Hagfish slime exudate stabilization and its effect on slime formation and functionality

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ABSTRACT
Hagfish produce vast amounts of slime when under attack. The slime is the most dilute hydrogel known to date, and is a highly interesting material for biomaterial research. It forms from a glandular secrete, called exudate, which deploys upon contact with seawater. To study slime formation ex vivo and to characterize its material properties, stabilization of the sensitive slime exudate is crucial. In this study, we compared the two main stabilization methods, dispersion in high osmolality citrate/PIPES (CP) buffer and immersion in oil, and tested the influence of time, temperature and pH on the stability of the exudate and functionality of the slime. Using water retention measurements to assess slime functionality, we found that CP buffer and oil preserved the exudate within the first 5 hours without loss of functionality. For longer storage times, slime functionality decreased for both stabilization methods, for which the breakdown mechanisms differed. Stabilization in oil likely favored temperature-sensitive osmotic-driven swelling and rupture of the mucin vesicles, causing the exudate to gel and clump. Extended storage in CP buffer resulted in an inhibited unraveling of skeins. We suggest that a water soluble protein glue, which mediates skein unraveling in functional skeins, denatures and gradually becomes insoluble during storage in CP buffer. The breakdown was accentuated when the pH of the CP buffer was raised from pH 6.7 to pH 8.5, probably caused by increased denaturation of the protein glue or by inferior vesicle stabilization. However, when fresh exudate was mixed into seawater or phosphate buffer at pH 6-9, slime functionality was not affected, showing pH insensitivity of the slime formation around a neutral pH. These insights on hagfish exudate stabilization mechanisms will support hagfish slime research at a fundamental level, and contribute to resolve the complex mechanisms of skein unraveling and slime formation.

KEY WORDS: Hagfish slime, Stabilization, Exudate storage, Vesicle rupture, Protein glue

INTRODUCTION
Hagfish defend themselves against predators with vast amounts of slime (Zintzen et al., 2011). The slime forms when a glandular secrete, called exudate, is released from ventrolateral pores into the surrounding seawater. The exudate contains two major functional components, mucin vesicles (Fig. 1A) and thread skeins (Fig. 1B), as well as residual fluid, which is co-secreted with the skeins and vesicles. The contact with water combined with convective mixing (Winegard and Fudge, 2010) triggers the slime formation (Fig. 1C), causing the skeins to unravel and release their long, keratin-like threads. Simultaneously, the mucin vesicles swell and burst and release mucin-like glycoproteins, which interact with the threads and together form an ephemeral underwater network that physically entraps large amounts of water compared to other mucus hydrogels (Fig. 1D). The secreted slime is a unique biomaterial as it is the most dilute and fastest-forming hydrogel known to date (Fudge et al., 2005). The protein threads have similar properties to spider’s silk (Fudge et al., 2003, 2005) and its viscosifying properties in extensional flow were suggested to be beneficial when attacked by suction feeders (Böni et al., 2016b). The slime threads are of high interest as they are flexible and tough, making them a promising source for novel fibers in clothing and biomedical applications. The hagfish mucins constitute a functional and relatively easily accessible source of mucins, which could be of particular interest for research on marine mucus or serve as a model for secreted mucin-like glycoproteins.

To obtain exudate from hagfish, the surrounding area of the slime pores is mildly electro-stimulated (Movie 1). The sensitive exudate is immediately stabilized either in a high osmolality buffer or by immersion in oil. The latter method was first used by Ewaldt et al. (2011). The authors stabilized the exudate under mineral oil to study whole slime mechanics using rheology. Following a similar approach, medium-chain triglyceride (MCT) oil was found to have superior stabilization and handling properties to mineral oil (Böcker et al., 2016), mainly because handling with a micropipette is easier as the sample remains fluidized.

The stabilizing effect of a high osmolality buffer was first described by Downing et al. (1981b), who found that exudate is stabilized in 1 M (NH₄)₂SO₄. The stabilization criteria were further investigated by Salo et al. (1983), who reported that hagfish exudate is also stabilized in 0.5-1 M sodium citrate, as well as in 3 M sodium tartrate and sodium acetate, and were later systematically tested by Luchtel et al. (1991), who found that high osmolarity solutions of sulfate and phosphate were also able to stabilize the exudate. Piperazine-N,N’-bis(2-ethanesulfonic acid (PIPES, 0.1 M), PMSF (a serine protease inhibitor), as well as small amounts of EDTA, glycerol and MgCl₂ were added to the stabilization buffer by Spitzer et al. (1988). A simpler version of this buffer (0.9 M citrate/0.1 M PIPES, pH 6.7), to which 0.02% NaN₃, as well as a protease inhibitor cocktail, can be added to ensure bacterial and enzymatic stability (Böni et al., 2016b) became the widely used standard, largely favored by the facilitated separation of skeins and vesicles in citrate (Salo et al., 1983). The stabilization mechanism in buffer remains elusive, but the presence of high molarities of di- and trivalent anions was found to be important for stabilization of mucin vesicles. In addition, the stability criteria for skeins seem intimately
linked to those of vesicles. The mucin vesicles are known to be stable for days in buffers with the appropriate ion composition and an osmolarity of ≥900 mOsmol/l (Luchtel et al., 1991), but thread skeins quickly lose their ability to unravel in CP buffer (Bernards et al., 2014). The effect of ionic strength and ionic composition needed for stabilization of hagfish exudate was studied in depth to stabilize mucin vesicles (Herr et al., 2010; Luchtel et al., 1991), but the influence of time, temperature and pH during storage of both main stabilization techniques was not investigated, although crucial for hagfish slime research, because stabilization of hagfish slime is directly linked to the complex slime formation mechanism.

In this study, we applied water retention measurement as an easy, robust and quantitative method to assess hagfish slime functionality, which is intimately linked to skein unraveling. This is of particular advantage when other techniques to gauge the material properties of fluids, gels and soft solids, such as rheology, are not applicable due to difficult sample handling, low torques or inhomogeneous structures. With this method, we investigate the impact of storage time, temperature and pH on exudate functionality, and discuss putative mechanisms which reduce or even lead to a loss of the slime-forming functionality, further on referred to as degradation. We found that exudate faces different pathways of degradation when stored in oil or in buffer. Furthermore, we provide evidence that the skeins of the Atlantic hagfish (Myxine glutinosa) contain a water-soluble glue (Bernards et al., 2014), which likely denatures during storage. We propose that the denatured glue is the main cause of ceased unraveling of hagfish skeins when stored in buffer over longer times. This work will support future research on hagfish slime by facilitating the choice of stabilization method, and by describing the degradation processes during storage.

**RESULTS AND DISCUSSION**

**Water retention to assess hagfish slime functionality**

Fresh unstabilized hagfish exudate at its natural concentration (∼0.02 wt%) (Böni et al., 2016a; Fudge et al., 2005) was able to gel the whole volume of 20 ml seawater (Fig. 2A), of which subsequently ∼14 ml (initial load, IL) could be lifted (Fig. 2B). After draining for ∼1 min under the influence of gravity, the slime lost around half its initial load. Once the water has drained, the slime can hardly rehydrate and the structure irreversibly collapses (Fig. S2) because the protein threads cluster and the slime loses its sieve-like structure (Fudge et al., 2005). The IL is a good measure of the effectiveness of water absorption and allows the convenient comparison of effects such as a varying exudate concentration or structure collapse (Fig. S2). Other authors similarly used a ‘removable mass’ to assess slime functionality and investigate the influence of dithiothreitol (DTT) (Koch et al., 1991) and stirring on slime formation (Lim et al., 2006), or measured the water egress from the slime to investigate the physical water entrapment and sieve-like properties (Fudge et al., 2005). The soft and wet character of biomaterials such as hagfish slime, in particular, often imposes substantial challenges to characterization methods applied in materials science (Ewoldt et al., 2014). Assessment of material properties of hagfish slime was so far mainly done by rheological measurements (Böcker et al., 2016; Böni et al., 2016a,b; Ewoldt et al., 2011), and by measuring the water egress (Böni et al., 2016a; Fudge et al., 2005). Although rheology allows the study of
mechanics and flow behavior, the technique is limited by the softness of the slime and by potential inhomogeneities. Water retention measurements, in contrast, provide simple, but quantitative and robust information about the effectiveness of water uptake and the water-holding capacity, which are both critical criteria for hydrogel functionality. Water retention measurements therefore permit assessment of the functionality of hagfish slime, and the study of effects such as storage, which are otherwise hard to assess.

**Influence of stabilization method and time on slime functionality**

Currently there are two main approaches to stabilize hagfish exudate: immersion in MCT oil and dispersion in a high osmolarity citrate/PIPES (CP) buffer (Fig. 3A). To evaluate if one method is superior to the other, water retention measurements were performed 5 hours after sampling (Fig. 3B). Both stabilization methods resulted in an almost indistinguishable slime functionality, as all possessed water retention properties similar to natural unstabilized hagfish slime. As a third stabilization method, heptane was tested, which also resulted in water retention properties similar to the other two methods. Heptane is intriguing due to its volatility, allowing removal of the stabilant at room temperature without leaving residues, which is not possible with MCT oil and CP buffer. Although handling was more difficult and stability lasted only ~2 days, heptane is a promising method for short-term stabilization. In contrast to short-term stability, however, extended storage resulted in a loss of functionality and showed marked differences between the MCT oil and the CP buffer (Fig. 3C,D). Whereas MCT samples showed a rather sudden onset of functionality loss, the CP samples underwent a more gradual breakdown. After 5-10 days the MCT samples started to clump and did not deploy anymore when in water. The time for the onset was strongly dependent on each individual sample as often for biological samples, but also on the mechanical and temperature history of the sample. In contrast, exudate in CP buffer showed a gradual loss of functionality, which was found to be accompanied by a reduced skein unraveling. However, after a storage of 2 weeks the water retention properties markedly decreased, which could be linked to a reduced skein unraveling. From these observations it can be inferred that the principal mechanism of degradation is different for the stabilization methods. This hypothesis was further supported by the finding that the storage lifetime of a MCT sample could be extended when it was transferred to CP buffer before clumps occurred, giving it a second life. Stabilization and storage of slime exudate is crucial in hagfish slime research. We therefore studied the two putative degradation mechanisms by varying temperature and pH to determine the influence of storage on the exudate samples.

**Temperature induced mucin vesicle swelling and rupture**

Exudate (20 μl covered with 200 μl MCT oil) was heated in an Eppendorf tube in a water bath to 37, 42 and 50°C for 5 min to test the influence of temperature on hagfish exudate stability and on slime formation (Fig. 4A). Whereas heating to 37°C had a minor impact, heating to 42 and 50°C resulted in almost completely inhibited slime formation. Heating MCT exudate under a microscope revealed that the sample gelled as soon as the heat wave reached the vesicles, suggesting that the vesicles swelled and ruptured at elevated temperatures (Fig. 4B,C; Movie 2). The ruptured vesicles released their mucin, forming a firm gel with the little available liquid originating from the residual fluid. The gel fails to swell rapidly when in water and also prevents a subsequent unraveling of the gel-embedded skeins. It is likely that the surrounding MCT oil favors swelling and rupture of the vesicles.
at elevated temperatures given their putative phospholipid bilayer membrane (Luchtel et al., 1991). To investigate the existence of a phospholipid bilayer, lipid analysis was performed (Fig. 4D). Substantial amounts of phosphatidylethanolamine (PE) and phosphatidylcholine (PC), and minor amounts of cholesterol (Chol) and lysophosphatidylcholine (LysoPC), were found, adding further evidence for the presence of a phospholipid membrane (Luchtel et al., 1991). A surrounding non-polar phase such as MCT oil likely favors an exchange of membrane phospholipids with the oil phase, leading to a break in vesicle membranes. This effect is more favored at higher temperatures (Hardy et al., 2013; Seantier et al., 2005), as membranes become softer when temperatures come close to the melting temperature of the lipids (Garcia-Manyes et al., 2005). The lipid analysis also showed the presence of triglycerides (TG), diglycerides (DG) and free fatty acids (FFA). Salo et al. (1983) found that hagfish mucin vesicles contain ∼4.8% lipids. As speculated by the authors, it is likely that non-membrane lipids are needed for the formation of a functional mucin gel. A positive effect of lipids on mucins is known as they can associate to mucins by hydrophobic interactions via the hydrophobic domains of mucin glycoproteins (Gong et al., 1990; Lai et al., 2009). Thereby, lipids were found to increase the viscosity and viscoelasticity of mucin (Lai et al., 2009; Zhang et al., 2015) and support their capability to form gels (Rogunova et al., 1997). The appearance of the heated and clumped sample was similar to old (>2 weeks) MCT samples, which were also gelled. However, the clump formation during storage in MCT oil is more likely to be a slow osmotic-driven swelling and rupture of mucin vesicles, given that the residual fluid of the exudate does not have obvious stabilizing effects on vesicles (Herr et al., 2010). It should be noted that clumping can also be induced when the samples are mechanically stressed, e.g. with a micropipette.

Vesicles stabilized in CP buffer do not rupture when mechanically stressed with a micropipette, when stored in the fridge for over 6 months or when heated to 50°C. The high ionic strength of the buffer likely keeps the glycoproteins in a stiff, condensed state, which makes merging of adjacent vesicles energetically unlikely. The polyanionic nature of mucins makes their conformation highly sensitive to ionic strength (Verdugo, 2012), which strongly reduces swelling of anionic gels at elevated salt levels (Khare and Peppas, 1995; Vasheghani-Farahani et al., 1990). Also, the high ionic strength of the CP buffer could stabilize the membrane due to a binding of the buffer ions on the phospholipids (Garcia-Manyes et al., 2005). Although the vesicular structure did not visually break at 50°C, the mucin probably denatured and aggregated inside the vesicle and lost the ability to swell, as the ability to form a viscous solution when mucin vesicles were mixed with water was lost. When the sample was heated to 42°C the vesicles were still able to form a viscous solution. Furthermore, heating exudate in CP to 42°C completely inhibited skein unraveling, which was not observed at 37°C. These results are in line with the findings of Bernards et al. (2014), who still observed unraveling at 35°C for Eptatretus stoutii skeins. Our observations show also that the unraveling of M. glutinosa skeins is highly temperature sensitive. We suggest that heat sensitivity of the skeins either originates from the presence of a protein glue that denatures at elevated temperatures and thus cannot mediate unraveling anymore, or that heat causes adjacent threads to stick together, thereby inhibiting unraveling.

**Influence of pH on storage and on slime formation**

The pH of the CP buffer does not match the physiological pH values of hagfish blood (∼pH 8) (Wells et al., 1986), the residual fluid of the exudate (pH 7.3) (Herr et al., 2010), nor the pH of seawater.
We therefore investigated if an increased pH of the CP buffer has beneficial effects on storage (Fig. 5A). From the tested pH values (pH 8.5, 7.4 and 6.7), pH 6.7 (being the commonly used pH for CP buffer) showed the highest initial load, the highest degree of skein unraveling and thus the best slime functionality after 7 days of storage. In contrast, it was found that when fresh MCT oil-stabilized exudate was mixed into seawater or phosphate buffer between pH 6 and pH 9 (Fig. 5B,C; Fig. S3A), the slime showed similar water retentions, implying that the reduced functionality at higher pH originates from the storage. At pH extremes (pH 4.5 and pH 10) no functional slime formed. At low pH, the skeins did not unravel and aggregated with the mucins (Fig. S3B). At high pH, the mucins formed a tacky solution and many skeins did not unravel resulting in a weak network, which was unable to entrap water when lifted out of the water. These results show that skein unraveling is pH-dependent and that an increased pH of the CP buffer decreases the slime functionality by limiting the amount of unraveled skeins.

We have two hypotheses as to why a lower pH stabilizes the exudate better. First, pH 6.7 and possibly even lower pH values close to pH 6 (~pH 8) could be beneficial for mucin gel condensation inside the vesicle. Mucin granules are considered to have a low intraluminal pH (~pH 6) (Ambort et al., 2012; Kesimer et al., 2010; Perez-Vilar et al., 2005). A low storage buffer pH, potentially close to the isoelectric point (pI) of mucin, is likely beneficial in keeping the mucin condensed. Close to the pI the number of counterions is minimal and therefore the osmotic pressure in and around the mucins decreases, resulting in a limited swelling capacity (Flory, 1953; Khare and Peppas, 1995). A similar observation was made for mucin granules from human cervical cells, which showed a significantly decreased swelling velocity at pH 6.5 compared to pH 7.4 (Espinosa et al., 2002). Increasing the pH gradient from the CP buffer to the vesicle intragranular pH could thus result in compromised integrity of the mucin meshwork inside the granule (Perez-Vilar, 2007; Perez-Vilar et al., 2005) and cause partial swelling. These would inhibit vesicle swelling at the time of slime deployment and thus also limit skein unraveling, as both processes are suggested to be intimately linked (Luchtel et al., 1991; Winegard and Fudge, 2010). The second hypothesis is that a higher pH denatures the seawater soluble glue of the skeins faster. As most of the skeins are stripped from their plasma membrane when secreted through the slime pore (Downing et al., 1981a) they lack protection when exposed to the stabilization buffer. It is likely that the protein glue slowly denatures and becomes insoluble at elevated pH during storage.

Extended storage in buffer denatures seawater soluble glue of thread skeins

MCT oil-stabilized hagfish skeins spontaneously unraveled in seawater (Fig. 6A; Movie 3). The absence of flow and the high local viscosity due to the concentrated mucin around the skeins likely slowed the unraveling down. The spontaneous unraveling suggests the existence of a seawater-soluble glue in M. glutinosa skeins, which dissolves and mediates unraveling by releasing the stored spring energy of the stored spring energy of the thread, as similarly observed for E. stoutii skeins (Bernards et al., 2014). Further evidence for the existence of a seawater soluble glue is provided in the SEM images in Fig. 6B. The images show a skein in the process of unraveling. In the inter-thread spacings, filamentous structures could be seen, which were also observed for E. stoutii skeins (Bernards et al., 2014). At high magnification the filamentous
structures look like they are part of the rough surface of the skein, which is about to dissolve. Old skeins that were exposed to CP buffer for longer times fail to unravel in water, even in the presence of strong flow. However, when those old skeins were subjected to a trypsin solution they unraveled naturally similar to freshly harvested skeins, i.e. starting at the apical end of the skein (Fig. 6C; Movie 3) (Bernards et al., 2014). Similar to Bernards et al. (Bernards et al., 2014), we also observed that trypsin removed the glue faster than digesting the thread. We suggest that the protein glue denatures during storage and therefore becomes insoluble, being the main cause for a ceased unraveling after prolonged storage in CP buffer. Trypsin, cleaving on the C-terminal of lysine and arginine amino acid residues (Rodriguez et al., 2008) seems to be able to digest the denatured protein glue, thus re-initiating unraveling in water. It is possible that the glue is denatured by oxidation, by high ionic strength, or by other unfavorable conditions. The fact that elevated temperatures and non-physiological pH inhibit skein unraveling support the existence of a protein glue, as the protein is likely to denature. An alternative hypothesis is that adjacent loops of the thread stick to each other during storage, thereby inhibiting unraveling. The trypsin-driven unraveling would also support this mechanism as trypsin could digest the parts of the thread that are stuck together and liberate stored strain energy. Here, we note that our findings stand in slight contrast to the current theory of Winegard and Fudge (2010), who state that the unraveling of *M. glutinosa* skeins requires mucin strands and flow. Our movies revealed that seemingly neither flow nor mucin strands (the old skeins were washed with MilliQ and DTT prior to trypsin unraveling) are required for unraveling. We do not doubt the necessity of flow and mucin strands for the formation of a functional slime network, but we propose that flow and mucin strands are rather needed to propagate the spontaneous unraveling by mucin strands attaching to the unraveling threads and not for initialization of unraveling. However, further investigations are required to decouple the influence of single parameters such as flow, mucin and glue dissolution on skein unravelling, and on the complex mechanism of slime formation.

**Summary**

In this study, the two main stabilization methods for hagfish exudate, immersion in MCT oil and dispersion in CP buffer, were compared. An overview of the major advantages and disadvantages of both stabilization methods are shown in Table 1.

Using water retention measurements to assess the functionality of hagfish slime, it could be shown that for short storage times (<5 hours) both stabilization methods produced slime networks equal to fresh unstabilized hagfish exudate. As a novel stabilization method heptane was tested, which also preserved the functionality but was more difficult in handling than MCT oil as it had a drying effect on the exudate. Nevertheless, heptane could be used for applications where the stabilant is to be removed as it quickly evaporates. Therefore, depending on the purpose of exudate stabilization, a different stabilization technique has to be chosen.

Furthermore, we studied the boundary conditions necessary for a successful stabilization of exudate for laboratory experiments. Longer storage times caused the samples to degrade, and the MCT oil and CP buffer methods showed different breakdown mechanisms. MCT samples formed clumps after ~5–7 days, probably due to osmotic driven swelling and rupture of the mucin vesicles. The mucin vesicles and the thread skeins were found to be highly sensitive to elevated temperatures, which possibly accelerates vesicle rupture. Rupture is likely favored by the non-stabilizing surrounding residual fluid and by phospholipids in the membrane, as they interact with the surrounding oil phase, causing

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**Table 1. Advantages and disadvantages of the two major exudate stabilization methods**

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<tr>
<th>Advantage/Disadvantage</th>
<th>CP buffer</th>
<th>MCT oil</th>
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<tr>
<td>Advantages</td>
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<tr>
<td>Inhibition of bacterial and enzymatic degradation</td>
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<td>Accurate concentration</td>
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<td>Separation of skeins and vesicles</td>
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<td>In natural environment (residual fluid)</td>
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<td>Long-term storage of vesicles</td>
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<td>No additional ionic strength</td>
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<td>Disadvantages</td>
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<tr>
<td>High ionic strength</td>
<td>Sensitive to mechanical stress and room temperature</td>
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<td>Skeins cease to unravel</td>
<td>No bulk separation of skeins and vesicles</td>
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<tr>
<td>Difficult concentration determination</td>
<td>No inhibition of bacterial and enzymatic degradation possible</td>
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**Fig. 6. Spontaneous unraveling of *M. glutinosa* skeins is mediated by a water-soluble protein, which becomes insoluble during storage in buffer.**

(A) Spontaneous unraveling of a functional *M. glutinosa* skein surrounded by mucin vesicles in seawater in the absence of flow (MCT oil-stabilized sample). (B) SEM of *M. glutinosa* skeins that were unraveled with MilliQ water without stirring on a SEM slide (left). (Note that this image is reproduced from Fig. 1B.) The unfinished unraveling shows small bridging filaments between the thread parts (middle, closer view on right). (C) Old skein unravels in the presence of trypsin. The unraveling starts at the apical end of the skein (arrow).
leakage of the mucin and a subsequent gelation of the system. The gelled system does not form a slime anymore when in contact with seawater as the thread skeins are trapped in a dense mucin gel matrix. CP buffer-stabilized samples, on the other hand, showed a gradual loss of functionality over time, which could be linked to reduced skein unraveling. At long buffer exposure times, fewer skeins unraveled and therefore less water was retained. We propose that a seawater soluble glue, which holds the threads together and mediates unraveling, denatures during storage in the buffer and thus likely becomes insoluble. Evidence for the presence of such a glue as similarly observed for E. stoutii skeins (Bernards et al., 2014) was provided by trypsin-induced unraveling of old skeins as well as by electron microscopy images. The suggested increasing insolubility likely causes a gradual loss of skein unraveling and thus decreases slime functionality. When the pH of the CP buffer was raised from pH 6.7 to pH 8.5 the functionality further decreased. It could be shown that the negative influence of the higher pH was only observed after storage, but not when fresh exudate was mixed into seawater of higher pH. The reasons why a higher storage pH reduces slime functionality remain elusive, but it is likely that a higher pH denatures the water-soluble glue faster or has inferior stabilizing properties on the mucin vesicles.

Our findings underline the importance of rigorous cooling of MCT exudate samples and show that a stabilization buffer pH of 6.7 and possibly lower is beneficial to preserve the functionality of CP exudate samples. The observed degradation processes provide valuable guidelines for the choice of appropriate stabilization for hagfish exudate and will foster the uncovering of the complex mechanisms of slime formation.

MATERIALS AND METHODS
Exudate sampling and stabilization
Atlantic hagfish (M. glutinosa) were kindly provided by the Atlantershavsparken in Ålesund, Norway. Sampling was performed according to the approved ethical application by the Forskedsutvalget (FOTS ID 6912) based on a similar protocol to that described by Herr et al. (2010). In brief, hagfish were anesthetized in fresh seawater using a 1:9 mixture of clove bud oil (Sigma-Aldrich) to ethanol at a concentration of 1 mL/L. Once sedated, the hagfish were transferred to a dissection tray and blotted dry. Slime exudate was obtained by mild electric stimulation (80 Hz, 18 V, HPG1, Velleman Instruments, Gavere, Belgium) on the ventral side (Movie 1). The released exudate was then collected and stabilized in MCT oil (Delios V, BASF, Ludwigshafen, Germany), heptane (VWR, Oslo, Norway), or in a high osmolarity CP buffer consisting of 0.9 M sodium citrate and 0.1 M PIPES at pH 6.7, 0.02% NaN₃ (VWR, Oslo, Norway), and protease inhibitor (Sigmafast, Sigma-Aldrich). All samples were immediately stored at 4°C. The fish were transferred to a recovery bath after sampling. Import of the samples was approved by the Norwegian Food Safety and Veterinary Office (FSVO) and export was approved by the Norwegian Seafood Council.

Water retention
The cohesiveness of hagfish slime mediated by its long fibers allows the lifting of the slime mass. The slime network formation efficiency can therefore be directly evaluated by lifting up the entire slime, as the network formation is based on the ability of the threads and mucins to form a cohesive slime. Slime for water retention measurements was prepared by placing MCT- or CP-stabilized exudate on the bottom of a 20 mL glass flask with a micropipette. Sterile filtered (0.2 μm PA-20/25 filter, Machery-Nagel, Düren, Germany) seawater from Norway was poured in and mixing was performed according to Ewoldt et al. (2011), by gently sloshing the water over the sample heads eight times. Water retention measurements were performed similar to described by Bøni et al. (2016b). An in-house built mixing device (Fig. S1) was attached onto a laboratory scale and a video camera (Alpha 5100, Sony, Tokyo, Japan) was placed in front of the scale to optically monitor the weight change over time. The mixed slime was transferred from the glass flask to a small beaker and placed on the scale, and then the mixing device lowered into the slime and rotated 10 times to wrap up the slime mass. The wrapped slime was then lifted, arrested in the upper position, and the water egress was recorded gravimetrically. The exudate concentration of the measurements was determined according to the assumption of Ewoldt et al. (2011) (density of the exudate is close to 1 g/mL, as ~60% of the exudate mass is water). MCT oil samples could be accurately pipetted with a micropipette as the exudate sank to the bottom of the Eppendorf tube (Movie 1). To ensure an accurate concentration of exudate in CP buffer, exudate from MCT oil was pipetted into a defined volume of CP buffer for stability and pH tests. For pH experiments, the pH of seawater was adjusted with HCl and NaOH, respectively. All experiments were performed in triplicate.

Microscopy
Light microscopy images were captured on a Nikon Diaphot (Nikon, Tokyo, Japan) and analyzed with the NIS elements D3.0 software. Rupture of mucin vesicles at different temperatures was investigated by mounting a heating plate onto the microscope. The heating plate was preheated to the corresponding temperature for 5 min. A microscopy slide with 1 μl exudate from MCT oil and covered with a coverslip was mounted on the heating plate. The time needed for the vesicle halo to burst was used as rupture time. All experiments were performed in triplicate. The value for rupture at 4°C was determined by measuring the time needed for MCT samples to clump in the fridge. Slime unraveling with trypsin (from porcine pancreas, Sigma-Aldrich; 30,000 units/ml in 5 mM potassium phosphate buffer, pH 6.5) was performed on old skeins (8 months) that were stabilized in CP buffer and did not unravel anymore. The skeins were washed with 100 mM DTT to remove mucins and then dialyzed against MillIQ water (24 h, 3×) and freeze dried. Non-freeze-dried skeins were also investigated and showed the same unraveling behavior.

SEM
For scanning electron microscopy (SEM), fresh unstabilized exudate was placed on SEM plates and a few drops of MillIQ water were added onto the sample without stirring. The samples were air dried for 48 h and subsequently sputter coated (Bal-Tec SCD 050 sputter coater, Leica Microsystems, Wetzlar, Germany) with a 3 nm thick platinum layer. SEM was performed on a LEO 1530 (Carl-Zeiss SMT AG, Oberkochen, Germany). For cryo-SEM, heptane-stabilized exudate samples were snap frozen with a high pressure freezer (Bal-Tec HPM100, Leica Microsystems) and subsequently freeze fractured (Bal-Tec BAF600, Leica Microsystems). SEM was conducted on a LEO 1530.

Lipid analysis
Vesicles for lipid analysis were obtained similar to the protocol of Salo et al. (1983). Exudate stabilized in CP buffer was filtered through a series (60 and 20 μm) of nylon mesh filters (Merck, Darmstadt, Germany) to separate the mucin vesicles from the skeins. The vesicle solution was washed three times with CP buffer and subsequently concentrated by repeated centrifugation (2960 g for 30 min). The concentrated vesicles were then decomposed according to the protocol of Bligh and Dyer (1959). A mixture of chloroform methanol, chloroform and nanopure water, at a ratio of 2:2:1, were added to the sample while homogenizing with a colloidal mill (Ultra Turrax, IKA, Staufen im Breisgau, Germany). The homogenized sample was centrifuged (2960 g for 10 min), the chloroform phase was collected, and the contained lipids were concentrated by bubbling N₂ through the sample. Lipid classes were qualitatively analyzed by a Nexera/Prominence HPLC system (Shimadzu, Kyoto, Japan) coupled to a LTII evaporative light scattering detector (ELSD, Shimadzu) according to Olsson et al. (2012) with the following modifications. The sample was dissolved in a mixture of eluents (eluent A:eluent B=1:1 by vol.) to a 0.05-0.1 mg/ml concentration and separated on Reprosil-Pur 120 CN column (250×4.6 mm, 5 μm) equipped with a guard column (ReproSil-Pur 120 CN, 10×4.6 mm, 5 μm) (Dr Maisch GmbH, Ammerbuch, Germany) kept at a constant temperature (26.0°C). A binary gradient elution at a constant flow rate of 1.0 mL/min, and consisting of eluents A=n-hexane and B=toluene:methanol:acetic acid:
triethylamine=60:40:0.2:0.1 (by wt) was used with the following timetable: at 0.0-3.0 min 95:5 (%A:%B), at 8.0 min 60:40, at 14.0 min 50:50, at 15.0 min 5:95. The ELSD evaporation temperature was 30°C and the gas pressure (N₂) was kept at 3.20 bar. The injection volume was 10 µl. All solvents were of LC grade (Merck and VWR). The lipid classes were identified by comparison with retention times of commercial analytical standards (Avanti, NuCheck and Sigma-Aldrich).

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Competing interests
The authors declare no competing or financial interests.

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