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From reductionism to synthesis: The case of hagfish slime

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ABSTRACT

Reductionist strategies aim to understand the mechanisms of complex systems by studying individual parts and their interactions. In this review, we discuss how reductionist approaches have shed light on the structure, function, and production of a complex biomaterial – hagfish defensive slime. Hagfish slime is an extremely dilute hydrogel-like material composed of seawater, mucus, and silk-like proteins that can deploy rapidly. Despite being composed almost entirely of water, hagfish slime has remarkable physical properties, including high strength and toughness. While hagfish slime has a promising future in biomimetics, including the development of eco-friendly high-performance fibers, recreating hagfish slime in the lab has been a difficult challenge. Over the past two decades, reductionist experiments have provided a wealth of information about the individual components of hagfish slime. However, a reductionist approach provides a limited understanding because hagfish defensive slime, like most biological phenomena, is more than just the sum of its parts. We end by providing some thoughts about how the knowledge generated in the last few decades might be synthesized into a working model that can explain hagfish slime structure and function.

1. Reductionism and slime

A reductionist strategy aims to understand complex systems by analyzing the function of their constituent parts and their interactions (Brigandt and Love, 2017). Over the last two decades, we have been using reductionist strategies to understand the defensive slime of hagfishes. At first glance, a defensive secretion that consists of seawater, mucus, and silk-like threads might not seem complex enough to warrant such a "divide-and-conquer" approach. However, we have discovered that hagfish slime, like most biological phenomena, possesses layers of hidden complexity that underlie its structure and function. The polymer physicist J.D. Ferry published one of the first detailed papers on hagfish slime and he was clearly vexed by it: "The heterogeneity of the slime and its irreversible contraction render it unsuitable for study of mechanical properties in relation to its composition and structure" (Ferry, 1941). Since then, we have learned a great deal about this unique biomaterial, and much of this knowledge has come from using a reductionist approach to study it at several levels of organization, including molecules and subcellular structures, cells, and tissues, as well as the whole organism and behavior. We will discuss all these advances and conclude by discussing the limits of reductionism as it pertains to hagfish slime, and how a synthetic approach is now being applied to more deeply understand the mechanisms underlying the structure, function, and biogenesis of this material.

Hagfishes defend themselves from gill-breathing predators by producing large volumes of fibrous slime in a fraction of a second when they are attacked (Lim et al., 2006; Zintzen et al., 2011). The slime clings to an attacking predator's mouth and gills and distracts the predator so that the hagfish can escape. Much of the research on hagfish slime from the last four decades has aimed to understand the structure and function of the slime, how it deploys so quickly, and how it results in such a high volume of material. This research has relied on several laboratory techniques that allow researchers to study the different slime components in isolation. Ferry (1941) was the first to anesthetize hagfishes (using ether) and to empty the slime glands using electrical stimulation. Downing et al. (1981a, 1981b) improved on Ferry's methods by providing a new method of hagfish anesthesia (tricaine methanesulfonate), and also by showing that exudate could be stabilized and stored in solutions containing high concentrations of polyvalent anions such as citrate or sulfate. This so-called "aqueous stabilization buffer" also allowed the secreted products from gland mucous cells (mucous vesicles) to be separated from the fibrous products of gland thread cells (thread skeins) via simple filtration through a mesh that retains the relatively large thread skeins and allows the much smaller mucous vesicles to pass through. Downing et al. (1981a, 1981b) provided detailed protocols for the electrical stimulation of slime glands to release

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exudate and Winegard and Fudge (2010) introduced the use of clove oil for hagfish anesthesia (McCord et al., 2020; Winegard and Fudge, 2010).

Other methodological innovations paved the way for further progress in understanding hagfish slime. Koch et al. (1991) introduced the "removable mass" assay as a simple way of measuring the efficacy of hagfish slime formation under varying conditions by measuring how much slime could be removed from a beaker with a metal hook. Using this assay allowed them to probe the importance of the two components of the slime by varying the ratio of threads to mucous vesicles in citrate-stabilized slime and by chemically disrupting the mucus with the reducing agent dithiothreitol (DTT), which breaks disulfide bonds. Lim et al. (2006) employed the removable mass assay to assess the importance of hydrodynamic mixing for the proper setup and subsequent collapse of the slime. Fudge et al. (2003) used a technique from the early spider silk biomechanics field - tensile testing with a glass rod force transducer - to measure the tensile behavior of individual slime threads under a variety of conditions. Downing et al. (1981a) developed a geometric model to estimate the length of slime threads based on the dimensions of intact thread skeins, and Fudge measured thread length more directly by measuring the extensibility of completely unraveled slime threads and back-calculating the resting length from material properties measured previously. Luchtel et al. (1991) probed the function of hagfish slime mucous vesicles by stirring fresh exudate into solutions with varying ionic compositions and observing slime formation as well as appearance under a microscope. Herr et al. (2010) developed a flow-through assay that allowed them to immobilize and stabilize mucous vesicles under a microscope and observe their behavior as they were exposed to seawater and a wide range of chemical and ionic conditions. Similar devices have since been used to study thread skein unraveling under a variety of conditions (Bernards et al., 2014, 2018; Jain et al., 2019). Ewoldt et al. (2011), Chaudhary et al. (2019), and Böni et al. (2018a, 2018b); Böni et al., 2017; Böni et al., 2016) developed and applied a variety of biophysical techniques for probing the mechanical behavior of whole slime and isolated mucus. Our current understanding of hagfish slime, which we describe below, would be far less complete without these methodological innovations, but there is still more work to be done to fully understand this unusual biomaterial.

2. Slime composition

Hagfishes produce slime from numerous paired glands along their ventrolateral surface. The slime is formed when the slime glands eject a concentrated suspension, known as exudate (Fig. 1), into seawater via a process known as holocrine secretion (Koch et al., 1991). The exudate has three main components: fluid, thread skeins (coiled threads), and mucous vesicles. While the volume of exudate released in a typical sliming event is relatively miniscule, it rapidly expands once released from the glands to more than 10,000 times its original volume (Fudge et al., 2005; Fudge et al., 2015; Chaudhary et al., 2019). Hagfish can produce about 1 L of dilute slime in a typical sliming event while utilizing only a handful of their over ~160–200 slime glands (Fudge et al., 2005; refs for slime gland numbers). Hydrated slime is composed of approximately 99.996% seawater, 0.002% mucin, and 0.002% threads, making it an extremely dilute biological material (Fudge et al., 2005).

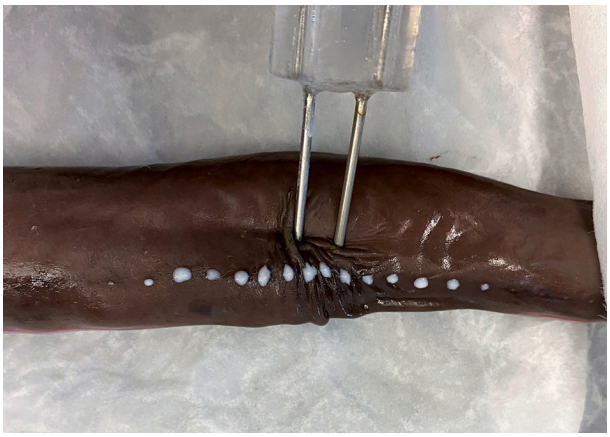

Fig. 1. Slime exudate released from slime gland pores after electrostimulation. The white exudate is visible outside of the slime gland pores lining the ventrolateral surface of an anesthetized *E. stoutii* after the glands discharged their contents. Note the muscle contractions near the two electrodes of the stimulator wand.

2.1. Slime gland exudate fluid

Roughly a third of the volume of exudate is a clear fluid that contains substantial concentrations of ions and organic osmolytes and which can be obtained by centrifugation of exudate. The ionic component resembles the intracellular fluids of osmoconforming marine animals, with relatively high K^+ concentrations and low Na^+, Cl^- and Ca^{2+} concentrations (Herr et al., 2010), which suggests that much of the fluid originates from the cytoplasm of thread and mucous cells that rupture during their ejection from the glands. Organic osmolytes, particularly methylamines including trimethylamine oxide (TMAO), trimethylglycine (betaine), and dimethylglycine (DMG), are found in high concentrations (490 mM in total) in the exudate fluid. While the function of such high levels of methylamines is not fully understood, they have been shown to inhibit unraveling of the thread skeins in vitro, and they therefore may be involved in the stabilization of the skeins in the slime glands. (Jain et al., 2019). In contrast, methylamines do not appear to be involved in stabilization of mucous vesicles (Herr et al., 2010).

3. Hagfish slime threads

Hagfish slime threads consist primarily of intermediate filament (IF) proteins (Koch et al., 1994, 1995). They have a resting length of approximately 10–17 cm and can extend to ~34 cm before breaking (Fudge et al., 2005). In the Pacific hagfish (*Eptatretus stoutii*) hydrated threads have a maximum diameter of approximately 3.0 μm, with the diameter tapering down to ~1.5 μm at the apical end, and ~ 1.0 μm at the basal end of the gland thread cells (GTCs) that produce them (Fudge et al., 2005; Fudge and Schorno, 2016). It is currently not known whether slime threads from other species of hagfishes have similar dimensions.

The tensile properties of the slime threads differ between wet and dry conditions. In their native, hydrated state, the threads are rubberlike, with a Young's Modulus (E) of 6 MPa (Fudge et al., 2003), whereas dry threads are considerably stiffer, with a modulus similar to that of hard plastic (E = 3.6 GPa; Fudge and Gosline, 2004). Wet threads also have far greater extensibility (220%) compared to dry threads (100%). However, dry threads are stronger (strength = 530 MPa) and tougher (toughness = 240 MJ/m^3) than wet threads (strength = 180 MPa, toughness = 130 MJ/m^3). Fudge et al. (2003) elucidated the structure of the slime threads using x-ray diffraction and Congo Red staining. As in α-keratins, aligned α-helices dominate the thread structure. Unlike mammalian keratins, however, hydrated slime threads possess rubberlike elasticity up to strains as high as 35%. At strains greater than this, they undergo plastic deformation, which corresponds to the unzipping of hydrogen bonds within α-helices in intermediate filament proteins. The subsequent formation of ß-pleated sheets by these same proteins imparts remarkable strength and toughness to these fibers, and thus the slime (Fudge et al., 2003).

3.1. Cellular origins of slime threads

Thread skeins are produced in the slime glands within gland thread cells (GTCs), where they are packaged within the cytoplasm (Downing

et al., 1981a; Fernholm, 1981; Winegard et al., 2014; Fudge and Schorno, 2016). A single thread occupies most of the volume of a mature GTC (Fernholm, 1981; Winegard et al., 2014). Mature threads are primarily composed of two intermediate filament (IF) proteins, α and γ, which assemble into 12-nm diameter filaments in the GTC cytoplasm (Fig. 2). Immature threads also contain microtubules (MT), which disappear later in thread maturation. The α protein is a type II keratin homolog and the γ protein shares features of both type I and type III IFs (Koch et al., 1994; Koch et al., 1995; Schaffeld and Schultess, 2006; Fudge et al., 2015). These IF proteins are most likely synthesized in the cytoplasm on the apical side of the nucleus where there is a high density of both mitochondria and polysomes (Downing et al., 1984; Winegard et al., 2014; Fudge et al., 2015). Koch et al. (1995) sequenced the genes for these IF proteins in *E. stoutii* and found both the α (66.6 kDa, native pI 7.5) and γ (62.7 kDa, native pI 5.3) protein subunits have threonine-rich central rod domains that form α-helices, which in turn assemble in a 1:1 ratio into coiled coil dimers. Higher level assembly into 12-nm IFs is currently not described in this system.

Threads in very young GTCs consist of a bundle of about ten IFs, with a collective diameter of 30 nm (Winegard et al., 2014). Thread girth increases as more IFs are added, along with MTs (Fig. 2). At some point in thread development, a striking rearrangement of IF proteins occurs, with IFs appearing to disassemble and then reassemble with their neighbors into a single condensed IF superstructure (Downing et al., 1984; Winegard et al., 2014). This transition of IF packing appears to coincide with a fundamental change in the assembly mechanism, with new material added to the periphery of the growing thread not as intact 12-nm IFs, but instead as IF protein subunits. In the last stage of IF development, MTs are lost and IF proteins further compact, filling in the spaces once occupied by the MTs (Winegard et al., 2014). Compared to other intracellular polymers, mature slime threads are enormous, with a cross-sectional area that is over 60,000 times greater than a single IF.

3.2. 3D Structure of thread skein

Each skein consists of a single ~15-cm long thread that is coiled into an approximately 150 μm long and 75 μm wide ellipsoid skein (Fig. 3; Downing et al., 1981a; Fernholm, 1981; Spitzer and Koch, 1998). Within the skein, the thread is organized into ~500 loops arranged into about 15 conical loop arrangements, which appear as distinct layers on the skein surface (Winegard et al., 2014). Each loop has a basal segment that runs for ~60 degrees along the skein periphery and contributes to the cabled appearance of the skein surface and the layering mentioned above. Loops are staggered relative to their neighbors, with the oldest loops at the apical end of the GTC, while younger loops are closer to the nucleus at the basal end (Winegard et al., 2014). The GTC nucleus appears to serve as the template for the three-dimensional morphology of the thread loops, which are the repeating structural units of the skein (Winegard et al., 2014). As the thread grows, it appears to be confined by the previous layer of thread loops and the apical nuclear surface. The ascending and descending portions of the conical loop conform to the nuclear surface, while the circumferential runs at the base of each conical loop are formed in the groove where the nucleus and plasma membrane converge. As the thread matures, the nucleus drastically reduces in size and retreats toward the basal end of the GTC (Fig. 3), leading to less conical, shorter loops. While the exact mechanism of coiling is still unknown, the fact that successive loops are staggered in a clockwise manner suggest a wheel-like mechanism, although this hypothesis has yet to be rigorously tested (Winegard et al., 2014; Fudge and Schorno, 2016).

3.3. Unraveling of thread skeins

Mature GTCs lose their cell membrane when they are ejected from slime glands (Downing et al., 1981a; Fernholm, 1981), releasing a naked thread skein. Based on in vitro observations of thread skeins under a microscope, skein unraveling is affected by ionic composition as well as the presence of methylamines, which are abundant in slime gland exudate. In the absence of ions, skeins unravel radially in an uncontrolled manner and form a network that entraps only a limited volume of water (Böni et al., 2018a, 2018b). High Na^+ and Cl^- concentrations lead to more controlled skein unraveling, but in the absence of Ca^{2+} ions, slime formation is also impaired. While Ca^{2+} ions are necessary for an expanded thread network that fully retains water (Böni et al., 2018a, 2018b), the underlying mechanisms are unclear. One possibility is that this effect is mediated by the presence of Ca^{2+}-activated transport proteins in the mucous vesicle membrane (Herr et al., 2014), with impaired mucus deployment having secondary inhibitory effects on skein unraveling ((Winegard and Fudge, 2010).

Bernards et al. (2014) showed that isolated skeins from Pacific hagfish (*Eptatretus stoutii)* unravel spontaneously and energetically when they are exposed to seawater. This was an unexpected result given that skeins from the Atlantic hagfish (*Myxine glutinosa*) generally do not unravel spontaneously in seawater. The unraveling process in *E. stoutii*

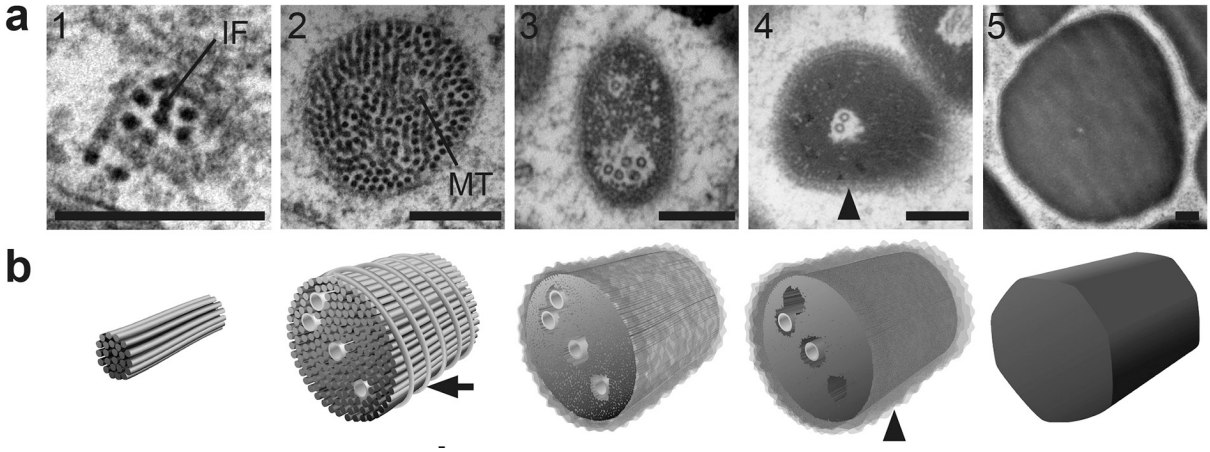

Fig. 2. Slime Thread Development. (a) Transmission electron micrographs (TEM) of slime thread formation. The thread in panel (1) is from a very immature GTC and consists of a bundle of a small number of 12-nm IFs. Threads increase in girth with the addition of more IFs and MTs (2). IFs become more tightly packed (3), which causes an electron lucent halo (asterisk) to form around the MTs. As IFs compact further, a fluffy rind (arrowheads) appears on the surface of the thread (4), which likely indicate that IF subunits or proteins are directly added to the thread. In the last stage (5), MTs and the fluffy rind are lost as IF proteins compact further, filling spaces previously occupied by MTs (5). Scale bars are 200 nm. (b) Illustrations of the panels in (a). Panel 2 shows a wrapping filament (arrow) of unknown identity, which winds around the developing thread at regular intervals. Figure adapted from Winegard et al. (2014).

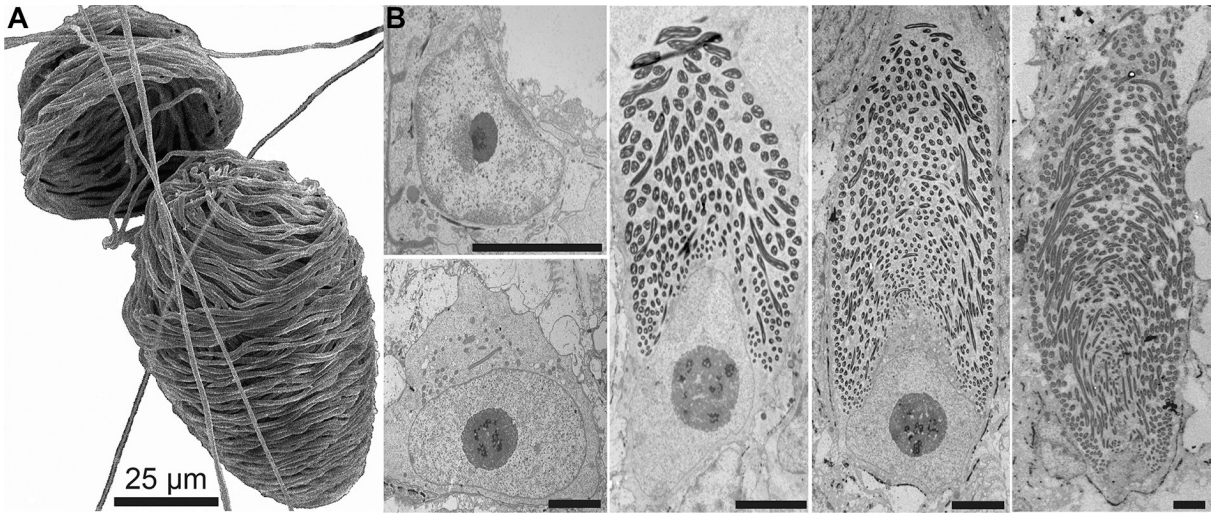

Fig. 3. Structure of gland thread cells. (A) Scanning electron micrograph (SEM) of a coiled thread from mature *M. glutinosa* GTC that is broken open, revealing the staggered loop organization (partially highlighted in red), which form conical looping layers that spiral around the skein (one layer is highlighted in purple). (B) TEM images showing the progression of hagfish GTC development. Immature GTCs lack a slime thread and have prominent nuclei. As the slime thread increases in length and diameter, the nucleus decreases in size and recedes to the base of mature cells. Scale bars are 5 μm. Figure adapted from Winegard et al. (2014). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

skeins appears to be mediated by the dissolution of a seawater-soluble protein adhesive that helps hold the loops of thread together within a skein and the subsequent release of mechanical energy stored in the coiled thread. The most persuasive evidence for this hypothesis is the fact that the protease trypsin is able to induce skein unraveling in both species under conditions where unraveling is normally is inhibited (low salt and high temperature). A more recent study showed that the difference in the behavior of *E. stoutii* and *M. glutinosa* skeins likely has to do with differences in the properties of their skein glue, with *M. glutinosa* possessing glue that is less soluble in seawater (Bernards et al., 2018). The discovery of skein glue may also explain the high concentration of methylamines in slime gland exudate. Recent work showed that skein unraveling is inhibited by the presence of the osmolytes TMAO and betaine, which are the two methylamines that are present in the highest concentrations in the slime glands (Jain et al., 2019). The inhibitory effect of these compounds, which are both known to generally make proteins less soluble (i.e. they are kosmotropes) is likely due to their stabilizing effects on skein glue proteins.

Given that spontaneous unraveling observed in vitro occurs over timescales of seconds to minutes, it is unclear exactly what its significance is to the behavior of the slime under natural conditions, where deployment is known to occur in 100–400 ms (Lim et al., 2006; Zintzen et al., 2011). Winegard and Fudge (2010) showed that *M. glutinosa* skeins require vigorous hydrodynamic mixing for unraveling to occur and proposed that the presence of long mucus strands facilitates unraveling by transducing hydrodynamic mixing forces down to the small length scale of intact skeins (~150 μm; Fig. 4). A recent theoretical treatment of skein unraveling by Chaudhary et al. (2019) demonstrated that skein unraveling may be accelerated by the pinning of skeins at a surface, such as the jaws or gills of a predator or even the hagfish itself.

4. Hagfish slime mucus

The mucous vesicles that are released from slime glands interact with seawater to produce the mucous component of hagfish slime. The concentration of mucus in hagfish slime (0.02 mg/mL) is 2–3 orders of magnitude more dilute than most other biological mucous secretions (Fudge et al., 2005). Hagfish slime mucus consists of 77% protein, 12% carbohydrates (primarily sulfated mucopolysaccharides), 5% lipid, and 6% sulfate by dry weight, indicating that it is likely made up primarily of glycoproteins (Salo et al., 1983). This composition is highly unusual for mucous secretions, which typically have much higher carbohydrate contents of up to 80%. Although they are frequently referred to in the literature as "mucins", it is currently unknown whether the glycoproteins that make up hagfish slime mucus are related to molecules from the MUC family (Moniaux et al., 2001). Transcriptomic analyses that are now underway of genes expressed in the slime glands should soon reveal the identity of the proteins that make up the backbone of hagfish slime mucus glycoproteins.

4.1. Mucous vesicles

Mucous vesicles are produced in gland mucous cells (GMCs). Unlike the GTCs, which only produce one thread per cell, GMCs produce several thousand disk-shaped mucus vesicles, which are formed in the Golgi apparatus (Luchtel et al., 1991). Like GTCs, GMCs rupture when released from slime glands, causing mucous vesicles to be released into the external environment (Fernholm, 1981; Herr et al., 2010). This form of secretion – holocrine secretion – is unusual for animal mucus, which typically is secreted via a merocrine mode in which vesicles fuse with the plasma membrane and release naked mucin granules via exocytosis (Koch et al., 1991; Luchtel et al., 1991; Spitzer and Koch, 1998). Holocrine secretion likely evolved in hagfish slime glands due to selective pressures for rapid and copious release of mucus for defense (Herr et al., 2014).

The rapid deployment of hagfish slime suggests that mucous vesicles undergo a dramatic transformation when they interact with seawater and multiple studies have attempted to understand the mechanisms underlying this transformation. Downing et al. (1981a) and Luchtel et al. (1991) stirred fresh exudate into solutions with varying ionic compositions and concentrations and assessed whether competent slime was produced. They discovered that most salt solutions resulted in slime formation, but solutions containing high concentrations of polyvalent anions such as citrate inhibited slime formation. Microscopic examination revealed that anions such as citrate inhibit slime formation by inhibiting vesicle swelling. Luchtel et al. (1991) suggested that these patterns could be explained by a vesicle membrane that is permeable to all ions except polyvalent anions. This hypothesis was subsequently refuted by observations of vesicle stabilization in citrate solutions, even when the vesicle membrane was disrupted with detergent (Herr et al., 2014). Recent work suggests that the stabilizing effects of polyvalent anions is due to the polycationic nature of the mucous glycoproteins in

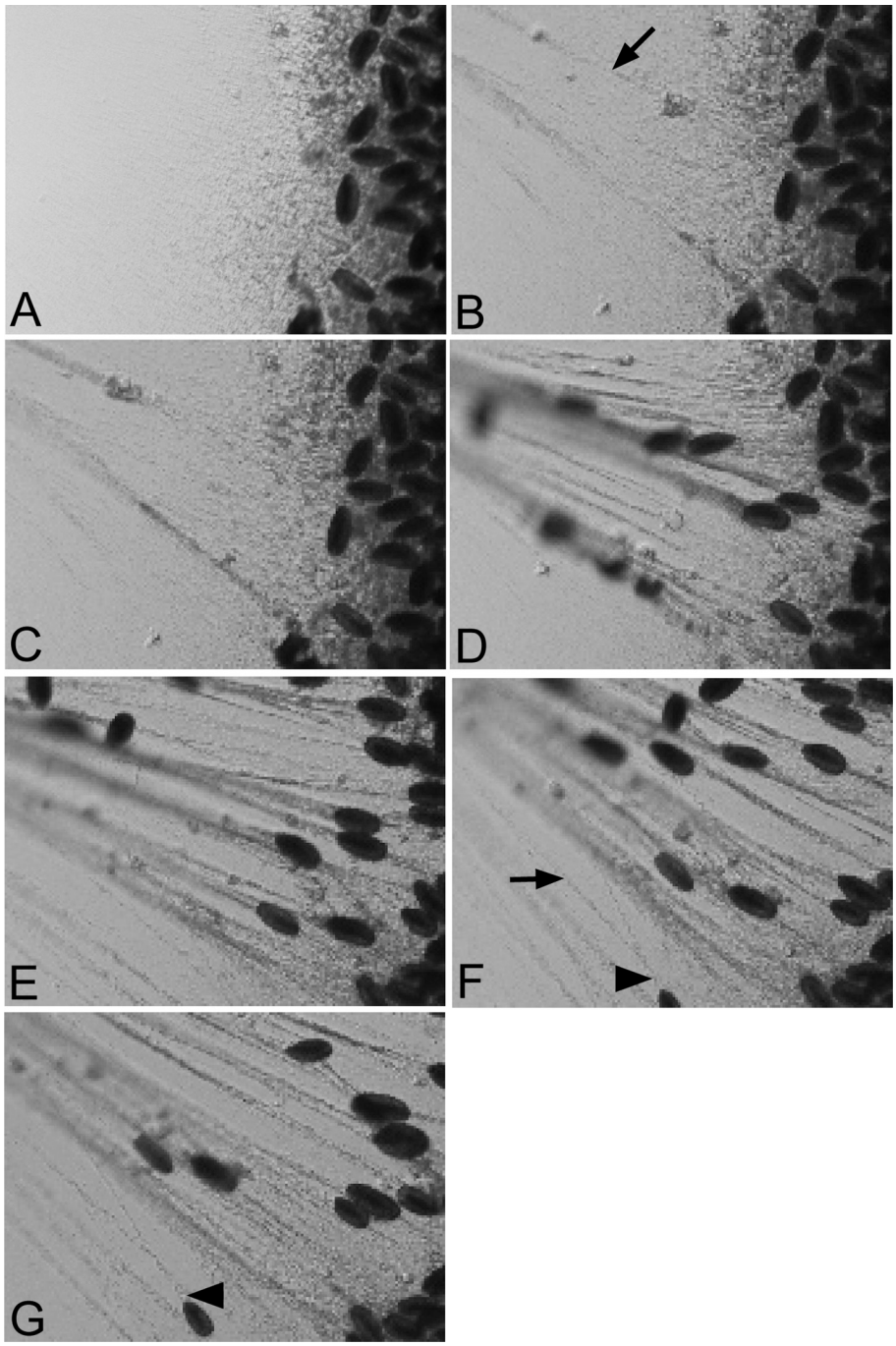

Fig. 4. Mucus strand formation from slime exudate exposed to suction flow. (A) Condensed exudate prior to flow, with intact skeins on the far right surrounded by mucous vesicles (paler, granular material). (B,C) Mucus strands form from the mucin vesicles as flow initiates. (D,E) Mucous strands attached to thread skeins and pull them along with flow. (F,G) Skein unraveling initiates. Arrowheads indicate an unraveling thread skein and arrows indication mucous strands. Figure taken from Winegard and Fudge (2010) with permission.

hagfish slime, which is puzzling given that all other mucus molecules are believed to be polyanionic (Fudge et al., 2020).

While the evolution of holocrine secretion undoubtedly increased the quantity of defensive mucus deployment in hagfishes, it also posed its own challenges, as vesicles were ejected into the environment with an intact membrane, which had the potential to slow the movement of water and ions involved in vesicle swelling and rupture. Indeed, the vesicle membrane has been shown to mediate stabilization and rupture. When membranes are intact, citrate-stabilized vesicles exposed to seawater in a flow chamber exhibit a bimodal distribution of swelling, with some that begin to swell early and relatively slowly and others that swell rapidly after a delayed start (Herr et al., 2014). However, when the membranes are disrupted with detergent, variability in swelling decreases dramatically, suggesting that the membrane plays a role in regulating swelling and rupture. A large fraction of citrate-stabilized vesicles require Ca^{2+} ions to swell and rupture (Herr et al., 2014), a trait that may be mediated by Ca^{2+}-activated channels in the vesicle membrane (Herr et al., 2014). Given that Ca^{2+} concentrations in cells are low, the relatively higher concentrations in seawater may act as a trigger for rupture. Aquaporins are also found in vesicle membranes and facilitate rapid vesicle swelling in seawater, as vesicles treated with the aquaporin inhibitor Hg^{+} show strongly diminished swelling rates (Herr et al., 2014). The appearance of aquaporins in the vesicle membrane may have coincided with the appearance of holocrine secretion and the need to move water through the membrane quickly, which is required for rapid slime deployment.

4.2. Mucus material properties

The astoundingly low concentrations of mucus and threads in hagfish slime (about 20 mg/L for each) can best be understood by recognizing that the slime does not irreversibly bind seawater, but instead appears to trap it in small channels. This idea is supported by the fact that when a newly produced mass of slime is lifted out of water into air, most of the water will run out of the slime after a few minutes, leaving only about 5% of the original volume (Ferry, 1941; Fudge et al., 2005). While it is not entirely clear how the slime achieves a network structure that results in this viscous entrainment behavior, it is clear that the mucus plays an important role. It is also clear from the very low concentration of mucus that the mucous glycoproteins cannot be homogeneously dispersed and still have an effect on the mechanics. Furthermore, the observation of mucus strands by Winegard and Fudge (2010) and their seeming importance for skein unraveling (Fig. 4) suggests that these strands possess some elastic properties. The heterogenous distribution of the mucus presents difficulties for measuring the mechanical properties of the network, especially given that handling the slime appears to change its structure and properties. Nonetheless, researchers have tried to measure the material properties of hagfish slime mucus in the absence of threads, and they have generally done so using citrate-stabilized exudate in which thread skeins are removed via filtration. Mucus solutions can then be produced by dialyzing away the stabilizing salts or by diluting them. Fudge et al. (2005) showed that hagfish slime mucus solutions possess viscosity (measured using an Ostwald viscometer) similar to seawater at native concentrations, with higher concentrations of mucus (up to 15× higher) resulting in only modest increases in viscosity. Rementzi et al. (2019) measured viscoelasticity in hagfish slime mucus solutions in shear using a cup and bob apparatus and found that at very high concentrations, hagfish slime mucus exhibits mechanical properties in shear that are similar to those of whole slime (Rementzi et al., 2019).

5. Slime glands

5.1. Histology and structure

Depending on the species, extant hagfishes have approximately 70–130 pairs of slime glands (Fudge et al., 2015; Miyashita et al., 2019). Each gland, typically 2–3 mm in diameter, connects to an evenly spaced pore on the hagfish's ventrolateral surface via a short duct (Fig. 5). During holocrine secretion, GTCs and GMCs rupture and their contents are squeezed through the gland duct and pore. It is unclear whether the cells rupture in the gland as they are squeezed toward the duct, or if their passage through the narrow duct itself causes rupture. Both GTCs and GMCs originate from undifferentiated cells in the basal epithelial lining of the slime glands. As these cells grow and mature, they move away from the edge of the gland toward the center (Schorno et al., 2018a), though GTCs take longer to mature than GMCs. A thin collagenous capsule surrounds the gland epithelium (Lametschwandtner et al., 1986; Fudge et al., 2015). This capsule is surrounded by a thin layer of striated muscle, known as the *musculus decussatus*. When this muscle contracts, it increases pressure within the glands, forcing the exudate out of the pore into the external environment (Fudge et al., 2015).

5.2. Emptying and refilling

The contents of the slime glands make up approximately 3–4% of a hagfish's body mass when full (Fudge et al., 2005). Electrical stimulation of a slime gland and ejection of exudate (Fig. 1) correspond with a decrease in gland size, with glands that are exhausted having an approximately 3.5× smaller cross-sectional area (Schorno et al., 2018a; Schorno et al., 2018b). Histological examination of glands stimulated multiple times to exhaustion reveals that some GTCs and GMCs are retained in these glands (Schorno et al., 2018b). After exhaustion, slime

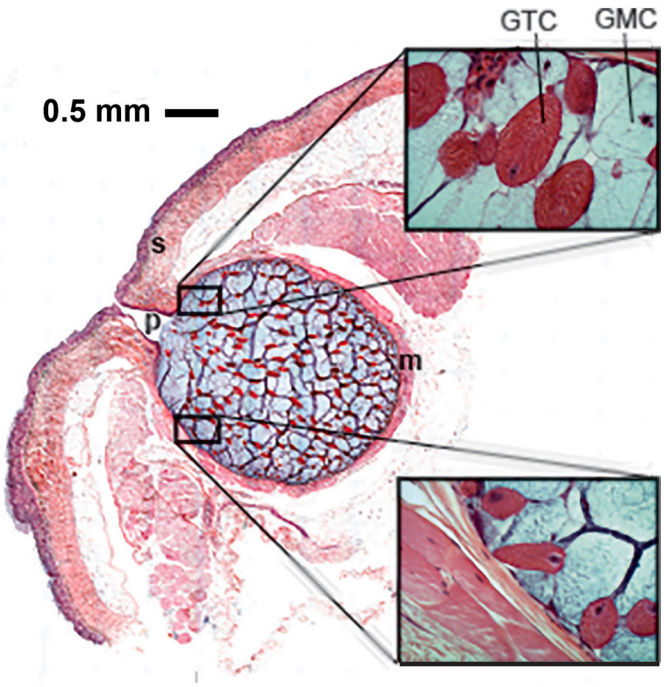

Fig. 5. Structure of slime gland. Hematoxylin and eosin-stained cross-section of a hagfish (*M. glutinosa)* slime gland showing gland thread cells (GTC), gland mucus cells (GMC), gland pore (p), striated muscle encapsulating the gland (m) and skin (s). The upper inset shows a mature GTC within the gland lumen, while the lower inset shows an immature GTC near the gland epithelium. Figure adapted from Winegard et al. (2014).

glands steadily increase in size as they refill (Schorno et al., 2018a). Refilling is a relatively slow process, with Pacific and Atlantic hagfish taking about 3–4 weeks to completely refill their slime glands (Schorno et al., 2018b). GTCs appear to be the limiting factor in refilling, with GMCs replenishing and maturing more quickly (Schorno et al., 2018a). GTCs originate in the gland epithelium and increase in size and maturity as they migrate away from the gland periphery to the center of the gland (Schorno et al., 2018a). In spite of this gradient of GTC size and maturation, multiple electrical stimulations of full slime glands result in expulsion of mature skeins that are remarkably homogeneous in size, suggesting that immature thread cells at the gland periphery are retained during exudate release (Schorno et al., 2018a), at least when glands are fully recharged.

6. Whole slime structure and properties

Hagfish slime is an extremely dilute hydrogel-like material, composed of 99.996% seawater, 0.002% mucin, and 0.002% threads (Fudge et al., 2005), which is 1000× more dilute than typical mammalian mucus. *E. stoutii* produces about 1 L in a typical sliming event, but it is estimated that they could produce up to 24 L of slime if they released exudate from all of their glands (Fudge et al., 2005). Hagfish slime is not a fiber-reinforced composite like tendon, nor is not a true gel, as it does not permanently trap water; the threads and mucus instead act as a fine sieve that slow down and entrain seawater, confining it to channels between the threads and mucus (Fudge et al., 2005). This allows the slime to act as a coherent mass over short time scales, but because most of the water is trapped and not bound per se, water rapidly drains out of the slime if it is pulled out of water and suspended in air (Fudge et al., 2005). Similarly, the network will collapse and decrease in volume if the slime is mechanically disturbed (Ferry, 1941: Fudge et al., 2005).

Hagfish slime is one of the softest known biomaterials, with a storage modulus of $G' \approx 0.02$ Pa for timescales $0.1\ s \leq t \leq 10s$ (Ewoldt et al.,

2011). The viscoelastic time-dependent properties of the slime are independent of the slime concentration and unlike other materials, with a constant power law exponent ($\alpha = 0.18 \pm 0.01$), constant relative damping tan $\delta = G''/G' \approx 0.2$–0.3, and an overall stiffness that scales linearly with concentration ($\sim c^{0.99\pm0.05}$; Chaudhary et al., 2019). This suggests that the structure of whole hagfish slime remains self-similar over multiple orders of magnitude of concentration and may point to a fractal network organization. One of the complexities of its mechanical behavior is that elongational strain causes it to stiffen, whereas shear causes it to soften (Böni et al., 2016). These seemingly divergent properties can be understood if one considers the mechanical behavior of any thin fiber, which can be stiff in tension, but offers little resistance to bending or compression. The consequences are that slime threads dominate the slime's mechanics when loaded in tension, while the mucus dominates in shear when the threads offer little resistance to deformation. Indeed, at high concentrations, hagfish mucus exhibits rheological properties in shear that mimic those of whole slime (Rementzi et al., 2019).

7. Ecological function

The primary ecological function of hagfish slime is defense, particularly against predatory fishes. Baited cameras have captured footage of a few shark species and several bony fishes attempting to consume hagfishes, but instead receiving mouthfuls of slime (Fig. 6; Zintzen et al., 2011). When a fish bites a hagfish, it releases slime exudate. Thrashing movements of the hagfish and/or water flow resulting from suction feeding or biting from a predator cause the slime to rapidly expand in the oral and buccal cavity, which disturbs the predator, allowing the hagfish to escape (Fudge et al., 2015; Zintzen et al., 2011). The slime entangles on the gill filaments and gill rakers, presumably impairing the fish's ability to respire, although a drop in respiratory function after sliming has never been measured (Lim et al., 2006). Because the slime is not a true gel but acts via viscous entrainment, it does not completely shut down water flow over the gills. However, it drastically slows the flow and thus likely impairs gas exchange. Viscous entrainment may make hagfish slime more effective in defense than if it were a true gel by making it more difficult to dislodge from gills. If the slime were a true gel, it could allow for a build-up of pressure that a fish could use to dislodge the slime plug. It is also softer than most hydrogels, which likely aids in its ability to conform and adhere to the gills. Whether hagfish slime is fatal to predatory fishes who attempt to eat a hagfish is currently unknown. Several marine avian and mammalian predators consume hagfishes without major consequences (Martini, 1998), likely because they possess respiratory surfaces (lungs) that are not fouled with slime when they eat a hagfish.

8. Connections with other fields

One of the hazards of a reductionist approach is that by dissecting a physiological phenomenon into its parts and examining each in detail, one risks losing sight of the big picture and/or complex interactions that might exist among the parts. One might therefore conclude that reductionism leads to a relentless march toward focusing on smaller and less significant components and provides no opportunities for synthesis. This point of view ignores one of the most powerful benefits of a reductionist approach, and that is that it leads researchers to do experiments and make observations that can lead to entirely new and unexpected lines of inquiry. Our work on the material properties of hagfish slime threads provides several examples of this phenomenon.

8.1. Intermediate filaments in living cells

Some of the earliest biophysical measurements that were made on hagfish slime threads came from simple tensile tests that allowed us to generate a stress-strain curve for this unusual silk-like material (Fudge et al., 2003). While the motivation of these experiments was to understand the mechanical behavior of the defensive slime and its constituents, the results unexpectedly led to a rich new line of inquiry in the area of cell mechanics. Earlier, Koch and colleagues characterized the two dominant slime thread proteins (α and γ) and identified them as belonging to the family of intracellular cytoskeletal proteins known as

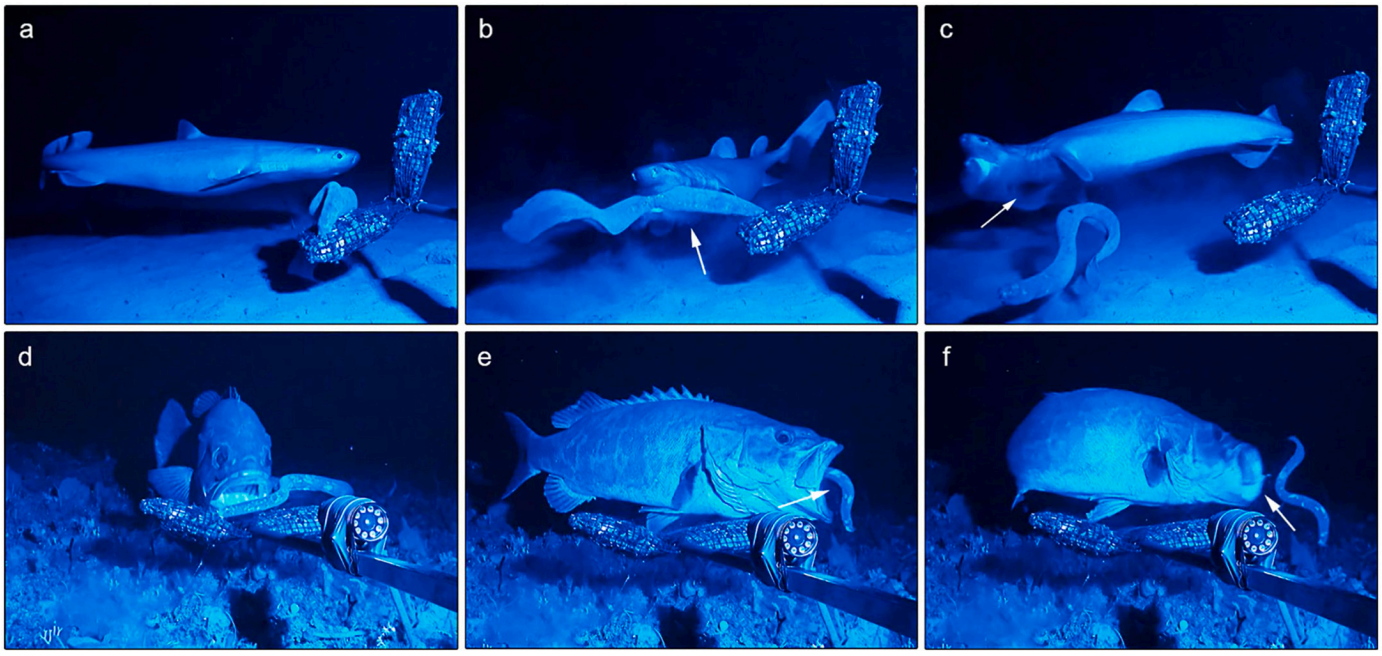

Fig. 6. Hagfish slime is an effective defense against gill-breathing predators. A seal shark *Dalatias licha* (a–c) and a wreckfish *Polyprion americanus* (d–f) bite hagfishes in an attempt to consume them. The predators approach their potential prey (a,d) and then bite or attempt to swallow the hagfishes (b,e), but the hagfishes have released their defensive slime (arrows) into the predators' mouths in less than 0.4 s. (c,f) The predators choke on the slime, releasing the hagfish and gagging in an attempt to remove the slime from their mouth and gills. Figure taken from Zintzen et al. (2011) with permission.

IFs. While little was known about the tensile properties of individual IFs in cells, it was widely assumed that they had an elastic modulus that was similar to that of the other two main cytoskeletal elements, F-actin and MTs (2–4 GPa) (Howard, 2001). The stiffness of hydrated α-keratins like wool, which consist mainly of aligned IFs, is in the same range (2 GPa), which only reinforced this assumption. The tensile properties of slime threads did not conform to these assumptions, however, showing rubberlike elasticity, a conformational transition, and extreme extensibility that was dramatically different from the tensile properties of F-actin, MTs and alpha-keratins (Fudge et al., 2003). These insights, along with evidence from biophysical explorations of other IFs and IF networks, ultimately transformed cell biologists' understanding of IF mechanics and led to the idea that IFs could be involved in protecting cells from large strains (Kreplak and Fudge, 2007; Fudge et al., 2008; Beriault et al., 2012; Hu et al., 2019; Fleissner et al., 2020).

8.2. Keratin biomechanics

Hard α-keratin is a composite material made of numerous keratinized epidermal cells glued together to form tough structures such as wool, hooves, and claws. Within keratinized cells, IFs are embedded within an isotropic, high-sulfur matrix (Feughelman, 1959; Fraser et al., 1986; Fudge and Gosline, 2004;). While hagfish slime threads and hard α-keratins are both comprised primarily of IFs, they can have dramatically different mechanical properties. Most striking is the difference between their Young's moduli under hydrating conditions, with keratin fibers such as wool having moduli that are about 500 times higher than wet slime threads; hydrated slime threads are also five times more extensible than wool. In air, the properties of dry slime threads and keratin fibers converge, showing similarly-shaped stress-strain curves and moduli. To reconcile these puzzling observations, we proposed that alpha-keratins exhibit surprisingly low hydration sensitivity because the keratin matrix is cross-linked around the IFs under dehydrating conditions. The matrix has the effect of mechanically resisting the swelling of IFs under hydrating conditions, thereby keeping them effectively dry and therefore stiff (Fudge and Gosline, 2004). This "matrix squeeze" hypothesis explains several aspects of keratin and IF mechanics, and a recent attempt to test it using a comparative approach has found further evidence in support of it (Greenberg and Fudge, 2013). These insights into the structural mechanics of mammalian keratins can be traced directly back to our earlier work on the material properties of hagfish slime threads, which revealed the properties of relatively pure IFs in the absence of a keratin matrix.

8.3. Baleen mechanics

Whale baleen is a tough, keratinous tissue that makes up the feeding structures that mysticete whales use for filter feeding. Unlike keratinous epidermal appendages in other mammals, baleen cannot undergo stiffening via air-drying. The matrix squeeze hypothesis described above predicts that the keratin IFs in whale baleen should not be able to achieve the same levels of stiffness that are achieved in keratins produced by terrestrial mammals. Measurements of baleen material properties are consistent with this prediction and raise the possibility that whales use alternative mechanisms to stiffen their baleen (Szewciw et al., 2010). One strategy for stiffening baleen bristles is calcification, with the sei whale (*Balaenoptera borealis*) exhibiting the most highly calcified keratin of any mammal (Szewciw et al., 2010). These insights into baleen structure and function arose out of the work on keratin mechanics described above.

8.4. IFs as inspiration for biomimetic materials

The impressive material properties of hagfish slime threads have made them a source of inspiration for biomimetic development (Fudge et al., 2010). Negishi et al. (2012) and Böni et al. (2018a, 2018b) successfully created films and fibers using solubilized IF proteins derived from hagfish slime threads. Fu et al. (2015, 2017) created materials from recombinant α and γ proteins from Pacific hagfish. Pinto et al. (2014) produced fibers from recombinant vimentin proteins that self-assembled into a gel of entangled 10-nm IFs. Zingerman-Koladko et al. (2016) and Khayat et al. (2020) produced fibers from recombinant nuclear lamin proteins (i.e. the IF proteins found inside the nucleus) from the nematode *Caenorhabditis elegans*. While none of these attempts has managed to replicate the impressive material properties of native hagfish slime threads, the properties are improving as more researchers work on the problem of producing eco-friendly alternatives to petroleum-based fibers.

9. From reductionism to synthesis

For several years we have employed reductionism to learn more about the two primary components of hagfish slime – mucous vesicles and slime threads. Much of this knowledge has come from studies in which vesicles or thread skeins were collected from slime glands and stabilized in a citrate buffer that inhibits deployment. Stabilized slime components can then be separated and studied independently in custom microscope flow chambers under a variety of conditions. One of the dangers of a reductionist approach is that isolated components might not behave as they do under more natural conditions. In the case of hagfish slime mucous vesicles, one worry is that the use of citrate buffer masks the natural behavior of the vesicles. One possible effect is that when we "replace" the buffer in a flow chamber, the vesicles remain surrounded in a boundary layer of citrate buffer even after the chamber volume has been flushed with the test solution of interest. It is also possible that the buffer alters the properties of the vesicles in an irreversible way. If either of these is the case, then we are not observing the natural deployment behavior of the vesicles. For these reasons, we have begun to do experiments with freshly collected exudate introduced to a flow chamber within seconds after it is released from a slime gland. These (unpublished) data are showing vesicle behavior that is quite different from what we have seen previously in stabilized vesicles. While these experiments have the potential to provide a more authentic view of what happens when hagfish slime exudate encounters seawater during a predatory attack, they pose new technical challenges, such as how to capture the rapid deployment of vesicles under the microscope and how to maintain focus when seawater is added to the chamber. The first of these challenges can be solved by using a high-speed video camera, the second has yet to be overcome.

We wish to raise one more limitation of reductionism, which is that reductionism is good for taking things apart, but offers little in regards to putting things back together. For the case of hagfish slime, we now know a lot about how mucous vesicles and thread skeins behave in a microscope flow chamber when they are exposed to seawater, and we know how various physical and chemical conditions affect those behaviors. However, all of this information will not spontaneously self-assemble into a coherent theory of hagfish slime physiology. For example, if we want to answer the question, "how does hagfish slime exudate transform in a fraction of a second in a predator's mouth into the final slime product," most of the data we have collected on the effect of temperature, salt, organic osmolytes, aquaporin inhibitors, chelators, reducing agents, etc. will not give us the answer.

To answer such an open-ended question requires employing the creative sides of our brains and generating hypotheses about how we think this process works. Our current working model is that the mucous vesicles deploy extremely rapidly when they contact seawater, forming a soft elastic network in which intact thread skeins are embedded. Hydrodynamic mixing forces generated by the thrashing hagfish and the biting or suction-feeding predator deform the mucus, which loads the embedded skeins in tension, causing them to unravel. This hypothesis makes several testable predictions about the relative timing of mucous vesicle and skein deployment, as well as the strength of the mucus

network relative to the force required to pull the skeins apart. Measuring mucus and thread deployment kinetics and the forces required to pull a thread out of an intact skein might seem like "reductionist" experiments, but in this case we are using them as part of a larger effort to test a synthetic hypothesis.

In discussing our efforts to understand hagfish defensive slime, we have identified and illustrated several advantages and disadvantages of using a reductionist approach. On one hand, breaking a system up into parts makes it easier to study each one, and it can sometimes lead to new observations and unexpected breakthroughs. On the other hand, studying the parts in isolation might affect the behavior of those parts for a variety of technical reasons, and it is rarely obvious how to put the parts back together again. Our take home message therefore is that reductionism alone cannot form the basis of a fruitful research program and that true progress requires connecting the information gained from reductionist experiments to higher-level synthetic explanations.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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