Transient Binding and Dissipation in Cross-Linked Actin Networks

O. Lieleg,[1] M. M. A. E. Claessens,[1,2] Y. Luan,[1,3] and A. R. Bausch[1]

[1]*Lehrstuhl für Biophysik E27, Technische Universität München, Germany*
[2]*MESA, Faculty of Science and Technology, University of Twente, The Netherlands*
[3]*School of Pharmacy, Shandong University, People's Republic of China*

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In contrast with entangled actin solutions, transiently cross-linked actin networks can provide highly elastic properties while still allowing for local rearrangements in the microstructure-on biological relevant time scales. Here, we show that thermal unbinding of transient cross-links entails local stress relaxation and energy dissipation in an intermediate elasticity dominated frequency regime. We quantify the viscoelastic response of an isotropically cross-linked actin network by experimentally tuning the off rate of the transiently cross-linking molecules, their density, and the solvent viscosity. We reproduce the measured frequency response by a semiphenomenological model that is predicated on microscopic unbinding events.

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For living cells it is of utmost importance not only to withstand mechanical strains but also to allow for a constant restructuring of the cellular microstructure. This remodeling is facilitated by employing transiently cross-linking proteins. Despite this fact, research on cytoskeletal networks focused on the static plateau elasticity [1–3]. It is yet to be resolved how transiently cross-linking proteins affect the frequency response of cross-linked actin networks in the elasticity dominated intermediate frequency regime. Transiently cross-linking proteins can be characterized by an off rate k_{off}, which typically corresponds to frequencies in the intermediate regime of several mHz up to a few Hz [4–6]. In a comparable frequency regime, cells show increasing viscous dissipation [7,8]. This suggests that transient cross-links might trigger an important relaxation mechanism in the cytoskeleton. In general, in polymer networks distinct molecular mechanisms can lead to a relaxation of an external (or internal) stress on different time scales; depending on whether energy is stored or dissipated, elastic or viscous behavior can be evoked [9,10]. Many synthetic polymer networks are covalently cross-linked resulting in a predominantly elastic response over a broad frequency range—the covalent cross-links suppress the single polymer diffusion present in entangled solutions. Therefore, the frequency dependent viscoelastic behavior of a covalently cross-linked polymer network is expected to reach a constant level of elasticity at low frequencies while viscous effects become negligible. In contrast, if transient cross-links are present (i.e., physical cross-links based on electrostatic interactions or van der Waals forces), a pronounced minimum and maximum in the viscous dissipation is observed in a frequency range of 0.01–10 Hz [11]. This feature in the viscous dissipation is always accompanied by a decrease in elasticity at low frequencies; nevertheless, the viscoelastic response in this frequency regime is dominated by the network elasticity. Moreover, the ''stickiness'' of polymer sidegroups gives rise to additional friction processes in both asymptotic frequency regimes [12]. However, in the case of transiently cross-linked cytoskeletal networks, the molecular mechanisms responsible for the behavior at intermediate ($f \approx k_{\text{off}}$) and low frequencies ($f < k_{\text{off}}$) are poorly understood. A molecular understanding of these mechanisms is urgently needed to quantify the mechanical properties of living cells.

Here, we show that thermal unbinding of transient cross-links results in a stress release mechanism in cross-linked actin networks. This stress release mechanism decreases the static network elasticity and at the same time increases the viscous dissipation in the network. The time scale of this stress release is set by the lifetime of the cross-linking molecule and can therefore be tuned independently from high-frequency fluctuations of single actin filaments. To quantitatively explore the impact of transient binding effects for the viscoelastic response of cytoskeletal networks, we make use of a well-defined model system: isotropically cross-linked actin networks are investigated using rigor heavy meromyosin (HMM) as the cross-linking protein. In this reconstituted actin network the elasticity is determined by the density of actin filaments and cross-links and thus by only one length scale: the distance between cross-link points [2,13]. Moreover, the transient cross-links are characterized by a single unbinding rate. This makes this system an ideal candidate for a detailed investigation of transient binding effects.

G-actin is obtained from rabbit skeletal muscle following [14], stored, and polymerized into filaments as described before [13]. HMM is prepared from Myosin II by chymotrypsin digestion and tested using motility assays as in [15]. In the experiments the molar ratio R between HMM and actin, $R = c_{\text{HMM}}/c_a$, is varied. The formation of cross-linked rigor networks is recorded as described in [2], and the viscoelastic response is recorded in the linear regime as described in [13]. Adenosine

5′-(β, γ-imido)triphosphate (AMP · PNP) or glycerol is added before the actin polymerization is initiated; adenosine triphosphate (ATP) depletion is rheologically recorded. Glutaraldehyde is added together with HMM to prepolymerized actin filaments. The resulting solution is gently mixed with a cut pipette tip to avoid breaking of the filaments and then loaded into the rheometer (Physica MCR 301, Anton Paar, Graz, Austria) for structural equilibration and ATP depletion.

Proteins such as fascin [3], α-actinin [16], filamin [17], or rigor HMM [2,13] create noncovalent cross-links in actin networks. In these networks, the viscous dissipation $G''(f)$ exhibits a pronounced minimum at a frequency $f_{\min}$, which exact position depends on the cross-link density (Fig. 1 and supplementary information [18]). The elastic response of cross-linked actin-rigor-HMM networks differs from the expectation for covalently cross-linked networks as the elastic modulus $G'(f)$ is constant for sufficiently large frequencies $f > f_{\min}$ but decreases significantly at lower frequencies. In the low-frequency regime, the viscous dissipation reaches a maximum around $f_{\max} \approx 0.03$ Hz (Fig. 1 and supplementary information [18]). This time scale is independent of the cross-link density; however, the maximal amount of low-frequency energy dissipation increases linearly with the cross-link density.

To shed light on the molecular origin of this viscoelastic response, the possible molecular mechanisms involved should be tuned independently. A mechanism of energy dissipation that is always present in polymer networks is friction due to viscous drag of individual filaments. The viscosity of the solvent can be increased by the addition of glycerol. As a consequence, the time scale of the single filament relaxation regime should be shifted according to the increase in solvent viscosity. Indeed, for the cross-linked network the minimum in the viscous dissipation is relocated as the viscosity of the solvent is increased by glycerol [Fig. 2(a)]. However, the addition of glycerol does not affect the elastic network response over the whole frequency range. It is important to note that the viscous dissipation depends on the solvent viscosity only at frequencies $f > f_{\min}$ while, e.g., the maximal dissipation at $f_{\max} \approx 0.03$ Hz remains unchanged. This agrees with pre-

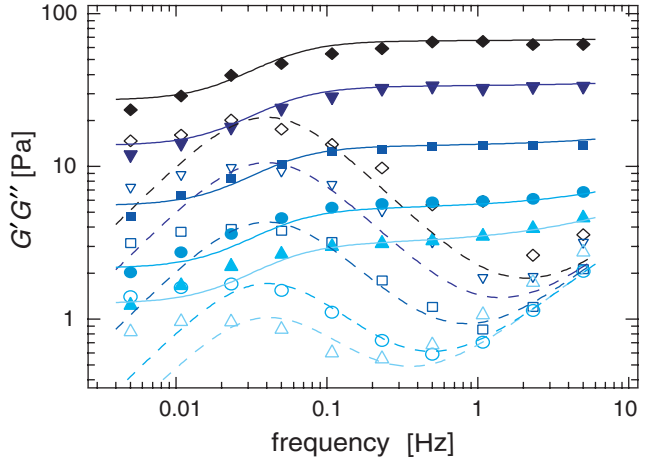

FIG. 1 (color online). Elastic (solid symbols) and viscous (open symbols) response for actin-rigor-HMM networks as a function of frequency [$c_a = 19$ μM, $R = 0.0076$ (upright triangles) up to $R = 0.143$ (diamonds)]. The solid and dashed lines represent a global best fit of the model described in the main text. All parameters are constant with the exception of the cross-link density N. Here, $N \sim R^{1.1}$ is used in excellent agreement with the experimental finding $G_0 \sim R^{1.2}$ (supplemental information [18]).

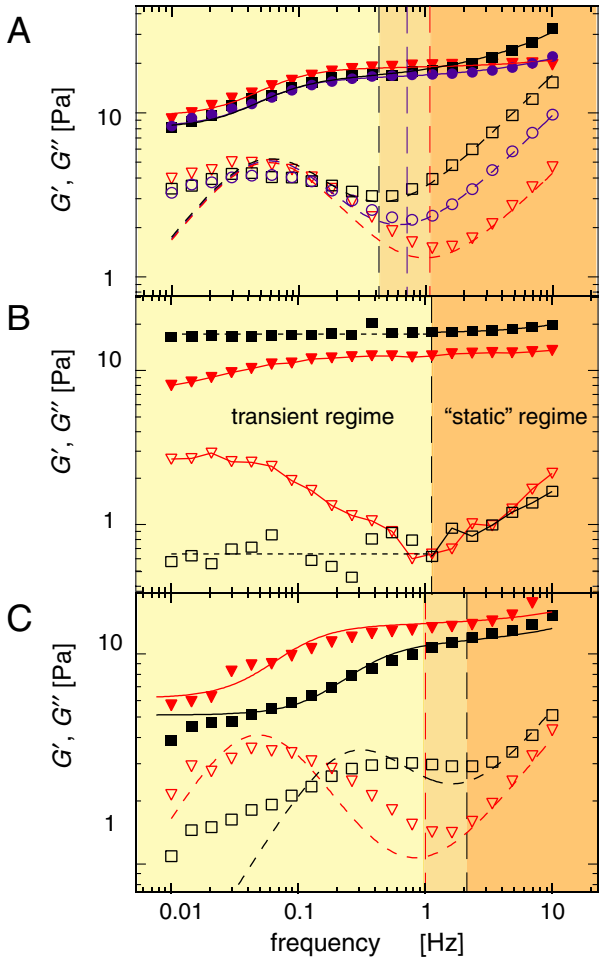

FIG. 2 (color online). Elastic (solid symbols) and viscous (open symbols) response for actin-HMM networks ($c_a = 9.5$ μM, $R = 0.1$). The solid and dashed lines in panels (a) and (c) represent a global best fit of the model as discussed in the main text. Parameters are adjusted as they are controlled experimentally (see supplemental information [18]). (a) Distinct amounts of glycerol [0% (triangles), 25% (circles), and 50% (squares)] are added to increase the solvent viscosity; (b) 0.1% glutaraldehyde is added to fix the actin-HMM bond. The resulting viscoelastic response (squares) is compared to a nonfixed network (triangles). The dotted lines are constant fits to guide the eyes. (c) 2 mM AMP · PNP (squares) is added to a standard ATP sample (triangles) to tune the off rate of the actin-HMM bond. (a)–(c): The vertical lines represent the transition from the transient cross-link regime to the “static” cross-link regime as described in the main text.

vious results [2,19] which also indicated that single filament fluctuation is not sufficient to rationalize the complex dissipation behavior of a cross-linked polymer network. In fact, another molecular mechanism has to be considered. Recall that the maximum in the viscous dissipation is located around $f_{\max} \approx 0.03$ Hz independent of the cross-link density [Fig. 1(b)]. The cross-links are created by the protein HMM which is known to form a noncovalent bond to the biopolymer actin with a typical off rate $k_{\text{off}} \approx 0.09\ \text{s}^{-1}$ [4]. As this off rate is on the order of $f_{\max}$, the binding kinetics of the cross-linking protein HMM might give rise to additional mechanisms in the network accounting for the observed frequency dependence of the viscoelastic moduli for frequencies $f < f_{\min}$.

It was shown before that the bond between actin and actin binding proteins (ABPs) can be forced to unbind in the presence of mechanical forces [5,20]. However, the transient nature of an actin-ABP bond should also allow for spontaneous unbinding events in thermal equilibrium. Thus, thermal unbinding of distinct cross-links could be the molecular reason for the observed behavior of both viscoelastic moduli in the linear response regime. To verify this hypothesis, a fixation of the actin-ABP bond would be of use. Glutaraldehyde is able to create a covalent linkage between neighboring molecules by the formation of a Schiff base. It is commonly used for fixation purposes of cells and other biomaterials [21] and can be employed to create a chemical bond between HMM and actin. Indeed, as depicted in Fig. 2(b), the minimum in the viscous dissipation can be suppressed by the addition of 0.1% glutaraldehyde. At the same time, the decrease in the network elasticity at low frequencies observed in the absence of glutaraldehyde is almost completely suppressed. This suggests that the minimum in the viscous dissipation marks the frequency below which the transient character of the cross-links starts to dictate the viscoelastic response of the network.

The covalent fixation of an actin-ABP bond using glutaraldehyde has an extreme effect on the viscous dissipation. A more subtle method to affect the viscous response of a cross-linked polymer network might be given by only slightly changing the binding kinetics of the cross-linking molecule. At 13 °C, 2 mM AMP · PNP—a nonhydrolizable ATP analogon—increases the off rate of the actin-HMM bond from 0.09 s^{-1} up to 1.8 s^{-1} [4]. To quantitatively analyze how altered binding kinetics of the cross-linking molecule influence the viscoelastic response, a network with 2 mM AMP · PNP is compared to a network which contains only standard ATP. First, the addition of AMP · PNP to a cross-linked actin-HMM network changes the position of both the minimum and the maximum of the viscous dissipation [Fig. 2(c)]. However, for frequencies $f > f_{\min}$ the viscoelastic response seems almost unaffected. Second, the frequency at which the network elasticity starts to drop is also shifted with the increase in k_{off}. This strongly suggests that these two features have the same molecular origin.

The results presented so far imply that thermal cross-link unbinding triggers a relaxation mechanism which influences the elastic and the viscous properties of the network simultaneously. This calls for a simple semiphenomenological description to quantify the observed effects. In thermal equilibrium, an ensemble of N cross-links exhibits statistical unbinding events whose probability is determined by the cross-linker off rate k_{off}. This spontaneous unbinding can be described in analogy to a unimolecular reaction: $AB \overset{k_{\text{off}}}{\rightarrow} A + B$. Herein, the rebinding process is assumed to be fast enough to provide a constant equilibrium number of cross-links: the unbinding process is limited only by the lifetime of the actin-ABP bond and not by the reformation of new bonds. This results in an exponential decay of intact cross-links over time, $t \geq 0$: $N(t) \sim N \cdot e^{-k_{\text{off}} \cdot t}$, which can be translated into the frequency domain using a Fourier transformation yielding the complex function $\hat{N}(f)$. Since the linear relation $G_0 \sim N$ holds for actin-HMM networks (supplemental information [18]), the real part $\text{Re}(\hat{N}(f))$ represents the loss of elasticity due to cross-link unbinding:

$$G'(f) = G_0 - a \cdot \frac{N k_{\text{off}}}{\frac{k_{\text{off}}^2}{4\pi^2} + f^2} + b \cdot \left(\frac{f}{f_0}\right)^{3/4}, \qquad (1)$$

where the last term represents the fluctuation of single filaments in semiflexible polymer networks [22]. The time scale of this relaxation mode is set by the factor f_0, which is a function of the solvent viscosity η [10]. $\text{Im}(\hat{N}(f))$ contributes to the viscous part of the frequency spectrum where it competes with the single filament relaxation:

$$G''(f) = c \cdot \frac{N f}{\frac{k_{\text{off}}^2}{4\pi^2} + f^2} + d \cdot \left(\frac{f}{f_0}\right)^{3/4}. \qquad (2)$$

The key parameters are the cross-linker off rate which is known from independent experiments [4] and the number of cross-linking molecules; the prefactors a and c include the amount of energy that is released and dissipated by unbinding of a single cross-link, and b and d depend on the density of filaments in the network which is kept constant during a set of measurements. The best fit for the data set shown in Fig. 1 is obtained for $k_{\text{off}} \approx 0.3\ \text{s}^{-1}$, which is in excellent agreement with values determined by biochemical means [4] considering that cross-links exhibit two independent actin-HMM binding sites (supplemental information [18]). Not only is the maximal amount of dissipated energy $G''(f_{\max})$ quantitatively reproduced but so is the minimum in the viscous dissipation and their dependencies on k_{off} and N. The minimum in the viscous dissipation can thus be identified as a direct result of the competition between local stress release triggered by thermal cross-link unbinding and friction induced by fluctuations of single filaments. Importantly, the decay in the elastic response is also correctly reflected by the global fit. It can be seen that the mechanism of local stress release

becomes increasingly important when approaching $f \approx \frac{k_{\text{off}}}{2\pi}$, where the contribution of thermal cross-link unbinding, $\hat{N}(f)$, is most pronounced and the viscous dissipation becomes maximal. If the off rate of the cross-linking molecule is altered [Fig. 2(c)], the maximum in the viscous dissipation is shifted accordingly. The fits were obtained using literature values for k_{off} at 2 mM AMP · PNP [4,18]. In all experiments, at very high frequencies $f \gg \frac{k_{\text{off}}}{2\pi}$ the viscous response is unaffected by the stress release mediated by unbinding events. Vice versa, a variation of the solvent viscosity results only in a shift of the single filament relaxation regime without affecting the off rate of the cross-linking molecules. Consistently, only the known solvent viscosity needs to be considered to reproduce the viscoelastic network response in the presence of glycerol [Fig. 2(a)].

Local stress release triggered by thermal cross-link unbinding fully accounts for the dissipation in a frequency range of $\frac{k_{\text{off}}}{2\pi}$ up to at least 10 Hz. However, at frequencies $f < \frac{k_{\text{off}}}{2\pi}$ the macroscopic network elasticity is overestimated and the viscous dissipation is underestimated. It is clear that a third mechanism of relaxation has to be considered for the network response at very low frequencies $f < \frac{k_{\text{off}}}{2\pi}$ since the locally obtained frequency spectra exhibit a low-frequency regime resembling that of entangled solutions (supplemental information [18]). With increasing probability of unbinding events, a considerable amount of filaments are ''set free'' allowing for local filament reorientation or even reptation [12]. Although this local relaxation mechanism is partially masked on the macroscopic scale, it might still be necessary to account for it in future refined modeling approaches. It is important to note that the observed transient unbinding effects described here do not permanently change the material properties—underlining the thermal nature of the process. Transient cross-links provide maximal energy dissipation without permanently altering the microstructure of the network.

We have shown that the viscous dissipation in cross-linked actin networks at intermediate frequencies $f \approx k_{\text{off}}$ can be rationalized by a molecular stress release mechanism based on transient cross-linker unbinding. This mechanism superimposes dissipation mechanisms that are already known for semiflexible polymer solutions and networks. The frequency at which stress release by thermal cross-linker unbinding dominates over single polymer friction is set by the off rate of the cross-linking molecule, the cross-link density, and the viscosity of the solvent. This defines a mechanical transition regime: If the system is deformed with a frequency much faster than f_{min}, thermal cross-linker unbinding is too slow to significantly modify the viscoelastic response of the network, the cross-links appear to be ''static,'' and unbinding kinetics are more or less irrelevant [Fig. 2(b)]. However, if the deformation is imposed slow enough, the transient nature of the cross-links will dictate both the elastic and the viscous response of the network.

The transient binding and energy dissipation discussed here are indispensible for a detailed understanding and further development of adaptable biomaterials. Moreover, transient binding effects might also be employed by cells for mechanosensing tasks that take place on time scales comparable to the frequency range investigated in this study, especially since the nonstatic nature of actin-ABP bonds gives rise to a high sensitivity towards external or internal forces. Transient cross-linker binding allows for local reorganization processes and creates an adaptive network which is able to absorb mechanical shocks on the microscopic scale without causing structural failure. In fact, this might be an important advantage for living cells employing transiently cross-linked biopolymer networks instead of covalently cross-linked structures which would be much too brittle.

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