Origins of elasticity in intermediate filament networks

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  *Divalent Cations Crosslink Vimentin Intermediate Filament Tail Domains to Regulate Network Mechanics*,
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  *Elasticity in ionically cross-linked neurofilament networks*,
CHAPTER 8. INTERMEDIATE FILAMENT NETWORKS

Abstract

Intermediate filaments are principal structural elements found in abundance in the cytosol of all metazoan cells, where they form networks that contribute to cellular elasticity. We measure the linear and nonlinear viscoelasticity of reconstituted networks of two distinct intermediate filaments, vimentin and neurofilaments. Each network exhibits predominantly elastic behavior and strong nonlinear strain stiffening. Divalent ions behave as effective cross-linkers for both networks. The network behavior is consistent with the affine thermal theory for networks of semi-flexible polymers.

8.1 Introduction

The mechanical response of cells depends largely on the structure and elasticity of their cytoskeleton, consisting of a variety of biopolymer networks, including filamentous actin, microtubules, and intermediate filaments (IFs) \[1\]. While filamentous actin and microtubules have been extensively studied, much less is known about IFs, although some key parameters such as their persistence length have been measured \[2\]. Compared to actin and microtubules, IFs are more varied and specialized. Their networks are cytoskeletal components contributing to the elasticity of the cell: there are five families of IFs found in a variety of cell types, ranging from muscles to neurons. Intermediate filament networks exhibit pronounced nonlinear elasticity similar to that observed in actin networks that are cross-linked. However, there are myriad associated actin-binding proteins that lead to this cross-linking; by contrast, fewer cross-linking proteins have been identified for IFs \[3,4\]. Thus, the origin of the nonlinear elasticity in IF networks has not been identified.

Here, we investigate the elasticity of two different IF networks, vimentin and neurofilaments (NFs). The networks exhibit remarkably similar mechanical properties: they are weak elastic solids even at the lowest frequencies probed and they exhibit strong nonlinear strain stiffening over several decades in stress. This behavior requires cross-linking of the network and we show that divalent ions act as effective cross-linkers. By comparing the linear and nonlinear macroscopic behavior, we extract microscopic network parameters. These observations suggest a general design principle for regulating the elasticity of intermediate filament networks even in the absence of specific cross-linking proteins.

To explore the generality of this behavior, we use NFs, found only in neurons, and vimentin, found in nearly all mesenchymal cells. The main difference between these
IFs lies in the length of their negatively charged carboxy terminal tail domains; NF tail domains are much longer than those of vimentin and appear as sidearms extending from the NFs enabling the formation of lateral bonds between NFs [6]. The tail domains of vimentin are also thought to be important for inter-filament interactions, although their precise role is not as clear as in the case of NFs [7, 8]. Furthermore, optimal assembly is obtained at different ionic conditions, with NFs favoring a pH of 6.2, where vimentin does not assemble properly [9].

8.2 Materials and methods

Neurofilaments are purified from bovine spinal cords [10–12]: fresh tissue is homogenized, then centrifuged, after which the crude neurofilament pellet is purified overnight on a discontinuous sucrose gradient with 0.8 M sucrose (5.9 ml), 1.5 M sucrose (1.3 ml) and 2.0 M sucrose (1.0 ml). The purified neurofilament is then dialyzed for 76 hours and afterwards 120 µl aliquots are flash frozen in liquid nitrogen and stored at -80 °C. Human vimentin protein is expressed in Escherichia coli and purified from inclusion bodies [13]. The protein is stored at -80 °C in 8 M urea, 5 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM EDTA, 0.1 mM EGTA, and 10 mM methyl ammonium chloride. Twenty-four hours before use, we renature the protein from 8 M urea by stepwise dialysis (6 M, 4 M, 2 M) into a solution of 5 mM Tris-HCl, pH 8.4, 1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT. The protein concentration is determined using a Bradford assay with bovine serum albumin (BSA) as a standard.

The mechanical response of intermediate filament networks is measured with a stress-controlled rheometer using a 2° 20mm cone-plate geometry (HR Nano, Bohlin Instruments). Before rheological testing, neurofilament samples are thawed on ice, after which, varying concentrations of Mg$^{2+}$ are added. Vimentin polymerization is initiated by adding Mg$^{2+}$ and 1/10 of the final sample volume of 10X polymerization buffer (0.2 M Tris-HCl, pH 7.0, containing 1.6 M NaCl). The samples are quickly loaded onto the rheometer and polymerized between the rheometer plates for one hour at 25 °C, using a solvent trap to prevent drying. We measure the linear viscoelastic moduli $G'\(\omega\)$, $G''\(\omega\)$. In addition, we use large amplitude oscillatory measurements to qualify the network’s nonlinearity. However, to better quantify the network’s nonlinear behavior we utilize a differential measurement [14,15]. The system is held at a constant average (pre-)stress $\sigma$, while the differential response $d\gamma$ to a small additional oscillatory stress $d\sigma$ is measured. This measures the nonlinear tangent modulus $K = d\sigma/d\gamma$. 

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8.3 Results and discussion

To investigate the origin of elasticity in intermediate filament networks, we probe the frequency dependence of the linear viscoelastic moduli. Consistent with other biopolymer networks, both $G'$ and $G''$ depend weakly on frequency (from 0.01-10 Hz), with $G'$ much larger than $G''$, as shown in Fig. 8.1A. This suggests the existence of soft cross-linked gels. We characterize the linear elasticity by the plateau modulus, $G_0$, the value of $G'(\omega)$ at 0.1 Hz. Interestingly, for purely entangled networks of similar concentration, we would expect the moduli of the two IF networks to be comparable; the fact that NFs are about an order of magnitude stiffer can be accounted for by cross-linking. The networks also exhibit dramatic strain stiffening above a critical strain $\gamma_c$, as shown in Fig. 8.1B.

For both IFs, the elasticity depends strongly on the polymer concentration and also on the concentration of Mg$^{2+}$ added to the solution [16]. By analogy to prior studies of biopolymer networks [15, 17], we examine the dependence on IF concentration $c_{IF}$, at a fixed mole ratio, $R$ of Mg$^{2+}$ to IF. For both vimentin and NFs, the linear elastic modulus increases with filament concentration in a way similar to cross-linked F-actin networks, in that the modulus scales slightly stronger than quadratically with
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Figure 8.2 – Dependence of the linear elastic modulus, \( G_0 \), of vimentin with a concentration \( c_V \). In the absence of divalent cations, \( G_0 \sim c_V^{1.3} \), while in the presence of divalent cations, the concentration dependence becomes \( G_0 \sim c_V^{2.1} \).

filament concentration, as shown in Fig. 8.3A. This is consistent with a theoretical model for cross-linked semiflexible polymers, in which the elasticity is dominated by entropic filament stretching; in this affine thermal model, the filaments are considered to be entropic springs, leading to \( G_0 \sim c_{IF}^{11/5} \) [18]. In addition, we also find that \( G_0 \) scales directly with \( c_{Mg} \), as shown in Fig. 8.3B, demonstrating the role of Mg\(^{2+}\) in the network elasticity for both IFs. Interestingly, in the absence of divalent cations, \( G_0 \sim c_V^{1.28} \) (Fig. 8.2, open circles), which, within our measurement error, is consistent with \( G_0 \sim c_V^{7/5} \), as expected for an entangled solution of semiflexible polymers [19–21]. Taken together, these findings suggest that divalent cations behave as crosslinkers for IF networks.

To elucidate the role of Mg\(^{2+}\) as a network cross-linker, we investigate the nonlinear elastic regime of IF networks by probing the differential moduli [15]. Above a critical stress \( \sigma_c \), both IF networks display pronounced nonlinear stiffening with applied stress, exhibiting an approximate power law of 3/2, as shown in Fig. 8.4. This is consistent with theoretical expectations and previous experiments for cross-linked networks, where nonlinear elasticity results from the stretching out of thermal fluctuations of network strands between cross-links [15,17,18,22]. Interestingly, the predictions of the affine thermal model apply for networks cross-linked by molecular linkers and experiments have focused on actin-linkers such as scrin. The absence
Figure 8.3 – (Color online). A) $G_0$ as a function of $c_{IF}$ holding $R$ constant, where $R = 1000$ for neurofilaments (circles) and $R = 215$ for vimentin (squares). The solid line is obtained using a regression fit and depicts $G_0 \sim c_{NF}^{2.5}$ for neurofilaments, while the dashed line indicates $G_0 \sim c_{V}^{2.1}$. B) $G_0$ as a function of $c_{Mg}$ holding $c_{IF}$ constant, where $c_{IF} = 1$ mg/ml for neurofilaments (circles) and $c_{IF} = 2$ mg/ml for vimentin (squares).
of molecular linkers in these IF networks suggests that ions may be playing the role of permanent effective molecular cross-links, lending insight into the nature of ionic interactions in IF networks. Previously, these ionic interactions have been understood within a framework of condensed counterions and salt-bridging on polyelectrolyte brushes [23–25]. We hypothesize that Mg$^{2+}$ mediates attractive interactions between the negatively charged tail domains of the IFs thereby forming permanent cross-links analogous to molecular linkers. In particular, IF tail domains are likely collapsed flexible chains, and the cross-linking interactions would be mediated by a collection of divalent ions interwoven into the entangled tail domain structure. Although tail domains of a single chain will likely cross-link within themselves, only cross-linking between different chains will contribute to network elasticity. Theoretically we expect a universal form for the nonlinear elastic response of the networks for all protein and ion concentrations. The data sets from both neurofilament and vimentin networks can, indeed, be scaled onto a single master curve, which is in good agreement with the theoretical prediction shown by the solid line in the inset of Fig. 8.4 [15, 17]. Here, we fit each data set to the full theoretical curve, using two independent parameters: $\sigma$ is scaled by $\sigma_c$, while $K'$ is scaled by the linear elastic modulus $G_0$. For both networks, we find excellent agreement with the theoretical curve for approximately four decades in stress, although vimentin departs from the theoretical master curve at the highest stresses. This departure can be accounted for by considering the enthalpic contribution of filament backbone stretching [26], suggesting that the Young’s modulus of vimentin is less than that of neurofilaments, as discussed further below.

To further probe the mechanism of the network elasticity, we test the predicted relationship between the scale factors used above, $G_0$ and $\sigma_c$. Assuming an affine deformation, theory predicts the curve shown in the inset of Fig. 8.4, with

$$G_0 = 6\rho k_B T \frac{l_p^2}{l_c^3}$$

$$\sigma_c = \rho k_B T \frac{l_p}{l_c^2}$$

Here, $k_B$ is Boltzmann’s constant, $T$ is the temperature, $\rho$ is the filament density in length per volume, $l_p$ is the persistence length, and $l_c$ is the average distance between cross-links [15, 17, 18, 28]. Since $\rho \propto c_{IF}$, the model predicts that $c_{IF}^{1/2} G_0 \sim \sigma_c^{3/2}$, where the pre-factor should depend only on $k_B T$ and $l_p$. In particular, this relationship is predicted to be independent of $l_c$, so that even data sets with different cross-link densities should collapse onto a single curve. Both vimentin and NF networks agree with this data collapse and scaling for a variety of different filament and Mg$^{2+}$ concentrations, as shown in Fig. 8.5. In the case of NFs, the generality of this ionic cross-linking behavior is depicted by a qualitative collapse onto the same curve for other divalent ions such as Ca$^{2+}$ and Zn$^{2+}$. This scaling relates the linear elasticity.
Figure 8.4 – (Color online). $K'$ at 0.6 rad/sec as a function of $\sigma$ for vimentin and neurofilament networks: $[c_{\text{Mg}} = 2 \text{ mM (open symbols), } c_{\text{Mg}} = 8 \text{ mM (solid symbols) and } c_{\text{IF}} = 2 \text{ mg/ml (all symbols)}]$. The solid line shows a 3/2 power law. The inset shows the data sets rescaled by $\sigma_0$ and $G_0$ depicting the universal form of the stiffening response; the rescaled theory is indicated by the dashed red line, while the solid black line depicts the deviation due to enthalpic backbone stretching.
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Figure 8.5 – (Color online). The dependence of $c_{IF}^{1/2} G_0$ on $\sigma_c$. Vimentin data points are obtained with Mg$^{2+}$, while NF data points are obtained with Mg$^{2+}$ (closed circles), Ca$^{2+}$ (open circles), and Zn$^{2+}$ (crossed circles). The solid lines reflect regression fits. The affine thermal model predicts a power law of 3/2, shown for reference.

of the networks to the stress at which nonlinear behavior begins as the filaments approach their full extension; therefore, it is independent of the scaling of $K_0$ with $\sigma$ in Fig. 8.4. This provides strong evidence that the linear elasticity of IF networks is also governed by entropic stretching.

The relative shift between the two curves in Fig. 8.5. can be explained by a difference in persistence lengths of the filaments. Remarkably, we can precisely determine this persistence length directly from the macroscopic network behavior under shear. Using Eqs. (8.1) and (8.3) together with the bulk rheology yields

$$ l_p = \frac{1}{36} \rho k_B T \frac{G_0^2}{\sigma_c^3}, \quad (8.3) $$

where $\rho \approx 0.5 \times 10^{13} \text{ m}^{-2}$ for IF networks at a concentration of 4 µM. We find that for NFs, $l_p \approx 0.2 \mu$m, while for vimentin, $l_p \approx 0.5 \mu$m; both of these values are in good accord with previous experiments [2,29–31]. Furthermore, we are also able to determine the cross-linking lengthscale,

$$ l_c = 6l_p \frac{\sigma_c}{G_0}, \quad (8.4) $$
where we have obtained $l_p$ directly from bulk rheological parameters using Eq. (8.3). Direct determination of $l_c$ is difficult and has rarely been made in such systems [32]. Here, we find that $l_c \approx 0.3 \mu m$ for NFs and $l_c \approx 0.6 \mu m$ for vimentin. This is consistent with our hypothesis that the filaments are cross-linked on the scale of their persistence length and thus the networks can be characterized as semiflexible.

To further examine the behavior of Mg$^{2+}$ as a cross-linker, we consider the scaling of $l_c$ as a function of both $R$ and $c_{IF}$ (Eq. (8.4)). Cross-linking occurs on the scale of the entanglement length $l_e$, which scales as $c_{IF}^{-2/5}$ [18, 33]. Since our data suggest that Mg$^{2+}$ is effectively a cross-linker, we expect $l_c$ to scale with $R^{-x}$ for some exponent $x$ [15, 17]. Consistent with the theoretical prediction, the data do exhibit an approximate scaling, $l_c \sim R^{-x} c_{IF}^{-y} = c_{Mg}^{-x} c_{IF}^{-x+y}$; $x \approx 0.23$ for both IFs and $y \approx 0.4$ for vimentin and $y \approx 0.5$ for neurofilaments, measured at fixed $R$, as shown in Fig. 8.6. Interestingly, the larger value of $y$ for NFs is consistent with the correspondingly stronger concentration dependence of $G_0$ as observed in Fig. 8.3. This may be a consequence of denser cross-linking; then we expect $G_0 \sim c_{NF}^{5/2}$ [18]. Furthermore, as a consistency check for the value of $x$, we can also determine it directly from the $c_{Mg}$ dependence of $G_0$. Inserting a scaling of $l_c \sim R^{-x}$ into Eq. (8.1) gives $G_0 \sim c_{Mg}^{3x}$, holding $c_{IF}$ fixed. Based on the scaling of $G_0$ in Fig. 8.3B, we find $x \approx 0.20$, in good accord with the value measured directly from the $c_{Mg}$ dependence of $l_c$. Both the excellent agreement with theory and the internal consistency of our measurements provides convincing evidence that Mg$^{2+}$ effectively cross-links IF networks. Moreover, we find similar behavior for Ca$^{2+}$ and Zn$^{2+}$.

The values we observe for $G_0$ and the maximum stress are consistent with most previous experiments [1, 12], although one experiment with NFs yielded a smaller modulus and solution like behavior [34]. Remarkably, we can extract the microstructural parameters $l_p$ and $l_c$ directly from bulk rheology; values of $l_p$ are consistent with previous measurements [29], while values of $l_c$ are comparable to the expected mesh size $\xi \approx 1/\sqrt{\rho}$. Compared with vimentin, NFs consistently exhibit smaller values of $l_c$, suggesting a more densely cross-linked network; this may result from the longer NF tail domains and concomitantly stronger electrostatic interactions. Measurements based on probe particle motion in IF networks have suggested larger values of $\xi$ [34]; however, recent measurements yield results which are more consistent with the expected value [35].

At the very highest stresses, $\sigma/\sigma_c > 10$, the experimental data deviate significantly from the theoretical prediction (inset Fig. 8.4). This deviation could result from irreversible network fracture or failure; however, we find that in the high stress regime just below $\sigma_{max}$, the elastic behavior is fully reversible on the timescales of our measurements (Fig. 8.7). Alternatively, the observed behavior could result from slippage between crosslinks, as can occur in a solution or transiently-crosslinked system, such as F-actin solutions without permanent crosslinks [37]; however, such a
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Figure 8.6 – (Color online). A: the dependence of $l_c$ on $c_{IF}$. A power law of -0.5 is shown for reference. B: the dependence of $l_c$ on $c_{Mg}$. A regression fit results in a power law of -0.23 for both vimentin and neurofilament networks.

Figure 8.7 – Hysteresis test for vimentin networks at high applying stress. The entire linear and nonlinear elastic response of vimentin networks are highly reversible; the time between each data point is 1 – 2 minutes.
data collapse (Fig. 8.4) is not possible when the stiffening exponent varies with protein concentration, as observed for F-actin solutions \[37\]. Instead, we include the consequences of the extensibility of individual filaments. This is motivated by observations that many types of intermediate filaments are highly extensible compared to actin \([1,36,38]\) and appear straightened in human keratinocyte cells subjected to large uniaxial strains of 100% \[38\]. The extensional modulus depends on intrinsic properties of individual filaments, and should thus be independent of \(\ell_c\), whereas the linear entropic modulus scales as \(1/\ell_c^3\) \[18\]. Thus network response should be increasingly dominated by the enthalpic stretching mode for smaller values of \(\ell_c\) or for increasing values of \(\sigma\), where thermal fluctuations are pulled out thereby increasing the effective spring constant of the entropic mode. Consistent with this, the deviation of the data from the universal curve at high stress increases as \(\ell_c\) decreases, as seen more clearly in the inset of Fig. 8.8.

To make a more quantitative comparison, we extend the inextensible, entropic model by introducing an enthalpic stretch modulus, \(E\) \([26,33]\); this parameter describes the resistance of the filament to changes in its contour length, and is related to its intrinsic structure and material properties; for a linear elastic rod \(E\) represents the force required to stretch the rod to twice its original length. A value for \(E\) can be estimated by assuming the filament behaves as a homogeneous elastic rod of diameter, \(\approx 10\) nm, for which \(E \approx 4k_B T \ell_p/r^2 \approx 463\) pN. Interestingly this is comparable to the force required to unfold coiled-coil domains of vimentin dimers \([39]\), 200 – 300 pN. However, at the point where we observe network failure, we estimate that the filaments are stretched to less than twice their length. Moreover, the resulting filament tension is borne by the 16 dimers in cross-section \([40]\). Thus, these networks break upon application of forces on a per dimer basis that are much lower than required to unfold the coiled-coil domains. The resulting predictions using this extended theoretical treatment require no further fitting parameters, and are in excellent agreement with each set of experimental data for the entire strain stiffening curves, as shown in the inset of Fig. 8.8. We also determine the Young's modulus \(Y \approx 9\) MPa from the macroscoping rheological data using an affine theory for networks of extensible semiflexible polymers; this value is consistent with previously reported values of the keratin-like IF proteins from hagfish slime threads \([3,41]\). Thus, the full nonlinear behavior of vimentin networks is well described by this theory for crosslinked networks of stretchable, semiflexible polymers.

8.4 Conclusions

Vimentin and NF networks show striking similarity in their linear and nonlinear elastic behavior, both of which are governed by cross-linking due to divalent ions. Intrigu-
Figure 8.8 – Elastic response of vimentin networks results from stretching the entropic fluctuations of single semiflexible filaments at low to intermediate stresses; at high stress, enthalpic stretching of the individual filaments contributes to the nonlinear response. Each data set is rescaled by $\sigma_c$ and $G_0$, revealing a data collapse which reflects the universal form of the entropic model or inextensible theory (grey line). The departure between the entropic model and our experimental data depends on the average cross-linking distance, $\ell_c$: a larger departure is observed at high stress with decreasing $\ell_c$. (Inset) A zoomed in version of the main graph showing each set of the experimental data (colored symbols), which are in agreement with the modified theory (colored lines) that captures the behavior of networks in all stress regimes. These theoretical curves are calculated directly using the values of $\ell_p$ and $\ell_c$ obtained by determining $G_0$ and $\sigma_c$ together with Eqns. (8.3)-(8.4) and an extensional modulus for vimentin calculated from $E \approx 4k_B T \ell_p / r^2$. Thus, this calculation requires no further fitting procedure.
ingly, divalent ions play a role nearly identical to that of molecular cross-linkers in F-actin networks. This suggests that there is a strong affinity of the divalent ions to the IFs; thus, a large fraction of the ions must be bound hence becoming effective molecular cross-links. Despite the similarities in their mechanical behavior, the nonlinear rheology of vimentin shows a clear departure from that of NFs at the highest stresses; this results from the enthalpic contribution of filament backbone stretching [26]. By contrast NFs do not exhibit a measurable filament compliance, implying that their Young’s modulus is larger than that of vimentin; this is surprising given the similar molecular architecture of the backbone of the two IFs. This may reflect another important role of the tail domains: they might also affect the backbone stretching of filaments. However, this is based on macroscopic rheology, and direct force-extension measurements of individual filament stretching are needed to confirm this. Such filament extension experiments may also elucidate the nature of the electrostatic interactions that mediate effective molecular cross-linking between IFs.

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