Structure and dynamics of cross-linked actin networks

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Received 19th June 2009, Accepted 25th September 2009
First published as an Advance Article on the web 22nd October 2009
DOI: 10.1039/b912163n

The actin cytoskeleton, a network of protein-polymers, is responsible for the mechanical stability of cells. This biopolymer network is also crucial for processes that require spatial and temporal variations in the network structure such as cell migration, division and intracellular transport. The cytoskeleton therefore has to combine structural integrity and mechanical stability with the possibility of fast and efficient network reorganization and restructuring. Cells meet this challenge by using proteins to link filamentous actin (F-actin) and construct complex networks. The molecular properties of the cross-linking proteins determine to a large extent the (micro)structure, viscoelastic properties and dynamics of the resulting networks. This review focuses on the structural polymorphism that can be induced by cross-linking proteins in reconstituted F-actin networks and summarizes recent results on how the molecular properties of cross-linking proteins dictate the ensuing viscoelastic properties.

Cells assemble F-actin into structures ranging from dilute networks where filaments cross at large angles to bundles of closely packed parallel filaments. This polymorphism in F-actin organization is of vital importance as the different F-actin structures fulfill different roles in cells. Networks of cross-linked filaments constitute the cellular cortex and give cells their shape and mechanical stability,[1,2] while F-actin bundles provide support or create membrane protrusions in *e.g.* stereocilia, neurosensory bristles, microvilli and filopodia.[3–6] In spite of their mechanical function many of the F-actin assemblies are highly dynamic. Spatial and temporal reorganizations are essential for cytoskeletal processes such as cell motility, cell division and intracellular transport.[7,8] The regulation of these processes requires tight control over the network (dis-)assembly, morphology and mechanics.

To construct dynamic F-actin assemblies with specific morphologies and mechanical properties cells make use of actin binding proteins (ABPs). The wide range of existing ABPs as well as their simultaneous presence in the cytoskeleton hinder a direct correlation of network morphology and viscoelastic response *in vivo*. A bottom-up approach using well-defined *in vitro* model systems,[9] however, has proven very effective for unraveling the physical principles that determine the structural polymorphism in cytoskeletal networks. Furthermore, by systematically reconstituting actin networks with increasing complexity, the influence of biochemical and physical contributions to both the network mechanics and organization can be disentangled.

An integrated biophysical approach combining experimental techniques such as microscopy, macro- and microrheometry or scattering techniques with classical biochemical methods, theoretical modelling and simulations offers a large tool box for the characterization and analysis of complex actin filament assemblies. This extended tool box might be the main reason why cytoskeletal studies have experienced a renaissance lately. This

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review aims to summarize recent results which have significantly broadened our quantitative understanding of the microscopic organization and the complex viscoelastic behavior of cross-linked *in vitro* actin networks.

A. Structural polymorphism of actin filament assemblies

The detailed morphology of a given actin filament assembly results from actin binding proteins (ABPs) that use at least two F-actin binding sites to link distinct actin filaments using specific binding sites on the actin (Fig. 1). Multivalent cations and (natural or synthetic) polycationic molecules interact electrostatically with F-actin and can also control the morphology of F-actin networks.[10–13] Of course, cross-linking by polycations does not require specific sites on the negatively charged actin filaments. Although some of the approaches and physical principles described below may also be valid for these systems, in this review we will concentrate on ABPs.

One can distinguish two generic types of ABP-induced F-actin assemblies: networks of individual cross-linked actin filaments and actin bundles. In particular regions of the cytoskeleton either one of these assembly types may dominate or they may coexist forming a rather complicated composite phase. Understanding the local structures and rearrangements invoked by the different cross-linking proteins is the main goal of past and ongoing research. Ultimately, one aims for a multi-dimensional phase diagram where the effect of cross-linker length, cross-linker concentration, cross-linker binding domain and their respective binding affinity on the structure formation is determined. Such a detailed experimental description needs then to be matched with insights from theoretical concepts and simulations.[14–19]

It is evident that a critical concentration of crosslinking molecules is needed in order to have a major effect on the global structure and mechanics of a solution of actin filaments. Yet, below this threshold, some crosslinking molecules such as rigor-HMM and α-actinin already induce local heterogeneities.[20–22] The number and distribution of such heterogeneities increase with increasing concentration of the ABPs, until the ABPs are evenly distributed and trigger a global change in the network structure. This transition into the high ABP concentration regime and the resulting structures defy as yet a unifying description. Physical principles that have been put forward in order to rationalize transitions between different structural regimes are based on percolation models[21] or the comparison of different intrinsic network length scales.[20]

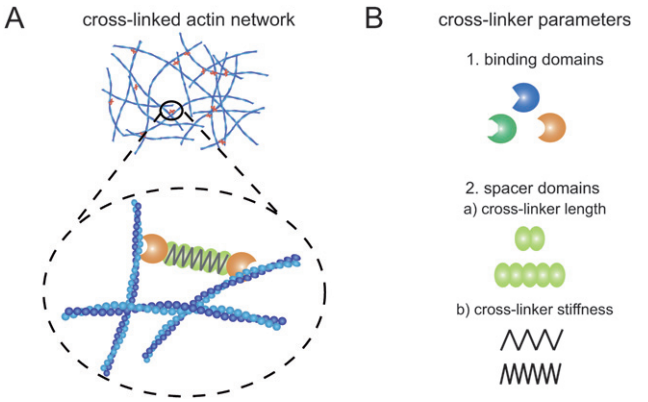

Fig. 1 (A) Many actin binding proteins (ABPs) possess at least two binding sites for F-actin and can thus cross-link actin filaments and organize them into complex networks. In reconstituted actin networks different actin/ABP mixtures can be studied while the biochemical network composition can be precisely controlled. This allows for disentangling physical and biochemical contributions to the structure and mechanical properties of cross-linked actin networks. (B) The biochemical and micromechanical characteristics of the cross-linking molecule dictate both the architecture and the viscoelastic properties of the formed network: the binding domains of distinct cross-linkers possess different affinities and on/off kinetics towards F-actin while the spacer domains can vary with respect to their number, size and stiffness thereby setting the cross-linker length as well as its shear stiffness. This review highlights how these microscopic cross-linker properties manifest themselves in characteristic meso- and macroscopic properties of the respective cross-linked F-actin networks.

Such approaches make it possible to determine the microscopic length scale that sets the structural transition in simple well-characterized systems such as isotropically cross-linked actin/rigor-HMM networks. F-Actin solutions are characterized by their entanglement length, isotropically cross-linked actin networks by the distance between cross-linking points. A quantitative criterion for the transition between F-actin solutions and cross-linked networks can therefore be given considering the two competing length scales. The dependence of the elastic modulus on the actin concentration and the particular length scales that characterize these two phases is known. Using these dependences one can obtain a constraint that describes the phase boundary (see ref. 23 and 20). The critical parameter which is held constant along the phase boundary of the entangled/cross-link transition is the average cross-linker distance.[20] This critical distance refers to a percolation threshold at which the cross-link density is high enough to evoke an overall network response that is no longer dominated by entanglements.

The transition from a filamentous network to a network of bundles can be quantitatively parameterized for the F-actin/fascin system[23] using a detailed description of the properties of actin/fascin bundles[25,26] as well as the appropriate modeling of the network elasticities.[23] This parameterization results in

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a simple criterion for the bundling transition that is qualitatively different from that of the cross-link transition described before. However, in this case a detailed microscopic interpretation of the transition is still lacking. Such a microscopic description would have to consider the tradeoff between the loss in entropy resulting from the packing of filaments into a well-ordered superstructure and the gain in enthalpy upon binding of the cross-linker proteins to the actin filaments.

For even more complicated phase transitions parameterizations of the transition boundary are not available yet. At our current stage of understanding, the network mesh size, the entanglement length and the cross-linker distance as well as the persistence length of actin filaments or bundles play an important role in the transition between different structural phases. Yet, it is a priori not clear which of these length scales dominate in the different structural regimes. This underlines the importance of a detailed microscopic characterization of the different network microstructures.

Concerning the network structures induced by different ABPs, intuition seems to match reality—at least in *in vitro* systems: small cross-linking proteins such as scruin,[27] fascin[23] or espin[28] tend to tightly pack actin filaments into parallel bundles. Larger cross-linking molecules such as α-actinin,[29,30] filamin,[31,32] *dicteostelium discoideum* filamin[33] or anillin[34] tend to induce a more complex phase behavior: while at low concentrations they cross-link actin filaments into networks or gels, at higher concentrations purely bundled phases or composite networks with a rather diverse geometry occur[35,36] (for a schematic overview see Fig. 2). Engineered cross-linkers in which two hisactophilin actin binding motifs are coupled by different numbers of *dicteostelium discoideum* filamin rod domains support this observation: the shortest constructs have the highest F-actin bundling propensity, larger distances between the binding domains result in a composite phase, a structure which is extremely difficult to quantify.[33]

Interestingly, the size of the cross-linking protein seems to have little or no effect on the ABP/actin ratio at which the structural transition occurs. This underlines that mainly the binding affinity and therefore the average distance between cross-links at a given actin concentration sets the transition to another structural phase. The type of phase is then set by the geometry and the resulting configurational freedom of the cross-linking protein. Thus, it seems to be the effective binding propensity which determines the resulting phase—analogous to adhesion phenomena, where the effective binding efficiency is set by the product of binding affinity and separation distance between distinct adhesion molecules.[37]

The effect of the ABP binding domain on the network structure has been less studied—as a matter of fact, most cross-linkers seem to have an affinity for F-actin with a typical dissociation constant of $K_d \sim 0.1$ mM and a dynamic off-rate on the order of $k_{off} \approx 1\ s^{-1}$ at room temperature.[38,39] Differences in binding affinity between different α-actinins have been shown to result in shifts in the bundling threshold concentrations.[29] As a result of the temperature dependence of the binding constant, $K(T) \sim \exp(-E_B/k_BT)$, transitions between different network phases can also be achieved by a variation of the temperature.[21,40] For an effective remodelling of the actin cytoskeleton it is essential that the unbinding rates of cross-linking proteins from actin filaments are in the msec regime. This avoids the necessity of complete

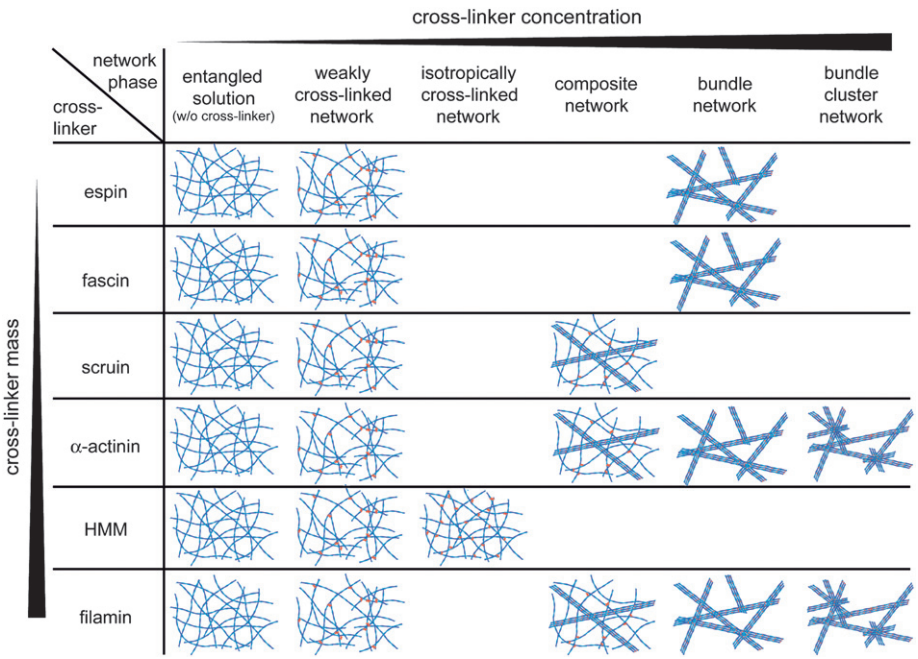

Fig. 2 In reconstituted actin networks a pronounced structural polymorphism is observed. The detailed architecture of a cross-linked actin network is set both by the type and concentration of the cross-linking molecule. At low cross-linker concentrations a generic weakly cross-linked phase occurs while relatively high cross-linker concentrations are required to induce a structural transition to an isotropically cross-linked, bundled or composite phase. At very high cross-linker concentrations clusters of actin bundles are observed for long and flexible cross-linking molecules. In contrast, small cross-linkers tend to form homogeneous purely bundled phases.

destruction/depolymerization of individual actin filaments and thus facilitates intracellular transport, cell division or locomotion.

The binding affinity of cross-linking proteins is often also sensitive to specific chemical stimuli.[41,42] Such stimuli may make it possible to switch between different network architectures and organize F-actin into highly localized mesoscopic structures. For some ABPs differences in binding affinity can be regulated by calcium binding[43] In the α-actinin superfamily many actin binding proteins contain a calcium binding EF domain—in the Ca^{2+} bound form actin binding is inhibited. The high histidine content of *dictyostelium discoideum* hisactophilin causes the binding of this protein to F-actin to be pH sensitive.[44,45] More than 20 proteins are known to affect the F-actin binding affinity of filamin. Interaction with these proteins may divide filamins into distinct subpopulations that can either adapt dynamically or are well anchored to the F-actin.[46] However, it is important to note that measuring the ABP binding affinities is extremely difficult as the results depend strongly on the experimental conditions.

Given the amount of different ABPs available, the structural polymorphism of reconstituted actin networks containing only a single ABP species is already pronounced. Yet, even more complex structures can be obtained when several ABP species are used simultaneously. It is still an open question which parameters and ABP properties decide if two cross-linking molecules modify the organization of an actin network independently or synergistically.[47,48]

The activity of molecular motors can additionally affect the phase behavior of F-actin networks.[49,50] Motor proteins such as myosin II can exert force on the F-actin network microstructure in a concerted manner and can thus actively induce fast transitions between ordered and disordered phases.[51,52] This could be an especially important mechanism, as large ABPs at sufficiently high concentrations cause F-actin networks to become kinetically trapped.[53,54]

In the absence of active molecular motors, *in vitro* actin networks are generally considered to represent equilibrated structures. However, it has recently been demonstrated that some *in vitro* actin networks build up internal stress during polymerization[53] while others become trapped in metastable configurations that can be cured by temperature treatment.[54] These findings support the idea that while some reconstituted actin networks are indeed well equilibrated, this is not necessarily the case for all ABPs or networks formed by polycations.[13] It is an important challenge for future investigations to further characterize and categorize cross-linked actin networks in terms of their putative out-of-equilibrium properties and to identify their detailed growth conditions and structure formation mechanisms.

So far we have discussed the influence of the cross-linkers on the overall organization of actin networks. Yet, the architecture of the cytoskeleton and thus its viscoelastic properties are also tuned on the level of single bundles.[55,56] The bending rigidity of an F-actin bundle is determined by the bundle dimensions and the competition between cross-linker shearing and F-actin extension/compression.[25,57] As a result, three regimes of mechanical response can be discriminated.[57] First, in the decoupled bending regime the constituent actin filaments bend independently because intervening cross-links do not resist shear. Second, in the fully coupled bending regime the filaments are rigidly held together by intervening cross-links that strongly resist shear. Third, there is an intermediate regime in which the bending rigidity of the bundle is determined by the shear stiffness of the cross-linking protein. The existence of these different bending regimes has direct implications for the buckling and entropic stretching behavior of F-actin bundles.[57] Moreover, these considerations give insight into how the material properties of the cytoskeleton impose mechanical limitations on the design of cytoskeletal processes.[58]

Surprisingly, one of the parameters that determines the bending rigidity of a bundle, the number of constituting filaments, is often well defined and limited in reconstituted actin bundles.[26,59] This may mean that reconstituted actin bundles represent kinetically trapped structures.[60] The limited size could, however, also result from a build-up of in plane shear elastic stresses.[61,62] For actin bundles cross-linked by fascin the energetic tradeoff between filament twisting and cross-linker binding has been put forward as a mechanism by which the bundle size can be adjusted.[26] By using more than one ABP cells possibly overcome this physical thickness limitation.[26] A second bundling protein may be necessary to integrate pre-existing small bundles into thicker ones or to improve the packing order in thick but disorganized bundles. Additional proteins may also be introduced into well organized bundles to lock the structure.[63,64]

B. Correlation of network microstructure and the viscoelastic network response

Changes in the F-actin network microstructure are generally associated with transitions in mechanical response regimes (Fig. 3A).[27,29,33,54,65–67] An increase in ABP concentration not only alters the structure of an F-actin network but can also enhance its elastic response up to 1000fold.[65,68] Generically, the plateau elasticity G_0 of an F-actin network at very low ABP concentrations is comparable to that of an F-actin solution without cross-linkers.[20,69] Only above a critical cross-linker concentration is a strong increase in G_0 with increasing ABP concentrations observed. The critical cross-linker concentration is found at an ABP/actin ratio $R = c_{ABP}/c_{actin}$ for most ABPs studied so far and coincides with the formation of bundles or composite phases.[23,27,29,33,54]

The effectiveness of a given APB in terms of fortifying the elastic network response is typically expressed by a scaling exponent x. This exponent is determined by fitting a power law $G_0 \sim R^x$ to the different mechanical response regimes—which is a difficult and critical approach, as these regimes rarely span more than one order of magnitude in the relative cross-linker concentration R. The scaling exponent changes with each structural transition and covers a range of $0.1 \leq x \leq 2$.[23,27,65,70] A quantitative understanding of these scaling exponents and their relation to the network microstructure is still in its infancy. This is mainly due to the fact that the experimentally obtained scaling relations classifying the macroscopic network response result from a combination of various microscopic parameters—and many of them are difficult to determine.

For a detailed description of the elastic response not only the network structure but also the micromechanical properties of its constituents have to be known. Moreover, one needs to

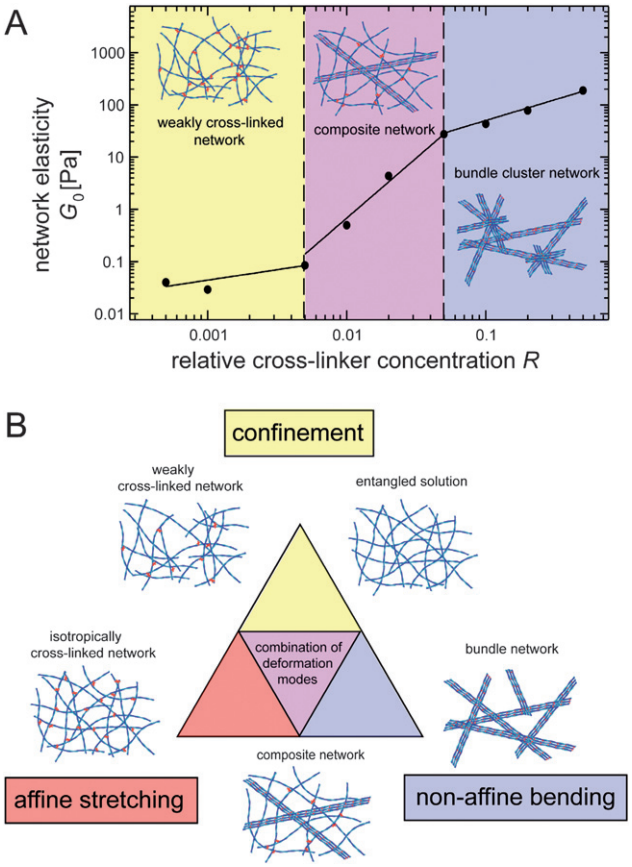

Fig. 3 (A) Transitions between different structural phases are always accompanied by transitions between different mechanical response regimes. These different response regimes can be visualized best by analyzing the plateau elasticity of the network, G_0, as a function of the relative cross-linker concentration, $R = c_{ABP}/c_{actin}$. For instance, the cross-linker α-actinin creates three distinct mechanical response regimes which correlate with a weakly cross-linked phase, a composite network phase and a bundle cluster phase. (B) The occurrence of different structural phases has far reaching physical consequences: the detailed network microstructure sets the local deformation mode and thus also the macroscopic network elasticity: entangled actin solutions and weakly cross-linked actin networks can be well understood in terms of confinement effects which are induced by surrounding actin filaments. At higher cross-linker concentrations, however, either affine stretching deformations or non-affine bending deformations have to be considered. For composite networks these two microscopic deformation modes might be equally important in determining the macroscopic network elasticity.

understand how stresses and strains are transmitted through the networks. Such detailed information on the local deformation field is difficult to acquire.[71] A major complication arises from the fact that—in contrast to flexible polymers—semi-flexible biopolymers such as F-actin are anisotropic and show a different response to forces perpendicular (bending) or parallel (stretching/compression) to the mean contour.[72] This anisotropy of the filaments in combination with complex heterogeneous network structures may cause local or mesoscopic deviations from a simple affine deformation field. Current theoretical modeling of the elasticity of both entangled and cross-linked semi-flexible polymer networks is based on assumptions how, and on which scale, the macroscopic deformation field translates to the highly anisotropic individual network constituents.[72–78]

So far three different approaches have been established to rationalize the elastic response of different types of F-actin hydrogels (Fig. 3B). The elastic modulus G_0 of entangled actin solutions lacking any cross-linker molecules can be described by the partial suppression of filament fluctuations by surrounding polymers.[79–81] While this confinement effect seems to also be a reasonable approximation for G_0 of the generic weakly cross-linked network phase at low cross-linker concentrations, different modelling approaches have to be employed if bundles are formed[23,27] or if the average distance between cross-linking points becomes smaller than the F-actin persistence length of 15 μm.[20,70]

In isotropically cross-linked networks affine stretching deformations seem to be predominant[70,73] while in purely bundled biopolymer networks non-affine bending deformations were proposed to determine the network elasticity.[23,72,82,83] Only in cases where the network architecture is simple and homogeneous—either purely bundled or purely single filaments—is it possible to directly relate model parameters to the experimentally observed network microstructure and the resulting network elasticity. In more heterogeneous networks it is still unclear which deformation mode sets the macroscopic and local elastic network response. One might speculate that in composite networks, which combine structural elements of both filamentous networks and bundles, the mechanics of single bundles and their density would decide if affine stretching or non-affine bending dominates or if both deformation modes are equally important.

The viscoelastic response of the biologically so important heterogeneous actin networks still poses a major challenge for experiment and theory alike, especially considering that the network microstructure sets the local deformation mode and with that the micro- and macroscopic elastic response of cross-linked actin networks. Determining and quantifying the network microstructure is a difficult task involving mainly microscopy and microrheology techniques. For successful modelling one will need to find suitable algorithms to produce such networks in a reliable manner—which becomes increasingly difficult if one needs to consider the growth conditions and history of kinetically trapped structures.

Besides determining the network organization and with that the local deformation mode and the plateau elasticity of the network, cross-linking proteins also affect the dynamic viscoelasticity of actin networks (Fig. 4).[24,84,85] At high frequencies, the network response originates from the longitudinal fluctuation modes of effectively independent polymers, *i.e.* individual actin filaments or bundles.[86,87] This high frequency behavior is therefore thought to be virtually identical in entangled and cross-linked F-actin solutions.[88]

The intermediate frequency regime between the high frequency crossover of both viscoelastic moduli and the rubber plateau (*i.e.* typically between 0.01 Hz and 100 Hz) is less well investigated—although the relevant time scales in this regime are probably of biological importance. It has recently been demonstrated that at low frequencies, *i.e.* in the mHz regime, the finite lifetime of the cross-linking molecules kicks in and dominates the viscoelastic response—especially the dissipation mechanism (Fig. 4B).[24,40,89]

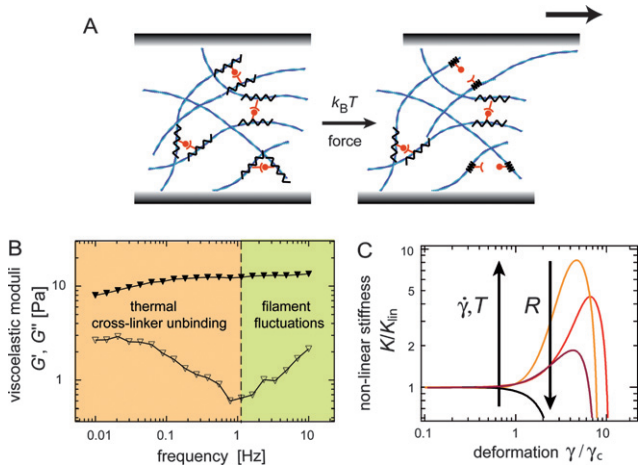

Fig. 4 (A) Actin networks possess transient cross-links which can unbind both in thermal equilibrium and under force. The stretched and relaxed springs indicate actin filaments in a cross-linked (elastic energy stored) and unbound (elastic energy released) configuration. (B) Viscoelastic frequency spectrum of an isotropically cross-linked actin/rigor-HMM network: thermal cross-linker unbinding entails local stress relaxation and triggers a loss in elasticity as well as a dissipation mechanism—both of which become increasingly important with increasing cross-link concentration. The time scale of the thermally activated cross-linker unbinding process is set by the microscopic off-rate of the cross-linking molecules which is typically in the mHz regime. At higher frequencies thermal fluctuations of distinct actin filaments or bundles dominate the viscoelastic frequency spectrum of cross-linked actin networks. (C) The non-linear stiffness of actin networks is highly sensitive to enforced cross-linker unbinding events. The detailed shape of the non-linear response is set by various parameters such as the relative cross-linker concentration R, the loading rate $\dot{\gamma}$ and the ambient temperature T.

With the natural crosslinker off- and on-rates, k_{off} and k_{on}, individual cross-linkers un- and rebind in thermal equilibrium. This cross-linker unbinding is an important stress release mechanism which entails a drop in the network elasticity and increases the viscous dissipation with decreasing frequency. Clearly, this characteristic of thermal ABP unbinding becomes more pronounced with increasing degree of network interconnectivity—thus being most pronounced in the case of isotropically cross-linked F-actin networks.[24,70]

The detailed structure of the microscopic interaction potential between actin filaments and cross-linking molecules dictates the response in this biologically relevant frequency regime. Besides the off-rate the binding energy and the position of the transition state ('bond length') influence the dynamic viscoelastic network response. Vice versa these key parameters of the interaction potential can—for simple network geometries—be obtained from macrorheological measurements[40] utilizing a simple semi-phenomenological model which is predicated on single cross-linker unbinding events.[24] Importantly, the transient nature of the cross-links guarantees structural and mechanical adaptability at long time scales and ensures an elastic network response at time scales that are short compared to the cross-linker off-rate.

In turn, the mechanical stability of cross-linked actin networks is limited by the strength and lifetime of the actin/cross-linker bond.[69] At high (external or internal) forces, the physical bond between a cross-linker and attached actin filaments is forced to unbind.[90–92] As a result, the interconnections in the network yield which may result in a redistribution of stresses. This can entail a complete failure of the network structure or permanent plastic deformations.

Although this forced unbinding might require rebinding of the cross-linker in order to preserve the network microstructure upon stress release it protects the actin filaments from rupturing. The forced unbinding of cross-linking proteins depends on the force loading rate[93,94] which might provide a mechanism that cells harness for mechanosensing: unbinding will result in the presentation of formerly unavailable binding sites, which in turn could trigger a signaling cascade.

Before yielding, the elastic response of cross-linked or bundled actin networks becomes non-linear. The degree and type of non-linearity (strain or stress hardening or weakening) can depend on many parameters (Fig. 4C): the network microstructure[23,66,70,95,96] and the cross-linker density R[23,24,65,70,97] play important roles in the nonlinear response. Purely or predominantly elastic networks are independent of the applied loading rates and thus their non-linear response can be successfully probed by various techniques. For more viscous networks, especially in frequency ranges where the cross-linker dynamics kicks in, or for entangled solutions it has turned out that viscous flow cannot be neglected and the network response strongly depends on both the loading rate and the detailed measurement protocol[32,69,98] as well as on the ambient temperature T.[99] Moreover, it was suggested that cross-linker unbinding,[69] stretching[100] or even unfolding[101,102] might add additional compliances to the mechanical response of F-actin networks under load which could further complicate the non-linear mechanics of F-actin networks. Thus, it remains quite a challenge to separate elastic, viscous and plastic contributions to the stress response in the different methods.

The many parameters involved have so far prevented the elucidation of the molecular origin of this complex non-linear behavior. A generic model for the non-linear network response would have to account for the microscopic differences in the network structures as well as for the subtle interplay of cross-linker unbinding events and the persistence length of actin filaments and bundles.

While the physical understanding of *in vitro* systems has progressed considerably, there is still a lot to do to approach the complexity found in nature. We are now in the position to start addressing more complicated systems, where not only mixtures of cross-linkers but also mixtures of different filament types found in the cytoskeleton can be studied and quantified. The controlled disassembly of complex cytoskeletal networks by molecular motors or depolymerization agents is another task which is yet to be achieved in *in vitro* model systems. In this context the finite life time of the cross-linking molecules will allow modulation of the conflicting needs of adaptability *vs.* mechanical stability in order to match the viscoelastic network properties to the changing needs of living cells. Which of these effects will dominate *in vivo* and how cells can control these mechanisms on a local scale will need much more attention in future studies.

This work was supported by Deutsche Forschungsgemeinschaft through the grant BA2029/7 and the DFG-Clusters of Excellence Nanosystems Initiative Munich (NIM)

and Munich-Centre for Advanced Photonics (MAP). O. Lieleg acknowledges a postdoc fellowship from the German Academic Exchange Service (DAAD).

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