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# Composition, morphology and mechanics of hagfish slime

Douglas S. Fudge*, Nimrod Levy, Scott Chiu and John M. Gosline

*Department of Zoology, University of British Columbia, 6270 University Boulevard, Vancouver, BC V6T 1Z4, Canada*

*Author for correspondence at present address: Department of Integrative Biology, University of Guelph, Guelph, ON N1G-2W1, Canada (e-mail: dfudge@uoguelph.ca)

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## Summary

**Hagfish slime consists of mucins and protein threads that are released from slime glands and mix with seawater to produce an ephemeral material with intriguing physical properties. We recently characterized the mechanics of the slime's fibrous component, and here we report the first mechanical properties of the mucin component and the slime as a whole. Our results suggest that hagfishes can produce remarkable quantities of the slime because it is almost three orders of magnitude more dilute than typical mucus secretions. Mechanical experiments using whole slime produced *in vitro* demonstrate that the slime threads dominate the slime's material properties and impart elasticity. Mucins impart viscosity at the strain rates tested and are important for rapid deployment of the slime. We also found that slime threads are tapered at both ends, which suggested to us that hagfish slime might best be modeled as a discontinuous fibre-reinforced composite. Our measurements demonstrate that the mucins are not capable of providing shear linkage between threads, but this is not necessary because the threads are long enough to span an entire slime mass. Our findings suggest that hagfish slime consists mainly of bulk seawater entrained between mucin-coated threads, and in this way functions more like a fine sieve than coherent mucus. These results are consistent with the hypothesis that the slime has evolved as a defense against gill-breathing predators.**

Key words: biomechanics, slime, mucus, hagfish, fibre-reinforced composite.

## Introduction

Hagfishes are notorious for their ability produce large volumes of slime when they are provoked or stressed (Downing et al., 1981a; Ferry, 1941; Strahan, 1959; Fig. 1). Hagfish slime differs from other animal slimes in that it contains not only slippery mucins, but also fine fibres, or 'slime threads', which are believed to lend it strength and cohesion (Downing et al., 1981b; Fernholm, 1981; Koch et al., 1991b). The slime is formed when specialized slime glands eject coiled threads (or 'skeins') and mucin vesicles into seawater (Fig. 2). While considerable work has been done on the slime threads and their constituent proteins (Downing et al., 1984, 1981b; Koch et al., 1995, 1994; Spitzer et al., 1984); very little is known about the mucin component or how the mucins and threads function together in whole slime.

In a recent paper, we reported the tensile properties of individual slime threads and demonstrated that they are soft and elastic at low strains, but ultimately strong and extensible, and remarkably tough (Fudge et al., 2003). We also found that the proteins in the threads undergo a dramatic conformational change at large strains in which predominantly α-helical proteins take on a more extended β-sheet conformation. These results for isolated slime threads led us to hypothesize that the mechanical behavior of whole slime is dominated by the threads, with the mucin component of the slime acting to mechanically link the threads together. More specifically, we wondered whether the behavior of whole slime could be modeled as a fibre-reinforced composite, in which forces are transferred between adjacent slime threads *via* shearing of the mucin component.

To answer this question we measured the mechanical properties of hagfish slime mucins and whole slime. We also made a variety of measurements required for a complete understanding of hagfish slime form and function, such as slime thread length and diameter, the concentration of mucins and slime threads, and the amount of slime exudate stored by hagfishes as a function of body mass. From our results we conclude that the mucin component of the slime is not capable of transferring significant forces between slime threads as in a typical fibre composite. Furthermore, experiments with whole slime demonstrate that slime threads indeed dominate the mechanical behavior, while the mucin component imparts viscosity and aids in the rapid deployment of the slime into its mature and fully hydrated state. Although the precise ecological function of hagfish slime is not known, our results are consistent with the hypothesis that hagfish release the slime in order to thwart attacks by gill-breathing predators.

Fig. 1. Slime production by a hagfish in seawater. Photo courtesy of Chris Ortlepp.

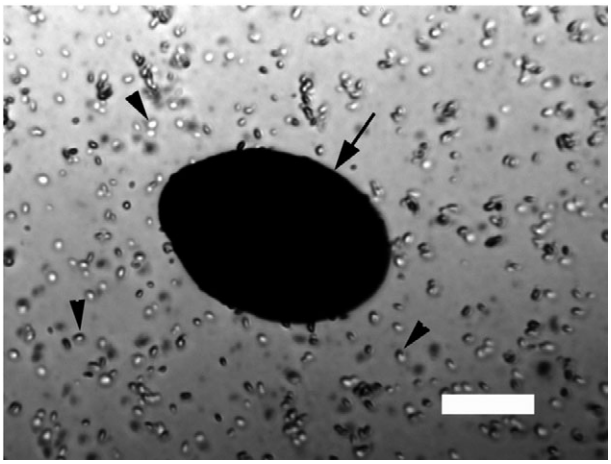

Fig. 2. Hagfish slime is formed from a concentrated exudate released by the slime glands. The exudate contains both coiled slime threads, or 'skeins' (arrow) and mucin vesicles (arrowheads) that rupture in seawater. Scale bar, 50 μm.

## Materials and methods

### *Experimental animals*

Pacific hagfish *Eptatretus stoutii* Lockington were obtained with assistance of staff at the Bamfield Marine Station in Bamfield, British Columbia, Canada. Traps were baited with herring and set in Barkley Sound on the bottom at a depth of approximately 100 m and left overnight. Hagfish were transported to the University of British Columbia where they were held in a 200 l aquarium of chilled seawater (34‰, 9°C) and fed monthly meals of squid in accordance with the regulations of the UBC Committee on Animal Care (protocol A2-0003).

### *Concentrations of threads and mucins in native slime*

Hagfish were induced to produce a single mass of slime in their 200 l aquarium by pinching them on the tail with forceps. Care was taken to insure that the slime was produced by hagfish swimming in the middle of the water column rather than close to the bottom or surface, which could have constrained slime hydration. The slime was gently collected by scooping it into a plastic kitchen colander lined with 53 μm nylon mesh. As the colander was lifted out of the aquarium, free water was allowed to run out of the bottom through the mesh, and the colander was tipped slightly to allow free water sitting on top of the slime to spill out. The contents of the colander were transferred to a bucket for subsequent measurement of slime volume. Threads were removed from the slime by twirling them onto a glass rod until they collapsed and squeezed out most of the entrapped mucins and water. This technique was very effective at collecting the vast majority of the threads in the slime, and concentrating them into a fibrous ring that could be easily handled for subsequent purification and drying. The ring of threads was placed back into the original slime solution to which a pinch of dithiothreitol (DTT) was added. Along with gentle heating of the slime (to about 60°C), DTT helped in the removal of mucins that were still bound to the threads. The threads were treated in this way until they were no longer slippery to the touch (about 30 min), at which point they were removed, rinsed with several changes of distilled water, and dried in an oven at 80°C for the determination of dry mass. Thread concentration was calculated by dividing the dry mass of threads by the total volume of the slime collected.

Mucin concentration was measured by dialysis of 50 ml of the remaining slime solution using Spectra/Por dialysis tubing with a 12–14 kDa cut-off (Spectrum Laboratories Inc., Rancho Dominguez, CA, USA). Mucin samples were dialysed four times against 5 l of distilled water in a cold-room (4°C) for 8 h. Preliminary trials demonstrated that the above procedure was adequate to lower the concentration of salts to a negligible amount. Mucin dry mass was obtained by drying 25 ml of the dialyzed solution in a drying oven at 80°C. Mucin concentration was obtained by dividing the mass of mucins by the volume of solution dried. Mucin concentrations were adjusted by subtracting out the concentration of material in the distilled water, which was measured by drying down 25 ml of the distilled water used for the dialyses. Mucin and thread concentrations were measured in this way for five independent slime masses.

The high ratio of salts to mucins in the slime made it technically challenging to accurately measure the mucin concentration. In contrast, because the slime threads are insoluble, they were easily separated from the slime and therefore their concentration could be measured more accurately. To confirm the mucin concentrations obtained *via* dialysis, we also measured the mucin concentration *via* a centrifugation technique we will refer to as 'slimatocrit', due to its similarity to the measurement of hematocrit. The premise

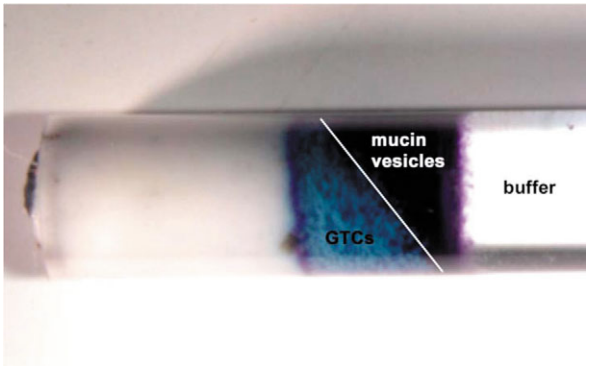


Fig. 3. Result of a typical 'slimatocrit' trial, in which slime exudate was stained with Toluidine Blue and spun in hematocrit tubes to measure the volume fractions of thread cells and mucin vesicles. Mucin vesicles (dark staining) and gland thread cell skeins (GTCs) make up about equal amounts of the exudate.

of this technique is that the volume ratio of mucin vesicles to threads can be measured by centrifuging slime exudate in hematocrit tubes. From these data, the concentration of mucins in the slime can be calculated from the thread concentration data. Slime exudate was collected from anaesthetized hagfish and transferred into an aqueous stabilization buffer (Downing et al., 1984) containing 0.5% Toluidine Blue. Stained and stabilized slime exudate was drawn up into 75 mm microhematocrit capillary tubes, and spun for 10 min in a hematocrit centrifuge. Centrifugation resulted in two distinct layers, a slime thread skein layer, and a Toluidine Blue stained mucin vesicle layer topped by stabilization buffer (Fig. 3). The relative volume of the layers was calculated from their dimensions, which were measured under a dissecting scope with a Filar eyepiece micrometer. Sixteen slimatocrit measurements were made in total.

### *Slime storage* vs *hagfish mass*

Measurement of the amount of exudate stored in slime glands was performed on hagfish that were anaesthetized as described in Fudge et al. (2003). Hagfish that released slime in the anesthesia bucket were rejected and placed back in the aquarium. Immediately after losing touch sensitivity, hagfish were removed from the anesthesia bucket, blotted dry and weighed. The animals were then placed on a chilled dissection tray where they were kept hydrated and cool, except for the area from which slime exudate was collected, which was rinsed with distilled water and blotted dry. Rather than trying to measure the mass of exudate expressed from every slime gland, five glands were chosen as representatives. Exudate was expressed from the glands *via* mild electrical stimulation as described in Fudge et al. (2003). Exudate from all five glands was collected with a spatula and transferred to a pre-weighed microcentrifuge tube. Glands were stimulated until exhausted of exudate. Wet and dry mass were measured to the nearest 0.1 mg using a Mettler H31 balance (Mettler Instruments, Zurich, Switzerland). Exudate samples were dried in a drying oven at 80°C until the mass was stable over time. The total mass of stored exudate was calculated by multiplying the pooled exudate mass by the total number of glands and dividing by five.

### *Slime thread length*

When ejected from the slime glands into seawater, slime thread skeins quickly unravel. When they are collected into stabilisation buffer, however, they retain their original ellipsoid shape and coiling. Thread length was measured by transferring single stabilized skeins to a seawater-filled test chamber, allowing it to partially unravel, and attaching its respective ends to two glass rods using techniques described in Fudge et al. (2003). The original intent of this setup was to slide one rod away from the other until the thread was just taut; the distance between the two rods would then reveal the length of the thread. This plan was confounded by the tendency of the coiled thread to unravel in stages, with the thread going taut at times when there were clearly large sections that had not yet unraveled. Because it was not possible to observe the glass rod under the microscope and simultaneously observe the entire length of the thread for clusters of thread loops, using tautness as an endpoint was abandoned. Instead, thread failure was used as an endpoint and the resting length calculated from the average failure strain of slime threads as reported in Fudge et al. (2003) ($\epsilon_{max}$=2.2). While the popping open of clusters of loops caused deflections of the glass rod, these were always transient and easily distinguished from the long steady deflection that occurred before failure of the thread. In essence, these length measurements were extensibility tests of entire slime threads.

### *Slime thread taper*

Diameter measurements were made at eleven equispaced positions along the length of intact slime thread skeins in distilled water. While most slime thread skeins rupture and lose their coiled structure in seawater, some remain mostly intact, and it was these that were used for diameter measurements. Slime thread skeins were visualized under high power (100× interference contrast oil immersion objective) on a Leitz polarizing microscope (Ernst Leitz Canada, Midland, ON, Canada) fitted with a Panasonic WV-BL600 video camera. Thread diameter was measured on captured images using Scion Image release v. 3b software (Scion Corp., Frederick, MD, USA).

To isolate skeins and prepare them for scanning electron microscopy (SEM) imaging, slime exudate was collected and stirred into stabilization buffer. Stabilized slime was filtered through 53 μm nylon mesh, which retained the skeins and allowed the mucin vesicles to pass through. After washing the filter disk with excess buffer, skeins were removed from the disk by gentle shaking with 10 ml of buffer in a capped vial. Skeins were fixed for 2 h in 4% glutaraldehyde in stabilization buffer, rinsed with fresh buffer, and then rinsed again with 0.2 mol $l^{-1}$ cacodylate buffer (pH 7.1). Skeins were transferred onto a Nucleopore Track-Etch membrane (13 mm diameter,

6 μm pore size, Corning, Acton, MA, USA) in-line with a 5 ml syringe. The cells were dehydrated with an ethanol series consisting of 5 ml of the following ethanol solutions: 30%, 50%, 70%, 80%, 90%, 95%, 100%, 100%. While still wet with 100% ethanol, the filter disk was transferred into a Balzer CPD 020 critical point drying apparatus (Bal-Tec, Manchester, NH, USA). Before critical-point drying, the ethanol was replaced by ten rinses with liquid carbon dioxide. Dried skeins were immediately transferred into a Nanotech Semprep 2 gold sputter coater, and coated under vacuum for 3.2 min. Images were collected using a Hitachi S4700 scanning electron microscope.

### *Mucin mechanics*

Our original intent was to characterize the viscoelastic properties of hagfish mucins, but after isolating mucins from the slime, it became clear that mucins in seawater at native concentrations possess no elastic properties, and if they have any effect on the properties of seawater at all, it is only to raise the viscosity. We therefore measured mucin mechanics using a simple Ostwald viscometer (Fisher Scientific, Hampton, NH, USA). Slime masses from five different animals were isolated and viscosity measured on three different 7 ml subsamples from each. Transit times through the viscometer were measured with a stopwatch to the nearest 0.01 s and averaged. The viscometer was mounted in a water bath maintained at 9°C. After introduction into the viscometer, mucin samples were allowed to equilibrate for 20 min before testing. Between trials, the viscometer was flushed with the following solvents: 10 ml distilled water ($dH_2O$; 3×), 10 ml 1 mol $l^{-1}$ HCl, 10 ml $dH_2O$, 5 ml acetone, 5 ml acetone, and then flushed with filtered air until dry. Mucin samples were obtained by collecting slime masses from the 200 l hagfish tank using a mesh-lined colander. Slime threads were removed by twirling them out onto a glass rod. Mucins bound to the threads on the glass rod were removed by gently massaging the threads until they were no longer slippery to the touch. Before testing, mucin samples were filtered twice through 53 μm mesh to remove particulates that might have interfered with the viscosity measurements.

The concentration-dependence of mucin viscosity was measured by preparing concentrated slime solutions in distilled water and diluting them to attain a concentration series that spanned two orders of magnitude. Concentrated stock solutions were prepared by stirring slime exudate from anaesthetised hagfish directly into a beaker of chilled distilled water. This resulted in a thick mixture containing both slime mucins and slime threads. Threads were removed by inserting a glass rod into the mixture and spinning the threads onto the rod. The remaining mucin solution was then vacuum-filtered on ice through two layers of 53 mm nylon mesh to remove any remaining slime threads or other particulates. Mucin stock solutions were prepared from four different hagfish and had an average mucin concentration of 470±67 mg $l^{-1}$ (mean ± S.E.M.), which was measured gravimetrically by drying down 2× 25 ml samples of the stock solution at 80°C. Mucin solutions in seawater were prepared by diluting mucin stocks with an equal volume of double strength artificial seawater (Coralife, Carson, CA USA) to obtain a solution half as concentrated as the distilled water stock. Further dilutions were made with full strength seawater. Viscosity measurements were made as described above.

### *Whole slime mechanics*

The extreme heterogeneity of the slime required a mechanical testing apparatus that could simultaneously quantify the viscous and elastic components of its mechanics but not destroy its delicate structure in the process. Ideally, the slime would be formed from exudate and seawater within the testing apparatus itself. In addition, the apparatus had to be large enough to allow for the complete unraveling of the slime threads. The apparatus shown in Fig. 4 fulfils all these criteria. We used an Instron model 5500 (Norwood, MA, USA) universal testing machine to move a 2 l beaker of hagfish slime up and down while forces were measured by a 100 g load cell from which hung a stationary plunger. The plunger consisted of a 40.5 mm nylon disk fitted with eight radial spikes that protruded 12 mm from the edge of the disk. The plunger was attached to the load cell *via* a 0.36 m stainless steel rod. The nylon disk was sandwiched between 3 mm thick lead disks that gave the plunger a total mass of 104 g and kept sufficient tension on the attachment rod so that compression forces could

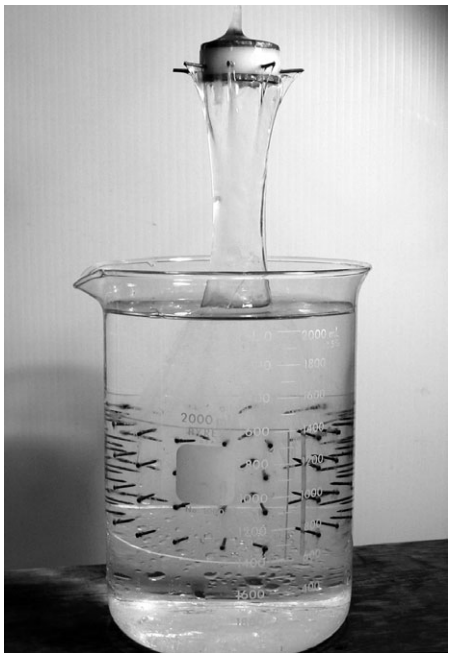

Fig. 4 Apparatus used for whole slime mechanical measurements. Fresh slime exudate was added to the top of a 2 l beaker and slime was formed by the gentle stirring caused by oscillations of the plunger. Hydration of slime exudate led to unraveling of gland thread cell skeins and their subsequent elongation and attachment to the inside of the beaker and the plunger. Force on the plunger was measured using a 100 g load cell.

be measured without the rod lifting off from the load cell. The inside surface of the beaker was modified by inserting a 62 mm wide plastic collar studded with 72 1.3 mm diameter nails that protruded 12 mm toward the centre of the beaker (Fig. 4). We found that after the slime formed within this setup, it became attached to both the plunger and the spiked collar. For each trial, the plunger was oscillated up and down in 2 l of 9°C seawater at a rate of 11.7 mm $s^{-1}$ over an amplitude of 100 mm and force and displacement data were collected at 100 Hz. After 50 s of data collection in seawater only, 100 mg of fresh slime exudate from an anaesthetised hagfish was added *via* a spatula to the top of the beaker. As the slime concentration measurements demonstrate later in this paper, this amount of exudate is enough to produce about 1 l of slime. Some trials were performed in the presence of 5 mmol $l^{-1}$ DTT, which reduces disulfide bonds within and between mucin molecules and has been shown to decrease the viscosity of mucus (Bell et al., 1985). Stress–relaxation trials were performed on slime that had been allowed to develop for 500 s of plunger oscillations. As the stainless steel rod oscillated up and down in the beaker, the length of rod immersed in the water changed over time. This resulted in a highly predictable buoyant force that in some trials represented a considerable amount of the force variation. We therefore measured the change in force on the plunger as a function of the degree of (static) immersion, which allowed us to remove the contribution of the buoyant force from our data. The relationship between rod immersion ($x$) in mm and static force on the load cell ($y$) in mN was $y$=0.026$x$ in seawater and $y$=0.024$x$ in distilled water. Preliminary trials revealed a predictable 30 Hz source of noise in the data that presumably arose from resonant oscillations of the force transducer. This noise was digitally filtered using a second order Butterworth recursive algorithm (Winter, 1979) with a 10 Hz cut-off. Filtering the data in this way had no effect on the low frequency events resulting from deformation of the slime. Raw data were additionally processed to remove a force spike that occurred at the extremes of the crosshead excursion. This spike arose from the rapid deceleration of the beaker of water at the turnaround point and had nothing to do with the material properties of the slime. About 0.1 s worth of data points were deleted from each half cycle of the force traces to remove these spikes.

### *Water egress from the slime*

Our mucin and thread concentration measurements suggested that hagfish slime is able to organize heroic amounts of water with very little material. In making these measurements, we collected the slime by lifting it out of an aquarium using a colander. This procedure took only a few seconds to perform. To test whether the slime could organize water on longer timescales, we measured water egress from slime formed *in vitro*. Slime was formed by stirring about 100 mg of fresh slime exudate into a 1 l plastic cylinder (diameter=88 mm, length=148 mm) that was nested in a 2 l beaker of seawater. The cylinder hung from a 5 kg load cell and was covered on the bottom with plastic 4 mm mesh. At the start of the experiment, the bridle connecting the cylinder to the load cell was slack. The beaker of water was quickly lowered well below the cylinder and the mass of the cylinder, plus water and slime was recorded at 10 Hz by the load cell. These measurements were performed with four different slime samples.

### *Congo Red staining*

In a previous study, we established that Congo Red (CR) staining can be used to detect an α→β transition in intermediate filament proteins from mechanically strained slime threads. Here we used CR as a way of evaluating the strains induced in slime threads as a result of mechanical perturbation of the whole slime in seawater. Hagfish were coaxed into producing a mass of slime in their aquarium as described above. 'Unperturbed' slime was gently collected from the aquarium in a shallow, mesh-lined colander with a glass slide on the bottom. The slime was allowed to drain and adhere to the mesh before being rinsed with a very gentle, continuous flow of tapwater for 15 min. The slime was then rinsed with distilled water, and allowed to completely dry onto the glass slide and the mesh. When dry, the glass slide was freed from the mesh by trimming the slime threads with a razor blade. The slime was stained in a 1% CR, 10% ethanol solution for 1 h, after which it was de-stained in distilled water for 5 min, followed by a gentle distilled water rinse. 'Perturbed' samples were prepared in the manner described above, except a ruler was pushed into the slime and moved back and forth 20× at an approximate frequency of 1 Hz and an amplitude of 10 cm before the sample was collected into the colander.

Values are reported as means ± S.E.M.

## Results

### *Hagfish slime is 99.996% seawater, 0.0015% mucin and 0.002% threads*

Dialysis and gravimetric analysis of naturally formed slime from five hagfish revealed a concentration of molecules greater than 12–14 kDa of 15±1 mg $l^{-1}$, while slime thread concentration was 20±3 mg $l^{-1}$. The average volume of slime produced from a single pinch on the tail was 0.91±0.17 l. For the 16 slimatocrit measurements that were made, the average ratio of mucin vesicle to gland thread cell (GTC) skein volume was 1.15±0.1, from which a mucin concentration of

Table 1. *Concentration of mucins in a variety of mucus secretions*

| Source | [Mucin] (mg $ml^{-1}$) | Reference |
|---|---|---|
| Hagfish slime | 0.02 | This study |
| Gastric mucus | 47 | Sellers and Allen, 1989 |
| Duodenal mucus | 38 | Sellers and Allen, 1989 |
| Colon mucus | 20 | Sellers and Allen, 1989 |
| Slug pedal mucus | 1–32 | Denny, 1979 |
| Human salivary mucus | 14 | Veerman et al., 1989 |
| Human gastric mucus | 30 | Pain, 1980 |

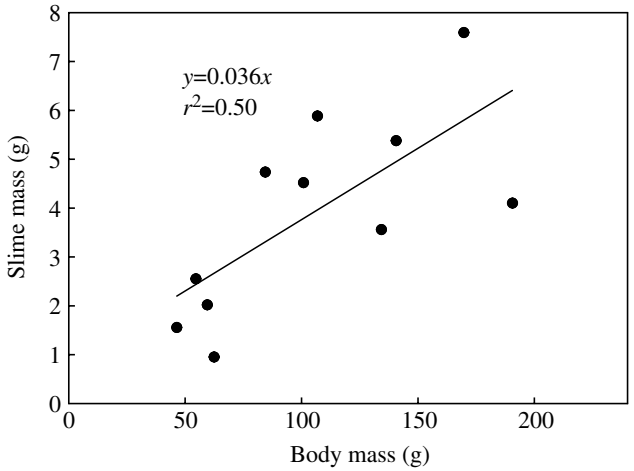


Fig. 5. Scaling of stored slime mass with body mass. The slope of the line suggests that stored slime represents 3–4% of the hagfish's mass.

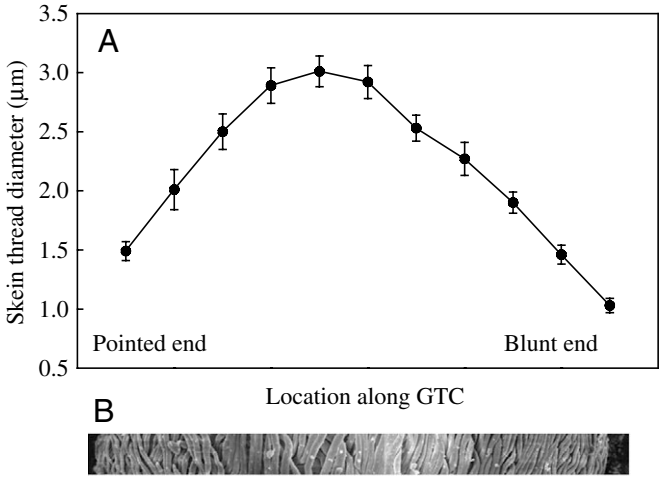


Fig. 6. (A) The diameter of the thread on the outside of intact slime thread skeins as a function of position along their longitudinal axis. Note the bi-directional taper. (B) A composite of four SEM images along a single thread cell that demonstrates how thread diameter tapers off at both ends of the cell (GTC).

17±3 mg $l^{-1}$ can be extrapolated, assuming that skeins and stabilized vesicles have the same density. Expressed as percentages (w/v), the mucin and thread concentrations are both about 0.002%. The mucin concentration of the slime is orders of magnitude lower than the mucin concentration in more conventional mucus secretions such as gastric mucus (Table 1).

*Stored slime represents 3–4% of hagfish body mass*

Collection of slime exudate from eleven anaesthetized hagfish revealed a significant positive linear relationship with body size ($P$=0.014, $r^2$=0.50), with a regression equation of $S$=0.036$M_b$, where $S$ is the total mass of stored slime (g) and $M_b$ is the hagfish mass (g). From this equation, one can conclude that slime exudate makes up about 3–4% of hagfish body mass (Fig. 5).

*Slime threads break at 34 cm and have a resting length of 10–17 cm*

The average length at failure for the ten slime threads tested was 34±1 cm. Because a small length of thread at either end was used to attach the slime thread to mounting rods, this value is a slight underestimate of failure length. If we assume that the extensibility of entire slime threads is the same as the extensibility as the thread segments tested in Fudge et al. (2003) (i.e. $\epsilon_{max}$=220%), then the resting length of slime threads is 10–11 cm. However, because whole slime threads are tapered, it is more likely that they will break at their narrowest point, with the thicker regions never reaching their maximum extensibility. Slime threads exhibit a threefold difference in diameter from their thickest to thinnest points (Fig. 6), and therefore a ninefold difference in cross-sectional area. This means that when the thinnest part of the thread is at the breaking stress (180 MPa), the stress in the thickest part will be only 1/9th of that, or 20 MPa, which corresponds to a strain of only 1.0. This means that the overall breaking strain of an entire thread should fall somewhere between 100% and 220%, giving a possible range of resting lengths of 10–17 cm. These values are consistent with Fernholm's estimate of 6–11 cm, and a bit lower than the estimate of 24–60 cm by Downing et al. (1981b).

*Slime threads are bi-directionally tapered*

Measurement of thread diameter within intact slime thread skeins using light microscopy revealed a distinct bi-directional taper (Fig. 6), in contrast to the uni-directional taper proposed by Downing et al. (1981b). Hydrated threads within skeins exhibited a maximum diameter of 3.0±0.4 μm, which occurred in the middle of the skeins. Thread diameter was 1.5±0.2 μm at the pointed end, and 1.0±0.2 μm at the blunt end. Inspection of thread diameter under SEM confirms this result (Fig. 6B).

*Mucin viscosity is low, even at high concentrations*

The average dynamic viscosity of the mucin solutions collected from slime masses produced in aquaria was indistinguishable from the viscosity of seawater controls ($1.44\times10^{-3}$ Pa s $\pm0.003\times10^{-3}$ Pa s). Mucin solutions prepared in distilled water exhibited a linear, but weak concentration dependence, with the viscosity of the stock solutions exceeding that of their hundredfold dilutions by an average factor of only 3.2 (Fig. 7). The viscosity of mucin solutions prepared in seawater exhibited even less concentration dependence, with a linear slope that was over 14× lower than the slope for mucins in distilled water. The viscosity of stock solutions in seawater exceeded that of their 50-fold dilutions by an average factor of only 10% (Fig. 7).

*Slime maturation is rapid in seawater and delayed by 5 mmol $l^{-1}$ DTT*

Fresh slime exudate from anaesthetised hagfish added to seawater did not instantly transform into mature slime, but

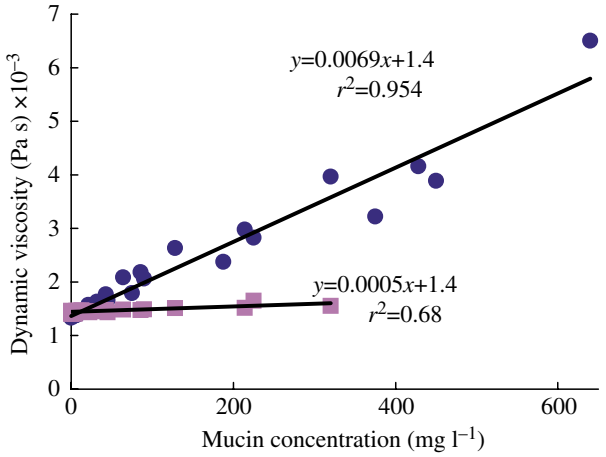


Fig. 7. Viscosity of mucin solutions prepared in distilled water (blue circles) and seawater (purple squares) as function of concentration. Note that the viscosity in seawater was low even at the highest mucin concentrations tested.

instead required some mixing by the oscillating plunger. In seawater, slime maturation was nearly complete after two full oscillations (34.4 s), as measured by the increasing forces exerted on the plunger. Slime maturation in distilled water was almost as rapid as in seawater, but in the presence of 5 mmol l$^{-1}$ DTT, maturation was delayed, taking about six cycles (103.2 s) on average (Fig. 8).

### *Slime threads dominate the material properties of hagfish slime*

Close inspection of the slime maturation process revealed that the increase in force generation by the slime over time corresponded with the unravelling of skeins and the subsequent entanglement of slime threads on the projections of the plunger and the inside of the beaker. Focusing in on a single oscillation for each treatment reveals almost identical force traces (Fig. 9). To test whether the forces measured were indeed a result of the straining of solid slime threads and not just a viscous phenomenon, stress–relaxation trials were performed after the initial 500 s period of slime maturation and oscillations. The three treatments (seawater, distilled water and 5 mmol l$^{-1}$ DTT in seawater) each exhibited distinct stress relaxation properties (Fig. 10). The forces generated by slime produced in seawater relaxed on average by about 74% after 500 s of relaxation. Although such a large relaxation suggests a significant viscous component to the peak forces generated by the slime, the fact that it still held significant force after 500 s confirms that a solid structure is involved in the forces measured. Slime formed in seawater in the presence of 5 mmol l$^{-1}$ DTT relaxed significantly less, losing only about 52% of the peak force after 500 s of relaxation. DTT cleaves disulfide bonds and has been shown to be an effective means of disrupting networks of mucins (Bell et al., 1985). In contrast, the proteins that make up the slime threads have been shown to be almost completely devoid of cysteine (Koch et al., 1995, 1994), thus one would

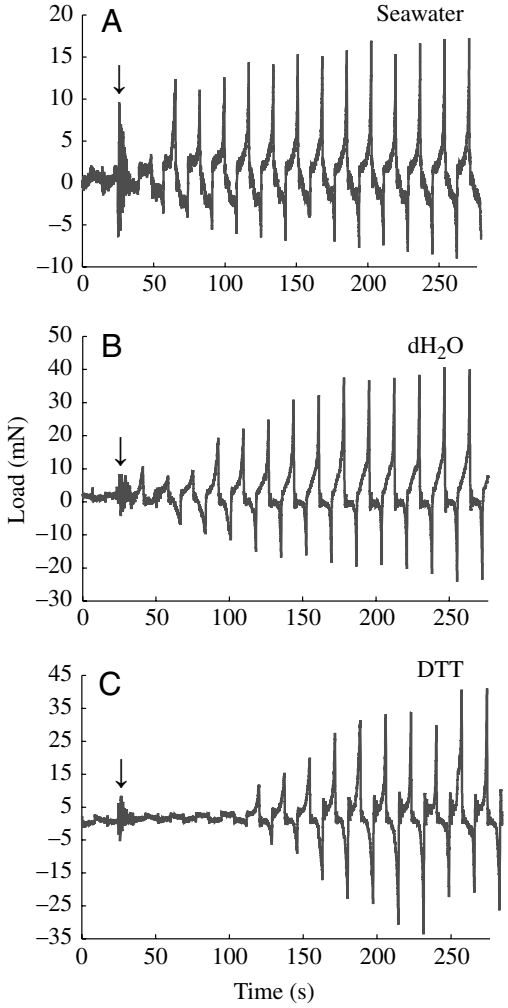


Fig. 8. Representative force traces showing slime development over time in (A) seawater, (B) distilled water (dH$_2$O), (C) seawater containing 5 mmol l$^{-1}$ DTT (DTT). Arrows denote when the slime exudate was added to the beaker of seawater.

expect DTT to have no effect on the thread mechanics. The slower force decay with DTT is consistent with our assertion that it is indeed the slime threads that are responsible for the majority of the forces generated by the slime in these oscillation experiments. This effect of DTT also suggests that some of the force generated by the slime in seawater can be attributed to the mucin component of the slime. It also suggests that the presence of cross-linked mucins allows the threads to slip more easily past each other and off the projections of the apparatus.

Hagfish slime is known to collapse when it is mechanically disturbed, with a massive loss of mucins and water (Ferry, 1941). We expected to see evidence of this process in our force traces over time. Interestingly, we saw no degradation of slime properties over the 500 s of data collection, and even extended trials of plunger oscillations (up to 3000 s) had no effect on the magnitude or shape of the force traces (data not shown). This was likely due to the limited volume of seawater used for these

measurements as well as the fact that slime threads that became entangled on the projections of the plunger and the walls of the beaker had little opportunity to collapse onto each other.

From the four trials performed in seawater, the average peak forces in the positive and negative directions were 24.6 mN and 12.6 mN, respectively, for a total peak force (± S.E.M.) of 37.2±7.6 mN. Because the mucin component exhibited no elastic properties and only marginally elevated viscosity, it is reasonable to conclude that these forces arose mostly from the straining of slime threads. The stability of the force traces over time suggests that the slime threads were able to recover between cycles, and this suggests that the strain they experienced was less than their yield strain of 35% (Fudge et al., 2003). If we assume that attached slime threads are extended to their yield strain (about 35%) during each cycle, then each thread will exert about 7 μN of force. Assuming that each thread contributes to the force in one direction only (which admittedly will not hold for threads whose minimum strain occurs close to the middle of the plunger excursion), then this corresponds to about 5300 slime threads. A typical slime mass contains about 24 000 slime threads, so this suggests either that the threads are strained less than 35% on average, and/or a majority of the threads were not ever brought into tension due to the orientation of their attachment. It is unlikely that many of the threads were strained to the breaking point, since a similar calculation predicts that only about 150 threads strained maximally could account for the forces measured. This is clearly much lower than the number of threads that could be seen with the naked eye and would require that the vast majority of threads were never put into tension.

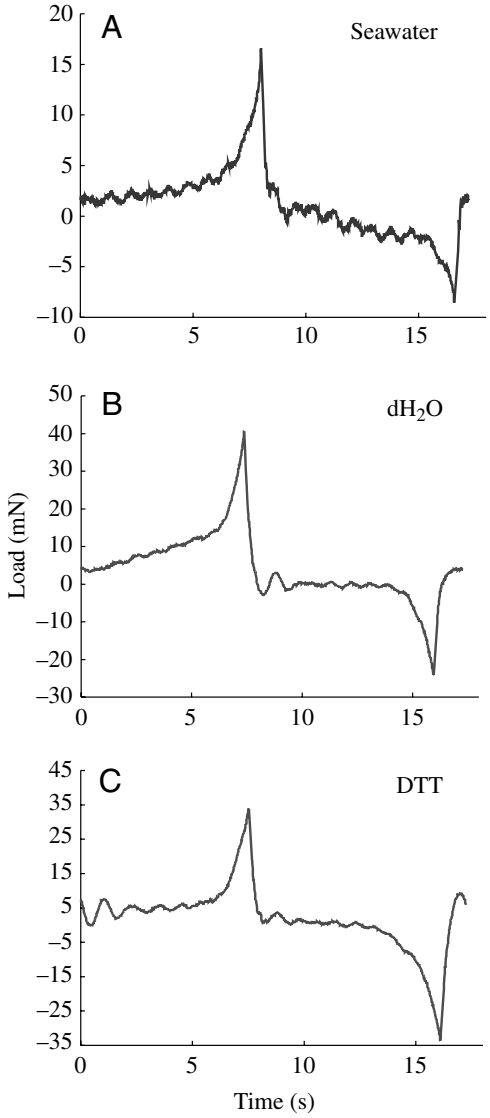


Fig. 9. Representative force traces for single plunger oscillations in (A) seawater, (B) distilled water ($dH_2O$) and (C) seawater containing 5 mmol $l^{-1}$ DTT.

### *Hagfish slime binds water loosely*

Measuring the flow of water out of hagfish slime formed within a 1 l cylinder revealed that the slime is not able to organize large volumes of seawater over timescales of more than a few seconds. When the 2 l beaker was dropped away from the hanging mesh-bottomed cylinder containing the slime, an average of 1100±110 g of slime was trapped in the cylinder. The rate of water egress from the slime was rapid at first (340±80 ml $s^{-1}$ over the first 0.5 s) and declined rapidly over time (Fig. 11). The decrease in egress rate resulted from the decrease in the pressure head as the water level dropped in the cylinder, and presumably the collapse of the slime and obstruction of the mesh by the threads. Curiously, the rate of egress was not linear when plotted on a semi-log scale, showing a distinct bump where the flow rate increases and then decreases again (Fig. 11). After 250 s of hanging in air, the

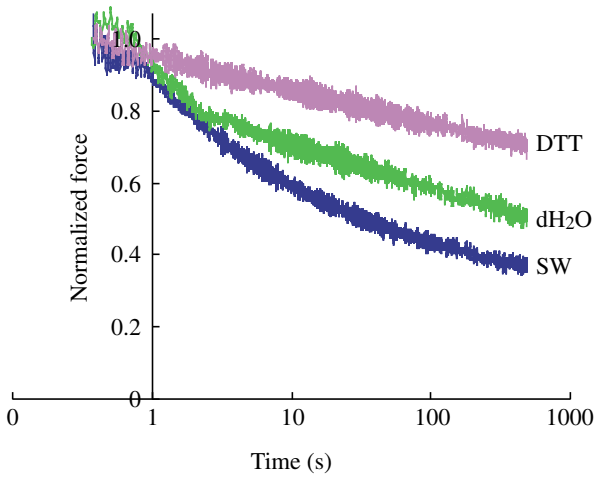


Fig. 10. Representative curves of stress–relaxation of whole hagfish slime in the same apparatus as described in Fig. 7. The slime was strained by a 50 mm movement of the plunger within the slime and held for 500 s. Stress relaxation in seawater (SW) and distilled water ($dH_2O$) was more rapid at first and then settled into a slower rate of force decay. In seawater containing 5 mmol $l^{-1}$ DTT, this initial rapid force decline was absent.

average mass left in the cylinder for the four trials was only 62±13 g or about 5.6% of the initial mass.

*Slime perturbation induces an α→β transition in slime thread proteins*

Slime threads from both unperturbed and perturbed slime bound CR, but only threads from perturbed slime exhibited strong and extensive CR metachromasia (Fig. 12). The few threads that did exhibit CR metachromasia in unperturbed samples were likely strained during collection or rinsing. Threads within perturbed slime also showed a tendency to form parallel bundles, whereas unperturbed samples did not. The presence of bundles is consistent with previous observations of bundles in perturbed slime and in the slime found near hagfish eggs (Koch et al., 1991a).

## Discussion

We found that mucins and threads occur at vanishingly low concentrations, which helps explain how hagfishes can produce such large volumes of slime. Slime threads have an average breaking length of about 34 cm, are bi-directionally tapered, and their constituent proteins undergo an α→β transition when the slime is disturbed. Experiments with whole slime indicate that the slime threads dominate the mechanics and impart elasticity, while the mucins impart additional viscosity and assist in the rapid deployment of the slime into its mature state. In the following sections, we will synthesize these data into a coherent model of hagfish slime structure and mechanics and speculate on the ecological function of the slime in light of these findings.

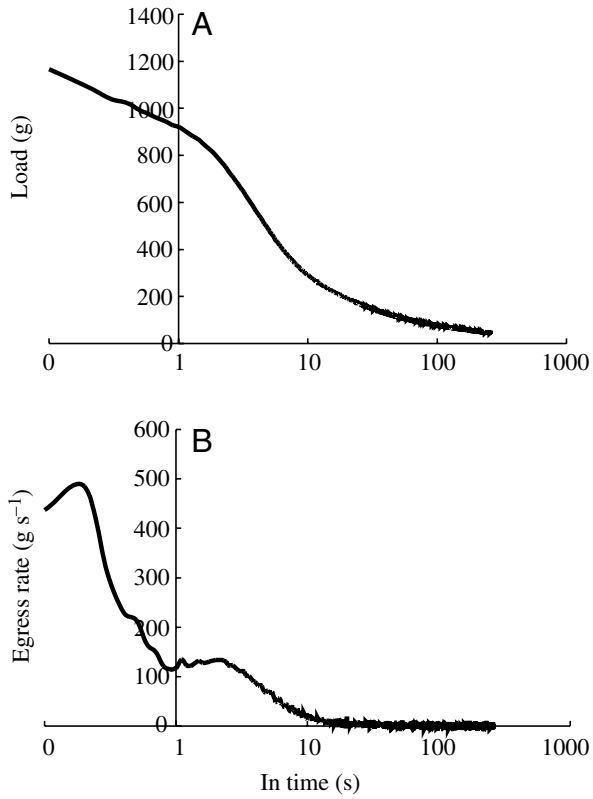


Fig. 11. Lifting about 1 l of slime into air using a 4 mm mesh lined cylinder resulted in massive water loss from the slime. (A) Water left in the beaker as a function of time measured as load on the force transducer. (B) Rate of water egress from the slime calculated from the same data. These results demonstrate that the slime is not able to organize water over long time scales.

*Hagfish slime is 1000× more dilute than other mucus secretions*

Gravimetric and 'slimatocrit' measurements indicate that the mucins in hagfish slime are present at only 15 mg l$^{-1}$, which is over 1000× more dilute than typical mammalian mucus secretions (Table 1). Along with the slime storage data reported in Fig. 5, the mucin concentration data can be used to

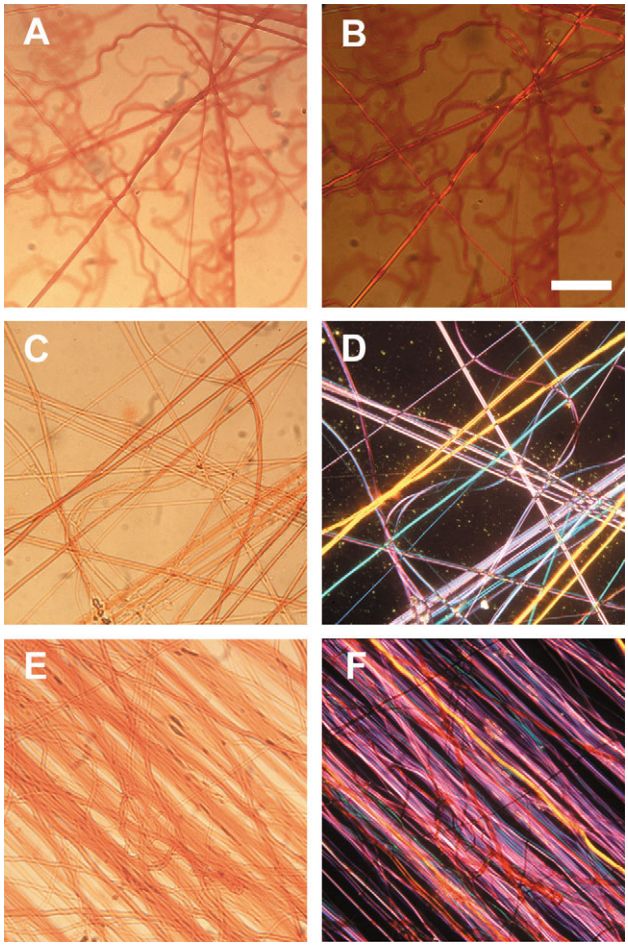


Fig. 12. Congo Red (CR) staining of hagfish slime. Slime threads (but not mucins) bound CR, but only threads from slime that was physically perturbed showed metachromasia. (A) Bright field image of slime threads from unperturbed slime. (B) Dark-field (polarisers crossed) image of same threads. Note the lack of CR metachromasia in most of the threads. (C) Bright field image of threads from perturbed slime. (D) Same as C, dark field. (E) Bright field image showing bundling of slime threads in perturbed slime. (F) Same as E, dark field. Bar, 10 μm.

calculate the maximum volume of slime that a typical hagfish can produce. A typical *E. stoutii* weighs about 60 g, so according to Fig. 5, it should have about 2.2 g of slime exudate in its arsenal. Of these 2.2 g, about 66% of the mass is water, leaving about 0.73 g of dry mucins and threads. The concentration data show that mucins and threads make up approximately equal amounts of the dry mass of the slime, which implies that about 0.36 g of the stored exudate is dry mucins. At a final mucin concentration of 15 mg $l^{-1}$, this amount of mucin could be used to make about 24 l of slime, or about 400× the hagfish's own volume. In contrast, if the mucins in hagfish slime were as concentrated as they are in typical mammalian mucus (about 30 mg ml), the hagfish would be able to produce only 12 ml of slime.

Our estimate of 24 l is far higher than Strahan's measurement of 0.5 l, but closer to the estimates of Koch et al. (1991b) and Goode and Bean (1895) (about 7–8 l). Strahan's estimate came from placing hagfish in only 1 l of water. Because the vast majority of slime volume is seawater, holding the animal in such a low volume imposes an artificial ceiling on the maximum volume that can be measured. Indeed, the average slime volume we measured in our 200 l aquarium from a single pinch on the tail was 0.91 l, and this surely was only a fraction of the animal's maximum capacity.

Why is the mucin concentration in hagfish slime so low? From an evolutionary standpoint, one would expect selection to favour hagfish that can produce functionally competent slime with as little energetic investment as possible. Because it is mostly water, mucus may not seem like an energetically expensive material to make, but for many marine organisms, mucus production represents a large portion of their energy budget. From 13–80% of the energy intake of gastropods and chitons is used in the production of mucus (Denny, 1989). In light of these facts and the fact that 3–4% of a hagfish's wet mass is slime, it is not surprising that selection has favoured hagfish that can produce slime as cheaply as possible. The question of how hagfishes make such dilute slime will be addressed later in the Discussion.

### *Hagfish slime is not a fibre-reinforced composite*

The bi-directional taper of the slime threads depicted in Fig. 6 is reminiscent of the taper exhibited by collagen fibrils in tendon, which is generally attributed to collagen's role as fibrous reinforcement in these structures (Trotter and Koob, 1989). Although they are quite long, collagen fibrils do not span an entire tendon, and so stress must be transferred to adjacent fibrils *via* shear of the proteoglycan matrix. Composite theory predicts that the tensile forces borne by the fibrils decrease toward the fibril ends and are highest in the middle. Thus, the most economical use of collagen protein is to make fibrils that are tapered at both ends. Could this explain the taper of hagfish slime threads?

If hagfish slime behaves as a fibre-reinforced composite-like tendon, then shear transfer between adjacent slime threads must be significant. The critical fibril aspect ratio ($s_c$=fibril length divided by radius) at which reinforcement is effective in a fibre composite is determined by the ultimate stress ($\sigma_{fu}$) of the fibril divided by the yield stress of the matrix in shear ($\tau_{my}$) or:

$$s_c = \sigma_{fu} / \tau_{my} . \quad (1)$$

If the aspect ratio of the reinforcing fibrils is greater than $s_c$, then force transfer will be adequate to load the fibrils maximally, and they will strengthen the composite. If the aspect ratio is lower than $s_c$, the fibrils will not be loaded maximally, and they will be less effective at reinforcing the composite. From Fudge et al. (2003) we know that the ultimate stress of slime threads in seawater is 180 MPa, and from the present study we know that the aspect ratio of the threads (length/radius) is about $10^5$ (10 cm/1 μm). From Eqn 1 we can calculate the magnitude of the matrix yield stress that would be required for the threads to be fully loaded in tension by stress transfer from an elastic matrix, which is about 1.8 kPa ($\tau_{my}=\sigma_{fu}/s_c$=180 MPa/100 000). This value is over 3 orders of magnitude higher than the yield stress of conventional mucus ($\tau_{my}$ 2 Pa; Majima et al., 1983). The conclusion of this analysis is that even if hagfish slime mucus were as concentrated and elastic as gastric mucus (which it most certainly is not), it would still not be able to provide effective shear linkage between threads. Perhaps it is not surprising that mucins do not provide shear linkage given that the slime threads are long enough to span an entire mass of slime and therefore do not require any linkage for mechanical continuity.

CR staining demonstrates that during modest deformations of the slime, as might occur while a hagfish or predator thrashes within it, most of the threads are loaded to stresses higher than the yield stress (Fig. 12), effecting an α→β transition in the thread intermediate filament (IF) proteins. How do the threads get stretched past their yield point if the mucins do not link them together? One possibility is that the slime threads are so long that pulling one through water generates drag forces that accumulate as significant tension in the thread. The drag force $D$ on a long cylinder pulled through seawater can be calculated as:

$$D = 2\pi\mu Ul / [\ln(l/a) - 0.807] \quad (2)$$

(Vogel, 1994), where μ is the dynamic viscosity ($1.5\times10^{-3}$ Pa s), $U$ is the velocity (assumed to be 0.1 m $s^{-1}$), $l$ is the length (estimated as 0.15 m) and $a$ is the diameter of the thread (estimated as 2 μm). For a thread of these dimensions pulled through seawater at this speed, the drag force is about 13 μN. For comparison, the yield force of a 2 μm diameter thread is about 10 μN (Fudge et al., 2003), so it is theoretically possible that part of a slime thread could be transformed via this mechanism. Because the tension at any point in the thread will be cumulative as a result of drag forces downstream, only the upstream end will experience this magnitude of drag. If one considers that a significant portion of the thread has a diameter of only 1 μm and that the yield force for these segments is only about 2.5 μN, then this could account for even more of each thread being transformed. In addition, slime threads tend to catch on neighbouring threads so that a single thread pulled at

0.1 m $s^{-1}$ likely experiences much higher drag than that arising from skin friction of the thread alone. Furthermore, the forces required to accelerate the volume of mucins and water entrained between threads could also contribute to the draw transformation of the threads when the slime is deformed.

### *A model of hagfish slime structure and mechanics*

What is the function of the mucins if not to link the threads? Koch et al. (1991b) demonstrated that isolated slime thread skeins stirred into seawater in the absence of mucin vesicles fail to produce anything resembling naturally formed slime. Instead, the slime threads collapse into a small fibrous clot that binds relatively little water. In addition, whole slime exudate containing both skeins and mucin vesicles fails to produce viable slime in the presence of the disulfide breaker DTT (Koch et al., 1991b). Here we demonstrated that DTT also delays slime development and affects the viscoelastic properties of the slime. These results suggest that the presence of cross-linked mucins is important to the function of the slime. And yet, the extremely low concentration of mucins and their low viscosity indicate that they can't possibly exist as a cross-linked network that permeates the entire volume of slime. The only way to reconcile these facts is if the distribution of mucins in the slime is heterogeneous, with discrete networks of mucins at relatively high concentrations dispersed in seawater with relatively few mucins. If this is indeed the case, then to understand how the slime works, it is important to know the size of these mucin networks.

A natural answer to the question of network size comes from looking at the vesicles in which the mucins are packaged. Ejected slime exudate consists of slime threads skeins and mucin vesicles from ruptured gland mucus cells. Mucin vesicles are ellipsoids with a major axis length of about 7 μm (Luchtel et al., 1991) and it is not difficult to imagine that the mucins within a vesicle are cross-linked into a discrete networks that would be dispersed by DTT. If mucin vesicles swell but remain as intact networks of mucins, how are they distributed in the slime? Mucin vesicles are known to bind readily to slime threads (Koch et al., 1991b), and it is possible that the ratio and dimensions of slime threads and vesicles has evolved to optimize the interaction between the two. The ratio of slime thread surface area to the number of vesicles supports this idea. We estimate the ratio of mucin vesicles to slime thread skeins to be about 5700:1. Given that the threads are about 150 mm long and are tapered with a middle diameter of 3.0 μm and end diameters of 1.0 μm and 1.5 μm, the surface area of a single thread is about 700 000 $\mu m^2$. Each mucin vesicle should therefore have about 120 $\mu m^2$ of slime thread surface area on average on which to bind. This means that all of the condensed mucin vesicles (each with a projected surface area of about 21 $\mu m^2$ assuming major and minor axes of 7, 3 and 3 μm) could bind to a thread with room to spare. If the vesicles swell to a degree that is typical of mucus granules (i.e. 200% increase in radius; Verdugo, 1991), they will cover more of the thread surface. This process is depicted in Fig. 13.

The decent match between the number and size of swollen mucin vesicles and slime thread surface area is consistent with the mucin-thread interaction proposed in Fig. 13. However, this model makes a startling prediction about the structure of the whole slime: the vast majority of the volume of hagfish slime is bulk seawater. If we make the crude assumption that the threads are 150 mm long, equally spaced and parallel to one another, a 1000 ml cylinder of slime will have a diameter of about 92 mm. From the thread length and diameter data presented here, it is possible to calculate the volume of a typical slime thread as about $5.5\times10^5$ $\mu m^3$. If the thread has a hydrated density of 1.38 g $cm^{-3}$, then its mass is about 0.75 μg. At a concentration of 20 mg $l^{-1}$, this translates into about 27 000 slime threads in a 1 l mass of slime. Assuming that the mucin vesicles swell as depicted in Fig. 13, then this means that each mucin-coated thread will have a radius of about 10 μm, and the total volume taken up by all of the mucin-coated threads will be about 1.1 ml. This represents 0.11% of the volume of a 1000 ml slime mass, and suggests that about 99.9% of the slime is bulk seawater. It is interesting that the threads and mucins together occupy only about 1/1000th the volume of the slime according to this model, because this is the factor by which the concentration of other mucus secretions exceeds that of hagfish slime (Table 1). Moreover, this suggests that the mucins coating the slime threads exist at hydrated concentrations comparable to those in other mucus secretions.

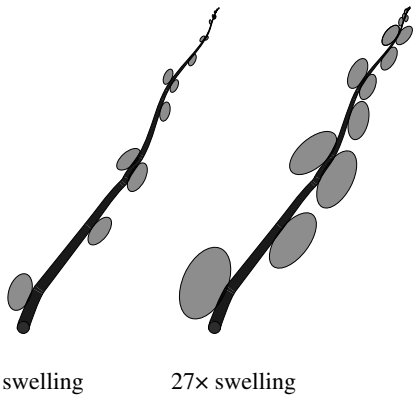


Fig. 13. The ratio of mucin vesicles to thread surface area suggests that the threads are capable of binding every mucin vesicle. Here we depict an elongated slime thread with bound mucin vesicles before and after swelling (by a factor of three in all dimensions).

If most of the slime is bulk seawater, how does the slime manage to be as coherent as it is? The simplest answer is that the water is confined to channels between the slime threads, and the flow through these channels is slow enough to make the slime act as a coherent structure over short timescales. Indeed, one of the things one notices when the slime is lifted out of water is that water streams out of it, reducing its volume substantially. Ferry (1941) noted this phenomenon, and estimated that the slime collapses to 1/50th of its original volume when it is handled. The results of our water egress experiment (Fig. 11) support the idea that the slime contains

channels of bulk seawater. The take-home message from these trials is that in its most expanded state, the slime does not immobilize water like a gel, but only slows it down. A few calculations demonstrate that our egress data are consistent with the structure of slime proposed above.

If we assume, as we did above, that a slime mass consists of equally spaced and parallel mucin-coated slime threads that are 150 mm long, then we can estimate the average distance between slime threads to be about 500 μm. The rate of water flow through a pipe $Q$ can be calculated using the Hagen–Poiseuille equation (Vogel, 1994):

$$Q = \pi \Delta P r^4 / 8\mu l \,, \quad (3)$$

where $\Delta P$ is the pressure gradient determined from the height of the water column in the 'pipe' formed by the vertically oriented threads, $r$ is the pipe radius, $\mu$ is the dynamic viscosity, and $l$ is the pipe length. If we estimate the channel radius as half the distance between threads (i.e. 250 μm), this yields a flow rate for seawater of 0.011 ml $s^{-1}$, and a total flow rate for all 24 000 channels of about 300 ml $s^{-1}$. This is remarkably similar to the average initial flow rate of 340±80 ml $s^{-1}$ (mean ± S.E.M.) measured during the egress trials. Of course, as the pressure head drops and the slime contracts, the flow rate will plummet, and this is what we observed.

According to this new model of hagfish slime structure, the slime is not as much a coherent material as a very fine three-dimensional sieve that can trap water over short timescales, but over longer timescales simply slows it down. Thinking about the slime in this way is remarkably consistent with the hypothesis that the slime functions as a defence against gill-breathing predators (Fernholm, 1981; Martini, 1998). If the slime indeed evolved as something that could bind to gills and disrupt respiratory flow, one would not necessarily expect it to bind water irreversibly; slowing it down would be sufficient. Furthermore, the tendency of the slime to contract over time will decrease the distance between threads, and dramatically reduce the flow rate, which varies according to the fourth power of pipe radius.

### *Conclusions*

In this paper we demonstrate that hagfish slime is remarkably dilute and consists mostly of bulk seawater entrained between mucin-coated threads. Although the threads that permeate the slime exhibit a bidirectional taper, we have shown that the mucin component does not provide shear linkage between threads as in a fibre-reinforced composite. The slime owes its coherence mainly to the thousands of slime threads that are long enough to span the entire structure. We propose that the main functions of the mucins are to bind to the slime threads and aid in its rapid deployment, although the exact mechanism of the latter is unknown. The slime is unlike a gel in that it does not bind water over long timescales, and instead appears to function as a fine three-dimensional sieve that may have evolved as a deterrent to gill-breathing predators and competitors.

### List of abbreviations

| | |
|---|---|
| $a$ | slime thread diameter |
| CR | Congo Red |
| $D$ | drag force |
| $dH_2O$ | distilled water |
| DTT | dithiothreitol |
| GTC | gland thread cell |
| IF | intermediate filament |
| $l$ | length |
| $r$ | pipe radius |
| $s_c$ | fibril length in a fibre-reinforced composite |
| SEM | scanning electron microscopy |
| $U$ | velocity |
| $\Delta P$ | pressure head |
| $\epsilon$ | extensibility |
| $\mu$ | dynamic viscosity |
| $\sigma_{fu}$ | ultimate fibril stress in a fibre-reinforced composite |
| $\tau_{my}$ | matrix yield stress in shear |

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