Motility of $CoFe_2O_4$ nanoparticle-labelled microtubules in magnetic fields

B.M. Hutchins, M. Platt, W.O. Hancock and M.E. Williams

Abstract: Kinesin motor proteins transport intracellular cargo unidirectionally along microtubule tracks. This intracellular transport system is being explored as a novel method of assembly, separation, and transport in nano- and micro-scale systems; however, facile techniques for patterning and directing microtubules remain limited. Biotinylated 20 nm diameter $CoFe_2O_4$ magnetic particles and neutravidin bridging molecules are used to label microtubules containing varying amounts of biotin. The effect of magnetic nanoparticle labelling on the speed of microtubule gliding across kinesin-covered surfaces is investigated both with and without applied magnetic fields. Microtubule gliding speed decreased with an increasing amount of attached magnetic cargo, but very lightly decorated microtubules were only slightly affected. All magnetically tagged microtubules could be drawn from the solution by application of a magnetic field, however, the presence of a field did not significantly affect gliding speed or direction. These findings provide new insight for the ultimate use of external magnetic fields as a tool to control the kinesin/microtubule system in carrying out concerted nanobiological tasks.

1 Introduction

Motor proteins are responsible for the transport and delivery of intracellular cargo at rates of up to microns per second. In cells, kinesin motor proteins hydrolyse ATP and use this energy to move along 25 nm diameter cylindrical tubulin polymers (e.g. microtubules) that thus act as intracellular conduits [1]. *Drosophila melanogaster* conventional kinesin motors travel unidirectionally from the slow growing (minus) end to the fast growing (plus) end of the microtubule with an average speed of 0.8 μm/s in vitro [2, 3]. A single kinesin motor can take hundreds of 8 nm steps and travel a micron or more before disassociating and diffusing away from the microtubule surface [4, 5]. It has been hypothesised that engineered motor proteins such as kinesin could be used in vitro in bio-MEMS devices to transport and assemble non-biological cargo. To do so requires synthetic modification of the proteins to enable attachment of artificial and nanoscale materials, and the development of external means by which placement, assembly and direction may be controlled.

To monitor the transport of single kinesin motor proteins on microtubules, single molecule experiments that involve TIRF microscopy [6, 7] or the attachment of bulky, micron-scale cargo [8] are typically required. We instead choose to use the inverse experiment, depicted in Fig. 1*a*, known as a gliding assay, in which microtubules are transported along a motor-functionalised surface. Using this type of assay allows the study of motor functionality, using readily available fluorescence microscopy techniques. We and others have demonstrated that chemical modification of kinesin and microtubule proteins can provide a means of selective labelling for attachment of quantum dots [9–12], silica [5, 13] or polymer [11] beads, and other inorganic nanoparticles [14, 15]. These labelling schemes provide new opportunities to use biomolecular motors for directed assembly of complex nanostructures as well as possible applications in protein tracking and force measurements [5, 13, 16]. In the first efforts to harness these motor proteins in bio-NEMS devices, microtubules have been controlled by confining microtubule motility in narrow or enclosed channels [17–20], utilising electric fields for separation and transport [21, 22], or redirecting microtubules with viscous fluid forces [23]. Limited by fabrication costs and rigid experimental configurations, these techniques still require improvement before such devices can be made widely applicable. Magnetic labelling is a complementary tool that does not suffer from these limitations, providing a facile, inexpensive and adaptive methodology to exert control over microtubules.

Recent work in our laboratory demonstrated the static patterning of $CoFe_2O_4$ magnetic nanoparticle-labelled microtubules onto kinesin-covered surfaces, using relatively weak applied magnetic fields [15]. Even in the absence of applied magnetic fields, the aligned microtubules possessed reduced gliding speeds relative to microtubules that had been allowed to diffusively reach the surface. Although initial experiments implicated the attachment of bulky groups on the microtubules as the source of the reduced motility, further study was required to better understand and optimise the system for use in bio-MEMS applications. In this report, we present a detailed investigation of the binding and motility of magnetically-functionalised microtubules, and extend these studies to examine the effect of applied magnetic fields on gliding speed and direction. This new technique is an enabling methodology for the

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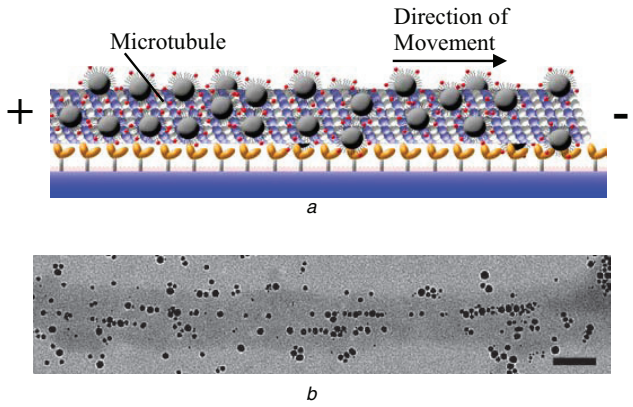

Fig. 1 *Gliding assay*

a Scheme of a gliding assay, in which a microtubule with attached magnetic nanoparticles is transported by a kinesin motor functionalised glass surface

b Corresponding transmission electron microscopy (TEM) micrograph.

Scale bar is 100 nm

assembly, manipulation and separation at the nanometer scale and may find significance in controlling the transport direction of motor proteins at the nanometer scale in vitro.

2 Experimental

2.1 Chemicals and reagents

All chemicals were purchased from standard commercial sources and used without further purification. Kinesin and tubulin proteins were isolated from natural sources (described subsequently) by standard methods [2, 24].

2.2 Synthesis of $CoFe_2O_4$ nanoparticles

$CoFe_2O_4$ nanoparticles were prepared as previously described [25] by the high temperature decomposition of $Fe(acac)_3$ and $Co(acac)_2$ in the presence of coordinating ligands. These particles were then purified by repeated washes with ethanol and encapsulated in 5% biotin-tagged micelles [15, 26]. After purification by ultra-centrifugation (to remove empty micelles), the nanoparticles were used at a concentration of 3 mg/ml in freshly prepared BRB12 [12 mM 1,4-piperazinediethanesulfonic acid (PIPES), 1 mM $MgCl_2$, 1 mM ethylene glycol-bis(2-aminoethyl-ether)-*N*, *N*, *N*′, *N*′-tetraacetic acid (EGTA, pH 6.8)].

2.3 Kinesin and microtubule preparation

HexaHis-tagged *D. melanogaster* conventional full length kinesin was purified according to published methods [2] and used at a concentration of 5 μg/ml. Tubulin was purified from freshly harvested bovine brain tissue and labelled with rhodamine or biotin using standard techniques [24]. Preparation of biotin-functionalised microtubules was performed by copolymerisation of biotinylated tubulin and rhodamine containing tubulin (25% rhodamine-tagged tubulin and 75% purified unlabelled tubulin) at 37°C in BRB80 buffer (80 mM PIPES, 1 mM $MgCl_2$, 1 mM EGTA, pH 6.8) with 1 mM guanosine triphosphate, 4 mM $MgCl_2$ and 5% v/v dimethylsulfoxide for 20 min. The microtubules were diluted 100-fold (320 nM tubulin dimer) with BRB80 buffer containing 10 μM paclitaxel.

Microtubules were labelled with $CoFe_2O_4$ nanoparticles by incubating 10 μl of paclitaxel-stabilised microtubules (320 nM tubulin dimer) with 10 μl neutravidin (0.5 mg/ml) for 5 min, followed by addition of 5 μl of stock nanoparticle solution (3 mg/ml). 10 μl of the solution was added to freshly prepared motility solution (90 μl BRB80 containing 0.2 mg/ml casein, 10 μM paclitaxel, 20 mM glucose, 20 μg/ml glucose oxidase, 8 μg/ml catalase and 0.5% BME) with or without 1 mM ATP, as noted in the text.

2.4 Magnetic surface capture and motility studies

All motility experiments were performed in flow cells constructed from two pieces of double-sided tape sandwiched between two coverslips. Prior to each experiment, a new flow cell was constructed and the interior surfaces were perfused with 0.5 mg/ml casein for 5 min, followed by a 5 min exposure to 5 μg/ml hexaHis-tagged kinesin in buffer (BRB80, 0.2 mg/ml casein, 1 mM ATP). Magnetic preconcentration on the surface was accomplished by adding magnetic nanoparticle labelled microtubules in buffer without ATP to the flow cell, and placing a cube magnet (0.5 cm side length) under the flow cell, as seen in Fig. 2. After 5 min, the slides were imaged by fluorescence microscopy to assess the surface coverage. Microtubule movement was then initiated by the addition of 1 mM ATP into the flow cell and subsequently monitored by fluorescence microscopy both in the presence and the absence of the magnet.

2.5 Instrumentation

Fluorescence microscopy was performed on an upright Nikon E600 microscope (1.2 NA, 60× water immersion objective) coupled to a Genwac GW-902H CCD camera. Video was recorded with a Panasonic AG-MD835 video cassette recorder onto VHS videotapes for offline analysis. Transmission electron micrographs were obtained on a JEOL JEM 1200 EXII operating at 80 kV with an attached high-resolution Tietz F224 digital camera.

The 5 mm cube NdFeB permanent magnet (Engineered Concepts, Inc., Birmingham, AL, USA) that was employed had a field strength of 0.45 T. Field gradients were measured with a hand-held gaussmeter (Magnetic Instrumentation, Inc.; Model 907).

Data sets were analysed for significance using Student's *t*-test, at a 95% confidence interval, following standard methods.

3 Results and discussion

To develop methods by which externally applied forces can be used to control the operation of motor protein systems, biotin-functionalised $CoFe_2O_4$ magnetic nanoparticles were coupled to the surfaces of microtubules. Application of a magnetic field gradient generates a force on the magnetically-tagged microtubules sufficient to create aligned arrays of mobile nano-biological hybrids [15], and

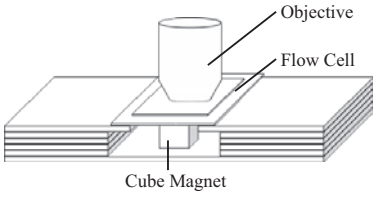

Fig. 2 *Experimental arrangement for magnetic surface preconcentration and directed motility experiments*

A 60× water immersion objective was used to focus on the bottom of the flow cell (larger coverslip)

Magnetic microtubules were preconcentrated with a 5 mm cube magnet

was expected to be sufficiently strong to alter their transport trajectory. However, functionalising microtubule surfaces with bulky labels can perturb the normal functioning of the kinesin–microtubule system. To understand, predict and control motor binding and motility with this hybrid nano-biological system, we undertook a series of detailed experiments to elucidate the effect of the neutravidin-linked magnetic nanoparticles and applied magnetic fields on binding and mobility.

3.1 Effect of labelling on microtubule motility

Standard microtubule gliding assays were performed using fluorescence microscopy to visualise the kinesin-driven transport of rhodamine-labelled microtubules in the presence of 1 mM ATP. To attach nanoscale $CoFe_2O_4$ magnets, hetero-labelled microtubules were prepared by copolymerising varying ratios of biotinylated tubulin and rhodamine-labelled tubulin. Magnetic, biotinylated $CoFe_2O_4$ nanoparticles were bound to biotinylated microtubules by the addition of neutravidin to give a standard biotin–neutravidin–biotin sandwich. Thus, these copolymerised microtubule constructs contain both magnetic nanoparticles and non-reactive rhodamine fluorescent tags, as pictured in Fig. 1. Because $CoFe_2O_4$ nanoparticles of this size are superparamagnetic at room temperature [25], in the absence of a magnetic field, these particles do not possess internal fields that would lead to their aggregation, making them ideal for use as magnetic tags. The quantity of attached magnetic material is not precisely known, but is assumed to be proportional to the amount of biotinylated tubulin used during the polymerisation. The transmission electron microscopy image in Fig. 1*b* of a sample of magnetically-labelled microtubules shows lines of particles that are separated by 20–25 nm, the approximate diameter of a single microtubule [27]. The exact particle attachment locations are difficult to determine because of protein denaturation during the process of imaging. However, no aggregation of the magnetic nanoparticle labels is observed, and the distinct individual particles appear to be distributed (somewhat inhomogeneously) along the microtubule.

We previously demonstrated that magnetic fields are useful for increasing both the surface concentration (e.g. magnetic preconcentration) and the degree of alignment of magnetic nanoparticle-tagged microtubules [15]. In those initial experiments, both nanoparticle attachment and magnetic preconcentration (without ATP in solution) appeared to reduce motility rates, once motor function was restored by addition of ATP. We therefore sought to conduct a more detailed study of the relationships between the extent of functionalisation (e.g. % biotin-labelled tubulin), magnetic fields and microtubule motility.

The speed that microtubules moved over a kinesin-functionalised surface was monitored using fluorescence microscopy for each of the microtubule biotinylation percentages. A series of experiments were designed to differentiate between changes in motility that might arise from magnetic labelling of microtubules, preconcentration of microtubules on a surface and the presence of a magnetic field. In the first set of gliding assays, the movement of microtubules with varying levels of magnetic functionalisation was observed without application of a magnetic field. During these assays, not all of the observed microtubules on the kinesin-covered surface were motile. Fig. 3 shows that the percentage of motile microtubules decreases linearly with the extent of biotinylation (and amount of attached magnetic $CoFe_2O_4$ cargo). Furthermore, analysis of the motile microtubules (Fig. 3, closed circles) indicates

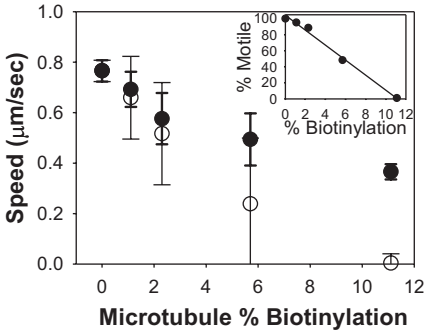

Fig. 3 *Effect of biotin-induced magnetic particle labelling (% biotin–tubulin) on transport speed of microtubules with differing biotin content*

Open circle indicates all visible microtubules, and for closed circle indicates only motile microtubules
Inset: percent of motile microtubules against biotin content

that the average gliding speed also decreases dramatically as the percentage of magnetic nanoparticle-labelled tubulin increases, and decreases to zero when non-moving microtubules are included in the analysis (Fig. 3, open circles). As a control, the unlabelled microtubules (0% biotinylated tubulin) are all motile and have gliding speeds expected for this motor construct [2, 3].

The decrease in the number of microtubules bound to the kinesin-covered surface (i.e. binding events) limits the ability to monitor statistically significant numbers of microtubules, particularly in the case of the most heavily decorated (11% biotin–tubulin) microtubules. Fig. 4*a–e* contains fluorescence microscopy images showing the reduction in the number of microtubules bound to the motors as the biotinylated tubulin percentage increases. This limited affinity for kinesin imposes a limit on the maximum amount of magnetic cargo that can be attached to the microtubules. To define the maximum limit of useful magnetic functionalisation, to determine the cause of this trend and to rule out statistical errors based on small sampling size, the motility assay was repeated with the same $CoFe_2O_4$-labelled microtubules but following magnetic preconcentration/alignment onto the motor-covered surface in the absence of ATP. A small cube-shaped NdFeB magnet with a field strength and gradient of 0.45 T and 45 T/m, respectively, was positioned directly beneath (~175 μm, coverslip thickness) the kinesin-covered surface as shown in Fig. 4. Attraction of the $CoFe_2O_4$-labelled microtubules to the higher field gradient is expected to draw them towards the glass surface: the images in Fig. 4*f–j* reveal an increase in the surface coverage as a result of the applied magnetic field.

Using the magnetically preconcentrated microtubules on the kinesin surface, ATP was added to initiate motor transport, the magnet was removed and motility was monitored. We again observed that the microtubule gliding speed depended strongly on the amount of $CoFe_2O_4$ labelling. The average speeds of labelled microtubules that were magnetically preconcentrated are given in the second column of Table 1 ('preconcentrated + no field'), and compared with the same microtubules that diffused to the surface in the absence of a field ('diffused + no field'). The microtubule motility again decreases as the quantity of magnetic cargo increases, although a slight decrease in speed was also observed for certain label densities after magnetic preconcentration. Because these sets of data were acquired using microtubules moving in the absence of an applied field,

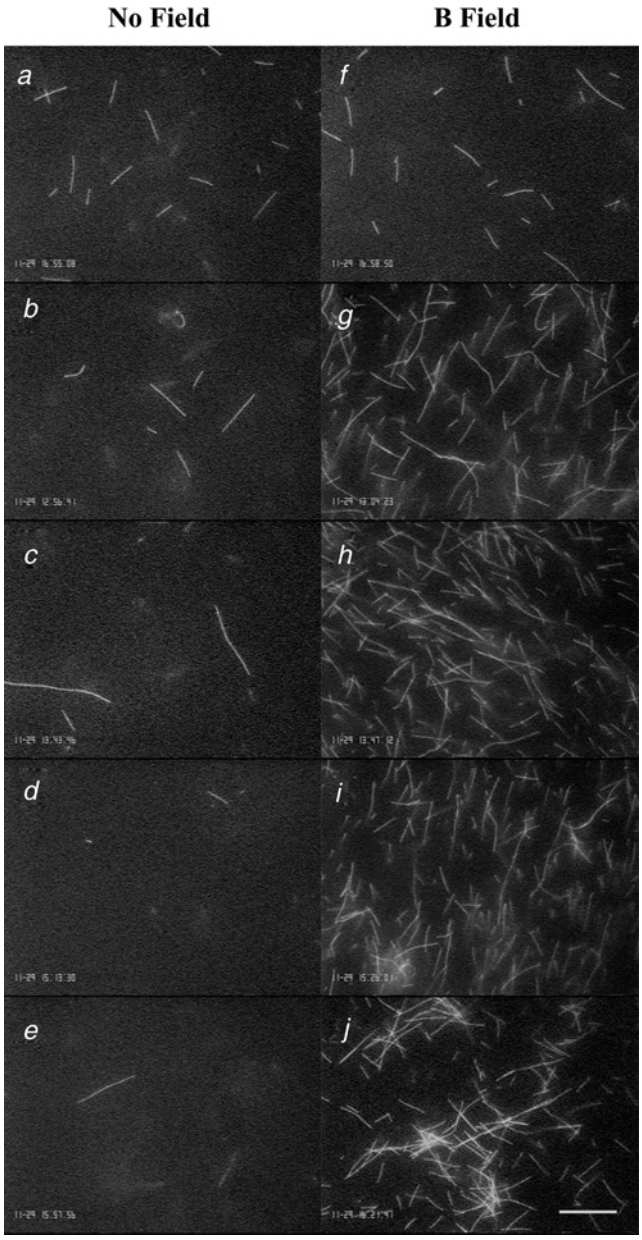

Fig. 4 *Fluorescence microscopy images of microtubules with different percentages of biotinylated tubulin, bound to a kinesin-coated glass coverslip*

a and *f* 0%
b and *g* 1.1%
c and *h* 2.3%
d and *i* 5.7%
e and *j* 11.1%
Samples were observed in (*a*–*e*) the absence and (*f*–*j*) presence of a magnetic field (applied for 3–5 min)
Scale bar is 20 μm

this latter difference implies that the act of magnetic preconcentration affects the kinesin–microtubule function.

Several mechanisms for the effect of magnetic preconcentration were considered. Excess (unbound) nanoparticles present in solution during the magnetic capture process are also attracted to the motor-covered surface by the magnetic field, and it is possible that they inhibit kinesin binding. However, in control experiments using biotinylated particles and 5.7% biotin–tubulin microtubules without added neutravidin (i.e. unlinked magnetic particles in solution with microtubules), the microtubule gliding speed was not significantly diminished in the presence of the $CoFe_2O_4$ particles (0.68 ± 0.05 μm/s, $N = 29$). As these particles were exposed to the kinesin surface in the presence of a magnetic field, this result indicates that the presence of non-specifically bound or unbound particles does not interfere with motor function, even when the motor surface is essentially coated with free (unlinked) nanoparticles.

Whereas motor deactivation due to ATP starvation has been reported on time scales similar to those used for the surface capture procedure [28], control experiments performed with identical ATP concentrations using non-labelled microtubules (i.e. no magnetic particles or neutravidin) gave an average speed of 0.72 ± 0.05 μm/s, ($N = 45$). This value is equivalent to native rates within experimental error, ruling out ATP starvation as a root cause. Another possibility is that the forces associated with attracting the magnetically labelled microtubules with the field gradient result in irreversible deformation of the microtubule or the underlying motors. This mechanism is difficult to experimentally assess, however, it seems unlikely given that when microtubules are transported across the surface and bind to new (and uncompressed) motors, their speeds do not change. For magnetic preconcentration to affect the speed of a gliding microtubule, compression forces would have to disrupt the entire motor surface even if microtubules had not landed there. Pressure-induced distortions of (at least) the motors are therefore thought to be negligible and unimportant. One possibility that cannot be overlooked is that for higher microtubule labelling, the preconcentration step selects for those filaments with larger numbers of attached particles, whereas the diffusive loading approach selects for microtubules with fewer attached particles (and higher kinesin affinity). In this scenario, the slower speeds observed following magnetic preconcentration would reflect a larger number of particles per microtubule.

Blocking access to the kinesin surface and thus reducing the number of motors bound to each microtubule is not expected to affect the gliding speed, because in gliding assays, the speed is essentially invariant over three orders of magnitude variation in motor surface density [2]. Conversely, steric blocking of kinesin binding sites on microtubules is expected to affect the gliding speed, as hindrance of the motors' path along the microtubule could cause stalling of individual motors, resulting in a net drag force on the microtubule. Previous studies have shown that biotinylation and functionalisation by other small molecules do not affect microtubule gliding speeds [15, 24]. In contrast, slowly detaching kinesin heads (~8 nm), neutravidin (~5 nm) and $CoFe_2O_4$ particles (~20 nm) do cause noticeable decreases in gliding speeds [15, 30]. Recent work in which microtubules were labelled with cargo of differing sizes suggested that motility is reduced when the cargo approaches or exceeds the size of the motor [11]. Conventional kinesin is a processive motor, meaning that the two heads coordinate to take hundreds of steps before detaching from the microtubule. Hence, the most straightforward explanation for this roadblock-induced slowing is that the trailing motor head remains bound to the microtubule until the leading head finds its next binding site, but if this binding site is blocked, the motor waits there before eventually detaching [2, 29]. This results in a competition between active motors pulling the microtubule forward and stalled microtubules resisting forward movement. A second factor that may contribute to the slow speeds is that the onrate of motors to heavily labelled microtubules is also slowed [29], which results in fewer active motors pulling against the stalled motors.

Table 1: Gliding speed of magnetically labelled microtubules as a function of applied field

% Biotin	Speed,[a] $\mu m\ s^{-1}$			
	Diffused + no field[b]	Preconcentrated + no field[c]	Diffused + field applied[d]	Preconcentrated + field applied[e]
0.0	0.77 ± 0.04 (53)	0.62 ± 0.07 (16)[f]	0.76 ± 0.04 (52)	0.56 ± 0.09 (7)[f]
1.1	0.69 ± 0.07 (40)	0.41 ± 0.05 (43)[f]	0.66 ± 0.08 (42)	0.41 ± 0.05 (57)[f]
2.3	0.58 ± 0.10 (26)	0.49 ± 0.09 (40)	0.54 ± 0.08 (42)	0.45 ± 0.06 (40)
5.7	0.49 ± 0.10 (14)	0.37 ± 0.11 (16)	0.50 ± 0.14 (41)	0.26 ± 0.07 (11)[f]
11.1	0.37 ± 0.03 (2)	–[g]	–[g]	0.12 ± 0.07 (7)[f]

[a]Gliding speed for motile microtubules, reported as mean ± the standard deviation. Values in parentheses are the number of observed moving microtubules
[b]Microtubules bound and observed without an applied magnetic field
[c]Microtubules preconcentrated with an applied magnetic field, and motility observed after removing field
[d]Microtubules exposed to surface in absence of magnetic field, and motility observed with field subsequently applied
[e]Microtubules loaded and observed in the presence of a magnetic field
[f]Statistically different from 'diffused + no field' by *t*-test
[g]No motile microtubules were observed

In addition to the above slowing mechanism, the image in Fig. 4*j* further suggests that, at least at high label densities, some microtubule aggregation is present when magnetic fields are applied. It is possible that the magnetic field gradient increases collisions between magnetically-labelled microtubules as they are pulled towards the surface, and therefore increases the probability of neutravidin- or magnetic nanoparticle-mediated microtubule cross-linking. Aggregation would serve to further block-binding sites on the microtubule surface and inhibit motility. Hence, the ease with which the heavily $CoFe_2O_4$-labelled microtubules are preconcentrated and aligned on kinesin-covered surfaces is counterbalanced by reduced motor transport speed.

3.2 Effect of applied magnetic fields during transport of labelled microtubules

As even small permanent magnets can produce forces sufficient to induce alignment and enhanced microtubule surface densities [15], we next investigated how motility is affected by the presence of a magnetic field. Using the cell in Fig. 2, the NdFeB cube magnet (also used for preconcentration) was placed ~175 μm from the motor-covered surface, such that a substantial component of the field gradient is oriented orthogonal to the surface. In this orientation, the magnetic field causes a downward force on the microtubule (perpendicular to the transport direction), which may result in a drag force on the microtubule or may deform the motors and/or microtubules.

The last two columns of Table 1 contain a compilation of the motility data for magnetically labelled microtubules transported on kinesin-covered surfaces in the presence of a magnetic field. In the first of these two columns, magnetically labelled microtubules were allowed to bind to the kinesin surface in the absence of an applied field (e.g. by diffusion to the surface). After binding to the motor-covered surface, the magnet was placed below the coverslip. Analysis of the resulting motility data (Table 1, 'diffused + field applied') reveals that the gliding speeds are equivalent to microtubules that had not been exposed to the magnet (Table 1, 'diffused + no field'). Hence, microtubules that diffused to the surface were not significantly impacted by the presence of the magnet. The strong attraction of microtubules containing 11.1% biotinylated tubulin to the magnet results in crowding on the surface and no motile microtubules were observed (Table 1, 'diffused + field applied').

The final test was to determine whether microtubules that had been magnetically preconcentrated on the surface were affected by the external magnetic field. Following preconcentration, the flow cell was perfused with ATP to initiate motor transport while keeping the magnet in place, and speeds were measured before and after removing the magnet. The average speeds in the presence of the magnet are listed in the fourth column of Table 1 ('preconcentrated + field applied'), and the speeds following removal of the magnet are listed in the second column ('preconcentrated + no field'). For the three lowest biotin (and hence particle functionalisation) levels, removing the magnet had little or no influence on the speed. In contrast, for the 5.7% biotin-labelled microtubules, removal of the magnet resulted in an apparent increase in the gliding speed, although there is no strong statistical difference because of the variances of the two data sets. This result may suggest that a sufficiently large force normal to the surface slows the kinesin–microtubule interaction, but we further discount this possibility for two reasons. First, if this were the case for 5.7% biotin-tagged microtubules, then at least some influence of the external field would be expected for the 2.3% biotin-labelled microtubules. Secondly, it is possible that the preconcentration step selects for the more particle-laden microtubules. This subpopulation has slower gliding speeds and interacts with fewer kinesin motors, so that they readily diffuse away following removal of the magnet; the resulting surface contains microtubules with a higher mean gliding speed. This scenario is consistent with the 11.1% biotin microtubules, which are observed on the surface only in the presence of the magnet, but are bound so weakly that they diffuse away when the magnet is removed. Taken together, these observations lead us to conclude that the magnetic fields that pull microtubules towards the kinesin surface have little effect on the kinetics of kinesins transporting these microtubules.

Further, the forces produced by magnetic field interactions were insufficient to control the direction of microtubule movement, regardless of magnet or flow cell geometry, resulting in the same random movement of microtubules seen for samples in the absence of an applied magnetic field. Hence, although the magnetic preconcentration step resulted in aligned microtubules on the surface, perfusing ATP into the system to initiate transport resulted in randomisation of microtubule directions within roughly 1 min. Improving the microtubule and flow cell

constructions are the subject of ongoing work, with the goal of interfacing these optimised magnetic structures with microchannels to achieve the nano-biotechnological transport applications envisioned for these systems.

4 Conclusions

The manipulation of these hybrid nano-biological systems by external magnetic fields requires a thorough understanding of the interplay between particle attachment and motor function. Whereas greater levels of particle functionalisation enhanced the effects of external magnetic fields on the labelled microtubules, kinesin binding and transport of these particle-laden microtubules were diminished. Furthermore, microtubule transport speed and direction were not substantially affected by the presence of a magnetic field regardless of the number of magnetic labels. Magnetically labelled microtubules provide a new tool for in vitro investigations of the role of microtubules and motors in important cellular processes such as cell division, axonal transport and flagellar motility.

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