Filament length tunes elasticity in flexibly crosslinked actin networks


CHAPTER 4. FILAMENT LENGTH TUNES ELASTICITY IN ACTIN-FILAMIN GELS

Abstract

Networks of the cytoskeletal biopolymer actin crosslinked by the compliant protein filamin form soft gels that stiffen dramatically under shear stress. We demonstrate that the elasticity of these networks shows a strong dependence on the mean length of the actin polymers. This behavior is in agreement with a model of rigid filaments connected by multiple flexible linkers (see chapter 3). This model allows us to estimate loads on individual cross-links, which we find to be less than 10 pN. We contrast the filament length dependence we observe in actin-filamin gels with the behavior of actin networks with short rigid linkers.

4.1 Introduction

The actin cytoskeleton is a composite intracellular biopolymer network. To tune the mechanical properties of the cytoskeleton for such diverse processes as cell division, locomotion, and shape change, a large number of actin binding proteins organize network structure [1]. Nucleating and capping proteins regulate the polymerization of monomeric actin into filamentous actin (F-actin). Crosslinking proteins bind the actin filaments together to form elastic gels or bundle structures, such as in stress fibers and filopodia. Motor protein assemblies control tension within the networks by pulling on actin filaments crosslinked to the network [2–4]. Even though the important molecular components are known, relatively little is understood of how this large ensemble of proteins collectively contributes to the mechanical response of the cytoskeleton.

Investigating the origins of the mechanical response of the complex and composite structure of the cytoskeleton presents a major challenge in biophysics. One approach has been to study reconstituted in vitro F-actin networks in the presence of purified binding proteins [3, 5–9]. Reconstituting the network allows precise control of its chemical composition and systematic investigation of its properties. A ubiquitous feature of these networks is that they stiffen strongly with increasing applied shear [5, 10]. When F-actin is crosslinked by small rigid crosslinks, the stiffening arises from the properties of the filaments themselves. F-actin is a semiflexible polymer with a persistence length of 17 µm [11]. Thermal bending undulations in the F-actin give rise to a decrease in its end-to-end distance; application of a force stretches out these undulations. For small extensions the force is proportional to the extension, whereas for large extensions approaching the contour length, the force diverges, leading to strain-stiffening [12]. Both the linear and nonlinear network elasticities are consistent
with the theoretical predictions for a network of semiflexible polymers, provided the
deformation is affine \([5,7,13]\). However, this picture of network mechanics implicitly
assumes that the elasticity is controlled by one component, the actin filaments. It
ignores any contribution of the crosslinking proteins; these can be both large and
compliant, and therefore can themselves contribute to the elasticity.

One example of a large and flexible crosslink is filamin (Fig. 4.1B), which is
abundant in cells. Filamin crosslinks F-actin into orthogonal networks in the cortex,
connects F-actin to integrins, and may play a role in mechanotransduction \([14–18]\).
Reconstituted actin networks with filamin can form isotropic networks mimicking
the actin cortex of living cells, as shown in Fig. 4.1A,C, or (partially) bundled net-
works at high filamin concentrations (Fig. 4.1D,E). Actin-filamin networks exhibit a
mechanical response that is qualitatively different from networks formed with rigid
crosslinks \([6,8,19,20]\). Filamin-F-actin networks are compliant, weakly elastic solids.
Nevertheless they can support large shear stresses because of their pronounced non-
linear strain-stiffening. Their nonlinear behavior is inconsistent with predictions for
an affinely deforming network with rigid crosslinks \([5,6,20]\). In comparison to net-
works with rigid crosslinks, networks crosslinked by filamin exhibit mechanical prop-
erties that more closely mimic the properties of cells \([3,6,19]\). Recent experimental
reports show that the unusual nonlinear elasticity of these networks is consistent
with a model of rigid polymers connected by multiple flexible crosslinks \([20–22]\).
The rigid polymer of length \(L\) constrains the deformation profile of the \(n\) flexible
crosslinks bound along its length, as illustrated in Fig. 4.2; thus, polymer length is
predicted to be an important parameter controlling the linear and nonlinear proper-
ties of the network \([22]\). Indeed, the linear viscoelasticity and rupture stress of F-actin
networks crosslinked by filamin are sensitive to the addition of gelsolin \([20,23]\), an
actin capping and severing protein that shortens the average filament length. While
these previous data support the view that F-actin length affects the rheology of these
networks, fully elucidating the physical principles of this mechanism demands a more
systematic investigation of the linear and nonlinear behavior of filamin-gelsolin-F-
actin networks.

In this chapter, we investigate the mechanical response of networks of F-actin
cross-linked by filamin as we systematically decrease \(L\) by adding gelsolin. Using bulk
rheology we show that the linear modulus increases proportional to \(L^2\). The critical
strain, which marks the onset of stiffening, decreases with increasing \(L\). In the non-
linear regime, the maximum stress before breaking is proportional to \(L\). These results
are contrasted with the rheology of networks formed with rigid crosslinks to demon-
strate that these behaviors are unique features of the filamin-F-actin system. Thus, we
show that the linear and nonlinear elastic behavior of F-actin crosslinked by filamin
is indeed tuned by varying \(L\), in a manner that is consistent with the theoretical pre-
dictions for a network of stiff polymers connected by flexible linkers (see chapter 3).
Figure 4.1 – (a) Electron micrograph of a fixed and rotary-shadowed filamin-F-actin network at $c_A = 1 \text{ mg/ml}$, $L = 15 \mu m$, and $R_F = 0.005$. Scale bar= 100 nm. (b) Electron micrograph of rotary-shadowed filamin molecules. Scale bar= 50 nm. (c)-(e) Confocal images of various networks. Scale bar= 5 $\mu m$. $c_A = 0.5 \text{ mg/ml}$ with $L = 15 \mu m$ and (c) $R_F = 0.002$ or (d) $R_F = 0.01$, or (e) $L = 1 \mu m$ and $R_F = 0.04$. 
4.2 MATERIALS AND METHODS

The model quantitatively explains the dramatic nonlinear stiffening of filamin-F-actin networks, providing fundamental insight into its origins. In addition, this model provides an estimate of the maximum load experienced by individual filamin cross-links, which is less than 10 pN, too small to result in significant unfolding of filamin Ig-like domains.

4.2 Materials and methods

4.2.1 Proteins

We purify monomeric (G) actin from rabbit skeletal muscle [24], followed by gel-filtration (HiLoad 16/60 Superdex 200pg, GE Healthcare). Aliquots of purified G-actin in G buffer (2 mM Tris HCl, 0.2 mM ATP, 0.2 mM CaCl2, 0.2 mM DTT, 0.005% NaN3, pH 8.0) are frozen in liquid nitrogen and stored at −80°C. Recombinant human filamin A is purified from Sf9 cell lysates [25]. Recombinant human plasma gelsolin is purified [26] or purchased (Biogen, Cambridge, MA). For rigidly crosslinked networks, we incorporate biotinylated actin monomers (Cytoskeleton, Denver, CO) that can be crosslinked by NeutrAvidin protein (Pierce, Rockford, IL).
4.2.2 Network formation

We form networks with an actin concentration, \( c_A = 0.5 \) mg/ml, unless otherwise noted, and control network microstructure by varying the molar ratio of filamin dimers to actin monomers, \( R_F \). We regulate the actin filament length distribution with gelsolin. The molar ratio of gelsolin to actin monomers, \( R_G \), sets the mean actin filament length \([27]\). Samples are prepared by mixing solutions of 10x polymerization buffer (20 mM Tris-HCl, 20 mM MgCl\(_2\), 1 M KCl, 2 mM DTT, 2 mM CaCl\(_2\), 5 mM ATP, pH 7.5), gelsolin, filamin, and G-actin.

For rigidly crosslinked networks, biotinylated actin monomers are incorporated in actin filaments at a molar ratio of biotinylated G-actin to non-biotinylated G-actin, \( R_B \). Crosslinking is mediated by NeutrAvidin protein. Samples are prepared by mixing 10x polymerization buffer, gelsolin, biotinylated G-actin, and G-actin. After 3 min NeutrAvidin at a 1:1 molar ratio to biotinylated actin is gently mixed in.

The sample is loaded into a microscopy chamber, consisting of two cover slips with a 1 mm spacer, or between rheometer plates and polymerized for 1 hour at 25°C.

4.2.3 Characterization of f-actin length distribution

To characterize the actin filament length distribution, we polymerize 0.3 mg/ml F-actin in the presence of gelsolin. After 1 hour, the filaments are labeled and stabilized with a 1:1 molar ratio of Alexa-488 phalloidin and incubated at 25°C for 30 min. The filaments are diluted to a concentration of 2 nM, and 5 \( \mu \)L of the suspension is pipetted onto a cover slip functionalized with poly(acrylamide-co-diallyldimethylammonium chloride). A second coverslip is placed on top and the sample sealed. Nearly all filaments stick to the coated coverslip. Immobilized filaments are imaged using a confocal microscope (Leica SP5); image pixel size is 160 nm. Filament contour lengths, \( l \), are measured manually in ImageJ; the minimum distance measurable using this method is 0.5 \( \mu \)m. For each gelsolin concentration, the width of the distribution of filament lengths is nearly equivalent to the mean (data not shown).

For network formation, we polymerize F-actin in the presence of gelsolin and filamin. In previous work, addition of alpha-actinin to gelsolin-regulated F-actin narrowed the width of the length distribution without significantly affecting the mean length \( L \) \([28]\). Similarly, we expect that filamin should not significantly change the values of \( L \) we measure here.

4.2.4 Imaging

For confocal microscopy, samples are fluorescently labeled by polymerizing in the presence of 0.6 \( \mu \)M Alexa-488 phalloidin and examined (Leica, TCS SP5). For transmission electron microscopy a 10 \( \mu \)L drop of assembled network is applied to a 400
4.2. MATERIALS AND METHODS

Figure 4.3 – Mean F-actin length $L$ as a function of the molar ratio of actin to gelsolin, $R_G^{-1}$. $L$ decreases from its unregulated value (dashed line) as gelsolin is added. The solid line denotes linear scaling.

mesh carbon-coated nickel grid and incubated for 30 s, stained with 1% uranyl acetate, rinsed by passing a drop of distilled water over the grid, air dried, and imaged (JEOL 2100).

4.2.5 Rheology

We use a stress-controlled rheometer with 40-mm stainless steel parallel plates and a 160 µm gap (AR-G2, TA Instruments or C-VOR, Bohlin Instruments). We polymerize samples in situ and use a solvent trap and apply a thin layer of low viscosity mineral oil around the sample to minimize evaporation. We confirm that the results are independent of gap and reproducible within and between different protein preparations.

The linear viscoelastic response is measured by applying a frequency-dependent, sinusoidal stress, $\sigma \sin(\omega t)$, and measuring the strain, $\gamma \sin(\omega t + \delta)$. We maintain $\gamma < 2\%$ to ensure linear response. The elastic modulus is

$$G'(\omega) = \frac{\sigma}{\gamma} \cos(\delta); \quad (4.1)$$

the viscous modulus

$$G''(\omega) = \frac{\sigma}{\gamma} \sin(\delta). \quad (4.2)$$
We measure the response in the nonlinear regime with a differential or "prestress" measurement; a small amplitude oscillatory stress, $\delta \sigma$, is superposed on a steady prestress, $\sigma_0$, to measure the differential modulus,

$$K^*(\sigma_0, \omega) = \frac{\delta \sigma}{\delta \gamma} \bigg|_{\sigma_0}. \quad (4.3)$$

The elastic and viscous components are $K'$ and $K''$, respectively. We confirm there is no time dependence in $K'$ at various levels of prestress and minimal hysteresis in $K'(\sigma_0)$ (see chapter 5), as shown in Fig. 4.4. In a complementary strain ramp approach, we increase the strain at a fixed rate and measure the resulting stress. Both $\sigma(t)$ and $\gamma(t)$ are smoothed using a cubic spline algorithm to compute the differential modulus

$$K = \frac{d \sigma}{d \gamma}, \quad (4.4)$$

by applying a numerical derivative to the stress-strain curve.

### 4.3 Results and discussion

#### 4.3.1 F-actin length distribution in the presence of gelsolin

Within the cell, the contour lengths, $\ell$, of actin filaments are highly regulated. Typical lengths range from a hundred nanometers to a few microns [29, 30]. In vitro, high enough concentrations of pure monomeric actin will polymerize spontaneously in the presence of divalent salt and ATP. Yet, these in vitro filaments are typically much longer than those in the cell, with contour lengths that can be up to 50 $\mu$m (data not show).

To better mimic the conditions in cells, we use the F-actin capping and severing protein gelsolin to vary the mean length, $L = \langle \ell \rangle$, of our in vitro actin filaments. To characterize the filament length distribution in the presence of gelsolin, we image a diluted sample of F-actin stabilized with fluorescent phalloidin. For the ratio of gelsolin to actin, $R_G = 0$, the unregulated F-actin has a mean length of $L = 14.8 \mu$m. Upon adding a small amount of gelsolin, $R_G = 1 : 3700$, the length distribution is dominated by the presence of the gelsolin and $L$ decreases to 10.4 $\mu$m. Increasing $R_G$ decreases $L$ further. We find that $L$ scales linearly with $R_G^{-1}$, as shown in Fig. 4.3; it varies as $L = (330R_G)^{-1}$, with $L$ measured in microns. This is consistent with a model where each gelsolin molecule associates with one actin filament. Each actin monomer adds 2.7 nm to the filament length [31], so that one micron of filament is composed of 370 monomers, predicting $L = (370R_G)^{-1}$. Some inactivation of gelsolin during storage may account for the slightly larger observed filament lengths compared to the prediction. These findings are consistent with previous studies of actin filament length distributions [27, 28, 32].
Figure 4.4 – (Color online) (A) To measure the nonlinear differential elastic response at a particular prestress, $\sigma_0$, a small, oscillatory stress is superposed on a static stress $\sigma_0$ and the resulting oscillatory strain is measured. (B) For a typical actin-filamin network with $c_a = 0.5 \text{ mg/ml}$, $R_p = 0.005$, and $L = 15 \mu m$ we show that there is no time dependence in $K'$ at various levels of prestress $\sigma_0$, which are shown in panel (C). $\sigma_0$ can be increased to just below the maximum stress supported by the network and decreased again with minimal hysteresis in the differential elastic modulus, $K'(\sigma_0)$. 

$K'(\text{Pa})$

$\sigma_0 (\text{Pa})$

$0.01 \quad 0.1 \quad 1$

$1 \quad 10 \quad 10$

$\text{Time (s)}$

$100 \quad 200 \quad 300 \quad 400 \quad 500$
4.3.2 Microstructure of filamin-gelsolin-F-actin networks

We form in vitro networks of actin filaments whose lengths are regulated with gelsolin and which are crosslinked by filamin. In vitro, filamin efficiently crosslinks F-actin into orthogonal networks, which are soft but support large stresses [6,14]. These networks mimic several key features of cell mechanical properties [3,6,19]. The microstructure of these networks varies as we change $L$ and the molar ratio of filamin to actin, $R_F$.

For $R_F \lesssim 0.01$ the networks are a homogeneous mesh of F-actin as seen by electron microscopy (Fig. 4.1A) and confocal microscopy (Fig. 4.5A and B). For $R_F > 0.01$ large bundles appear within the mesh (Fig. 4.5C-D). The value of $R_F \approx 0.01$ above which bundles appear is roughly independent of $L$ [20]. From electron microscopy, the bundles appear as loose, branching structures with diameters $\sim 100$ nm (Fig. 4.5C, inset). These observations are consistent with reports for networks with filamin from chicken gizzard [23,33,34]. We confirm this bundling transition by tracking the thermal motion of particles within the networks (data not shown). Varying $L$ has little effect on the visual appearance of the non-bundled networks (Fig. 4.5A-B). However, in the bundled networks, F-actin partitions more readily into the bundles at high $R_G$, forming networks of pure bundles without a background F-actin mesh, as visible in confocal microscopy (Fig. 4.5D) or detectable by particle tracking. This may be due to increased diffusion and decreased entanglements for shorter filaments, allowing them to more easily associate into bundles [33].

4.3.3 Linear response

To probe the mechanical properties of the filamin-gelsolin-F-actin networks, we use a stress-controlled rheometer. For an actin concentration $c_A = 0.5$ mg/ml and $L = 15$ $\mu$m, a weakly crosslinked network having $R_F = 0.001$ is a soft, viscoelastic solid (Fig. 4.6A, squares). The elastic modulus $G'$ is two- to three-fold larger than the viscous modulus $G''$, and $G'(\omega)$ increases as a weak power-law with the frequency, $\omega$, over a broad frequency range. This network is only slightly stiffer than purely entangled actin (triangles). Increasing $R_F$ further to 0.01 only modestly increases $G'$ and has little impact on the frequency response. This is in contrast to F-actin with rigid crosslinking induced by addition of NeutrAvidin to networks with a small fraction, $R_B$, of biotinylated actin monomers incorporated into the F-actin. Increasing $R_B$ leads to a drastic increase in the stiffness of the network, as shown in Fig. 4.6B; this is accompanied by a decrease in the slope of the weak power-law frequency response of $G'(\omega)$, consistent with more solid-like behavior.

In the filamin networks, as we systematically decrease the mean filament length $L$ from 10 to 2 $\mu$m by adding increasing amounts of gelsolin, $G'$ decreases from 1 to 0.2 Pa (Fig. 4.6C). For rigidly crosslinked networks, $G'$ also decreases with $L$ (Fig. 4.6D). For both types of crosslinks, the slope of $G'(\omega)$ does not vary drastically...
Figure 4.5 – Microstructure of filamin-gelsolin-F-actin networks. (A-B) For $R_F \lesssim 0.01$ networks are a homogeneous mesh of F-actin. (C-D) Large bundles are present at high $R_F$. A) Confocal image, $R_F = 0.002$, $R_G = 0$ ($L = 15 \mu m$). B) Confocal image, $R_F = 0.01$, $R_G = 1 : 370$ ($L = 1 \mu m$). C) Confocal image, $R_F = 0.01$, $R_G = 0$. Inset: TEM image. D) Confocal image, $R_F = 0.04$, $R_G = 1 : 370$. Inset: Confocal image at lower magnification to show network connectivity. Scale bars are 10 $\mu m$ for confocal images and 0.5 $\mu m$ for TEM image.
Figure 4.6 – (Color online) Linear viscoelasticity of crosslinked F-actin networks. Elastic moduli $G'$ (solid) and viscous moduli $G''$ (open). Filamin crosslinked networks are soft, viscoelastic solids that become stiffer with increasing $R_F$ or $L$: A) $L = 15 \mu m$ with various $R_F$ and C) $R_F = 0.01$ with various $L$ (in $\mu m$). Rigidly crosslinked networks become stiffer with increasing $L$ and significantly stiffer and more solid-like with increasing $R_B$: B) $L = 15 \mu m$ with various $R_B$, and D) $R_B = 0.01$ with various $L$.

with $L$.

4.3.4 Dependence of the modulus on filament length

To quantify the changes in the elasticity of these networks as we decrease $L$, we plot $G_0$, defined as $G_0 = G'\big|_{\omega=0.1 \ \text{Hz}}$, as a function of $L$ (Fig. 4.7A). For $R_F = 0.001$, $G_0$ is 0.2 Pa for the networks with the shortest filaments, $L = 1 \sim 2 \ \mu m$. As we increase $L$ to 15 $\mu m$, $G_0$ increases to 0.5 Pa. For increasing values of $R_F$, $G_0$ starts out at roughly the same value for short filaments, but increases more strongly with $L$. Interestingly, at each $R_F$, $G_0$ increases stronger than linearly with $L$.

This strong dependence on $L$ is not expected from an affine theory [12] that has been used to describe the linear and nonlinear elasticity of actin crosslinked with point-like rigid crosslinks such as heavy meromyosin and scruin [5, 7, 13]. In this
theory network elasticity is governed by the thermal compliance of the semiflexible F-actin polymers; thermal fluctuations of the F-actin get stretched out as the network is deformed \([12]\). This model predicts:

\[
G_0 = 6\rho k_B T \frac{\ell_p^2}{\ell_c^3}
\]  

(4.5)

where \(\rho\) is the linear density of polymer, \(k_B\) is Boltzmann’s constant, \(T\) is the temperature, \(\ell_p\) is the persistence length of F-actin, and \(\ell_c\) is the distance between crosslinks. Thus, in this theory the network elasticity is controlled by the distance between crosslinks rather than the length of the actin filaments, in disagreement with our results for filamin-F-actin.

Alternatively, the elasticity of our networks can originate from the compliant nature of the filamin crosslinks. The large 160 nm chain between the actin binding domains of a filamin protein is quite flexible and can be modeled as a linear polymer with \(\ell_p = 20\) nm \([35]\). As a result, a filamin crosslink is soft compared to an F-actin segment of length \(\ell_c\), which ranges from 0.3 to 2 \(\mu\)m. This suggests that the compliance of the network is governed by the flexible crosslinks. Thus, we propose a model in which the actin polymers are treated as rigid rods linked by many flexible linkers (see chapter 3), as depicted in Fig. 4.2A. When the network surrounding a rigid rod is deformed, the linkers get stretched by an amount that increases linearly in the distance from the center of the rod, as shown in Fig. 4.2C. Provided the network deformation is uniform on the length scale of \(L\):

\[
G_0 = \frac{1}{8} \rho nkL \sim R_F L^2,
\]  

(4.6)

with \(k\) the stiffness of the flexible crosslinks and \(n\) the average number of crosslinks per actin filament \([22]\). The explicit \(L\) dependence arises as a direct result of the non-uniform deformation profile of the crosslinks. The average number of crosslinks per actin filament is proportional to both \(R_F\) and \(L\) and is given by \(n = 370R_F L\); thus, the overall prediction is that \(G_0\) will increase proportional to \(R_F L^2\).

To test this mechanism, we plot \(G_0\) as a function of \(R_F L^2\) (Fig. 4.7C). The data for different crosslinking densities collapses onto a single curve. For \(R_F L^2 \geq 0.1\), \(G_0\) scales nearly linearly with \(R_F L^2\), consistent with the prediction. This supports the model of crosslink dominated elasticity. Below \(R_F L^2 = 0.1\), which corresponds to \(n = 7\) crosslinks for a 5 \(\mu\)m filament, the values of \(G_0\) are roughly equivalent to the elasticities we measure for F-actin solutions in the absence of crosslinking, as shown by the gray bar in Fig. 4.7C. This suggests that the linear elasticity of these weakly crosslinked networks is dominated by the solution elasticity, not by the crosslinks. The threshold of \(R_F L^2 = 0.1\) corresponds to typical physiological conditions \((L = 2 \mu m, R_F = 0.02)\) \([36,37]\), suggesting that by spatially or temporally regulating \(L\),
Figure 4.7 – Dependence on $L$ of the linear elastic modulus measured at a frequency of 0.1Hz, $G_0$. A) For filamin networks $G_0$ increases stronger than linearly with $L$. $R_F = 0.001$ (white), 0.002 (light gray), 0.005 (gray), 0.01 (black). B) Rigidly crosslinked networks show qualitatively different behavior. $R_b = 0.01$. C) $G_0$ for networks crosslinked with filamin at different $R_F$ collapse onto a single curve when plotted vs. $R_F L^2$ with nearly linear scaling above $R_F L^2 = 0.1$. The shaded bar represents the range of moduli measured for F-actin solutions with $2 < L < 7 \mu m$. 
cytoskeletal elasticity could be adjusted from essentially that of entangled F-actin to a network with tunable stiffness. By contrast, the dependence of $G_0$ on $L$ for the rigidly crosslinked networks is of a qualitatively different form; $G_0$ increases linearly with $L$ for small $L$ but approaches a plateau for large $L$ (Fig. 4.7B). Simulations of 2D [38] and 3D [39] stiff polymer networks reveal a dependence on $L$ qualitatively similar to our results. The departure from the plateau for decreasing $L$ in simulations has been attributed to an increase in the non-affinity in the deformation of the network, where the affine thermal theory is expected to break down.

### 4.3.5 Nonlinear response

The dependence of $G_0$ on $R_F$ and $L$ is consistent with network elasticity that is governed by the filamin crosslinks. We further test the origin of the elasticity by measuring the nonlinear elastic properties of the filamin-F-actin gels with two complementary techniques: strain ramps and prestress measurements.

**Strain ramps**

In the first approach, we increase the strain, $\gamma$, at a fixed rate and measure the resulting stress, $\sigma$. From the derivative of the stress-strain curve, $K = d\sigma/d\gamma$, we quantify the nonlinear behavior. This technique has been used to study nonlinear behavior of both entangled and crosslinked F-actin networks [40–42]. For a filamin crosslinked network with $L = 15 \mu m$ and $R_F = 0.01$, $K$ normalized by its initial value, $K_0$, is equal to 1 for small strains (Fig. 4.8A). At the critical strain, $\gamma_c = 0.06$, $K/K_0$ increases above 1, and the network begins to stiffen. It stiffens 30-fold before breaking at $\gamma_m = 0.9$. Networks with shorter filaments initially display weakening behavior, where $K/K_0$ decreases below 1, due to their lower network connectivity, but eventually stiffen. As we decrease $L$, $\gamma_c$ increases markedly, as shown in Fig. 4.8A.

By contrast, rigidly crosslinked networks with $L > 5 \mu m$ stiffen at small strains, independent of $L$ (Fig. 4.8B). Networks with $L \leq 2 \mu m$ do not stiffen and display weakening behavior. This is consistent with a transition from stiffening behavior arising from pulling out fluctuations in F-actin filaments, where

$$\gamma_c = \frac{1}{6} \frac{\ell_c}{\ell_p}$$

is set only by $\ell_c$ and $\ell_p$, to weakening behavior, where the network becomes too sparsely connected to stiffen. However, the strong dependence of $\gamma_c$ on $L$ for filamin-F-actin gels is inconsistent with such a nonlinear response arising from thermal fluctuations of the actin filaments being stretched out. We propose instead that the nonlinear response for filamin-F-actin originates from the stiffening behavior of the crosslinks. Single molecule experiments indicate that filamin proteins stiffen markedly...
CHAPTER 4. FILAMENT LENGTH TUNES ELASTICITY IN ACTIN-FILAMIN GELS

Figure 4.8 – (Color online) Nonlinear stiffening in strain ramps with a rate of 0.1s$^{-1}$. The derivative of the stress-strain curve, $K$, normalized by its initial value, $K_0$, as a function of strain. A) Filamin with $R_F = 0.01$ and $L = 15$ (short dash), 10 (long dash), 7 (dash-dot), 5 (solid), 2 (dot) µm. For the network with $L = 15$ µm, $K/K_0 = 1$ at small strains before beginning to stiffen above $\gamma_c = 0.06$. Networks with shorter filaments initially display weakening behavior, where $K/K_0$ decreases below 1, due to their lower network connectivity, but stiffen at higher strains where the slope of the curve becomes positive. $\gamma_c$ increases with decreasing $L$. B) Rigid crosslinks with $R_B = 0.01$ and $L = 10$ (dash), 5 (solid), 2 (dot) µm. Networks with long filaments display stiffening behavior that is independent of $L$, while networks with short filaments display weakening behavior. Insets: Same data plotted vs. stress.
as they are stretched towards their contour length \( \ell_0 \) [47]. When the network surrounding a rigid rod is deformed strongly, linkers bound at the ends of the polymers are stretched most, as depicted in Fig. 4.2C. These linkers will be the first to reach full extension and stiffen, setting the critical strain at which the network begins to stiffen. These end-bound linkers reach full extension at a strain [22]:

\[
\gamma_c = 4 \frac{\ell_0}{L}.
\] (4.8)

The \( L \)-dependence arises because the amount an end-bound crosslink must stretch to accommodate a given macroscopic network strain increases with the length of the rigid rod to which it is bound.

Plotting \( \gamma_c \) as a function of \( L^{-1} \) in Fig. 4.9A, the dependence of \( \gamma_c \) on \( L^{-1} \) is in stark contrast to the stiffening behavior of rigidly crosslinked networks, which display no dependence of \( \gamma_c \) on \( L \). The increase of \( \gamma_c \) with increasing \( L^{-1} \) is qualitatively consistent with the prediction of the model. We see similar behavior for \( \gamma_m \) (Fig. 4.9B), suggesting that the non-uniform deformation profile of the linkers prevails up to large strains. Interestingly, the \( \gamma_c \) data from the two systems coincide at small \( L^{-1} \) (Fig. 4.9A). In this limit of large \( L \) the model of rigid rods with flexible linkers predicts that the smallest of strains would lead to stiffening. However, this model relies on the linkers being the softest mode in the system. When the prediction for stiffening by the linkers would yield a lower \( \gamma_c \) than by the F-actin segments themselves, this picture breaks down, and it is no longer valid to assume the F-actin behave as rigid rods. In this limit, the compliance of the F-actin would contribute to the stiffening behavior of the system, consistent with our observation.

**Prestress measurements**

In our second technique for probing nonlinear response, we apply a steady prestress, \( \sigma_0 \), and probe the differential elastic modulus, \( K'(\sigma_0, \omega) \), with a small oscillatory stress. This technique has been used in crosslinked F-actin networks to study nonlinear stiffening behavior [5, 6, 13, 42]. Rigidly crosslinked F-actin networks display stiffening with \( K' \sim \sigma_0^{3/2} \) [5]; we see the same behavior for networks crosslinked by biotin-NeutrAvidin (Fig. 4.10B). This is consistent with the predictions for the affine thermal model in which the nonlinear response is due to pulling out thermal bending fluctuations in the semiflexible actin filaments within the network [5, 12].

Our model of rigid filaments connected by multiple flexible linkers predicts a different stiffening behavior that arises from the stiffening of the filamin crosslinks. The theoretical model is extended to the nonlinear regime by employing a self-consistent effective medium approach [20–22]. In this approach, the linkers are bound on one side to the rigid rod and on the other to an elastic continuum with a nonlinear elasticity that is required to self-consistently represent a uniform and isotropic collection.
Figure 4.9 – Dependence of the critical and maximum strains on $L$. Filamin with $R_F = 0.01$ (solid), rigid crosslinks with $R_B = 0.01$ (open). A) $\gamma_c$ of the filamin networks increases with increasing $L^{-1}$. In contrast, $\gamma_c$ for rigidly crosslinked networks is independent of $L$ with mean value denoted by the solid line. B) $\gamma_m$ vs. $L^{-1}$. 

86
of such elements. As the network is deformed the linkers get stretched and stiffen one-by-one as they approach full extension and start pulling back on the effective medium. This model predicts $K' \sim \sigma_0$ in the limit of a dense network.

To test this prediction, we measure $K'_{\sigma_0}$ for the networks crosslinked by filamin. For a network with $R_F = 0.003$ and $L = 15 \, \mu m$ (solid circles, Fig. 4.10A), $K'$ increases with $\sigma_0$ above a critical stress, $\sigma_c = 0.1 \, Pa$, and reaches a stiffness, $K'_m = 10 \, Pa$, before breaking at $\sigma_m = 1 \, Pa$. Networks with higher $R_F$ and $L$ stiffen more and support larger stresses. For these networks, $K'$ increases more strongly than linear in $\sigma_0$ just above $\sigma_c$, whereas at high $\sigma_0$, $K' \sim \sigma_0$ (Fig. 4.10A). This unusual stiffening behavior is in agreement with the prediction of the model. Rescaling $K'$ by its initial value and $\sigma_0$ by $\sigma_c$, the $K'_{\sigma_0}$ data for networks formed with different $R_F$ and $L$ collapse onto a single curve, provided the network is not highly bundled [20]. Our rescaled data agrees well with the nonlinear response calculated with the effective medium model (Fig. 4.11), with only one fit parameter that represents the coupling of a rigid rod to the effective medium. In contrast, the rescaled data from networks rigidly crosslinked by biotin-NeutrAvidin fall on a separate curve, which is well described by the prediction of the affine thermal theory of crosslinked semiflexible networks (Fig. 4.11). These data support the model of crosslink dominated elasticity in the filamin-F-actin networks.

Interestingly, although the filamin-F-actin networks are all quite compliant, the maximum stiffness before breaking, $K'_m$, increases strongly with $R_F$, suggesting that network failure is due to filamin unbinding [20,45]. Thus, the overall magnitude of stiffening, $K'_m/G_0$, increases with $R_F$ (Figs. 4.10A and 4.12). Of these networks the highly bundled ones show the most dramatic stiffening (open symbols). The opposite behavior is observed for the rigidly crosslinked networks; $G_0$ increases significantly with $R_B$, while $K'_m$ is nearly independent of $R_B$ (Figs. 4.10B and 4.12), presumably because network failure is due to F-actin rupture [13].

### 4.3.6 Dependence of maximum stress on filament length

Assuming crosslink unbinding as the dominant failure mode for these networks, a scaling argument based on the theoretical model predicts how $\sigma_m$ scales with $c_A$, $R_F$, and $L$. On the microscopic level, the crosslink will unbind from F-actin at a force, $f_m$. With multiple crosslinks per filament, the crosslinks act in parallel, and the total rupture force per filament increases linearly with $n$. From the density of filaments and assuming an isotropic orientation of filaments within the network, the maximum stress is [21,22],

$$\sigma_m = \frac{1}{45} \rho nf_m$$

(4.9)
CHAPTER 4. FILAMENT LENGTH TUNES ELASTICITY IN ACTIN-FILAMIN GELS

Figure 4.10 – Nonlinear stiffening in prestress measurements. A) Filamin networks with $R_F = 0.003$ and $L = 1$ (squares), 5 (triangles), 15 $\mu$m (solid circles); $R_F = 0.005$ with $L = 15$ $\mu$m (diamonds); $R_F = 0.01$ with $L = 15$ $\mu$m (open circles, bundled network). $K'(\sigma_0)$ is independent of prestress, $\sigma_0$, for small prestresses before beginning to increase with $\sigma_0$ at a critical stress, $\sigma_c$. Networks with higher $R_F$ and $L$ stiffen more and support larger stresses before breaking. Line denotes linear scaling predicted by the model. B) Rigidly crosslinked network with $L = 15$ $\mu$m and $R_B = 0.0003$ (diamonds), 0.001 (inverted triangles), 0.003 (triangles), 0.03 (circles), 0.3 (squares). These networks also display stiffening behavior, but the maximum stiffness and stress are roughly the same for every sample. Line denotes $K \sim \sigma^{3/2}$ scaling predicted by the affine thermal model.
4.3. RESULTS AND DISCUSSION

Figure 4.11 – Rescaled nonlinear stiffening of non-bundled networks with filamin (black) or rigid crosslinks (light gray) at various $R_F$ or $R_B$, $L$, $c_A$. Filamin data is consistent with model of rigid rods connected by flexible linkers [21] (gray line), while rigid crosslinking data is consistent with model of semi-flexible filaments connected by rigid linkers [5] (dark gray line).

Thus, as we increase $L$ at fixed $R_F$, the number of filamins per actin filament will increase, leading to a scaling prediction

$$\sigma_m \sim n \sim R_F L \quad (4.10)$$

We first look at $\sigma_m$ measured in prestress experiments. In Fig. 4.10A, we see that $\sigma_m$ supported by the $R_F = 0.003$ networks increases as we increase $L$. To quantify this, we plot $\sigma_m$ as a function of $L$ in Fig. 4.13A. Above a critical value of $L$, the maximum stress increases with $L$. This critical value of $L$ decreases with increasing $R_F$. Similarly, for fixed $L$, $\sigma_m$ increases roughly linearly with $R_F$ over a broad range of $R_F$ (Fig. 4.12). For the highest values of $R_F$, where the networks are highly bundled, $\sigma_m$ increases dramatically (open symbols, Fig. 4.12). For $R_F$ below a critical value, $\sigma_m$ is roughly independent of $R_F$. This critical value of $R_F$ decreases with increasing $L$ [20].

We can collapse all the filamin data onto a single curve by plotting $\sigma_m$ as a function of $R_F L$ [20], as shown in Fig. 4.13B. For $R_F L > 0.01$, $\sigma_m$ grows nearly linearly with $R_F L$, consistent with the prediction of the model. For smaller values of $R_F L$, the network is rather weakly connected and breaks at very low levels of stress. The value of $R_F L \approx 0.01$ corresponds to $n \approx 4$. At physiological conditions $n \approx 15$—suggesting that the cytoskeleton operates in a regime where it has high enough connectivity to
Figure 4.12 – Maximum stress and stiffening for crosslinked networks: A) Maximum stress as a function of $R_F$ for filamin crosslinked networks with $L = 15 \mu m$ (black circles) or $L = 5 \mu m$ (black triangles) and as a function of $R_B$ for rigidly crosslinked networks with $L = 15 \mu m$ (gray circles). Gray line denotes mean maximum stress for rigidly crosslinked networks having $R_B > 0.001$. B) Magnitude of stiffening as a function of crosslinking ratios for networks with $L = 15 \mu m$. Filamin vs. $R_F$ (black circles), rigid crosslinks vs. $R_B$ (gray circles). (A-B) Non-bundled networks (solid symbols) and bundled networks (open symbols).
4.3. RESULTS AND DISCUSSION

Figure 4.13 – (Color online) Scaling of the maximum stress, $\sigma_m$, with $L$ for non-bundled filamin networks. A) The value $\sigma_m$ increases with $L$. From prestress measurements (circles) or 0.1 $s^{-1}$ strain ramps (squares): (white), 0.003 (light gray), 0.005 (gray), 0.01 (black). B) $\sigma_m$ for samples of different compositions collapse onto a single curve when plotted vs. $R_F L$. Above $R_F L = 0.01$ the data scale roughly linearly with $R_F L$. $L = 15$ (circles), 10 (diamonds), 7 (inverted triangles), 5 (triangles), 2 (pentagons), 1 $\mu$m (squares). Inset: $\sigma_m$ grows as the logarithm of loading rate in stress ramps for networks with $L = 15 \mu$m.
support large external stresses or internal tensions compared to purely entangled F-actin without rupturing. The linear scaling with $R_F L$ or $n$ suggests that failure of these networks is indeed determined by unbinding of crosslinks $[20,45]$. In contrast, $\sigma_m$ for rigidly crosslinked networks is nearly independent of $R_B$ for $R_B > 0.001$ (Fig. 4.12); this is consistent with rupture of F-actin at network failure $[13]$.  

To further quantify the variations in the nonlinear response close to network failure, we investigate the dependence on all control parameters $R_F, R_G, \text{ and } c_A$. For $c_A = 0.5 \text{ mg/ml}$ (black) and $L = 15 \mu \text{m}$ (circles) with sparse cross-linking ($R_F = 0.0003$), $\sigma_m$ is threefold larger than for purely entangled actin (Fig. 4.14a). The maximum stress is independent of $R_F$ up to 0.001, as shown by the $R_F$ dependence in Fig. 4.14a. Upon increasing $R_F$ further, $\sigma_m$ increases nearly linearly with $R_F$, up to a maximum of 5 Pa before the networks become bundled. Once bundles appear, $\sigma_m$ continues to increase with $R_F$ (open symbols, Fig. 4.14a). For $L = 7 \mu \text{m}$ (inverted triangles), $\sigma_m$ for all $R_F$ is smaller than for the longer filaments. For $L = 1 \mu \text{m}$ (squares), $\sigma_m$ is 0.3 Pa and independent of $R_F$ up to 0.01, before the network becomes bundled. Increasing $c_A$ to 1.0 mg/ml (purple online) and further to 1.5 mg/ml (green online) at fixed $R_F$ and $L$ increases $\sigma_m$. 

We first focus on low cross-linking densities, in the absence of bundles. Above, we found a naive estimate of the maximum stress at network failure would be $\sigma_m \sim n c_A$ (Eq. (4.9)). However, this does not account for the three-body nature of filamin-F-actin cross-linking: the probability to form an effective cross-link requires binding to two actin filaments. This adds a factor of $\xi^{-1} \sim c_A^{1/2}$ which measures the linear density of neighboring actin filaments along a particular filament. The scaling prediction then becomes $\sigma_m \sim c_A^{3/2}$. To test this prediction, we scale $\sigma_m$ from Fig. 4.14a by $c_A^{3/2}$, $\tilde{\sigma}_m = \sigma_m/c_A^{3/2}$. When plotted as a function of $n$, the data for the non-bundled networks do indeed collapse onto a single curve, as shown by the closed symbols in Fig. 4.14c. For $n \gtrsim 3$, $\tilde{\sigma}_m$ has a nearly linear dependence on $n$, in agreement with the prediction of the model. The somewhat stronger than linear scaling with $n$ may indicate additional cooperativity beyond our simple model. For $n \lesssim 3$, the networks are weakly connected and support only very small shear stresses, nearly independent of $n$. The scaled data for the bundled networks also collapse, but onto a separate curve, which has a larger magnitude and somewhat weaker $n$ dependence than the non-bundled networks (Fig. 4.14c, open symbols). A similar collapse is observed for $\tilde{K}_m' = K_m'/c_A^{3/2}$, when it is plotted as a function of $n$, as shown in Fig. 4.14d. These results further confirm our hypothesis that $n$ is a key control parameter for the network mechanics. 

The approximately linear dependence of $\sigma_m$ on $n$ in Fig. 4.14 suggests that network failure corresponds to a particular force per filamin cross-link. This failure is likely due to filamin unbinding from actin. From the schematic in Fig. 4.2, the maximum tension in a typical actin filament occurs at its mid-point, and is given by the
Figure 4.14 – (a) Maximum stress, $\sigma_m$, and (b) maximum differential modulus, $K'_m$, vs $R_F$. (c) Scaled maximum stress, $\tilde{\sigma}_m = \sigma_m / c_A^{3/2}$, and (d) maximum differential modulus, $\tilde{K}'_m = K'_m / c_A^{3/2}$, vs $n$. $c_A = 0.5$ mg/ml (black), $c_A = 1.0$ mg/ml (purple), and $c_A = 1.5$ mg/ml (green). $L = 1 \mu$m (squares), $L = 2 \mu$m (pentagons), $L = 5 \mu$m (triangles), $L = 7 \mu$m (inverted triangles), $L = 10 \mu$m (diamonds), and $L = 15 \mu$m (circles). Lines denote linear scaling with $n$. 
CHAPTER 4. FILAMENT LENGTH TUNES ELASTICITY IN ACTIN-FILAMIN GELS

sum of forces applied by the filamins bound on each side of the mid-point. For large $n$, these forces should increase linearly away from this mid-point, which leads to an average tension $\langle \tau \rangle = nf_0/6$, where $f_0$ is the maximum force experienced by a filamin. For an isotropic network, the shear stress is given by $\sigma = \frac{2}{15}\rho \langle \tau \rangle_m$, where $\rho \sim \xi^{-2}$ is the density of polymer length per volume and $\langle \tau \rangle_m$ refers to the average tension along actin filaments oriented in the direction of maximal network extension. These are the filaments expected to be under the greatest tension. For a 1 mg/ml network, $\rho = 40 \mu m^{-2}$, which sets $\langle \tau \rangle_m \approx 2 \text{ pN}$ for $\sigma_m = 10 \text{ Pa}$. This force is the result of multiple filamins $n$, as noted above. Thus, the load on any individual filamin is less than 2 pN at network failure under the conditions of our experiments, corresponding to loading rates of $0.1 - 1 \text{ pN/s}$. This is comparable to rupture forces for the actin-cross-linker bond measured for a number of actin binding proteins [7, 43–45], but is far below the 50 – 100 pN forces required for full unfolding of individual Ig domains in filamin [47]. Indeed, recent single molecule studies indicate that filamin unbinding is favored over unfolding at loading rates below 50 pN/s [45]. Thus, we believe that network failure is a result of filamin unbinding and that filamin Ig domain unfolding is unlikely [46].

We can also determine $\sigma_m$ from the strain ramp measurements. In the inset to Fig. 4.8A, we see that $\sigma_m$ also increases with $L$ for strain ramps conducted at a rate of $0.1 \text{ s}^{-1}$. Plotting $\sigma_m$ determined in this way as a function of $L$ (squares, Fig. 4.13A) for $R_F = 0.01$, we find that these measurements of $\sigma_m$ show similar scaling as the prestress measurements do, and at this strain rate the values from the two methods nearly match. More generally, we expect that $\sigma_m$ will depend on the rate of the measurement. The unbinding force for a single crosslink is expected to increase as the logarithm of the loading rate [41,48]. Indeed, in an analogous macroscopic measurement, we find that $\sigma_m$ increases as the logarithm of the loading rate on the network, again consistent with crosslink unbinding at network failure (inset, Fig. 4.13B).

4.4 Conclusions

The linear and nonlinear elastic behavior of filamin-gelsolin-F-actin networks support a model of crosslink dominated elasticity. The F-actin behaves as a rigid filament that constrains the deformation profile of the flexible crosslinks bound along its length; this leads to the unusual $L$ dependence in the rheology of these networks. Our data suggest that the lengths of actin filaments within crosslinked cytoskeletal networks may be an important determinant of cell mechanics.

Large, flexible crosslinks like filamin form compliant gels that can nonetheless support stresses that are orders of magnitude larger than those of purely entangled F-actin. The stiffness of these networks can be tuned over a broad range by external
stress or internal tension [3, 6, 20]. In contrast, rigid crosslinks form networks with a linear stiffness that is highly tunable by increasing the crosslink concentration, but show less dramatic nonlinear stiffening and tend to break at smaller strains [5, 7]. Interestingly, the mechanical response of F-actin networks can be tuned between these two cases by systematically varying the molecular weight of a crosslink [8].

Many physiological crosslinks are smaller and expected to be more incompliant than filamin; within the cell these crosslinks typically organize F-actin into bundles rather than orthogonal meshworks. For example, the α-actinin dimer forms an antiparallel rod of ≈ 30 nm, and fimbrin has two actin binding domains in tandem and is only ≈ 12 nm. Indeed, rheological studies show that α-actinin-F-actin networks have highly tunable linear stiffness [49], suggesting α-actinin behaves predominantly as a rigid crosslink. This also suggests that the cell may use large, compliant crosslinking proteins like the 160 nm long filamin dimers precisely because of the unique mechanical properties of the networks they form. In support of this view, filamin-F-actin networks mimic many key rheological features of cells [6, 19]. This highlights the potential value of these results in providing insight into the behavior observed in cells. Our results will serve as an important first step in developing more sophisticated models of cytoskeletal mechanics. Moreover, the model can be used to estimate the conditions required for forced unfolding of cross-links inside living cells [50].

4.5 Acknowledgements

This work was performed in collaboration with K. E. Kasza, G. H. Koenderink, Y. C. Lin, W. Messner, E. A. Millman, F. Nakamura, T. P. Stossel and D. A. Weitz. Most of the experiments were performed by K.E.K, some were performed by C.P.B.. C.P.B. gratefully acknowledges the hospitality of Harvard University where most of this work was performed. We thank C. Storm for helpful discussions.
Bibliography


