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Effect of weightlessness on colloidal particle transport and segregation in self-organising microtubule preparations

James Tabony *, Nathalie Rigotti, Nicolas Glade, Sandra Cortès

Commissariat à l'Energie Atomique, DSV, Institut de Recherches en Technologies et Sciences pour le Vivant, CEA-Grenoble, 17 rue des Martyrs, 38054 Grenoble, Cedex 9, France

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Abstract

Weightlessness is known to effect cellular functions by as yet undetermined processes. Many experiments indicate a role of the cytoskeleton and microtubules. Under appropriate conditions *in vitro* microtubule preparations behave as a *complex* system that self-organises by a combination of reaction and diffusion. This process also results in the collective transport and organisation of any colloidal particles present. In large centimetre-sized samples, self-organisation does not occur when samples are exposed to a brief early period of weightlessness. Here, we report both space-flight and ground-based (clinorotation) experiments on the effect of weightlessness on the transport and segregation of colloidal particles and chromosomes. In centimetre-sized containers, both methods show that a brief initial period of weightlessness strongly inhibits particle transport. In miniature cell-sized containers under normal gravity conditions, the particle transport that self-organisation causes results in their accumulation into segregated regions of high and low particle density. The gravity dependence of this behaviour is strongly shape dependent. In square wells, neither self-organisation nor particle transport and segregation occur under conditions of weightlessness. On the contrary, in rectangular canals, both phenomena are largely unaffected by weightlessness. These observations suggest, depending on factors such as cell and embryo shape, that major biological functions associated with microtubule driven particle transport and organisation might be strongly perturbed by weightlessness.
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Keywords: Bio-complexity; Weightlessness; Particle transport and segregation; Self-organisation; Reaction–diffusion; Microtubules

1. Introduction

Gravity is known to intervene in some biological processes. For example, plant roots grow along the gravity direction [1]. In some species, at a critical moment during the early stages of embryogenesis, it intervenes in the processes that determine the direction of the axis of the body of the organism which subsequently develops [2–5]. In astronauts, weightlessness has been reported to cause a decrease in bone mass and modified immune responses [6–8]. Experiments on various cell types exposed to conditions of weightlessness – in particular those of the osteo and immune systems – show modifications in cellular functions [9–11]. However, because the accelerating force produced by gravity is considered insufficient to intervene in the biochemical and reactive processes which occur within a cell or embryo, these observations have not been satisfactorily accounted for.

One possible manner by which gravity may participate in some biological processes is by way of its symmetry-breaking intervention in some reactive self-organising mechanisms. In *complex* systems comprised of populations of strongly coupled elements, new so-called *emergent* phenomena develop by way of the non-linear dynamics by which elements are coupled together and behave as a collective ensemble [12–14]. In many *complex* systems, self-organisation occurs as a major *emergent* property. A feature of some systems is that self-organisation is strongly affected by weak external factors that break the symmetry of the process and so modify the collective behaviour.

Biological processes are largely based on chemical and biochemical reactions. Solutions of reacting chemicals do not normally self-organise. Nevertheless, it has been established, both theoretically [15,16] and experimentally [17–19], that this is not always the case and that some reactive systems can self-

* Corresponding author. Tel.: +33 4 38 78 96 62.
E-mail address: james.tabony@cea.fr (J. Tabony).

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organise by behaving as a *complex* system in which molecular species are coupled together in a non-linear manner by a combination of reaction and diffusion [20]. In addition, it has been predicted theoretically that in some cases the presence at a critical moment early in the process of a weak external factor, such as gravity or a magnetic field, which breaks the symmetry of the process can determine the self-organised morphology that subsequently develops [21].

Under appropriate conditions the formation *in vitro* of microtubules shows this type of behaviour [22,23]. In these preparations, self-organisation also results in the collective transport and organisation of any colloidal and sub-cellular particles initially present in the reaction mixture [24]. No other biological agents, such as molecular motors, are present. Here we show that a brief early period of weightlessness can strongly inhibit these transport phenomena. We used two different methods to attenuate the effects of gravity; free-fall conditions produced in a sounding rocket of the European Space Agency and clinorotation [25,26]. Both methods resulted in equivalent behaviour. In large centimetre-sized containers, exposure to 13 min of weightlessness prevented the collective particle transport that self-organisation causes [27]. In miniature cell-sized containers under normal gravity conditions, we observed that collective particle transport can lead to a strongly segregated particle distribution. In these small containers, the effect of weightlessness depends strongly on their shape. In square wells, weightlessness inhibited both self-organisation and the transport and organisation of colloidal particles. However, in canal-shaped containers, weightlessness had no major effect. Hence, in this case, the asymmetric shape of the container is sufficient to break the symmetry of the process and permits the development both of self-organisation, and collective particle transport and segregation.

2. Microtubule self-organisation and collective particle transport

Microtubules are tubular shaped supra-molecular assemblies, several microns long, having inner and outer diameters of 16 nm and 24 nm, that arise from the self-assembly of the protein, tubulin, by way of reactions involving the hydrolysis of guanosine triphosphate (GTP) to guanosine diphosphate (GDP) [28,29]. They may be formed *in vitro* by warming a solution of purified tubulin in the presence of excess GTP from about 7 °C to 36 °C. A series of chemical reactions occur; GTP is hydrolysed to GDP, and microtubules form within 2–3 min. The reaction then continues by processes in which tubulin molecules are added to the growing end of a microtubule whilst others are liberated from its shrinking end. The reaction dynamics at opposite ends of a microtubule are different; consequently, often one end grows whilst the other end shrinks.

For appropriate reaction dynamics, *in vitro* preparations self-organise over a period of several hours to form stationary macroscopic patterns of microtubule orientation and concentration [22,30]. In spectrophotometer cells ($40 \times 10 \times 1$ mm), a series of equidistant (0.5 mm) stripes form. Each individual stripe contains within itself, another series of stripes of about 100 μm separation and these stripes in turn contain further levels of organisation of about 20 μm, 5 μm and 1 μm separation.

Gravity intervenes in the self-organising process [31]; striped morphologies occur when the experiment is carried out in sample containers that are vertical, but a different pattern arises when microtubules are assembled the same containers lying flat [32]. The final stationary morphology depends upon the direction of the sample with respect to gravity at a critical "bifurcation" time early in the process (6 min) before any pattern is visible [33]. Self-organisation does not occur when preparations are exposed to conditions of weightlessness for the first 13 min of the process [27].

Experiments show that self-organisation arises from reactive processes involving the growth and shrinking of individual microtubules. For appropriate microtubule reaction dynamics, 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. We postulate that neighbouring microtubules will preferentially grow (and new microtubules nucleate and form) into regions of high tubulin concentration whilst avoiding those of low concentration. The chemical trails produced by individual microtubules will then modify and determine the direction of growth of their neighbours. Thus neighbouring microtubules are coupled together and the overall population forms a *complex* system that progressively self-organises by a reaction–diffusion process. For self-organisation to occur both experiments, and numerical simulations of the process outlined above, require in addition, the presence of a weak external factor that induces a weak directional bias on the action of individual microtubules [34,35]. This breaks the symmetry of the reaction–diffusion process and so affects the subsequent collective behaviour. Gravity is believed to act by interacting with density fluctuations caused by microtubule disassembly, thus inducing an asymmetry in transport which in turn leads to the preferential growth and hence orientation of microtubules along its direction.

Using an analytical theoretical approach, likewise based on a reaction–diffusion-asymmetric transport process involving the reactive growing, shrinking, and nucleation of individual microtubules, Portet et al. [36] came to similar conclusions. More recently, Baulin et al. [37] consider theoretically a rather more simplified situation in which the reactive shrinking and growing of individual microtubules is limited to the complete disassembly of individual microtubules accompanied by the nucleation and formation of new ones. They also predicted the formation of aligned arrays of microtubules – though not patterns of microtubule concentration – which would self-organise under the effect of a weak external orienting factor.

In miniature cell-sized containers, the morphology that forms is strongly dependent on sample geometry [38]. Close to a boundary, microtubules growing perpendicular to the boundary must stop when they arrive at it. On the contrary, those growing parallel to it do not suffer from this inhibition. Hence, the presence of the boundary favours microtubules growing parallel to it. For small samples of suitable geometry, the orienting effect

produced this way intervenes in and modifies self-organisation. Elongated forms particularly favour the self-organising process.

The self-organising process also results in collective particle transport [24]. When 1 μm diameter colloidal polystyrene particles are added to the initial preparation of tubulin and GTP, then about 15 min into the self-organising process, the beads all start to move in the same direction at speeds of several μm per minute [34]. Molecular motors are not present in these preparations. The rate of particle transport increases with increasing reaction rates and ceases when self-organisation is complete after about 5 h. This behaviour is independent of whether the added particles are polystyrene beads, phospholipid vesicles, purified chromosomes, or isolated nuclei. In addition, because the speed of particle transport is dependent upon the local concentrations of free tubulin and microtubules, the process also leads to the accumulation of particles into the regions of the self-organised structure where high microtubule concentrations develop. So as well as being transported, the colloidal beads are also themselves organised by the self-organising process.

Numerical simulations of the self-organising process by Glade et al. [34] furnish a possible explanation as to how collective particle transport might arise. These simulations predict at different time intervals during self-organisation, the formation of parallel fronts of oriented microtubules which cross the reaction space at speeds of several μm per minute [24]. Growing microtubules are known to be able to exert a force on objects in their path. Hence, the observed particle transport could arise by the collective force thus generated. However, the travelling fronts are comprised of variations of at least 30% in microtubule concentration. Since the microtubule preparation is extremely viscous, they also correspond to viscous waves of several thousand Poise. Such travelling waves of concentration and viscosity would be quite capable of transporting colloidal sized particles along with them. At present, we cannot distinguish as to whether the travelling fronts displace the particles by a pushing effect from the microtubules within them, or by way of the viscosity and concentration effects associated with them. The fact that the speed and direction of the travelling fronts compares favourably with the experimental speed and direction of colloidal particle transport is consistent with them being responsible for the observed behaviour.

3. Materials and methods

Tubulin was isolated from bovine brains, then purified on a phospho-cellulose column 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 the desired concentration of 10–12 mg ml^{-1} in the presence of excess GTP. The latter was provided either directly at an initial concentration of 2 mM, or at a concentration of 1 mM in the presence of an enzyme regenerating system comprised of acetyl kinase (0.83 μg ml^{-1}) and acetyl phosphate (20 mM). There was no difference in behaviour between the two cases. In some experiments, preparations also contained the fluorophore, 4′,6-diamidino-2-phenylindole (DAPI). DAPI fluorescence is approximately eight times higher when associated with microtubules than when associated either with free tubulin or in buffer solution [39]. The concentration employed (8 μM) is sufficiently low as to not affect self-organisation. In these experiments, the intensity of the DAPI fluorescence is proportional to the microtubule concentration [30].

In many cases, samples also contained colloidal polystyrene particles of either 1.1 μm (Sigma LB-11) or 0.8 μm diameter (Sigma LB-8). In some cases, to facilitate their observation, 1.0 μm diameter (Sigma L2278) fluorescently stained particles were used. The concentration of particles was adjusted to give the number density shown in Fig. 3-III. Other samples contained chromosomes isolated from *HeLa* cell cultures at interphase using established procedure [40].

Preparations were placed at 7 °C: i); in rectangular glass cells, 40 mm × 10 mm by 1 mm: ii); to a height of 8 cm in 4 mm internal diameter glass tubes: iii); in miniature cell-sized containers. The latter were made as previously described [38] by moulding poly-dimethyl siloxane (PDMS) elastomer [41] onto a template comprised of islands of resin, of chosen shape and size, standing out from a flat surface. The cured PDMS sheet, when peeled away from the mould, contains numerous small wells of the desired shape and size. This sheet is then placed on a microscope cover slide positioned in the lower part of a metallic support cooled to 7 °C. A drop of cold tubulin solution is added, the sample degassed, another cover slide placed on top, and the whole is secured in place with the upper part of the pre-cooled metal support.

Microtubule formation was instigated by rapidly warming the preparations from 7 °C to 36 °C and observing them under a microscope with a Zeiss Achroplan LD20 objective. Depending on the experiment, observations were made with one or more of the following methods; I) through crossed polars; II) through crossed polars with a wavelength retardation plate at 45°; II) from the fluorescence (excitation 360 nm, emission 460 nm) of the added DAPI; III) from the fluorescence (excitation 575 nm, emission 610 nm) of the polystyrene particles. Images were recorded at regular intervals of approximately 2 min for the next 3–5 h.

4. Results

There are several methods by which the effects of gravity can be removed or attenuated. One method is space-flight. Close to the earth, terrestrial gravity is still present. However, in a free-falling object, its effects are nullified. In sounding rockets, or orbiting craft such as the space-shuttle, conditions of weightlessness of between 10^{-2} g to 10^{-4} g are readily obtained [9].

On the ground, a simple method to reduce the effects of gravity is rotation of the sample about the horizontal axis (clinorotation) [25,26]. One of the major effects of gravity is to interact with any density differences present in the sample to cause a buoyancy force that may displace higher density regions

along the gravity direction. In this case, clinorotation continually changes the 'direction of fall' so that the net directional transport which gravity causes is cancelled out. In practice, speeds of rotation about 60 rpm are often effective. However, the centrifugal forces sample rotation causes also limits the attenuation of the gravity effects that may be achieved. For example, at 60 rpm, the centrifugal force 1 mm, 4 mm and 10 mm, off the axis of rotation are 4×10^{-3} g, 1.6×10^{-2} g, and 4×10^{-2} g, respectively. Using this simple devise we have shown that clinorotation is as effective as free-fall space-flight conditions in inhibiting *in vitro* microtubule self-organisation [42].

Here, we report both space-flight and clinostat experiments. Unfortunately, because of damage to the experimental module on landing, we obtained only partial results from the space-flight experiment. As the experiment could not be readily repeated we carried out a similar experiment under conditions of clinorotation, and then extended these observations to those we had been unable to make in the space-flight experiment.

4.1. Experiments in large centimetre-sized containers

4.1.1. Sounding rocket experiment

Thirteen minutes of weightlessness was obtained in a free-falling rocket of the European Space Agency (Maxus 5). An experimental module was constructed by the Swedish Space Corporation and DTM on behalf of the European Space Agency. It contained two sample compartments. In one compartment, microtubules were assembled under conditions of weightlessness, whereas in the other they were assembled on a 1 *g* centrifuge. In each compartment, 20 samples were placed around the perimeter of a wheel that could be moved to position consecutive samples between a microscope objective (×20) and an illumination system. The mechanism of the positioning devise was such that samples were repositioned before the objective with an accuracy of about 10 μm. In addition, to determine the exact position of particles in the sample cell, independently of any variation of the position of the sample cell in the microscope field of view, different symbols about 10 μm wide were engraved on the inner glass surface of the sample cell.

A routine method for monitoring the extent and assembly kinetics of microtubule formation from tubulin is via the solutions optical density at around 350 nm [29]. For one sample on the on-board 1 *g* centrifuge, and two samples in the weightlessness compartment, the optical density of the preparation was recorded for the first 13 min. For practical reasons we used a wavelength of 370 nm.

Tubulin samples as described in the section 'Materials and methods' were placed in glass cells, 40×10×1 mm, and thoroughly degassed. Half of the samples contained 0.8 μm diameter polystyrene beads and the other half purified chromosomes. Ten hours before the launch, samples were installed in the flight module at 7 °C. Additional samples, kept separate at 7 °C, were used in ground-control experiments started 2 h before the launch.

As soon as conditions of weightlessness were obtained at time, $t=0$ min, approximately 2 min after launching the sounding rocket, samples were rapidly warmed to 36 °C and the on-board 1 *g* centrifuge switched on. It was stopped 13 min later, just prior to payload re-entry. At this time, the sample morphology which develops is already determined and the subsequent behaviour is unaffected by payload re-entry and the return to normal gravity conditions [27]. At time *t*, $t=30$ min, shortly after the payload landed ($t=18$ min), the microscope illumination was switched on, and images of consecutive samples recorded for the next 2.5 h.

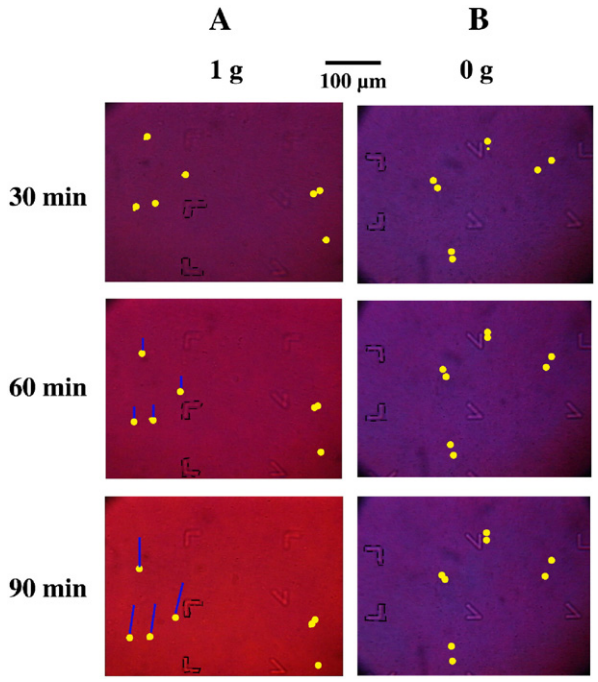

Fig. 1. Effect of 13 min of weightlessness produced in a free-falling sounding rocket, on the collective transport of 0.8 μm diameter polystyrene particles by microtubule self-organising processes. Preparations were contained in optical cells measuring 40×10×1 mm. Microtubules were formed, when conditions of weightlessness (time, $t=0$ min) were obtained, by warming preparations containing tubulin and excess GTP from 7 °C to 36 °C. A); shows photographs viewed through crossed polars and a wavelength retardation plate of a reference sample assembled on the 1 *g* 'on-board' centrifuge at times, $t=30$ min, $t=60$ min and $t=90$ min. Some of the colloidal particles present are highlighted in yellow. Particles are transported over approximately 80 μm and some of their trajectories (from their position at $t=30$ min) are indicated. B) are photographs, recorded at the same times, of a sample exposed to weightlessness for the first 13 min. There is no particle transport when gravity effects are substantially reduced by free-fall conditions. To unambiguously determine relative particle positions, symbols were engraved on the inner glass surface of the sample cell. Two of them have been highlighted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Unfortunately, the rocket was launched under adverse meteorological conditions. Due to high winds in the recovery zone, the payload was damaged on landing; the heating unit failed and cold air entered through rents in the fuselage. The sample temperature, which was 36 °C just after impact, slowly dropped and had fallen to 16 °C by the time the payload arrived at the launch site ($t=180$ min). Recordings of the sample temperature showed that heating of the samples to 36 °C had occurred as planned and the samples had remained at this temperature until time, $t=20$ min, 2–3 min after impact. At

times t, $t=30$ min, $t=60$ min and $t=90$ min, the sample temperature was 34 °C, 28 °C and 25 °C respectively.

For the samples on the 1 g on-board centrifuge, the optical density recording (370 nm) for the first 13 min showed that microtubules had assembled to the same extent and with the same kinetics as observed in the ground-control experiment. For samples assembled under conditions of weightlessness, the optical density recordings showed no significant differences compared with these. Hence, although gravity strongly effects self-organisation, it does not modify either the level, or kinetics, of microtubule assembly from tubulin. When removed from the module after 3 h ($t=180$ min), the optical density of the samples, proportional to microtubule concentration, were 60–70% of those in the ground-control experiment. This indicated that the microtubules had disassembled by 30–40% due to the progressive decrease in temperature in the flight module from 36 °C to 16 °C.

Fig. 1 shows images recorded on the on-board microscopes from samples on both the '1 g centrifuge' and 'weightlessness' compartments at times, $t=30$ min, $t=60$ min, and $t=90$ min. At these times, the sample temperatures were 34 °C, 28 °C and 25 °C respectively. Between times, $t=30$ min to $t=60$ min, the decrease in temperature from 34 °C to 28 °C would not have resulted in any significant microtubule disassembly. As can be seen in Fig. 1B, during this time, for samples assembled in the 'weightlessness' compartment, there was no significant displacement of colloidal particles. On the contrary (Fig. 1A), for samples assembled on the on-board 1 g centrifuge, particles moved by about 30 μm over this 30 min interval. By time, t=90 min, in spite of the progressive decrease in temperature to 25 °C, for samples assembled on the on-board 1 g centrifuge, particles had continued to move. The average rate of particle displacement was about 1.5 μm per minute compared with 3 μm per minute in ground-control experiments (but maintained at 36 °C). Reducing the temperature from 36 °C to 30 °C reduces the rate of particle movement. By time, $t=90$ min, for the samples assembled on the 1 g centrifuge, the parts of the preparation under observation had started to show an optical birefringence indicating that despite the temperature decrease some microtubule orientation and self-organisation was still occurring. For samples assembled under conditions of weightlessness, no birefringence developed during this time, thus indicating that self-organisation was not taking place, and there was no particle transport. We observed no qualitative difference in behaviour between samples containing polystyrene beads and those containing purified chromosomes.

4.1.2. Clinostat experiments

Microtubule formation was instigated by placing samples containing 1 μm diameter polystyrene particles at time, $t=0$ min, in a clinostat rotating at 60 or 30 rpm installed in a hot-room at 36 °C. Reference samples under normal gravity conditions were simultaneously prepared by placing another sample at a different position in the clinostat, but which did not undergo rotation. Samples were removed from the clinostat after 15 min, and observed for the next 5 h on a microscope hot stage maintained at 36 °C. Images were recorded every 2 min

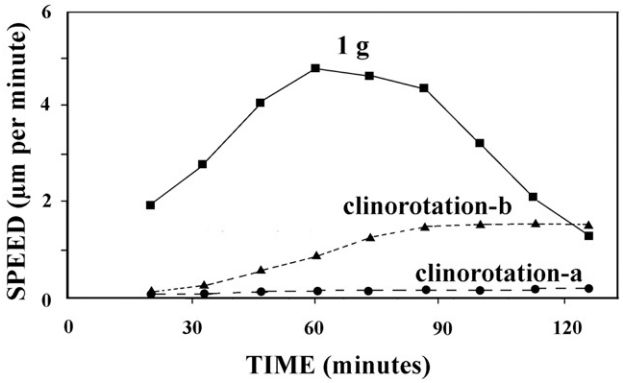

Fig. 2. Transport of 1 μm diameter polystyrene particles by microtubule reaction–diffusion processes under normal and reduced gravity effects as produced by clinorotation. Microtubule preparations were formed by warming a solution of 10 mg ml^{-1} tubulin in the presence of excess of GTP and polystyrene beads. Variation in the rate of particle transport for samples under normal (1 g) gravity conditions; B), subject to weightlessness produced by clinorotation at 60 rpm in a 4 mm internal diameter tube for the first 15 min (clinorotation-a); clinorotation at 30 rpm in a rectangular cell, 40 × 10 × 1 mm (clinorotation-b).

and the position of individual particles monitored. Experiments were carried out in tubes of 4 mm internal diameter, and with rectangular sample cells (40 × 10 × 1 mm), the same as used in the space-flight experiment.

The reference samples, not subject to weightlessness by clinorotation, self-organise as already reported [24]. Particles are transported through the preparation, along the direction of microtubule orientation that develops, at an average rate of about 3 μm per minute (Fig. 2). Transport stops when self-organisation is complete.

In 4 mm diameter tubes rotating at 60 rpm, self-organisation does not occur [42]. In this case, as for the sounding rocket experiment, we did not observe any particle transport (Fig. 2). In rectangular cells rotating at 60 rpm, where the centrifugal field at the periphery of the cell (4×10^{-2} g) is higher, we observed both self-organisation and particle transport; albeit at a slower rate than under normal gravity conditions. For a rate of clinorotation of 30 rpm, the centrifugal force at the edge of the sample is significantly less (1×10^{-2} g) than at 60 rpm. Experiments carried in rectangular cells at this rate of rotation resulted in samples where there was almost no self-organisation and little particle transport (Fig. 2). From these observations we deduce that the threshold value at which gravity triggers both self-organisation and particle transport is approximately 10^{-2} g.

4.2. Experiments in miniature cell-sized containers

We also carried out experiments in miniature cell-sized containers. We examined self-organisation and colloidal particle distribution for two different geometries, 175 μm square wells (70 μm deep) and rectangular canals, 200 μm by 5000 μm (70 μm deep). In these containers, microtubule self-organisation under normal gravity conditions has already been described [38]. We extended these observations by adding fluorescently stained colloidal particles to the preparation and studying the effect that self-organisation has on their distribution. We then

studied the effect on these phenomena of 15 min of weightlessness as produced by clinorotation (60 rpm).

In large centimetre-sized containers, for samples not subject to weightlessness, the particle transport which self-organisation causes also leads to their organisation When self-organisation is complete, the particle distribution is no longer uniform, but instead coincides with the self-organised microtubule pattern [24]. In miniature containers, we found that this effect was strongly enhanced. For example, in square wells (Fig. 3A-III), at the end of self-organisation, a large proportion of the particles are concentrated into one part of the sample, with few in the remainder. Comparison with the DAPI fluorescence image (Fig. 3A-II), where the intensity is proportional to microtubule concentration, shows that the particles have been transported to, and accumulated in, the region of high microtubule concentration. The initially uniformly distributed particles have, in this case, been separated into strongly segregated regions of high and low particle density by the self-organising process.

When experiments were carried out under conditions of weightlessness for the first 15 min, then as for centimetre-sized containers, neither self-organisation nor particle transport occurred, and the particle distribution remained uniform (Fig. 3B).

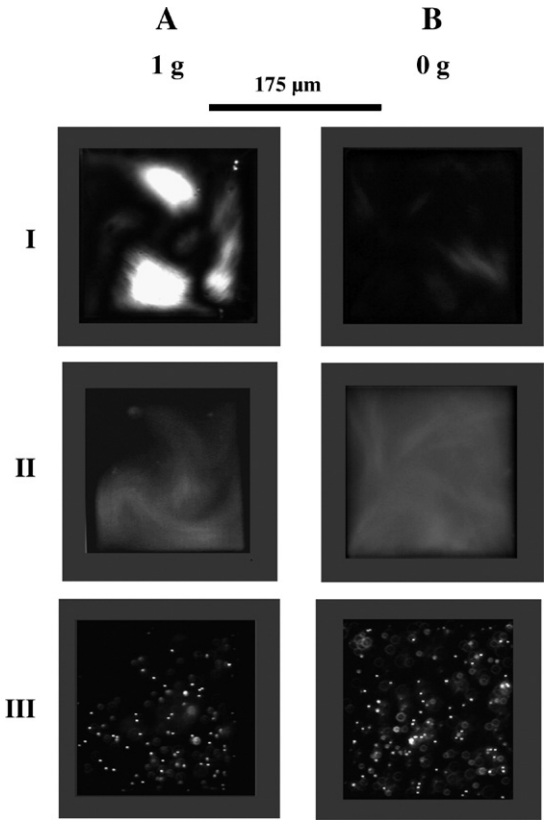

Fig. 3. Self-organisation and particle distribution in miniature cell-sized square shaped containers under normal gravity conditions (A) and subject to weightlessness produced by clinorotation for the first 15 min (B). Images were recorded; I) through crossed polars, indicating variations in microtubule orientation; II) from DAPI fluorescence, indicating variations in microtubule concentration; III) from 1.0 µm diameter fluorescent polystyrene beads present. Under normal gravity conditions, self-organisation leads to the transport and segregation of particles into regions of high microtubule concentration. These phenomena are strongly inhibited when samples are exposed to a brief period of weightlessness.

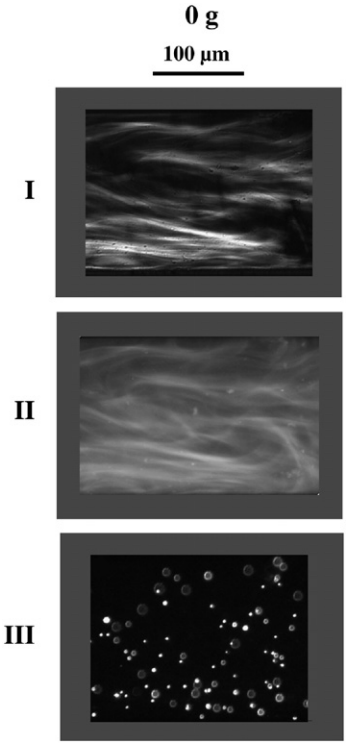

Fig. 4. Self-organisation and particle distribution in miniature canal-shaped containers (200 µm by 5000 µm) under conditions of weightlessness produced by clinorotation for the first 15 min. Images of part of a canal were recorded; I) through crossed polars; II) from DAPI fluorescence; III) from fluorescence of 1 µm diameter polystyrene beads present. Contrary to what occurs in square wells, in this case, self-organisation and particle transport and organisation are not suppressed by weightlessness. This difference in behaviour is thought to arise from the microtubule orienting effect produced by the shape of the miniature container and which induces self-organisation.

In canal-shaped samples, self-organisation under normal gravity conditions results in the development, along the long axis of the sample, of several parallel regions of high microtubule concentration and orientation. Once again particles are transported to and accumulate within these regions (Fig. 4). However, because the morphology is comprised of several regions of high microtubule concentration running parallel to the long axis, the effect is less noticeable than for square wells.

For this sample geometry, contrary to square wells, when experiments were carried out under conditions of weightlessness for the first 15 min, both self-organisation, and particle transport and organisation, occurred. We observed no significant differences compared with the behaviour at 1 *g* (Fig. 4).

5. Discussion and conclusions

In large centimetre-sized containers, microtubule driven particle transport and organisation were found to be strongly gravity dependence. Colloidal polystyrene particles and chromosomes both behaved in a similar manner. The fact that transport phenomena do not occur when self-organisation is not triggered by gravity proves that they are directly caused by the

self-organising process. From the value of the centrifugal field at which the phenomena start to develop, we estimate the threshold level for triggering this behaviour as approximately 10^{-2} g. The fact that the assembly kinetics measured under conditions of weightlessness are the same as for samples at 1 *g* shows that weightlessness has no significant effect on the kinetics of microtubule assembly.

The comparison of the space-flight with the clinorotation experiments confirm that clinorotation is an effective method for attenuating gravity effects. The method, when applicable, has substantial practical advantages over space-flight experiments, being simple, inexpensive, rapid, not subject to accidents and technical failures, and readily repeated. The clinostat experiments reported here took only a few weeks to carry out, compared with the sounding rocket experiment that took three to five people over 18 months full-time preparation, cost a substantial sum of money, and gave only partial results. Had we not carried out the clinostat experiments, then because of the drop in temperature from 36 °C to 16 °C (resulting from damage to the experimental module on landing) almost no reliable conclusions could have been drawn from the space-flight experiment.

The experiments in miniature cell-sized containers show that the accumulation and organisation of particles into regions of high microtubule concentration is strongly enhanced by their small size. In square wells, self-organisation resulted in an almost complete segregation of particles into regions of high and low particle density. In this case, a brief period of weightlessness inhibited both self-organisation and associated particle transport. Thus, in cellular and embryo-sized containers, weightlessness is capable of having a substantial effect on these phenomena. Nevertheless, the behaviour is strongly dependent on sample shape. The elongated shape of a canal is, even under conditions of weightlessness, sufficient to trigger both self-organisation and particle transport.

Microtubules have two major biological functions; they organise the cell interior and they are responsible for the directional transport from one part of the cell to another of sub-cellular particles such as vesicles and chromosomes. Major cellular activities are perturbed when these processes do not occur normally.

The results presented here show that in addition to self-organisation, weightlessness may under suitable conditions also have substantial effects on the transport and organisation of colloidal and sub-cellular particles which self-organisation causes. Furthermore, these transport processes remain inhibited for some time after normal gravity conditions are restored. Hence, if the type of reaction–diffusion process considered here also occurs *in vivo* – in cells and embryos – then exposure to brief periods of weightlessness may under appropriate circumstances, have major biological consequences. Whether or not this is the case will also depend upon a variety of symmetry-breaking factors – such as the shape of a cell or embryo – which may, or may not, also be present. Depending on circumstances, these factors might either oppose or reinforce the action of gravity. Hence, in some cases, the effect of weightlessness may be to strongly inhibit microtubule organisation and particle transport; in other cases it may perturb and slow them down; in other cases the effect may be negligible. Considerations of this type can account for why different cell types show differing sensitivities towards weightlessness. Based upon this argument, one might expect that circular or spherical shaped cells and embryos would show a stronger gravity dependence than strongly elongated cells such as neurons. Cells of the immune system, many of which are spheroidal, are often reported as showing a gravity dependence [10,43]. The early stages in development of *Xenopus*, zebra fish and chicken embryos show a gravity dependence [2,3,44] and they are likewise close to spherical.

Experiments on human epithelial (MCF-7), human lymphocyte (Jurkat), glial, rat utricular hair cells, and thyroid carcinoma cells, cultured under conditions of weightlessness show substantial reductions in microtubule organisation compared with those at 1 *g* [10,43,45–50]. These observations, consistent with the effect of weightlessness on the self-organisation of microtubules *in vitro*, thus raise the possibility that they arise from similar physical chemical processes. If this is the case, one might expect that microtubule driven particle transport and localisation would be similarly affected by weightlessness. Some important biological processes dependent on these phenomena are cell-division, signalling pathways, exo and endo-cytosis, and transport and localisation of mRNA containing vesicles. Based upon this argument, for the cellular systems where microtubule organisation is inhibited by weightlessness, we predict that weightlessness will also lead to the following changes in cell function: retarded cellular division and reduced growth rates, inhibited signal transduction, and impaired protein localisation and synthesis.

During the review process, one of the referees drew our attention to, and asked us to discuss, a recent article by Higashabita et al. [51] reporting that weightlessness in bovine brain microvascular endothelial cells leads to a disorganisation of actin stress fibres. The formation of these stress fibres is associated with specific, membrane located, Rho GTPases, in turn activated by guanine nucleotide exchange factors (GEF). The authors report that weightlessness affected the GEF protein in such a way that Rho GTPase activation was diminished. They concluded that the disorganisation of actin stress fibres which weightlessness causes is a result of this effect. However, they did not consider the physical chemical mechanism as to how gravity might bring about such a change.

It is known that the actin and microtubule networks of the cytoskeleton are interconnected in part via Rho GTPases [52]. For example, Putnam et al. [53] report that the effect of externally applied mechanical strain on the distribution and activation of Rho GTPases requires changes in the microtubule cytoskeleton. Tensile strain decreased the amount of membrane associated Rho GTPase by 70%. Other workers have shown there is a functional relationship between both Rho GTP'ase, GEF protein, and microtubules that requires an intact microtubule cytoskeleton [52,54,55]. The fact that both proteins lost their localisation to the cell periphery upon microtubule depolymerisation suggested that a microtubule dependent transport process was involved [52,54,55].

Thus, in bovine brain microvascular endothelial cells, were weightlessness to result in a disorganised microtubule network and inhibited microtubule driven transport, then the localisation of both GEF and Rho GTPase molecules to the cell periphery could be lost and so lead to the consequences reported by Higashabita et al. However, as actin shows a type of reaction dynamics, of growing and shrinking rods, similar to microtubules, it is also conceivable, and cannot be excluded, that the disorganised actin network observed by Higashabita et al. is a direct effect of weightlessness on the actin cytoskeleton. It would be interesting to investigate the effect of weightlessness both, on microtubule organisation and microtubule driven transport and localisation in bovine brain microvascular endothelial cells, and on actin organisation *in vitro*.

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