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A New Model of Hagfish Slime Mucous Vesicle Stabilization and Deployment

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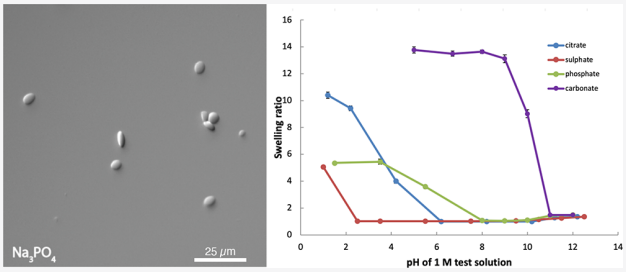
ABSTRACT: Hagfishes thwart predators by releasing large volumes of gill-clogging slime, which consists of mucus and silk-like fibers. The mucous fraction originates within gland mucous cells, which release numerous vesicles that swell and rupture when ejected into seawater. Several studies have examined the function of hagfish slime mucous vesicles in vitro, but a comprehensive model of their biophysics is lacking. Here, we tested the hypothesis that vesicles contain polyanionic glycoproteins stabilized by divalent cations and deploy in seawater via exchange of divalent for monovalent cations. We also tested the hypothesis that vesicle swelling and stabilization are governed by "Hofmeister effects". We found no evidence for either hypothesis. Our results show that hagfish mucous granules are only stabilized by multivalent anions, and pH titration experiments underscore these results. Our results lead us to the conclusion that the hagfish slime mucous gel is in fact polycationic in nature.



INTRODUCTION

Hagfishes are a group of bottom-dwelling marine animals that defend themselves from fish predators by producing large volumes of fibrous slime when they are attacked.[1−3] The slime consists mainly of seawater plus two components, mucus and threads, which are ejected from the hagfish's slime glands.[4−7] Slime threads, which are silk-like fibers with a length of approximately 150 mm, originate within specialized "thread" cells in the slime glands.[4,5,8] Within thread cells, the slime thread is produced and coiled into a compact ellipsoid structure, known as a "skein".[1,4,9] The mucous fraction of the slime is produced within large mucous cells in the slime glands known as gland mucous cells, which produce numerous mucous vesicles that fill the cytoplasm in mature cells.[5,10] An attack by a predator triggers the ejection of threads and mucus from the slime glands, which is effected by contraction of a thin layer of striated muscle surrounding each slime gland. Ejection of mature thread and mucous cells from the slime gland causes rupture of their plasma membranes and the release of naked skeins and numerous mucous vesicles.[4,5] Formation of the defensive slime involves unraveling of the skeins and swelling of the mucous vesicles, which together produce a complex material that is exceptionally good at clinging to and clogging gills.[2,3] Multiple studies have explored the chemical and physical properties of hagfish slime and the factors that affect those properties.[2,7,11−13]

Downing et al.[5] showed that mucous vesicles and skeins from hagfish slime glands can be collected from anesthetized hagfishes via gentle electrical stimulation of the muscle layer surrounding each slime gland. After stirring the gland exudate into a stabilization buffer containing 0.9 M sodium citrate, the mucous vesicles can be separated from the skeins via filtration. The mucous vesicles are disc shaped, with a diameter of approximately 3−7 μm and a thickness of 1−2 μm. Because the vesicles are released not by exocytosis but instead via rupture of the entire mucous cell, they are ejected from the slime gland with their lipid bilayer membranes intact.[10] Salo et al. showed that the glycoproteins in hagfish slime mucous vesicles contain approximately 12% carbohydrate by weight,[14] which is far less than the carbohydrate content of vertebrate mucins, which can be as high as 75%.[15] Hagfish slime glycoproteins, like mucins, are believed to be polyanionic.[16] Although they are often referred to as mucins, the identity of the protein backbone of hagfish slime glycoproteins has not yet been determined.

Aside from the fundamental studies described above, research on hagfish slime mucous vesicles has aimed to answer two questions about these structures: how are the vesicles stabilized in a condensed state in the mucous cells and how do they deploy in seawater? In other mucus-secreting systems, polyanionic mucins are condensed within mucus-producing cells by divalent cations such as Ca^{2+}.[17−19] Divalent cations are

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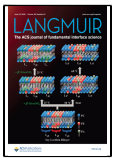

believed to act as charge shielders that allow negative charges on mucin glycans to pack tightly together and also act as cross-linkers that form a mechanical bridge between adjacent negative charges. Luchtel et al. stirred fresh exudate from hagfish slime glands into a variety of solutions and found that the only solutions capable of keeping the vesicles condensed were those containing substantial concentrations of multivalent anions, such as sulfate and phosphate.[10] This led them to conclude that the vesicle membrane is impermeable to multivalent anions and permeable to most other salts and solutes. Herr et al. developed a flow-through assay for studying the behavior of individual hagfish slime mucous vesicles in vitro and showed that not only is Ca^{2+} incapable of condensing vesicles but also it seems to be required for vesicle rupture.[20] Herr et al. also showed that the fluid fraction of hagfish slime gland exudate (or "supernatant") contains high concentrations of methylamines such as TMAO and betaine but found that these compounds are not able to keep the vesicles condensed and therefore likely perform a different function in the gland.[20,21] Furthermore, Herr et al. showed that the supernatant itself is also not capable of keeping the vesicles in a condensed state,[20] suggesting that the chemical microenvironment of gland mucous cells is different from supernatant. Taken together, the results described above suggest that the condensation mechanism of mucus molecules in hagfish slime gland mucous cells may differ from the condensation mechanism in other mucus systems.

The flip side of the stabilization question is how do the vesicles deploy in seawater? Luchtel et al. showed that most solutions, aside from those containing multivalent anions, are capable of forming mucus when they are mixed with slime gland exudate.[10] It is also clear that vesicle rupture is not driven by osmosis, as hagfishes are osmoconformers, and therefore, their tissues and body fluids possess an osmolality that is the same as seawater.[22,23] Using their flow-through assay to study the behavior of citrate-stabilized vesicles exposed to seawater, Herr et al. showed that the exudate appears to contain two different kinds of vesicles that differ in their timing and rate of rupture.[20,24] These experiments also showed that the water channels known as aquaporins are present in the vesicle membrane that accelerate vesicle swelling. Herr et al. suggest that rupture in the majority of vesicles may be mediated by Ca^{2+}-activated, nonselective pores in the vesicle membrane.[24]

In other mucus secreting systems, rapid swelling of mucous granules is believed to be governed by a "Jack-in-the-box" mechanism that involves exchange of Ca^{2+} ions for Na^{+} ions.[25,26] With their single positive charge, Na^{+} ions are far less effective at charge shielding than Ca^{2+} and they are not capable of forming electrostatic cross-links between adjacent negative charges. Thus, Na^{+}/Ca^{2+} exchange leads to a sudden increase in repulsion among negatively charged mucins and rapid swelling.[25,26] Osmotic effects likely further drive swelling, with two Na^{+} ions exchanged for every Ca^{2+}. Although a Jack-in-the-box mechanism has been proposed for hagfish slime mucous vesicle deployment,[20] some experimental results are difficult to reconcile with such a model, namely, that Ca^{2+} does not seem capable of condensing hagfish mucous vesicles, and the only salts capable of condensing the vesicles contain multivalent anions. In the current study, we provide new data on the behavior of individual vesicles from freshly collected hagfish slime gland exudate and on the behavior of mucous granules obtained by disrupting their vesicle membranes. To test hypotheses about biophysical and biochemical mechanisms underlying the function of hagfish slime mucous vesicles, these experiments were carried out using a wide variety of salts at varying concentrations and over a large pH range. On the basis of these new results, we propose a new model of mucous vesicle function in hagfish slime that simultaneously explains both stabilization and deployment of these structures.

METHODS

Animals, Anesthesia, and Slime Exudate Collection. Experiments were conducted at two locations, the University of Guelph in Guelph, Ontario, Canada, and Chapman University in Orange, California, United States. For the experiments conducted in Guelph, Pacific hagfish (*E. stoutii*) were obtained from the Bamfield Marine Science Center (Bamfield, British Columbia, Canada) and maintained in the Hagen Aqualab within 2000 L Environmentally Controlled Aquatic Recirculation System (ECARS) tanks with recirculating artificial seawater (34‰) at 10 °C. For experiments conducted at Chapman, Pacific hagfish were collected within 10 km of Moss Landing, CA and maintained in recirculating 900 L tanks of seawater (34‰) maintained at 10 °C.

To anesthetize hagfish prior to exudate collection, a 1:9 dilution of clove oil in ethanol was added to 3 L of chilled artificial seawater for a final concentration of 50 μL clove oil L^{-1}.[20] Hagfish were placed in the clove-oil anesthetic until they stopped responding to touch, or about 20−30 min. To collect slime gland exudate, the skin around several slime glands was rinsed with deionized water and gently wiped dry. Mild electrical stimulation using a GRASS Instruments SD9 stimulator (15 V, 80 Hz; Quincy, MA) of the skin above the slime glands caused contraction of the muscle layer around the glands and expression of exudate onto the skin. For experiments involving fresh vesicles, exudate was collected with a Teflon-coated spatula and transferred directly to the experimental chamber. For experiments involving stabilized vesicles, exudate was stirred into a stabilization buffer (SB) solution composed of 0.9 M sodium citrate and 0.1 M PIPES, pH 6.7.[4,8] After exudate collection, hagfish were transferred to a bucket with fresh, chilled seawater, where they typically recovered from the anesthetic within seconds to minutes. All experimental protocols were approved by the University of Guelph Animal Care Committee (Protocol #2519).

Flow-through Assays. To observe the behavior of slime gland exudate and individual vesicles under the microscope, we constructed custom flow-through chambers based on a design by Herr et al.[20] The chambers were constructed by drawing two parallel lines of petroleum jelly containing 0.1 mm diameter glass beads approximately 6 mm apart onto a glass slide, onto which a coverslip (18 × 18 mm) was placed.[20,24] This created a chamber with a volume of approximately 32 μL. For trials involving fresh exudate, a 1−2 μL drop of exudate was placed in the center of the chamber and covered with a coverglass. For experiments involving mucous granules, a suspension of stabilized vesicles was prepared by filtration of SB-stabilized exudate through a nylon mesh with a 40 μm pore size, which retained the much larger thread skeins and allowed the smaller vesicles to pass through.[10,27] To standardize the number of vesicles used in a given assay, the concentration of mucin vesicles in stabilization buffer was quantified using spectrometry, with absorbance measured at 350 nm with an Ultrospec 3100 pro spectrophotometer (Biochrom Ltd., Cambridge, England).[24] Samples were diluted to a concentration of 100 vesicles/μL and stored at 4 °C before use. To remove the influence of the vesicle membrane and investigate the properties of the mucus gel more directly, isolated vesicles were exposed to 0.1% Triton X-100 detergent in SB for 90 min before testing. We refer to vesicles exposed to detergent hereafter as "granules". A 30 μL aliquot of stabilized granules was added to each chamber, and the granules were given 2 min to settle and adhere to the glass slide. The chamber was then flushed with 60 μL of SB to wash away any loose granules by pulling it through to the other side via capillary action using a piece of filter paper. Adhered granules were then exposed to 30 μL of experimental solution using the same method.[20,24] The swelling

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Langmuir 2020, 36, 6681−6689

behavior of granules was observed from time lapse videos captured by a monochrome digital camera (Q-imaging Retiga Exi Fast1394) on a Nikon Eclipse 90i microscope (Nikon Instruments Inc., Melville, NY) with a 20× DIC objective. The time lapse videos were set to capture 5 frames per second (fps) for 30 s, followed by 1 fps for 160 s.[20,24] Granule perimeters were traced before and after exposure to a given test solution within NIS-Elements A.R. 3.0 software (Nikon Instruments Inc.), and granule areas were calculated using the Measurement module of NIS-Elements.

Fresh Exudate Trials. Experiments using fresh exudate were carried out using a Zeiss Axio Imager M2 microscope with an Axiocam 506 monochrome camera and ZenPro v 2.3 software (blue edition), and the exudate was visualized using a 40× DIC objective lens. For each trial of a given test solution, 4−5 locations on a slide were chosen to follow throughout the assay. Each solution was tested with exudate from three different hagfish with a total of 60−90 vesicles analyzed for each solution. Because vesicles from fresh exudate did not readily adhere to glass microscope slides, we coated the slides with polystyrenesulfonate (M_w = 10^6, Sigma-Aldrich) by dipping them in a 10 mM solution and letting them dry before use. Fresh exudate was exposed to unbuffered solutions over a range of concentrations of the following salts, with the average pH value of the 1 M solution given in parentheses. Monovalent anions and cations: NaCl (7.1), KCl (7.3), NaF (7.9), $NaNO_3$ (7.7), and $NaCH_3COO$ (8.3). Divalent cations: $MgCl_2$ (5.4) and $CaCl_2$ (6.2). Divalent anions: $(NH_4)_2SO_4$ (5.0), $MgSO_4$ (6.6), Na_2SO_4 (7.9), and NaH_2PO_4 (7.9). Trivalent anions: Na_3PO_4 (11.5) and Na citrate (8.4). The pH of these solutions was not adjusted to avoid adding buffer salts that might have their own effects on stabilization or swelling. pH effects were carefully controlled in two other sets of experiments (see below). For salts that did not have stabilizing effects, the concentration was increased in 1 M intervals until a stabilizing concentration was found or the solubility limit was reached. For stabilizing salts, we lowered the concentration by 0.5 M intervals to find the lowest concentration that could still stabilize. In addition, we conducted trials with sucrose, artificial seawater (ASW) (Instant Ocean, 34 g/L, ion composition (g/L): Cl^- 18.7, Na^+ 10.4, SO_4^{2-} 2.6, Mg^{2+} 1.2, Ca^{2+} 0.4, K^+ 0.4, HCO_3^{-1} 0.2)[28] and nanopure water. To probe the influence of the vesicle membrane on swelling in the various solutions, we exposed fresh vesicles to solutions containing 0.1% Triton X-100 and performed the flow-through assay as described in the section above. For each trial, the effect of the test solution was recorded by observing the behavior of the vesicles before and after exposure to the test solution. Trials in which vesicles swelled to the point where they could no longer be seen were scored as "burst", whereas those in which vesicles retained their approximate size and shape were scored as "stable".

Hofmeister Experiments. To test the hypothesis that vesicle behavior is governed by Hofmeister effects, granules were exposed to solutions covering a wide range of positions on the Hofmeister series. The Hofmeister series provides a relative scale for the tendency of various ions to cause proteins to precipitate or dissolve in aqueous solutions. Salts that promote precipitation are referred to as kosmotropic, and those that promote dissolution are referred to as chaotropic. The following salts, in order of most kosmotropic to most chaotropic, were used to test the Hofmeister hypothesis: NH_4F, NH_4 acetate, NH_4Cl, $Na_3Citrate$, Na_3PO_4, Na_2SO_4, NaF, Na acetate, NaCl, NaI, and NaBr. All solutions were buffered with 5 mmol·L^{-1} Tris base and adjusted to a final pH of 8.0 using Dowex ion-exchange resin to lower the pH in sodium salts or 1.0 mol·L^{-1} HCl to lower the pH of ammonium salts, or 1.0 mol·L^{-1} NaOH to raise the pH. All solutions were adjusted to have a final osmolarity between the range of 2795 and 2805 mOsm using a Vapro Vapor Pressure Osmometer (model 5520, Wescor Inc., Logan, UT).

pH Series Experiments. Mucus granules were exposed to a pH series in the presence of four multivalent anions that are known to stabilize vesicles: citrate, phosphate, sulfate, and carbonate. Dowex ion-exchange resin was used to lower the pH of sodium salts. Dowex resin exchanges H^+ ions for Na^+, thus lowering the pH without changing the anion concentration. For all of the polyvalent anions used, at pH values above the anion's highest pK_a, negative charge was at its maximum. At lower pH, charge decreases, with transitions between trivalent (if applicable), divalent, and monovalent ions represented by the pK_a values of the anion. The distribution of anionic species as a function of pH in the four salts examined is provided in Figure S1. All solutions were buffered with 5 mmol·L^{-1} Tris base and adjusted to have a final osmolarity in the range of 2795−2805 mOsm·L^{-1} using a Vapro Vapor Pressure Osmometer (model 5520, Wescor Inc., Logan, UT). Mucous granules were prepared as described above for the Hofmeister experiments from 10 different hagfish, with granule area measured for at least 5 granules from each hagfish at each pH level. Granules in a given flow-through chamber were exposed to only one of the pH levels in a given salt series. Granule areas were measured as described above.

Data Analysis. To extract a quantitative value for the transition pH for the anions in each solution, we fitted the swelling data to a logistic curve of the form $y = \frac{L}{1 + e^{-k(x-z)}} + l$ where L represents the maximum asymptote height of the curve (13.51, 5.40, 5.05, and 9.91 for the sodium salts of carbonate, phosphate, sulfate, and citrate, respectively). The parameter k represents the vertical slope at the transition pH, z represents the horizontal shift of the curve, and l represents the curve's minimum asymptote (1.0 for all solutions. A swelling ratio of 1.0 indicates no change in area and thus is the smallest ratio possible). The k parameter was assigned a constant value of 4.2 after a series of least-squares nonlinear regressions was performed for each replicate data set to estimate which k value resulted in the smallest total fit error (sum of squares) for all of the salts. k values were tested over a range of 1.0−10.0 in 0.1 increments. The value k = 4.2 gave the smallest fit error value for all four solutions (0.31, 0.36, 0.25, and 0.76 for the curves of sodium carbonate, phosphate, sulfate, and citrate, respectively). A nonlinear least-squares regression was then performed to estimate the most accurate transition pH value (parameter z) for each individual salt from this model equation. All regressions were done using scripts written in Python. Mean estimated transition pH values were then compared using a one-way ANOVA followed by a Tukey HSD post-hoc test.

RESULTS

Swelling of Mucous Vesicles from Fresh Exudate. Exposure of fresh vesicles to a variety of salts over a wide range of concentrations revealed that 1 M solutions containing polyvalent anions were consistently effective at stabilizing vesicles (Table 1) (Figure 1). Out of the five salts that stabilized, sodium citrate was the most effective because it stabilized at concentrations as low as 0.5 M. Solutions containing divalent cations were able to stabilize vesicles but only at very high concentrations of 3 M for $CaCl_2$ and 5 M for $MgCl_2$. These high concentrations of $CaCl_2$ and $MgCl_2$ were only able to stabilize the vesicles if their membranes were intact; solutions of these salts containing the membrane-disrupting detergent Triton X-100 caused the vesicles to burst.

Hofmeister Effects. To test the idea that the swelling of hagfish slime mucus is governed by Hofmeister effects, isolated granules (vesicles without the membrane) were exposed to 11 salt solutions containing ions representing a wide range of positions on the Hofmeister series (Table 2). Overall, our results were not consistent with a Hofmeister mechanism of stabilization and swelling, with the biggest inconsistency being that kosmotropic salts (i.e., ammonium salts of fluoride, acetate, and chloride, and sodium salts of fluoride and acetate) all resulted in swelling of the granules. Other trials were generally consistent with the Hofmeister predictions, with sodium salts of iodide and bromide, at the chaotropic end of the Hofmeister series, resulting in swelling. Sodium salts of citrate, phosphate, and sulfate, at the kosmotropic end of the Hofmeister series, were capable of keeping the granules in a

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Table 1. Effects of a Variety of Solutions on Freshly Collected Hagfish Slime Mucous Vesicles[a]

solute	Effect of 1 M solution	+Triton X-100	stabilizing concentration
monovalent cations monovalent anions			
NaCl	burst	burst	none
KCl	burst	burst	none
NaF	burst	burst	none
$NaNO_3$	burst	burst	none
Na acetate	burst	burst	none
divalent cations monovalent anions			
$CaCl_2$	burst	burst	3.0 M
$MgCl_2$	burst	burst	5.0 M
divalent anions divalent cations			
$MgSO_4$	stable	stable	1.0 M
monovalent cations multivalent anions			
$(NH_4)_2SO_4$	stable	stable	1.0 M
Na_2SO_4	stable	stable	1.0 M
Na_3PO_4	stable	stable	1.0 M
Na citrate	stable	stable	0.5 M
other			
sucrose	burst	burst	none
ASW (34‰)	burst	burst	N/A
dH_2O	burst	burst	N/A

[a]For the middle two columns, data are for hagfish slime exudate that was exposed to 1 M salt solutions in the absence or presence of 0.1% Triton X-100 detergent, which disrupts membranes. Only salts containing multivalent anions were able to stabilize vesicles, and they did so in the presence of detergent as well. The last column provides the concentration (if any) at which a given salt was able to stabilize in the absence of detergent.

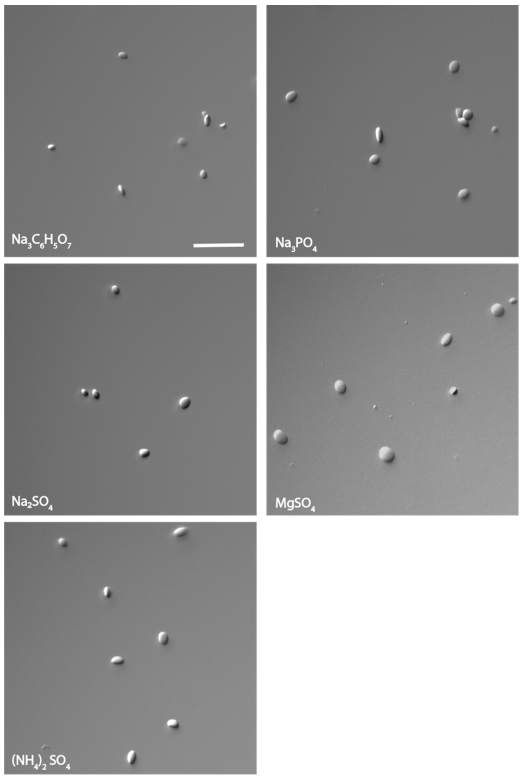

Figure 1. Fresh hagfish slime mucous vesicles in five stabilizing solutions. Fresh vesicles were exposed to a variety of salt solutions to determine if they stabilized the vesicles or caused them to burst. Shown here are vesicles that were exposed to each of five solutions (sodium citrate, sodium phosphate, sodium sulfate, magnesium sulfate, and ammonium sulfate, concentration 1 M) that all had stabilizing effects. Images for solutions that did not stabilize are not shown, as the vesicles are not visible after they burst. Scale bar is 25 μm.

condensed state. We found that there was little variability in swelling behavior within a given test solution, with all granules either swelling or remaining condensed.

pH Effects in Salt Solutions with Polyvalent Anions. Results from the Hofmeister trials revealed that the only salts capable of condensing the mucus gel were those containing polyvalent anions. To further test the idea that the condensing effects of these salts arise directly from their possession of multiple negative charges, we exposed mucous granules to four polyvalent anion solutions over a wide pH range. Granules showed a high degree of swelling at low pH and little to no swelling at higher pH values (Figure 2A). At pH values above 10, minor swelling (1.27−1.49-fold area increase) was observed (Figure 2B). The pH values where the low-pH transition occurred differed widely among the four solutions. Fitting the swelling data to a logistic model curve allowed us to quantitatively estimate the transition pH for each salt. Estimates of the transition pH for sodium citrate, sodium sulfate, sodium phosphate, and sodium carbonate were 4.03 ± 0.02, 1.77 ± 0.005, 5.41 ± 0.07, and 10.08 ± 0.04, respectively (Table 3), all of which were significantly different from each other (TukeyHSD, $p < 0.05$). A linear regression of the transition pH values against the second lowest pK_a values for each respective anion showed a strong positive relationship (R^2= 0.9543, p = 0.0231) (Figure 2A).

DISCUSSION

Experiments using fresh slime gland exudate revealed that vesicles in stabilizing solutions are considerably smaller than those observed in exudate in the absence of stabilizing salts. It is not clear if these differences were caused by condensation of the vesicles by the stabilizing salts, swelling of the vesicles in fresh exudate, or a combination of the two. Herr et al. found that stabilized vesicles exposed to an excess of slime gland supernatant swell in vitro,[20] but it is unclear if this swelling is the vesicles returning to their original size within the gland or if this swelling is more akin to the changes that occur during deployment in seawater.

Luchtel et al. demonstrated that solutions containing polyvalent anions such as citrate, phosphate, and sulfate are the only ones that are capable of stabilizing hagfish slime mucous vesicles.[10] They suggested that the stabilizing effects of these ions arise because they are impermeant to the vesicle membrane, whereas all other particles (monovalent anions and cations, multivalent cations, and even neutral molecules such as sugars) are permeant. Our results demonstrate that the stabilizing effects of polyvalent anions cannot be explained by membrane impermeability, as these solutions also stabilize vesicles whose membranes have been disrupted with detergent. Instead, it appears that polyvalent anions have a direct stabilizing effect on the mucous gel.

We tested the hypothesis that stabilization and swelling of hagfish slime mucous glycoproteins is governed by Hofmeister effects, i.e., that they are kept in a condensed and stabilized state in the slime gland via exposure to molecules at the kosmotropic end of the Hofmeister series, and that swelling

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Table 2. Effects of Chaotropic and Kosmotropic Salts on Hagfish Slime Mucous Granules[a]

Salt	Hofmeister predicted effect	Observed effect
NH_4F	stabilizing (chaotropic)	swelling
NH_4 acetate		swelling
NH_4Cl		swelling
Na citrate		stabilizing
Na_3PO_4		stabilizing
Na_2SO_4		stabilizing
NaF		swelling
Na acetate		swelling
NaCl		swelling
NaI		swelling
NaBr	Swelling (kosmotropic)	swelling

[a]Granules were prepared by treating vesicles stabilized in a citrate-based stabilization buffer with 0.1% Triton X-100 to remove any influence of the vesicle membrane. Salts on the extreme kosmotropic end of the Hofmeister series caused granule swelling, which rules out Hofmeister effects as a viable explanation of vesicle swelling and stabilization. Numerical data are not provided because there was no variability in the swelling response among vesicles within a given treatment, either all of the granules swelled or they remained in a condensed state. Observed effects are summaries of observations of mucous granules prepared from three different hagfish.

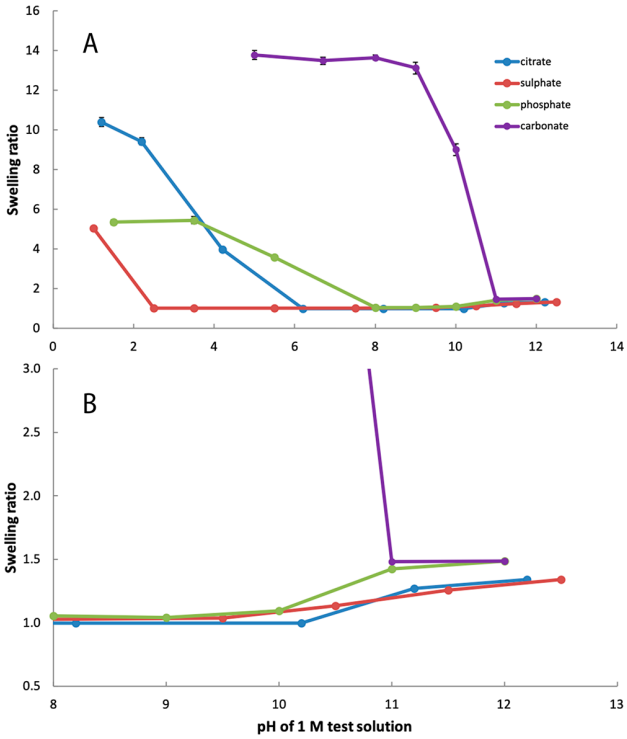

Figure 2. Effects of four multivalent anionic salts on hagfish mucous granules over a large range of pH. Granules were prepared by collecting slime gland exudate into a 0.9 M sodium citrate stabilization buffer, filtering out the thread skeins, and disrupting their membranes via exposure to Triton X-100 detergent. (A) Granules were generally condensed at high pH and swollen at low pH, with the swelling transition at low pH differing substantially among the four salts tested. These data suggest that swelling at low pH is governed by protonation of the stabilizing anions. (B) Detail of the data shown in A. Swelling at high pH was much smaller in magnitude and was far more uniform among the four salts both in magnitude and in the pH where swelling was observed. These results suggest that swelling at high pH was the result of deprotonation of positively charged groups on the mucous glycoproteins.

Table 3. pK_a Values for Each of the Four Multivalent Anions Used for the pH Series Experiments and the Transition pH where Swelling of Mucous Granules Occurred[a]

multivalent anion	pK_a values	swelling transition pH
sulfate	−3.0, **1.90**	1.77 ± 0.01
citrate	3.13, **4.76**, 6.39	4.03 ± 0.02
phosphate	2.12, **7.21**, 12.67	5.41 ± 0.07
carbonate	6.40, **10.31**	10.08 ± 0.04

[a]Swelling transition pH was estimated by fitting the swelling data to a logistic curve and performing a nonlinear least-squares regression to find the transition pH value that provided the best fit. We found that the pH at which swelling initiated was different for each salt, and this pH corresponded with the second lowest pK_a for each anion (bold text). These data suggest that swelling at low pH is not governed by direct effects on the mucous polymers but instead by changes to the titratable groups on the multivalent anions.

and deployment involves loss of these stabilizing effects. Our results show that salts from both extremes of the Hofmeister series, e.g., the kosmotrope ammonium acetate and the chaotrope NaBr, are capable of swelling hagfish slime mucous granules, which undermines Hofmeister effects as a plausible mechanism underlying hagfish slime mucous vesicle function. Consistent with Luchtel et al.'s results,[10] the only ions that stabilized hagfish slime mucous granules were those containing polyvalent anions, such as phosphate, citrate, carbonate, and sulfate, and these are not at the extreme stabilizing end of the Hofmeister series.

We reasoned that if the stabilizing effects of polyvalent anions arise from their possession of multiple negative charges, then they should show strong pH effects. More specifically, protonation of these anions at lower pH should cause them to lose their ability to stabilize. We indeed found that solutions of sulfate, phosphate, citrate, and carbonate all lose their ability to keep the mucous granules stabilized when pH is lower than a critical value, with the critical value differing substantially among the four salts. How can we explain the fact that swelling occurred at a different pH in the four salts examined? If the key to stabilizing the mucous granules is sufficient concentrations of anions possessing two or more charges then one would expect the multivalent anions to lose their ability to stabilize at the pH where the monovalent form begins to dominate and the concentration of the divalent form dwindles, or in other words, somewhere close to the second lowest pK_a for each salt. We found a strong correlation between the swelling transition pH for each salt and its second lowest pK_a (Table 3). The seeming contradiction between our pH results and those of Luchtel et al.[10] and Böni et al.,[29] who showed that low pH can inhibit slime formation, might be explained by the fact that they were using fresh exudate, and therefore, the vesicles still possessed their membranes. At such a low pH, it is likely that channels and pores in the membrane would have been

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Langmuir 2020, 36, 6681−6689

denatured, which could have disrupted the ion exchange needed for proper deployment.

As proposed originally by Verdugo, rapid swelling of vertebrate mucous granules can be explained by a Jack-in-the-box mechanism in which divalent cations such as Ca^{2+} are exchanged for monovalent cations such as Na^{+} in these systems.[18] According to this theory, polyanionic mucin polymers are condensed within vesicles in goblet cells by high concentrations of charge-shielding and cross-linking Ca^{2+} ions. Exocytotic release of mucin granules exposes them to extracellular fluid, which contains relatively low $[Ca^{2+}]$ and relatively high $[Na^{+}]$, leading to ion exchange. Exchange of Ca^{2+} for Na^{+} has three important effects: (1) The charge difference between the two ions leads to a 2:1 molar ratio of Ca^{2+} leaving and Na^{+} entering, creating an osmotic imbalance that pulls water into the granule and causes swelling. (2) Ca^{2+} is far more effective as a charge shielder than Na^{+}, and therefore, exchange of the two ions leads to a dramatic increase in electrostatic repulsion among neighboring negative charges on the mucin. (3) The double positive charge of Ca^{2+} also makes it capable of acting as a cross-linker, meaning that nearby negative charges stick to each other when a Ca^{2+} ion is between them. In contrast, Na^{+}, with its single positive charge, is not able to form stable cross-links between neighboring charges of −1. In other mucus systems, the Jack-in-the-box hypothesis is supported by evidence of high concentrations of Ca^{2+} in mucin granules as well as a rapid efflux of Ca^{2+} preceding granule swelling.[25,26] We reasoned that if hagfish mucous deployment is governed by similar mechanisms, then we should be able to inhibit the swelling of mucous granules (or recondense them) by exposing them to high concentrations of Ca^{2+} or Mg^{2+}. While we did find that very high concentrations of $MgCl_2$ and $CaCl_2$ (3−5 M) were able to inhibit swelling of fresh mucous vesicles, this ability disappeared if the vesicle membrane was disrupted with detergent. We found that even saturated solutions of $MgCl_2$ and $CaCl_2$ were not able to condense hagfish slime mucous vesicles in the presence of detergent.

Although it has been assumed up to this point that hagfish slime mucus consists of polyanionic mucin-like molecules and that the mucus gel is stabilized within gland mucous cells via divalent cation counterions,[16,24] the data we present here are not consistent with this model. Polyanionic mucous granules should remain condensed in high concentrations of divalent cations, and we see no evidence of stabilization, even at extremely high concentrations of $MgCl_2$ and $CaCl_2$. Furthermore, we found that multivalent anions are capable of condensing hagfish slime mucous granules, which is also not consistent with the behavior of a polyanionic mucous gel.[17,19] These results have led us to consider the hypothesis that the hagfish slime mucus gel possesses multiple positive charges, i.e., it is polycationic. This hypothesis is the same as the Jack-in-the-box mechanism, with the only difference being the sign of the charges on the polymers and the counterions (Figure 3). According to this hypothesis, high concentrations of multivalent anions keep the polymers in a condensed state in the gland mucous cells and swelling is triggered by the exchange of multivalent anions for monovalent anions such as Cl^{-}. Such a model would explain why the only salts capable of condensing hagfish slime mucous granules are those containing di- or trivalent anions. It also explains why titration of multivalent anions with acid, thereby converting them to monovalent ions, leads to swelling. Furthermore, it explains why $MgCl_2$ and $CaCl_2$ are not able to condense hagfish slime mucous granules, even at very high concentrations.

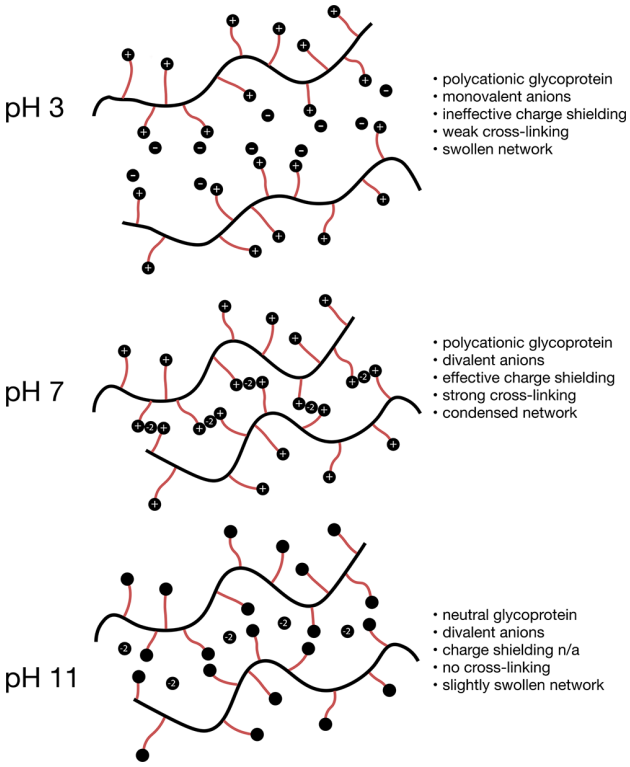

Figure 3. Schematic of pH effects in hagfish slime mucous granules. pH effects of 1 M solutions containing titratable anions such as citrate can be explained by electrostatic effects such as charge shielding of positive charges on the mucous glycoproteins and cross-links formed between adjacent positive charges via their mutual attraction for a divalent anion. For simplicity, only two adjacent glycoproteins are shown and only the counterions present between the two polymers are shown. We propose that swelling of mucous vesicles during their deployment in seawater is governed by a process similar to that shown in the pH 3 scenario, with the main difference being that the sudden increase in electrostatic repulsion among polymers is triggered by exchange of divalent anions for monovalent anions rather than a decrease in pH.

The polycationic mucus hypothesis explains why hagfish slime mucous granules swell at low pH, and why they do so at a pH that corresponds to the stabilizing anion's lowest pK_a. How then can we understand the relatively subtle swelling effects we observed in all four salt solutions at values greater than pH 10? We propose that the large amount of swelling at low pH (range of maximum area increase from about 4−14 fold) is caused by protonation effects on the stabilizing anions, whereas the more subtle swelling at high pH is caused by direct effects on the glycoprotein itself. High pH may have caused deprotonation of positively charged groups on the mucus polymers and a corresponding loss of positive charge. While neutralizing these charges would reduce the electrostatic repulsion among the polymers, it would also abolish the cross-linking effects of divalent anions, allowing the polymers to drift apart via diffusion. In this way the subtle swelling at high pH can be understood as arising from a loss of cross-linking and an absence of the electrostatic repulsion that drives swelling at low pH and in vivo. The idea that swelling at high pH is governed by direct effects of pH on the mucus polymers

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Langmuir 2020, 36, 6681−6689

is supported by the fact that the swelling occurs at nearly the same transition pH and in the same magnitude in all four salts. These results also provide clues about the nature and possible identity of the charged groups on the mucus glycoproteins. If these groups are positively charged at most pH values and neutral at pH values above 10, this considerably narrows down the list of chemical moieties that might be the charge bearers in hagfish slime mucous glycoproteins. Candidates for the chemical groups responsible for the positive charges on hagfish slime mucus polymers include amino acids such as lysine (pK_a 10.54) and arginine (pK_a 12.5), where the latter makes up 1.4% of oxidized hagfish slime mucus residues.[11]

While the polycationic mucus hypothesis explains the behavior of hagfish slime mucous granules under a variety of conditions, it is a rather extraordinary claim given that all other mucous systems are believed to be polyanionic in nature. It therefore raises the question of why selection would favor such a system. One possibility is that the silk-like threads that form the other major component of the slime are negatively charged and that positively charged mucus sticks better to the threads and forms a more robust slime. On the basis of the amino acid composition of the two proteins that make up the vast majority of the slime threads, α and γ, the net charge of α/γ dimers at the pH of seawater (8.2) is predicted to be −19, suggesting a net negative charge for the slime threads (Prot pi v. 2.2.19.136, https://www.protpi.ch).

Another possible factor is that deployment speed in seawater may be faster for positively charged mucous polymers. Because hagfish slime mucus is released via holocrine secretion involving rupture of the gland mucous cells, the glycoproteins ejected into seawater are still in possession of their plasma membranes. Also, because they are isosmotic with seawater, deployment cannot rely simply on osmosis and therefore must involve the flux of ions across the membrane, presumably through channels or pores. If deployment speed is a premium, as we believe it is for hagfish slime, then it is likely that electrostatic repulsion (ala Verdugo's Jack-in-the-box) plays an important role as it can drive swelling faster than diffusion alone. The reverse Jack-in-the-box mechanism we propose posits that two monovalent anions are exchanged for each divalent anion, which leads to an osmotic imbalance, a loss of charge shielding, and a loss of cross-linking within polycationic polymers. All three of these effects would contribute to sudden swelling of the mucous granule. Consideration of the concentration of monovalent and divalent ions in seawater suggests that a reverse Jack-in-the-box may be more effective than one involving polyanionic mucus. First, the concentration of divalent cations (63 mM total for Mg^{2+}, Ca^{2+}, and Sr^{2+}) in seawater is higher than that for divalent anions (28 mM for SO_4^{2-}). This means that the loss of charge shielding and cross-linking that arises from exchange of monovalents for divalents will be more complete in a polycationic system than in a polyanionic system. Second, the concentration of monovalent anions in seawater (547 mM total for Cl^-, Br^-, and F^-) is higher than that for monovalent cations (479 mM total for Na^+, K^+), suggesting a stronger diffusional drive into the vesicles for the former. For these reasons, swelling of polycationic polymers should be faster in seawater than it is for polyanionic polymers, although it would be good to test this assertion using polymer particles of known charge and size.

While the hypothesis that hagfish mucous polymers are cationic in nature explains a wide variety of anomalous results, including some presented in the current study, it contradicts the assertion by Böcker et al. that the glycoproteins are negatively charged.[16] These researchers based their conclusion on two observations: one from an experiment measuring the zeta potential of hagfish slime mucous polymers and another where they blended hagfish slime exudate with polymers of known charge. In the case of the zeta-potential measurements, they report a value of −33 mV. While it is fairly straightforward to collect zeta-potential data, interpretation can be complex. Zeta potential is a measure of the surface charge of particles, and it is therefore possible to measure negative zeta potentials for positively charged particles that are decorated by negative counterions and vice versa. The other evidence cited by Böcker et al. is that hagfish slime exudate forms aggregates when blended with positively charged chitosan polymers but not when blended with negatively charged κ-carrageenan.[16] It is important to note that these experiments were done by blending slime gland exudate, which contains both mucus and threads, into the two polymer solutions. Thus, the collapse seen in chitosan might have been caused by aggregation of negatively charged slime threads, and not mucus, with the positively charged chitosan. At any rate, the deployment of slime gland exudate in an aqueous solution is a highly complex phenomenon that could be affected by many different variables aside from the charge of the polymers, and thus, we believe that it is difficult to infer the charge on hagfish slime mucous glycoproteins from these exudate aggregation results.

If the cells that produce mucous vesicles in hagfish slime glands arose from more typical mucus-secreting cells that secrete anionic mucins, it is difficult to imagine how this might have evolved via gradual selection. The reason for this is that a gradual transition from negatively charged glycoproteins to positively charged ones would have to pass through a maladaptive valley of uncharged polymers, which would lack the ability to swell rapidly via electrostatic mechanisms. It is therefore difficult to imagine how natural selection, which lacks foresight, would be able to push the glycoproteins in the direction from a polyanionic to a polycationic state. One possibility is that the transition was not gradual and that the flipping of the charge from negative to positive occurred via a single mutation that resulted in substitution of a positively charged moiety for a previously negative one. Gland mucous cells are believed to have arisen via modification and invagination of specialized mucus cells in the hagfish epidermis known as large mucous cells, which resemble gland mucous cells and become packed with mucous vesicles when they are mature. It would be interesting to explore the properties of the vesicles produced by large mucous cells to see if they contain a polycationic glycoprotein gel or if they are more similar to conventional mucous cells. If large mucous cells also possess cationic mucus, then this trait may have evolved originally for a different reason besides increased adhesion to slime threads or increased deployment speed.

CONCLUSIONS

Here, we tested the hypotheses that the mucus in the defensive slime of hagfishes consists of a network of polyanionic glycoproteins. We also tested the idea that the stabilization and swelling of these molecules is governed by Hofmeister effects. Our results contradict both of these hypotheses and instead lead us to conclude that the mucus consists of a network of polycationic molecules that are likely stabilized within the slime glands with anionic counterions. Future work should aim to identify the protein backbone of hagfish slime

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Langmuir 2020, 36, 6681−6689

mucous glycoproteins, the identity of the glycans, the source of the positive charges on these molecules, and the negative counterions that stabilize these molecules in the slime glands. A deeper understanding of the mechanisms whereby the mucous vesicles are stabilized and deployed will no doubt be useful for current biomimetic efforts to produce synthetic materials that can replicate the unusual material properties of hagfish slime.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.langmuir.0c00639.

Relative concentrations of charged species within sulfate, citrate, phosphate, and carbonate solutions as a function of pH, plotted with mucous granule swelling data (PDF)

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Notes

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■ REFERENCES

(1) Fernholm, B. Thread cells from the slime glands of hagfish (Myxinidae). *Acta Zool.* **1981**, *62*, 137−145.

(2) Lim, J.; Fudge, D. S.; Levy, N.; Gosline, J. M. Hagfish slime ecomechanics: testing the gill-clogging hypothesis. *J. Exp. Biol.* **2006**, *209*, 702−710.

(3) Zintzen, V.; Roberts, C. D.; Anderson, M. J.; Stewart, A. L.; Struthers, C. D.; Harvey, E. S. Hagfish predatory behaviour and slime defence mechanism. *Sci. Rep.* **2011**, *1*, 131.

(4) Downing, S. W.; Spitzer, R. H.; Salo, W. L.; Downing, J. S.; Saidel, L. J.; Koch, E. A. Threads in the hagfish slime gland thread cells: organization, biochemical features, and length. *Science* **1981**, *212*, 326−328.

(5) Downing, S. W.; Salo, W. L.; Spitzer, R. H.; Koch, E. A. The hagfish slime gland: a model system for studying the biology of mucus. *Science* **1981**, *214*, 1143−1145.

(6) Koch, E. A.; Spitzer, R. H.; Pithawalla, R. B.; Downing, S. W. Keratin-like components of gland thread cells modulate the properties of mucus from hagfish (*Eptatretus stouti*). *Cell Tissue Res.* **1991**, *264*, 79−86.

(7) Fudge, D. S.; Levy, N.; Chiu, S.; Gosline, J. M. Composition, morphology and mechanics of hagfish slime. *J. Exp. Biol.* **2005**, *208*, 4613−4625.

(8) Fudge, D. S.; Gardner, K. H.; Forsyth, V. T.; Riekel, C.; Gosline, J. M. The mechanical properties of hydrated intermediate filaments: insights from hagfish slime threads. *Biophys. J.* **2003**, *85*, 2015−2027.

(9) Winegard, T.; Herr, J.; Mena, C.; Lee, B.; Dinov, I.; Bird, D.; Bernards, M.; Hobel, S.; Van Valkenburgh, B.; Toga, A.; Fudge, D. Coiling and maturation of a high-performance fibre in hagfish slime gland thread cells. *Nat. Commun.* **2014**, *5*, 3534.

(10) Luchtel, D. L.; Martin, A. W.; Deyrup-Olsen, I. Ultrastructure and permeability characteristics of the membranes of mucous granules of the hagfish. *Tissue Cell* **1991**, *23*, 939−948.

(11) Böni, L. J.; Zurflüh, R.; Baumgartner, M. E.; Windhab, E. J.; Fischer, P.; Kuster, S.; Rühs, P. A. Effect of ionic strength and seawater cations on hagfish slime formation. *Sci. Rep.* **2018**, *8*, 9867.

(12) Rementzi, K.; Böni, L. J.; Adamcik, J.; Fischer, P.; Vlassopoulos, D. Structure and dynamics of hagfish mucin in different saline environments. *Soft Matter* **2019**, *15*, 8627−8637.

(13) Ewoldt, R. H.; Winegard, T. M.; Fudge, D. S. Non-linear viscoelasticity of hagfish slime. *International Journal of Non-Linear Mechanics* **2011**, *46*, 627−636.

(14) Salo, W. L.; Downing, S. W.; Lidinsky, W. A.; Gallagher, W. H.; Spitzer, R. H.; Koch, E. A. Fractionation of hagfish slime gland secretions: partial characterization of the mucous vesicle fraction. *Prep. Biochem.* **1983**, *13*, 103−135.

(15) Smith, B. F.; LaMont, J. T. Hydrophobic binding properties of bovine gallbladder mucin. *J. Biol. Chem.* **1984**, *259*, 12170−12177.

(16) Böcker, L.; Rühs, P. A.; Böni, L.; Fischer, P.; Kuster, S. Fiber-enforced hydrogels: hagfish slime stabilized with biopolymers including κ-carrageenan. *ACS Biomater. Sci. Eng.* **2016**, *2*, 90−95.

(17) Shashoua, V. E.; Kwart, H. The structure and constitution of mucus substances. II. The chemical constitution of *Busycon* mucus. *J. Am. Chem. Soc.* **1959**, *81*, 2899−2905.

(18) Verdugo, P. Hydration kinetics of exocytosed mucins in cultured secretory cells of the rabbit trachea: a new model. *Ciba Found Symp.* **2008**, *109*, 212−225.

(19) Espinosa, M.; Noe, G.; Troncoso, C.; Ho, S. B.; Villalon, M. Acidic pH and increasing [Ca2+] reduce the swelling of mucins in primary cultures of human cervical cells. *Hum. Reprod.* **2002**, *17*, 1964−1972.

(20) Herr, J. E.; Winegard, T. M.; O'Donnell, M. J.; Yancey, P. H.; Fudge, D. S. Stabilization and swelling of hagfish slime mucin vesicles. *J. Exp. Biol.* **2010**, *213*, 1092−1099.

(21) Jain, G.; Starksen, M.; Singh, K.; Hoang, C.; Yancey, P.; McCord, C.; Fudge, D. S. High concentrations of trimethylamines in slime glands inhibit skein unraveling in Pacific hagfish. *J. Exp. Biol.* **2019**, *222*, jeb213793.

(22) McFarland, W. N.; Munz, F. W. A re-examination of the osmotic properties of the pacific hagfish, *Polistotrema stouti*. *Biol. Bull.* **1958**, *114*, 348−356.

(23) Currie, S.; Edwards, S. L. The curious case of the chemical composition of hagfish tissues — 50 years on. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **2010**, *157*, 111−115.

(24) Herr, J. E.; Clifford, A. M.; Goss, G. G.; Fudge, D. S. Defensive slime formation in Pacific hagfish requires Ca2+-and aquaporin-mediated swelling of released mucin vesicles. *J. Exp. Biol.* **2014**, *217*, 2288−2296.
(25) Verdugo, P.; Deyrup-Olsen, I.; Aitken, M.; Villalon, M.; Johnson, D. Molecular mechanism of mucin secretion: I. The role of intragranular charge shielding. *J. Dent. Res.* **1987**, *66*, 506−508.
(26) Verdugo, P.; Aitken, M.; Langley, L.; Villalon, M. J. Molecular mechanism of product storage and release in mucin secretion. II. The role of extracellular Ca^{+2}. *Biorheology* **1987**, *24*, 625−633.
(27) Spitzer, R. H.; Downing, S. W.; Koch, E. A.; Salo, W. L.; Saidel, L. J. Hagfish slime gland thread cells. II. Isolation and characterization of intermediate filament components associated with the thread. *J. Cell Biol.* **1984**, *98*, 670−677.
(28) Christy, M. T.; Dickman, C. R. Effects of salinity of tadpoles of the green and golden bell frog (*Litoria aurea*). *Amphibia-Reptilia* **2002**, *23*, 1−11.
(29) Böni, L. J.; Zurflüh, R.; Widmer, M.; Fischer, P.; Windhab, E. J.; Rühs, P. A.; Kuster, S. Hagfish slime exudate stabilization and its effect on slime formation and functionality. *Biol. Open* **2017**, *6*, 1115−1122.

https://dx.doi.org/10.1021/acs.langmuir.0c00639
Langmuir 2020, 36, 6681−6689