Viscoelasticity of Isotropically Cross-Linked Actin Networks

R. Tharmann, M. M. A. E. Claessens, and A. R. Bausch
E22-Biophysics, Technische Universität München, James Franck Straße, 85748 Garching, Germany
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Despite their importance for the proper function of living cells, the physical properties of cross-linked actin networks remain poorly understood as the occurrence of heterogeneities hamper a quantitative physical description. The isotropic homogenously cross-linked actin network presented here enables us to quantitatively relate the network response to a single filament model by determining the dominating length scale. The frequency dependence of the linear response and nonuniversal form of the nonlinear response reveal the importance of cross-linker unbinding events.

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The forces that are generated and transmitted in cellular processes, such as, e.g., motility and division, are closely connected to the viscoelastic behavior of the cytoskeleton. Elucidation of the origin of the viscoelasticity of cytoskeletal networks is therefore of great interest for the understanding of cell mechanics. The semiflexible polymer actin is one of major constituents of the cytoskeleton and *in vitro* reconstitution of actin networks with a different degree of complexity has proven to be a promising approach for studying the underlying physical principals (see, e.g., [1] and references therein). Until now, physically entangled solutions [2,3] and cross-linked networks [4–8] of F-actin have been the most studied cytoskeletal model systems for investigation of how micro- and macroscopic stresses and strains are mediated to the single molecule level. The elastic response of F-actin solutions is well described by a tube model being dominated by the spatial confinement of the thermal bending undulations [2,9]. The interaction length scale in this problem is the Odijk or entanglement length L_e [10], which is set by the filament stiffness and concentrations. Unlike the case of solutions, single polymers in cross-linked networks are subject to stretching forces directed along their backbone. The simplest model describing the elastic response of such networks assumes an affine deformation of single polymer strands between cross-links [11]. *In vivo* a myriad of actin binding proteins (ABPs) changes not only the mechanical properties of the network, but also its structure substantially. Cross-linking proteins generally induce microphase separation into all kinds of clusters and bundles [4–6,12], which make it difficult to unambiguously identify the deformation modes, length, and time scales that determine the mechanical response of the network. Homogeneously cross-linked networks that can be described by simple models can usually only be found at low cross-linker concentrations [5]. However, to quantitatively test theoretical predictions it is necessary to have a model system of isotropically cross-linked biopolymers in which the important length scales can be varied over a wide range. A thorough study of model networks with such well-defined architecture and well-understood elastic elements is mandatory to reliably test and firmly establish the highly attractive single filament models for the elastic response of semiflexible polymer networks.

Here, we show that in the presence of the molecular motor heavy meromyosin (HMM) in the rigor state actin filaments form a homogeneous and isotropically cross-linked network without any embedded bundles or other mesostructures; these networks are thus an ideal system to test predictions based on single filament models. The linear elastic response of rigor-HMM/actin networks is of entropic origin and fully determined by the distance between cross-linked points L_c, which can directly be inferred from the experimental data. The frequency dependence of the linear response and nonuniversal form of the nonlinear response indicate the significance of unbinding of cross-linking proteins for the viscoelastic response of the network. Moreover, determination of L_c allows the extraction of the characteristic force necessary to rupture the actin-rigor-HMM bond from macroscopic measurements.

Actin is prepared from rabbit skeletal muscle and treated as described before [8]. Gelsolin is prepared from bovine plasma serum and used to adjust the filament length to 21 μm. HMM is prepared from Myosin II by chymotrypsin digestion and tested using motility assays as in [13]. The molecular motors were added in a molar ratio r_{ac} (c_a/c_{HMM}) to the sample before initiation of actin polymerization at room temperature 21 °C. The transition from active to rigor HMM is followed by recording the elastic response G' at 0.5 Hz of the actin/HMM network in time. With the initial ATP concentration (0.1–2.5 mM) the active-rigor transition takes place ~20 minutes after the actin polymerization is equilibrated and a plateau in $G'_{0.5\ \mathrm{Hz}}(t)$ is reached [13]. Rheological measurements are performed with a rheometer (Physica MCR301, Anton Paar, Austria), in plate-plate geometry ($r = 25$ mm) with a gap size of 160 μm and a sample volume of 517 μl. The moduli $G'(f)$ and $G''(f)$ are detected between $f =$ 4 Hz–5 mHz. To determine the stress, σ, in the network as a function of the applied strain, γ, the sample is sheared in rotation at a constant rate of 2%/s [8]. The derivative of the smoothed $\sigma(\gamma)$ curves yields the tangential modulus K'

at a given γ. Fluorescence video microscopy is performed using reporter filaments in a ratio 1 reporter-filament/500 unlabeled filaments and samples for transmission electron microscopy are prepared as described before [8].

In the absence of ABPs, F-actin solutions form isotropic homogeneous solutions. Both fluorescence and transmission electron microscopy do not show any sign of structural changes in the F-actin network when filaments are cross-linked by rigor-HMM, after ATP depletion in the solution (Fig. 1). In contrast to other actin binding proteins rigor-HMM does not promote the formation of actin bundles; even at high rigor-HMM concentrations a network with an isotropic homogenous mesh is found, which is confirmed by multiparticle tracking of embedded colloidal probe beads. The degree of cross-linking, and therefore L_c, can be tuned by varying the rigor-HMM concentration.

The frequency dependent viscoelastic response of the rigor-HMM/actin networks confirms the absence of structural changes. With increasing rigor-HMM concentration the elastic modulus G' of the F-actin networks increases, and only at low frequencies an increased dissipation is observed [Fig. 2(a)]. Above a threshold in cross-link density $r_{ac} < 200$ the frequency dependence of the elastic modulus G' remains unchanged; only the absolute value of the modulus increases with the rigor-HMM concentration. This makes it impossible to construct a master curve out of the G' and G'' data in contrast to what has been observed for actin networks cross-linked by scruin or fascin [14,15]. To determine which length scale dominates the elastic response of the cross-linked network, the elastic plateau modulus G_0 is determined at the minimum in G''. For all actin concentrations, G_0 scales with r_{ac}, as $G_0 \sim r_{ac}^{-1.2}$ [Fig. 2(b)]. At very high cross-linker concentrations ($r_{ac} < 3.3$) G_0 decreases with r_{ac}. Saturation of all possible HMM binding sites along the filament prevents cross-linking and thus results in a decrease of G_0. This suggests that the minimal cross-linker distance L_c of rigor-HMM molecules is set by the entanglement length L_e; a further decrease of the distance between bound rigor-HMM molecules has no effect on the network elasticity.

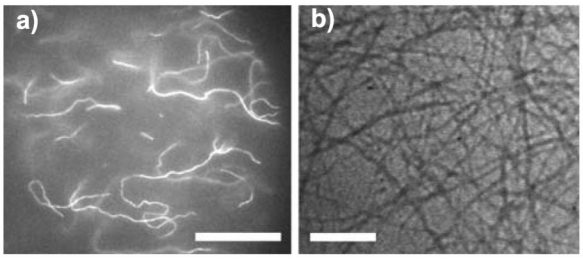

FIG. 1. (a) Fluorescent micrograph of reporter filaments in an actin network in the presence of rigor-HMM ($r_{ac} = 5$, $c_a = 23.8\ \mu$M, scale bar 10 μm). (b) Electron micrograph of composite rigor-HMM/actin network ($r_{ac} = 5$, $c_a = 9.5\ \mu$M, scale bar 0.5 μm). The addition of rigor-HMM does not result in the formation of actin bundles.

Assuming that the elasticity of the network results from stretching out the thermal excess length on a filament of length L_c, the plateau modulus G_0 can be described by [11]

$$G_0 \sim \frac{\kappa_0^2}{k_B T \xi^2 L_c^3}, \tag{1}$$

where, $L_c \geq \xi$, $\xi = 0.3 c_a^{-1/2}$ is the mesh size of the network, κ_0 the bending modulus, k_B the Boltzmann constant, and T the absolute temperature. In the absence of structural changes cross-links can only appear at intersection points of the actin filaments; the averaged distance between cross-links L_c is therefore given by c_{HMM} and L_e:

$$L_c \sim c_{\mathrm{HMM}}^{y} L_e \sim c_{\mathrm{HMM}}^{y} \left(\frac{\kappa_0}{k_B T}\right)^{1/5} c_a^{-2/5}, \tag{2}$$

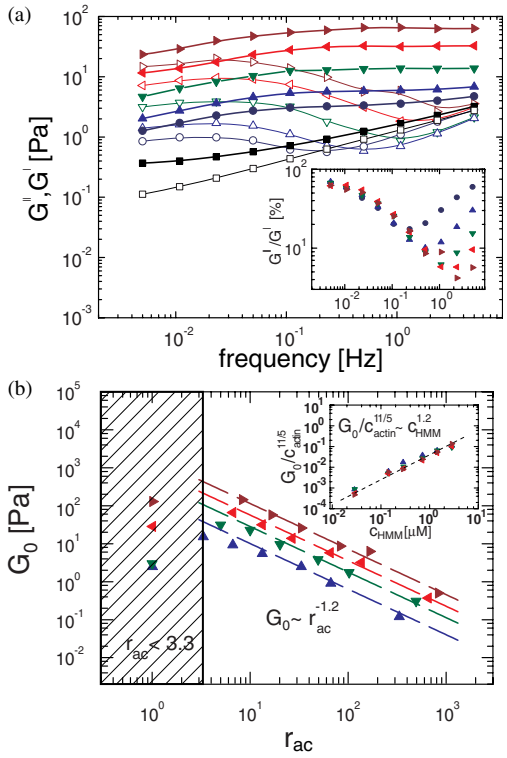

FIG. 2 (color online). (a) The frequency dependence of the moduli G' (full symbols) and G'' (open symbols) for a rigor-HMM/actin network at $c_a = 19\ \mu$M and ■: $r_{ac} = 660$, ●: $r_{ac} = 132$, ▲: $r_{ac} = 66$, ▼: $r_{ac} = 26$, ◀: $r_{ac} = 14$, ▶: $r_{ac} = 7$. With increasing HMM concentration the shape of $G'(f)$ and $G''(f)$ remains unchanged; a distinct minimum in $G''(f)$ is observed for $r_{ac} < 200$. Inset: the frequency dependence of $\tan(\delta) = G''/G'$ for the same rigor-HMM/actin networks. At low frequencies an increasing creep independent of c_{HMM} is observed. (b) The plateau modulus G_0 as a function of the cross-linker density. For all actin concentrations $G_0 \sim r_{ac}^{-1.2}$ (▶: $c_a = 23.8\ \mu$M, ◀: $c_a = 19\ \mu$M, ▼: $c_a = 14.3\ \mu$M, ▲: $c_a = 9.5\ \mu$M). At $r_{ac} < 3.3$ the filaments become saturated with HMM and as a result G_0 decreases slightly. Inset (symbols correspondingly): when $G_0(c_{\mathrm{HMM}})$ is normalized by $c_a^{11/5}$ all data points collapse onto one curve indicating that the network response is dominated by L_c.

where y is a dimensionless scaling parameter related to the fraction of cross-linking rigor-HMM molecules. Equation (1) can therefore be rewritten as

$$G_0 \sim \frac{\kappa_0^{7/5}}{(k_B T)^{2/5}} (c_{\mathrm{HMM}}^{y})^{-3} c_a^{11/5}. \quad (3)$$

Normalizing $G_0(c_{(\mathrm{HMM})})$ with the actin concentration dependence $c_{\mathrm{actin}}^{11/5}$ results in a collapse of all data points on one single curve for which $\frac{G_0}{c_a^{11/5}} \sim c_{\mathrm{HMM}}^{1.2}$ [Fig. 2(b), inset]. Thus the elastic plateau modulus of the cross-linked rigor-HMM/actin network can be described by the affine deformation of single filaments between two cross-linking points alone. The compliance of the individual filaments is dominated by their thermal fluctuations and the compliance of the individual cross-linking molecules does not alter this picture.

For a semiflexible polymer network in which the mechanical response is dominated by the affine stretching of filaments, the elasticity of the network should, with decreasing L_c, become nonlinear at smaller strains. The onset of the nonlinear response regime γ_{crit} is predicted to scale with the thermal excess length between cross-linking points as $\gamma_{\mathrm{crit}} \sim \frac{k_B T L_c}{\kappa_0}$ [11]. When the rigor-HMM/actin network is probed at small strains the tangential modulus K' is independent of γ and corresponds to the measured G_0 until at γ_{crit} the network strain hardens [Fig. 3(a)]. Above the maximal strain, γ_{max}, the network is no longer able to maintain its integrity resulting in strain weakening and fracture. With increasing c_{HMM} the onset of the nonlinear stress-strain relation γ_{crit} indeed shifts to smaller deformations [Fig. 3(a)]. The critical strain scales with $\gamma_{\mathrm{crit}} \sim c_{\mathrm{HMM}}^{-0.4}$ and, consistent with the observed plateau moduli [Fig. 3(b)], the critical stress increases as $\sigma_{\mathrm{crit}} \sim c_{\mathrm{HMM}}^{0.8}$. The network response is mainly set by the rigor-HMM concentration and thus dominated by the averaged L_c.

At the lowest r_{ac} ($r_{\mathrm{ac}} = 3.3$), the saturation in G_0 can be interpreted as an indication that all entanglement points are cross-linked [Fig. 2(b)]. Furthermore, we assume a prefactor of unity in the theoretical scaling expression for L_e, the distance between entanglements: $L_e = (\xi^2 l_p^{1/2})^{2/5}$, which we show is consistent with our observations. This allows the determination of the prefactor a and the direct calculation of L_c from γ_{crit}: $L_c = a \frac{\kappa_0 \gamma_{\mathrm{crit}}}{k_B T}$. For the rigor-HMM/actin system a is determined to be 1.6 ± 0.07 and thus L_c is obtained for all actin and rigor-HMM concentrations [Fig. 3(b), inset]. In general, the onset of the nonlinear response regime does not necessarily depend only on L_c: the appearance of γ_{crit} was reported to depend strongly on the length or compliance of the cross-linking protein [7,16]; a probably contains this information. Now that L_c is known, the prefactor of Eq. (3) can be determined from $G_0(r_{\mathrm{ac}})$ to be approximately equal to 9, which is in excellent agreement with the theoretical prefactor of 6 [17]. The transition in the shape of the frequency dependent response that was observed for cross-linker ratios $r_{\mathrm{ac}} <$ 200 can now be attributed to a critical length scale L_c^* at which the network response can no longer be understood from the spatial confinement of bending undulations [2] but is dominated by the stretching of thermal undulations of single filaments [11].

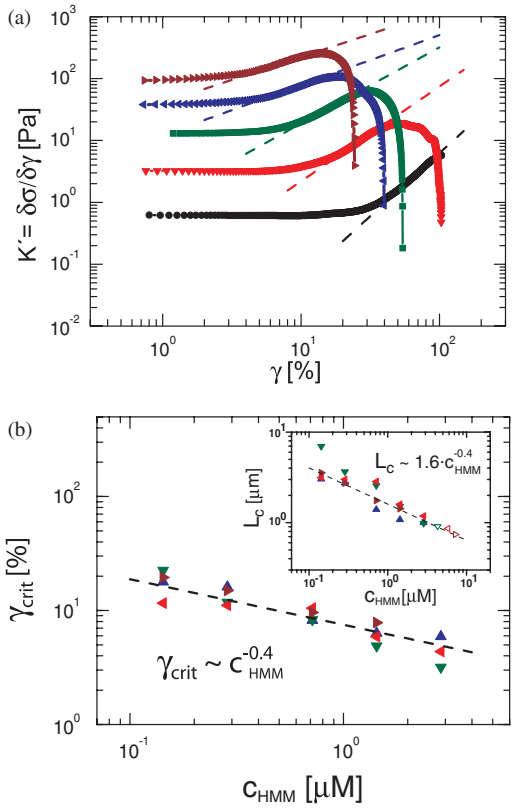

FIG. 3 (color online). (a) The differential elastic modulus K' as a function of the deformation. With increasing rigor-HMM concentrations the slope in the nonlinear response regime clearly decreases ($c_a = 19$ μM and ●: $r_{\mathrm{ac}} = 660$, ▼: $r_{\mathrm{ac}} = 132$, ■: $r_{\mathrm{ac}} = 26$, ◀: $r_{\mathrm{ac}} = 14$, ▶: $r_{\mathrm{ac}} = 7$). (b) When γ_{crit} is plotted against c_{HMM} a scaling of $\gamma_{\mathrm{crit}} \sim c_{\mathrm{HMM}}^{-0.4}$ is observed for all actin concentrations (▶: $c_a = 23.8$ μM; ◀: $c_a = 19$ μM; ▼: $c_a = 14.3$ μM; ▲: $c_a = 9.5$ μM). Inset (symbols correspondingly): the distance between cross-links L_c as a function of c_{HMM} obtained from γ_{crit} assuming that $L_c = L_e$ at $r_{\mathrm{ac}} = 3.3$.

At strains larger than γ_{crit}, where the network strain hardens, the scaling exponent x in $K'(\gamma) \sim \gamma^x$ clearly decreases with increasing c_{HMM} [Fig. 3(a)]. This observation does not agree with the assumption that the strain hardening of the network can be understood from the full force extension relation of a single actin filament [14,18]. The values of x vary from 2 at low rigor-HMM concentrations to almost 0 at high actin and rigor-HMM concentrations. The apparent disappearance of the nonlinear response at high concentration of rigor HMM might be the result of the rupturing of rigor-HMM bonds. The nonlinear response regime connects γ_{crit}, σ_{crit}, determined by the thermal length of the polymer, and γ_{max}, σ_{max}. The major network reorganization responsible for the change in mechanical response at σ_{max} might result from the forced

unbinding of cross-links at a critical force. Assuming that within a mesh the forces are distributed among four cross-links the maximal force a single rigor-HMM cross-link can hold is given by $\sigma_{\max} L_c^2/4$ and was found to be approximately 8 pN for all actin and rigor-HMM concentrations. This is in good agreement with the 9.2 ± 4.4 pN measured in optical tweezers experiments for the unbinding force of single rigor-HMM molecules [19,20]. For applied strain rates between 1%/sec and 15%/sec ($\sim$1–10 pN/sec), only a very weak dependence of $\sigma_{\max}$ on the strain rate is observed, which is in excellent agreement with single molecule studies, where at such loading rates only a very weak dependence is reported [20]. Consistent with the single filament model γ_{crit} is also independent on the shear rates applied (1%/sec to 15%/sec).

Changing deformation modes is another possible cause for the nonuniversal, gradually changing strain-hardening behavior. At high c_a and c_{HMM} the network elasticity might no longer be determined by the affine stretching of filaments; the bending of the actin segments between the cross-linked points perhaps dominates the nonlinear response instead [21].

The finding that unbinding of rigor-HMM actin bond plays a role in the network mechanics is confirmed by the frequency dependent viscoelastic response. The response at low frequencies resembles vaguely a simple Maxwellian body suggesting that the elastic component creeps slowly due to transient unbinding events. In the frequency dependence of $\tan(\delta) = G''/G'$ two regimes can be distinguished: at high frequencies $\tan(\delta)$ decreases with decreasing c_{HMM} while at low frequencies $\tan(\delta)$ is independent of the rigor-HMM concentration [Fig. 2(a), inset]. The minimum in $G''(f)/G'(f)$ might characterize the time scale of first ABP unbinding events: when the sample is probed at lower frequencies an increasing number of cross-linkers unbind and increasingly local plastic deformations of the network occur until maximal plastic deformation at around 0.03 Hz is reached. Considering that the unbinding of rigor-HMM is strongly dependent on the time scale, load, ADP, and salt concentration, the frequency range in which unbinding events occur is in good agreement with the literature values for τ [22]. The lifetime of the bond between actin and other cross-linking molecules such as, e.g., α-actinin, is reported to be $\tau \approx 20$ s [23]; it is therefore remarkable that creep has not been observed before. However, cross-linking proteins generally form networks that, depending on the ABP concentration, both bundle and cross-link actin filaments simultaneously. The loss modulus of these mixed networks might very well be dominated by the presence of bundles masking the effect of ABP unbinding and dominating their viscoelastic response.

We reported that the elastic response of isotropically cross-linked rigor-HMM/F-actin networks is dominated by the entropic stretching of filaments between cross-linked points with a well-defined distance L_c. The nonlinear response of such a network strongly depends on the cross-linker concentration and does not follow a universal scaling relation. The slope of $K'(\gamma)$ in the nonlinear response regime is determined by the thermal length of the actin filaments and the maximal force single cross-links can hold before bond rupture. Also the long time behavior is indicative of transient network reorganizations attributable to cross-linker unbinding events. Considering the high affinity of rigor-HMM for F-actin (almost four order of magnitude higher than for α-actinin [24]) this strongly suggests that also for other systems ABP unbinding rather than unfolding [25] affects the rheology of cross-linked actin networks. The detailed understanding of a purely cross-linked isotropic network and of a purely bundled network [8,15] provide a benchmark for addressing the challenge to describe the mechanics of composite networks, which are characterized by structural heterogeneities [5–7,12].

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[1] A. R. Bausch *et al.*, Nature Phys. **2**, 231 (2006).
[2] B. Hinner *et al.*, Phys. Rev. Lett. **81**, 2614 (1998).
[3] M. L. Gardel *et al.*, Phys. Rev. Lett. **91**, 158302 (2003); J. Liu *et al.*, Phys. Rev. Lett. **96**, 118104 (2006); M. M. A. E. Claessens *et al.*, Nature Phys. **2**, 186 (2006).
[4] J. Y. Xu *et al.*, J. Biol. Chem. **275**, 35 886 (2000).
[5] M. L. Gardel *et al.*, Science **304**, 1301 (2004).
[6] J. H. Shin *et al.*, Proc. Natl. Acad. Sci. U.S.A. **101**, 9636 (2004).
[7] B. Wagner *et al.*, Proc. Natl. Acad. Sci. U.S.A. **103**, 13 974 (2006).
[8] R. Tharmann *et al.*, Biophys. J. **90**, 2622 (2006).
[9] H. Isambert *et al.*, Macromolecules **29**, 1036 (1996).
[10] T. Odijk, Macromolecules **16**, 1340 (1983).
[11] F. C. Mackintosh *et al.*, Phys. Rev. Lett. **75**, 4425 (1995).
[12] M. Tempel *et al.*, Phys. Rev. E **54**, 1802 (1996).
[13] J. Uhde *et al.*, Phys. Rev. Lett. **93**, 268101 (2004).
[14] M. L. Gardel *et al.*, Phys. Rev. Lett. **93**, 188102 (2004).
[15] O. Lieleg *et al.*, cond-mat/0611752; (to be published).
[16] M. L. Gardel *et al.*, Proc. Natl. Acad. Sci. U.S.A. **103**, 1762 (2006).
[17] F. Gittes *et al.*, Phys. Rev. E **58**, R1241 (1998).
[18] C. Storm *et al.*, Nature (London) **435**, 191 (2005).
[19] T. Nishizaka *et al.*, Nature (London) **377**, 251 (1995).
[20] B. Guo *et al.*, Proc. Natl. Acad. Sci. U.S.A. **103**, 9844 (2006).
[21] P. R. Onck *et al.*, Phys. Rev. Lett. **95**, 178102 (2005); C. Heussinger *et al.*, Phys. Rev. Lett. **96**, 017802 (2006).
[22] T. Nishizaka *et al.*, Biophys. J. **79**, 962 (2000).
[23] H. Miyata *et al.*, Biochim. Biophys. Acta **1290**, 83 (1996).
[24] S. B. Marston, Biochem. J. **203**, 453 (1982); D. H. Wachsstock *et al.*, Biophys. J. **66**, 801 (1994).
[25] B. A. DiDonna *et al.*, Phys. Rev. Lett. **97**, 068104 (2006)