule morphology. (a) Single straight microtubule with MAPs—periodic structural features on microtubule wall labeled with white arrow. Bar is 0.05 μm. (b) Multitude of straight microtubules may spontaneously form patterns of aligned microtubules (labeled with white arrow). Formation of patterns of aligned microtubules is responsible for appearance of biphasic kinetics graphs (Buljan *et al*. 2009). The turning point between monophasic and biphasic kinetics may be considered as the beginning of ordered phase formation (Buljan *et al*., 2009). Bar is 5.0 μm. (c). Microtubule assembly kinetic graphs for the set of MTP concentrations [ml/mg] (0.5, 1.0, 2.0, and 10.0). C Curved microtubule morphology in the presence of excess of 1 mM CaCl2. (a) Single curved microtubule with MAPs—periodic structural features on microtubule wall labeled with white arrow. Bar is 0.05 μm. (b). Multitude of curved microtubules—no alignment observed. Bar is 5.0 μm.

However, we have shown that divalent cations such as calcium may convert straight microtubules into the ones of curved morphology.[6] For the purpose of this work, we have produced similar calcium effect but at a different MTP and calcium concentration (Fig. 1(C)). We have observed that if the excess of calcium (1 mM $CaCl_2$) was introduced into the solution before microtubule assembly commenced, then the microtubules were predominantly curved and much longer (Fig. 1C-b) than microtubules without calcium excess (Fig. 1B-b).

B. Demix of microtubules self-organized and non-self-organized phases—a qualitative observation of their durability versus mechanical disruption

In order to participate in fundamental processes, i.e., cell shape maintaining, signaling, energy transduction, and substance translocations, microtubules cellular self-organizations have to exhibit a certain degree of structural robustness as well as responsiveness to different stimuli. In order to obtain quantitative measures of these qualities, a rheological study is required. However, at this stage of our research, we can do some preliminary and qualitative observation of intrinsic durability of demixed phases against mechanical disruption.

On the other hand, from the technical experimental point of view, it is important to have some insight into the durability of this system because to view the microtubule morphology and its spatial organization by electron microscopy, one has to perform quite disruptive preparation procedure. Thus, it is important to know if spatial microtubule organization can be preserved, at least in part, after these preparative procedures.

The arbitrary microtubules self-organized pattern, observed by birefringence, was left to establish a stationary state at 60 min after microtubule assembly initiation at 3 mg/ml of MTP and at 36 °C (Fig. 2(A)). The pattern (a) was exposed to vigorous mechanical perturbation: a tip of Pasteur pipette was immersed to the bottom of a couvette, then it was pulled, left to right, from one side of couvette to the other side, and back. Comparing the resulting stationary pattern (b) with initial pattern (a), one can see local dislocations, reshaping, fragmentation, and so on of ordered (white) and non-ordered (dark) phases. Although the initial pattern was disrupted, that is, the individual, white and dark, regions have changed locations and shape, yet the total size of each reagion was preserved to a great extent. The estimated ratio of the size of the total ordered phase (the total sum of white regions sizes) and not ordered phase (dark regions) is preserved nonetheless. Therefore, this may indicate that although the initial ordered and non-ordered phase pattern might be broken at the macroscopic scale, at the level of micron-domains, the type of ordering which is characteristic of each phase might be preserved. This observation indicates that by carefully taking a small amount of solution from the white and the dark regions separately, it is possible to observe the differences of microtubules spatial order and morphology in these two regions (phases) (see Figs. 2(B-a) and 2(B-b)). For these purposes, we have taken samples from non-birefringence (white square in Fig. 3(A-f)) and birefringence regions (black square in Fig. 3(A-f)). Electron microscopy of the non-birefringence region (white square) shows the presence of dominant irregular clumps of curved microtubules multitude, accompanied by a minor number of straight dispersed microtubules Fig. 2(B-a). However, an electron microscopy image of a sample from a birefringence region (black square) shows the multitude of straight and ordered microtubules, which are accompanied by a lower number of curved and non-ordered microtubules Fig. 2(B-b). Hence, although each phase may be contaminated by a minor presence of the other phase, as shown by electron microscopic probes, the spatial separation, i.e., demixing of these two phases, is still remarkable.

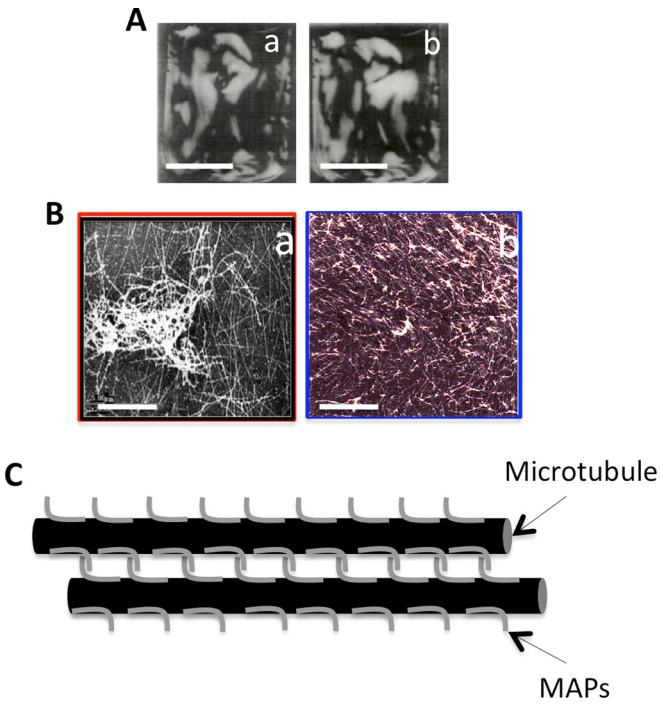


FIG. 2. The intrinsic durability in the system composed of macroscopically demixed microtubules self-organized and non-organized phase. Macroscopically self-organized phase of multitude of microtubules is spontaneously breaking the symmetry of homogenous space, from which different rudimentary morphological patterns may rise which show remarkable intrinsic durability against mechanical disruption: A. (a) An arbitrary global self-organized pattern in stationary state, 60 min after microtubule assembly initiation, at 3 mg/ml and 36OC. The pattern (a) has undergone strong mechanical perturbation: a pippette was immersed to the bottom of cuvette, then it has been pulled left-right from one side of couvette to the other side and back. Bar is 5 mm. (b). The resulting stationary pattern is different from the initial pattern (a), but the pattern (a) is not completely diminished; moreover, the ratio of the size of ordered phase versus a non-ordered phase is preserved. Bar is 5 mm. B Electron microscopy images of the probes taken from the sample in Fig. 3(A-f) at locations: (a) non-birefringence region (white rectangular), and (b) birefringence region (black rectangular). Bars are 5 μm. C Model: two straight microtubules in an aligned configuration. Numerous MAPs: they are attached to the outer surface by one of their two ends while they perpendicularly protrude from the surface by their other end. MAPs serve as spacers and joiners for neighboring microtubules. They may ratchet microtubules alignment due to their electrostatic mutual interactions, and increased effective excluded volume of the microtubules. Outer diameter of microtubule is 25 nm, while MAPs protrusion is about 15 nm, thus, it follows that the effective diameter of microtubule + MAPs is 55 nm.

C. Demix of self-organized and non-self-organized phase as a function of the MTP concentration—the critical MTP concentration threshold

1. Birefringence observations

The samples of different MTP concentrations (in mg/ml) Figs. 3(A) and 3(B): (b) 0.5, (c) 1.0, (d) 1.5, (e) 3.0, (f) 4.0, and (g) 5.0) were prepared and left for 60 min at 36 °C in order for microtubules assembly to achieve stationary state. We then recorded macroscopic birefringence patterns (Fig. 3(A)). As a reference sample, pure buffer (a) was used. The first appearance of the macroscopic birefringence pattern (white spots) was observed approximately at 1 ± 0.05 mg/ml MTP concentration. We observed that as long as MTP concentration increased, the birefringence spot, i.e., self-organized pattern, extended over the sample volume, while non-birefringence, i.e., non-self-organized pattern shrinked. It is likely that microtubules begin to self-organise even at lower concentrations, but such self-organisations are below the level that can be manifested at macroscopic scale, at least in terms of birefringence recording. Quite generally, at higher MTP concentration, multiple scattering may interfere with the birefringence pattern caused purely by anisotropic phase in solution. However, it has been shown that in microtubule system, multiple scattering has negligible interference with birefringence pattern.[10]

2. Light scattering observations

Light scattering is a sensitive function of the presence of anisotropic (ordered) phase of rod like particles in the solution.[84] We employed light scattering in transmission mode to determine the critical MTP concentration at which the microtubule self-ordered phase appears. The samples of MTP were prepared in the range of 0.4 mg/ml-5 mg/ml. In each concentration case, the sample has been left up to 60 min in a couvette at 36 °C, so that microtubule assembly and its self-organization can reach stationary state. Then the light intensity, after being passed through the sample, was recorded by a detector in a forward geometry. Furthermore, the light intensity composed of transmitted and forwarded scattered components was recorded by the forward positioned detector. Importantly, the forward scattered component is created by the ordered phase *only*.[84] The results are shown in the form of the graph in Fig. 3(B), which represents MTP concentration dependence of the observed light intensity. The graph has nonlinear form—that is biphasic, i.e., it is composed of the two linear components (Fig. 3(B)). Each component of the graph is fitted to linear form with the goodness of fit defined by the range of the least squares (R2); $0.9 \leq R^2 \leq 1$. Therefore, the turning point occurs at an MTP concentration of 1.0 ± 0.06 mg/ml. The turning point, between two components, is determined by the forward scattered light intensity that comes from the ordered phase exclusively.[6,84] Hence, we understood that this turning point reflects the MTP concentration threshold at which highly crowded conditions occurred, and self-organized microtubule phase was formed and demixed from non-self-organized phase. In other words, it is possible that at a turning point a demix as bifurcation process occurred which led to spatio-temporal separation of microtubules of different morphology (straight from curved). Furthermore, after passing through the turning point, the initially homogenous system was transformed into heterogeneous one, constituted by two different, and yet complementary phases (self-organized and non-self-organized). Hence, the turning point has the character of a bifurcation point, and demix has character of bifurcation process.

D. Demix of mirotubules self-organized and non-self-organized phases versus time and transient periodic patterns formation and disappearance

Our observations revealed that self-organized phase of straight microtubules may occur very early (within the first few minutes) during the microtubule assembly (Fig. 1(A-b)).

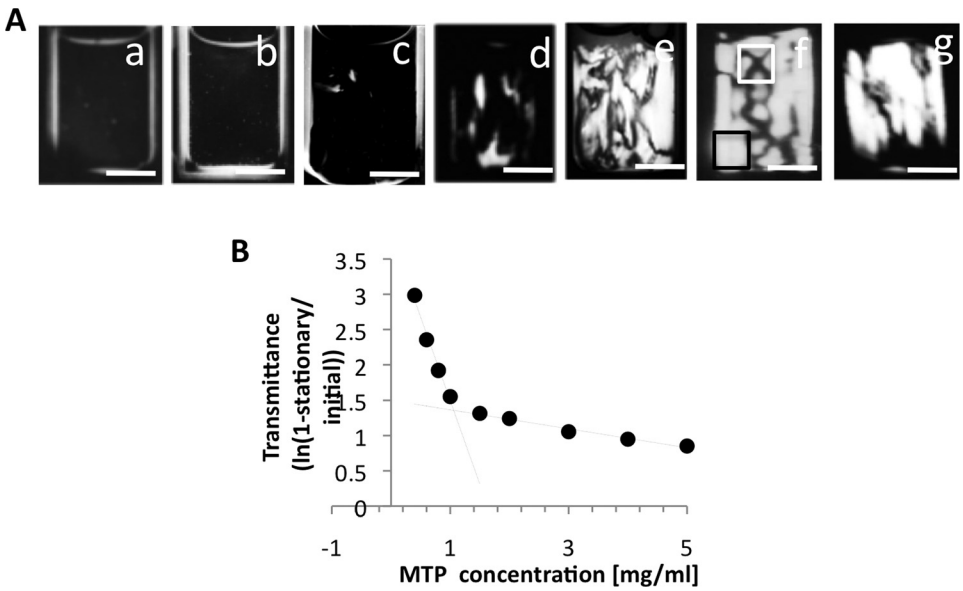


FIG. 3. The spatial extent of microtubules spontaneous self-organized, and non- self-organized phase, *in vitro*, versus the concentration of microtubule protein. To see the relation between the spatial extent of microtubule self-organized and non-self-organized phase versus the concentration of microtubule protein concentration, we have used birefringence phenomena. White and black regions correspond to self-organized and non-self-organized phase, respectively. A. Birefringence was observed at temperature of 360 °C, and at stationary state of microtubule assembly, i.e., at 60th min after assembly initiated. Buffer only was used as reference sample (a). Different MTP concentrations (in mg/ml) were applied: (b) 0.5, (c) 1.0, (d) 1.5, (**e**) 3.0, (f) 4.0, and (g) 5 mg/ml. The first birefringence spot was observed at 1.0 mg/ml of MTP concentration. The bar is 5.0 mm. B Intensity of transmitted light is sensitive to the presence of self-organized phase within the system: see Materials and Methods (Buljan *et al.*, 2009). Light (300 nm) transmittance intensity was measured at stationary state of the same MTP preparation as in (A) in MTP concentrations interval 0.2–5.0 in mg/ ml. The corresponding graph shows strong nonlinearity; it is biphasic. A linear least squares fit is shown for each phase of the graph (R2); 0.9! R2!1. The turning point between two phases occurs at MTP concentrations 1.0 ± 0.06 mg/ml.

Then, as birefringence experiment shows, it continues during stationary state (60 min and on) (Fig. 3(A)). During this period, the microtubules phases, the self-organized and the non-self-organized, were globally demixed and randomly distributed as different domains throughout the space of the sample. We observed that the higher concentration of MTP proportionally yielded the increase in the region of self-organized phase at the expense of non-self-organized phase. Interestingly, in terms of symmetry, no finer feature of locally differentiated pattern of these phases was found during this period.

We first have extended observation of demix throughout the later stage of stationary state, from 90 to 305 min. Surprisingly, we have observed *transient* periodic patterns during this period (Figs. 4(A-a)–4(A-f)). In this experiment, four periodic patterns randomly occurred. They have endured for different time intervals: from half an hour to approximately 2 h, and then they slowly declined (Figs. 4(B)–4(E) and Table 2 in Fig. 5(B)).

We were interested to see if periodic patterns may eventually reappear spontaneously. Therefore, we further extended the observation time to 12 h. Importantly, although birefringence reveals the presence of demixed phases during the second extended observation time, the periodic patterns have not been observed (Fig. 4(A-g)).

As per Fig. 4, we were able to record each of the four patterns in at least two of their different stages. Each of these stages corresponds to two sub-sequent moments of the same pattern. Thus, we were able to measure the scale of periodic spacing and its rate of change, on four randomly formed transient periodic patterns over a period of 5 h using images from Figs. 4(B)–4(E). The results show overall increase of periodic scale versus time: Table 1, Fig. 5(A), and overall graph in Fig. 5(C). We were also interested in the rates of these changes, and how the temperature changes may eventually affect these rates. Therefore, temperature was held constant (36 °C) during formation and development of the two stages of the first pattern, at 90th and at 105th min (Figs. 4(A-a) and 4(A-b)). The temperature was kept at the same level (36 °C) during the formation of the first stage of the second pattern (Fig. 4(A-c)). It was then decreased for 5 °C and held constant at 31 °C, till the end of the experiment. Thus, the second stage of the second pattern was developed at 31 °C, as were also all stages of third and fourth patterns. The rate of changes of periodicity spacing scale was changing (increasing) during these observations (Fig. 5(C)). We have compared the rate of changes between the two stages of each pattern with the corresponding changes between the two stages of the first pattern (Fig. 5(D)). The rate of changes of scale of periodicity for the second, third, and fourth patterns is respectively, twice, six, and ten times higher than the corresponding rate of the first pattern. We associated the rate of change for the second pattern to the fall in temperature during the development of the second stage of this pattern. However, further rate of changes occured during the constant temperature (31 °C) and cannot be associated with the effect of the temperature changes. We may speculate that except temperature, the only other factor that can affect these later rates of changes of periodicity scale, could be GTP-depletion.

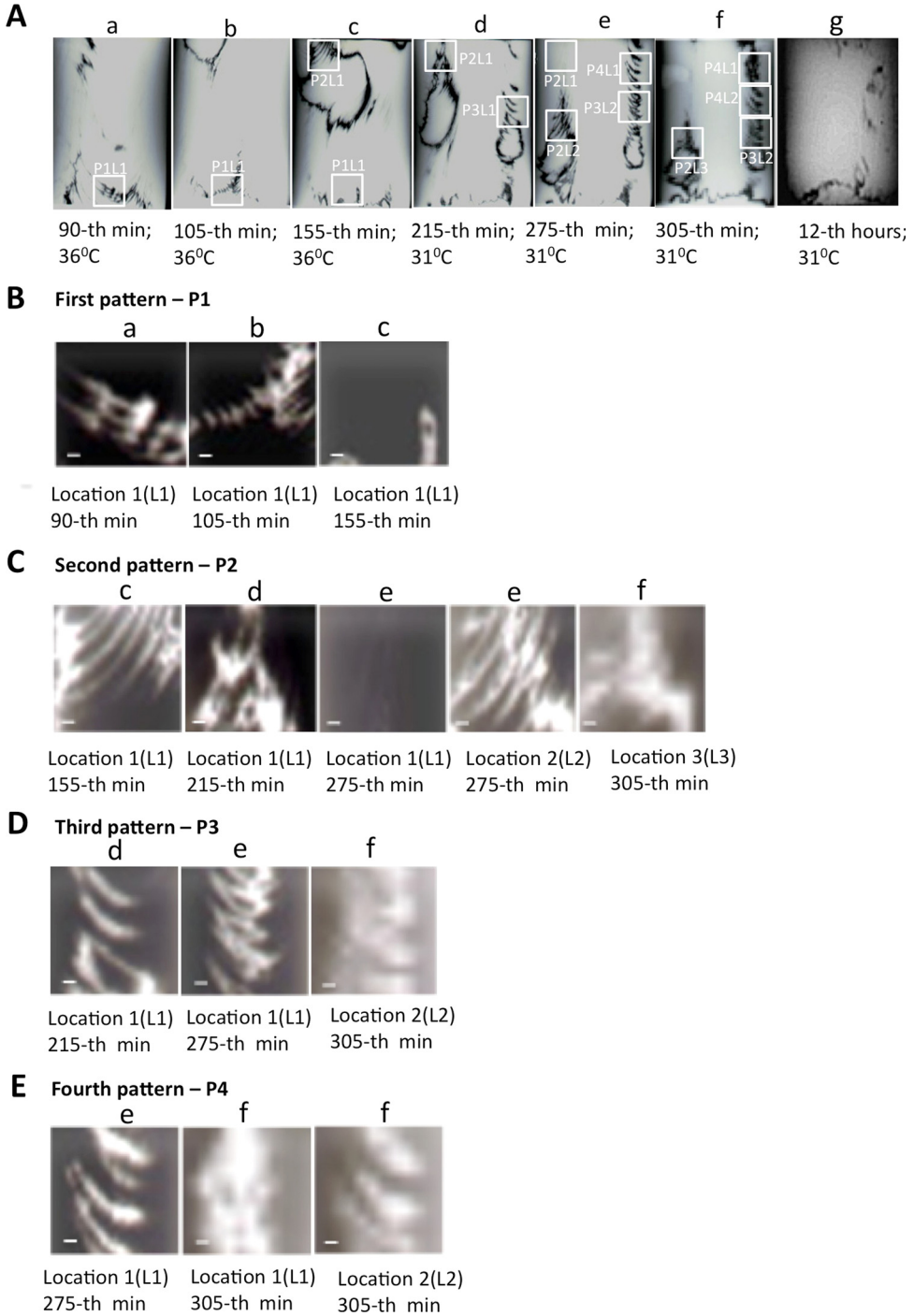


FIG. 4. The dynamic of transient periodic patterns in the crowded mix of straight and curved microtubules A. The spontaneously formed periodic patterns with spatial periodicity was observed at 90th min after microtubules assembly initiated, at 360 °C, and 5 mg/ml MTP, and observed during the next 215 min. The changes of patterns were recorded at different stages: (a) 90th min, (b) 105th min, (c) 155th min, (d) 215th min, (e) 275th min, and (f) 305th min. Bar is equal 1 mm. Temperature was constant, i.e., 36 °C from stage (a) up to (b), then it was decreased by 50 °C and held constant up to stage (f). Periodic patterns occurred randomly in different locations, they moved by diffusion at another locations and slowly degraded. We have followed four different periodic patterns, labeled with white rectangular, named "the first," "the second," "the third," and "the fourth," and marked, respectively, as P1, P2, P3, and P4 rectangular. Different locations of patterns are labeled as L1, L2, and L3. Each rectangular captures the area of the same size. B. First pattern P1 (a) At 90th min: location 1(L1)—the first periodic pattern observed. (b) At 105th min: location 1(L1)—the first pattern is still periodic but changed. (c) At 155th min: location 1(L1)—the first pattern is degraded. C. Second pattern P2 (c) At 155th min; location 1(L1)—the second pattern observed. (d) At 215th min: location 1(L1)—the second pattern left location (1) due to diffusion, but the pattern from other location is brought in by diffusion. (e) At 275th min: location 1(L1)—no any pattern, all left due to diffusion. (e) At 275th min; location 2(L2)—the second pattern located but partially degraded. (f) At 305th min, location 3(L3)—the second pattern is degraded. D. Third pattern P3 (d) At 215th min; location 1(L1)—the third initial periodic pattern observed. (e) At 275th min: the third pattern left the location due to diffusion, and the pattern from other location is brought in by diffusion. (f) At 305th min: location 2(L2)—the third pattern located but degraded. E. Fourth pattern P4 (e) At 275th min; location 1(L1)—the initial pattern observed. (f) At 305th min: the initial pattern left the location due to diffusion, and the pattern from other location is brought in by diffusion. (f) At 305th min: location 2(L2)—the initial pattern located but partially degraded.In each image B, C, D, and E, the bar is 0.1 mm.

A

Table 1

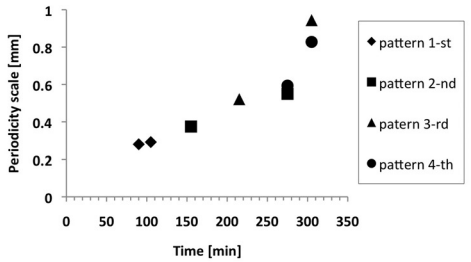
Time [min]	Temperature [C°]	Average periodicity [mm] First pattern	Second pattern	Third pattern	Fourth pattern	Standard deviation
90	36	0.2798				0.0137
105	36	0.2916				0.0218
155	36		0.3748			0.0332
215	31			0.5214		0.0609
275	31		0.5499			0.0495
275	31				0.5938	0.0639
305	31			0.9443		0.1016
305	31				0.8278	0.1231

B

Table 2

Pattern	Duration time [min]
The first	*130
The second	120
The third	90
The fourth	30

C



D

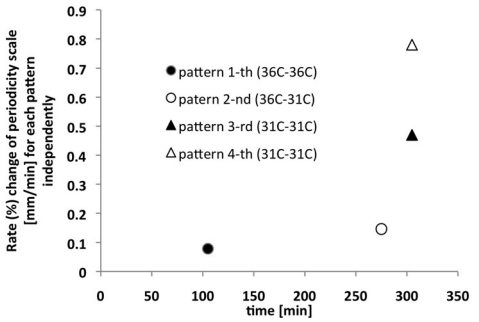


FIG. 5. Transient patterns dynamic. A. The data collected from the four transient patters, which randomly occurred and disappeared (see Fig. 4). B. Duration of different transient patterns. Duration was estimated as the time between moments of initial (sharpest) and final (the blurriest) stage of pattern. Initial and final stage corresponds to the values, less then 0.065 or higher then 0.130, respectively, for the standard deviation of measured average pattern periodicity. If the standard deviation was above 0.130, we consider that pattern is degraded. If pattern disappears before reaching the final stage of critical (highest) standard deviation (*see Table 2; the first pattern), the moment of its final stage was calculated as average value of the time of its last record and the time of recorded disappearance. C. The overall graph represents the transient pattern scale of periodicity changes versus time. It is constituted by the developments of four individual patterns data taken as they occur during the time of observation. D. This graph is constructed by the rates of percentile changes of the scale of periodicity [mm/min] between two stages of each pattern. These rates are faster at longer time scale at which pattern occurs. When compared, the rate associated to the second, third, and fourth pattern are, respectively, twice, sixth, and tenth times higher than the rate of the first pattern. The rate associated to the second pattern is likely controlled by the fall of temperature (from 360 °C to 310 °C), while the rates of the third and fourth patterns are the most likely controlled by the GTP depletion.

IV. EMPIRICAL MODEL

Here, we are going to formulate the model. This model will present the rationale for the data obtained in this work with regards to microtubule spatio-temporal patterning that is due to long demix (also known as spinodal decomposition) and microtubule transient periodic patterning in a system constituted by a multitude of straight and curved microtubules. The kind of spatial-temporal pattern that will be developed, if it is developed at all, in a system of multitude of straight and curved microtubules, depends on a particular combination of rate constant of reactions included as well as rate constant of diffusion of GTP-tubulin diffusion in particular. This is determined by the chemical and physical conditions of the system.

A. Spinodal decomposition

As our experimental data have revealed, the process of microtubule spatio-temporal self-organization (patterning), in a closed system of multitude of straight and curved microtubules, may span from an early stage of microtubule assembly 2-3rd min and up to 12 h into the assembly. The main organizational patterns, in a system of straight and curved microtubules, appeared as domains of aligned straight microtubules and irregular entanglements in a multitude of curved microtubules. At an earlier stage, these patterns have appeared randomly mixed regardless of microtubule morphology (Fig. 6). However, the patterns developed by microtubules of different morphologies undergo slow demix. In the final stage, all patterns associated to each of two different microtubule morphologies are completely segregated in two phases. This demix is proceeding, at constant temperature, in closed system. It is going during the period when the energy of chemical source (GTP) is present in the system, but is also proceeding well behind, i.e., when the GTP is already consumed. Hence, we have recognized this demix as a slow isothermal spinodal decomposition, which is guided by cross-diffusion between straight and curved microtubule multitudes in conjunction with excluded volume. The long preservation span of patterns, during spinodal decomposition (up to 12 h Fig. 4(A-g)), may indicate that a strong effect of excluded volume and mutual interactions between perpendicularly protruded projections of MAPs, plays a ratcheting role on spinodal decomposition. We have speculated that in the final stage, as well as in a previous stage, the relative positions of domains are controlled by strong excluded volume effects, and mutual electrostatic interactions of MAPs protrusive parts (Figs. 2(C) and 6).

B. Transient spatio-temporal periodic pattern formation

We have observed formation and disappearance of transient periodic patterns (further referred to as "pattern(s)"), which consisted of periodically arranged strips of multitudes of ordered and non-self-ordered microtubules.

The possible mechanism of transient spatio-temporal periodic pattern formation is illustrated in Fig. 7. The transience of patterns is in accordance with the fact that our experimental system is closed. This indicates that pattern occurrence and disappearance is dependent on the available energy. Furthermore, our observation shows that the appearance of patterns coincides with energy dissipation due to inorganic phosphate (P_i) liberation during GTP-hydrolysis in GTP-tubulin subunits located in a microtubule wall. Thus, the system is dissipative.

Reactive-diffusion scheme of microtubule growth consists of GTP-tubulin addition at the both tips of microtubule ends, and a somewhat lagging irreversible hydrolysis (dephosphorylation) of GTP in GDP at GTP-tubulin subunits in a microtubule wall.

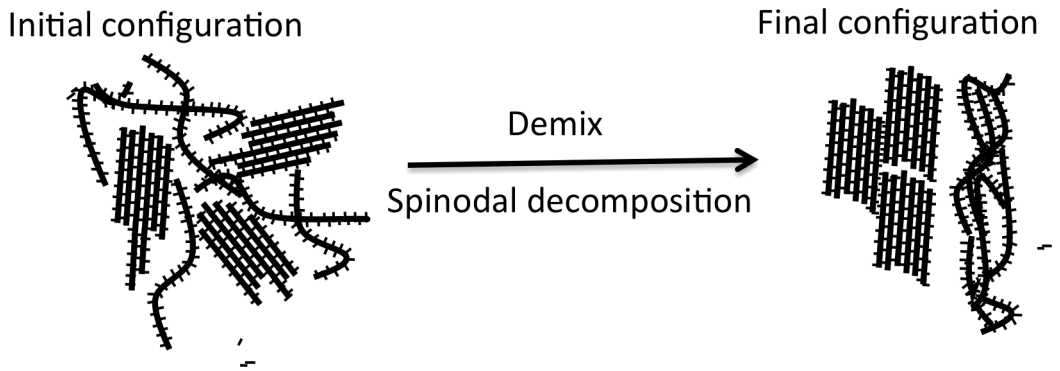


FIG. 6. Long (12 h) running demix or a slow isothermal spinodal decomposition segregates aligned domains of straight microtubules from multitude of curved microtubules. The process is energy independent, but is driven by cross-diffusion in conjunction with excluded volume. MAPs are present in the system. At some initial stage, domains of aligned straight microtubules and curved microtubules are distributed randomly and mixed evenly. Hence the system is homogeneous. The final stage is reached after a long running demix (spinodal decomposition), and it is constituted by the two phases which are well separated spatially. One phase is constituted of all domains of aligned microtubules, while the other phase is constituted of all multitudes of entangled curved microtubules. The system is inhomogeneous.

Irreversible hydrolysis transfigures homogeneous microtubule wall into heterogeneous, and changes a linear dynamic of growth into a non-linear while dissipation of energy occurs. After the irreversible GTP-hydrolysis occured, a part of chemical free energy is dissipated via release of inorganic phosphate (P_i) into solution. On the other hand, the GDP-tubulin remains built into the wall lattice and is accompanied by a part of energy (obtained from hydrolysis) stored as mechanical strain in the wall lattice. That part of chemical energy powers the phenomena known as "dynamic instability" (stochastic switch from microtubule assembly to disassembly and vice versa). Thus, GTP-hydrolysis is considered to be the core of non-equilibrium microtubule growth and is therefore consistently considered as main contributor to nonlinear dynamic and dissipative character of microtubule growth. Importantly, if an autocatalytic reaction is present in the reaction-diffusion system, it may change the system substantially.[86] GTP-hydrolysis is indeed autocatalytic reaction, which is the necessary general condition for periodic pattern formation.[87]

The addition of GTP-tubulin is composed of GTP-tubulin association and dissociation. The rate of GTP-tubulin diffusion has to be high enough in order to supply enough of the GTP-tubulin (i.e., to hold local concentration of GTP-tubulin (next to the tip) above the critical concentration) necessary for microtubule growth. However, a certain conditions may appear that impair the rate of GTP-diffusion in the system constituted of multitude of straight and curved microtubules. The GTP-tubulin diffusion may fulfill its role differently for straight and curved microtubule multitudes. Impaired rate of GTP diffusion, including what was mentioned above in regard to reaction-diffusion scheme of microtubule assembly, may lead to important spatio-temporal pattern formation.

Let us now consider the details of our model from the stage when straight microtubules were aligned in domains (Fig. 7(a)). Domains are always formed by restricted number of microtubules. This number can vary under different experimental conditions. Under our experimental conditions, the number ranged from 2 to 18 microtubules.[7] Furthermore, the average length of microtubules in a domain was shorter than those out of domain. In particular, the average length of curved microtubules was always significantly longer than of microtubules in the domain (Figs. 1(B) and 1(C)).[7] If the rate of reactions is constant, then these data indicate that the rate of local free diffusion of GTP-tubulin is apparently impaired with respect to aligned and nonaligned microtubules multitudes.

Domain formation is ongoing while GTP-tubulin is being added to microtubule ends. This will continue while the concentration of GTP-tubulin is above the critical threshold for microtubule assembly. The neighbouring microtubules in a domain also consume GTP-tubulin for their growth. It is obvious that they mutually compete for GTP-tubulin during this process. The level of GTP-tubulin is replenished by its diffusion in the vicinity of the microtubule ends. However, the progress of a domain's ongoing formation is arrested, once the number of neighbouring microtubules reaches a certain critical value. Hence they collectively take out the GTP-tubulin, within the vicinity of their ends, faster than it can be supplied, to that location, by the given rate of diffusion. Because of the same reason, the average length of domain microtubules will be restricted. Here, we see a certain kind of feedback mechanism: the collective of domain microtubules effectively impair the local diffusion, and in turn, impaired diffusion will lead to decreased rate of GTP-tubulin addition. As a result, the number of microtubules in a domain is restricted as is their length. Because of the intrinsic asymmetry of a microtubule body, and intensive consumption of GTP-tubulin from the frontal zones, a particular local pattern of GTP-tubulin concentration distribution is developed. We have illustrated this very simplistically in Fig. 7(B). In front of microtubule tips, the concentration of GTP-tubulin may fall bellow the critical concentration necessary for microtubule assembly, while in the zones, which lay aside of microtubule body, this fall does not lead GTP-tubulin concentration below the critical threshold. Hence, individual microtubules with distant tips may continue to grow in these zones. The tips of curved microtubules are often distant, so they do not compete for GTP-tubulin as the collective of domain microtubules does. This situation is illustrated in Fig. 7(C).

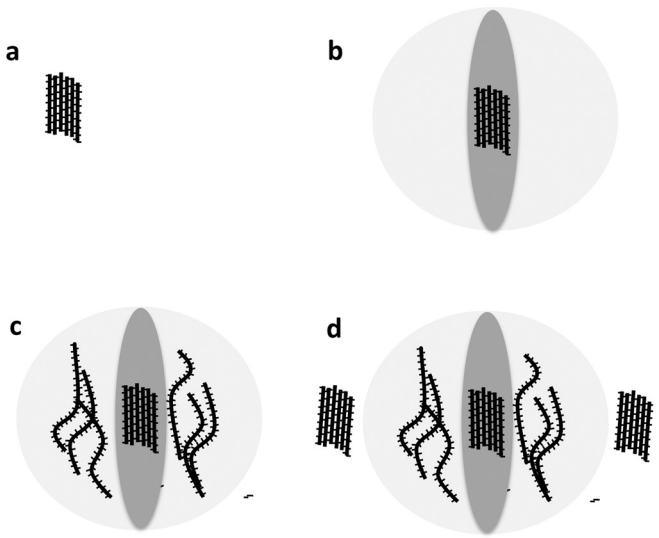


FIG. 7. Transient periodic pattern formation in a closed system and in a heterogeneous microtubule solution space. (a) Formation of a domain of aligned (further just "domain") straight microtubules is proceeding until there is enough GTP-tubulin to be added to the microtubule ends. During domain formation, microtubules undergo dynamic instability, which is powered by energy obtained from the GTP-hydrolysis. By stochastically causing shrinking and extension of the microtubule length, the dynamic instability enables the microtubules to undertake numerous complex readjustments in order to occupy an optimal location in domain. Dynamic instability is responsible for maintaining the given number of the microtubules in domain. If some microtubule (new comer) which is formed apart from the domain approaches, it will be eliminated by dynamic instability. Formation of domain is arrested when the number of neighboring microtubules reaches certain critical value. Neighboring microtubules consume the GTP-tubulin for their growth, and they mutually compete for the GTP-tubulin during this process. This consumption by microtubules is replenished by the GTP-tubulin diffusion. (b) As the number of neighboring microtubules in domain is increasing, competition is increased as well for the consumption of the GTP-tubulin. At some critical number of neighboring microtubules, the rate of a collective consumption of the GTP-tubulin may overtake the rate of the GTP-tubulin diffusion. The zone in front of domain of straight aligned microtubules may be depleted of the GTP-tubulin. Hence, in this zone, the concentration of GTP-tubulin is decreased below the critical threshold for microtubule assembly. Therefore, the growth of microtubules in domains will be stopped and the growth of the domain will be arrested as well. But, the reached size of the domain may be preserved due to effects of the excluded volume and the mutual interactions of the MAPs. On microtubule sides, there will appear the zones where the concentration of the GTP-tubulin is decreased to a lesser degree. However, this decrease may not lead to the concentration of the GTP-tubulin dropping below the critical threshold. (c) Microtubules with the lower rate of a collective consumption may still continue to grow in these zones. This is the case of a curved microtubules. Since the tips of a neighboring curved microtubules are positioned at a distance, these microtubules, as a collective, have a lower rate of consumption of the GTP-tubulin than aligned straight microtubules in domains. (d) On both sides of domain of straight aligned microtubules, but further behind the multitude of growing entangled curved microtubules is the zone in which diffusion of the GTP-tubulin can pump enough GTP-tubulin, so that the microtubules with a high collective rate of consumption can continue to develop. Indeed, these are the conditions that enable the transient periodic pattern to be formed.

The zone in which diffusion of GTP-tublin, at its given rate, can supply enough GTP-tubulin is located at the both sides of domain of straight aligned microtubules, but further behind the multitude of growing curved microtubules. Hence, microtubules with a high collective consumption rate can continue to develop the new domains in these zones.

Since dynamic instability is continuously proceeding during the transient periodic spatio-temporal pattern formation, it constantly leads to stochastic microtubules to shrink (or to their total destruction) and extension (including new regrowth). In this way, dynamic instability enables domain microtubules to undertake numerous complex and fine readjustments to occupy, in terms of excluded volume, the optimal location in a domain. Dynamic instability induces exchange between straight and curved microtubules outside of a domain. In other words, it induce constant cross-diffusion between the multitude of straight and curved microtubules.

V. DISCUSSION

A. Microtubule assembly: Straight and curved microtubules, spontaneous self-organization and demix, turning point, critical threshold, and durability

Our system was initially constituted of a MTP. Here, MTP denotes $\alpha\beta$-tubulin dimmer + MAPs. Microtubules start to assemble from a complex GTP-$\alpha\beta$-tubulin when an appropriate concentration of a GTP is inserted into the MTP system (Figs. 1(A-a) and 1(A-b)). Once the assembly commenced, straight and curved microtubules were formed (Fig. 1B-b) and 1(B-c)). A few minutes into the assembly, a multitude of formed straight and curved microtubules undergo spontaneous demix above the turning point (Fig. 1(A-b)). Phases of straight microtubules possess an intrinsic anisotropy, while a phase of a multitude of curved microtubules is intrinsically isotropic. Therefore, anisotropic and isotropic phases are observed by birefringence as a white and a dark zone, respectively (Fig. 3(A)). In terms of a MTP concentration, a critical threshold at which demix reaches a stationary state is 1.0 mg/ml concentration of MTP (Fig. 3(B)).

In order to better understand the relation between the microtubule morphology and a type of a phenomenological self-organization, we have tested a durability of a spatio-temporal microtubule self-organization in a stationary state (Fig. 2). Since the spatio-temporal pattern of a microtubule self-organization could be destroyed during sampling stage for electron-microscopy study, we have performed qualitative test of a durability of patterns. Our experimental test shows that although sampling may partially destroy patterns, a certain part of them may also remain unaffected. For this reason, we did an experiment which shows a significant durability of a microtubule spatial patterns versus strong mechanical perturbation (Figs. 2(A) and 2(B)). Surprisingly, a significant durability of aligned domains of straight microtubules can be explained by the strong ratcheting effect exerted by the excluded volume on the spontaneous microtubule self-organization. In particular, this applies to the microtubule system where MAPs are present (such as in our system). Microtubule itself is a long (measured in microns) hollow cylinder with an outer diameter of 25 nm. If MAPs are present, they are partially attached by one of their two ends to the outer surface of a microtubule wall, while their other end is protruding from the surface into the solution (perpendicularly to the wall surface) Figs. 1(B), 1(C), and 2(C). It is well known that they serve as spacers between the neighboring microtubules.[37] MAPs increase the effective excluded volume of microtubules by increasing their outer diameter up to 55 nm. As noted earlier, it is likely that a strong excluded

volume is ratcheting microtubule alignment. In conclusion, strong effect of excluded volume and MAPs-MAPs direct mutual interaction may preserve alignment configuration against variety of destructive forces.

B. Demix as a bifurcation process

We have found that a system constituted of a multitude of straight and curved microtubules possesses an intrinsic bifurcation point at which crowded conditions occur, which in turn enable microtubules of different morphology and intrinsic order to undergo bifurcation process know as spontaneous demix. As far as we understand, and as mentioned in Introduction, demix is spatio-temporally bifurcation process in which cross-diffusion in conjunction with excluded volume, plays a pivotal role.[1,39] A multitude of straight microtubules appears intrinsically ordered and easily detected as an anisotropic phase. However, curved microtubules are mainly entangled forming irregular clumps, which is due to huge heterogeneity in modes of their curvatures. If the curvatures were of the same mode, even the curved microtubules would be straightforwardly ordered, but in different fashion, like in Herzfeld's systems.[88]

It is important to note that neither bulk flow nor external temperature gradient was imposed onto the system. Hence, any local motion, including domains, within the system is due to internal, i.e., local, heterogeneity of the system. Therefore, effects of a bulk flow or convection on the process of self-organization as such should be neglected, and the system may be considered as a reaction-diffusion system—as we will discuss further.

C. The possible mechanism(s) implicated in demixing of mirotubules of different morphology and transient periodic patterns formation

1. Long term demix of straight and curved microtubules: Spinodal decomposition

Our results have shown that in the system of multitude of straight and curved microtubules, microtubules of different morphology start to demix from an early stage of microtubule assembly (a few minutes after assembly commences) and is still recorded at 12th h. The multitude of straight microtubules undergoes a self-organization and forms a spatio-temporal ordered phase, which is detected by birefringence as anisotropic phase. The multitude of curved microtubules undergoes a non-self-organization forming different entanglements, which are detected as non-birefringence isotropic phase. Chemical energy of GTP is largely dissipated during the early stage (few hours after assembly commences) of demix. Hence this, energy independent, process can proceed and be maintained by cross-diffusion in conjunction with excluded volume. Therefore, the most likely candidate for such long running demix could be a slow isothermal spinodal decompisition.[40] Spinodal decomposition is switched by local fluctuations of the components.[89] As far as microtubule system is concerned, cross-diffusion in conjunction with excluded volume and surface charge of microtubule wall may generate abundance of local athermal fluctuations including fluctutations of straight and curved microtubules.[1,27,38] In conclusion, these local athermal fluctuations may switch on the isothermal spinodal decomposition, which can demix curved and straight microtubules during the long run.

2. Transient periodic patterns formation

Apart from the pure demix of multitudes of straight and curved microtubules, we found an additional phenomena. In a very late stage of the stationary state of microtubule assembly, i.e., between 90 and 305 min after microtubule assembly has been initiated, we detected transient periodic patterns in our system. To explain these patterns formation and disappearance, one should remember that our system is thermodynamically closed. The GTP is the unique source of chemical energy in the system and is undergoing irreversible depletion process due to irreversible GTP hydrolysis into GDP, which cannot energetically support microtubule growth. Therefore, transiency of these patterns indicates that they are energetically dependent on GTP, and their cessation is associated with the GTP-depletion. To understand the appearance of periodic features in these patterns, we may invoke reaction-diffusion theories, which predict periodic spatio-temporal patterns formation due to spatio-temporal concentrations periodic variations of reactants. However, if the GTP is present in the system, then it powers the dynamic instability, which causes microtubule to partially disassembly and reassembly.[17,20] This will create local fluctuations in GTP-tubulin and GDP-tubulin. As we mentioned earlier, these fluctuations will affect the growth and relative orientations of surrounding microtubules. In addition, the relative orientation of surrounding microtubules will be strongly affected by excluded volume. As long as the number concentration of microtubules is higher, this effect will be more pronounced. Therefore, local fluctuations in GTP-tubulin and GDP-tubulin in conjunction with the excluded volume may cause local microtubule displacement, which in case of straight microtubules leads to microtubules local ordering, i.e., to development of parallel alignments. However, it is expected that microtubule displacement is spatio-temporally different in the multitude of curved microtubules, which leads curved microtubules to be entangled, and segregated from the multitude of straight and aligned microtubules. Indeed, once the multitudes of straight and curved microtubules are demixed, it is expected that they are ratcheted by excluded volume in conjunction with the local fluctuations of GTP-tubulin and GDP-tubulin concentrations. As a consequence, these two morphologically different microtubule multitudes are irreversibly organized in spatio-temporal terms.

Let us see how the periodicity of these patterns may originate in this system. If the system is reaction-diffusion system, the periodicity of a formed patterns is approximately equal to the distance over which relevant molecules (in our system GTP-tubulin and GDP-tubulin) diffuse before reacting. The periodicity of the pattern formed is approximately determined as the ratio of the reaction rate and diffusion constant.[90] The increase of the reaction rate will shorten the time required for the relevant molecule to diffuse before

reacting, thus, the periodicity will decrease. This has been specifically demonstrated for the microtubule system in which GTP is regenerated by the conversion of inorganic phosphate (liberated due to hydrolysis of GTP) into organic phosphate contained in GTP.[91] The rate of conversion of inorganic to organic phosphate is convenient measure of the rate of energy dissipation in microtubule system.[28] In that research, it has been shown that by the increase of temperature from 30 °C to 35 °C, the reaction rate of conversion of inorganic phosphate into organic increases by factor of 2.1. Under the same conditions, the average periodic spacing distance between microtubules stripes decreases by the factor of 1.47. Surprisingly, in our experiments, the temperature decrease from 36 °C to 31 °C has caused the increase in the average periodic spacing by the factor of 1.4671. It should be mentioned that our system is different than that of the authors mentioned above. In our system, GTP was not regenerating, while in their GTP was regenerating. However, the excellent agreement in the independently obtained result can be explained by the fact that we have performed experiment by decreasing temperature during the period when plenty of GTP was still present in the system—otherwise periodic patterns would not be formed at all. It is important to mention that according to the reaction-diffusion theories, the square root of the increase rate (2.1) of phosphate conversion is approximately equal to the obtained factor (1.47) of periodic spacing decrease.[90] Therefore, this finding in turn confirms that reaction-diffusion processes are in the background of periodic patterns formation in our microtubule system.

The result concerning the temperature dependence of macroscopic periodicity scale of periodic patterns is in a reasonable qualitative agreement with others, obtained in the study of the biological system of Acetabularia Whorls.[92] It has been shown that in the absence of bulk flow and convection, specific initial (intrinsic) conditions may enable formation of a symmetric stable spatial pattern with a variety of geometries.[93]

3. Cross-diffusion in the system constituted by multitudes of straight and curved microtubules

Since our data strongly indicated that microtubules spatio-temporal pattern formation is guided by cross-diffusion in conjunction with excluded volume, we decided to pay more attention to see how cross-diffusion may occur in microtubule system.

It has been proposed that cross-diffusion may occur in the systems with a significant surface electrostatic, and excluded volume.[1] Both factors are strongly present in microtubule system. Hence, it is reasonable to expect that significant cross-diffusion may occur in microtubule system. Briefly, microtubule is a long (at micron scale) hollow cylinder. Its intrinsic building block is $\alpha\beta$-tubulin dimmer, which is highly net charged at its perpendicularly protruded C-terminus (-10 in terms of electrons), and possesses a significant permanent dipole moment.[38] It is well known that this net negative charge is strong enough to form a zone of exclusion for molecules (negatively charged) and even other surrounding microtubules. The outer diameter of microns long microtubules amounts approximately 55 nm due to MAPs presence in our system. Hence, the excluded volume should have a significant effect. In biological cells, MAPs are regularly present. They are present in our *in vitro* system as well. Therefore, the excluded volume in conjunction with cross-diffusion should be considered a critical driving force in microtubule spatio-temporal pattern formation. It has been already shown that the excluded volume is the key factor in self-organization of long rods.[32,34,35,94] However, it was recently shown that the local cross-diffusion between different chemical species may be induced by differences in their excluded volumes.[1,39] Therefore, in a system of multitude of microtubules of different morphology, cross-diffusion may be largely induced by huge differences in excluded volume between straight and curved microtubules. In other words, excluded volume in conjunction with cross-diffusion may critically guide spatio-temporal patterns formation in this system.

Furthermore, concerning spatio-temporal patterning in biological systems, an important question has been recently elaborated in regard to durability of spatio-temporal patterns versus noise in biological systems, and in particular in inductive signaling mechanism in neuronal growth.[95,96] We have shown experimentally that the microtubule spatio-temporal patterns, including spinodal decomposition of multitude of straight and curved microtubules formed by cross-diffusion in conjunction with the excluded volume is quite persistent versus time and versus strong disruption by mechanical force. Therefore, this persistence of microtubules multitudes spatio-temporal patterning may be critical in maintaining spatial information processing (including inductive signaling in neuronal growth) against noise during morphological development and growth.

In summary, the results reveal that rudimentary morphological pattern development in microtubule multitude is proceeding via macroscopic symmetry breaking of initially homogenous system and spatio-temporal reintegration of newly developed subsystems with intrinsically different morphology and local symmetry. The key stages of these events are: microtubule crowdedness, the critical threshold, bifurcation by entropy driven demixing processes, and spatio-temporal pattern formation due to cross-diffusion in conjunction with excluded volume.

Although demonstrated in the relatively simple mesoscopic closed system of acentrosomal straight and curved microtubule multitudes *in vitro*, the conditions of spinodal decomposition and transient periodic spatio-temporal patterns formation may have more general, yet critical importance in morphological pattern development in complex and open biological systems, including those relevant to tumorigenesis.

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