Cross-Linker Unbinding and Self-Similarity in Bundled Cytoskeletal Networks

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The macromechanical properties of purely bundled *in vitro* actin networks are not only determined by the micromechanical properties of individual bundles but also by molecular unbinding events of the actin-binding protein (ABP) fascin. Under high mechanical load the network elasticity depends on the forced unbinding of individual ABPs in a rate dependent manner. Cross-linker unbinding in combination with the structural self-similarity of the network enables the introduction of a concentration-time superposition principle—broadening the mechanically accessible frequency range over 8 orders of magnitude.

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The cytoskeleton is a highly complex, dense and heterogeneous network which offers the cell the crucially needed dynamic adaptability of its mechanical behavior [1]. For this purpose each cell exploits the whole range of accessible building blocks such as actin filaments, microtubules, and intermediate filaments, as well as their associated binding proteins to form localized cytoskeletal structures adapted to their special needs [2]. An important feature the cytoskeleton has to fulfill is the ability to withstand deformations on short time scales while still allowing the cell to adjust and rearrange for processes taking place on longer time scales.

The semiflexible nature of actin filaments themselves results in a separation of length scales and therefore time scales as already observed for *in vitro* entangled actin solutions [3,4]. The addition of actin-binding proteins (ABPs) alters the structure of entangled solutions depending on the molecular structure of the cross-linking molecule used into isotropically cross-linked networks [5], purely bundled networks [6,7], or heterogeneous composite phases [8,9]. Concomitant with the structural change new length and time scales are introduced. While the microstructure of such complex reconstituted actin networks mainly determines the elastic response [10–12], the length dependence of the persistence length of individual bundles has to be considered as well [6,13,14]. Additionally, the typical lifetime of an actin–cross-linker bond might influence the frequency behavior of cross-linked actin networks.

Here we show that the viscoelastic behavior of bundled actin-fascin networks crucially depends on molecular unbinding events of actin-fascin bonds. On intermediate time scales comparable to the cross-linker off-rate the nonlinear mechanical response is highly sensitive to the applied strain rate. On the same time scale, the frequency dependence of the viscoelastic moduli shows a generic behavior which is a signature of unbinding events and allows creating a master curve. Such a superposition of cross-linker concentration and time enables us to determine the viscoelastic properties over almost 8 decades in rescaled frequency.

Globular actin (G-actin) is obtained from rabbit skeletal muscle and stored in lyophilized form at $-21\,^{\circ}\mathrm{C}$ [15]. For measurements the lyophilized actin is dissolved in deionized water and dialyzed against G-buffer (2 mM Tris, 0.2 mM ATP, 0.2 mM $CaCl_2$, 0.2 mM DTT and 0.005% NaN_3, *p*H 8) at $4\,^{\circ}\mathrm{C}$. The G-actin solutions are kept at $4\,^{\circ}\mathrm{C}$ and used within seven days of preparation. The average length of the actin filaments is controlled to 21 μm using gelsolin obtained from bovine plasma serum following [16]. Recombinant human fascin (55 kD) was prepared by a modification of the method of [17] as described in Ref. [18]. In the experiments the molar ratio R between fascin and actin, $R = c_f/c_a$, is varied over almost three decades.

The viscoelastic response of actin-fascin networks is determined by measuring the frequency-dependent viscoelastic moduli $G'(\omega)$ and $G''(\omega)$ with a stress-controlled rheometer (Physica MCR 301, Anton Paar, Graz, Austria) within a frequency range of three decades. Approximately 520 μl sample volume are loaded within 1 min into the rheometer using a 50 mm plate-plate geometry with 160 μm plate separation. To ensure linear response small torques (≈ 0.5 μNm) are applied. Actin polymerization is carried out *in situ*, and measurements are taken 60 min after the polymerization was initiated.

Above a critical concentration of the ABP fascin, actin filaments are organized into a homogeneous cross-linked network whose building blocks are bundles only. These bundles are interconnected to each other, the degree of cross-linking among individual bundles depends on the fascin concentration (see Ref. [6] for details and images of the bundle network). The nonlinear elasticity of fascin networks in the purely bundled phase is probed best by applying a constant shear rate $d\gamma/dt$ to avoid viscous creep which in principle is not neglectable in these networks. The resulting stress σ is reported and from the smoothed $\sigma(\gamma)$ relation the differential modulus $K = \partial\sigma/\partial\gamma$ is calculated. With increasing cross-linker concentration R the nonlinear response exhibits a transition from a strain-hardening regime to strain-weakening as depicted in Fig. 1(a). As with increasing R the bundle stiffness as

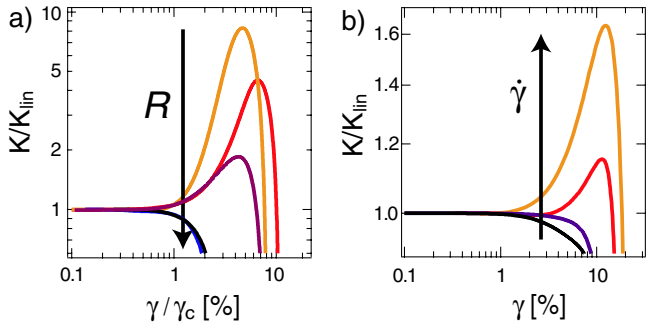

FIG. 1 (color online). Differential modulus K normalized by its value in the linear regime as a function of strain. The nonlinear response can be tuned from hardening to weakening at a fixed strain rate $d\gamma/dt = 6.25\%\ \mathrm{s}^{-1}$ by varying the fascin concentration ($R = 0.02, 0.05, 0.1, 0.2, 0.5$) as shown in (a) as well as for fixed $R = 0.1$ tuning the strain rate ($d\gamma/dt = 0.125, 1.25, 6.25, 12.5\%\ \mathrm{s}^{-1}$) as depicted in (b). γ_c defines the onset of the nonlinear response and marks the critical deformation at which the differential modulus K deviates by 5% from its value in the linear regime.

well as the connectivity of individual bundles is enhanced, at high fascin concentrations the macroscopic deformation will lead to forced unbinding of interconnecting fascin molecules even before nonlinear behavior can be evoked.

On the other hand, the same effect can be achieved by applying different strain rates to a bundle network with fixed microstructure ($R = 0.1$) as shown in Fig. 1(b). Varying the strain rate by 2 orders of magnitude leads to a continuous change in the degree of strain hardening. This underlines the nonuniversal behavior of the nonlinear response of semiflexible polymer networks as also reported for purely entangled actin solutions [19] and isotropically cross-linked networks [5]. Even if the molecular mechanism responsible for this strain hardening remains yet to be understood, the nonlinear response of semiflexible polymer networks can be tuned by many factors including the polymer bending rigidity, cross-link density, binding kinetics, and compliance of the cross-linker, as well as the strain rate. When the bundle network is sheared with very low strain rates, detaching of fascin molecules is very likely on the time scale of the experiment ($\approx$100 s in the case of $d\gamma/dt = 0.125\%\ \mathrm{s}^{-1}$). Therefore, the remaining number of connection points between fascin bundles might become too low to evoke strain hardening as in the case of higher shear rates. This result suggests that the elastic response of purely bundled actin networks might be equally determined by the cross-linker concentration and the chosen time scale of the experiment. The nonstatic nature of actin-fascin bonds introduces the cross-linker off-rate as an important time scale. For actin-fascin bonds in filopodia the off-rate was determined to be $k_{\mathrm{off}} \approx 0.07\ \mathrm{s}^{-1}$ *in vivo* [20]; however, this value might be different in an *in vitro* experiment. For a single molecular bond a linear increase of the maximal loading force with the logarithm of the loading rate, $f_{\max} \sim \ln(df/dt)$, would be expected from the Bell prediction [21,22].

To this end we have conducted shear rate experiments in such a way that the nonlinear response is completely tuned from strain hardening to weakening. To further investigate the dependence of $f_{\max}$ on the loading rate, a weakly bundled network is chosen where the density of bundle interconnection points is low ($R = 0.05$). For this network type strain hardening is observed for all strain rates applied (Fig. 2). The maximum strain the bundle network can endure without being irreversibly damaged is independent from the strain rate, $\gamma_{\max} \approx (22 \pm 1)\%$. The maximum stress $\sigma_{\max} = \sigma(\gamma_{\max})$ the bundle network can withstand depends logarithmically on the strain rate $d\gamma/dt$ as depicted in the inset of Fig. 2. The maximum force a single fascin molecule connecting two bundles can hold is given by $f_{\max} \sim \sigma_{\max} l_c^2$ where l_c denotes the average distance of two neighboring bundle interconnection points being fixed for a given R. For the data presented here the proportionality $df/dt \sim d\gamma/dt$ holds at the point of rupture $\gamma_{\max}$ (see supplementary information [23]). Therefore the Bell prediction is reproduced surprisingly well—indicating that forced unbinding of actin-fascin bonds gives rise to the observed strain rate dependence. As the macroscopic deformation is transmitted in a nonaffine way to the bundle cross-linking points [6,24], not all interconnecting fascin molecules will be loaded with the same force during a strain rate experiment. Surprisingly, the Bell prediction is still fulfilled.

Unbinding of fascin molecules between single bundles should also be observable in the low frequency behavior of the viscoelastic moduli. Indeed, a pronounced minimum in $G''(\omega)$ can be found in the linear response of such networks. From the frequency spectra we roughly estimate the time scale for these events to be on the order of several seconds, which in general matches the typical off-rates measured for various actin-binding proteins [25,26]. The

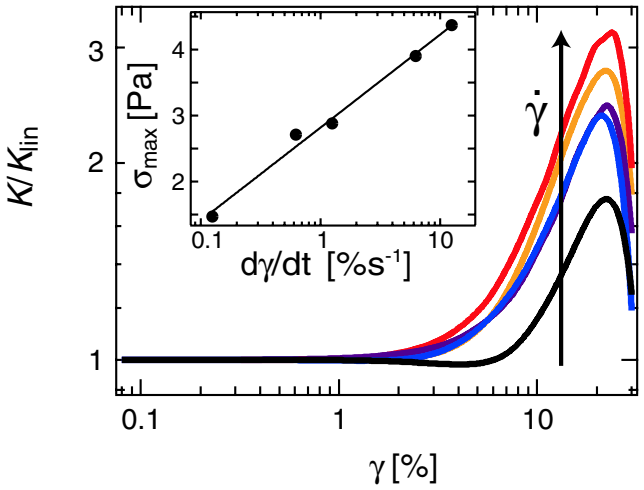

FIG. 2 (color online). Differential modulus K normalized by its value in the linear regime as a function of strain. A weakly bundled network ($R = 0.05$) is examined at different strain rates ($d\gamma/dt = 0.125, 0.625, 1.25, 6.25, 12.5\%\ \mathrm{s}^{-1}$). The inset shows the maximum stress $\sigma_{\max}$ vs the strain rate.

measured frequency spectra of actin-fascin networks show two subpopulations in dependence on R: Interconnected bundle networks exhibit a plateaulike storage modulus $G'(\omega)$ while the loss modulus $G''(\omega)$ reveals a well-defined minimum which shifts to higher frequencies with increasing fascin concentration. Networks below the bundling transition (low R), however, feature frequency spectra that do not exhibit a minimum in $G''(\omega)$ but nevertheless highly resemble each other.

To obtain appropriate parameters for a putative generalization process an impartial criterion is needed. Therefore for each frequency spectrum the minimum position in the loss modulus $[\omega^*|G''^*(\omega^*)]$ together with the corresponding value of the storage modulus is determined for samples in the bundle phase. For samples below the bundling transition ω^* is obtained by creating a ''best match'' overlay of the spectra. Normalizing the frequency spectra by these values results in a master curve which shows two different regimes corresponding to distinct network types. Therefore, the master curve is discussed best in two parts: First, at low rescaled frequencies the master curve corresponds to networks in the purely bundled phase [Fig. 3(a), rescaled $G'(\omega)$ shifted up for clarity]. This regime contains a pronounced plateau region in the storage modulus $G'(\omega)$ accompanied by a clear minimum in the loss modulus $G''(\omega)$. Secondly, networks before the bundling threshold also show a common frequency dependence similar to a purely entangled actin solution [Fig. 3(b)].

For an actin concentration of $c_a = 9.5\ \mu$M the threshold ratio R^* was determined to be $R^* \approx 0.01$ [6]. Indeed, the scaling parameters obtained for the master curve construction follow a conjoint power law $G'^* \sim (\omega^*)^x$, $G''^* \sim (\omega^*)^x$ in the purely bundled phase, while dropping off this relation for values $R < R^*$ [see inset of Fig. 3(b)]. For the pure bundle phase a linear relation between the scaling parameters, $x = 1$, is obtained. This is an indication of self-similarity as also observed in other soft matter systems [27]. Fascin networks with $R < R^*$ can also be rescaled with a common power law between the scaling parameters, however $x \approx 1/2$. As in this regime the tube model can be applied to describe the mechanical response [6], the addition of few fascin molecules seems to only slightly modify the network properties, and does not result in a self-similar structure.

The corresponding part of the master curve, $\omega/\omega^* > 10^3$, shows a regime $\sim\omega^{0.5}$ in both viscoelastic moduli right before the crossing-over, $\omega_{\text{cross}} \sim 1/\tau_e$, where τ_e denotes the entanglement time. While the molecular origin of this behavior is still unclear, it is expected for cross-linked semiflexible polymer networks in an intermediate frequency regime resulting from tensions in the network [4]. It is also reported for entangled actin networks from two-point micro-rheological experiments [28] and interpreted to be due to the diffusive dissipation of long-wavelength longitudinal fluctuations. Only at very short time scales $t \ll \tau_e$, and therefore very high frequencies beyond the crossing-over of $G'(\omega)$ and $G''(\omega)$, i.e., around

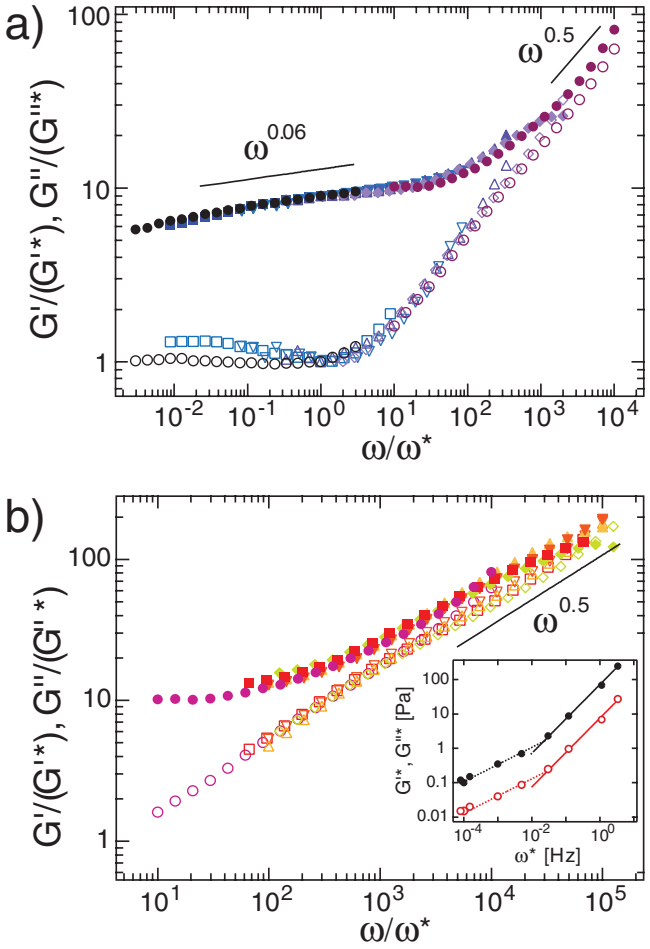

FIG. 3 (color). Master curve for actin-fascin networks (a) in the bundle phase ($R = 0.5$, 0.2, 0.1, 0.05, 0.02, 0.01) and (b) before the bundling transition ($R = 0.01$, 0.005, 0.002, 0.001, 0) as described in the text. Closed symbols denote G', open symbols denote G''. The inset shows the dependence of G'^* and G''^* on ω^* (full line: $\sim(\omega^*)^1$, dotted line: $\sim(\omega^*)^{1/2}$).

1 kHz, a scaling $\sim\omega^{0.75}$ due to undisturbed relaxations along single filaments would be expected [4,29,30] and is also detected experimentally [31]. Rescaling the loss factor $\tan(\delta) = G''(\omega)/G'(\omega)$ [23] shows unambiguously that the $\omega^{0.5}$ regime observed for actin-fascin networks is indeed an analytical power law as the Kramers-Kronig relations are fulfilled. Possibly, the frequency behavior at intermediate times might be strongly dependent on the network architecture as a regime $\sim\omega^{0.75}$ has been described for actin networks in a composite phase, as obtained by the cross-linker scruin [32].

Thus the mesoscopic network structure does not only determine the plateau modulus but also the frequency behavior of semiflexible polymer networks. Despite the fact that for composite networks a master curve description was reported [32], this is not a generic feature for cross-linked actin networks. Purely isotropically cross-linked networks [5] do not allow a master curve construction based on a concentration-time superposition. There, the

network elasticity is enhanced by simply decreasing the cross-linker distance l_c—without any overall change in the network structure. Thus self-similarity does not apply for isotropically cross-linked networks. The master curve construction applied here is only possible if one intrinsic parameter determines structure *and* mechanical behavior at the same time, which is the case for purely bundled actin-fascin networks. Raising the fascin concentration R ''magnifies'' all system properties including the mechanical parameters of its constituting bundles [6,13]. This creates a self-similar network that can be described by the principle of cross-linker concentration-time superposition introduced here.

Still, thermal and enforced unbinding of interconnecting fascin molecules could lead to subtle changes in the network structure. The increase of $G''(\omega)$ at frequencies lower than ω^* is quite weak, which stands in marked contrast to isotropically cross-linked networks as they are formed by heavy meromyosin in the rigor state. There, a steep uprise of $G''(\omega)$ below the minimum position is reported. This difference is attributable to the fact that the mechanical response of an isotropically cross-linked network is dominated by the number of cross links, corresponding to l_c. Thus unbinding results in a structural and mechanical transition from a cross-linked network to an entangled solution. In contrast, for a purely bundled network the linear response is dominated by the bundle properties and the nonaffine deformation mode. Only at large time scales $t \gg 1/\omega^*$ it will be slightly affected by the cross-linking degree.

In summary, the binding kinetics of the cross-linking molecules and the network structure determine the network elasticity at high deformations. Forced unbinding of cross links under mechanical load can tune the nonaffine response on intermediate times. Moreover, the self-similar structure of purely bundled actin-fascin networks allows a generalization of their frequency behavior. The concentration-time superposition introduced here broadens the measurable frequency window over 8 orders of magnitude simply by altering the typical network length scales tuning R—fully equivalent to the principle of temperature-time superposition used for classical flexible polymer networks. Thus, the chosen time scale crucially affects the mechanical network response as distinct length scales dominate the viscoelastic behavior. As a consequence a generic broad distribution of relaxation times has to be considered describing the frequency behavior of cross-linked semiflexible polymer networks and living cells [33,34]. The self-similar *in vitro* system presented here provides a benchmark to address the challenging question of how single filament and bundle properties, network structure, and binding kinetics of ABPs interplay to provide the high adaptability, which is known for the cytoskeleton of living cells. The observed forced and rate dependent unbinding of cross-linking molecules may turn out to be an important mechanism cells employ for mechanosensing tasks. Therefore, the described effects may be more generic for an understanding of adaptable biomaterials.

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