

Research



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Mechanisms of gill-clogging by hagfish slime

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Hagfishes defend themselves from gill-breathing predators by producing large volumes of fibrous slime when attacked. The slime's effectiveness comes from its ability to clog predators' gills, but the mechanisms by which hagfish slime clogs are uncertain, especially given its remarkably dilute concentration of solids. We quantified the clogging performance of hagfish slime over a range of concentrations, measured the contributions of its mucous and thread components, and measured the effect of turbulent mixing on clogging. To assess the porous structure of hagfish slime, we used a custom device to measure its Darcy permeability. We show that hagfish slime clogs at extremely dilute concentrations like those found in native hagfish slime and displays clogging performance that is superior to three thickening agents. We report an extremely low Darcy permeability for hagfish slime, and an effective pore size of 10–300 nm. We also show that the mucous and thread components play distinct yet crucial roles, with mucus being responsible for effective clogging and low permeability and the threads imparting mechanical strength and retaining clogging function over time. Our results provide new insights into the mechanisms by which hagfish slime clogs gills and may inspire the development of ultra-soft materials with novel properties.

1. Introduction

Hagfishes are eel-like marine animals that produce large volumes of dilute, fibrous slime when attacked by predators [1–4]. The slime has been shown to thwart fish predators by lodging in their mouths and clogging their gills, which causes them to abort their attack [2,4]. Hagfish slime consists primarily of water, but also contains strong protein threads and mucus [1,5,6]. Although the three-dimensional structure of the slime has not been resolved, it has been hypothesized to consist of two elaborate and interpenetrating networks of mucus and threads [6].

The physical properties of hagfish slime offer some insights into the function of this unique biomaterial. Hagfish slime is remarkably dilute, with mucus concentrations of approximately 15 mg l^{-1} , which is three orders of magnitude less concentrated than human salivary mucus [7]. Slime threads are sparse, with concentrations of about 20 mg l^{-1} . The dilute nature of hagfish slime (approx. 35 mg l^{-1} total for mucus plus thread solids) corresponds with an ability to trap up to 26 000 times its own weight in seawater [8]. By contrast, the best superabsorbent hydrogels only absorb up to 3000 times their weight [9]. From a mechanical point of view, the slime's properties are highly unusual. When handled, the slime readily deforms and adopts the shape of any container, and yet the silk-like slime threads allow it to support its own weight out of water. The storage modulus for hagfish slime in shear is 0.02 Pa, making it one of the softest biomaterials known [10]. Individual slime threads

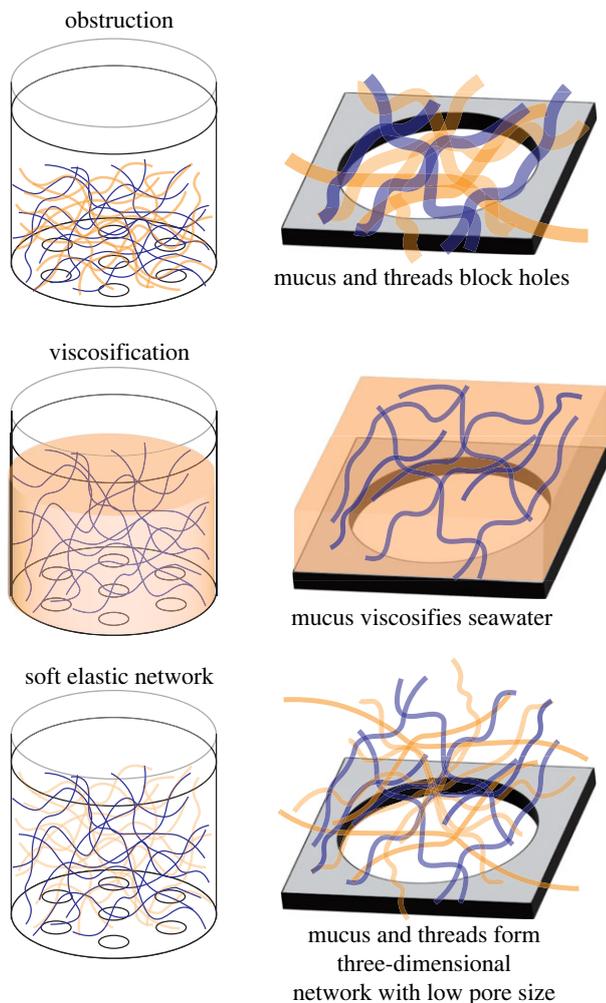


Figure 1. Graphical schematic of three hypotheses for how hagfish slime might clog. The obstruction hypothesis posits that threads (blue) and mucus (orange) in the slime slow the flow by blocking the holes through which water typically flows, similar to how hair can clog a shower drain. The viscosification hypothesis claims that deployment of mucous vesicles viscosifies seawater in the slime, which results in slower flow through holes. The soft elastic network hypothesis claims that resistance to flow through the three-dimensional network of mucus and threads within the slime itself is mainly responsible for the slime's ability to clog.

possess robust tensile material properties, with a rubberlike Young's modulus of about 6 MPa, and a tensile strength of 180 MPa in water [5].

Video evidence reveals that release of slime exudate by hagfishes in the wild after they are attacked is remarkably effective at preventing further attacks [4]. Laboratory experiments with isolated fish heads demonstrate that the slime is effective at clogging fish gills [2], which may impair gas exchange for the predator if the slime remains on the gills for an extended period. The mechanism of clogging, however, is not known. We explored three possible mechanisms, which are not mutually exclusive, by which hagfish slime might act as a clogging agent (figure 1). One possibility is that the solid components of the slime, i.e. the threads, and perhaps the mucus, obstruct the holes of the structure they are clogging. Another possibility is that the slime gland exudate viscosifies the seawater that it mixes with and thus makes it more difficult to flow through a porous structure like gills. A third possibility is that the constituent slime threads and mucus form a soft elastic network with a very low internal pore size

size, and it is the resistance to the flow of seawater by this continuous porous structure that is responsible for clogging.

Here, we conducted a variety of experiments to evaluate these hypotheses, including quantifying the clogging performance of hagfish slime as a function of concentration, comparing the slime's performance with other materials with known thickening properties, measuring the effects of hole size and the relative contributions to clogging of the mucous and thread components. Our results are consistent with the idea that the slime clogs because it is a continuous soft elastic network with a very small (i.e. less than 10–300 nm) average internal pore size.

2. Methods

2.1. Animal care and slime gland exudate collection

Pacific hagfish (*Eptatretus stoutii*) were collected from Barkley Sound, British Columbia for experiments at the University of Guelph, and from Monterey Bay for experiments at Chapman University. Hagfish were anaesthetized using clove oil (150–200 mg l⁻¹) in 34‰ artificial seawater (ASW) (Instant Ocean, Blacksburg, VA, USA), and slime exudate was collected from several glands using mild electrical stimulation (60 Hz, 1 ms duration, 18 V) using the methods described in McCord *et al.* [11] and Jain *et al.* [12]. Exudate, which contains undeployed mucous vesicles and thread skeins, was collected using a Teflon-coated spatula and stored in stabilization buffer (0.5–0.9 M sodium citrate, 0.1 M PIPES buffer, pH 6.7) [13] at 4°C. Stabilization buffers containing high concentrations of polyvalent anions such as citrate are known to keep skeins and mucous vesicles in a condensed state, with subsequent dilution with seawater causing skein unravelling and vesicle swelling. After exudate collection, hagfish were allowed to recover in a bucket with fresh ASW and were returned to their holding tank after they resumed normal posture and were able to react to physical stimulation. Exudate was always used as soon as possible, and never more than 48 h after collection. Mucus-only and threads-only samples were prepared via filtration of stabilized slime exudate using Fisherbrand Sterile Cell Strainers with a 40 µm mesh size, which retains skeins and allows the smaller mucous vesicles to pass through.

2.2. Measurement of mucus concentration

We found that the concentration of exudate had a strong effect on the clogging properties of the resultant slime, and we, therefore, used a modification of the spectrophotometric protocol developed by Salo *et al.* [14] to quantify and set the concentration of mucous vesicles in each suspension prior to deployment in seawater. A standard curve was created by measuring the absorbance at 350 nm for a concentrated suspension of vesicles and six dilutions of that suspension. The concentration of mucous vesicles in each suspension was measured using a TC20 cell counter (Bio-Rad, Hercules, CA, USA) to capture an image of vesicles in 0.4 µl of suspension, and the number of vesicles was counted using ImageJ software. Mucus dry weight was measured for seven different vesicle suspensions by subjecting them to dialysis (12–14 kDa molecular weight cut-off) against 21 of distilled water over 72 h at 4°C, with five changes of the water over that time. After dialysis, the material inside the dialysis tubing was dried and weighed. These data allowed us to convert easily obtained spectrophotometric data to a mucus dry weight, so that the mucus concentration for a given sample could be accurately measured and adjusted as needed for standardization.

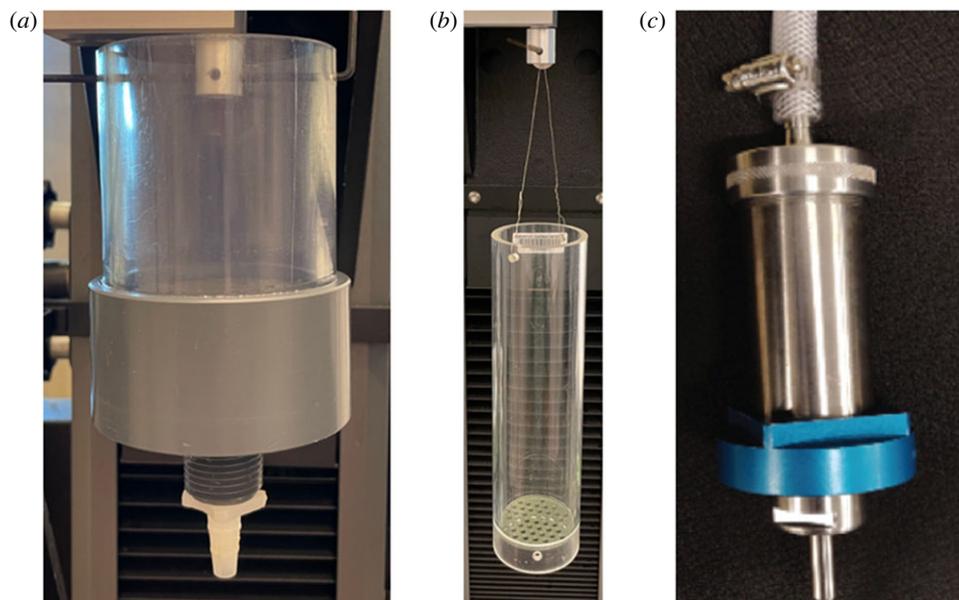


Figure 2. Custom experimental devices used to measure clogging performance (*a,b*) and Darcy permeability (*c*). For the clogging performance assays, samples were poured into a cylinder hanging from a force transducer with a porous mesh at the bottom. Drain rate was measured by monitoring the weight of the cylinder over time as water drained out of it. The cylinder in (*a*) was used for the comparisons between hagfish slime preparations and the three other materials. The simpler device in (*b*) was used for measuring the effects of flushing, hole size and mixing. For the Darcy permeability trials (*c*), seawater was pushed through a slime sample held between two highly porous plates using an applied pressure, with flow rate measured by monitoring the weight of the water that accumulated in the container below.

2.3. Sample preparation

We found that the properties of the slime depended strongly on the method of sample preparation and, therefore, samples were standardized in the following way. For whole slime, mucus-only and threads-only samples of varying concentrations, 50 ml of chilled ASW was added to a 250 ml beaker and then a concentrated suspension of whole slime exudate, mucous vesicles or thread skeins in stabilization buffer was pipetted into the beaker, followed by another 50 ml of chilled ASW. Slime deployment was triggered by pouring the resulting mixture between beakers a total of four times.

To evaluate the clogging ability of hagfish slime and hagfish mucus, we compared them with other materials such as xanthan gum, hydrated psyllium husk and the high molecular weight polymer, poly(ethylene oxide) (PEO). Xanthan gum samples were prepared by hydrating pure powdered xanthan gum (Bob's Red Mill Natural Foods, Inc., Milwaukie, OR, USA) in deionized (DI) water. Solutions with a total volume of 100 ml were prepared at varying concentrations by adding incremental amounts of xanthan powder to the water while mixing with a magnetic stir bar at low speed. Following each incremental addition, mixing speed was significantly increased to facilitate hydration and prevent clumping. Each xanthan solution was mixed until it was homogeneous. The most concentrated solution ($10\,000\text{ mg l}^{-1}$) took approximately 12 min to prepare. Psyllium husk (Nutricost, Vineyard, UT, USA) and PEO solutions (molecular weight 900 kDa; Thermo Scientific, Waltham, MA, USA) were prepared in the same way, with the most concentrated psyllium solution ($25\,000\text{ mg l}^{-1}$) taking approximately 6 min to prepare and the most concentrated PEO solution ($30\,000\text{ mg l}^{-1}$) taking approximately 7 min to prepare.

2.4. Clogging assay and calculation of drain rate

Clogging performance was measured with two custom devices. The first device (figure 2*a*) was used for trials using whole hagfish slime, mucus-only, threads-only and the three other materials (xanthan, psyllium, PEO). This first device consisted of a 51.9 mm inner diameter polycarbonate cylinder with a

stainless-steel porous disc (0.60 mm thick, 1.35 mm circular holes) at its bottom. This device tapered below the porous disc to an outflow pipe of diameter 13.8 mm, which connected to another tapered adaptor with an outflow diameter of 4.6 mm. The second device (figure 2*b*) had a simpler design that omitted the complicated tapers. This device consisted of a 37.2 mm inner diameter polycarbonate cylinder with a porous plastic 3D-printed disc (thickness 1.2 mm, pore diameter 1.3 mm) at the bottom and was used for trials investigating the effects of flushing, hole size and mixing. The cylinders were hung from a 100 N load cell on an Instron Model 5943 universal testing machine, which provided load and time data at a sampling rate of 10 Hz. At the start of a trial, data collection was initiated, and a prepared sample was poured into the cylinder, with load values decreasing over time as the sample drained out of the bottom. Drain rate, D (weight per time), was measured from load versus time curves as the average between the time interval of 1–10 s after the sample was added to the cylinder. The first second of data was omitted from this analysis, because the load values were dominated by a spike caused by the inertial impact of the sample against the bottom of the cylinder. Good clogging performance resulted in slow drainage over time, or a low drain rate value, while poor clogging performance resulted in fast draining over time, or a high drain rate value. To test the hypothesis that slime threads are important for resisting the effects of gill flushing by a predator, we also conducted clogging trials with whole slime and mucus-only samples and then attempted to flush the slime away with additional 100 ml volumes of seawater. A drain rate was calculated for each flush. For flushing trials, whole slime samples had mucus concentrations ranging from 8.5 to 25 mg l^{-1} ($N=5$) and mucus-only samples ranged between 14 and 90 mg l^{-1} ($N=6$).

Average drain rate D for varying concentrations, c , of whole slime, mucus-only slime and the three other materials resembled sigmoidal response curves. That is, above a critical concentration the drain rate dramatically decreased. For these experiments we fitted the data with an empirical log-logistic model,

$$D = D_{\infty} + \frac{D_0 - D_{\infty}}{1 + (c/\alpha)^{\beta}}, \quad (2.1)$$

where $(D_0, D_\infty, \alpha, \beta)$ are model parameters interpreted as low-concentration drain rate (D_0), high-concentration drain rate (D_∞), critical concentration for significant decrease (α) and power-law exponent (β). We performed model fitting with custom-written R scripts.

2.5. Hole size effects

The three mechanistic hypotheses about how hagfish slime clogs make divergent predictions about how the slime's clogging performance should change when the hole size of the mesh is changed. If the slime clogs primarily via blocking the holes, then one would expect that the hole size of the mesh should have strong effects on clogging performance, as larger holes would be more difficult to block. The viscosifying hypothesis also predicts that clogging performance should be sensitive to hole size, with smaller holes causing slower drain rates, even for a constant total hole area. If, however, the slime reduces flow primarily because of its own inherent hydrodynamic resistance, then changing the hole size of the supporting mesh should have little effect on clogging performance, at least at scales where the mesh hole size is much greater than the internal pore size of the slime. To test these hypotheses, we quantified the effect of hole size on drain rate. To do this, we created six 3D-printed discs that fit into the bottom of our drain rate cylinder. Each disc had the same porosity in terms of the total area of the holes, but differed in the number and diameter of the holes. Discs each had a diameter of 39.60 mm, hole diameters for the six discs were: 9.00, 6.37, 4.20, 3.00, 2.11 and 1.35 mm, and the thickness of all discs was 1.20 mm. Total hole area for each disc was 318 mm². Slime from two hagfishes was used for these experiments and 10 trials were performed for each of the six different hole sizes. To compare these data with data for a viscous fluid, identical trials were run with glycerol ($N=5$ for each hole size) (Fisher Scientific, Mississauga, ON, Canada, 99.5).

2.6. Mixing effects on mucus drain rate

To better understand the role mucus plays in slime development and collapse, which have been explored in previous laboratory studies, we conducted mixing trials using mucus-only samples [1,2,15]. These experiments were based specifically on protocols described in Lim *et al.* [2], which investigated the effect of mixing on whole slime. Lim *et al.* [2] used 'removable mass', or the mass of slime that could be lifted out of a beaker with a metal hook, as a way of assessing slime quality, but removable mass measurements are not possible in the absence of slime threads because the mucus alone cannot support its own weight in air. We, therefore, assessed mucus quality by measuring drain rate. To quantify the effects of mixing, 5 ml of mucus stock suspension was added to 45 ml of 4°C ASW in a 100 ml beaker, which was then agitated on a horizontal shaker plate at 200 r.p.m. for a set time interval (0, 10, 30, 60, 120 or 180 s). To isolate the effects of mixing from time in contact with ASW, we conducted trials in which the mucus sample was simply added into the beaker with ASW with no additional mixing for the same time intervals. Drain rate for each sample was measured as described above. Mucus concentrations were measured prior to trials using the assay described above and the suspensions were diluted accordingly, so that all samples had an average mucus concentration of 40 mg l⁻¹.

2.7. Darcy permeability measurements

Hydrodynamic (Darcy) permeability is an intrinsic property of porous media that quantifies the ease of flow through the material due to an applied pressure difference. It can be used to infer the effective pore size and connectivity within a material.

The apparent Darcy permeability κ is defined from

$$Q = \kappa \frac{A}{\mu L} \Delta P, \quad (2.2)$$

where Q is the volume flow rate passing through the sample, ΔP is the pressure difference driving the flow, μ is the viscosity of fluid (water) flowing through the porous structure, L is the thickness of the slime sample and A is the cross-sectional area.

Permeability κ has dimensions of length-squared and conceptually arises from the internal geometry of the porous structure. Specifically, κ can be quantitatively related to the size of geometric constrictions inhibiting viscous flow. With knowledge of pressure drop and flow rate, we calculate an apparent Darcy permeability of the slime sample as

$$\kappa = \frac{\mu L Q}{A \Delta P}. \quad (2.3)$$

To quantitatively measure permeability, we pushed water through a volume of whole slime under controlled pressure drops (not originating from gravitational effects) using a custom-built set-up shown in figure 1c. A pressure holder (XX4004740, EMP Millipore co., MA, USA) fitted with a pressure filter was used to hold a fixed volume of sample. The liquid water column was then allowed to flow through the whole slime sample under an externally applied pressure from a compressed nitrogen gas reservoir. Since the gravitational pressure head was negligible compared with the applied pressure, we assumed that the pressure difference, ΔP , driving the flow was constant. For each trial, pressure was applied at four different levels: 6.25, 12.50, 18.75 and 25.00 psi (43, 86, 129, 172 kPa in standard units), with the flow rate at each pressure measured by collecting and weighing the outflow from the filter as a function of time using a 100 N load cell on an Instron Model 5943 universal testing machine.

3. Results

3.1. Hagfish slime clogs at remarkably low concentrations

Hagfish slime reduced flow through a mesh (i.e. clogged) at very low concentrations of a few tens of milligrams per litre (figure 3). Drain rate values plateaued at slime concentrations that are remarkably similar to the concentrations measured in native hagfish slime (35 mg of mucus and threads per litre; [1]). Clogging trials using whole slime as well as mucus-only samples (in which the slime threads were removed) revealed that the mucus clogs just as well as whole slime at most concentrations (figure 3). Furthermore, samples containing only slime threads produced high drain rate values ($0.201 \pm 0.027 \text{ N s}^{-1}$, $N=7$) and displayed virtually no clogging ability, indicating that the mucus is necessary for clogging. Compared with the xanthan gum, psyllium husk and PEO solutions, hagfish slime clogged the mesh (i.e. had similarly low drain rates) at concentrations that were two to three orders of magnitude lower (figure 3).

3.2. Slime without threads is easily washed away

The dominant importance of mucus to clogging performance led us to wonder whether the threads have a function other than clogging. We hypothesized that the threads are involved in keeping the slime from being flushed away from a predator's gills. In our flushing trials, the clogging performance of mucus-only samples was severely diminished after only a

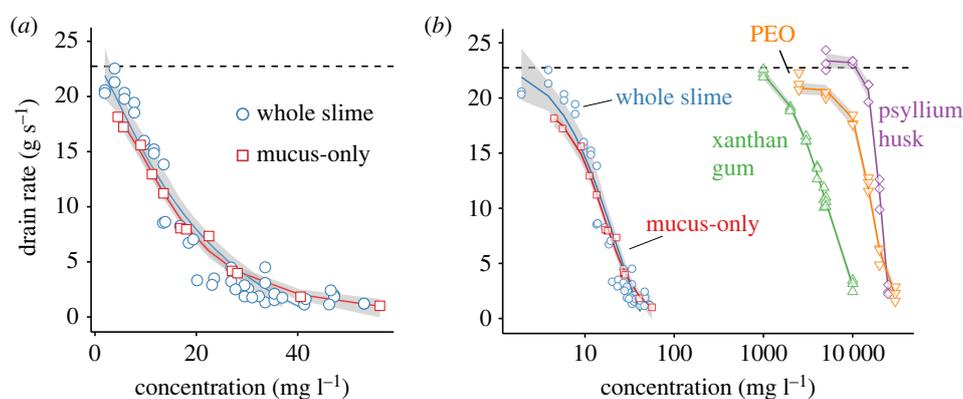


Figure 3. Clogging performance of hagfish slime components and other materials. (a) Drain rate of whole slime and mucus-only samples through a porous disc with 1.30 mm holes. Each point represents one clogging trial at a given concentration of slime or mucus. Data are plotted as a function of the total concentration of mucus and slime threads. The close correspondence of the two curves and the fact that unravelled threads alone do not clog (data not shown) suggests that the mucus is primarily responsible for clogging. The dashed line represents the average drain rate value for seawater in this apparatus. (b) Drain rate as a function of concentration for: whole slime; mucus-only; two other hydrogels, xanthan gum and psyllium husk; and the high molecular weight polymer, PEO. Concentration is plotted on a logarithmic scale due to the large differences in clogging ability between hagfish slime and the other materials. Data are fitted with the log-logistic model of equation (2.1). Grey shaded areas represent standard error of the mean (s.e.m.). Data for whole slime and mucus-only samples are the same as in (a).

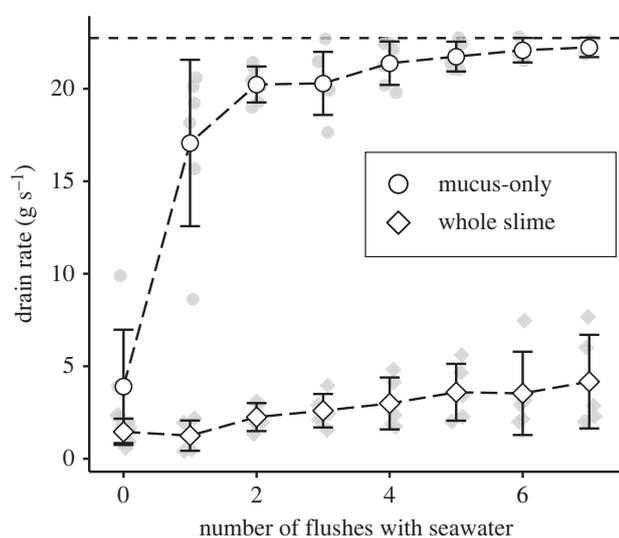


Figure 4. Drain rate as a function of the number of seawater flushes for whole slime and mucus-only samples. Coloured dots represent mean drain rates and error bars represent standard deviation. Data from all individual trials are plotted in grey in the background. For most mucus-only trials, a single seawater flush was nearly sufficient to return the drain rate to that of seawater, whereas meshes clogged with whole slime maintained low drain rates even after several flushes with seawater. Whole slime samples had mucus concentrations ranging from 8.5 to 25 mg l⁻¹ ($N = 5$), and mucus-only samples ranged between 14 and 90 mg l⁻¹ ($N = 6$).

single flush of seawater, whereas whole slime was remarkably good at maintaining its clogging performance over several rounds of flushing and after seven total flushes (figure 4).

3.3. Drain rate of hagfish slime is insensitive to hole size

As expected, the viscous fluid glycerol showed strong effects of hole size, with the lowest drain rates occurring for the smallest holes. By contrast, the drain rate for hagfish slime was relatively unaffected by hole size even up to the highest hole diameter of 9 mm (figure 5).

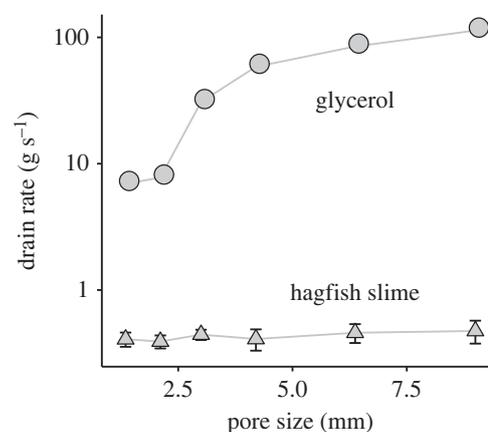


Figure 5. Drain rate plotted on a semi-log-scale as a function of hole size for glycerol (averages from $N = 5$) and hagfish slime with a mucus concentration of 30 mg l⁻¹. Error bars represent standard deviation. The insensitivity of drain rate to hole size in whole slime samples undermines the hole size and viscosifying hypotheses and supports the soft, porous network hypothesis.

3.4. Slime development is similar for mucus-only and whole hagfish slime

We measured the drain rate of mucus-only samples that were subjected to varying amounts of mixing and found a similar pattern of set-up and collapse to that seen in whole slime. Drain rate decreased and then collapsed until about 80 s of mixing, and then increased with subsequent mixing (figure 6). Similar trials with mucus in the absence of mixing produced a generally linear trend of decreasing drain rates (figure 6). A lack of mixing resulted in poor clogging performance when contact time between stabilized slime and seawater was short, but at contact times of 180 s, clogging performance in the absence of mixing approached the best performance observed in mixed samples.

3.5. Hagfish slime has an extremely low hydrodynamic permeability

We used a custom pressure-driven system to measure the Darcy permeability of hagfish slime over a wide range of

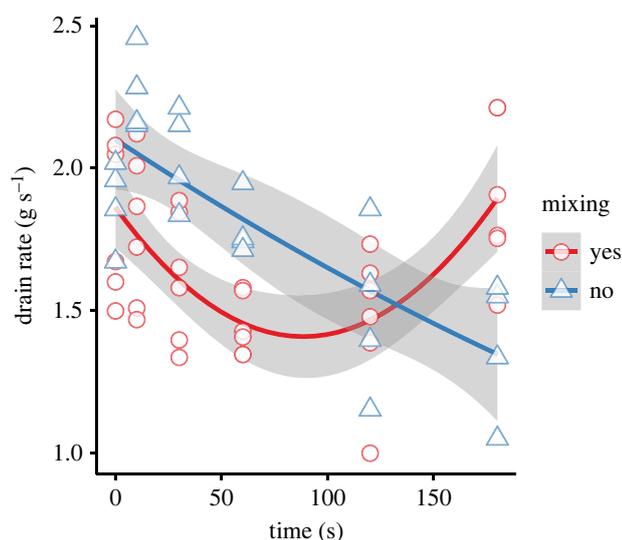


Figure 6. Drain rate of mucus-only samples in the presence and absence of mixing. Values represent results from individual trials. Trend lines represent moving average; grey shaded areas represent standard error of the mean (s.e.m.). All samples had an average mucus concentration of 40 mg l^{-1} .

concentrations. Figure 7a shows the raw data from one such experiment. The calculated volume flow rate was roughly constant for the duration of experiments when the applied pressure was held constant. As described in the Methods, with knowledge of pressure drop and flow rate, we calculate an apparent Darcy permeability of the slime sample. Figure 7b shows the apparent Darcy permeability of slime samples as the concentration c of exudate is varied. A power-law trend $\kappa \sim c^{-1}$ is observed across two orders of magnitude in concentration. A best-fit power-law exponent of -1.18 ± 0.04 was obtained for the range of concentrations explored. Extrapolating such a power-law trend to physiological concentrations, which are slightly lower, $c \sim O(10^{-2}) \text{ wt}\%$, we estimate the permeability of hagfish slime at its physiological concentrations to be $\kappa \sim O(10^{-13}) \text{ m}^2$. The apparent permeability of the set-up without slime (e.g. due to the supporting mesh) was orders of magnitude higher than the values shown in figure 7, hence our measurements should be representative of the intrinsic permeability of the slime.

4. Discussion

4.1. Mucus is key to the superlative clogging of hagfish slime

When compared with thickening agents like xanthan gum, psyllium husk and the polymer PEO, hagfish slime was able to effectively clog at concentrations two to three orders of magnitude lower (figure 3). Our results suggest that both whole hagfish slime and mucus-only hagfish slime can effectively clog at extremely dilute concentrations, with performance reaching a plateau at concentrations approaching those in naturally produced hagfish slime (figure 3). The conservation of clogging ability in the absence of slime threads suggests that the mucus plays a primary role in clogging. Any contribution from the slime threads depends on the mucus already being present, as unravelled slime threads alone are unable to effectively clog, even at high concentrations. These observations, plus the fact that slime threads are probably

more energetically demanding to produce than mucus [16], led us to wonder what the selective pressures were that led to the presence of threads in hagfish slime. Flushing trials revealed that slime without threads is easily washed away, suggesting that the threads probably function to keep the slime together and attached to the gills of predators to maintain clogging.

4.2. Mechanism of clogging

Superior clogging by hagfish slime raises questions about how exactly clogging is achieved. We imagined three mechanistic hypotheses, which are not mutually exclusive: obstruction of holes, viscosifying seawater and creation of a soft elastic network with small average internal pore size. The obstruction of holes hypothesis predicts that clogging performance of the slime should diminish as the size of the holes in the mesh is increased. We found that the drain rate was in fact mostly insensitive to the hole size, which undermines the hole obstruction hypothesis. The viscosifying of seawater hypothesis also predicts that the flow rate should be sensitive to the hole size of the mesh, as a viscous fluid will encounter more resistance to flow from numerous small holes compared with fewer large ones. Our results for the viscous fluid glycerol follow this pattern, but whole hagfish slime do not. This is consistent with Fudge *et al.* [1], who showed that preparations of hagfish mucus in seawater at their native concentration have a viscosity that is indistinguishable from seawater, and even concentrations 50 times higher have only modest positive effects on viscosity. All of these results undermine the viscosifying hypothesis. Unlike the other two hypotheses, the soft elastic network hypothesis states that the primary resistance to flow created by hagfish slime originates within the slime itself and not via its interaction with the external boundary mesh. This hypothesis predicts that flow rate should not change as the hole size is increased, as long as the slime can be supported on the mesh well enough not to slip through. Our results are consistent with this prediction and demonstrate that the slime can span holes as large as 9 mm in diameter and probably larger, which should be enough to span the distance between gill arches, even in large piscivorous fishes. This is perhaps not surprising, given that the threads give the slime the strength to span holes, and their resting length is estimated to be about 150 mm [1]. Taken together, these results allow us to reject the hole obstruction and viscosifying hypotheses, and provide support for the soft elastic network hypothesis.

4.3. Permeability, pore size, structure and deployment

If hagfish slime clogs because it is a soft elastic network, what is the nature of that network? Our measurements of the Darcy permeability of hagfish slime provide some insight. The permeability κ of a porous structure generally scales as $\kappa \sim \xi^2$, where ξ is the average internal pore size of the slime network. Thus, based on our experimental measurements of $\kappa \sim 10^{-16}$ – 10^{-13} m^2 , a mean pore size of approximately 0.01–0.3 μm is expected for hagfish slime (the larger size at physiological concentration) [17]. This estimate is much smaller than the pore size in the network of slime threads within hagfish slime, which has been estimated to range from approximately 10 to 500 μm [18]. Further, we can calculate the expected Darcy permeability of just the slime threads within hagfish slime by modelling them as a network of randomly oriented cylindrical fibres,

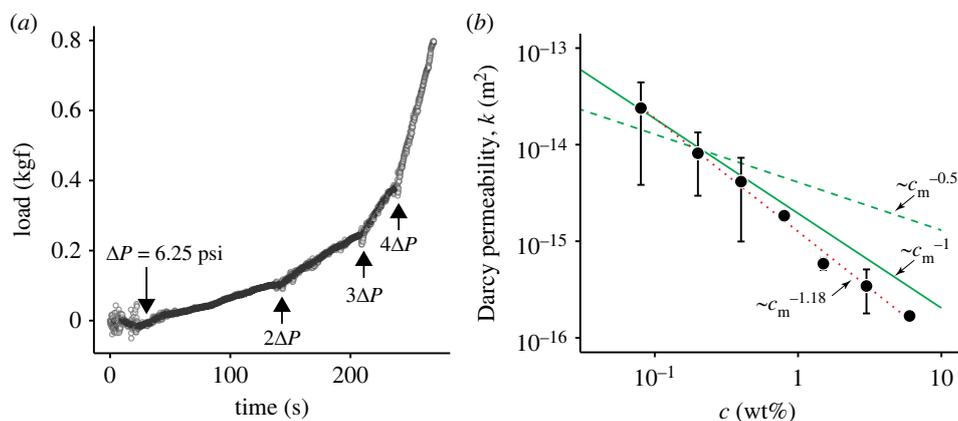


Figure 7. Permeability of hagfish slime. (a) The mass of the solvent that flowed through the slime under varying applied pressure is plotted as a function of time for different applied pressures. (b) The Darcy permeability, κ , of hagfish slime varied with the concentration of the slime. The dotted line shows the best-fit power-law dependence of permeability on mucus concentration, $\kappa \sim c^{-1.18 \pm 0.04}$. For comparison, we plot a scaling estimate for semi-dilute polyelectrolyte unentangled solutions in a no salt/weak salt regime ($\kappa \sim c^{-0.5}$). A scaling law derived for a concentration-independent scale and structure of mucus (details in Discussion), closely follows the experiments $\kappa \sim c^{-1}$.

with the permeability given by $\kappa = a^2 [(1/2)(\pi/\varphi_t)^{1/2} - 1]^2 [0.71e^{-0.52\varphi_t}]$, where φ_t is the volume fraction of fibres and a is the fibre diameter [19]. Since hagfish slime is ultra-dilute, i.e. φ_t is close to zero, we can approximate the above relationship as $\kappa \sim a^2/\varphi_t$. For the hagfish thread network, $a \approx 2 \mu\text{m}$, and $\varphi_t \approx 10^{-4}$ – 10^{-2} for the range of concentrations explored in our permeability experiments. This gives a range of permeability $\kappa_t \approx 10^{-10}$ – 10^{-8} m^2 . This overestimates the experimentally measured permeability by over five orders of magnitude. This analysis leads us to the same conclusion that it is the mucus and not the threads that must be responsible for the vast majority of the clogging performance exhibited by hagfish slime.

The above analysis leads us to the conclusions that the effective internal pore size in hagfish slime is about 10–300 nm and that the mucus component is responsible for forming this porous network. What then is the structure of the mucus network? At one extreme, we can consider the possibility that mucus molecules are homogeneously dispersed in the slime. This arrangement seems highly unlikely given the extremely low concentration of the mucus (15 mg l^{-1}). At such a low concentration, fully dispersed mucus molecules should be well below their critical overlap concentration. For example, for the neutral polymer PEO with a molecular mass of 600 kDa, the overlap concentration is estimated to be about 4700 mg l^{-1} [20]. At concentrations lower than this, adjacent molecules will generally not be close enough to interact with their neighbours and, therefore, are unlikely to impart significant viscosity or elasticity to the solution. Clearly then, we can reject the idea that the mucus molecules, which are present at a concentration of approximately 15 mg l^{-1} , are homogeneously dispersed.

Furthermore, if we treat the mucus as spherical mucin glycoproteins of size ξ as described in Dobrynin *et al.* [21], we can construct a simple scaling law for the scaling of κ as c_m is varied, where c_m is the mucus concentration. For a suspension of isolated spheres, the permeability can be described as $\kappa = 2r^2/9\varphi$, where r is the sphere diameter [22]. For a semi-dilute unentangled polyelectrolyte mucus solution, the mucin blob size scales as $\xi \sim c_m^{-1/2}$. Thus, the volume fraction of mucus solutions scales as $\varphi \sim c_m \times c_m^{-1/2 \times 3} = c_m^{-1/2}$, where the two contributions appear because of the linear increase in the number of blobs with concentration, and the second factor is

the scaling of volume of individual blobs (assuming a sphere). Assuming $\xi \sim r$ and the scaling for volume fraction φ from above, the scaling for permeability can be written as $\kappa \sim c_m^{-1/2 \times 2}/c_m^{-1/2} = c_m^{-1/2}$. This clearly underpredicts the observed concentration dependence in experiments as shown in figure 7b. Thus, a model for hagfish mucus treating it as semi-dilute unentangled polyelectrolyte solution does not agree well with our experimental observations.

If the mucus is not homogeneously distributed in the slime, then it must be heterogenous, but what is the arrangement of molecules and supramolecular structures? Koch *et al.* [13] and Fudge *et al.* [1] considered a ‘bead on a string’ model in which swollen mucous vesicles decorate deployed slime threads, but this model has been disproven by microscopy of fluorescently labelled deployed slime, which reveals no such morphology. Winegard & Fudge [6] showed that deployed mucous vesicles can be drawn into mucus strands that appear to possess significant elasticity and are also involved in the unravelling of thread skeins. We propose that these elongate mucus strands are a key feature of hagfish slime structure. Furthermore, we propose that mucus strands form an efficient volume-filling network by adopting a fractal morphology. Other studies by us and others [18,23] have led to similar conclusions of self-similarity within hagfish slime, although this idea has yet to be confirmed by high-resolution three-dimensional characterizations of the mucus network. Further, the measurements of Rementzi *et al.* [23] suggests that as the mucus concentration changes, the characteristic mesh size of mucus macromolecules remains unchanged, and the length of mucus filaments increases instead. The analysis described above states that the blob size ξ is concentration-independent, hence the volume fraction of mucus solution scales linearly with concentration, i.e. $\varphi \sim c_m \times c_m^{0 \times 3} = c_m$. The permeability of such mucus networks then can be described with a similar relation as the arrays of randomly oriented arrays of cylinders in the limit of ultra-dilute volume fraction, $\kappa \sim a^2/\varphi_t$. Thus, permeability scales as $\kappa \sim c_m^{-1}$, and thus agrees closely with our data in figure 7b.

The idea of a fine network of soft elastic mucus strands leads to the question of how the network is formed, especially given the remarkable speed of hagfish deployment, which has been estimated to produce about 0.9 l of slime in 100–400 ms [2,4]. Many suspension feeding animals,

such as tunicates and larvaceans [24] produce fine mucus network structures with pore sizes even smaller than those estimated for hagfish slime, but they produce these mucus nets at a far more leisurely pace (i.e. minutes). Previous work on hagfish slime deployment has demonstrated that vigorous mixing is a necessary condition for proper deployment of exudate into competent slime [2,6,25]. Lim *et al.* [2] used stabilized whole slime exudate and a removable mass assay to show that mixing facilitates slime development, but further mixing causes slime collapse. This behaviour was also mentioned by Ferry [15]. We replicated these experiments using mucus-only samples, using drain rate as a measure of slime quality rather than removable mass, because mucus-only samples cannot support their own weight in air. Our data followed a similar pattern as Lim *et al.* [2], who showed that mixing increases removable mass up to a point, with further mixing resulting in decreases in removable mass. We found that mixing helped slime development, and that mucus-only clogging performance decreased after a certain amount of mixing (figure 6). We previously assumed slime collapse to be primarily mediated by the aggregation of slime threads, but these new data suggest that disruption and/or aggregation of mucus may also play a role in mixing-induced collapse. If, as we suggest above, hagfish slime consists of a fine network of soft elastic mucus elements, then it is not difficult to imagine collapse of the mucus network with excessive mixing. It should be noted that both Lim *et al.* [2] and our experiments were conducted using stabilized slime components, which slows the rate of slime development after exposure to seawater. Thus, the timescale of the changes shown in figure 6 are not representative of the slime's natural behaviour. How exactly turbulent mixing might lead to a fractal network of mucus strands is currently unknown.

4.4. Biological implications

Footage of hagfishes being attacked in the wild reveals that diverse fish predators, including biters and suction feeders from Osteichthyes and Chondrichthyes, have difficulty ridding themselves of slime, even after multiple forceful flushing attempts [4]. While the high aspect ratio of the threads probably allows them to get tangled on the gill filaments and gill arches, adhesive forces may also play a role. It is currently unknown if fish predators who get slimed by hagfishes can recover or if the interaction is fatal, but our data suggest that the slime has the potential to remain on fish gills for several minutes. We should point out that fishes in the wild have been shown to employ backflushing when trying to rid their gills of the slime, whereas our clogging assay only tested the effects of flushing with seawater in the same direction as the original flow. If backflushing is more effective at removing

the slime or even making it less effective at clogging, then it is likely that some predators are able to survive after getting hagfish slime on their gills.

5. Conclusion

In this study we have shown that hagfish slime is remarkably good at clogging and can effectively clog at far lower concentrations than other polymers and thickening agents. We have also shown that mucus plays a primary role in the clogging function of hagfish slime, while the silk-like slime threads are important for retaining the slime's ability to clog over time. We have quantified the Darcy permeability of hagfish slime for the first time, and these measurements suggest an effective pore size of 10–300 nm. Our results support the hypothesis that the slime's superior clogging is primarily the result of its low permeability and is not a result of other mechanisms such as obstructing holes or viscosifying seawater. The mechanisms elucidated here could one day inspire a new class of ephemeral materials that can provide a barrier to flow that sets up quickly and subsequently collapses and/or biodegrades.

Ethics. University of Guelph Animal Use Protocol no. 2519.

Data accessibility. All data are available at the Chapman University Digital Commons (<https://doi.org/10.36837/chapman.000428>).

The data are provided in electronic supplementary material [26].

Authors' contributions. L.T.: data curation, investigation, methodology, writing—original draft, writing—review and editing; G.C.: conceptualization, data curation, investigation, methodology, writing—original draft, writing—review and editing; G.J.: data curation, investigation, supervision; A.L.: investigation, supervision; A.H.: data curation, formal analysis, investigation, methodology, visualization; A.N.: data curation, investigation, methodology, project administration, validation; Y.Z.: data curation, formal analysis, visualization; R.H.E.: conceptualization, data curation, funding acquisition, methodology, resources, supervision, writing—original draft, writing—review and editing; D.S.F.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, supervision, visualization, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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