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Untangling the Mechanics of Co-entangled Cytoskeletal Networks

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Abstract

Active networks of interlinked protein filaments comprising the cytoskeleton largely control cellular mechanics and cell architecture. By forming cytoskeleton networks that combine motile, semiflexible actin with rigid, supportive microtubules, cells maintain structural integrity and shape while being able to flow and move. To elucidate the complex mechanical processes that arise between interacting networks of actin and microtubules within cells, we create a suite of randomly-oriented, well mixed networks of actin and microtubules by co-polymerizing varying ratios of both proteins in situ. We use optical tweezer microrheology in order to characterize the nonlinear mesoscale mechanics of in vitro co-entangled actin-microtubule composites. To perturb each composite far from equilibrium, we use optical tweezers to displace a microsphere a distance greater than the median contour lengths of the filaments at a speed much faster than their intrinsic relaxation timescales. During perturbation, we simultaneously measure the force the filaments exert to resist the strain and the subsequent force relaxation after strain. We find the presence of a large fraction of microtubules ($\phi_T > 0.7$) is needed to substantially increase the resistive force, which is accompanied by large heterogeneities in force response. Actin minimizes these heterogeneities by reducing the composites mesh size and supporting microtubules against buckling. Composites also undergo a sharp transition from stress-softening to stress-stiffening dynamics when the fraction of microtubules exceeds 0.7. The induced force following strain relaxes via two time-dependent power-law decays. The first decay phase arises from actin bending fluctuation, with scaling exponents that increase proportionally with the fraction of actin. Alternatively, the second phase is independent of composite composition, with a scaling exponent of $\sim 0.4$ indicative of actin filaments reptating out of deformed entanglement constraints.
Introduction

The cytoskeleton is a complex network of protein filaments that gives eukaryotic cells structural integrity and shape while enabling cell motility, division and morphogenesis. Multifunctional mechanics and processes within cells are possible because of the varying sizes, stiffnesses and interactions between different cytoskeletal proteins (1-4). Actin and tubulin are two such proteins that form filaments of similar lengths (~10 µm) but very different stiffnesses. Actin monomers polymerize into ~7 nm wide semiflexible actin filaments, with persistence lengths of $l_p \approx 10$ µm. Contrastingly, tubulin dimers polymerize into 25 nm wide hollow, rigid tubes, called microtubules, with persistence lengths $l_p \sim 1$ mm (5, 6). At high concentrations, both actin filaments and microtubules sterically interact to form entangled networks. These entangled networks can be characterized by concentration dependent mesh sizes. The mesh size for actin and microtubule networks are $\xi_A = 0.3/c_A^{1/2}$ and $\xi_T = 0.3/c_T^{1/2}$, respectively, where $c_A$ and $c_T$ are the corresponding protein concentrations in units of mg/ml and the resulting mesh sizes are in units of microns (7-9). The dynamics of entangled filaments can be described by the tube model or reptation model, where each filament is confined to a tube-like region formed by surrounding constraining filaments (10, 11). According to the tube model, actin filaments are forced to relax induced stress by diffusing curvilinearly (i.e. reptating) out of the deformed tube (10, 11). The timescale for the process, often termed disengagement, is on the order of minutes to hours for actin and microtubules (12-14). Entangled actin can also partially relax due to faster mechanisms such as bending fluctuations (15-17).

Steric interactions between actin and microtubules directly influence cell shape and mechanics by regulating filament mobility and providing coordinated reinforcement to the cytoskeleton (6, 18). Cell motility and cytokinesis relies on the structural interactions and
physical crosstalk between actin and microtubules (6, 19). Further, while individual microtubules buckle under substantial compressive forces (20-23), physical interactions with the elastic network of actin in the cytoskeleton allow microtubules to bear large compressive loads within the cell (6, 13, 20, 24).

Studies of composite networks of actin and microtubules are further motivated by materials engineering, in which soft elastic networks are often reinforced with stiff fibers or rigid rods. By tuning the concentration of rigid rods relative to the flexible filament network, bulk properties of composites can be optimized to create lightweight materials with high strength and stiffness (19, 25). Such composites offer enhanced control over large-scale mechanics. Additionally, these composites have increased failure limits, which are tuned by the differences between the mechanics of the two composite constituents. Finally, composites both in nature and in industry often exhibit emergent properties, where the resulting mechanical properties are not a simple sum of the single-component network mechanics.

Regardless, in vitro studies of cytoskeleton networks thus far have largely focused on single-component systems of either actin or microtubules (7, 26-28). At the same time, the microscale differences between microtubules and actin likely result in marked differences in stiffness, strength, and viscoelasticity of cytoskeleton composites as compared to networks of one constituent (6). A previous passive microrheology study of a composite comprised of an 1:1 actin:tubulin ratio showed that while entangled actin solutions appeared incompressible, when microtubules were added the resulting composite displayed a finite compressibility resulting from the high stiffness of microtubules (13, 29). This same study showed that the crossover to viscous behavior that microtubule networks exhibit at low strain frequencies was not observed for actin solutions or equimolar composites. Additionally, a previous nonlinear bulk rheology
study of crosslinked actin networks doped with low concentrations of microtubules (~3 – 10x lower than the actin concentration) showed that the addition of microtubules led to nonlinear strain-stiffening as compared to the signature strain-softening behavior of entangled and weakly-crosslinked actin networks (19, 30, 31). The authors explained this shift as a result of stiff microtubules suppressing bending modes and local non-uniform fluctuations of semiflexible actin filaments, leading to enhanced stretching and affine deformation. Although these few studies revealed important emergent properties in actin-microtubule composites, they were limited in the parameter space of composite makeup. Instead of polymerizing both proteins together, these composites were created by adding pre-polymerized microtubules to actin. This method can induce flow alignment and shearing of microtubules, as well as bundling of actin filaments, preventing the formation of a truly isotropic, well integrated, co-entangled composite. Further, these studies have probed the bulk response resulting from large-scale nonlinear strains, and the microscopic linear response due to passively diffusing microspheres. Yet, the relevant lengthscales in actin-microtubule composites are in between these two scales, with persistence lengths of ~10 µm to 1 mm, filament lengths of ~5 – 20 µm, and mesh sizes on the order of a micron. Thus, the question remains as to how the relative concentrations of actin and microtubules impact the mechanical properties of composites.

Here we create co-polymerized, co-entangled actin-microtubule composites and systematically vary actin:tubulin molar ratios. We characterize the nonlinear *mesoscale* mechanics of composites by pulling optically-trapped microspheres 30 µm through the composites at a rate much faster than the system relaxation rates while measuring the local force induced in the composites by the strain. These measurements perturb the composites far from equilibrium, and are uniquely able to probe possible buckling, rupture, and rearrangement events,
as well as micro- and mesoscale spatial heterogeneities in the composites. We find composites with more microtubules than actin exhibit lower initial stiffness than actin-dominated composites and subsequent stress-stiffening as opposed to the softening displayed in actin-dominated composites. We also find that high concentrations of microtubules are needed to substantially increase composite resistive force and induce more pronounced heterogeneities in force response, which we show arises from the smaller mesh size of actin relative to microtubules. The force relaxation following the strain exhibits a unique two-phase power-law decay for all composites. The initial fast decay, arising from actin fluctuations, is increasingly suppressed as the relative concentration of microtubules increases. Conversely, the second phase slow decay, is robust to changes in network composition and is indicative of entangled filaments diffusing out of non-classical entanglement tubes.

Methods

Actin-microtubule composites are formed with rabbit skeletal actin, porcine brain tubulin, and rhodamine-labeled tubulin purchased from Cytoskeleton (AKL99, T240, TL590M), and Alexa-488-labeled actin purchased from Thermofisher (A12373). To form composites, unlabeled actin monomers and tubulin dimers were suspended in an aqueous buffer containing 100 mM PIPES, 2 mM MgCl$_2$, 2 mM EGTA, 1 mM ATP, 1 mM GTP, and 5 μM Taxol to a final protein concentration of 11.3 μM (Figure 1A). This buffer was optimized to achieve co-polymerization and stabilization of actin and microtubules. To image composite architecture, 0.13 μM of pre-assembled Alexa-488-labeled actin filaments, at a 1:1 labeled:unlabeled monomer ratio, and 0.19 μM pre-assembled rhodamine-labeled microtubules, at a 1:10 labeling ratio, were also added to the solution as tracer filaments (Figures 1A,B and 2). For microrheology measurements (Figure
a sparse amount of 4.5 μm microspheres (probes, Polysciences Inc.), coated with Alexa-488 BSA (Invitrogen), were added. The final mixture containing unlabeled actin monomers, unlabeled tubulin dimers, pre-polymerized Alexa-488 actin filaments, rhodamine-labeled microtubules, and microspheres was pipetted into a sample chamber. Sample chambers were made from a glass slide and coverslip, separated by ~100 μm with double-sided tape to accommodate ~20 μL, and sealed with epoxy. Co-polymerization of both proteins was achieved by incubating the sample for 1 hour at 37 °C prior to measurement. This sample preparation method results in the formation of a well-integrated and stable co-entangled composite (Figure 1B and Figure 2). The molar fraction of tubulin in composites, $\phi_r = \frac{[\text{tubulin}]}{([\text{actin}]+[\text{tubulin}])}$ where $[\text{actin}]$ and $[\text{tubulin}]$ are the molar concentrations of each protein, was varied from 0 to 1 while total protein concentration (labeled and unlabeled actin and microtubules) was held fixed at 11.6 μM. The lengths of actin and microtubules were 8.7 ± 2.8 μm and 17.8 ± 9.7 μm, respectively, independent of $\phi_r$ (Figure 3).

The optical trap used in microrheology measurements was formed using an IX71 fluorescence microscope (Olympus) fitted with a 1064 nm Nd:YAG fiber laser (Manlight) focused with a 60x 1.4 NA objective (Olympus). A position-sensing detector (Pacific Silicon Sensor) measured the deflection of the trapping laser. Trapping laser deflection is proportional to the force acting on the trapped probe according to Hooke’s law, $F=k_i x$, where $F$ is force, $k_i$ is trap stiffness coefficient, and $x$ is strain distance. Trap stiffness, $k_i$, was calibrated using the Stokes drag method (32-34). During force measurements, a probe embedded in the composite was trapped and moved a distance $x = 30$ μm at a constant speed of 20 μm/s relative to the sample chamber using a nanopositioning piezoelectric stage (Mad City Labs) (Figure 1C,D). Laser deflection and stage position were recorded at a rate of 20 kHz using custom-written
Labview code. Post-measurement data analysis was done using custom-written MATLAB software. Displayed force curves in Figures 4 and 6 are averages over an ensemble of 25 different trials, $i$, with each $i^{th}$ trial located in a different region of the network separated by distances greater than 100 µm.

**FIGURE 1 Schematic of experimental approach.** (A) Cartoon of molecular components used in actin-microtubule composites. The tubulin molar fraction, $\phi_T = \frac{[\text{tubulin}]}{([\text{actin}]+[\text{tubulin}])}$, is varied from 0 to 1 with total protein concentration fixed at 11.6 µM. (B) To display network architecture, a small fraction of actin and microtubules are labeled with Alexa-488 (green) and rhodamine (red), respectively. The image shown is a two-color laser scanning confocal image of a $\phi_T = 0.5$ composite. (C) For microrheology measurements an optically-trapped microsphere probe embedded in the composite is displaced a distance $x = 30$ µm (Strain, magenta) at a speed of 20 µm/s while the resistive force, $F_r(x,t)$, that the composite exerts on the probe is measured (Force, blue). (D) Sample microrheology data showing the resistive force (blue) and probe position (magenta) during (Strain) and following (Relaxation) probe displacement. Data shown is for the $\phi_T = 0.5$ composite.
FIGURE 2 Co-polymerizing actin and microtubules creates isotropic, co-entangled networks. Two-color laser scanning confocal imaging of actin networks ($\phi_T = 0$), microtubule networks ($\phi_T = 1$), and an equimolar composite of the two ($\phi_T = 0.5$), collected and processed as described in Methods. Total protein concentration is 11.6 µM in all images, with 0.13 µM of actin and microtubules labeled with Alexa-488 (green) and rhodamine (red), respectively. (A) Single frame 512x512 images from time-series collected at 30 fps, with scale bars of 50 µm. (B) Standard deviation of time-series comprised of 5400 frames, which shows the extent to which filaments are fluctuating. (C) Single 64x64 pixel images from 64-voxel z-stacks, with scale bars of 10 µm. (D) Binary skeletonization of the standard deviation of z-stacks to show network connectivity and architecture.
FIGURE 3 Measured actin and microtubule contour lengths within composites. Images were taken on the surface of the sample chamber and only filaments that were adhered to the surface (visibly immobile) were measured (as shown in inset). Fluorescent labeling and image acquisition methods are the same as those used in Figure 2. A total of 350 microtubules and actin filaments in each composite were measured using Fiji (as depicted in the inset with the yellow (actin) and blue (microtubule) segmented lines). Measured lengths of microtubules and actin are 18.8 +/- 9.7 µm and 8.7 +/- 2.8 µm, respectively. The distribution shown is for a φr = 0.5 composite, but similar length distributions were measured for all composites.

To visualize network architecture and mobility (Figure 2) and measure filament lengths (Figure 3), we used a Nikon A1R laser scanning confocal microscope with 60x objective to collect 2D images, 3D images, and 2D time-series of composites. Post-imaging analysis was carried out using ImageJ/Fiji. To characterize filament mobility within composites, 512x512 pixel time-series, with pixel size of 0.41 µm, were recorded at 30 fps for 3 minutes (Figure 2A,B). We then computed the standard deviation of each pixel of the time-series over time to
obtain a single 512x512 image that shows filament fluctuations (Figure 2B). To characterize network architecture, 3D images were constructed by collecting 64-voxel z-stacks with a voxel size of 0.41 µm to create a (26 µm)$^3$ cube (Figure 2C,D). We then computed the standard deviation of each voxel over z, resulting in a single 64x64 pixel image. We converted this image to binary which we then skeletonized to display network connectivity and structure (Figure 2D).

**Results and Discussion**

Though actin and microtubules coexist within cells, the standard in vitro polymerization and network formation conditions for the two proteins are incompatible. Of the few previous studies that have investigated actin-microtubule networks, all of them have used pre-polymerized and stabilized microtubules before adding to actin monomers to polymerize the final network (3, 13). This technique can lead to flow alignment and rupturing of microtubules upon pipetting into actin monomer solutions and/or the experimental sample chamber. Instead, we sought to assemble unperturbed co-polymerized networks of actin and microtubules entangled with one another within an experimental sample chamber. To do so, we designed hybrid buffers and polymerization methods detailed in Methods. As shown in Figure 2, our methods result in isotropic, well-integrated networks of sterically interacting, co-entangled actin and microtubules.
FIGURE 4 Mesoscale force response of actin-microtubule composites display a $\phi_T$-dependent crossover from stress-softening to stress-stiffening. (A) Ensemble-averaged resistive force ($<F_i(x)>$) exerted on moving probe by actin-microtubule composites of varying tubulin molar fraction $\phi_T$ (listed in legend). Data shown is an average over 25 individual, $i$, measurements. (B) Differential modulus $K(x)$ of composites, calculated as the derivative of the average force with respect to strain distance $x$ ($K(x) = d<F_i(x)>/dx$). (C) Differential modulus values averaged over strain distances of $x = 1 \mu$m (black) and $x = 10 \mu$m (grey), and normalized by the corresponding value at $x = 0$ ($K_0$) for each composite ($K_{avg}/K_0$). Strain distances averaged over are shown as dotted vertical lines in (A). As shown, composites transition from stress-softening ($K_{avg}/K_0 < 1$) to stress-stiffening ($K_{avg}/K_0 > 1$) when the tubulin molar fraction $\phi_T$ exceeds 0.5.

We use active microrheology to measure the local force response of actin-microtubule composites with varying tubulin molar fractions, $\phi_T$, subject to nonlinear strains. The data presented in Figure 4 shows the ensemble-averaged force, $<F_i(x)>$, exerted on optically trapped
microspheres as they are pulled a distance $x$ (30 µm) through each composite. As shown, introducing higher fractions of tubulin to composites increases resistive force (Figure 4A) as well as stiffness (Figure 4B,C). The differential modulus, $K(x) = d\langle F(x) \rangle/dx$, quantifies the degree of network stiffness, where increasing and decreasing $K(x)$ values during strain signify stress stiffening and softening, respectively. As shown in Figure 4B, composites comprised of mostly actin (tubulin fraction $\phi_T \leq 0.5$) are initially relatively stiff but quickly soften. However, beyond $\phi_T = 0.5$, composites undergo a stark transition, displaying an initially soft/viscous force response followed quickly by stress stiffening (Figure 4B). We can quantify the degree of stiffening/softening during the strain by averaging $K(x)$ over different strain distances $x$, which we denote as $K_{avg}$, and normalizing $K_{avg}$ by the initial ($x = 0$) measured value, $K_0$ (Figure 4C).

For strains up to $x = 1$ µm, the average differential modulus increases by an order of magnitude from its initial value for composites with mostly microtubules ($\phi_T \geq 0.7$), while it drops by an order of magnitude for $\phi_T \leq 0.5$ composites. As the strain continues (i.e. $x$ increases) all composites exhibit increased softening as the force response approaches a viscous steady-state regime, as indicated by the measured forces approaching $x$-independent plateaus (Figure 4A) and the reduced $K_{avg}/K_0$ values for $x = 10$ µm compared to those for $x = 1$ µm (Figure 4C).

These results are qualitatively in line with previous bulk rheology results for crosslinked actin networks doped with microtubules. These studies found that the addition of microtubules led actin networks to stress-stiffen rather than soften, due to microtubules suppressing actin bending modes (7, 19, 35). However, even for weakly-crosslinked actin networks, these studies found that microtubule fractions as low as $\phi_T \approx 0.3$ induced stiffening (19), as opposed to $\phi_T > 0.5$ in our experiments. Because crosslinking in actin networks can suppress bending modes and lead to stress stiffening (36), this difference likely arises from the lack of crosslinking in our
composites. Further, it has been suggested that microtubules are able to propagate mechanical loads long distances across the cytoskeleton, enhancing the large-scale elastic response of otherwise flexible networks (29). Thus, the differences between our results and those of macrorheology may also be due to the fact that actin-microtubule composites can exhibit lengthscale-dependent nonlinear stress response, such as softening at the micro- and mesoscales but stiffening on a macroscopic scale. In fact, previous studies have shown that even entangled networks of actin alone can display scale-dependent nonlinear elasticity with stress-stiffening at the microscale and stress-softening in bulk (37).

Increasing the fraction of tubulin increases the average resistive force during strain as well as the heterogeneities in force response. Microscale heterogeneities are shown in the individual force curves by “bumpiness” or increased noise. Large-scale heterogeneities can be seen by comparing the force curves for all $i$ trials measured in different regions of the sample (separated by $>100 \, \mu m$). Figure 5A, which shows the individual force curves, $F_i(x)$, for all $i$ measurements for actin networks ($\phi_T = 0$), microtubule networks ($\phi_T = 1$), and $\phi_T = 0.5$ composites, clearly shows that increasing the fraction of microtubules increases heterogeneity at both scales.
FIGURE 5 Microtubules increase average resistive force and heterogeneity of force response during strain. (A) Force curves for all 25 measurements, $i$, of composites with tubulin molar fractions of $\phi_T = 0$ (red), 0.5 (green) and 1 (purple). Distribution of force values during strain (i.e. noise in force curves) as well as trial-to-trial variability are much higher for 100% microtubules compared to composites. (B) Standard deviation of the force values for each trial, $\Delta F_i$, shows increasing microscale heterogeneity in the force response during strain with more microtubules. (C) Average force during strain, $\langle F_{i\text{-avg}} \rangle$, for each composite (black), as well as the corresponding percent range in strain-averaged force, $\Delta_{\text{avg}} = 100\% (F_{i\text{-avg,max}} - F_{i\text{-avg,min}})/(2 \langle F_{i\text{-avg}} \rangle)$ (grey) as a function of $\phi_T$ (bottom axis) and ratio of microtubule mesh size to actin mesh size $\frac{\xi_M}{\xi_A}$ (top axis). As shown, both the average and range in force values are surprisingly unaffected by the presence of microtubules until a tubulin fraction of 0.9. At these molar fractions ($\phi_T \leq 0.7$), the mesh size of actin remains smaller than microtubules ($\frac{\xi_M}{\xi_A} > 1$) despite the fact that the tubulin molarity is equal to or larger than that of actin for $\phi_T \geq 0.5$. The dotted vertical line shows when the mesh sizes for both filaments are equal ($\frac{\xi_M}{\xi_A} = 1$). (D) Illustration of equimolar actin-microtubule composite ($\phi_T = 0.5$). As depicted, the actin mesh size is $\sim 2\times$ smaller than the microtubule mesh, with $\xi_A = 0.6 \mu m$ and $\xi_M = 1.1 \mu m$. 
To quantify the microscale heterogeneity (i.e. the noisiness in force curves) and its dependence on tubulin fraction, we calculate the standard deviation of $F_i(x)$ values during the strain for each $i^{th}$ trial, which we denote as $\Delta_xF_i$. As shown in Figure 5B, $\Delta_xF_i$ remains relatively small and unchanged until a tubulin fraction of $\phi_T \approx 0.7$ after which the standard deviation values significantly increase. The large peaks and dips in force curves responsible for the increased $\Delta_xF_i$ in microtubule-dominated composites are indicative of microtubule buckling events. Previous studies have shown microtubules buckle under large compressive loads (22, 23), except when they are integrated within the cytoskeletal matrix (20, 24, 29, 38). In particular, by coupling with the surrounding elasticity of the actin network, the load bearing capacity of microtubules increases, suppressing microtubule buckling (6, 24, 29). Thus, we interpret the displayed reduced standard deviation and elimination of large dips in the force during strain for $\phi_T < 0.7$ composites as due to the elastic actin network providing reinforcement to microtubules against buckling.

To evaluate the large-scale heterogeneities, we average each $F_i(x)$ over the strain $x$, which we signify as $F_{i-\text{avg}}$, and calculate the ensemble-averaged $F_{i-\text{avg}}, \langle F_{i-\text{avg}} \rangle$, as well as the corresponding percent range, $\Delta_{\text{avg}} = 100\%\left(F_{i-\text{avg},\text{max}} - F_{i-\text{avg},\text{min}}\right)/(2\langle F_{i-\text{avg}} \rangle)$. As shown in Figure 5C we find that the average force, $\langle F_{i-\text{avg}} \rangle$ is ~3x larger for microtubule networks than that for actin networks and the corresponding percent range ($\Delta_{\text{avg}}$) is doubled. Surprisingly, the resistive force and heterogeneities do not increase smoothly as $\phi_T$ increases. Instead they remain relatively unchanged until the tubulin fraction is larger than 0.7. To understand these results, we calculate the predicted mesh size for single-species actin and microtubule networks at each concentration present in composites. Interestingly, when actin and tubulin are at equal molar concentrations (i.e. $\phi_T = 0.5$) the actin mesh size is ~2x smaller than that for the microtubules ($\xi_M/\xi_A \approx 2$), as depicted in Figure 5D. As displayed in the top axis of Figure 4C, $\xi_M/\xi_A$ remains larger than 1 up
to $\phi_T \approx 0.8$, with the actin mesh size ranging from ~6 to 1.25 smaller than that of microtubules. Only at tubulin molar fractions of 0.9 and 1 do we see a marked increase in average force and range in force as the tubulin mesh size becomes smaller than that for actin ($\xi_M/\xi_A < 1$). Thus, up to $\phi_T \approx 0.8$ a tighter, more entangled actin network pervades the system and suppresses the heterogeneities caused by larger, more rigid microtubules. In other words, the effective mesh formed in the co-entangled composite is dominated by actin rather than microtubules until tubulin comprises ~80% of the proteins present in the composite (Figure 5C,D).

We also characterize the relaxation of induced force following strain for all composites. As shown in Figure 5A, the force relaxations for all composites display two phases of power-law relaxation in time ($\langle F_i \rangle \sim t^{-\alpha}$), an initial fast decay followed by a slow decay that begins at ~0.6 s. Scaling exponents for the fast decay decrease roughly linearly, from $\alpha_1 \approx 1.7$ to 0.5, with increasing fractions of tubulin, and essentially disappear for microtubule networks ($\phi_T = 1$) (Figure 6B,C). Conversely, the exponents for the slow decay are largely insensitive to composite composition, displaying an average exponent of $\alpha_2 \approx 0.4$ independent of tubulin fraction (Figure 6C).
FIGURE 6 Force relaxation of actin-microtubule composites following strain exhibits two-phase power law decay. (A) Relaxation of ensemble-averaged induced force following strain as a function of time for composites of varying tubulin fractions $\phi_T$. Color scheme is as in Figures 3 and 4. Induced force relaxes via two distinct power law decays: an initial fast decay, $\langle F_i \rangle \sim t^{-\alpha_1}$, followed by a slow decay, $\langle F_i \rangle \sim t^{-\alpha_2}$ that begins at $\sim$0.6 s (vertical dotted line). The dashed lines are fits of the data to power laws with exponents $\alpha_1$ (fast decay) and $\alpha_2$ (slow decay). (B) Zoomed-in fast decay curves with black lines corresponding to scaling exponents of 1.7 and 0.4. (C) Scaling exponents measured for fast ($\alpha_1$) and slow ($\alpha_2$) decays. The fast scaling exponent decreases proportionally with decreasing actin concentration (i.e. increasing $\phi_T$), and becomes indistinguishable from the slow scaling when $\phi_T = 1$. Conversely, slow relaxation is independent of composite composition, with an average exponent of $\alpha_2 \approx 0.4$.

These unique relaxation characteristics are in opposition to expected dynamics for entangled polymer systems. The force relaxation in entangled polymer systems is typically described by a sum of exponential decays due to distinct relaxation mechanisms with well-
separated corresponding timescales (15, 39). The slowest of these relaxation mechanisms is predicted to be that of the entangled polymers diffusing out of deformed entanglement tubes (i.e. disengagement). However, previous studies have shown that entangled networks of both actin and DNA can exhibit power-law force relaxation when subject to nonlinear strains (40, 41). In these studies, force relaxation of entangled actin transitioned from exponential to power-law when the strain rate exceeded a critical value (~2x slower than the rate used here). The power-law exponent of ~0.5 could be explained as arising from non-classical disengagement of entangled filaments from strain-dilated entanglement tubes (40, 42, 43). Namely, the nonlinear strain dilates the entanglement tubes, so following strain, tubes shrink back to their original size on the same timescale that filaments reptate out of the deformed tubes. The result is that, as each filament attempts to reptate out of its tube, the characteristic disengagement time grows longer, thereby producing power-law decay. Our second relaxation phase, with scaling exponent close to the previous finding of 0.5 for actin networks, can likewise be explained by this non-classical disengagement mechanism. Further, the insensitivity of $\alpha_2$ to $\phi_T$ indicates that entanglements rather than filament flexibility drive the relaxation. As the overall concentration of all composites is the same, the density of entanglements is also similar. Thus, the disengagement timescales and mechanisms, which are determined largely by entanglement density, remain constant as well.

Conversely, the fast relaxation has a clear dependence on composite composition. As $\alpha_1$ scales roughly linearly with the fraction of actin in composites, this fast mode clearly arises from actin filaments in the network. Entangled actin networks have been shown to relax via several mechanisms that are faster than disengagement, including relaxation of bending fluctuations (15, 17). These mechanisms arise from the semiflexible nature of actin, in contrast to rigid microtubules that are highly resistive to bending and Brownian fluctuations. As described above,
it has been suggested that microtubules can reduce non-affine bending modes of actin, suppressing fluctuations and non-affine deformations during strain (7). This effect is apparent in the ability of microtubules to promote stress-stiffening in composites (Figure 4). As such, our measured fast relaxation mode can be understood as arising from actin bending fluctuations that microtubules increasingly suppress as $\phi_T$ increases.

**Conclusion**

We use optical tweezers microrheology to characterize the mesoscale force response of co-entangled composites of actin and microtubules, which we create by *in situ* co-polymerization of actin monomers and tubulin dimers using custom-designed hybrid conditions. By systematically varying the relative concentrations of actin and microtubules (quantified by the molar fraction of tubulin $\phi_T$), we show that composites exhibit a wide range of mechanical properties that can be tuned by $\phi_T$. Our collective results demonstrate that microtubules suppress actin bending fluctuations, enabling composites to stiffen in response to strain and relax the induced force more slowly; while actin supports microtubules against buckling by providing a soft semiflexible mesh that permeates the larger microtubule mesh and partially absorbs induced stress. These results demonstrate the synergistic ways in which thin, semiflexible actin filaments and thick, rigid microtubules can sterically interact to enable the wide range of mechanical processes and properties the cytoskeleton exhibits. Our quantitative analysis of the dependence of mechanical properties on composite composition also provides important new insights into how composite materials can be tuned to display user-defined mechanics, and how cells can likewise tune their mechanical properties by using varying cytoskeletal filament networks. This study provides the groundwork for future investigations on in vitro cytoskeletal composites that
include crosslinking proteins, intermediate filaments, and motor proteins. These future studies will expand the phase space of mechanical properties that these bio-inspired composite materials can exhibit, and shed light onto the role that each of these additional components play in the mechanics of the cytoskeleton.

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