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Stress-Dependent Elasticity of Composite Actin Networks as a Model for Cell Behavior

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Networks of filamentous actin cross-linked with the actin-binding protein filamin *A* exhibit remarkable strain stiffening leading to an increase in differential elastic modulus by several orders of magnitude over the linear value. The variation of the frequency dependence of the differential elastic and loss moduli as a function of prestress is consistent with that observed in living cells, suggesting that cell elasticity is always measured in the nonlinear regime, and that prestress is an essential control parameter.

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Mechanical force generation and transmission is essential in the proliferation, differentiation, division, and migration of living cells. The importance of such forces has inspired *in vivo* studies of the mechanical behavior of cells [1–4]. These have revealed an intriguing observation: a wide variety of cells exhibits properties well described by soft glassy rheology (SGR), a theory of linear viscoelasticity developed to characterize soft solids with glasslike behavior [3]. However, the rationale for the applicability of this theoretical approach and the origin of the variation of behavior within the SGR model is completely unknown. The complexity of *in vivo* studies of cells has inspired complementary *in vitro* studies of networks of filamentous actin (*F*-actin) [5–9]; it is a major constituent of the cellular cytoskeleton, which, to a large degree, determines the mechanical properties of cells. However, the rheological properties of *in vitro F*-actin networks are quite different from those of cells: the magnitude of the elastic modulus typically underestimates that measured in cells, often by several orders of magnitude. In addition, unlike the linear behavior assumed for cells [1–3,10], the viscoelasticity of *F*-actin networks is linear only for very small deformations; like most semiflexible biopolymers [11], it rapidly becomes strongly nonlinear with increasing deformation [5,9,12,13]. One possible cause for this discrepancy is the fact that, *in vivo*, *F*-actin is cross-linked with a variety of actin-binding proteins (ABP's), which are essential in determining the elastic properties of the cytoskeleton. However, *in vitro* experimental measurements of the elasticity of *F*-actin networks formed under physiological conditions and cross-linked with physiologically important ABP's are sorely lacking, and there has been no attempt to relate the behavior of such networks to the SGR behavior observed for cells.

In this Letter, we describe the properties of *F*-actin networks formed at physiological concentrations, and cross-linked with the physiologically relevant ABP, filamin *A* (FLNa). FLNa cross-links are highly compliant, and have little effect on the linear mechanical response of the network. However, we find dramatic nonlinearities in the mechanical properties of the networks under large applied stress. In the nonlinear regime, the elastic modulus increases by as much as a factor of 40 with very little change in applied strain. Because of this extreme nonlinearity, a more accurate determination of the properties is obtained by measuring the differential or tangential elastic modulus; this can increase by nearly a factor of 300 over its linear value. Moreover, the differential viscoelastic response of these networks as a function of applied prestress is similar to that observed in cells. These results demonstrate that the dynamical mechanical properties of a cell can be mimicked with these *in vitro* networks, and highlight the potential importance of nonlinear elastic response for cells.

FLNa is typically present in nonmuscle cells at a molar ratio to actin, R, of 1:50 to 1:100 [14]. It is a homodimer consisting of an actin-binding domain, 24 β-sheet repeats, and two unstructured sequences of 32 amino acids [14]. Atomic force measurements show that, at small forces, FLNa can be modeled as a wormlike chain with a persistence length of 20 nm [15]. At higher forces of 50–100 pN, the β-sheet repeat sequences reversibly unfold, doubling the contour length [15]. We use recombinant human FLNa purified from Sf9 cell lysates [16] and globular actin purified from rabbit skeletal muscle. To form the *in vitro* networks, we gently mix solutions of FLNa with 10x actin polymerization buffer (20 mM Tris-HCl, 20 mM $MgCl_2$, 1 M KCl, 2 mM DTT, 2 mM $CaCl_2$, 5 mM ATP), and then add *G*-actin. The solution is mixed gently and loaded within 10 sec into a stress-controlled rheometer (CVOR, Bohlin Instruments) with 40-mm-parallel-plate geometry and 140 μm plate separation; the total sample volume is 230 μL. We confirm the formation of homogeneous and isotropic network microstructure by utilizing multiparticle tracking. We use a solvent trap to minimize evaporation and confirm the results are independent of geometry.

We measure the elastic modulus, G', and the loss modulus, G'', of 12 μM *F*-actin as we vary R. To ensure linear response, we maintain the applied stress below 0.01 Pa. At

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the lowest FLNa concentration, $R = 1/2000$, G' and G'' have similar magnitudes and are weakly frequency dependent, as shown by the closed and open squares, respectively, in Fig. 1. At the lowest frequency probed, G' slightly dominates the response and is twofold larger than that of a pure F-actin solution [17]. Upon increasing R to $1/1000$, G' increases an order of magnitude to 1 Pa and becomes less frequency dependent, as shown by the solid triangles in Fig. 1. The magnitude of G' is remarkably insensitive to further increases in R, as shown in the inset to Fig. 1; this is in sharp contrast to F-actin networks formed with rigid cross-links, where G' increases significantly, varying as $G' \sim R^3$ [13]. Moreover, the dissipation is significant as G'' remains comparable to G' as the FLNa concentration is increased; this also contrasts sharply with rigidly cross-linked F-actin networks, where the relative magnitude of G'' to G' decreases with increased R [12].

To investigate the nonlinear mechanical response of these networks, we measure the elastic modulus at 0.5 Hz, $G'_{0.5}$, as a function of the maximum applied stress, σ. For $R = 1/2000$, there is no observable nonlinear behavior; G' is independent of σ up to 0.1 Pa and decreases for $\sigma > 0.1$ Pa, as shown by the closed squares in Fig. 2(a). This behavior is similar to that observed for entangled F-actin solutions.

Similar linear elastic behavior is observed for low σ upon increasing R to $1/1000$. However, in this case, $G'_{0.5}$ increases monotonically above a critical stress, $\sigma_c \approx 0.5$ Pa and critical strain, $\gamma_c \approx 15\%$; the network exhibits stress stiffening and the increase in $G'_{0.5}$ is nearly linear with stress. Ultimately the network ruptures and $G'_{0.5}$ decreases dramatically; this occurs at a stress, $\sigma_{\max} \approx 3$ Pa, and strain, $\gamma_{\max} \approx 50\%$. This transition between stress weakening and stress stiffening in cross-linked networks is consistent with previous results for cross-linked F-actin networks [8,13].

As R is increased further, we observe similar stress stiffening. For all R, σ_c, γ_c, and $\gamma_{\max}$ remain roughly constant, as indicated by the arrows in Figs. 2(a) and 2(b). However, $\sigma_{\max}$ increases dramatically, as indicated by the solid, up-pointing arrows in Fig. 2(a). For $R = 1/100$, $G'_{0.5}$ increases nearly linearly with applied stress from 0.5 Pa to 70 Pa resulting in a dramatic increase of $G'_{0.5}$ to 40 times its linear value. Moreover, this occurs with very little change in the strain, as shown by the solid circles in Fig. 2(b). The stress-stiffening behavior of these networks is roughly 20-fold larger than any previously reported [5,8,9]. Furthermore, in contrast to covalently cross-linked F-actin networks, σ_c and the maximum strain of the linear regime of these networks are both rather insensitive to changes in R [13]. Instead, the parameter that is most sensitive to variations in R is $\sigma_{\max}$; these networks can withstand stresses 10–100 times larger than G'. Because the mechanical response of these networks is differs from those of rigidly cross-linked F-actin networks, we hypothesize that the FLNa cross-links significantly contribute to the network properties.

To directly compare the behavior of these networks with that of cells, we replicate physiological conditions of F-actin in the cortical cytoskeleton. We use gelsolin to shorten the filaments to 1 μm, use physiological concentrations of F-actin (48 μM) and ratios of FLNa ($R = 1/100$) [16]. For these networks, G' is 0.1 Pa; this underestimates the elasticity of cells by 10^3–10^4. Since G' is rather insensitive to variations in R or c_A, we instead hypothesize that the applied stress is the appropriate parameter to account for the large discrepancy between *in vivo* and *in vitro* measurements.

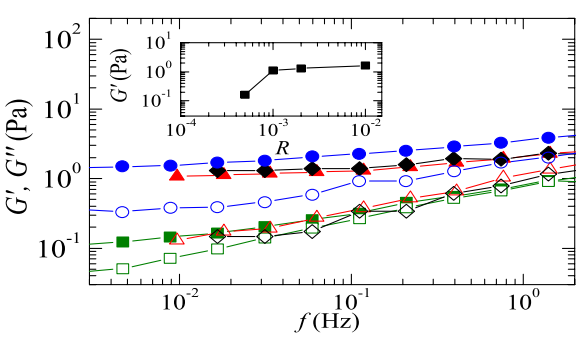

FIG. 1 (color online). The frequency dependence of G' (closed symbols) and G'' (open symbols) for 12 μM-F-actin (unregulated length) networks cross-linked with FLNa where R is varied from $1/2000$ (squares), $1/1000$ (triangles), $1/500$ (diamonds), and $1/100$ (circles). Inset: G' at 0.01 Hz as a function of R.

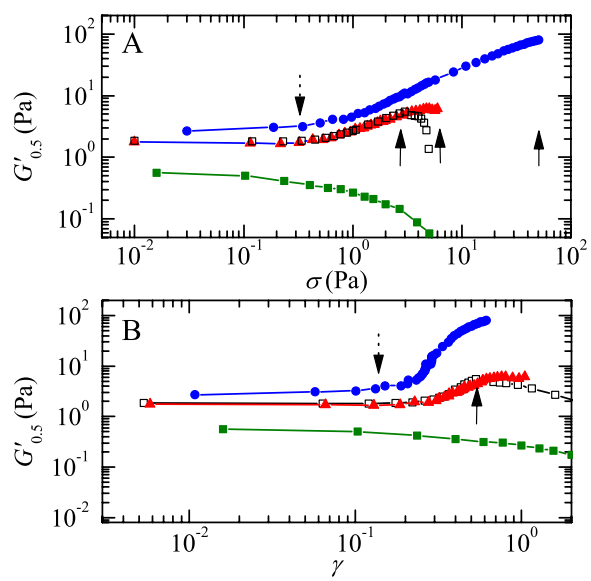

FIG. 2 (color online). (a) G' as a function of σ_0 at 0.5 Hz for 12 μM F-actin (unregulated length) cross-linked with FLNa with $R = 1/2000$ (closed squares), $1/1000$ (open squares), $1/500$ (closed triangles), and $1/100$ (closed circles). Dashed arrow indicates σ_c. Solid arrows indicate $\sigma_{\max}$. (b) G' as a function of strain, γ. Dashed arrow indicates γ_c. Solid arrow indicates $\gamma_{\max}$.

To more precisely determine the elasticity given the near divergence with strain, we use a differential measurement; we superpose a small amplitude oscillatory stress, $\delta\sigma(\omega) \sim |\delta\sigma|e^{i\omega t}$, on a steady stress, σ_0, and measure the response, $\delta\gamma(\omega) \sim |\delta\gamma|e^{i\omega t}$. We confirm that the response is linear for all σ_0 provided $|\delta\sigma| \leq \sigma_0/10$. We determine the complex differential or tangential modulus, $K^*(\omega, \sigma_0) = [\delta\sigma(\omega)/\delta\gamma(\omega)]|_{\sigma_0}$. When $\sigma < \sigma_c$, $K'(\omega)$ and $K''(\omega)$ are independent of σ_0 and are identical to $G'(\omega)$ and $G''(\omega)$. By contrast, when $\sigma > \sigma_c = 0.1$ Pa, the magnitudes of $K'(\omega)$ and $K''(\omega)$ become very sensitive to σ_0; however, changes in frequency dependences are small, as shown in Fig. 3. By the time σ_0 is increased to 27 Pa, K' increases to 100 Pa, reflecting an increase of nearly 10^3 from its linear value. At large σ_0, the tangential modulus of the networks is comparable to values of the elastic modulus reported for cells.

There is further analogy between these networks and cells: we measure the frequency dependence of the differential elastic modulus for different σ_0, and find $K'(\omega) \sim \omega^{x-1}$ where x is a function of σ_0, decreasing from 1.2 to 1.05 as σ_0 increases from 3 to 27 Pa. At 0.1 Hz, $\ln K'$ is inversely proportional to x, as shown in Fig. 4(a). Moreover, the ratio, K''/K', decreases linearly from 0.4 to 0.2 as σ_0 is increased, as shown in Fig. 4(b). This is strikingly similar to the rheological behavior of living cells [3,18]: the frequency dependence of cells is also a weak power law, whose exponent varies between about 1.15 and 1.35. Moreover, the variations of $\ln G'$ and G''/G' as a function of x in cells are identical to those seen between $\ln K'$, K''/K', and x in these *in vitro* networks [3]. It is this dynamic mechanical response that is the striking signature of a soft glassy solid [19]. While the SGR model provides an excellent phenomenological fit to our data, it does not offer insight into the relationship with σ_0. For this, a more specific model of the constituents of this composite network is required [20].

This suggests that the elastic modulus measured in cells is analogous to the linear, differential modulus of *in vitro* networks when they are in a highly nonlinear, stress-stiffened state under a steady prestress. Well adhered cells typically exert a stress of 200–1000 Pa on their substrate [4]; applied stresses to probe the mechanical response of cells are typically of the order of 20 Pa [1]. Thus, it is likely that nonlinearity in the differential mechanical response of living cells is only apparent at larger stresses.

Our *in vitro* model demonstrates that the nonlinear mechanical response of a physiological network of F-actin cross-linked with FLNa is sufficient to capture the complex mechanical behavior of living cells. This provides a rationale for the variations in mechanical behavior of cells: it suggests that the variations in the frequency dependence and magnitude reflect the nonlinear elasticity of the actin cytoskeleton due to variations in the magnitude of prestress of individual cells. Thus, cell prestress is the dominant parameter determining the elasticity of cells, just as it is in determining the elasticity of the networks. Our data suggest that the relationship between rheology and cellular prestress observed in cells [21] may arise from the inherent nonlinear mechanical response of the cytoskeletal actin network.

The utility of this *in vitro* model system is that it greatly facilitates understanding the underlying mechanical behavior of these composite FLNa-F-actin networks which is qualitatively different from other F-actin networks [13]. In the linear regime, the mechanical stiffness is fairly insensitive to large variations in the concentration of FLNa cross-links. In this case, the role of the cross-linkers is to maintain network integrity at low frequencies in a network of shortened actin filaments. The magnitude of the linear elasticity of the network is similar to that of entangled

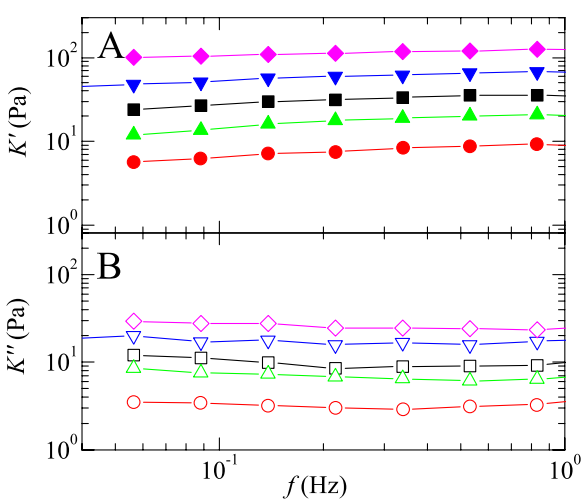

FIG. 3 (color online). (a) K' (solid symbols), and (b) K'' (open symbols) for 36 μM F-actin (2 μm length) cross-linked with FLNa ($R = 1/100$) as a function of σ_0: 3 Pa (circles), 6 Pa (triangles), 9 Pa (squares), and 14 Pa (upside down triangles) and 27 Pa (diamonds).

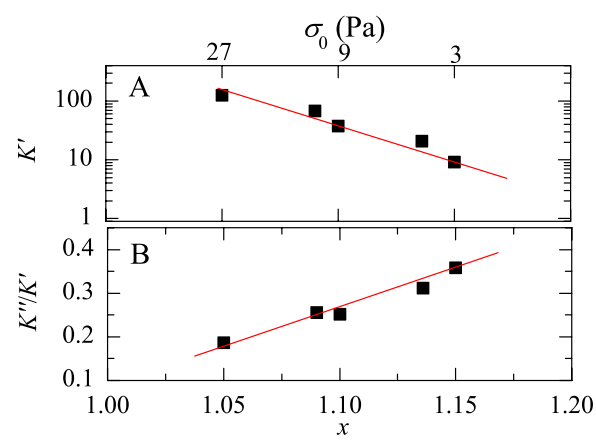

FIG. 4 (color online). (a) K' as a function of x (bottom) and σ_0 (top) (b) K''/K' as a function of x (bottom) and σ_0 (top).

F-actin solutions [22] rather than those of rigidly cross-linked F-actin networks [13]. By contrast, the maximum stress the networks can withstand is extremely sensitive to the degree of cross-links, suggesting that the nonlinear behavior is dominated by the properties of the FLNa cross-linkers, rather than by the F-actin filaments. The differential elastic modulus depends on the amount of prestress, and can increase significantly over its linear value; this nonlinearity is far greater in magnitude, and more abrupt with strain than previously observed for networks of semiflexible polymers [11,13]. These results demonstrate how specific actin-cross-linking proteins can qualitatively alter the mechanical properties of composite F-actin networks. The mechanical response of the composite FLNa-F-actin networks eludes either the current theoretical models for entangled F-actin solutions or those for rigidly cross-linked F-actin networks. Instead, we speculate that the FLNa cross-links mediate the dynamic nonlinear mechanical response of these networks through an interplay between the nonlinear divergence of the stiffness of individual semiflexible FLNa cross-links with a force- and time-dependent unfolding of β sheets within the FLNa molecule [15] as well as binding kinetics of the FLNa molecule with F-actin. Alternatively, current models of prestressed structures suggest that their mechanical response depends very weakly on the elasticity of the individual elements [23].

The remarkable similarity to the mechanics of living cells demonstrate that their complex structure and behavior can be replicated with an *in vitro network* of shortened F-actin filaments cross-linked with FLNa, provided prestress is applied. Thus models that capture cytoskeletal elasticity may not require the complex dynamic responses of cells; instead their behavior might reflect the materials properties of the cytoskeletal network. These results also highlight the critical importance of the nonlinear elastic behavior in the mechanical properties of these networks. Moreover, they suggest that all measurements of the mechanical properties of cells may, in fact, be differential measurements of a nonlinear material, subjected to a constant prestress [23]. Future work is required to identify how the mechanical behavior of the 2D cortical cytoskeleton will differ from our 3D networks. Further experimental work is also necessary to develop a microscopic model of the nonlinear elasticity of the filamin A-actin networks.

The collection of cytoskeletal proteins provides an exceptional experimental toolbox to build soft materials with exquisitely tunable mechanical properties. Understanding how the mechanical properties of single proteins are manifested at the cellular level is essential to building a mechanical framework of cellular behavior.

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