1 Introduction:
The structural and mechanical integrity of the cell
1.1 Cytoskeleton and extracellular matrix

The cell is a composite, highly dynamic and heterogeneous system. Its shape, mechanical functions, and ability to adapt to changing circumstances are all controlled from within, by the cytoskeleton (CSK). The CSK is a network composed of three different filament types: actin microfilaments (MFs), microtubules (MTs) and intermediate filaments (IFs) (Fig. 1.1). These filaments contribute in distinct ways to the overall mechanical behavior of cells. Moreover, they may collectively balance preexisting forces inside the cell via local compression and tension [147, 278]. Actin and intermediate filaments are able to resist internal tension by stretching their bent shape. The stiffer microtubules, on the other hand, can bear larger compressive loads. The efficacy of this system is ensured by the presence of accessory proteins that link the filaments to each other and to other cell components. Unlike conventional, polymeric materials, the cytoskeleton can actively generate forces by means of active filament (de)polymerization and the action of motor proteins. As a result, cells can autonomously adapt their shape and mechanical behavior [196].

![Figure 1.1. Schematic representation of a cell embedded in an extracellular matrix (ECM). Actin microfilaments (MFs) are attached to cell adhesion sites and contribute with myosin motors to cellular contraction. Microtubules (MTs) span the cell interior and provide tracks for transport of vesicles, proteins, and organelles by motor proteins. Intermediate filaments (IFs) link the nucleus (grey shaded oval) with the cell periphery, creating a supporting framework that distributes stresses.](image)

Cellular functions are highly dependent on the surrounding extracellular matrix (ECM). This fibrous matrix composed of various macromolecules produced by cells provides an anchoring support to cells and helps to bind cells together to form tissues. The ECM is mainly composed of fibrous proteins such as collagen, elastin, fibronectin and laminin that strongly interact with each other [142]. The cytoskeleton inside cells is physically anchored to the ECM by means of
transmembrane proteins known as integrins. This connection enables cells to control the spatial organization of the ECM by the application of contractile forces, while at the same time contributing to ECM-dependent changes in cell shape [301].

1.2 Biopolymers: supramolecular protein architectures

Proteins are made up of one or more long polypeptide chains, which are linear chains of different amino acids in a specific sequence that is encoded in an organism’s genes. This sequence dictates how the polypeptide chains fold. There are various folding motifs, of which the α-helix or β-sheet are most prevalent. Proteins typically fold into a globular form (e.g. actin, tubulin) or fibrous form (e.g. intermediate filaments, fibrin, collagen). Self-assembly of many identical protein molecules driven by specific non-covalent interactions leads to formation of filaments constituting the cytoskeleton and the ECM.

The supramolecular architecture of the CSK and ECM fibers is specific for each filament type. Actin and microtubules are polar structures made of globular protein subunits held primarily by longitudinal bonds (Fig. 1.2 A-B). The weaker lateral bonds allow individual protofilaments to twist around each other to form a helical lattice in case of actin and helical tube in case of MTs. These relatively weak interactions make the filaments largely inextensible and rather fragile. Weak assembly allows, however, for rapid turnover of individual subunits and thereby helps to dynamically rearrange the cell’s interior, which is necessary for cell locomotion as well as for intracellular transport. Fibers from the ECM, like collagen and fibrin, are on the other hand more stable, and their supramolecular architecture is reinforced by covalent bonds between their protein subunits.

The hierarchical architecture of protein biopolymers confers remarkable mechanical properties. Intermediate filaments, for instance, are extremely extensible due to their rope-like structure (Fig. 1.2 C). IFs lack the overall structural polarity that is characteristic for actin filament and microtubules and their multiple protofilament organization makes them easier to bent and more difficult to break compared to actin and microtubules. Fibers from the IF family can be stretched up to 4 times their original length [163], even though the lateral bonds that connect subunits are relatively strong. The high extensibility of IFs is a consequence of their multilevel architecture that is sensitive to an applied force. Under increased levels of deformation, stretching of individual protofilaments followed by molecular unfolding takes place [241]. A similar fiber stretching mechanism is observed for fibrin, which is the main blood clotting factor and an extracellular matrix protein. Due to a half-staggered organization of the fibrin molecules in protofibrils and further hierarchical assembly into fibers (Fig. 1.2 D), a succession of different structural levels is achieved. This, in return, results in a high extensibility of individual filaments [191] that is further enhanced by flexible polypeptide domains that connect the protofibrils [82].

Complex structural hierarchy does not always favor high extensibility of
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Figure 1.2. Supramolecular architecture of various protein filaments. Microtubules (A) and actin filaments (B) are polar filaments built up from globular protein subunits (actin and tubulin). Intermediate filaments (C) do not posses polarity due to an antiparallel arrangement the fibrous protein subunits. Collagen fibres (D) are self-assembled from triple helical tropocollagen molecules in a quarter-staggered arrangement. Collagen microfibrils laterally and longitudinally associate into fibrils, and fibrils bundle to form fibres. Fibrin fibers (E) are twisted bundles of loosely coupled protofibrils, which are made of fibrin molecules in a half-staggered arrangement.
filaments. Collagen, the most abundant ECM protein in human tissue, is built from three long polypeptide chains wound into a triple helix. This tropocollagen molecule binds side-by-side and end-to-end to other tropocollagen molecules to form collagen fibrils (Fig. 1.2 E). Due to rigid connections between individual molecules, collagen fibers, like actin filaments and microtubules, are not very extensible. Yet, collagen’s hierarchical organization allows for viscous sliding of subunits and results in very tough filaments [45].

1.3 Nonlinear elasticity of biopolymer networks

Differences in the structure and self-assembly of subunits endow filaments with distinct mechanical properties. The largest difference between different filament types is evident from their highly disparate bending rigidities. Actin filaments are rather flexible filaments with diameter of $d \sim 7$ nm and the persistence length of $l_p \sim 17$ µm [97]. Due to the structural organization these filaments are hard to stretch and easy to break. Hollow cylindrical microtubules ($d \sim 25$ nm and $l_p \sim 5$ mm) [97] are strong and rigid but fragile, while rope-like intermediate filaments ($d \sim 9$ nm and $l_p \sim 1$ µm) [182, 210] are easy to bend but hard to break. By contrast, ECM filaments have diameters that are at least 10-fold larger than those of cytoskeleton filaments. Nevertheless, those filaments can still be classified as a semiflexible polymers since their persistence length is comparable to their contour length, $L_0$.

In living cells biopolymer filaments form crosslinked and bundled structures that are much stronger than individual filaments. In vitro reconstituted networks of different filament types, both from the cytoskeleton and ECM, have distinct mechanical properties and dynamics. However, these networks all show a common response to an applied stress: their network stiffness remains constant at small deformations but it increases nonlinearly at high stresses [285]. This unusual strain-stiffening response of biopolymers makes them different from most synthetic polymers and is thought to help protect cells and tissues from mechanical damage.

1.3.1 Enthalpic and entropic contributions to network elasticity

The degree and the type of elastic nonlinearity in biopolymer networks depend on the network microstructure [177, 57], cross-linker density [177, 94] and processes such as cross-linker unbinding [175], stretching [41] and unfolding [71]. The stress-stiffening behavior has been shown to originate either from the entropic (thermal, $E$) elasticity of individual filaments [195] or from the enthalpic (athermal, mechanical, $M$) elasticity arising from network rearrangement or filament buckling [224]. The former corresponds to a more uniform (or affine, $A$) strain field, while the latter corresponds to a highly non-uniform (nonaffine, $NA$) strain field. Which of these two regimes is expected
depends on network connectivity and density. The nonaffine regime is characteristic of sparsely crosslinked networks, as shown by 2D [72, 118, 311, 67] and 3D computer simulations [49, 144]. In this limit, the shear modulus scales linearly with bending modulus of the filaments, $\kappa$ [166, 262]. There are, however, no simple analytical models that can quantitatively describe the elasticity in this regime. The affine regime applies to densely crosslinked, homogenous networks. Since all filaments experience exactly the same deformations, the network elasticity can be calculated analytically from the density of the fibers and their single-fiber stretching rigidity, $\mu$, by orientationally averaging over all fibers [285, 94]. This analytical model predicts that the network stiffness scales with protein concentration, $c$, according to $G_0 \sim c^{11/5}$ for a thermal (AE) system [195] and as $G_0 \sim c^1$ for an athermal (AM) system [119]. Any nonaffinity will tend to lower the network stiffness by increasing the number of degrees of freedom in the system.

1.3.2 Nonaffinity crossover point

Sparsely crosslinked, heterogeneous networks exhibit a spatially inhomogeneous strain field even when the applied shear is uniform. The filaments therefore undergo reorientations rather than alignment in the shear direction as predicted by continuum elastic theory. This results in the elastic energy being predominantly stored in bending deformations and networks that are overall softer since only few filaments can store elastic energy. Nonaffine deformations can occur not only in case of low density networks, but they also occur in heterogeneous bundle systems [177, 134, 133] and were postulated affect even the mechanical behavior of high density F-actin networks [94].

The transition between NA/A regimes of deformation depends strongly on the density of filaments and three different filament length scales: the filament contour length, $L_0$, the mean crosslink distance, $l_c$, and the bending length, $l_b$, that relates the bending and stretching moduli of filaments according to $l_b=(\kappa/\mu)^{1/2}$ [119]. In 2D random networks of monodisperse rods, the distribution of stress between stretching and bending modes is captured by the so-called nonaffinity length, $\lambda$, [119]. Scaling by this length universally captures the effect of changing $l_c$ and the filament stiffness. The ratio of $L/\lambda$ can therefore be used to establish what regime the network is in.

Recent lattice simulations and analytic theories have shown that, in addition to crosslink density and filament length, there is one more key parameter that defines the affine transition point, namely the network connectivity, $z$ [40]. In case of a local connectivity of $z=3$ that corresponds to a branched network, the deformation field is expected to crossover from nonaffine to affine at a dimensionless ratio of bending to stretching rigidity, $\kappa_{bend}/\kappa_{stretch}l_c^2$ of $10^{-2}$. Below this crossover point, fiber bending dominates, while above it, affine stretching dominates, in accordance with prior theoretical and computational studies. However around this crossover point, a remarkably broad intermediate regime exists. In this mixed stretch/bend regime, the shear modulus scales simultaneously with $\kappa$ and $\mu$.

The presence of other filamentous components that differ in length and rigid-
ity can also have effect on the A/NA crossover point. It was shown in polydisperse networks that the presence of stiffer filaments homogenizes the initially nonaffine strain field of monodisperse system in the linear mechanical regime, increases the shear modulus and shifts the mechanics of the network from nonaffine to affine even at low network densities [11, 10, 181]. This mechanism is only apparent close to the NA/A transition regime, while deep in the nonaffine regime stiff filaments redistribute nonaffine deformations making the system even more nonuniform [10]. In this case there is, however, no simple definition of the crossover point.

1.4 Outstanding questions

Most of the existing theoretical models that are commonly used to describe the nonlinear response of biopolymer network to large deformations treat the individual filament as isotropic inextensible rods with constant diameter. While some types of filaments, such as actin and microtubules, are indeed rather inextensible, the majority of biopolymer filaments from the cytoskeleton and ECM show a high extensibility that can be accounted for by their complex internal architecture. Recently the affine entropic model extended to include enthalpic stretching of the filament backbone [285] was used to explain this high extensibility of intermediate filament networks in the nonlinear mechanical response [180]. It still remains unclear, however, how various levels of organization of supramolecular filaments contribute individually to the overall nonlinear mechanics of those biopolymer networks.

The structural hierarchy of biopolymer filaments ensures high network functionality and the presence of multiple structural levels decreases the probability of catastrophic failure. Mutations in the basic structural units, however, cause defects in polymer assembly and can lead to pathological conditions [46]. Most human diseases related to mutations in IFs family and ECM proteins result from formation of filaments with a distorted internal architecture. Changes in intermolecular adhesion generally reduce network resilience and cause premature network failure by breaking, for example, hydrogen bonds that are crucial to defining the structure of the protein building blocks. These changes at the molecular level destabilize the whole filament structure, making fibrillar packing less regular and possibly causing protein unfolding, sliding of molecules against each other, or breaking of crosslink bonds much earlier than anticipated by Nature.

In some cases, structural defects can also enhance internal order. The highly ordered hierarchical structure of amyloid plaques, for instance, results in robust structures in which the dissipation of mechanical stresses is prevented. A full understanding of the contribution of the hierarchical structure to the mechanical stability of biopolymers and their nonlinear network mechanics is therefore crucial for future treatment strategies to remedy or even prevent disease states.
1.5 Scope of this thesis

The goal of this thesis is to elucidate the role of the supramolecular, internal architecture of protein filaments that build the cytoskeleton and extracellular matrix in the macroscopic nonlinear elasticity of the networks they form. This high nonlinear elastic response to external forces is thought to help protect cells and tissues from mechanical damage. To this end, we combine macroscopic rheology to characterize the nonlinear elasticity with imaging and scattering techniques to characterize the morphology and supramolecular structure of different biopolymer filaments. The experimental approach is described in Chapter 2.

In Chapter 3, we study the nonlinear elasticity of the major blood-clotting protein fibrin in relation to its supramolecular architecture. We show that in the limit of so-called “coarse fibrin clots”, which are composed of fibers that are thick bundles of thin, semiflexible protofibrils, fibrin clots exhibit a strong strain-stiffening response to an applied steady stress, with a complex dependence on stress. We can account for this response in terms of a loose semiflexible bundle model, where small stress causes entropic stretching of the fibrin bundles, moderate stress leads to filament backbone stretching, and large stress causes entropic stretching of flexible domains inside the fibers. These consecutive stretching processes under increasing levels of load ensure a high resistance of fibrin clots to large deformations.

In Chapter 4, we investigate the influence of bundle size on the nonlinear elasticity of fibrin clots. We characterize the nonlinear elasticity in the limit of so-called “fine fibrin clots”, which are composed of thin bundles of only 3 protofibrils. Similar to coarse gels, the fine clots also show a dramatic stress-stiffening response with a complex functional dependence on stress. In analogy to the coarse clots in Chapter 3, we can describe the nonlinear response by an affine entropic model if we include backbone extension and shear-induced alignment of the protofibrils. We show that the persistence length and stretch modulus of the protofibrils are fully consistent with the same parameters deduced from the rheology of coarse clots. Therefore, Chapters 3 and 4 together strongly support an affine entropic model for fibrin rheology, independent of bundle size.

In Chapter 5, we provide a complementary analysis of the nonlinear elasticity of coarse fibrin clots based on large amplitude oscillatory shear (LAOS). We show that the strain response to an imposed sinusoidal stress is highly non-sinusoidal, indicating strong nonlinearity. We quantify the departure from linearity by various methods, including Fourier Transform analysis and Lissajous plots. We find that these methods have comparable sensitivity to the onset of stress-stiffening as measurements of the differential modulus that we used to probe the nonlinear behavior of fibrin networks in Chapter 3 and 4.

In Chapter 6, we shift the focus to the rheology of a different type of extracellular matrix protein, namely collagen. In most tissues, collagen fibers consist predominantly of collagen type I, which forms heterotypic fibrils with another collagen, namely type V. This co-assembly is thought to provide a mechanism for diameter regulation. We show that increasing levels of collagen V strongly reduce the stiffness of hybrid collagen networks compared to homotypic
collagen I gels. It is, however, difficult to quantitatively interpret the influence of collagen V content on the mechanics of heterotypic collagen networks, since the network appears to deform in a nonaffine manner. There is not yet an analytical theory available to give quantitative predictions of the elastic modulus in this regime. We tentatively ascribe the influence of collagen V to changes in the interaction among tropocollagen molecules within or between fibers. Our results suggest that collagen composition can be used as an alternative control parameter to design collagen-based biomaterials.

In Chapter 7, we turn to cytoskeletal biopolymer networks. We implement a new in vitro model system that helps pave the way for future systematic studies of the influence of interactions between different filament types on composite network mechanics. In vivo, all three of the cytoskeletal filaments, actin microfilaments, microtubules (MTs) and intermediate filaments (IFs), are known to colocalize and strongly interact with each other. In vitro studies of composite actin-IF or actin-MT networks indicate that the disparate bending rigidities of the filaments and interactions between them can lead to surprising non-additive effects on network elasticity. We set up for the first time a composite network of MT and vimentin (a member of the IF family that is present in all mesenchymal cells) and identify buffer conditions that allow for simultaneous assembly of both proteins. Rheological data show that the stiffness and nonlinear response of this composite system are intermediate between those of the pure components.

Finally, in Chapter 8, we describe a new shear cell device that can be used to visualize changes in the microstructure of biopolymer networks under large shear by confocal microscopy. This device permits application of large oscillatory and steady shear strains while simultaneously imaging the sample through the glass bottom plate. The device can be used to quantify nonaffinity in the strain field by tracking fiducial markers such as probe beads embedded inside the network, or to directly observe fiber bending and stretching or network rupture at large stress. Such measurements are critical to obtain a full understanding of the influence of the multiscale structure of biopolymer networks on their remarkable nonlinear elasticity.