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Molecular control of stress transmission in the microtubule cytoskeleton

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ABSTRACT

In this article, we will summarize recent progress in understanding the mechanical origins of rigidity, strength, resiliency and stress transmission in the MT cytoskeleton using reconstituted networks formed from purified components. We focus on the role of network architecture, crosslinker compliance and dynamics, and molecular determinants of single filament elasticity, while highlighting open questions and future directions for this work.

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1. Introduction

The transmission and generation of forces within the cell are critical for numerous biological processes, acting as mechanical signals that control motility, division, differentiation and death [1,2]. It is known that cells detect physical interactions using anchored proteins that shift conformation under force, revealing or creating novel binding sites [3,4]. These changes promote the recruitment of additional proteins (e.g., signal transduction or cytoskeletal proteins) that then further propagate the mechanical information throughout the cell [5]. There has been substantial progress in identifying and characterizing such molecular mechanosensors, however, the mechanisms by which such locally generated forces and signals connect to tissue and organism function remain poorly understood [6–10]. One effective route of force transmission control is achieved through spatiotemporal reorganization of the cytoskeleton, and it is clear that even individual cytoskeletal filaments can play critical roles in transmitting stress over cellular length scales (Fig. 1) [11–15].

Here, we focus on stress transmission within the microtubule (MT) cytoskeleton, which is unique in both its extreme stiffness and in its ability to rapidly reorganize by dynamic growth and shrinkage [16–19]. In cells, MT organization and dynamics are important for myriad biological processes, ranging from forming and maintaining neural growth cones and axonal bundles [20,21], to axonal branching and neural pathfinding [22,23], to the flow of actin in motile and developing cells [24,25], to the regulation of protein synthesis [26]. MTs maintain parallel MT bundles in development to form the mitotic spindle in the early stages of cell division, and in later stages of mitosis, MT-mediated mechanical signals

control the synchronous separation of chromosomes and cleavage furrow localization to form the two daughter cells [27]. In interphase, MTs form sparse networks that project radially outward from the nucleus providing essential tracks for intracellular transport by kinesin and dynein motor proteins [28–31]. And, in the lamellae, MTs and actin filaments interact to promote dynamic network growth and motility [32–35]. The mechanical properties of MTs are critical to these functions: MTs in cells have been observed to bend and buckle [36], and modeling has shown that their flexural rigidity must be included to accurately describe cytoskeletal networks in cells [37]. In many cases, MT-mediated mechanical stress is an important signaling mechanism: tension promotes MT outgrowth at focal adhesion sites [38], regulates MT turnover and organization [39], and silences spindle assembly checkpoints to enable cell cycle control [40].

In contrast to the many open questions regarding the detailed relationships between MT mechanics and function on the cellular level, the gross structural properties of individual microtubules and their dynamic assembly process are reasonably well understood [41,42]. MTs self-assemble from dimers of α- and β-tubulin, given the appropriate pH, temperature and the presence of Mg^{2+}-GTP. The assembly process starts when dimers assemble head-to-tail into protofilaments that rapidly polymerize laterally to create a hollow tube with inner diameter $d_i \sim 12$ nm and outer diameter $d_o \sim 25$ nm. The growth is polar: the fast growing MT end (the 'plus' end) terminates in β subunits while the slowly growing ('minus') end terminates in α subunits. Each dimer is a catalytically active GTPase, and in solution, each subunit binds GTP tightly. After addition to the polymer, the β subunit hydrolyzes its GTP, inducing a slight bend in the dimer that tends to destabilize the filament as the lowest energy conformation is now splayed slightly away from the filament axis. If the rate of dimer addition exceeds that of hydrolysis, then a 'cap' of GTP-rich tubulin dimers remains

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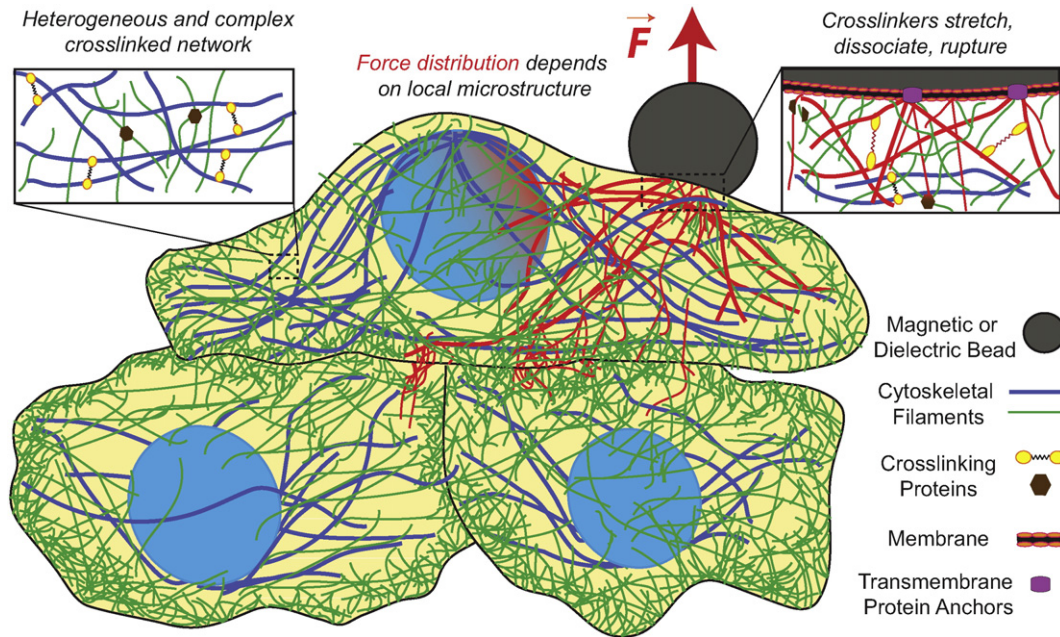
Fig. 1. Schematic of force transmission within a tissue. Here, a magnetic or dielectric bead is coupled to the outer membrane of an animal cell through a transmembrane protein that connects directly to the cytoskeleton (*i.e.*, *via* an integrin binding site). Magnetic or optical tweezers are used to apply forces to the bead, which in turn stretches, bends and (in the limit of very large forces) breaks the entangled and crosslinked filaments inside the cell. The filament rigidity and crosslinking allow forces (represented by red shading) to be transmitted over large length scales, and in some cases, to other cells.

at the plus-end, frustrating the conformational change and preventing MT depolymerization. If the rate of hydrolysis is faster, then the cap length shrinks to zero, and the filament quickly disassembles. This process, called 'dynamic instability' allows rapid reorganization of the MT cytoskeleton and is critical to the formation and function of the mitotic spindle, and numerous other biological processes [19].

The large filament diameter imparts extremely high stiffness to the polymeric microtubule. The average contour length varies but is typically of order ~1–10 μm, whereas the persistence length L_p (the average length over which the filament direction tends to decorrelate due to thermal fluctuations) is orders of magnitude greater, ~1–10 mm [17, 18,43–45]. The persistence length is given by the ratio of flexural rigidity EI and thermal energy: $L_p = EI/k_BT$ where k_B is Boltzmann's constant and T is temperature. If we assume that the MT is isotropic and continuous, EI can be expressed as the product of the geometric moment of inertia, I, which for a hollow cylinder is: $\frac{\pi}{16}(d_o^4 - d_i^4)$, and E, the Young's modulus. The extreme stiffness of MTs provides unexpected pathways for stress transmission, strength, and resiliency [46,47]. In contrast to most cellular and virtually all synthetic materials, MTs are nearly athermal. This means that their contour length and end-to-end lengths are roughly equal, leaving little to no 'excess length' to provide entropic stiffness at low strain. Rather, the MT filaments themselves bend and stretch under even small applied forces. In this rigid limit, the single-filament stiffness and the single-molecule crosslinking properties are critically important to determining the ability of networks to transmit stress, and forces applied to one point can propagate over distances much larger than a mesh size in an MT network. The study of MT networks thus provides new insight into the role of stiff filaments, as well as stiff bundles of semiflexible filaments (*i.e.*, actin), without the challenges of heterogeneity in bundle diameter and intra-bundle interactions and compliance that make the characterization and modeling of networks of bundles difficult. Additionally, the unique properties of MTs, which are at least an order of magnitude stiffer than carbon nanotubes (L_p of 20–170 μm) [48] and many orders of magnitude stiffer than lyotropic liquid crystalline polymers (L_p

of 30–120 nm) [49], drives technological interest in their use in novel materials [50–52].

2. Microtubule network mechanics

In vitro studies of purified cytoskeletal networks provide an excellent opportunity to explore the physical principles underlying the static and dynamic features of living matter, and have provided important insight into the molecular control of force transmission in cells [53–59]. A large number of prior studies explored entangled, crosslinked, and bundled F-actin networks and have established both the importance of the chain semiflexibility and the role of crosslinkers and architecture in determining local and bulk mechanics [58,60–71]. More recently, efforts have focused on mechanics of networks of MTs [47,72], as well as composite networks, composed of more than one type of filament, often in the presence of one or more crosslinking or motor proteins [58–61, 73–77]. The experimental approaches employed in these studies typically include confocal or electron microscopy to visualize network architecture and some form of rheological measurement to determine the network response to applied load. Local measurements have proven particularly useful since the network heterogeneity exists on the structural length scales of the cells, so simple coarse-grained models have limited power. The high filament rigidity and the large pore sizes prevent use of simple thermal microrheology tools, so active microrheology approaches are favored. These studies have shown that the nearly athermal MT networks have unique physical properties, which are important to both their role in the cellular cytoskeleton, as well as efforts to create synthetic materials with the extraordinary properties of living matter.

2.1. Crosslinker binding drives heterogeneous network formation

In vitro, entangled MT networks form homogeneous, isotropic materials, whereas crosslinked MT networks are heterogeneous, containing tubulin-rich and tubulin-poor regions that span large distances and

are reminiscent of functional cytoskeletal structures [46,47]. Their self-assembly is driven by a diffusion-limited aggregation (DLA), in which heterogeneity increases with crosslinking density for relatively small crosslinking densities, then the structures become insensitive to crosslinker concentration [47]. Heterogeneity arises from kinetic trapping during self-assembly, which is intrinsic to the densely crosslinked networks, and is largely insensitive to the crosslinker compliance. Similar structures and phenomena were observed using kinetically trapped actin/filamin bundle networks, where both the fractal dimension of the clusters and the cluster mass distribution has been explored [69]. Homogeneous and isotropic crosslinked networks are only expected at very low crosslinker densities for most crosslinker types. Densely crosslinked actin/myosin (HMM) networks appear to be exceptions, and are commonly used as model systems for isotropic network formation [61,65,70].

2.2. Entangled MT networks are soft elastic solids

Several prior studies investigated the rheological properties of entangled networks of MTs [78–80]. The most comprehensive study using conventional rheological approaches demonstrated that networks of fairly short MTs (characteristic length <3 μm, approximately equal to the mesh size) were soft elastic solids and that despite the lack of chemical crosslinking, these networks displayed transient attractive filament–filament interactions that enhanced elasticity and suppressed reptation-induced terminal relaxation [72]. These results were later supported by microrheological measurements using magnetic tweezers to perform spatially-resolved measurements of the displacements of ~5-μm beads under applied force [47]. Again, the entangled MT networks, now consisting of much longer filaments of ~20–25 μm, were found to be predominantly solids that showed an instantaneous network compression under application of up to ~30 pN of force, with modest time-dependent network reorganization occurring at longer times. When confocal microscopy was used to observe the loaded MT networks, no obvious long-length scale flow of material was observed, suggesting that the subtle reorganization of entanglements and adhesive contacts dominates the creep response. The origins of the transient adhesive attractions are not well understood, but these may arise from divalent crosslinking mediated by the Mg^{2+} ions that are required for coordination of the GTPase activity of tubulin. Attractive interactions have been reported for other rigid rod suspensions, for example, of surfactant-stabilized single walled carbon nanotubes, as well as networks of semiflexible actin and neurofilaments, suggesting these may be a common feature of rigid and semiflexible filament systems [72, 81–86].

Under microscale loading, the entangled MT networks exhibited an extended linear regime, with modest strain stiffening at high strains, above ~70% [47]. Although strain stiffening has been observed for many biopolymer networks, it is usually attributed to an entropic-to-enthalpic transition that is not possible in the rigid MTs [63]. Instead, it is possible that for large localized forces, there is an accumulation of filaments in front of the bead that increases the local polymer concentration and leads to a nonlinear increase in stiffness. The threshold force associated with the onset of stiffening is ~15 pN, a modest force that could easily be applied by a small number (~3–4) of kinesin motors *in vivo* during cargo transport or mitosis, suggesting this effect may have physiological relevance. An accumulation of filaments under load was previously reported in magnetic-bead based microrheometry measurements of entangled actin networks [87]. However, the actin remodeling led to strain softening and force-induced network fluidization, suggesting that unlike MT gels, entangled actin networks cannot support large stresses without some form of crosslinking. Strain softening was also observed in a suspension of very short MTs, with a characteristic length approximately equal to the mesh size, for strains above ~10% [72]. This suggests that strain-induced stiffening require either long MTs that can

form truly entangled gels or the application of localized stresses that can lead to the microscale reorganization and densification of the network.

2.3. Crosslinked MT networks are stiffer, stronger, and dominated by crosslinker kinetics

When a modest number of crosslinks are introduced, there is an increase in gel stiffness and suppression of strain-stiffening, although long-time network rearrangement is still observed, and the length scale of penetration of the deformation field is substantially increased. This suggests that long range stress transmission can be controlled by inclusion of a small number of crosslinking bonds, and that even sparse connections between filaments can play an essential role in regulating cell mechanics.

When networks are highly crosslinked using rigid streptavidin–biotin (S–B) bonds, they respond very differently to applied force, becoming highly nonlinear as a small number of crosslinkers bear stress until they are loaded to failure [46]. In such rigidly-bonded systems the time-dependent network rearrangements are directly linked to the force-induced unbinding of crosslinkers causing the creep velocity to increase exponentially with force [88]. Surprisingly, the network retains its elastic modulus even under conditions of significant plastic flow. This suggests that crosslinker breakage is balanced by the rapid formation of new bonds, and is perhaps one origin of microscopic self-healing in the cytoskeleton.

The elastic energy is dissipated partially through unbinding/rebinding events as indicated by small unrecovered displacement when the force is removed. However, this displacement is equal to or smaller than the distance moved in the creep regime, which is inconsistent with simple models of dissipation arising from hydrodynamics or filament contour fluctuations. The lack of dissipative mechanisms is also apparent in loading history tests. It is likely that the long persistence length of the MTs drives the network back to its initial configuration, even when individual bonds have been broken at high force. The model that emerges for such rigidly-bonded rigid filaments is one of a reconfigurable, but dominantly elastic network (Fig. 2). In this limit, individual bonds can be loaded to failure, but the energy stored in those bonds can be transferred to neighboring intact bonds, allowing the network to maintain its elastic nature, and minimizing the loss of mechanical information to the thermal bath. This contrasts with most simple pictures of soft gel mechanics, in which creeping flow is assumed to arise from viscous dissipation and the distance moved during this 'flow' regime is assumed unrecoverable when the loading force is removed. In rigid MT networks, the lack of dissipation and ability for dynamic bonds to reform even in the presence of plastic deformation allows for resiliency and fatigue-resistance that is rarely observed in synthetic soft materials.

The importance of single-filament mechanics and single bond breakage events to network rheology has previously been shown for isotropic actin gels [61,70], suggesting that modulation of filament and crosslinker properties is a robust means of controlling polymer mechanics. Reversible stress softening has also been observed in dendritic actin networks and in that case was attributed to the reversible Euler buckling of individual filaments under compression [89]. For MTs in highly crosslinked networks, buckling requires extremely high forces (~1–100 nN), which were not experimentally accessible, and are well-beyond the internal forces experienced by most cells. Thus, it is unlikely that buckling plays an important role in the rheology of highly crosslinked MT networks. Instead, the crosslinker unbinding and rebinding kinetics control the timescales of rearrangements, and the crosslinker compliance controls the strain to failure, which in turn controls the volume of engaged filaments near the bead and the overall network strength.

3. Single filament microtubule mechanics

It is clear from the network mechanics studies that the extreme rigidity of single MTs is essential to the fatigue resistance and self-
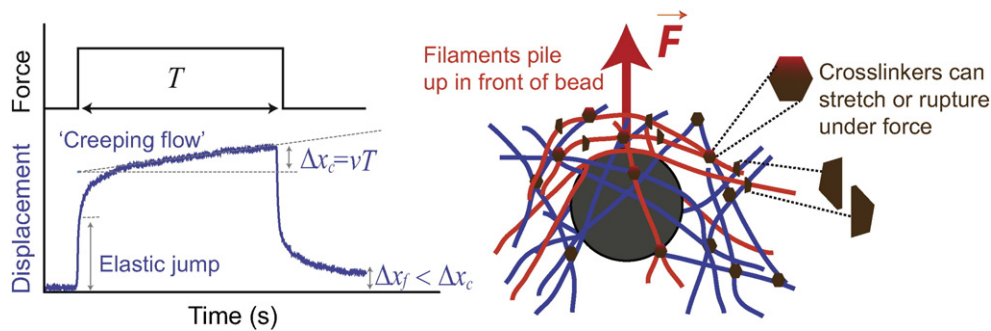
Fig. 2. Schematic of typical force loading curve and network rearrangements. A typical force–displacement curve for a crosslinked MT network. Under sudden application of force, the network responds elastically, then at longer times, a 'creeping flow' regime is seen where displacement increases linearly with time. For rigidly-crosslinked MTs, the bead speed is directly related to the crosslinker unbinding time, and only a small number of bonds are loaded to failure to allow local yielding of the material. In contrast to viscous systems, the stored energy that is lost during bond rupture can be transferred to other intact bonds, minimizing total dissipation and promoting fatigue resistance and resiliency.

healing properties of crosslinked MT gels. Single filament mechanics is an active area of research, and numerous experimental and computational studies have investigated structural origins of MT filament mechanics. Yet many questions still remain about the molecular determinants of individual filament stiffness, and in particular how stiffness is controlled by small molecule inhibitors of MT dynamics (*i.e.*, taxane and epothilone compounds) as well as MT-associated proteins (MAPs).

3.1. Structural understanding of MTs

Electron microscopy has long been used to determine the structures of tubulin and microtubules [90]. The structure of dimeric tubulin has been solved at atomic resolution [91], and although cryoelectron microscopy has not yet elucidated the polymer structure to atomic resolution, it has been possible to dock the known tubulin structure into lower resolution polymer data in order to recreate the microtubule structure [92–94]. Small angle X-ray scattering (SAXS) has been used to measure structural features including the regulation of protofilament number by the MT-associated protein tau [95], as well as the effects of small-molecule inhibitors of dynamic instability (*i.e.*, the taxane compound paclitaxel) on interprotofilament interactions [96]. Both methods typically require averaging over many static filaments, precluding measurement of heterogeneities, and making it nearly impossible to measure dynamic, time-resolved changes in MT structure. More recently, atomic force microscopy (AFM) has been used to measure the diameter of bare MTs as well as MTs decorated with tau or kinesin [97]. High-speed AFM methods may have the time-resolution to measure dynamic changes in structure, although to our knowledge these have not yet been reported [98]. It has been shown that in growing microtubules the protofilaments follow a straight line (which can in turn rotate around the filament axis due to a small helical pitch), but in depolymerizing microtubules the protofilaments curl outward, providing structural evidence for dynamic instability [99,100]. Other work comparing the conformation of tubulin dimers bound to either the non-hydrolyzable GTP analog GMPCPP or the post-hydrolysis GDP further support this by showing that the GTP hydrolysis state is the determining factor of protofilament curvature [101]. A recent study investigating the effects of post-translational modifications of tubulin found no gross changes in the MT structure for deacetylated or acetylated microtubules, suggesting the acetylation must impart a very local change in the tubulin dimer that may be important for some biological processes but not overall shape and organization [102]. Despite the high spatial resolution of many of these tools, the inability to measure dynamic structural changes makes it particularly difficult to measure the unique and dynamic structural properties of the growing microtubule plus-end, as well as the action and binding of numerous MT-associated proteins, both of which are essential to numerous biological functions [103].
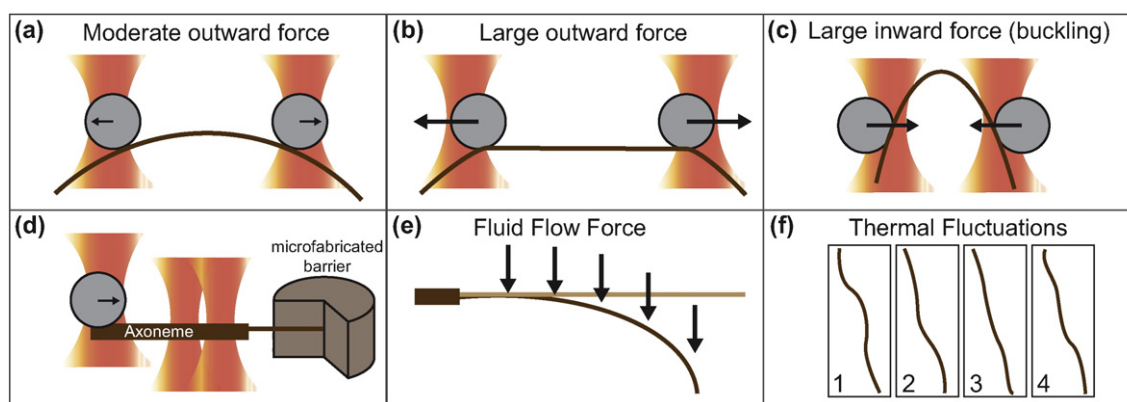
3.2. Methods to determine microtubule stiffness

Generally speaking, it is difficult to connect MT structure to the mechanical and assembly properties of MTs. However, there have been several successful attempts to do so. A three-dimensional model that incorporated the stress and strain of the protofilament conformations in the GMPCPP and GDP cases successfully recapitulated structure, polymerization rates, and depolymerization rates at the plus-end [104]. Additional all-atom simulations have also connected MT structures to continuum mechanics models [105]. Experimentally, MT flexural rigidity is typically measured by applying known forces to bend MTs and measuring the resulting curved conformation (Fig. 3). Active bending has been achieved using optical trapping [106–108] or flow [109]. One novel method used microfabricated barriers to deflect the growing end of a single MT polymerized from the end of an axoneme held steady in a keyhole optical trap [110]. This method is unique in its ability to measure the stiffness and force production of MTs that are dynamically growing. A common alternative is to observe the passive bending of the microtubule in solution due to thermal forces. This method, which requires only visualization, not application of force, was first introduced in 1993 [18] and has subsequently been used by many others (reviewed in [17]) to measure biopolymer stiffness. In most implementations the filament contour is traced in every frame of a movie of a thermally-excited, fluctuating MT. The spectral components of the contours are estimated (typically using a Fourier series expansion), and a mechanical model (*e.g.*, worm-like chain) is used to determine the stiffness from the variance in the spectral components from the ensemble of images.

While straightforward, this approach can be limited by high background noise, small fluctuation amplitudes, and stochastic fluctuations in the fluorophore labeling density that lead to errors in the analysis. Errors can be reduced by combining the first and second steps into one by using a global fitting routine that expresses the entire contour using the same spectral basis used in the variance analysis [44]. Use of Chebyshev polynomials to represent the contour rather than a Fourier series removes the need for periodic boundary conditions, further improving the analysis. Together, these improvements reduce image analysis errors to less than ~10% for typical image quality [44]. We recently extended this type of analysis to investigate heterogeneous flexural rigidity, using a sliding window method to resolve spatial differences in stiffness within a single MT [45]. Although the spatial resolution is limited (the smallest reasonable window was ~25% of the contour length), this is the first non-contact method to allow stiffness heterogeneities to be measured.

3.3. The challenges of measuring MT stiffness

Various methods of determining microtubule flexural rigidity have been employed by a number of groups, and a comparative analysis of

Fig. 3. Methods for determining microtubule stiffness. Optical trapping is used to exert forces (represented by black arrows) on micrometer diameter spheres that have been attached to a stabilized microtubule in several ways: (a) a moderate outward force induces a curvature in the microtubule, while at a larger outward force (b) the curvature is mostly at the attachment points. (c) Similarly an inward force will cause curvature and eventually buckling of the microtubule. This can be a useful measure of the failure limit of a microtubule under compressive load. (d) Multiple optical traps can be used to exert force on an axoneme directing it against microfabricated barriers. Force generation during polymerization can then be measured. (e) A known force due to fluid flow can be applied to a microtubule and the induced curvature can be used to determine microtubule stiffness. (f) Many images of microtubule thermal fluctuations can be captured and filament tracing used to determine an ensemble of microtubule conformations, which then can be used to calculate filament stiffness.

these results is examined thoroughly in [17]. It is clear that microtubule stiffness is sensitive to a large number of parameters: tubulin source [111], polymerization rate [112], presence of GTP or GTP analog [113, 114], small molecule stabilizers [114–119], and microtubule associating proteins (MAPs) [95,97,113,114,120–122]. While there appear to be consistent stiffness values reported within a particular research group for a fixed condition, the values vary significantly among different laboratories, even under seemingly similar MT preparation conditions. This is an issue that has yet to be entirely resolved.

There are additional issues that make MT stiffness quantification challenging. For a particular MT preparation, the experimental values of stiffness reported, even by a single laboratory, show a broad and non-Gaussian distribution [114,119], which some groups have described as log-normal [111]. The distribution is typically wide, with as much as an order of magnitude variation from maximal to minimal values, much larger than the errors expected from the image processing and spectral analysis steps, which have been estimated to be less than 10% [44]. This suggests that the variation is due to actual heterogeneity of MTs within a preparation. It is known that protofilament number varies slightly among MT ensembles, mostly varying between 12–14 for typical conditions, although in some chemical preparations values as high as 17 or as small as 9 have been observed [117]. Since *EI* is proportional to d^4, this diameter variation could account for some of the observed variations in stiffness. There is also a broad distribution of microtubule lengths, so any coupling between length and stiffness could contribute to the stiffness variations. Although most studies have not reported a correlation between length and stiffness, there are examples of contour length-dependent stiffness [123]. Finally, it is possible that the MTs possess defects such as dislocations or missing dimers that could lead to unexpected and large variations in their mechanical properties. For most *in vitro* preparations, MT growth and shrinkage dynamics are suppressed through the use of small molecule stabilizers such as paclitaxel. Such stabilizers may also maintain partially deteriorated or misassembled microtubules causing a change in overall stiffness [102]. It has further been shown that MT stiffness decreases when the rate of polymerization is increased [112]. One interpretation of this result is that rapidly formed structures tend to have more defects which reduce their stiffness and strength.

It has not yet been possible to measure the stiffness of dynamic MTs *in vivo*, so the presence and importance of the variation of MT properties within cells and tissues is not known. However, from *in vitro* studies, it is clear that the single filament stiffness can be very sensitive to polymerization conditions and the presence of MT-associated proteins (MAPs), and it is very possible that cells use the regulated modulation of single filament properties to control stress transmission within the cytoskeleton.

3.4. The effects of small molecule inhibitors of tubulin depolymerization on MT mechanics

Although the origins of intrinsic variation in MT stiffness under seemingly identical conditions are not fully understood, there are many preparation conditions in which the population average for MT stiffness shifts significantly, and this has allowed the effects of nucleotide conditions and small molecule inhibitors to be quantitatively assessed.

GTP is the natural tubulin nucleotide and is most commonly used in *in vitro* assays, but there are also several nonhydrolyzable or slowly-hydrolyzable GTP analogs, such as GMPCPP and GTPγS, which allow the effects of nucleotide type on MT mechanics to be investigated. MT inhibitors, such as paclitaxel, have medical use as cancer chemotherapeutics, since they arrest dynamic depolymerization and therefore inhibit mitosis. But, they are also implicated in chemotherapeutic peripheral neuropathy (CIPN), in which patients experience severe nerve pain which often requires them to reduce or discontinue treatment. Since MTs are enriched in neural tissue, particularly in the nerve axon, there is much interest in understanding what role paclitaxel may play in non-mitotic, MT-rich cells. Paclitaxel is also frequently used for *in vitro* studies to prevent MT disassembly.

Studies have consistently shown that when MTs are polymerized with GMPCPP they are ~3× stiffer as compared to GTP-polymerized MTs stabilized with paclitaxel [17,113,114]. Unlike GTP-polymerized microtubules, GMPCPP-polymerized microtubules are stable with or without paclitaxel, presenting an opportunity to test the effects of paclitaxel on MT mechanics. One study tested addition of paclitaxel to GMPCPP-MTs after polymerization and found no change in stiffness [113]. Another study confirmed this result while also finding that when paclitaxel is present during polymerization of GMPCPP-microtubules, the stiffness is significantly reduced to nearly half that of the no paclitaxel case, showing that even order of addition (during or after polymerization) is an important factor affecting microtubule flexural rigidity [114]. GTPγS-polymerized microtubules are less stable than GMPCPP-microtubules so paclitaxel is generally included for stabilization. The stiffness of GTPγS-polymerized microtubules (in the presence of paclitaxel) has been found to be comparable to the stiffness of GTP-polymerized microtubules [113,114].

Another set of small molecule stabilizers and chemotherapeutic compounds, epothilone-A and -B, are known to have an overlapping tubulin binding site with paclitaxel [124]. When MTs were formed with

epothilone-A, the persistence length was reduced by 20% and when epothilone-B was used, the reduction was 40% as compared to paclitaxel stabilized MTs [119]. This is surprising considering that epothilone stabilized microtubules have been found to have a slightly larger average number of protofilaments [125], which would favor a stiffer microtubule. If and how these changes in the stiffness of individual MTs impact biological function in neuronal cells and whether these changes are tied to CIPN remains an important open question.

3.5. How do MAPs affect microtubule stiffness?

Microtubule associating proteins (MAPs) are also important for regulation of microtubule dynamics and function, and MAPs can also modulate MT network organization to control long-term transmission of stress. Among MAP proteins, the most well-studied is tau, a neuronal MAP that promotes MT stability, and whose dysfunction has been linked to numerous neurodegenerative diseases [126]. Only one study so far reported L_p with tau present during polymerization (before stabilization with paclitaxel) and found that it had a large stiffening effect, increasing L_p by a factor of four, while adding tau after polymerization resulted in no significant stiffening [113]. Another study looked at the effects of 3R and 4R tau added post-polymerization on paclitaxel or epothilone stabilized microtubules, and found ~30–40% reduction in stiffness when paclitaxel or epothilone-A were used, but very little change in stiffness for epothilone-B-stabilized MTs, which suggests that different types of stabilizers can induce different, possibly synergistic effects [119]. The third study, added tau post-polymerization as well, but did not include paclitaxel at all. This group found that many different tau constructs all independently increased microtubule stiffness compared to tau-free microtubules [120]. The likely resolution to this apparent discrepancy is that tau can stiffen microtubules in the absence of paclitaxel, and that addition of paclitaxel after tau does not have any further effect. However, when paclitaxel is added first, it 'locks-in' the microtubule stiffness and the subsequent addition of tau can no longer cause stiffening. Again, this is an 'order of addition' effect, similar to that observed when GMPCPP and paclitaxel are both used to stabilize MTs in the absence of MAPs. The emerging rule of thumb is that the stabilizer that acts first wins, and that addition of subsequent stabilizers have little or no effect on stiffness.

Another particularly interesting MAP is EB1, which specifically binds to the growing plus-end of microtubules. EB1 acts as a master coordinator of the activity of numerous other MAPs at the dynamic plus-end, and is implicated in spindle assembly and misregulated in several cancers [127]. Studies have indicated that EB1 targets the plus-end by recognizing the GTP hydrolysis state of tubulin, and moreover that when nonhydrolyzable GTP analogs are used to form the MTs, EB1 will bind along the entire filament length [128]. This allows synthesis of homogeneous EB1-coated MTs of fixed, static length and enables mechanical measurement of the effects of EB1 on MT stiffness. Using thermal analysis of EB1-coated MTs, we recently showed that the addition of EB1 to GMPCPP microtubules stiffens the MTs in a concentration dependent manner, doubling the persistence length at 200 nM EB1 (13.1 mm) as compared to the value for GMPCPP microtubules lacking EB1 (6.7 mm) [114]. When both GMPCPP and paclitaxel were used to stabilize the MTs and then EB1 added, this EB1-dependent stiffening effect was abolished, even with 500 nM EB1 in solution. This suggests that when paclitaxel is present, it suppresses the ability of EB1 to subsequently act on the tubulin and MT structure. Equilibrium EB1-binding measurements ruled out the possibility that less EB1 bound in the presence of paclitaxel, so the differences in mechanical response arise from differences in the pattern or strength of the binding, rather than a change in the overall concentration. This has important implications for the use of paclitaxel both as a chemotherapeutic and in biophysical measurements, and suggests caution when using paclitaxel to investigate MAP function.

Several other MAPs have also been tested for their ability to alter microtubule stiffness. XMAP215 increases the rate of microtubule polymerization by an order of magnitude and inhibits destabilization of the plus-end by another MAP, XKCM1. The analysis of the thermal bending fluctuations of XMAP215-coated MTs using images obtained by video-enhanced DIC microscopy, demonstrated that XMAP215 did not change microtubule stiffness, despite reducing the curvature of tubulin protofilament rings. This work was done without paclitaxel stabilization [121]. MAP4 is somewhat analogous to tau, except that it is not neuron-specific, rather it is expressed ubiquitously in many tissues. A recent study showed that MAP4 does not change the mean MT stiffness in the presence of taxol. It does, however, reduce the filament-to-filament stiffness variation; the width of the measured distribution was roughly half that of MTs polymerized without MAP4 [113]. A study using hydrodynamic flow to actively bend taxol-free MTs and characterize their stiffness found that MAP65, a microtubule crosslinker (bundler) reduces microtubule stiffness by a factor of four. Surprisingly, they also reported that MAP65-coated microtubule bundles had the same mean persistence length regardless if they consisted of two or three microtubules [122].

3.6. What are the possible mechanisms for stiffness change?

There are clearly numerous factors that affect microtubule L_p (Fig. 4). Various studies have reported instances where either small-molecules or MAPs can increase, reduce, or not affect microtubule flexural rigidity. In principle, this inventory of MT interactions should provide a mechanistic understanding of how stiffness is altered and regulated, but in practice these measurements can be very difficult to interpret and compare. This limits our ability to understand how cells may modulate local stiffness to enhance or suppress force transmission.

If we assume that a microtubule can be reasonably approximated as a homogeneous hollow cylinder, there are two possible mechanisms for changing flexural rigidity, EI, which in this limit is given by the product of the Young's modulus and the geometric moment of inertia. The Young's modulus, E, is changed by altering material properties. In the case of MTs this could be achieved through changes in the inter- and intra-protofilament interaction energies. By contrast, the geometric moment of inertia, I, is altered by changing the shape, and particularly, by changing the inner or outer diameter. Given the fairly large MT lattice constant (~8 nm) and MT surface roughness, it is not obvious that such a continuum model would be useful. However, a recent report using data from ensemble-averaged cryo-electron micrographs of MTs consisting of 11–16 protofilaments to build pseudoatomic models of their structure, found that inter-protofilament interactions are relatively unchanged between microtubules of differing protofilament number [93]. This suggests that E and I may be truly separable components of microtubule mechanical stiffness, and that continuum modeling is sufficient.

The differences in L_p between microtubules polymerized with GTP or GMPCPP likely have contributions from changes in both E and I. We know from cryoelectron microscopy studies that GMPCPP-bound protofilaments have a straight conformation and GDP-bound protofilaments (that comprise the majority of a microtubule polymerized with GTP-tubulin) have a curved conformation which would change the internal stress of the microtubule and could alter E [101]. Further details come from a cryo-EM study of the differences in interprotofilament interactions of GTP-taxol and GMPCPP polymerized microtubules. It found an additional lateral interprotofilament contact in GMPCPP microtubules that likely increases E, explaining the larger stiffness of those microtubules [129]. Additionally, GMPCPP-polymerized microtubules have a different distribution of protofilament numbers. While polymerization with GTP-tubulin (without paclitaxel) leads to a roughly even split between 13- and 14-protofilament microtubules, for GMPCPP-MTs, nearly all (96%) of the
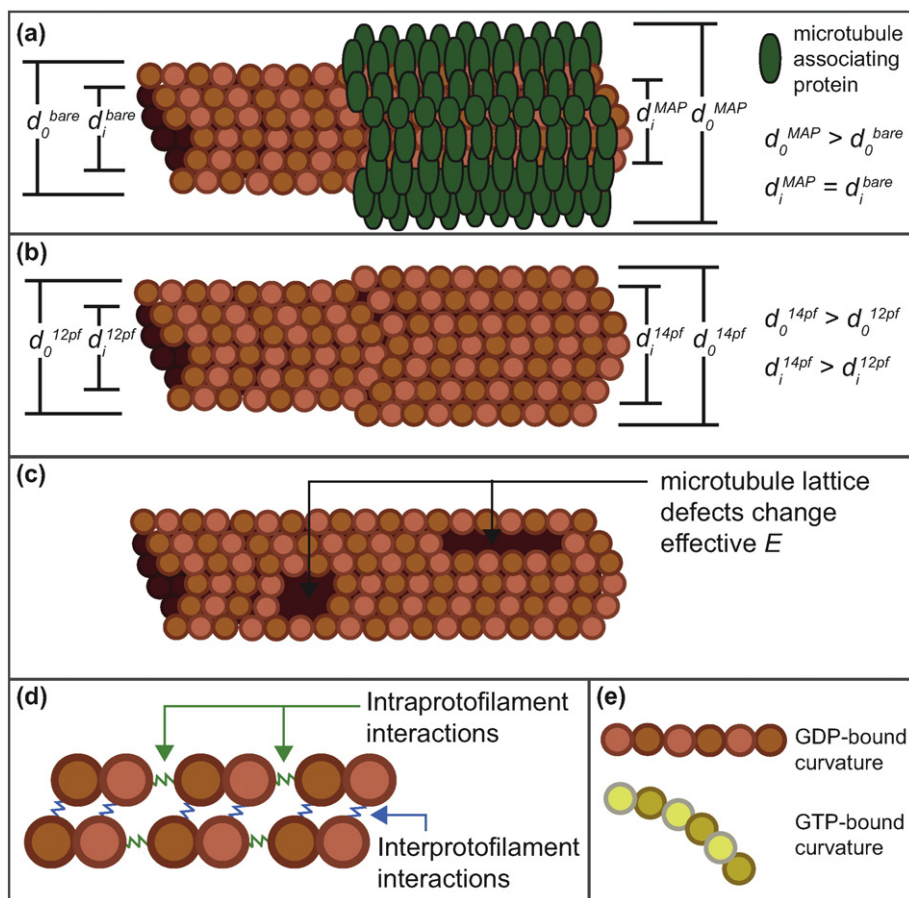
Fig. 4. Mechanisms for stiffness change in microtubules. (a) MAPs (microtubule associating proteins, represented as green ellipses) bind to lattice surface and increase effective outer diameter. (b) Increase in protofilament number increases both inner and outer diameters. (c) Microtubule lattice defects decrease effective Young's modulus. (d) Polymerziation conditions or MAP binding can affect intra- and inter-protofilament interactions changing the overall Young's modulus. (e) Nucleotide state of the tubulin dimers (GTP or GDP) changes the protofilament curvature which affects the internal stress of a polymerized microtubule.

microtubules have 14 protofilaments [101]. The increase in the average radius increases I, leading to an increase in L_p.

Studies on paclitaxel-stabilized microtubules also show a change in intrafilament interactions, which would impact E. Large-scale molecular simulations of paclitaxel binding have shown increased flexibility where β-tubulin binds to the α-tubulin of the next tubulin dimer, resulting in a relaxation in internal stress due to GTP hydrolysis [115]. High resolution cryo-electron microscopy structures have also shown that paclitaxel-polymerized microtubules have a similar structure to GMPCPP-polymerized microtubules, leading to the same reduction in internal stress [92]. Another study of protofilament curvature using atomic force microscopy found that both GMPCPP protofilaments and taxol-stabilized protofilaments had less curvature than GDP protofilaments, another piece of data confirming the change in intrafilament interactions. Microtubules polymerized with paclitaxel have been found to have an average protofilament number of 12, smaller than without paclitaxel [130]. Surprisingly, it has been shown that even prepolymerized microtubules will, on average, lose one protofilament after the addition of paclitaxel [131], suggesting a dynamic arrangement of the microtubule lattice along the entire length, not only at the ends.

The detailed mechanisms explaining how MAPs change microtubule stiffness are so far largely unknown. Tau has been found in a SAXS measurement to increase the diameter of the microtubules themselves even at low tau:tubulin binding stoichiometry [95]. At such low binding stoichiometries, many tubulin dimers remain unbound to tau, yet there is still an overall increase in diameter. A study of microtubule steady-state dynamics also showed strong tau effects at low tau:tubulin stoichiometries [132]. These studies suggest that tau may have an allosteric

effect on the microtubule, affecting a structural or mechanical change over many tubulin dimers. Polymerization of microtubules in the presence of Mal3, a yeast homolog of EB1, yields almost exclusively 13 protofilament microtubules, and showed a slightly different arrangement of tubulin dimers [133], revealing that EB1 changes intra- and inter-protofilament interactions to some degree. The presence of both geometric and energetic differences may explain why the distribution of MT stiffness is similar in the presence and absence of EB1, despite the reduction in protofilament number variation. It was also determined that EB1 can induce MT stiffening at low EB1:tubulin binding ratios, perhaps indicating the possibility of allosteric interactions similar to tau [114].

3.7. Future directions and new frontiers

One major remaining challenge in understanding force transmission in the MT cytoskeleton is the role of dynamics on mechanics and force transmission. It is clear that molecular interactions, including binding of filaments by MAPs and crosslinking proteins, can dramatically alter filament and network properties. However, it is becoming increasing clear that such binding interactions are frequently short-lived, and it is unclear to what extent these interactions can or do play a role in the steady state behavior of the system [134,135]. New super-resolution microscopy methods or ultrafast AFM imaging may allow the dynamic motions of MTs and their binding partners to be assessed with better spatial and temporal precision, and may allow better predictive models of the dynamic MT structure to be put forward. Dynamic rearrangements of MTs are also driven by motor proteins and/or by the growth

and shrinkage of the filaments themselves. For active ATP-driven kinesin systems, very complex patterns of MT organization have been observed. Kinesin-driven pattern formation depends sensitively on component concentration: at low concentrations of tubulin, vortices, asters, or bundles of MTs form at low, intermediate or high kinesin concentrations, respectively [136]. The effects of these ATP-driven motors on network mechanics is not known, although a better understanding of the role of motors in generating and transmitting forces within cells is essential, particularly in studying the mechanics and strength of the mitotic spindle. Finally, nearly all mechanical measurements of both individual MTs and entangled and crosslinked networks have been performed on MTs stabilized with some form of small molecule inhibitor, leaving open important questions about how dynamic instability itself may lead to novel pathways for stress transmission and athermal fluctuations within cells.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

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