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Short communication

Brief exposure to high magnetic fields determines microtubule self-organisation by reaction–diffusion processes☆

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Abstract

A frequent feature of microtubule organisation in living systems is that it can be triggered by a variety of biochemical or physical factors. Under appropriate conditions, in vitro microtubule preparations self-organise by a reaction–diffusion process in which self-organisation depends upon, and can be triggered by, weak external physical factors such as gravity. Here, we show that self-organisation is also strongly dependent upon the presence of a high magnetic field, for a brief critical period early in the process, and before any self-organised pattern is visible. These results provide evidence that external physical factors trigger self-organisation by way of an orientational bias that breaks the symmetry of the reaction–diffusion process. As microtubule organisation is central to many cell functions, this behaviour provides a mechanism by which strong magnetic fields can intervene in biological processes.

Keywords: Complex systems; Dissipative structures; Bifurcations; Weak fields; Cellular organisation; Cytoskeleton

1. Introduction

Microtubules, a major component of the cytoskeleton, are tubular super-molecular assemblies several microns in length, having inner and outer diameters of 16 and 24 nm, comprised of the protein, tubulin [1,2]. They control the internal organisation of the cell and participate in many cellular functions by way of this behaviour. Microtubules can be formed in vitro by warming a solution containing purified tubulin and guanosine triphosphate, GTP, from about 4 to 36 °C. A series of chemical reactions occurs, tubulin assembles into microtubules within a few minutes, and GTP is hydrolysed to guanosine diphosphate, GDP. Once microtubules are assembled, this reaction continues by similar processes whereby the complex tubulin–GTP is added to the growing end of a microtubule whilst tubulin–GDP is liberated from the opposite shrinking end.

Within cells, biologists have established that microtubule organisation and reorganisation result from the chemical dynamics of the reactive processes associated with their formation and maintenance. Another characteristic feature of their behaviour is that organisation is often triggered by a variety of internal or external factors either biochemical or physical in nature. Since solutions of reacting chemicals do not normally self-organise, nor are they strongly dependent on external physical stimuli, this raises the question of the physical chemical processes by which this behaviour comes about.

Theoreticians have proposed that for some particular types of chemical reaction, macroscopic self-organisation might arise by a combination of reaction and diffusion [3–9]. Under suitable conditions they predicted that a stationary

☆ These experiments were carried out using the facilities of the Grenoble High Magnetic Field Laboratory, Grenoble, France.

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pattern should form comprised of periodic variations in the concentration of some of the reactants; and some chemical reactions are known to self-organise this way [10–15].

In addition to self-organisation, it has been predicted that such systems might also show bifurcation properties of the bi-stable type [9]. At a critical moment early in the development of the self-organised state, the system may bifurcate between different dynamic pathways. Kondepudi and Prigogine calculated that the presence at this critical time of a weak external factor such as gravity, could determine the morphology of the self-organised state that subsequently develops [16–18]. The magnitude of this external factor can likewise act as a bifurcation parameter.

Under appropriate conditions, in vitro microtubule preparations behave this way [19–22]. They self-organise by a combination of reaction and diffusion [23] and this self-organisation can be triggered by gravity at a critical moment early in the process, before any pattern is visible [22]. In this article, we show that microtubule self-organisation by reaction–diffusion processes is also strongly dependent upon the presence, at the same critical early period, of a high magnetic field.

2. Microtubule self-organisation

When microtubules are assembled in vitro under appropriate conditions in glass cells measuring 4 cm by 1 cm by 1 mm, then starting from an initially homogeneous solution, a stationary macroscopic pattern progressively develops over about 5 h. The structure is comprised of a series of stripes of about 0.5 mm separation. In each striped region (Figs. 2, 3), the microtubules are highly oriented at either 45° or 135° to the long axis of the sample cell, but adjacent stripes differ in having alternating orientations. Variations in the microtubule concentration also occur from stripe to stripe that coincide with the variations in orientation [24]. Additional stripes of about 100 μm separation occur within the 0.5 mm bands. These in turn, contain stripes of about 20 μm separation. At distances below this, other levels of organisation of about 5 and 1 μm separation are present [22,23].

Gravity intervenes in the self-organising process. When the experiment is carried out in sample cells that are positioned vertical, a series of horizontal stripes form, whereas when the microtubules are assembled in sample cells placed horizontal ‘flat down’ then concentric circles develop. In addition, the morphology which forms is determined by whether the sample is vertical or horizontal at a critical ‘bifurcation time’ early (6 min) in the self-organising process (5 h) before any pattern is visible. When microtubules were assembled under conditions of weightlessness for the first 13 min of the process, then self-organisation did not occur [22].

Under conditions of near weightlessness, when a small air bubble is pushed through the preparation shortly after the bifurcation time, a line of high birefringence immediately forms along its trajectory. Subsequently, self-organisation, limited in extent, develops perpendicular to this trajectory [25]. Hence, using sheer to orient the microtubules, at an early stage in the process, will instigate self-organisation. This observation suggests that close to the bifurcation time any process that orients the microtubules might trigger self-organisation. Microtubules possess a high anisotropy in their diamagnetic susceptibility[26] and their interaction with a magnetic field leads to a torque capable of orienting them. Bras et al. [26] estimated the value of the diamagnetic anisotropy, calculated that preparations could be oriented with a 7 Tesla magnetic field, and carried out experiments demonstrating that magnetic fields of between 4–11 Tesla orient microtubules over macroscopic distances. Vassilev et al. [27] had earlier reported that the growth of microtubules in a 0.02 Tesla magnetic field, or in a pulsed electric field of 25 mV cm^{-1}, leads to microscopic domains of oriented microtubules. Likewise, Stracke et al. [28] have reported the drift of individual microtubules in an electric field.

To test the hypothesis outlined above we oriented the microtubules, for 15 min at the beginning of the process, with a strong uniform magnetic field of between 1 to 15 Tesla. Under normal laboratory conditions, the morphology depends on the sample orientation with respect to gravity; stripes when vertical and concentric circles when horizontal ‘flat down’. Hence, depending on the directions of both gravity and the magnetic field, the magnetic field may either oppose or reinforce the effect of gravity. We expected that applying a strong magnetic field, for a brief initial period, along the long axis of a horizontal sample, might result in the formation of a striped ‘vertical’ morphology rather than in concentric circles. In this article, we report experiments showing this is the case.

3. Self-organisation results from reactive processes and not liquid crystalline interactions

An important feature of self-organisation is whether it results from reactive processes or from static interactions related to the liquid crystalline properties of the microtubule solution. Here we summarise some of the arguments [29,30] indicating that self-organisation arises from chemical processes and not static interactions. A simple experiment [31,32] is to form the self-organised structure as described above, then attenuate the hydrolysis of GTP to GDP whilst simultaneously destroying the structure by mixing. After mixing, the solution contains microtubules at the same concentration and temperature as before. If the self-organised structure arises from static interactions, such as occur in some liquid crystals, then the structure will reform after mixing. This is not the case. Microtubules disassemble when cooled to 4 °C. If the preparation is again warmed to 36 °C, then microtubules once again form and GTP is

hydrolysed to GDP. When we carried out this experiment, the striped self-organised structure also reformed [31,32]. Microtubules may also be assembled, either under different buffer conditions or in the presence of stabilising agents such as taxol, such that the reaction dynamics are very different. In these cases, although microtubules are present at the same concentration as when self-organisation occurs, there is no self-organisation. These simple experiments indicate that the striped structure arises via chemical processes associated with microtubule formation and maintenance, and not from static interactions between the microtubules.

A further strong argument against static interactions is the dependence of self-organisation on gravity at a critical moment early in the process. Static interactions, such as may occur in liquid crystals, are equally present under conditions of weightlessness as at terrestrial gravity. If static interactions were giving rise to self-organisation, then self-organisation would also occur under conditions of weightlessness. The absence of microtubule self-organisation in weightlessness [22,33] is a clear demonstration that self-organisation does not arise from static interactions. Likewise, if the structure involved phase separation under the action of gravity, then the structure would form if a brief period of low gravity were followed by 1 *g* conditions. The fact that this behaviour is not observed [22,33] eliminates this possibility.

Static interactions in liquid crystals, although they can give rise to macroscopic variations in orientational order, do not lead to macroscopic variations in concentration. On the contrary, the central prediction of reaction–diffusion theories is the formation of macroscopic variations in the concentration of reacting species. Both neutron small angle scattering and fluorescence imaging demonstrate that substantial macroscopic microtubule concentration variations are present in the self-organised preparations [24].

During the initial stages of self-organisation, the left and right hand sides of the sample show respectively either obtuse or acute microtubule orientations. The striped structure subsequently develops by zones of acute orientation forming in the half of the sample with obtuse microtubule orientation, and vice versa. In neutron small angle scattering measurements restricted to a horizontal band of the dimensions of an individual stripe, the formation of an individual stripe manifests itself as a change in the direction, from an acute to an obtuse arc, of the microtubule scattering on the detector. During this orientational re-ordering, the intensity of the scattering, which is proportional to the microtubule concentration, decreases, then rises, before declining again. Orientational re-ordering, which is itself the stripe forming process, is hence associated with a chemical wave involving different concentrations of microtubules and free tubulin crossing the sample area under investigation. In other words, the stationary pattern arises because microtubules disassemble and reassemble with different orientations and concentrations in alternating parts of the sample. This neutron scattering experiment clearly [21] shows that self-organisation is associated with the reaction dynamics of the microtubules.

An important parameter in reaction–diffusion systems is the rate of energy dissipation. This will be strongly dependent upon experimental variables, such as concentration and temperature. In microtubule preparations, the rate of hydrolysis of GTP to GDP has been determined using P^{31} NMR spectroscopy [19,21,31,32]. In a reaction–diffusion system of the Turing-type, the periodicity or wavelength of the structure corresponds to approximately the distance over which groups of molecules diffuse before reacting. The periodicity, L, is related to the reaction rate, R, and diffusion constant, D, by terms involving $L^2=R/D$ [27,35]. In agreement with this, in self-organised microtubule preparations, increasing the reaction rate by a factor of 2 resulted in a decrease of the spacing by a factor of 1.4; the square root of the increase in the reaction rate [21].

The rate of diffusion is not varied as readily as the reaction rate. One simple approach is to examine the effect on self-organisation of the addition to the initial reaction mixture of small quantities of gelling agents. Increasing quantities of gelling agent increase the viscosity of the preparation, and inhibit diffusion. When microtubule assembly was carried out in gels of increasing agarose concentration, the effect was to perturb and eventually inhibit self-organisation [31,32]. This, and other observations concord with a diffusive contribution to the self-organising process.

Another possibility, which has also been considered and rejected, is that the pattern might in some way involve a coupling of reactive processes with flow due to thermal gradients during the warming process. The microtubule preparations are gels of high viscosity ($\approx$5000 poise) [34]. This renders thermal convection difficult. In addition, samples prepared in a hot room at 35 °C, in which there was no thermal convection, gave patterns the same as samples prepared under conditions where the bottom of the sample was warmer than the top by 5 °C [20]. Microtubules may also be formed by mixing together separate solutions of tubulin and GTP pre-warmed to 35 °C. In this case, the self-organised structure that develops is the same as that obtained by mixing tubulin and GTP in the cold and then warming. Hence, thermal convection appears to play no part in the self-organising process.

4. Results

Tubulin was isolated from cow's brains, purified, transferred into buffer, and stored in liquid nitrogen as previously described [19,35]. Different aliquots of the same tubulin preparation were used for all experiments. Samples, at 4 °C and a concentration of 10 mg ml^{-1} in the presence of 2 mM GTP, were placed to a height of 2 cm in glass cells, 4 cm by 1 cm by 0.1 cm. Experiments were carried out in the M2

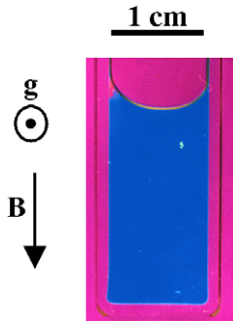

Fig. 1. Optical birefringence of microtubule preparation (10 mg ml^{-1}, 2 mM GTP) immediately after removal from a magnetic field of 13 Tesla, 15 min after instigating microtubule assembly. The sample was positioned horizontal in the field. The magnetic field was also in the horizontal direction. The sample was photographed through crossed linear polarisers, with a wavelength retardation plate between them at 45°. To highlight the uniform microtubule orientation, the sample was positioned with its long axis at 45° to the optical axis of the polarisers.

electromagnet at the Grenoble High Magnetic Field Laboratory, France. The magnetic field direction is along the horizontal and the intensity can be set between 1 to 13 Tesla. We constructed a sample holder that contained inserts for two sample cells. It was positioned in the magnet, so that samples were horizontal 'flat down', with one sample in the centre of the field and the other sample out.

Microtubules were assembled by placing cold samples of tubulin and GTP in the holder, preheated and maintained at 36 °C, positioned in the magnet as described above and operating at 13 Tesla. Initially, microtubules are not present in the preparation. They form and grow within the magnetic field. After 15 min, the holder was withdrawn from the magnet. At this time, no self-organised structure is apparent. The samples were photographed as described below (Fig. 1), and left for 5 h at 36 °C while the self-organised morphologies develop, then photographed again (Fig. 2).

Samples were observed between crossed polars with a wavelength retardation plate between them at 45°. In the absence of a sample, the birefringence of the wavelength retardation plate introduces a mauve interference colour. In sample regions, where microtubules are oriented in a direction opposed (135°) to the optical axis of the wavelength plate their birefringence subtracts from that of the wavelength plate and the interference wavelength decreases towards blue colours. Conversely, for regions where microtubules are oriented in the direction of the optical axis of the wavelength plate (45°), the overall birefringence increases and the interference colour shifts towards yellow. When self-organised preparations are viewed this way, the periodic variations in microtubule orientation appear as alternating yellow and blue stripes.

When the sample in the magnet was removed from the field after 15 min, it showed a strong uniform optical birefringence (Fig. 1) demonstrating that the microtubules were strongly aligned in the field direction. The microtubules have an average length of 5–10 μm [19,36] and the preparation has a high viscosity of several thousand poise. These factors inhibit any subsequent randomisation of orientation by thermal motions. At this time (15 min) the reference sample assembled outside the magnetic field did not show this birefringence. Five hours later, by which time self-organisation was complete, the reference sample had formed the normal 'horizontal' morphology. However, the sample subject to the horizontal magnetic field for the first 15 min, formed a striped morphology similar to that which forms when samples are prepared vertical outside of the magnet (Fig. 2). The magnetic field intensity may be considered as a bifurcation parameter and its value during the first 15 min of the process determines the subsequent behaviour, namely whether circles or stripes form. To determine the value of the magnetic field at which the bifurcation between the two morphologies occurs, the

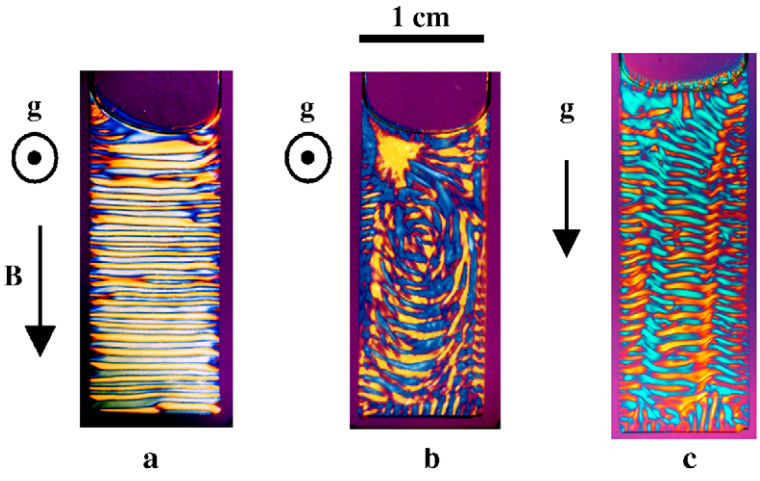

Fig. 2. The effect of exposure to a 13 Tesla horizontal magnetic field, for 15 min after instigating microtubule assembly, on the final self-organised microtubule morphology that develop 5 h later. Both samples were simultaneously positioned horizontal. The sample shown in (a) was placed in the magnetic field whereas sample (b) was outside of it. For comparison, (c) shows a sample prepared outside the magnet in the vertical position. Samples were photographed through crossed linear polarisers with a wavelength retardation plate between them at 45° with their long axis parallel to the optical axis of one of the polarisers.

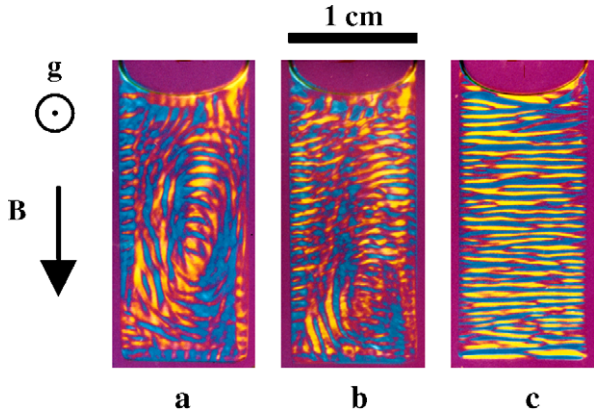

Fig. 3. Effect of different magnetic field strengths on the final self-organised microtubule morphology. Horizontal samples were exposed to a horizontal magnetic field for 15 min after instigating microtubule assembly. The magnetic field strengths were (a) 2 Tesla; (b) 4 Tesla; (c) 7 Tesla. Samples were photographed as described for Fig. 2.

experiment was repeated at different field strengths between 1 and 13 Tesla. As shown in Fig. 3, the transition between the two behaviours occurs at a field of approximately 4 Tesla.

5. Discussion

Experiments show that self-organisation contains both reactive and diffusive contributions and arises from chemical processes involving the continual growth and shrinkage of individual microtubules [23]. The complex, tubulin–GTP, is added at the growing end of a microtubule, and tubulin–GDP is liberated at the opposite shrinking end. This tubulin–GDP, whilst progressively diffusing out into the rest of the solution, is converted back to tubulin–GTP by excess GTP present, at which point it can be incorporated into the growing ends of neighbouring microtubules. New microtubules nucleate and grow in regions where the tubulin–GTP concentration is higher than a critical value.

Based on such a reaction–diffusion scheme, numerical simulations [36,37] predict, for suitably realistic reaction parameters, that the shrinking end of a microtubule leaves behind itself a chemical trail of high tubulin concentration. Likewise, the growing end produces a region depleted in tubulin. Because reaction rates increase with concentration, Glade et al. assumed that individual microtubules will preferentially grow into a region of high tubulin concentration whilst avoiding zones of low concentration. Numerical simulations predict [36,37] that when the chemical trails produced by individual microtubules are sufficiently strong, they modify and determine the direction of growth of their neighbours. In a large population of microtubules, initially equally distributed and un-oriented, the simulations predict the progressive development of oriented clumps of microtubules growing and shrinking along the same direction. Under these conditions, the direction of orientation within each microtubule clump is randomly determined and there is no macroscopic self-organisation over the whole sample. However, if at an early stage, there is a small asymmetry in the reaction–diffusion process that produces an orientational bias over the entire sample, then the simulations forecast the development of a self-organised structure. On a reaction space, 100 μm by 100 μm, containing 4.10^4 microtubules, they predict the formation, after 2–3 h of reaction time, of a self-organised structure comprised of regular bands of oriented microtubules of about 5 μm separation. This calculated structure compares well with the experimental structure over a similar distance scale. Portet et al. [38] have also carried out calculations of microtubule self-organisation by reaction–diffusion processes involving assembly and disassembly of microtubules and their conclusions are in keeping with those outlined above.

Although a slight orientational bias will suffice to trigger self-organisation, larger orientational effects, such as initially orienting the microtubules with a magnetic field, are calculated to have a similar effect. According to this explanation, any external factor that breaks the symmetry of the reaction–diffusion process by introducing an orientational bias over the entire sample will trigger self-organisation. The experimental results reported here, namely initially orienting microtubules with a strong magnetic field, are in agreement with this.

6. Conclusions

The results presented here demonstrate that microtubule self-organisation by reaction and diffusion is strongly dependent upon high magnetic fields. The behaviour is consistent with the hypothesis that self-organisation is triggered, as outlined above, by factors that cause an orientational bias that breaks the symmetry of the reaction–diffusion process. The application of a magnetic field, for a critical period early in the self-organising process, has an effect similar to a different orientation with respect to the gravity direction. Since, gravity is present under normal laboratory conditions, to observe an effect, the magnetic field must be large enough to overcome the effect of gravity. The magnetic field intensity acts as a bifurcation parameter. Under the conditions reported, the critical field strength is about 4 Tesla. The threshold at which gravity triggers self-organisation is between 10^{-2} to 10^{-1} g. Hence, if gravity were not present, we would expect that magnetic fields would trigger self-organisation at significantly lower field strengths of between 0.1 to 1 Tesla.

In cells, microtubule organisation, or reorganisation, by reactive processes, is frequently preceded by the presence of a biochemical or physical triggering factor. The in vitro processes outlined above provide one way by which physical stimuli, such as magnetic fields, might act. Biochemical processes based on this type of process could provide a novel mechanism for biological signal transduction and signalling. These remarks raise the question as

to whether such microtubule reaction–diffusion processes might also arise in vivo. If they do then cellular microtubule organisation might be modified by exposure to brief periods of high magnetic fields greater than 4 Tesla.

In support of this, it is known that cellular functions are modified when cells, in particular lymphocytes and osteoblasts, are cultured under conditions of weightlessness [39,40]. Many experiments point to an involvement of the cytoskeleton and microtubules. Recent experiments on cells cultured under low gravity conditions show modifications in cytoskeletal organisation. Human lymphocyte (Jurkat) [41], epithelial breast cancer cells (MCF-7) [42], utricular hair cells [43] and glial cells [44] cultured under conditions of weightlessness all show a disorganised microtubule network compared to 1 *g* control experiments. Microtubule organisation is central to many cellular functions and substantial changes, such as reduced growth rates and apoptosis, occur if it does not occur correctly.

Nuclear magnetic resonance imaging devises for medical purposes currently operate at around 2 Tesla and higher operating fields are envisaged. These fields are close to the bifurcation value of the magnetic field above which under the present conditions a modification in microtubule organisation might be anticipated. The behaviour described here provides a mechanism by which magnetic fields may intervene in fundamental biological processes. If the processes observed in vitro also arise in vivo, and are not corrected for by other cellular mechanisms, then brief periods of exposure to magnetic fields of higher than 4 Tesla may have significant biological consequences.

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