Control of non-linear elasticity in F-actin networks with microtubules

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We measure the elastic properties of composite cytoskeletal networks consisting of cross-linked actin filaments and microtubules. We show that even a small concentration of microtubules leads to dramatic and qualitative changes in the non-linear elastic properties of the actin filament networks. Specifically, we find that microtubules promote non-linear stiffening of F-actin networks because they are much stiffer than actin filaments and therefore suppress non-uniform strain. This finding may be relevant for interpretation of the mechanical behavior of cells, while also suggesting a new way to reinforce the non-linear elasticity of semiflexible polymer materials.

Introduction

The mechanics of eukaryotic cells are largely governed by biopolymer networks which make up the *cytoskeleton* and consist of semiflexible filaments interconnected by linker proteins. The underlying properties of such networks can be investigated using model networks of purified biopolymers; these exhibit novel material properties that are in stark contrast to those of synthetic polymers.[1–3] One of the most interesting properties of such biopolymer networks is their non-linear response to strain. For example, model networks consisting of pure filamentous (F-)actin and cross-linking proteins stiffen non-linearly due to entropic stretching of the filaments[4–6] or the cross-linker proteins.[7,8] Physiologically, this non-linear stiffening behavior may serve as a mechanism to protect cells from excessive deformation, or may enable them to tune their elasticity by generating tension with motor proteins.[9,10] Both experimental and theoretical studies of the material properties of cytoskeletal protein systems have largely focused on networks consisting of a single filament type, together with cross-linking or force-generating proteins.[1–3,5,10–15] These networks exhibit a rich mechanical behavior that varies with small changes in network concentration, connectivity and stiffness of the individual filaments.[5,16–19] However, the cytoskeleton of living cells is a *composite* material, consisting of three different filamentous components with widely different mechanical properties: F-actin, with a persistence length $l_P \approx 15$ μm, softer intermediate filaments (IFs) with $l_P \approx 1$ μm, and stiffer microtubules (MTs) with $l_P \approx 6$ mm.[20,21] It is known that structural interactions as well as crosstalk by biochemical signaling between these cytoskeletal filaments are involved in dynamic cellular processes such as cell motility and cytokinesis.[22] To date, however, there have been few *in vitro* studies probing the composite nature of the cytoskeleton and basic questions remain concerning the mechanical properties of such systems.[23–25]

The structural interactions between cytoskeletal filaments can be classified as non-specific interactions, such as steric entanglements, and specific interactions which are mediated by cross-linker proteins such as plectin.[26] Recent studies focusing on the non-specific interactions have shown that the actin cortex can reinforce microtubules against buckling[27,28] and that the addition of microtubules to F-actin networks confers a local compressibility.[25] An increased degree of control over the large-scale network mechanics is also expected in composite networks of different filament types with widely differing mechanics. It is commonly known in engineering that addition of stiff objects such as nanoparticle fillers in soft fiber networks reinforces the linear elasticity of the system.[29] Stiff rods such as carbon nanotubes have been shown to be especially effective due to their small size and perfect graphite structure.[30] Double-network gels have also been demonstrated to have both high mechanical strength and toughness.[31] However, how stiff fibers influence the non-linear elasticity of soft polymer matrices remains elusive. Such behavior is particularly important for cells, where stiff microtubules are embedded in networks of much less stiff F-actin or intermediate filaments.

Here, we create a model system incorporating F-actin and microtubules that demonstrates how even a small concentration of stiff microtubules leads to remarkable changes in the non-linear viscoelastic properties of F-actin networks. The microtubules provide a mechanism to control the non-linear stiffening response of actin, which can be understood theoretically in terms of the inhomogeneity of the strain field of the F-actin gel.[16,19,32] Pure F-actin gels stiffen non-linearly for uniform strains; such stiffening is not expected for non-uniform strains, for which filament bending predominates.[16] We conjecture that stiff microtubules can suppress non-uniform strains in soft F-actin gels and promote non-linear stiffening. We test this hypothesis by creating cross-linked F-actin gels with three different actin cross-linking proteins, to which we add varying amounts of microtubules. We show that the inclusion of microtubules can indeed result in strain stiffening of the composite material, even in the absence of any specific linkage of the microtubules to the surrounding F-actin gel. These observations can have important implications for the intracellular cytoskeleton, in which a dilute network of microtubules coexists with a denser meshwork of more flexible actin and intermediate filaments. Moreover, our work shows how the inclusion of rigid polymers can lead to

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a striking degree of direct control over the non-linear elastic properties of a network or softer polymers.

Results and discussion

Theoretical and numerical studies have shown that the strain response and deformation of semiflexible biopolymer networks depend sensitively on the molar ratio of cross-links to actin, R, and on the actin filament length, L.[16,17,19,33] At large R or for long L, the deformation is more affine, or uniform, and the response is dominated by stretching and compression of individual filaments, leading to strain stiffening.[3,5,33] Smaller R or shorter L leads to a smaller number of cross-links per filament, which increases the inhomogeneous strain and suppresses the stiffening. The deformation is thus non-affine and the mechanical response is expected to be dominated by bending of individual filaments [schematic in Fig. 1(a)].[19,34] We expect strain inhomogeneities, due to local rearrangements, to be suppressed upon addition of more rigid polymers [Fig. 1(b)]; stretching and compression of semiflexible filaments should then dominate bending and the network should strain-stiffen. As network connectivity decreases, a larger amount of rigid polymers will be required to suppress the inhomogeneities in the strain field.

Experimentally, we test this hypothesis by comparing the elastic properties of F-actin networks to those of composite F-actin–MT networks. We use three different actin cross-linking proteins, the rigid and irreversible cross-linker scruin,[5,35,36] biotinylated actin and NeutrAvidin, which provides an irreversible, but inert cross-link, and the flexible cross-linker filamin A (FLNa).[9,37] The degree of cross-linking is varied by altering the molar ratio of cross-linking protein to actin, $R_X = c_X/c_A$, where c_X is the concentration of the cross-linker (scruin, c_S, biotinylated actin, c_B, or filamin, c_F) and c_A is the concentration of actin. The molar ratio of NeutrAvidin to biotinylated actin is fixed at 1. The average actin-filament contour length, L, is regulated through addition of the actin severing and capping protein, gelsolin:[14] $L = (370R_G)^{-1}$, where R_G is the molar ratio of gelsolin to actin monomers.

We use confocal fluorescence microscopy to examine the morphology of the composite F-actin and MT systems and verify

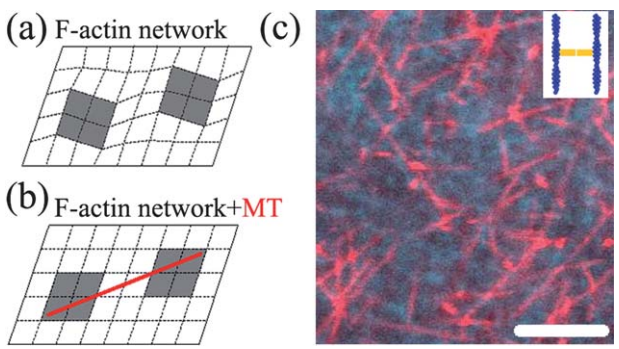

Fig. 1 Schematic of a protein composite network formed by cross-linked F-actin (gray) with interspersed MTs (red). Under shear, sparsely cross-linked F-actin networks without MTs deform non-affinely (a), while addition of MTs suppresses strain inhomogeneities making the deformation more affine (b). (c) Confocal microscope image of a fluorescently labeled composite network of MTs (red) and actin filaments (blue) cross-linked with scruin (yellow). $c_T = 5$ μM and $c_A = 12$ μM, and $R_S = 0.03$. Scale bar = 5 μm.

that the composite networks are homogeneous. We find that the MTs are randomly dispersed in the homogeneous F-actin networks, as illustrated for a network cross-linked by the protein scruin in Fig. 1(c). We observe neither bundling of actin filaments or MTs, nor phase separation of the components, although the MTs reptate freely in the actin matrix. Thus the elastic response reflects that of the composite architecture of an F-actin network interspersed with MTs.

All of the F-actin networks exhibit solid-like behavior with a roughly frequency-independent elastic modulus, $G'(\omega)$. F-actin–scruin networks with long L and low $R_S = 0.03$, close to the expected affine-to-non-affine transition,[5,16,19] exhibit strain weakening, as shown by open circles in Fig. 2(a). However, incorporating MTs at $c_T = 1$ μM leads to stress stiffening while it shows no significant influence on the linear elasticity, as shown by the solid circles in Fig. 2(a). When R_S is further lowered to 0.02 (open squares), this c_T is insufficient to modify the non-linear behavior (open diamonds); instead a higher MT concentration, $c_T = 5$ μM (solid squares), is required to suppress strain weakening. This supports our hypothesis: network connectivity decreases with decreasing R_S, so more MTs must be added to suppress local strain inhomogeneities. As a control, we test networks of MTs alone; they invariably fluidize at high strains, as shown by the gray lines in Fig. 2(a). This is in accordance with earlier measurements on pure microtubule solutions.[38,39] Hence, the strain-stiffening response of composite F-actin–MT networks results from a synergistic combination of components.

Even more pronounced behavior is observed in networks cross-linked with the highly compliant cross-linking protein, filamin A (FLNa).[7] Again, when MTs are incorporated (1 μM), even very sparsely cross-linked networks ($R_F = 0.002$) with $L = 2$ μm ($R_G = 1/740$) exhibit pronounced strain stiffening (solid triangles); by contrast, pure F-actin–FLNa networks exhibit strain weakening (open triangles).

To characterize the non-linear elastic response more exactly, we analyze the full strain response to the large amplitude oscillatory stress (LAOS);[40,41] this is a more sensitive means of detecting non-linearity and strain stiffening than measures of G' and G'', which use only the first harmonic of the measured response to an applied strain.[40] We use NeutrAvidin to irreversibly cross-link biotinylated F-actin networks. We determine the strain-stiffening index, $S(\gamma_0) = G_L/G_M$, where $G_L(\gamma_0) = \frac{\sigma}{\gamma}|_{\gamma=\gamma_0}$ and $G_M(\gamma_0) = (d\sigma/d\gamma)|_{\gamma=0}$, which provides a measure of the degree of non-linearity.[41] For networks with low biotinylated actin densities, $R_B = 0.03$, Lissajous plots obtained for $\omega = 0.6$ rad s^{-1} exhibit little non-linearity, as shown by the dashed line in Fig. 2(b); however, the networks do exhibit a slight strain weakening above $\gamma \approx 50\%$, and indicated by values of $S \leq 1$, as shown by open symbols in the inset of Fig. 2(b). By contrast, a strain-stiffening response is observed when MTs are added to the F-actin network, as clearly shown by the Lissajous plot in Fig. 2(b) and by $S \geq 1$, shown by solid symbols in the inset of Fig. 2(b). This demonstrates that embedded MTs reverse the strain-weakening response of F-actin networks.

To check the robustness of the MT-induced change in non-linear elasticity, we also perform alternative measurements using a differential measurement.[5,42] Previous work has shown that

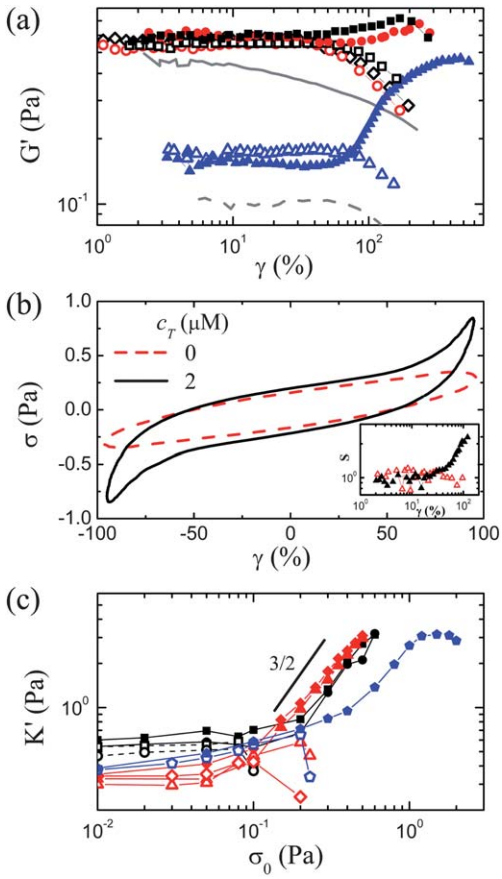

Fig. 2 (a) Addition of MTs at $c_T = 1$ μM leads to a strain-stiffening response for F-actin networks with scruin cross-link density close to the A–NA transition ($R_S = 0.03$, circles). For lower cross-link density ($R_S = 0.02$), $c_T = 1$ μM is insufficient (open diamonds); instead $c_T = 5$ μM (solid squares) is needed to reverse the strain weakening (open squares). F-actin–FLNa networks exhibit strain weakening at a ratio of $R_F = 0.002$ (open triangles); addition of MTs at $c_T = 1$ μM reverses this (solid triangles). MT networks alone exhibit strain weakening ($c_T = 5$ μM, solid line, and $c_T = 1$ μM, dashed line). (b) The Lissajous figure of stress–strain cycles for biotinylated F-actin networks ($R_B = 0.03$) are elliptical in the absence of MTs (dashed line), but show clear strain stiffening (at $\gamma \geq 50\%$) upon addition of MTs (solid line). (Inset) The elastic strain-stiffening index, S, as a function of the strain, γ ($c_T = 0$ μM, open symbols, and $c_T = 2$ μM, solid symbols). (c) K' as a function of σ_0 for F-actin networks cross-linked with FLNa (pentagons), biotinylated F-actin networks cross-linked with NeutrAvidin (waiting time in the pre-stressed state = 3 s, triangles; 20 s, diamonds), and scruin ($R_S = 0.02$, squares, and $R_S = 0.03$, circles). In the absence of MTs, F-actin networks exhibit weakening (open symbols); by contrast, in the presence of MTs the networks exhibit stiffening, with a power-law dependence, $K' \approx \sigma_0^{3/2}$ (solid symbols).

non-linear measurements on actin solutions using LAOS or strain ramps can be highly sensitive to the strain rate used,[41,43] where flow can appear to suppress non-linearity at lower shear rates. By contrast, differential measurements have been shown to be insensitive to such effects for cross-linked systems,[42] such as those that we study here. We determine the complex differential modulus, K^*, at $\omega = 0.6$ rad s^{-1}. At low cross-link densities for all three actin cross-linking proteins, the differential elastic modulus decreases with increasing levels of stress, as shown by open symbols in Fig. 2(c): $R_B = 0.03$, triangles and diamonds; $R_F = 0.002$, pentagons, $R_S = 0.02$, squares, and $R_S = 0.03$, circles. However, when MTs are added, we observe stress stiffening for all of these networks. The rigid cross-linkers scruin and biotin/NeutrAvidin both lead to a power-law stiffening of the form $K' \sim \sigma_0^{3/2}$, as shown by the solid black and red symbols in Fig. 2(c). This behavior is consistent with the exponent predicted and experimentally observed for affinely deforming networks of semiflexible polymers, each having an entropic force-extension behavior $dF/dl \approx F^{3/2}$.[5,33] To confirm that these experiments are not affected by viscous flow introduced by a steady pre-stress in weakly cross-linked networks, we vary the waiting time in the pre-stressed state before applying small oscillations. At $R_B = 0.03$, the differential elastic modulus has no obvious change with various waiting times, as shown by solid diamonds (20 s) and triangles (3 s) in Fig. 2(c). This behavior validates the pre-stress approach for studying the non-linear response of even weakly cross-linked networks, consistent with prior studies.[42]

In addition to varying the cross-linking fraction R, the connectivity of F-actin networks can also be altered by the average filament length L, while maintaining a constant R: decreasing L should produce the same qualitative effect as decreasing R.[44] We thus expect that more MTs are needed to suppress local non-affinity of F-actin networks with shorter filaments. Densely cross-linked biotinylated F-actin networks ($R_B = 0.05$) composed of shortened actin filaments with $L = 7$ μm ($R_G = 1/2600$) exhibit stress weakening, as shown by the open squares in Fig. 3. However, upon incorporation of 2 μM MTs, this network exhibits stress stiffening, as shown by the solid squares in Fig. 3. For $L = 5$ μm ($R_G = 1/1850$), an increased c_T of 4 μM is needed to reverse the stress-weakening response, as shown by the solid circles in Fig. 3. This confirms our hypothesis that high concentrations of rigid polymers are necessary to suppress the local inhomogeneities of networks composed of shorter actin filaments.

To capture the R_S, L, and c_T dependencies of the non-linear response, we summarize all the data in an R_G–R_S state diagram (Fig. 4). In the absence of reinforcing MTs, scruin cross-linked F-actin networks stiffen at large R_S and long L, shown by the black squares in the area labeled "A". Networks in this regime deform affinely[18] and their non-linear response is consistent with predictions for an affine response.[5,33] Decreasing the number of

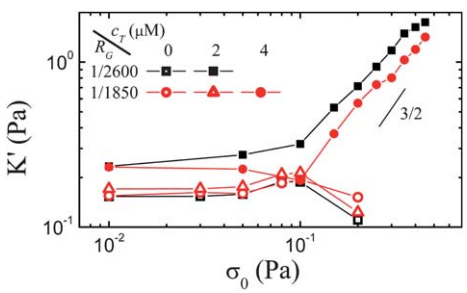

Fig. 3 NeutrAvidin cross-linked networks composed of filaments shortened by gelsolin exhibit stress weakening ($R_G = 1/2600$, open squares, and $R_G = 1/1850$, open circles). Adding MTs at $c_T = 2$ μM reverses this behavior and stress stiffening is observed for long L, $R_G = 1/2600$ (solid squares). For shorter L (larger R_G), addition of 2 μM does not reverse the stress-weakening response (open triangles), while 4 μM does (solid circles).

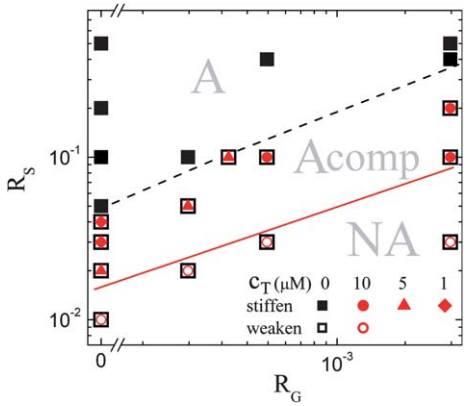

Fig. 4 State diagram summarizing dependence of the non-linear response of cross-linked F-actin–scruin networks on R_S, and R_G. In the absence of MTs, the dashed line separates the affine regime A, where networks exhibit strain stiffening, from the non-affine regime, NA, where networks exhibit strain weakening. Addition of MTs shifts the A/NA transition to the solid line; there is now a larger region marked "A comp", where MTs suppress non-affinity and networks thus exhibit strain stiffening. Close to the black line, $c_T = 1$ μM is sufficient (solid diamonds), while further away, $c_T = 5$ μM and 10 μM are required (solid triangles and circles).

cross-links per filament by either lowering R_S or increasing R_G results in strain weakening, as shown by the black open squares. In this regime, labeled "NA", network deformation is non-affine[18] and is expected to be dominated by filament bending.[16,17,19,32] The dashed line separates the affine and non-affine regimes in the absence of MTs. Upon addition of MTs, the transition between these two regimes shifts to lower values of R_S, as shown by the solid line. There is now a significantly larger region, labeled "A comp", where networks strain-stiffen, consistent with suppressing strain inhomogeneities in the composite network due to the stiff MTs. The threshold concentration of MTs required to achieve this effect depends on the distance from the boundary of the affine-to-non-affine transition. For the longest actin filaments, $c_T = 1$ μM is sufficient, while a ten-fold higher concentration is needed for very sparsely cross-linked F-actin networks or for networks with smaller L.

Experimental

We mix solutions of actin and cross-linking protein with 10× actin polymerization buffer (20 mM Tris–HCl, 20 mM $MgCl_2$, 1 M KCl, 2 mM dithiothreitol, 2 mM $CaCl_2$, and 5 mM adenosine 5′-triphosphate, pH 7.5) to initiate network formation and gently add pre-formed MTs to form the composite networks. The microtubules are pre-formed by mixing purified tubulin at a concentration of $c_T = 4$ mg mL^{-1} in 1 mM dithiothreitol, and tubulin polymerization buffer (80 mM piperazine-*N*,*N*′-bis[2-ethanesulfonic acid], 1 mM $MgCl_2$, and 1 mM ethylene glycol-bis[β-aminoethyl ether]-*N*,*N*,*N*′,*N*′-tetraacetic acid, pH 6.8) and 1 mM of non-hydrolyzable GTP analogue, guanosine-5′-[(α,β)-methyleno]triphosphate (GMPCPP), at 35 °C for two hours.

The composite networks are polymerized between the plates of a stress-controlled Bohlin CVOR rheometer for at least one hour at room temperature (25 °C) prior to mechanical testing. The complex shear modulus, $G^*(\omega)$, is obtained by applying a sinusoidal stress, $\sigma(\omega) = \sigma_0 \sin(\omega t)$, with variable amplitude σ_0 and frequency ω, and measuring the strain response, $\gamma(\omega) = \sigma(\omega)/G^*(\omega)$. At large strain amplitudes, the strain response to the imposed sinusoidal stress is non-sinusoidal. Thus, differential measurements represent a more accurate method to probe non-linearities.[5,42] A small amplitude oscillatory stress, $\delta\sigma(\omega) = |\delta\sigma|e^{i\omega t}$, is superposed on a steady pre-stress, σ_0, and the oscillatory strain response, $\delta\gamma(\omega) = |\delta\gamma|e^{i\omega t}$, is measured. In the linear regime, the tangent or differential modulus, $K^*(\omega,\sigma_0) = [\delta\sigma(\omega)/\delta\gamma(\omega)]|_{\sigma_0}$, is the same as the elastic modulus, $K' = G'$.

Fluorescent images of microtubule networks and F-actin networks are obtained on a laser-scanning confocal microscope (Zeiss LSM 510) using a 60×, NA = 1.2, water-immersion objective. Microtubules are polymerized from a mixture of tubulin and Alexa488-labeled tubulin at a 4 : 1 molar ratio, while fluorescently labeled F-actin networks are polymerized in the presence of 0.6 μM rhodamine phalloidin.

Conclusion

We find that a small concentration of microtubules can promote non-linear stiffening of F-actin networks. Importantly, this reinforcement does not depend on a direct elastic coupling of MTs to the surrounding network. This composite behavior is in contrast to most rubber-like materials composed of flexible polymers reinforced with rigid fibers such as carbon nanotubes,[30] yielding a larger linear elastic modulus and greater toughness. Such systems rely on a direct elastic coupling between fibers and the matrix: the surface of the fiber is treated in an oxidative process to improve the level of adhesion, and the strength of the bond between the matrix and the fiber is a controlling factor for the mechanical performance of these fiber-reinforced (FR) composites. Another example of an enhancement in mechanical strength is the so-called double-network (DN) gel, which consists of two cross-linked flexible polymer networks.[31] By contrast, we observe free movements of MTs in the presence of the cross-linked F-actin network, indicating that the MTs are not cross-linked to each other or bound to the F-actin matrix. Thus, the mechanism of reinforcement we observe is likely different from both FR and DN systems.

Our observations are consistent with the hypothesis that the high bending stiffness of the MTs suppresses bending of the surrounding actin network, thereby leading to an enhanced stretching response and consequent stiffening. It would be interesting in future to perform a confocal microscopy analysis of the network structure under shear, to directly verify this hypothesis.[18] Recent simulations have also shown some evidence for this synergy in the elastic properties of composites with dilute stiff fibers.[24] Those authors found that increased concentrations of stiff fibers led to both an enhancement of the stretching response relative to bending, as well as an increase in the linear shear modulus. Interestingly, we find that the reinforcement by MTs affects primarily the non-linear response, with only a small effect on the linear elasticity. Our non-linear results are in contrast with a recent report, in which the addition of F-actin reduced the resistance of a more flexible polymer network (neurofilaments) to large stress.[45] Since F-actin is substantially

more flexible than MTs, this suggests the importance of high fiber rigidity for the behavior we observe.

Our finding for reconstituted F-actin–MT networks is highly relevant for interpretation of the mechanical behavior of the intracellular cytoskeleton, in which a dilute network of MTs coexists with a denser F-actin or intermediate filament network. In the cell, the interactions are partly mediated by non-specific interactions, as in our model system, and partly by specific cross-linker proteins with binding domains for different filament types. For example, plectin which is a multidomain protein that binds intermediate filaments also contains actin or MT binding sites and is thus essential for maintaining cell integrity. It would be interesting to test how these specific interactions affect the mechanical properties of the cytoskeletal network *in vitro*.

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