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Microtubule self-organisation by reaction–diffusion processes in miniature cell-sized containers and phospholipid vesicles

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Abstract

Under appropriate conditions, in vitro microtubule preparations self-organise over macroscopic distances by a process of reaction and diffusion. To investigate whether such self-organisation can also occur in objects as small as a cell or an embryo we carried out experiments in miniature containers of cellular dimension. When assembled under self-organising conditions in wells of 120–500 μm, microtubules developed organised structures. Self-organisation is strongly affected by shape, being highly favoured by elongated forms. In wells of more complex shape, geometrical factors may either oppose or strengthen one another and so inhibit or reinforce self-organisation. Microtubules were also assembled within phospholipid vesicles of 2–5 μm diameter. Under self-organising conditions, we observed large shape changes from spheroids to long tubes (50–100 μm) and intertwined coils. We conclude that self-organisation of microtubules by reaction–diffusion processes can occur in containers of cellular dimensions and is capable of strongly deforming the cellular membrane.

Keywords: Complex systems; Self-organisation; Reaction–diffusion; Nano-containers; Vesicles; Microtubules

1. Introduction

The physical chemical processes by which order and organisation develop in biological systems, such as an embryo or a cell, are uncertain. At present, there are two main approaches to this problem; one involves static interactions between non-reacting elements, for example, liquid crystalline ordering, phase separation, molecular association, etc. In many cases of this type, the system is not maintained far-from-equilibrium.

The other approach is based upon the non-linear dynamics of continually reacting species. Normally solutions of reacting chemicals in a test-tube do not self-organise. Nevertheless, progressively over the last hundred years, researchers have shown that this is not always the case. Theoreticians [1–5] have proposed that some types of chemical reaction might show strongly non-linear reaction dynamics due to being sufficiently far-from-equilibrium. They predicted that in some cases this could result in macroscopic self-organisation. Some chemical systems originally discovered in the 1920s [6] and 1950s [7,8] have been shown to self-organise this way by a coupling of reaction and diffusion [9,10]. The patterns that develop are comprised of periodic variations in the concentration of some of the reactants. Such structures are often called reaction–diffusion or Turing-like structures; the latter after the British mathematician who was one of the first persons to propose such a mechanism in 1952 [3]. Nicolis and Prigogine called them 'dissipative' structures [5,11] because a continual dissipation of chemical energy is required to drive and maintain the system sufficiently far-from-equilibrium such that self-organisation occurs. It is this flux or dissipation of chemical energy that provides the thermodynamic driving force for self-organisation.

The fact that a reactive process may be associated with self-organisation does not necessarily mean that the self-organisation results from reaction and diffusion. For example, in some cases, reactive processes may be associated with the formation

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of supra-molecular assemblies, which can then self-order by way of static interactions. For example, tubulin and actin assemble via GTP and ATP hydrolysis, respectively to form microtubules and actin filaments. Under suitable conditions, such preparations can form oriented liquid-crystalline phases due to the static interaction between the supra-molecular elements. In these cases, reaction is the cause of assembly but it is not the cause of self-organisation. Under other conditions, such as the microtubule preparation we consider here [12–14], it is the reactive processes themselves, and which continue after supra-molecular assembly has occurred, that give rise to self-organisation.

In addition to self-organisation, it has also been predicted [15] that some types of reaction–diffusion system may be sensitive to weak external factors, in so much as their presence at a critical moment, or bifurcation time, early in the process, can break the symmetry of the process and determine the morphology of the state that subsequently develops. Kondepudi and Prigogine explicitly calculated that the presence of weak fields, such as gravity, or an electric or magnetic field could have this effect [15].

The spontaneous generation of positional information is a fundamental biological property. It has frequently been proposed that reaction–diffusion processes might provide an underlying physical–chemical basis for biological pattern formation. At present, many developmental biologists consider pattern formation and symmetry breaking in early embryogenesis more as a description of a sequence of events, involving sperm entry and genetic expression, rather than in terms of an underlying physical–chemical process by which order and pattern spontaneously develop from an initially unstructured starting point. There is no conflict between these two approaches, which are complementary.

Cellular organisation is largely controlled by microtubules [16]. These are tubular shaped super-molecular assemblies, comprised of the protein, tubulin, which have inner and outer diameters of 16 and 24 nm [17]. They are frequently several microns long and may be formed in vitro by warming a preparation of purified tubulin in the presence of guanosine triphosphate (GTP) from about 7 to 36 °C. Chemical reactions occur, GTP is hydrolysed to guanosine diphosphate (GDP), and within 2–3 min the tubulin assembles into microtubules [18]. Once microtubules form, reactions continue by processes involving GTP hydrolysis. There is hence a continual flux or dissipation of chemical energy through the system. Microtubules have two major roles; they organise and control the structure of the cytoskeleton, and they permit and control the directional movement of intracellular particles and organelles from one part of the cell to another. They participate in many fundamental cellular functions including, the maintenance of shape, motility, signal transmission [16,17] and they play a determining role in the organisational changes that occur during the early stages of embryogenesis [19]. Microtubules are a significant component of brain neuron cells and they make up the mitotic spindles that separate the chromosomes during cell division. In cells, biologists have established that microtubule organisation results from the chemical reactions associated with their formation and maintenance [16,17].

Under suitable experimental conditions, microtubule preparations self-organise in vitro by a process of reaction and diffusion [12,13,20–23]. When assembled in glass containers, measuring 4 cm × 1 cm × 1 mm, a series of stationary horizontal stripes of about 0.5 mm separation progressively develop over a period of approximately 5 h (Fig. 1A). In each striped band, the microtubules are highly oriented at either 45° or 135°, and adjacent stripes differ in having opposing orientations. This pattern of changes in microtubule orientation coincides with an identical concentration pattern; the microtubule concentration drops by about 30% and then rises again every time the microtubule orientation flips from acute to obtuse. Another series of stripes of about 100 μm separation occurs within each stripe, and these in their turn contain further sets of stripes of about 20, 5 and 1 μm separation. In addition, the morphology that forms is determined by the presence, or absence, of weak external factors such as gravity [23], shearing [24,25], magnetic fields [14], at a critical moment early in the process. These fields are believed to act by way of an interaction that causes a weak directional bias on the action

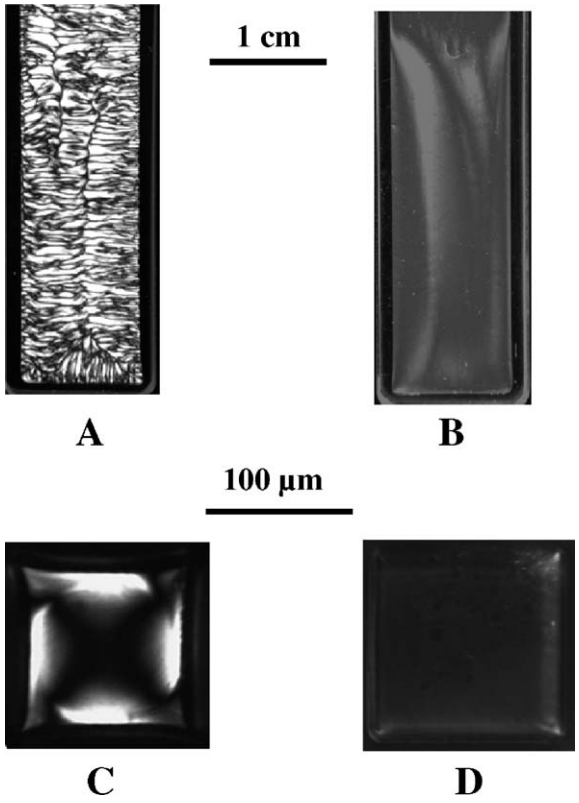

Fig. 1. Microtubules were assembled in buffer (see text) containing either 1 mM or 10 mM Mg^{++} ions. At 1 mM Mg^{++}, the microtubule reaction dynamics are such that self-organisation by reaction–diffusion processes occurs in centimetre-sized containers (A). On the contrary, at 10 mM Mg^{++}, the reaction dynamics do not permit self-organisation to develop beyond the extent shown in (B). Photographs (C) and (D) show the result of carrying out the same experiment (C; 1 mM Mg^{++} and D; 10 mM Mg^{++}) in miniature PDMS wells measuring 120 × 120 × 70 μm deep. The images show the final stationary morphologies, as viewed between crossed polarisers 5 h after instigating microtubule formation.

of individual microtubules [25,26]. This breaks the symmetry of the reaction–diffusion process and so affects the subsequent collective behaviour of the population. In other words, any factor that causes a weak orientational bias at the bifurcation time will affect self-organisation.

Various experiments, not described here, show that this behaviour comes about by the continual reactive processes involving GTP hydrolysis associated with microtubule formation and maintenance, and does not arise from phase separation or static interactions such as can give rise to lyotropic liquid crystalline phases. This evidence is summarised in Glade and Tabony [14].

The question arises as to whether microtubules might also self-organise this way in vivo. One of the limitations to comparing the in vitro behaviour outlined above, with the results of in vivo experiments, is the large size of the sample container (several cm) compared with the dimensions of many biological systems. Mammalian cells are often about 50 μm in size; plant cells are often somewhat larger (200 μm) and many embryos and seeds are approximately a millimetre in dimension. In addition, the walls of plant cells and embryos are essentially rigid, whereas those of mammalian cells are readily deformed.

We investigated whether microtubule reaction–diffusion processes can lead to self-organisation in cellular sized objects by assembling microtubules in miniature containers of from 2 to 500 μm in size. We assembled them in both poly-dimethyl siloxane (PDMS) elastomer sheets containing numerous small wells [27] and within phospholipid vesicles of 1,2 dioleoyl-*sn*-glycero-3[phospho-L-Serine] (DOPS) and 1,2 dioleoyl-*sn*-glycero-3 phosphocholine (DOPC). The first type of container is relatively rigid and the wells are from 120 to 500 μm in size. In this respect, they are similar to both plant cells and embryos. Phospholipid vesicles, on the other hand, are smaller (2–5 μm), are readily deformed, and resemble the membrane bilayer of a mammalian cell. We found that conditions that lead to self-organisation in centimetre-sized containers also result in self-organisation in miniature wells. The morphology that forms depends strongly on sample shape and dimensions; elongated forms particularly favour self-organisation. In containers of more complex form, geometrical factors either compete with one another and inhibit self-organisation, or on the contrary reinforce each other and further favour self-organisation. When microtubules are assembled in phospholipid vesicles of 2–5 μm diameter, self-organising conditions result in a substantial deformation of the vesicles from spheroids to long (50–100 μm) tubes and intertwined coils.

2. Experimental

Tubulin was isolated from cow brains, purified using standard procedures on a phospho–cellulose column [28] and transferred into buffer comprised of 100 mM MES (2-N morpholino ethanesulphonic acid),1 mM EGTA (ethylene glycol-bis-(B-aminoethyl) *N*, *N*, N^1, N^1 tetra-acetic acid), and 1 mM $MgCl_2$ in D_2O, at pH 6.75. After concentrating the tubulin to about 25 mg. ml^{-1}, aliquots were stored in liquid nitrogen. Before use, the solution was thawed to 7 °C then diluted in buffer to a tubulin concentration of 10 mg. ml^{-1}. Microtubules were assembled by warming the tubulin solution to 36 °C, in the presence of excess GTP. This was provided either directly at a concentration of 2 mM, or by way of a GDP to GTP enzyme regenerating system comprised of acetyl kinase (0.8 μg. ml^{-1}), acetyl phosphate (20 mM) and 1 mM GTP. This arrangement keeps the GTP concentration constant at 1 mM during the experiment. Experiments carried out using both methods of furnishing GTP showed the same behaviour. Some preparations contained $MgCl_2$ to a final concentration of 10 mM. In many cases, the fluorophore, 4′,6-diamidino-2-phenylindole (DAPI), was added to a final concentration of 8 μM. The molecule DAPI, possesses the property that its fluorescence is approximately eight times higher when associated with microtubules than when associated either with free tubulin or in buffer solution [29]. In these preparations, the DAPI fluorescence intensity is proportional to the microtubule concentration [22]. The concentration of DAPI employed here (8 μM) was sufficiently low as to not affect either self-organisation or the tubulin assembly kinetics.

Miniature wells of cellular dimensions were made in the following way [30]. The required sample shapes were drawn onto a quartz mask, and a template, comprised of islands of resin standing out from a flat surface, produced from it by lithographic methods. The template, measuring about 2×2 cm, was then used as a mould. Poly-dimethyl siloxane (PDMS) precursor and curing agent were mixed at room temperature to a mass ratio of 10 : 1, degassed with a water pump, poured over the mould, and degassed again. The silicone elastomer sheet of about 1 mm thickness was then cured overnight at 60 °C. When peeled away from the mould, it contains numerous small wells of the desired shape and size. This sheet was then cut to size, placed on a microscope cover slide positioned in the lower part of a metallic support and cooled to 7 °C. A drop of cold tubulin solution was added, and the sample gently degassed with a water pump. Another cover slide was then placed on top and secured in place with the upper part of the pre-cooled metal support. Microtubules were formed by placing the sample, lying flat, in a hot room at 36 °C. After several hours, the sample was placed on the hot stage (36 °C) of a Zeiss Akioscop microscope and observed with a Zeiss Achroplan LD 20 objective in the following ways: I) through crossed polars; II) from the fluorescence of the added DAPI (excitation 360 nm, emission 460 nm); III) with differential interference contrast (DIC) optics. In each experiment, to check that sample preparations self-organised in the manner previously described, samples were also made up in glass spectrophotometer cells, $4 \times 1 \times 0.1$ cm, and the self-organised morphology observed after 5 h.

There are several reports of the in vitro assembly of microtubules and actin in phospholipid vesicles [31,32]. We adapted the protocol described by Emsellem et al. [31]. Phospholipid vesicles comprised of DOPC and DOPS as supplied by Avanti Polar Lipids Inc. were made up to concentrations of 3 and 2 mg. ml^{-1}, respectively, in chloroform. The chloroform was then pumped off from 25 μl of this solution and the deposit re-hydrated for 2 h at 7 °C with 25 μl of the tubulin/buffer solution. In these cases, the

buffer solution also contained 20 μM rhodamine chloride and 8 μM DAPI. The solution was centrifuged at 100 ×*g* for 1 min and 15 μl of the supernatant diluted in 60 μl of 150 mM NaCl at 7 °C. Vesicles of 2–5 μm diameter form, which contain within them the tubulin/buffer solution. The NaCl buffer, which does not contain rhodamine chloride, remains outside the vesicles. The rhodamine chloride uniformly stains the tubulin/buffer solution within the vesicles. Its fluorescence (excitation 575 nm, emission 610 nm) can be used to readily detect, and confirm, that vesicles contain tubulin solution. Microtubules were then formed by placing the solution of vesicles in a water bath at 36 °C. It was left at this temperature for several hours before observing the vesicles under a Zeiss Akioscop microscope (Plan Neofluar 100 objective) by DIC optics, and rhodamine and DAPI fluorescence. DAPI fluorescence arises once the tubulin has assembled into microtubules and demonstrates that the vesicles under observation contain assembled microtubules.

3. Results

3.1. Self-organisation in miniature wells of different shape

Microtubule self-organisation requires specific reaction dynamics and does not occur under all buffer conditions. A simple way of modifying the behaviour is to increase the concentration of Mg^{++} ions in the buffer solution. With the buffer employed here the microtubule reaction dynamics are such that self-organisation occurs at 1 mM $MgCl_2$ but does not occur at 10 mM $MgCl_2$ (Fig. 1A, B). Microtubules assemble to the same extent in both cases.

Microtubules were assembled in miniature PDMS wells as described above. The well shapes we examined varied from squares and canals to forms that are more complicated. The largest and smallest dimensions were 500 and 120 μm, respectively, and the wells are about 70 μm deep. At 1 mM $MgCl_2$, stationary self-organised structures developed after approximately 3 h (Fig. 1C). However, self-organisation did not come about (Fig. 1D) when the experiment was carried out at 10 mM $MgCl_2$. This observation strongly suggests that the self-organised structure observed in the miniature wells results from the same reaction–diffusion processes that give rise to self-organisation in large sample cells.

Each PDMS elastomer sheet contains several hundred identical wells. The self-organised morphologies that form in individual wells all closely resemble one another. When observed through crossed polars, the contents of each well are of high optical birefringence. This indicates that the microtubules are strongly oriented and the patterns observed this way highlight variations in microtubule orientation. The DAPI fluorescence, whose intensity is proportional to microtubule concentration, indicate that substantial variations in microtubule concentration are also present in the structure. Preparations were also observed using differential interference contrast (DIC) optics in which the contrast results from a combination of factors related to both microtubule concentration and orientation.

Fig. 2 shows the structures that develop in square shaped containers of 175 and 235 μm. The patterns of microtubule concentration and orientation describe circles around the perimeter of the cell. They often show a swastika or spiral like appearance. We counted approximately as many spirals turning to the left as to the right. In addition, both the microtubule concentration and orientation are lower in the corners than elsewhere. In centimetre-sized containers, either striped or circular morphologies may arise depending on the

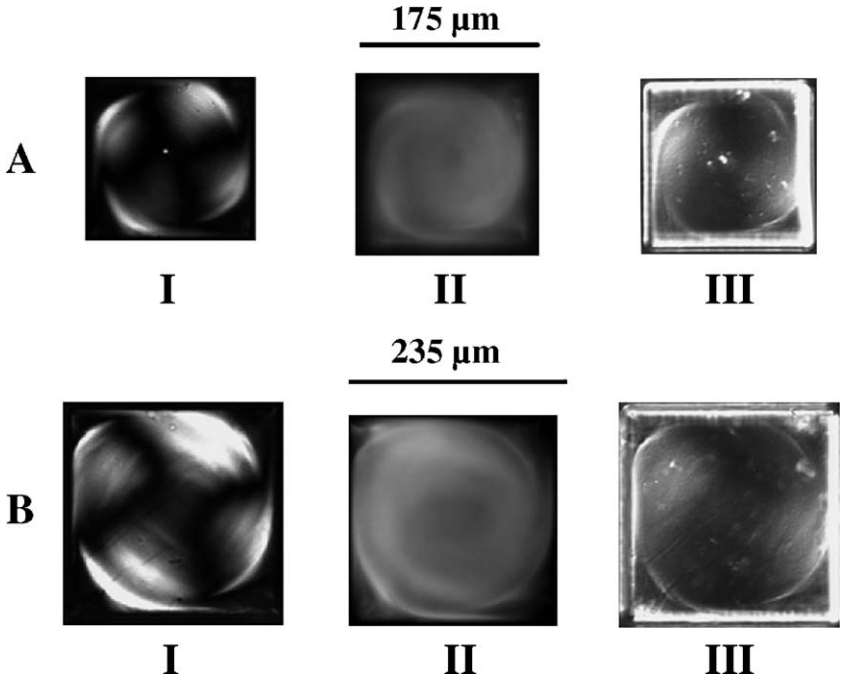

Fig. 2. Self-organised microtubule morphologies in square wells; (A) 175 × 175 × 70 μm; (B) 235 × 235 × 70 μm. Preparations were observed; I) through crossed polars positioned 45° to the axis of the well; II) by way of DAPI fluorescence; III) using DIC optics.

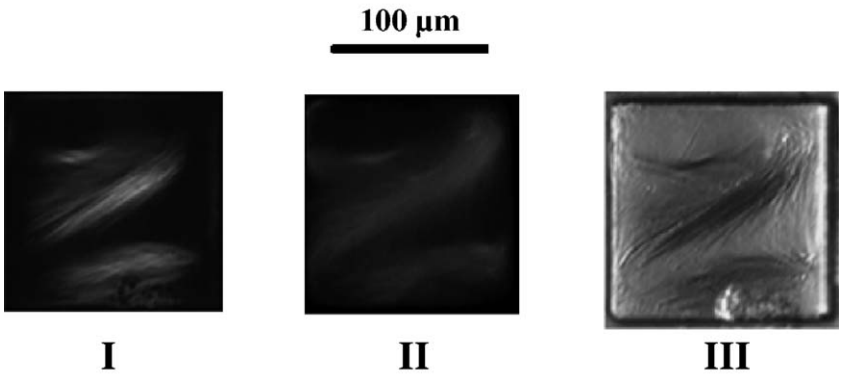

Fig. 3. Self-organised microtubule morphologies in square wells identical to those of Fig. 1C and D. In this case, the preparations were assembled with a higher initial rate of heating. The change in the initial reaction dynamics that this causes favours a slightly different type of morphology resembling the beginning of a striped arrangement. Preparations were observed; I) through crossed polars positioned 45° to the axis of the well; II) by way of DAPI fluorescence; III) using DIC optics.

orientation of the sample with respect to gravity at a critical time early in the process. The system is finely balanced between forming either of these morphologies. When the experiment in PDMS wells described above was carried out with a higher initial rate of heating (from 7 to 36 °C) then rather than forming circles, an S-like morphology develops (Fig. 3). In all of these cases, the self-organising process has spontaneously generated significant positional information within the cellular sized container.

Changing the geometry of the sample wells strongly affects the final morphology. In canal shaped containers, (Fig. 4A) many microtubules are oriented along the general direction of the long axis. The microtubule orientation alternates periodically between acute to obtuse whilst transiting through a parallel orientation close to the sample boundaries. The microtubule concentration shows a similar pattern, with regions of high and low concentration alternating along the canal. A similar type of structure develops in cylindrical capillaries of 300 µm diameter (Fig. 4B). Depending on the orientation of the cross polarisers with respect to the long axis of the sample (either 45° or 90°) then the pattern of birefringence shows either undulations as in Fig. 4AI or stripes as in Fig. 4B.

Additional effects arise when geometrical units are connected together. Depending on the exact geometry, different parts of the sample may either inhibit or reinforce self-organisation. Fig. 5A shows the effect of connecting a canal to one of the sides of a square. In this case, the strongly self-organised microtubules coming from the canal inhibit the weaker self-organisation that would have otherwise developed within the square. Fig. 5B shows the self-organised morphology that arises when two canals are joined orthogonal to one another. In the region away from the intersection, self-organisation occurs as for isolated canals. However, at the junction, where oriented microtubules arriving from orthogonal directions meet up, there is little or no self-organisation and the microtubule concentration is low.

The opposite behaviour comes about when the geometry is comprised of four squares connected at the corners of another central square (Fig. 5C). The effect of the connection on the four adjoining boundaries is to reinforce self-organisation. Strongly oriented microtubule arrays develop that penetrate well into the individual squares at an angle of approximately 45°. The corners of the squares joined in this way, are regions of high microtubule concentration and orientation and not the contrary.

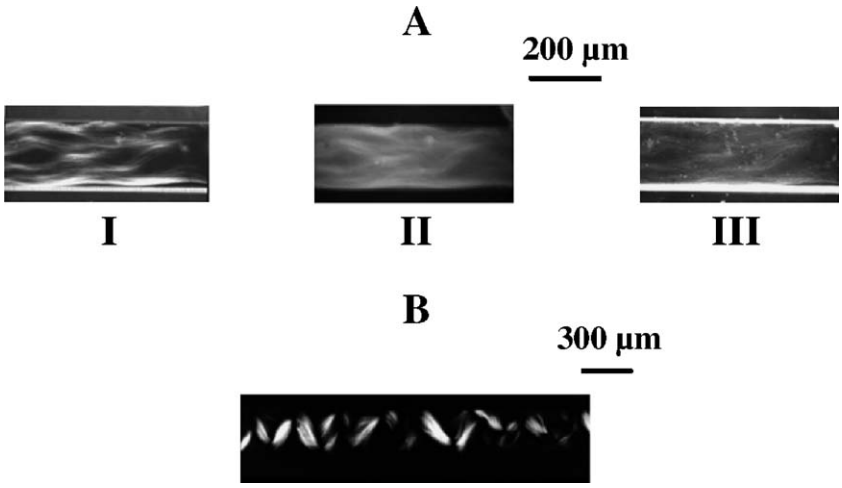

Fig. 4. Self-organised microtubule morphologies in; (A) canal shaped wells, 200 × 5000 × 70 µm deep. Preparations were observed I) through crossed polars positioned 45° to the axis of the well; II) by way of DAPI fluorescence; III) using DIC optics. (B) Shows the self-organised morphology that develops in a 300 µm diameter capillary when observed through crossed polars parallel to the long axis.

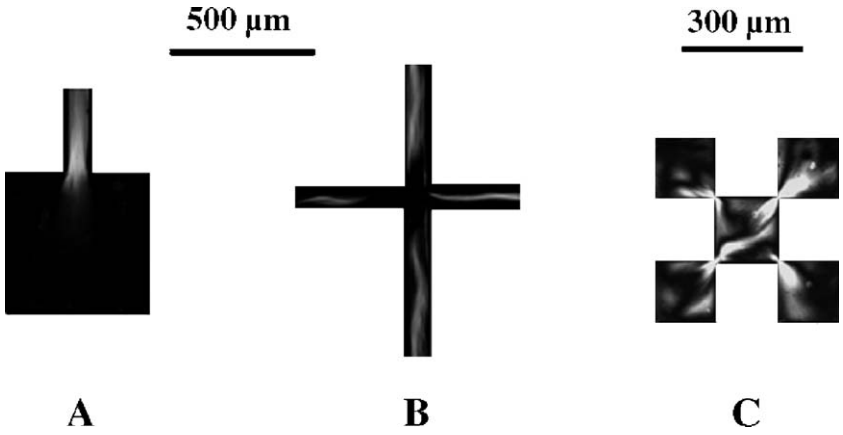

Fig. 5. Self-organised microtubule morphologies in more complex geometries. The geometry in (A) is comprised of a canal attached to a square. That in (B) is made up of two orthogonal canals. The geometry in (C) is comprised of four squares connected to the corners of another central square. For clarity, the boundary of the PDMS wells are indicated by the white background. Samples were observed through crossed polarisers.

3.2. Self-organisation in phospholipid vesicles

We also assembled microtubules in phospholipid vesicles of DOPS/DOPC under both self-organising (1 mM $MgCl_2$) and non-self-organising (10 mM $MgCl_2$) buffer conditions. Before assembling the tubulin into microtubules, the vesicles containing the unassembled tubulin were spheroids of 2–5 µm diameter. Thirty minutes after assembly, for both self-organising and non-self-organising conditions, the vesicles retained their initial size and shape. Three hours later, no changes in vesicle size and shape were observed in samples at 10 mM $MgCl_2$ (Fig. 6A). In this case, microtubule formation had no major effect on the vesicles. However, for samples in which self-organisation by reaction and diffusion does occur (1 mM $MgCl_2$), after three hours a significant number of vesicles showed dramatic shape changes. Many had developed long (about 50–100 µm) asymmetric linear tube-like shapes of about 2 µm diameter (Fig. 6B). We observed a significant number of bulbous shapes, such as shown in Figs. 6B and 7, suggesting that the long tubular forms observed arise by an elongation process. We also saw a number of more complicated structures in which tubes appeared to be folded into a helix or

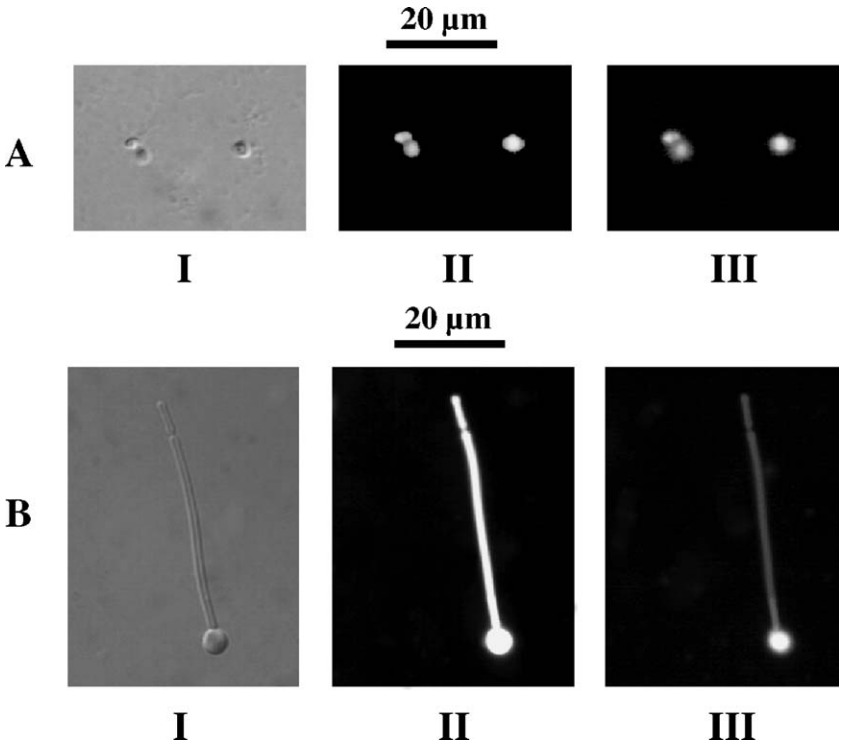

Fig. 6. Microtubules assembled in vesicles of DOPS/DOPC under (A) non-self-organising buffer conditions (10 mM Mg^{++}), and (B) self-organising buffer conditions (1 mM Mg^{++}). The tubulin preparation (10 mg. ml^{-1}) enclosed within the vesicles also contained rhodamine chloride (20 µM) and DAPI (8 µM). The photographs, taken three hours after instigating microtubule formation, were recorded by way of I) DIC optics, II) rhodamine epiflorescence, and III) DAPI epifluorescence. The rhodamine fluorescence arises from tubulin buffer contained within the vesicles, whereas the DAPI fluorescence arises from tubulin assembled into microtubules.

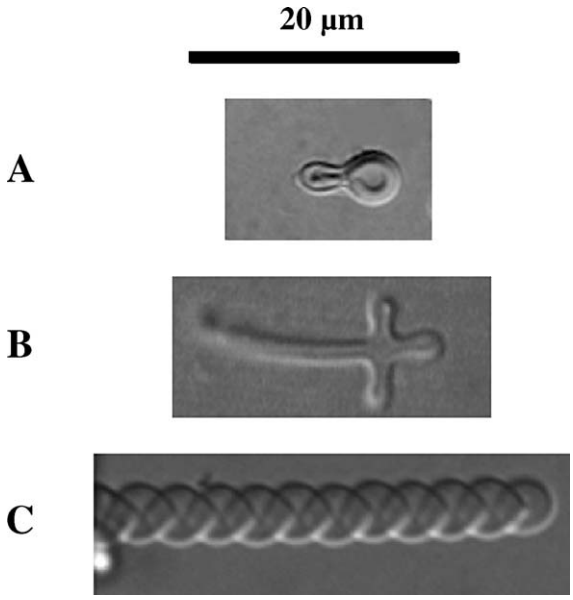

Fig. 7. Under the self-organising conditions shown in Fig. 6, vesicles of DOPS/DOPC also occasionally develop the types of morphology shown above. Samples were observed by way of DIC optics.

form a cross (Fig. 7). In addition, in some cases we recorded what could be a fusing together of spheroidal vesicles. We did not observe any such effects, either in vesicles not containing microtubules, or in vesicles containing microtubules assembled under non-self-organising conditions.

4. Discussion

4.1. Self-organisation in PDMS wells

Microtubules are chemically anisotropic and their reactivity at opposite ends differs in such a way that frequently they grow from one end whilst shrinking from the other. A microtubule incorporates molecules of the complex, tubulin-GTP, at its growing end and liberates tubulin-GDP from its shrinking end. The tubulin-GDP thus liberated progressively diffuses out into the rest of the solution whilst at the same time being converted back to tubulin-GTP by excess GTP present. At this point, the regenerated tubulin-GTP is again available for incorporation into the growing ends of neighbouring microtubules. In a similar manner, the growing end of a microtubule can cause a region depleted in tubulin-GTP. Microtubule self-organisation, as described here, is believed to occur by a combination of reaction and diffusion in which individual microtubules either grow into, or avoid, trails of high and low tubulin concentration caused respectively by the shrinking and growing ends of their neighbours [13,23,26].

For suitable reaction parameters consistent with experimental values, Glade et al. carried out numerical simulations [26,33] that forecast both the formation of these tubulin trails and that these trails can modify the rate and direction of growth of their neighbours. In a large population of initially equally distributed and un-oriented microtubules, the simulations predict the development of clumps of microtubules oriented along the same direction. Under these conditions, the microtubule orientation of individual clumps are not correlated and macroscopic self-organisation does not develop. If, however, at an early critical time, a small asymmetry in the reaction–diffusion process is present that produces a small orientational bias, then the orientation of the microtubules in the clumps become correlated and the simulations predict the development of a self-organised structure similar to the experimental pattern. According to this explanation, and in agreement with experiments, any external factor that breaks the symmetry of the reaction–diffusion process, by leading to a privileged direction of microtubule orientation or growth, will trigger self-organisation. Portet et al. [34] 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.

The shape and dimensions of the reaction space may also break the symmetry of the process and thus affect the behaviour of the microtubule population. This is illustrated by numerical simulations carried out using the algorithm and reaction parameters outlined above (see [26] for a full description). If boundary effects are minimised by using a continuous reaction space, then in the absence of an external symmetry breaking factor, macroscopic self-organisation does not arise [26]. When however, the simulation is carried out in a geometry comprised of two parallel boundaries, then the orienting effect produced by the boundaries is itself sufficient to bring about self-organisation. Even in the presence of other external symmetry breaking factors, such as gravity, this effect is sufficiently large that the overall morphology will be affected by the boundary conditions. Fig. 8 show the simulated self-organised morphology that develops in a 20 μm wide canal. The microtubules adopt an orientation in the general direction of the long axis of the canal and both their orientation and concentration show regular wave-like variations resembling those observed experimentally for a similar geometry (Fig. 4).

This behaviour comes about because a microtubule stops growing when it arrives at an impenetrable, rigid boundary. For the type of reaction dynamics adopted in the algorithm, a microtubule that can no longer grow, shrinks. Hence, close to a boundary, microtubules growing perpendicular to it are at a disadvantage compared with those growing parallel. The presence of a boundary thus favours the development of parallel microtubules and this affects self-organisation. This explanation accounts for why, in experiments carried out in miniature wells, microtubules often develop a privileged direction of orientation parallel to the boundary.

The fact that close to the boundary, perpendicular microtubules shrink and disassemble more readily than elsewhere, leads to a lower microtubule concentration here than further away. Hence, higher microtubule concentrations tend to develop towards the interior of the reaction space. In a region of slightly higher microtubule concentration, the tubulin-GTP concentration, regenerated from the shrinking ends of microtubules present, is also higher. This results in an increased rate of reaction, which in turn leads to a further increase in microtubule concentration. Higher microtubule concentrations liberate more tubulin, which results in more microtubules,

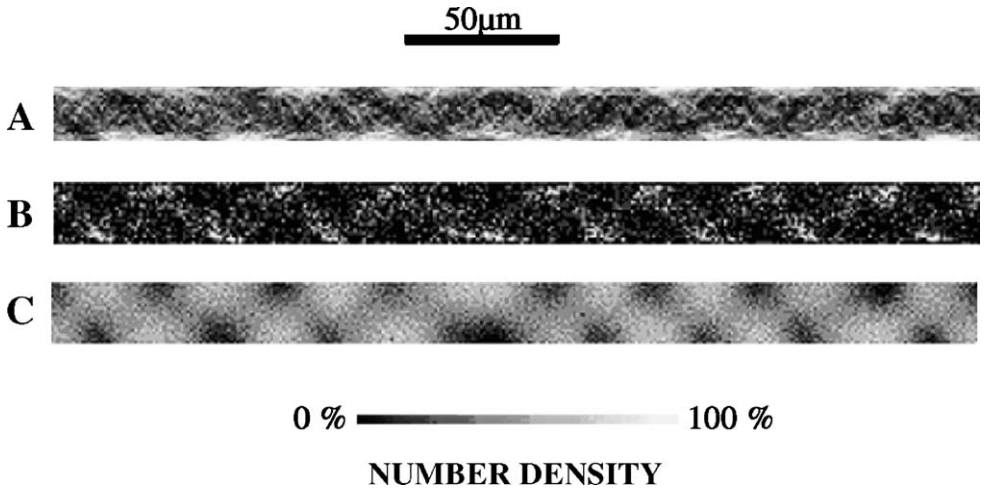

Fig. 8. Simulated self-organised morphology in a two-dimensional canal of 20 μm width. (A) Shows the distribution of microtubules; (B) is the same data, but in which only the extremities of the microtubules are represented. (C) Shows the distribution of tubulin-GTP. See text and Ref. [26] for full details of the algorithm and parameter values.

which leads to more tubulin, etc. Likewise, in regions of initially slightly lower microtubule concentration, the opposite behaviour occurs. The reaction–diffusion process thus gradually results in a reinforcement of the initially weak variations in concentration and orientation, and with time, a stationary pattern develops where regions of high microtubule concentration and orientation alternate with those of low concentration and orientation.

The presence of two orthogonal boundaries means that microtubules developing parallel to one boundary will partially disassemble when they encounter the other. Hence, the corners of a square can become regions unfavourable to self-organisation and of low microtubule concentration and orientation.

In the experiments on canal shaped samples, the level of microtubule organisation is significantly higher than for square shaped samples. This observation illustrates that self-organisation is both strongly dependent on sample shape and is favoured by elongated forms. Because the presence of a boundary favours the growth of microtubules oriented parallel to it, highly asymmetric shapes introduce a preferred overall direction of microtubule growth and orientation. As illustrated in the simulation described above, such a bias strongly favours self-organisation. The concordance of the simulated self-organisation with experiment, combined with the fact that experimental self-organised patterns do not develop under non-self-organising conditions supports the premise that the behaviour reported here arises from reaction and diffusion.

Sample geometries with orthogonal boundaries may cause a conflict in that the boundary direction changes abruptly by 90°. Microtubules, however, require a minimum distance before their orientation can change by such an amount. Hence, when a canal is connected to the side of a rectangle, the orientational effect caused by the canal boundary modifies the pattern of microtubule orientation and concentration that would otherwise developed in the square. Likewise, when two orthogonal canals meet, then at the intersection, oriented arrays of microtubules coming from orthogonal directions do not have sufficient space to make a 90° turn. In this region, self-organising effects from the two canals conflict with one another and lead to a zone of low microtubule orientation and concentration. The opposite occurs when squares are joined at their corners. The connections strongly promote microtubule organisation along the diagonal and the connected corners, instead of being regions unfavourable to microtubule formation and self-organisation, show the opposite behaviour.

Researchers have already reported the results of experiments on microtubules assembled in miniature containers. However, these preparations also contained either molecular motors [35] or centrosomes [36,37]. In these cases, the microtubule reaction dynamics, microtubule concentration and biological conditions all differ significantly from those used here. Microtubule organisation, when observed, is believed to arise from the molecular motors or centrosome organising centres present, and not from a reaction–diffusion process such as described here.

4.2. Self-organisation in phospholipid vesicles

Growing microtubules are known to be able to exert a force on objects in their path. During self-organisation by reaction and diffusion, macroscopic arrays of oriented microtubules develop that collectively transport large numbers of colloidal particles along the direction of microtubule orientation [38]. The process is hence capable of generating collective forces that may be sufficient to significantly deform a membrane bilayer. The fact that the shape and size changes described above do not occur either under non-self-organising buffer conditions, or since self-organisation by reaction–diffusion takes several hours, immediately after microtubule assembly, strongly suggest that the self-organising process itself causes them. The large shape changes observed could arise either by deforming a spherical vesicle and/or by the fusing together of several vesicles, and there is evidence that both processes occur. We observed a large number of morphologies in which a long protruberation extends from a bulbous end (Figs. 6B and

7). This suggests that long shapes observed arise by a deformation and extension of spheroidal vesicles. Depending on the lipid composition, Roux et al. have shown [39], that spherical vesicles are readily deformed into very long cylinders. The lipid composition of the vesicles used in this report is consistent with those permitting such a ready deformation.

There are several previous reports concerning the in vitro assembly of microtubules within phospholipid vesicles. Emsellem et al. observed a small deformation from spheroids to elliptical shapes [31]. In this case, the buffer conditions used were such that self-organisation by reaction and diffusion would not occur. Fygenson et al. used buffer conditions [40], which although not permitting macroscopic self-organisation, have reaction dynamics significantly closer to those used in this report. They observed a transition from spheroids of 5 μm diameter to tubes of 15 μm length. In both cases, the microtubule concentrations were approximately ten times lower than that used here.

5. Conclusions

We have assembled microtubules in sample containers of cellular dimensions and have shown that self-organisation by reaction–diffusion processes, similar to that which occurs in large centimetre-sized containers, also arises in these miniature containers. One of the advantages of this approach is that sample dimensions and shapes may be readily produced to mimic those found in cells and embryos. Experiments in such miniature containers permit a more meaningful comparison both with observations of microtubule organisation in vivo, and also with numerical simulations of self-organisation where computing power limits the size of the reaction space to a maximum of about 200×200 μm.

The generation of positional information is a basic phenomenon underlying embryogenesis and biological pattern formation. Normally, solutions of reacting chemicals in a test-tube do not self-organise. In the experiments reported here, containing initially only two reacting species (tubulin and GTP), the reaction–diffusion process leads to the spontaneous formation within the reaction space of patterns of microtubule orientation and concentration that contain a considerable quantity of positional information.

Sample shape and dimensions have a substantial effect on the self-organised morphologies. Elongated forms strongly favour self-organisation. In more complex shapes, different geometrical parts may either reinforce or inhibit self-organisation in an intricate manner and small shape changes can strongly affect the final pattern. Joining two squares by a short canal substantially modifies self-organisation and promotes the formation of aster-like arrays of microtubules reminiscent of those that occur during cell division where the two cells are still connected by a channel.

When microtubules are assembled under self-organising conditions in phospholipid vesicles, then dramatic changes in the size and shape of the vesicles occur. In these experiments, the presence of molecular motors was not necessary and the deforming force is produced by the collective growing and shrinking of the individual microtubules along the direction determined by the self-organising process. In such deformable containers, both sample shape and self-organisation mutually reinforce one another.

Organisation and re-organisation of microtubules, plays a substantial role in determining and modifying cell shape. The molecular basis by which reaction–diffusion processes leads to the type of behaviour described above hence raises the possibility that such processes may form an underlying physico-chemical basis for the way by which microtubules determine and modify cellular organisation and shape.

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