In vitro RECONSTITUTION OF COMPOSITE NETWORKS OF VIMENTIN AND MICROTUBULES

The shape of cells is defined by their internal cytoskeleton, which consists of microtubules, actin filaments, and intermediate filaments interconnected by crosslinking proteins and motors. This composite protein network enables cells to withstand mechanical forces, but also to actively generate forces. Studies of networks composed of either actin with intermediate filament or actin with microtubules indicate that the disparate bending rigidities of the filaments and interactions between them can lead to surprising non-additive effects on network elasticity. Composite networks of intermediate filaments, which are the most flexible among all cytoskeletal filaments, and microtubules, which are the most rigid, have not been studied yet. In this chapter, we reconstitute an in vitro system composed of microtubules and vimentin, which is a type III intermediate filament. Since both proteins require different buffer conditions for proper assembly, we used electron and fluorescence microscopy to image filaments formed in different buffers and identify conditions allowing for simultaneous polymerization of microtubules and vimentin. Confocal microscopy revealed that microtubules embedded in vimentin networks were bent, possibly due to forces exerted by polymerizing vimentin filaments. With the aid of rheology measurements we found that the mechanical properties of reconstituted microtubule-vimentin networks were intermediate between those of pure MTs and pure IFs. The new composite cytoskeletal model system created in this chapter paves the way for future systematic studies of the influence of filament interactions on cytoskeletal network mechanics.
7.1 Introduction

The mechanical properties of living cells are mainly determined by the cytoskeleton, a cytoplasmic biopolymer network consisting of actin filaments, microtubules (MTs) and intermediate filaments (IFs). IFs are a large superfamily with at least 65 distinct proteins in man, which are differentially expressed in a tissue-dependent and developmentally regulated manner [130, 126, 127]. IF proteins are categorized into six types (types I-VI), or, alternatively, in three assembly groups, known as groups I, II, and III, which can coexist as three separate IF systems within the same cell [125, 226, 89].

All three cytoskeletal filament types can be classified as semiflexible polymers with a persistence length, $l_p$, that has a similar magnitude as the contour length, $l_c$. However, they have highly disparate bending rigidities: MTs are the most rigid filaments with $l_p=5$ mm, actin filaments are more flexible with $l_p=15$ $\mu$m, and intermediate filaments are the most flexible with $l_p=0.5-1$ $\mu$m [32, 97, 264, 164, 209, 182, 138, 210, 18]. In vivo, the filaments are known to colocalize and strongly interact with each other. In fibroblasts, for example, association of IFs and actin [106], IFs and MTs [110], and actin and MTs [32] was found. Actin networks are often considered to be the main component responsible for the viscoelastic properties of cells [236] and for their stress-stiffening response to an imposed mechanical stretch [84, 292]. However, recent studies show that intermediate filaments also play a major role in cell mechanical resistance. For instance, keratin determines the mechanical strength of keratinocyte cells [194, 90], while vimentin contributes to the linear stiffness and stress-stiffening of fibroblasts [302, 234].

There are many indications that the three types of filaments influence each other’s organization and that interactions between them influence the overall mechanics of cells [55, 249, 60, 100, 227]. This crosstalk involves structural as well as regulatory interactions [171]. It is known, for instance, that the spatial arrangement of microtubules depends not only on microtubule organizing centers (MTOCs) [37], but also on the cytoplasmic environment. Microtubules growing in the peripheral actin-myosin cortex are bent by contractile myosin-driven forces [33]. Since the actin network is elastic, it suppresses large scale microtubule buckling and allows microtubules to bear enhanced compressive loads [32, 68]. This is consistent with the so-called tensegrity model, which states that cellular shape stability is achieved via a balance between actin filaments and IFs loaded under tension and MTs and thick actin bundles under compression [147, 278].

To obtain physical insight into the mechanisms by which the cytoskeleton controls cell mechanics, there have been many in vitro studies of purified cytoskeletal systems. Most of these studies have considered networks reconstituted from one filament type, either alone or with regulatory proteins such as crosslinkers and motors specific for that type. It was shown that crosslinked networks of actin as well as networks of IFs exhibit a rich mechanical behavior, which is tuned by filament and crosslinker density [94, 182, 180, 119, 118]. At low filament and crosslink densities, the network elasticity is dominated by nonaffine, bending modes and the networks generally strain-weak. At high
filament and crosslink densities, the elasticity is dominated by affine filament stretching and the networks strain-stiffen due to the entropic resistance of the filaments when their thermal slack is pulled out [195, 285]. The molecular structure of the crosslinking proteins also affects the network response, for instance by affecting the network architecture [176] or by directly contributing to the macroscopic network compliance [189, 41]. The complex, bundle-like internal architecture of IFs was shown to make them extremely extensible, in contrast to actin filaments, which are rather inextensible [240, 150]. Contrary to actin and IF networks, MT networks strain-weaken, independent of filament and crosslink density [183, 48]. Given their large bending rigidity, MTs likely behave as athermal rods, which bend and/or reorient when the network is sheared, rather than stretch.

In vitro systems of individual cytoskeletal network are unlikely to capture the full richness of the mechanics of the in vivo cytoskeleton. To date there have only been a handful of in vitro studies probing the mechanical consequences of the composite nature of the cytoskeleton. It has been shown that rigid MTs embedded in networks of more flexible actin filaments can change the nonlinear elastic response even at low MT density [181, 10]. The MTs suppress bending fluctuations of actin filaments, and thereby make the strain field more affine. MTs can thereby convert the strain-weakening response of sparsely crosslinked actin networks into an affine strain-stiffening response. It was furthermore shown by microrheology that MTs make actin networks compressible [229], which was rationalized by theoretical calculations [69]. Addition of stiff rods to an incompressible elastic medium such as an actin network reduces the effective Poisson ratio. MTs do not contribute to the linear elasticity of composite MT-actin network [181], suggesting that they interact only by steric repulsion with actin.

By contrast, addition of actin filaments to networks of vimentin or neurofilaments (NF), which both belong to the family of IFs, increases the linear elastic modulus over that of the two separate protein systems [76, 172]. This nonadditivity suggests the presence of attractive interactions between IFs and actin filaments. Indeed, it was shown that the C-terminal tail domain of vimentin mediates direct interactions with actin [76]. The composite networks also exhibited a weaker frequency dependence of the elastic modulus, which likewise indicates interactions between actin and vimentin. In networks composed of NFs and actin, the long side extensions of the NFs were shown to cross-bridge actin filaments and inhibit lateral alignment of NFs [172]. This was shown to reduce efficient cross-bridging among NFs, thereby reducing the mechanical resistance of composite actin-NF networks to large stresses compared to pure NF networks.

The mechanical behavior of composite networks of MTs and IFs has not yet been reported. Since MTs are at least 1000-fold more rigid than IFs, we anticipate rich mechanical behavior in this case. It has been proposed that IFs may increase the resistance of MTs to compressive loads by acting as an elastic support [38]. Moreover, IFs may potentially reinforce networks of MTs, which by themselves strain-weaken. Conversely, MTs may reinforce IF networks by acting as long and rigid inclusions. Reinforcement of soft networks by stiff elements such as carbon nanotubes increases the linear elasticity as well as the mechanical resistance when there are direct interactions between the com-
ponents [50, 4, 101]. Intriguingly, even in the absence of direct interactions, MTs can still promote strain-stiffening behavior of F-actin [181].

In this chapter, we reconstitute composite networks of microtubules and intermediate filaments from purified bovine brain tubulin and human recombinant vimentin. Vimentin is a class III and assembly group 2 type of intermediate filament, which occurs in nearly all mesenchymal cells [126]. Individual filaments and composite networks were assembled using different buffer conditions, in order to identify conditions that would allow for simultaneous polymerization of both filament types. Confocal and electron microscopy (EM) were used to visualize network and filament structures (see Chapter 2). The nonlinear viscoelastic properties of the networks were probed by large amplitude oscillatory shear tests in a plate-plate rheometer.

7.2 Sample preparation

7.2.1 Vimentin network purification and assembly

Human vimentin was expressed in Eschericia (E.) Coli and purified from inclusion bodies [129], followed by ion exchange chromatography on an anion exchange columns (DEAE-Sepharose) and a cation exchange column (CM-Sepharose, GE Healthcare life Sciences). The protein was eluted with a potassium chloride gradient. The vimentin was pure as assessed by SDS-PAGE gel electrophoresis, which revealed only a single band at the expected molecular weight of 54 kDa. Purified vimentin was stored at −80°C in a pH 7.5 buffer (5 mM Tris-HCl, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 10 mM methyl ammonium chloride (MAC)), which contained 8 M urea to keep the protein soluble. Before use, the urea concentration was reduced to 1 M by sequential dialysis against buffers (5 mM Tris-HCl, 1 mM DTT, 1 mM EDTA, and 0.1 mM EGTA, pH 8.4) of stepwise decreasing urea concentration (6 M, 4 M, 2 M). We used a cellulose membrane of 8-10 kDa molecular weight cut-off (Spectra/Pro) for the dialysis. Finally, the urea concentration was reduced to 0 M by dialyzing the sample overnight at 4°C against fresh dialysis buffer. Vimentin is present in this buffer in the form of tetramers, as observed by analytical ultracentrifugation [129]. Vimentin forms parallel coiled-coil dimers upon lowering the urea concentration to 6 M [129] (Fig. 7.1). The dimers are rod-shaped with a length of 45-50 nm. In 4.5 M urea, the dimers laterally associate into antiparallel tetramers. Due to this antiparallel arrangement, IFs possess no structural polarity, in contrast to actin filaments and MTs [218]. The final protein concentration was determined by a Bradford assay (Sigma Aldrich) with bovine serum albumin in water as a standard. Vimentin was polymerized by mixing vimentin tetramers in dialysis buffer with assembly buffer, as explained in the Section 7.3.
7.2.2 Composite vimentin-microtubule network assembly

Microtubules were pre-polymerized at few different concentrations from a tubulin stock solution (Cytoskeleton) in a 37 °C water bath for 1 hour, and stabilized with 10 \( \mu \text{M} \) taxol (Sigma Aldrich). Taxol-stabilized microtubules were mixed with vimentin tetramer solutions of varying vimentin concentration. Vimentin polymerization was then initiated by addition of 1/10 of 10X assembly buffer to the total sample volume and warming to 22 °C. For confocal imaging, unlabeled tubulin was mixed with rhodamine labeled tubulin (Cytoskeleton) in a 10:1 molar ratio. Vimentin was not labeled.

7.3 Results

7.3.1 Network and filament structure

\textit{In vitro} reconstitution of composite networks of two filament types is challenging since each filament type has individual requirements for native-like assembly. Intermediate filaments display a marked polymorphism depending on the temperature, pH, ionic strength, and specific salt and buffer types present during assembly [124, 284]. Similarly, the growth and dynamics of microtubules is highly sensitive to buffer conditions [303], temperature [92], and solution pH [244]. To optimize the conditions for both microtubules and vimentin assembly, we first screened different buffer conditions for the individual networks, checking the filament structure by EM and fluorescence microscopy.

Based on viscometry studies and EM, it is known that the main structural changes during vimentin assembly take place within the first hour of polymerization [310, 286, 228]. Upon mixing of vimentin tetramers with assembly buffer, the tetramers laterally associate into hexadecamers, which subsequently laterally associate to form Unit-Length-Filaments (ULFs) with a diameter of 16 nm. The ULFs anneal longitudinally to form short filaments, which lengthen over time [123, 275, 235] (Fig. 7.1 A). The diameter of mature IFs is \( \sim 10 \) nm, smaller than the diameter of individual ULFs [128], due to radial compaction during the first hour of the assembly process [128]. Combined with structural rearrangements involving changes in the relative position of tetramers with respect to each other [123], polymerization finally leads to the formation of 10 nm diameter filaments. Mass-per-length measurements by scanning transmission electron microscopy showed that the mass/length ratio of vimentin filaments formed by rapid dilution varies along the filament backbone between 24 and 40 molecules (or 6 to 10 tetramers) per cross-section [129, 128, 129]. In contrast, filaments formed slowly by dialysis against assembly buffer are smoother, with a constant mass/length ratio of 37 kDa/nm, or 29 molecules per cross-section [129].

We first assembled vimentin networks at different protein concentrations (0.14, 2 and 3 mg/ml) in a 45 mM Tris-HCl buffer of pH 7.0 with 50 mM NaCl. Similar buffer conditions (but with 25 mM Tris-HCl and pH 7.5) were previously shown to promote the formation of normal, 10 nm wide filaments [131]. Our EM imaging showed that the filaments were several micrometers long, gen-
Figure 7.1. Schematic showing different stages of intermediate filament assembly (A) and dynamic instability of microtubules (B). Pictures adapted from [100, 66].

...tly curved, and entangled (Fig. 7.2 A-C). We note that the density of filaments on the surface is not representative of the bulk density. For the 0.14 mg/ml network, the EM grid displayed a similar filament density everywhere, but for the 2 mg/ml and 3 mg/ml networks, most of the grid surface was too densely covered to identify filaments. The average diameter of the filaments was approximately 10 nm at all three concentrations, consistent with previous reports [123]. At the highest concentration of 3 mg/ml, the filaments appear more straight and aligned (Fig. 7.2 C), which may be caused by shear forces applied during sample deposition and/or drying onto the EM grid.

We next replaced the 45 mM Tris-HCl buffer with a sulfonate 1,4-Piperazinediethanesulfonic acid (PIPES) buffer of the same concentration but lower pH (6.8). This buffer is the most commonly used buffer for MTs and its replacement had a significant effect on the structure of the vimentin filaments (Fig. 7.3 A). After 1 hour of polymerization at 0.2 mg/ml vimentin, only short filaments accompanied by small subunits (white arrow) were visible. The filaments rarely overlapped and often had a branched and unwound structure (black arrows). Increasing the protein concentration to 2 mg/ml led to filament aggregation (Fig. 7.3 B), but the filament length remained small. When the PIPES concentration was lowered from 45 to 25 mM, and the pH increased to 7.0, we observed a striking change in filament morphology: long and entangled filaments were again formed, comparable to the filaments formed in Tris-HCl buffer (Fig. 7.3 C).

IFs assembly is highly dependent on ionic strength and usually polymerization takes place in the presence of sodium chloride [128]. Assembly of microtubules, on the other hand, requires magnesium ions, which have a catalytic effect on hydrolysis of tubulin-associated Guanosine-5’-triphosphate (GTP) [220].
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This energy source destabilizes the MT lattice, leading to dynamic instability: MTs are continually in either a growing or shrinking state [140, 300] (Fig. 7.1 B). Magnesium ions required by MTs are compatible with IF assembly, but act as crosslinks, causing an increase in network stiffness [180, 161, 182].

To test the influence of different salts on vimentin network structure, we assembled vimentin in the presence or absence of sodium and magnesium chloride. With no salt present, vimentin forms network with filaments that are ~24 nm in diameter (Fig. 7.4 A). This is more than twice the thickness of filaments assembled in the standard buffer, which contains 50 mM Na\(^+\) (Fig. 7.4 B). Addition of 4 mM Mg\(^{2+}\) to the buffer did not noticeably change the network or filament structure (Fig. 7.4 C). These results indicate that the main components of the MT buffer that affect vimentin assembly are the PIPES buffer and/or the pH.

**Figure 7.2.** Electron microscopy images of vimentin networks assembled at 22 °C with different protein content in buffer containing 45 mM Tris-HCl, 50 mM NaCl, pH 7.0. (A) 0.14 mg/ml vimentin, (B) 2 mg/ml vimentin, (C) 3 mg/ml vimentin. Scale bars, 200 nm.

**Figure 7.3.** Electron microscopy images showing the effect of different buffer conditions on assembly of 0.2 mg/ml (A, C) and 2 mg/ml vimentin networks (B). Networks were assembled at 22 °C in: (A) and (B) 45 mM PIPES, 50 mM NaCl, 1 mM EGTA, 4 mM MgCl\(_2\), pH 6.8, (C) 25 mM PIPES, 1 mM EGTA, 4 mM MgCl\(_2\), pH 7.0. Scale bars, 200 nm.
**Figure 7.4.** Electron microscopy images showing the effect of NaCl and MgCl$_2$ on assembly of 0.2 mg/ml vimentin network. Networks were polymerized at 22°C in: (A) 45 mM Tris-HCl, pH 7.0, (B) 45 mM Tris-HCl, 50 mM NaCl, pH 7.0, (C) 45 mM Tris-HCl, 50 mM NaCl, 4 mM MgCl$_2$, pH 7.0. Scale bars, 200 nm.

**Figure 7.5.** Confocal microscopy images of 1 mg/ml taxol stabilized microtubules. Filaments were polymerized at 37°C in buffer containing: (A) 80 mM PIPES, 1 mM EGTA, 4 mM MgCl$_2$, pH 6.8, (B) 25 mM PIPES, 1 mM EGTA, 4 mM MgCl$_2$, pH 6.8, (C) 25 mM PIPES, 50 mM NaCl, 1 mM EGTA, 4 mM MgCl$_2$, pH 6.8. The bright blobs are likely due to aggregated tubulin. Scale bars, 10 µm.

Microtubules are commonly reconstituted in MRB80 buffer, which contains 80 mM PIPES, 1 mM EGTA, 4 mM MgCl$_2$, pH 6.8. Under these buffer conditions, MTs polymerize into long, straight filaments, as shown in the fluorescence micrograph in Fig. 7.5 A. Owing to their large persistence length, microtubules (which have lengths of several microns) undergo only slight bending undulations. Decreasing the PIPES concentration to 25 mM, the concentration which is compatible with vimentin assembly, did not perceptibly change the appearance of the MTs (Fig. 7.5 B). Addition of 50 mM sodium ions, however, resulted in networks with filaments that were unusually thin (Fig. 7.5 C).

Since the dynamics of microtubules are known to be pH-sensitive [244], we tested the effect of pH on MT assembly. The optimum pH for MT polymerization was reported to be close to 6.8 [289]. Indeed, MTs assembled in 25 mM PIPES buffer of pH 6.8 formed a homogenous network with long and straight filaments (Fig. 7.6 A). Increasing the pH to 7.0 did not change the network structure.
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Figure 7.6. Confocal microscopy images of 1 mg/ml taxol stabilized microtubules, showing the effect of solution pH on MT assembly: (A) pH 6.8, (B) pH 7.0, (C) pH 7.4. Filaments were polymerized at 37 °C in buffer containing 25 mM PIPES, 1 mM EGTA, 4 mM MgCl₂. Scale bars, 10 µm.

Figure 7.7. Confocal microscopy images of 1 mg/ml taxol stabilized microtubules, showing changes in filament length over time. Filaments were polymerized at 37 °C in buffer containing 25 mM PIPES, 1 mM EGTA, 4 mM MgCl₂, pH 7.0 for (A) 15 min, (B) 30 min, (C) 45 min and (D) 1 hour. Scale bars, 10 µm.

(Fig. 7.6 B), but an even higher pH of 7.4, which is commonly used for vimentin assembly, inhibited MT assembly; even after 1 hour, only a few, short filaments were seen (Fig. 7.6 C). We next tested the influence of polymerization time on MT length under buffer conditions compatible with vimentin (25 mM PIPES, 1 mM EGTA, 4 mM MgCl₂, pH 7.0). Within the first 45 minutes of polymerization, the MT length increased with increasing polymerization time (Fig. 7.7 A-C). However, after 1 hour (comparable to the time scale of vimentin assembly), the networks consisted of filaments with an average length exceeding tens of micrometers (Fig. 7.7 D).

From all tested conditions, only the buffer containing 25 mM PIPES, 1 mM EGTA, 4 mM MgCl₂, and pH 7.0 supported proper assembly of homotypic networks of vimentin and MTs. To test whether the two filament types also assemble properly when mixed together, we imaged networks of vimentin mixed with 0.2 mg/ml MTs that had been pre-formed under the same conditions as vimentin. As revealed by EM, both filament types do indeed coexist (Fig. 7.8 A): long and relatively straight microtubules (see arrows) are sur-
rounded by a network of thinner and more curved vimentin filaments. Confocal microscopy of fluorescently labeled MTs embedded inside vimentin networks showed that the MTs are somewhat bent (Fig. 7.8 B-C), in sharp contrast with their straight appearance when assembled alone and observed in free solution (Fig. 7.7 D). This observation suggests that internal stresses in the vimentin network, which may build up during polymerization, distort the MTs. Compressive stresses on stiff filaments embedded in an elastic background may cause short wavelength buckling [32, 68, 158, 38]. Interestingly, fluorescently labeled actin filaments embedded in dark, entangled actin networks were also reported to be highly bent, which was tentatively ascribed to transient entropic trapping of bent filament segments in network void spaces [252].

7.3.2 Mechanics of vimentin-microtubule networks

To test the effect of MTs on the mechanical behavior of vimentin networks, we assembled networks of the separate filament types as well as composite networks in a buffer containing 25 mM PIPES, 1 mM EGTA, 4 mM MgCl₂, and pH 7.0 and measured the viscoelastic shear moduli by rheology. Experiments were performed at 22°C by large amplitude oscillatory shear tests (see Chapter 2), using a parallel plate geometry with a diameter of 40 mm and gap of 80 µm.

The stiffness of pure vimentin networks increased weakly with increasing protein concentration, scaling as $G' \sim c^x$ with an exponent of 0.2 (Fig. 7.9, black squares). $G'$ showed a similar concentration dependence when filaments were assembled in a conventional Tris-based vimentin buffer (45 mM Tris-HCl, 50 mM NaCl, pH 7.0), but with a ca. 1.9-fold larger magnitude (Fig. 7.9, green...
squares). The IF proteins vimentin, desmin, and neurofilaments, typically exhibit a weak concentration dependence with an exponent close to 1 in the absence of crosslinks [264, 180, 242] and a stronger dependence with an exponent of 2 in the presence of multivalent cations, which act as crosslinks [325, 182]. However, the exponent can also be much lower, depending on IF type and buffer conditions. For instance, exponents of 0.47 have been reported for vimentin, and 0.7 for desmin [264]. The origin of these variations in the concentration dependence is hitherto unexplained. It is possible that the variability is related to the degree of adhesiveness between the IFs, which is known to be sensitive to the salt concentration and pH. When assembled in the PIPES pH 6.8 buffer that produced short, branched and unwound filaments (Fig. 7.3 A-B), the network stiffness was independent of protein concentration (Fig. 7.9, yellow squares). Surprisingly, even at low protein content, where EM showed only small aggregates of short filaments, the networks were still predominantly elastic and $G'$ was even somewhat larger than for networks prepared in the other two buffers that both favored assembly of normal-looking filaments. This shows that it is important to combine rheology with EM when the assembly conditions are varied, to check the structure of the filaments.

Addition of 0.1 mg/ml preformed microtubules to networks of vimentin resulted in an increase in network elasticity compared to the pure vimentin net-

**Figure 7.9.** Dependence of the linear elastic modulus (at 0.5 Hz) on vimentin concentration in the presence of three different assembly buffers: optimal buffer for composite system (black squares; 25 mM PIPES, 1 mM EGTA, 4 mM MgCl$_2$, pH 7.0), non-optimal buffer for composite system (yellow squares; 45 mM PIPES, 50 mM NaCl, 1 mM EGTA, 4 mM MgCl$_2$, pH 6.8), and normal vimentin assembly buffer (green squares; 45 mM Tris-HCl, 50 mM NaCl, pH 7.0). EM images on top show the corresponding filament structure. Scale bars, 200 nm.
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Figure 7.10. Linear viscoelastic behavior of composite vimentin-microtubule networks. (A) Influence of MT addition on the linear elastic modulus of networks of 0.2 (black squares), 0.5 (green circles), and 1 mg/ml (black open triangles) vimentin. Orange squares show stiffness of pure MT networks. (B) Frequency dependence of the linear elastic (solid symbols) and viscous (open symbols) moduli for 0.5 mg/ml pure vimentin network (black circles), 0.5 mg/ml pure MT network (orange squares), and composite vimentin-MT network (both proteins at 0.5 mg/ml, yellow symbols).

For 1 mg/ml vimentin networks, $G'$ remained higher in the presence of MTs than for the pure vimentin network, but at 0.2 and 0.5 mg/ml vimentin, $G'$ became comparable to that of the pure vimentin networks at large MT concentration.

To test whether MTs change the dynamics of stress relaxation in composite networks, we measured the frequency dependence of the linear viscoelastic moduli. Pure networks of vimentin showed an essentially frequency-independent $G'$ over the entire range of frequencies probed, with no characteristic relaxation time. The loss tangent, which is the ratio $G''/G'$, was about 0.15 (Fig. 7.10 B). This behavior indicates that the vimentin networks are soft gels with solid-like behavior. In contrast, $G'$ of pure MT networks displayed a pronounced frequency dependence, and the loss tangent was 0.29. Composite networks had an intermediate rheological behavior, though closer to that of vimentin than to that of MTs: $G'$ was weakly dependent on frequency, and the loss tangent was 0.16.
We probed the nonlinear viscoelastic response of the networks by subjecting them to large amplitude oscillatory shear at a constant oscillation frequency of 0.5 Hz. Pure MT networks responded with a very small linear regime until a critical strain amplitude of \( \sim 1\% \), followed by a steady decrease of the elastic and viscous modulus with increasing strain amplitude (Fig. 7.11 A). This strain-softening behavior occurred for MT concentrations of 0.1, 0.2, and 0.5 mg/ml. These observations are consistent with earlier studies of MT rheology [183, 48], where the strain-softening behavior was ascribed to disruption of weak (nonspecific) adhesions between MTs and MT reorientation with increasing deformation. Networks composed of vimentin filaments showed two distinct types of nonlinear response depending on protein content. Dilute networks (0.2 and 0.5 mg/ml) strain-softened above a critical strain amplitude of \( \sim 10\% \) while denser networks (1 mg/ml) showed a strain-stiffening response (Fig. 7.11 B). However, the increase in network stiffness in denser networks was preceded by a small dip in \( G' \). This type of nonmonotonic behavior has also been observed for networks of desmin (a type III IF) [14]. Upon incorporation of MTs in the 1 mg/ml vimentin network, the strain-stiffening behavior was markedly suppressed. The elastic modulus was constant until a strain amplitude of \( \sim 10\% \), similar to the pure vimentin system, thereafter decreased, and then slightly increased again at a strain amplitude of about 200\%. The breakage strain was \( \sim 300\% \), similar to that of the pure vimentin network. The presence of MTs in the more dilute vimentin networks did not noticeably affect the nonlinear, strain-softening response.
7.4 Discussion and Concluding Remarks

In this chapter, we developed a novel \textit{in vitro} model system that will enable us in future to study the mechanical consequences of mixing different cytoskeletal filament types, specifically stiff microtubules and flexible vimentin filaments. We screened different polymerization buffer compositions to identify conditions that favor simultaneous polymerization of both filament types. We observed that both vimentin and MT assembly are very sensitive to buffer type and concentration, the presence of specific salts, and solution pH. We identified an optimal buffer composition (25 mM PIPES, 1 mM EGTA, 4 mM MgCl$_2$, and pH 7.0) that supports proper assembly of MTs as well as vimentin, as verified by electron microscopy. We performed first rheological experiments on both the pure and the composite networks in this buffer.

The vimentin networks behaved as weak viscoelastic solids with shear moduli of a few Pascal. The elastic modulus increased rather weakly with vimentin concentration, according to a power law with exponent 0.2. This increase is weaker than observed previously for entangled vimentin networks (exponents 0.5-1, \[264, 180\]) and crosslinked networks (exponent 2, \[325, 182\]), and weaker than theoretically predicted for networks of semiflexible polymers that are entangled (exponent 1.4, \[136\]) or crosslinked (exponent 2.2, \[195, 94\]). Prior studies of other IF systems (desmin) also showed a weaker than linear increase, with exponent 0.5 or 0.7 \[264\]. Vimentin networks are crosslinked by divalent cations such as Mg$^{2+}$, which was present in our buffer \[180\]. It will be interesting to perform more systematic measurements of the dependence on the rheology on vimentin concentration at different Mg$^{2+}$ concentrations. In our experiments, we used a fixed concentration of Mg$^{2+}$, meaning that the crosslinker to vimentin molar ratio decreased with increasing vimentin concentration. This changing crosslink density could possibly contribute to the weak dependence of $G'$ on vimentin concentration.

The enhancement of the linear elastic modulus of 1 mg/ml vimentin networks with different amounts of MTs suggests that there may potentially be direct interactions between vimentin and MTs. However, the evidence is not very strong at this stage. More systematic and extensive rheological measurements on composite networks with different compositions will be needed. Networks with equal weight concentrations of MTs and vimentin (both 0.5 mg/ml) had a stiffness comparable to that of the pure vimentin system of 0.5 mg/ml. This is consistent with prior measurements of mixtures of tissue-purified vimentin and MTs at a constant total protein concentration of 1 mg/ml \[152\].

Rheological measurements are an indirect indicator of filament-filament interactions. In future, it will be interesting to combine them with more direct experimental assays such as co-sedimentation assays and video microscopy tracking of filament diffusion in the composite networks. We are unaware of any literature on interactions between vimentin and MTs \[266\]. It have been reported however, that vimentin interacts with actin, through its tail domain \[53\], and that microtubules interact with neurofilaments via the long neurofilament side-arms \[137\].

In future it will be interesting to supplement the minimal model system
developed in this chapter with purified proteins that are known to crosslink MTs to vimentin in vivo, such as plectin [309, 290, 129], which is a member of the plakin family of cytolinkers. IFAP-300K protein [178, 132] is also a candidate cross-linker. Microtubule-associated protein MAP-2, which binds with high affinity to microtubules and projects long arms from the MT surface, has also been shown to interact with IFs [173, 120, 30] as well as actin [108, 107]. Motor protein such as kinesin and dynein, which in vivo are responsible for transporting IF precursor particles along MTs [121, 114, 237, 199, 329], could be used to study active turnover of the IF network in the presence of MTs and motor-mediated interactions [114, 174].

We observed little effect of the MTs on the nonlinear response of the vimentin networks. Networks of 0.2 and 0.5 mg/ml vimentin strain-softened, and this response was unchanged in the presence of MTs. Given the low protein density, the network deformation is likely nonaffine. With increasing strain amplitude, networks of 1 mg/ml vimentin first strain-softened slightly and then strain-stiffened. Addition of MTs made the initial strain-softening response stronger, but the subsequent strain-stiffening response remained. Thus, we did not observe reinforcement of the nonlinear response of vimentin networks by the MTs, in contrast with experiments on composite networks of actin and MTs [181]. However, more systematic measurements screening different vimentin and MT concentrations are needed to verify whether reinforcement can indeed be excluded. Another factor is the length distribution of the MTs, which is also expected to influence the transition between nonaffine and affine deformations, as shown by recent simulations [11]. It would be interesting to check how MTs of different lengths may affect the nonlinear response of the composite networks.

The data presented here are the first attempt to understand the physical principles that govern the mechanical behavior of composite IF-MT systems. A proper integration of IFs with other filament structures, including MTs and associated proteins, is necessary to maintain proper viscoelastic properties of living cells. At least 86 different human diseases arise from mutations in more than 50 different genes that encode the family of IF proteins [291, 223]. These mutations are likely to include defects in IF assembly or their interaction with other cytoskeletal components. The in vitro system developed in this chapter may help to bring us closer to understanding the role of molecular structure and interactions of cytoskeletal filaments in shaping cell mechanics.
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