

# From ultra-soft slime to hard $\alpha$ -keratins: The many lives of intermediate filaments

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**Synopsis** Intermediate filaments are filaments 10 nm in diameter that make up an important component of the cytoskeleton in most metazoan taxa. They are most familiar for their role as the fibrous component of  $\alpha$ -keratins such as skin, hair, nail, and horn but are also abundant within living cells. Although they are almost exclusively intracellular in their distribution, in the case of the defensive slime produced by hagfishes, they are secreted. This article surveys the impressive diversity of biomaterials that animals construct from intermediate filaments and will focus on the mechanisms by which the mechanical properties of these materials are achieved. Hagfish slime is a dilute network of hydrated mucus and compliant intermediate filament bundles with ultrasoft material properties. Within the cytoplasm of living cells, networks of intermediate filaments form soft gels whose elasticity arises via entropic mechanisms. Single intermediate filaments or bundles are also elastic, but substantially stiffer, exhibiting modulus values similar to that of rubber. Hard  $\alpha$ -keratins like wool are stiffer still, an effect that is likely achieved via dehydration of the intermediate filaments in these epidermal appendages. The diverse mechanisms described here have been employed by animals to generate materials with stiffness values that span an impressive eleven orders of magnitude.

## Introduction

The cytoskeleton of metazoan cells consists mainly of three filament types: F-actin filaments 6–7 nm in diameter, microtubules 24 nm in diameter, and intermediate filaments 10 nm in diameter (Alberts et al. 2007). While the sequence, structure, and function of F-actin and microtubules are highly conserved across vast evolutionary distances, intermediate filaments are notable for their diversity. In humans alone, there are 70 intermediate filament genes (Human Intermediate Filament Mutation Database, [www.interfil.org](http://www.interfil.org)) in six distinct classes, and considering that many intermediate filament proteins can copolymerize with several different partners, the number of unique 10 nm intermediate filaments in humans is likely in the hundreds. The diversity of intermediate filaments in other species is not as well-described, but is undoubtedly impressive given the diversity in humans.

While many of the functions of F-actin and microtubules are related to dynamic processes carried out by cells (e.g., muscle contraction, mitosis, intracellular trafficking) (Alberts et al. 2007), intermediate filaments appear to be suited to more

passive mechanical roles, and this likely has to do with their chemical stability. F-actin and microtubules exist in a dynamic equilibrium with soluble pools of monomers, and this, along with a dizzying number of proteins that can tip the equilibrium one way or another or modify the structure of the networks, allows these systems to respond rapidly to the needs and challenges of the cell. In contrast, intermediate filaments are far less soluble (Fey et al. 1984) and therefore well-suited to imparting passive mechanical integrity to cells and tissues. This is not to say that the intermediate filament network in living cells is static—it must disassemble and reassemble before and following cell division, and it is also known to remodel in subtle ways to changes in the physical and biochemical environment of the cell (Windoffer et al. 2004).

Cell biologists continue to find unique cellular functions for intermediate filaments (Kim and Coulombe 2007; Magin et al. 2007), but the one function that appears to unite all six classes is a structural one. Evidence from a variety of sources, including transgenic animal models and human

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genetic diseases caused by mutations in intermediate filament genes, demonstrates that faulty or absent intermediate filament networks often lead to fragile cells and tissues (Magin et al. 2004; Omary et al. 2004). Intermediate filaments also make up the fibrous component of a group of nonliving biomaterials known as  $\alpha$ -keratins, which include the soft outer layer of skin (*stratum corneum*) in amniotes, as well as harder mammalian structures such as hair, fur, nail, claw, hoof, and horn. In  $\alpha$ -keratins, intermediate filaments play an obvious structural role given that they make up the largest volume fraction of these nonliving keratinized tissues.  $\alpha$ -keratins are crucial for water conservation (*stratum corneum*) in amniotes and for heat conservation (hair and fur) in mammals and therefore these versatile biomaterials likely played a crucial role in the successful invasion of terrestrial habitats by vertebrates.

The mechanical properties of intermediate filaments have long been assumed to be the same as the properties of  $\alpha$ -keratin fibers such as wool, based on the fact that wool consists primarily of aligned intermediate filaments (Bray 2001; Howard 2001). Recent work, however, suggests that intermediate filaments in cells are radically different in their mechanics from wool fibers (Fudge et al. 2003; Kreplak et al. 2005; Kreplak and Fudge 2007). This insight has spawned new research into the diverse mechanical functions of intermediate filaments, which is the focus of this article. Here, we will highlight the mechanical behavior of four different materials, each of which is built from intermediate filaments, and each of which performs a unique mechanical function for the organisms that make them.

### Hagfish slime—an ultrasoft material

As a rule, intermediate filaments are strictly intracellular entities, whether they occur in living cells or the nonliving, keratinized cells within  $\alpha$ -keratins. Like much about their biology, however, hagfishes are an exception—they are the only animal known to secrete intermediate filaments, and they do so in their defensive slime (Downing et al. 1981a, 1981b). Hagfishes (Fig. 1) are best known for their ability to produce alarming quantities of slime when they are provoked or stressed, and they do this by ejecting slime exudate from epidermally derived slime glands that line both sides of the animal's body (Downing et al. 1984; Spitzer et al. 1984, 1988; Spitzer and Koch 1998). Hagfish slime differs from other slimes in that it contains not only slippery mucin-like



Fig. 1 All hagfishes, like this Pacific hagfish (*Eptatretus stoutii*) possess numerous slime glands from which they can release vast amounts of stringy slime when they are stressed or provoked.

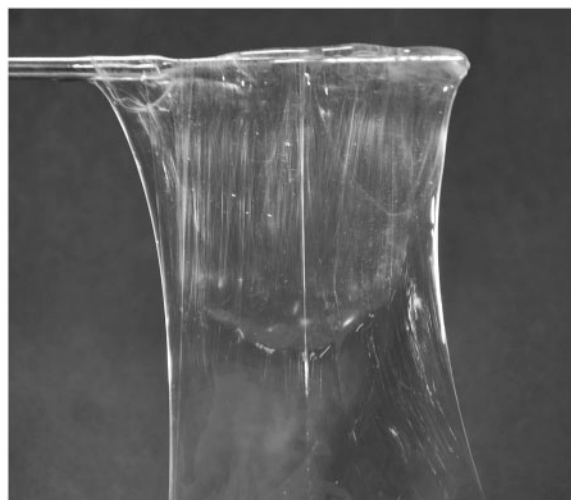


Fig. 2 Hagfish slime is an ultra-dilute assemblage of seawater, mucus, and fine protein threads. In this sample produced in the laboratory from slime collected from an anaesthetized hagfish, the fine web of entangled slime threads are visible.

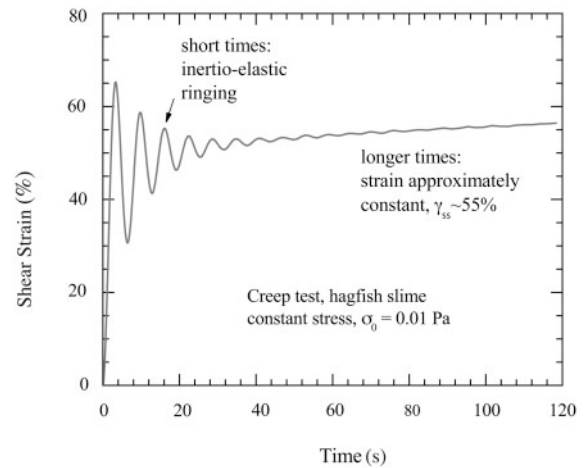
molecules, but also a fibrous component built from intermediate filaments (Fig. 2) (Koch et al. 1994, 1995). The fibrous component originates within specialized cells in the slime glands known as gland thread cells. These unique cells express intermediate filament proteins that assemble into 10 nm intermediate filaments, which then bundle into a single protein polymer thread that takes up the vast majority of the cytoplasmic volume in mature cells. The thread is about  $1\ \mu\text{m}$  at its narrowest and about  $3\ \mu\text{m}$  at its widest, and when it is fully unraveled, it can be about 15 cm long (Fudge et al. 2005). How exactly the cell manages to build a continuous and

mechanically coherent 15 cm long thread within the cytoplasm is at this point a complete mystery.

One of the most remarkable things about hagfish slime is how much of it can be made with so little starting material. A typical mass of slime released by a hagfish has a volume of about 900 ml, and this mass contains only 20 mg of slime threads and about 15 mg of mucins (dry weight) (Fudge et al. 2005). In contrast, typical mucus secretions contain about the same mass of mucins *per milliliter* (Sellers and Allen 1989). Furthermore, the so-called superabsorbent materials, of the kind that are used in disposable diapers, can absorb 50× their weight in water (Dutkiewicz 2002). In hagfish slime, the ratio is 26,000×. Do not look for hagfish slime in diapers any time soon, however, because there is an important difference between superabsorbent gels and hagfish slime that has to do with how the water is immobilized.

In superabsorbent materials, water is tightly bound to the polymers to form a weakly cross-linked gel, and this is why disposable diapers do not leak as long as their capacity has not been exceeded. In contrast, the water in hagfish slime is not tightly bound, and will leak out over the course of minutes under a suitable driving pressure (as when the slime is lifted into air) (Fudge et al. 2005). It is therefore not appropriate to talk of the water *absorbed* by hagfish slime, aside from the small volume taken up by condensed mucins that swell upon contact with seawater. Instead, it appears that bulk seawater is *entrained* within microscopic pores in the slime, and it is the viscous resistance to the flow of water through this network of pores that allows the slime to be lifted into air, at least momentarily.

The viscous entrainment of large volumes of water may at first appear like a strange function for a material that is ejected by an animal when it is attacked. This starts to make more sense, however, when one considers the most likely function of hagfish slime, which is to thwart attacks by gill-breathing predators (Lim et al. 2006). One of the things that makes teleost fishes such effective predators is their ability to capture prey via suction feeding, a process that is effected by a rapid expansion of the buccal cavity and a subsequent inrushing of water. Suction feeding is such a fast event that most prey typically have little chance of evasion once the attack has been detected. Hagfishes have evolved a counter-attack strategy that targets fishes where they are most vulnerable—the gills. We have shown that hagfish slime dramatically increases the hydrodynamic resistance of teleost gills, at least in freshly dead specimens, and the



**Fig. 3** Results of a creep test conducted with hagfish slime reconstituted from fresh exudate mixed with seawater in the laboratory. At short times the strain oscillates due to inertio-elastic ringing, in which the sample elasticity couples with the finite instrument rotational inertia to “ring” at a resonant frequency, just like a mass at the end of a spring. At longer times the strain is approximately constant,  $\gamma_{ss}$ , and this is representative of a primarily elastic material response.

likely result for a predator that gets a mouthful of slime is suffocation. The slime achieves this effect not by binding seawater tightly, but rather by simply decreasing the size of the pores through which the water must flow (Lim et al. 2006).

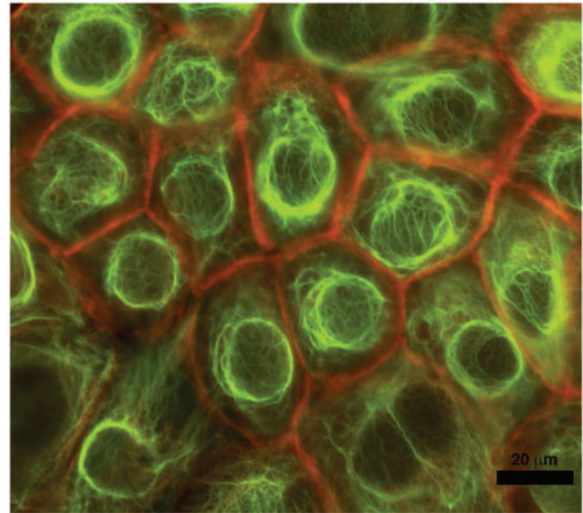
We recently began characterizing the material properties of mature hagfish slime using a torque-controlled cup-and-bob rheometer (Macosko 1994). We performed creep tests, in which a small constant load was imposed (shear stress  $\sigma_0 = 0.01$  Pa) and the resulting strain  $\gamma(t)$  was observed as a function of time. At long times, the strain approaches a constant,  $\gamma(t) \rightarrow \gamma_{ss}$ . The equilibrium elastic modulus of the sample can then be approximated by  $G_0 = \sigma_0 / \gamma_{ss}$ . We measured this elastic modulus to be  $G_0 = 0.02$  Pa (Fig. 3), which is about six orders of magnitude more compliant than materials like gelatin, making it one of the softest biomaterials known.

### Cytoskeletal networks—soft entropic gels

The ultrasoft slime produced by hagfishes is an exceptional material because of its extremely low modulus of elasticity, and also because it contains secreted intermediate filaments. In other organisms, however, intermediate filaments function exclusively *within* the cell, either as part of the cytoskeleton of a living cell, or as the fibrous component of the nonliving, fiber-reinforced  $\alpha$ -keratins. In the former case, cytoplasmic intermediate filaments form an

elaborate network of filaments that impart the cell with soft gel-like elasticity. To understand the origin of this elasticity requires some knowledge of the physical behavior of nanoscale filaments. Soft gels like gelatin are elastic because the collagen fibrils that make them up are flexible enough that collisions with solvent molecules make them bend. The overall effect of this thermal agitation on a long flexible polymer or filament is that its 3D conformation fluctuates over time. When a network of fluctuating filaments is deformed, the conformational freedom of the filaments is restricted and this represents a decrease in entropy of the network (Heidemann et al. 2000). The result is the development of an elastic restoring force that resists deformation and returns the gel to a state of maximum entropy when a load is removed. The tendency of a filament or polymer molecule to gyrate and therefore generate entropic gel elasticity can be quantified by a parameter known as the persistence length (or  $L_p$ ) (Gittes et al. 1993).  $L_p$  can be roughly understood as the distance over which two points on a filament remain spatially correlated over time as the filament gyrates due to thermal fluctuations. In isotropic materials,  $L_p$  is directly related to the bending stiffness of the filament,  $L_p = EI/kT$ , where  $EI$  is the flexural rigidity and  $kT$  is the thermal energy available to cause fluctuations (Boal 2002). Filaments with long  $L_p$  appear straight and rigid under the TEM, whereas filaments and molecules with short  $L_p$  appear wavy. Protein filaments with diameters on the scale of tens of nanometers typically have  $L_p$  on the scale of  $10^0$ – $10^3 \mu\text{m}$  (Gittes et al. 1993; Kojima et al. 1994; Kurachi et al. 1995; Felgner et al. 1996). If the filaments are longer than their  $L_p$ , they enjoy conformational freedom and tend to form filament networks that behave as soft entropic gels.

Intermediate filament proteins self-assemble *in vitro* and *in vivo* into high-aspect-ratio filaments with  $L_p$  values between 0.5 and  $1.0 \mu\text{m}$  (Hohenadl et al. 1999; Mücke et al. 2004), which is far less than the length of a typical filament. Suspensions of intermediate filaments therefore readily form entropic gels, and it is likely that this soft elasticity dominates the mechanical contribution of intermediate filaments at low cell strains (Fig. 4). Indeed, much of the work that has been done on the mechanics of intermediate filaments in cells has focused on the properties of entropic gels that have been constructed from purified intermediate filaments *in vitro*. From these studies we know that networks of intermediate filaments are soft and elastic, with elastic moduli in the range of 30 Pa (Janmey et al. 1991). Compared to gels made from



**Fig. 4** Deformation of intermediate filaments networks like the ones shown here result in a decrease in the entropy of the network and the development of an elastic restoring force. These human keratinocyte cells expressing GFP-tagged keratin 14 (green) were fixed and stained with rhodamine phalloidin, which highlights the cortical F-actin (red) in these cells.

F-actin and microtubules, intermediate filament gels are softer and far more extensible, and they also exhibit dramatic strain stiffening at high strains. The low modulus and high extensibility can be partly attributed to the low persistence length of the filaments, which results in a greater amount of “slack” in the network. The strain stiffening, however, is most likely a result of the tensile properties of the individual filaments themselves (Kreplak and Fudge 2007). In a recent paper on the mechanical response of the intermediate filament network in human keratinocytes to uniaxial strain, we showed that the network is capable of deforming elastically to strains up to 80%, with little or no visible damage to the network (Fudge et al. 2008). A significant portion of this elasticity can likely be attributed to soft entropic gel elasticity, whereby cell deformation results primarily in a decrease in the conformational entropy of the filament network, with very few filaments being loaded directly in tension.

### Hydrated intermediate filaments—rubber-like tensile elements

What happens then at higher strains, when more and more intermediate filaments are loaded directly in tension? To answer this question, it is important to know the tensile properties of intermediate filaments. Some of the best information we currently have about intermediate filament mechanics comes from



**Fig. 5** Hagfish slime threads are relatively pure bundles of intermediate filaments that exhibit rubberlike elasticity at strains up to 35%. Shown here are a number of thread cells, one of which has started to unravel. When fully unravelled, thread cells can reach lengths of 15 cm.

studying the material properties of hagfish slime threads, which are essentially pure bundles of intermediate filaments (Downing et al. 1984; Fudge et al. 2003). While they are small, with an average diameter of about  $2\ \mu\text{m}$ , they are much bigger than single 10 nm intermediate filaments, and they can be readily manipulated with a stereomicroscope and fine forceps (Fig. 5). Using a fine glass rod as a force transducer, we have conducted tensile tests of slime threads and have found them to be considerably more compliant and extensible than has been assumed for intermediate filaments. With an initial stiffness of about 6 MPa, slime threads behave like nanoscale rubber bands that can recover fully from strains up to 35% (Fudge et al. 2003). It is this rubber-like elasticity that likely accounts for the ability of the intermediate filament network in cells to continue to behave elastically long after filament bundles have been pulled completely taut and entropic gel elasticity is no longer relevant.

When hagfish threads are stretched to strains  $>35\%$ , they start to deform plastically, which simply means that they do not recover to their original dimensions even after the tensile load is released. We have shown using wide-angle synchrotron X-ray microdiffraction that this elastic/plastic transition corresponds to the point where  $\alpha$ -helical coiled-coils begin to extend and form stable  $\beta$ -sheet crystals with neighboring protein strands (Fudge et al. 2003).  $\beta$ -Sheet crystals are the structures that lend high strength to spider silk (Gosline et al. 1999) and their appearance in intermediate filaments makes them far stiffer near their breaking point than they are at low strains.

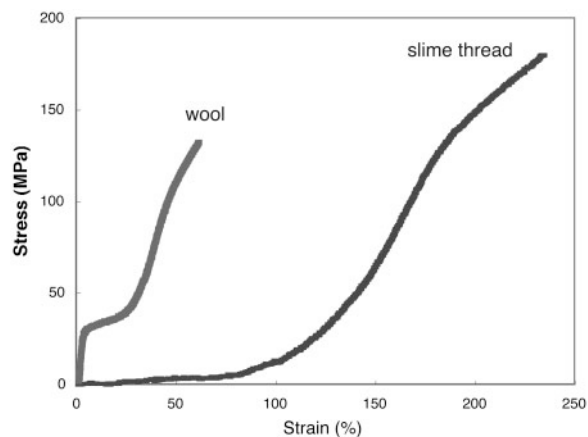
One of the most surprising properties of slime threads is their ability to deform to strains well over 200% before they fail. This kind of extensibility far exceeds that of F-actin and microtubules, which fail at strains well below 1% (Kishino and Yanagida 1988; Tsuda et al. 1996). These differences can be understood if one considers the molecular and supramolecular architecture of these three filament types. F-actin and microtubules are constructed from globular proteins held together by relatively weak intermolecular interactions, and there is little overlap of adjacent protein chains. As a result, when the rupture stress of the weakest adjacent proteins is reached, the filament fails. In contrast, intermediate filaments are constructed from staggered filamentous proteins with a great deal of overlap between neighbors. In addition,  $\alpha$ -helices represent a significant amount of “hidden length” that must be extended before the filaments can be broken. Recent work using atomic force microscopy has demonstrated that single intermediate filaments *in vitro* can deform to strains as high as 250% before breaking (Kreplak et al. 2005, 2008). These results not only reinforce the slime thread model as a valid way of exploring the mechanics of intermediate filaments, they raise the possibility that these filaments are virtually unbreakable at the kinds of strains normally encountered by cells.

$\alpha$ -Helices are held together by relatively strong hydrogen bonds (Pauling and Corey 1953) and these must be broken to plastically deform the filaments. Breaking these “sacrificial” bonds requires a great deal of energy and is one of the things that makes intermediate filaments tough. We have shown that intermediate filaments in cells appear to deform plastically at uniaxial cell strains  $>80\%$ , and this manifests as filament bundles that buckle when the cells return to their original size (Fudge et al. 2008). Together these results suggest that intermediate filaments in cells provide soft gel elasticity at low strains and rubberlike elasticity at moderate strains, but they are also able to absorb large amounts of energy at high strains. While their high extensibility and fracture mechanics may not be relevant to the biology of live cells, they may in fact serve a higher purpose under extreme mechanical loads. Cells are unlikely to survive deformations at which the intermediate filaments are stretched to strains  $>250\%$ , but perhaps the survival of a given cell is not so important. Under these conditions, intermediate filaments may function to stave off catastrophic mechanical failure of a tissue, which ultimately could mean the difference between survival and death for an organism.

## Hard $\alpha$ -keratins—stiff and tough

The concept that intermediate filaments could act as structural agents that continue to bear mechanical loads even after the cells that house them have been killed is not all that radical when one considers the case of the  $\alpha$ -keratins.  $\alpha$ -Keratins are biomaterials that are constructed from dead “keratinized” cells whose cytoplasm consists mostly of intermediate filaments bound up in a high sulfur protein matrix.  $\alpha$ -Keratins include relatively soft materials like the outer layer of epidermis in terrestrial vertebrates, the *stratum corneum*. When fully hydrated, soft keratins behave much like hagfish slime threads, but are not quite as extensible, an attribute most likely imparted by the keratin matrix.

What about hard  $\alpha$ -keratins such as hair, fur, nail, claw, horn, hoof, and baleen? These materials also are constructed primarily from intermediate filaments embedded in a keratin matrix, but they are obviously much stiffer. To be precise, the stiffness of a well-studied hard  $\alpha$ -keratin such as wool is about 2.5 GPa in the hydrated state, which is about as stiff



**Fig. 6** Comparison of the stress-strain curves for hydrated hagfish slime threads and hydrated wool fibers. In spite of the fact that wool is comprised primarily of aligned intermediate filaments, its initial stiffness is about 400 $\times$  higher than the stiffness of hydrated slime threads. This dramatic difference may be due to a partial dehydration of the intermediate filaments in wool and other hard  $\alpha$ -keratins.

as hard plastic. Compared to slime threads and *stratum corneum*, this is about 400–800 times stiffer (Fig. 6) (Park and Baddiel, 1972; Fudge et al. 2003). How can we explain this huge difference in stiffness in materials that are both constructed from the same filaments? The answer is likely intimately tied to the hydration level of the filaments. One clue to this puzzle is the fact that hard  $\alpha$ -keratins like wool are relatively hydration insensitive, with stiffness changing only by a factor of 2.7 between dry and wet specimens. Furthermore, the stress strain curve of dry slime threads is remarkably similar in shape to the curve for hard keratins like wool (Fudge and Gosline 2004). These observations, along with the fact that hard  $\alpha$ -keratins do not swell as much as slime threads do when they are hydrated, suggest that the intermediate filaments in hard  $\alpha$ -keratins are maintained in a dry state, even when the material is immersed in water (Fudge and Gosline 2004).

This conclusion raises the interesting questions of how the intermediate filaments are initially dehydrated, as well as how they remain that way. The most plausible mechanism is that the filaments, which assemble in the aqueous environment of the cytoplasm of keratinocytes, are air-dried during the keratinization process, and then locked in a dehydrated state by the cross-linking of the high sulfur matrix in which the filaments are embedded (Fudge and Gosline 2004). If this model is accurate, it raises further questions about how stiffening is achieved in  $\alpha$ -keratins that never have the opportunity for drying in air, such as whale baleen or the keratinous filiform papillae found on the tongues of mammals such as cats. We are currently working on how stiffening is achieved in whale baleen and exploring the possible contributions of covalent cross-linking and/or mineralization to this unique keratinous material.

## Conclusions

Intermediate filaments are relatively insoluble cytoskeletal filaments that over the course of metazoan evolution have been coopted for use in a huge diversity of biomaterials including ultrasoft hagfish slime, soft cytoplasmic gels, rubbery tensile elements,

**Table 1** Elastic moduli from the variety of intermediate-filament (IF) based materials discussed in this article

Material	Type	Stiffness (Pa)	Notes	References
Hagfish slime	Ultrasoft gel	$2 \times 10^{-2}$	Shear modulus	This article
IF networks	Entropic gel	$3 \times 10^1$	Shear modulus	Janmey et al. 1991
IF bundles	Rubberlike	$6 \times 10^6$	Tensile modulus	Fudge et al. 2003
Hard $\alpha$ -keratin	Stiff polymer	$3 \times 10^9$	Tensile modulus	Baden et al. 1974

as well as stiff and fibrous hard  $\alpha$ -keratins. To put this in sharper perspective, the intermediate-filament-based materials described here span a range of elastic moduli that differ by eleven orders of magnitude at the two extremes (Table 1). This impressive diversity arises from a variety of mechanisms and speaks to the versatility of intermediate filaments and the important roles they likely played in the evolution of animals.

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