Hagfish slime threads as a biomimetic model for high performance protein fibres

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Abstract

Textile manufacturing is one of the largest industries in the world, and synthetic fibres represent two-thirds of the global textile market. Synthetic fibres are manufactured from petroleum-based feedstocks, which are becoming increasingly expensive as demand for finite petroleum reserves continues to rise. For the last three decades, spider silks have been held up as a model that could inspire the production of protein fibres exhibiting high performance and ecological sustainability, but unfortunately, artificial spider silks have yet to fulfil this promise. Previous work on the biomechanics of protein fibres from the slime of hagfishes suggests that these fibres might be a superior biomimetic model to spider silks. Based on the fact that the proteins within these ‘slime threads’ adopt conformations that are similar to those in spider silks when they are stretched, we hypothesized that draw processing of slime threads should yield fibres that are comparable to spider dragline silk in their mechanical performance. Here we show that draw-processed slime threads are indeed exceptionally strong and tough. We also show that post-drawing steps such as annealing, dehydration and covalent cross-linking can dramatically improve the long-term dimensional stability of the threads. The data presented here suggest that hagfish slime threads are a model that should be pursued in the quest to produce fibres that are ecologically sustainable and economically viable.

(Some figures in this article are in colour only in the electronic version)

Introduction

The use of biological fibres by humans is an ancient practice—we have been spinning natural fibres into textile yarns and fabrics for over 8000 years (Jenkins 2003). The 20th century was witness to the invention of synthetic polymers like rayon, nylon and polyester, and with petroleum prices that were low and stable, sales of these synthetic polymers eventually eclipsed natural fibres and today represent two-thirds of the textiles produced worldwide. Synthetic fibres are attractive because of the high degree of quality control that can be brought to their production. For example, synthetics can be made thinner than the finest silks or thicker than the coarsest wools, and material properties can be controlled with impressive repeatability. Furthermore, continuous fibres can be made to be of virtually any length, eliminating much of the work associated with spinning and the nuisance of multiple fibre ends protruding from spun yarns.

As attractive as synthetic polymers are, their use is coming under close scrutiny due to the realization that petroleum reserves are finite and that oil prices are likely to rise steadily over the next few decades. Furthermore, with global environmental awareness at an all-time high, synthetic polymers have lost some of their lustre. Clearly, the synthetic fibre industry as it currently exists will ultimately decline and be replaced by an industry based on renewable feedstocks. The kinds of fibres that will be produced in this new industrial era (1) will be produced exclusively from renewable raw materials, (2) will not involve the use of toxic solvents, (3) will not generate toxic by-products, (4) will be completely...
including strength greater than steel and an energy to break kinds of spider silks have remarkable material properties for inspiration. In the late 1970s, it was discovered that certain properties that we have come to expect from synthetics. biodegradable and (5) will possess the consistent material properties that we have come to expect from synthetics.

One strategy for realizing this vision is to look to nature for inspiration. In the late 1970s, it was discovered that certain kinds of spider silks have remarkable material properties including strength greater than steel and an energy to break greater than most known materials (Denny 1976, Gosline et al 1999, Kitagawa and Kitayama 1997). Intense research on spider silk began with the goal of understanding it well enough to make it artificially on the industrial scale. Efforts were spurred on in the 1990s by the cloning of the first DNA sequences from spider silk genes (Guerette et al 1996, Hinman and Lewis 1992), which led to the expression of spider silk proteins (or spidroins) or parts thereof in convenient vectors such as bacteria. This work showed that spider silk protein fragments could not be spun into fibres using conventional spinning technologies (Seidel et al 1998, Winkler and Kaplan 2000), and suggested that spider silk mechanics arise in part by the complex mechanisms that occur in the silk gland and spinneret. Spider silk genes are also notoriously large and repetitive, which make them especially difficult to maintain in expression systems like bacteria (Arcidiacono et al 1998, Lewis et al 1996). The insolubility of spidroins also makes them very difficult to handle, and this is typically overcome using harsh and toxic solvents like hexafluoroisopropanol (HFIP), which is contrary to the goal of sustainable production. Unfortunately, the strong tendency of spidroins to form keratin materials like wool (Astbury and Street 1932, Astbury and Woods 1933, Bendit 1960, Paquin and Colomban 2007, Kreplak et al 2004), and more recently has been investigated for single intermediate filaments in vitro and in silico (Kreplak et al 2008, Qin et al 2010). This α → β transition occurs far more readily in hagfish slime threads than it does in hard α-keratins because slime threads lack the elastomeric matrix proteins that in α-keratins resist swelling in water and blocks of the full spidroin proteins and the formation of silk fibres that approach the mechanical performance of real spider silk (Karatzas et al 2007, Lazaris et al 2002), but neither holds much promise of delivering an economically viable product.

Interestingly, recombinant silk fibroins constructed with a modest number of identical fibroin repeats plus the native C-terminal domain are so strongly poised to form crystalline fibrils that when they are expressed in an insect cell-Baculovirus expression system, silk-like nano-fibrils form inside the insect cells where the proteins are synthesized (Ittah et al 2010). In a similar study, researchers expressed a fusion protein in bacteria that contained just four silk fibroin repeats plus the silk C-terminal domain, which were linked to a thrombin domain through a thrombin cleavage site (Stark et al 2007). Thrombin is a small, soluble protein that prevents the fibroin repeat from aggregating within the cell or once the fusion protein has been purified and concentrated. The addition of thrombin, however, releases the silk peptide, which then spontaneously aggregates to form fibres on the surface of the solution. Unfortunately, the properties of the fibres, when isolated and dried, are well below those of native spider silks.

Recent works on the mechanical properties of fibres isolated from hagfish slime suggest that these unique fibres may one day be replicated in a way that is environmentally sustainable and economically viable. These ‘slime threads’ consist of bundles of 10 nm protein nanofibres known as intermediate filaments (Koch et al 1994, 1995), which form part of the cytoskeleton in most animal cells. The genes for these proteins are far smaller than spidroin genes and not repetitive, making them more suitable for expression in bacteria. Another feature that makes them an attractive model is that they self-assemble from soluble precursors into 10 nm filaments in aqueous buffers. The key to the high strength and toughness of spider silk and hagfish threads is the β-sheet crystallites that simultaneously cross-link the protein molecules and arrange them into a structure in which ‘sacrificial bonds’ increase the energy required to break the material (Keten et al 2010, Mostaert and Jarvis 2007). Unfortunately, the strong tendency of spidroins to form β-sheet crystallites also makes them insoluble and difficult to work with (Seidel et al 1998, Winkler and Kaplan 2000). In contrast, the native state for intermediate filament proteins is α-helical coiled coils, which makes them far less likely to aggregate irreversibly and allows them to undergo self-assembly. Furthermore, stretching slime threads in water has been shown to induce a conformational transition in which the α-helices within aligned coiled coils are extended to form β-sheet crystals, which resemble those in spider silk (Fudge et al 2003). This α → β transition is well described in α-keratin materials like wool (Astbury and Street 1932, Astbury and Woods 1933, Bendit 1960, Paquin and Colomban 2007, Kreplak et al 2004), and more recently has been investigated for single intermediate filaments in vitro and in silico (Kreplak et al 2008, Qin et al 2010). This α → β transition occurs far more readily in hagfish slime threads than it does in hard α-keratins because slime threads lack the elastomeric matrix proteins that in α-keratins resist swelling in water and...
also provide a restoring force during and after longitudinal extension (Fudge and Gosline 2004).

We therefore hypothesized that slime threads stretched in water take on material properties that are similar to those of spider dragline silk. Here we present a mechanical characterization of draw-processed slime threads that is consistent with this hypothesis and which suggests that further exploration of the hagfish slime thread model is warranted.

Materials and methods

Animal care and slime collection

Slime threads from two species of hagfish were used for this study. For the tensile tests of control and stretched threads, slime threads were obtained from Atlantic hagfish (Myxine glutinosa). For all other trials, slime threads were obtained from Pacific hagfish (Eptatretus stoutii). M. glutinosa were collected at the Huntsman Science Centre in St Andrews, New Brunswick, Canada, and held at the Hagen Aqualab at the University of Guelph in artificial seawater (34%, 10 °C) and fed squid once per month to satiety (University of Guelph Animal Care Protocol 05R154). E. stoutii were collected at the Bamfield Marine Station in Bamfield, British Columbia, and held in a recirculating tank in artificial seawater (34%, 10 °C) at the University of British Columbia and fed squid once per month to satiety (UBC Animal Care Protocol A02-0003). Slime was collected from anaesthetized hagfishes as described in Fudge et al (2003).

Mechanical testing of slime threads

Individual slime threads were obtained via the controlled unravelling of thread skeins in distilled water as described in Fudge et al (2003). Unravelled lengths of slime threads were mounted on stainless steel pins in water and transferred into air by serial replacement of the water with ethanol as described in Downing et al (1984). Individual slime threads were obtained from Pacific hagfish (Eptatretus stoutii). M. glutinosa were collected at the Huntsman Science Centre in St Andrews, New Brunswick, Canada, and held at the Hagen Aqualab at the University of Guelph in artificial seawater (34%, 10 °C) and fed squid once per month to satiety (University of Guelph Animal Care Protocol 05R154). E. stoutii were collected at the Bamfield Marine Station in Bamfield, British Columbia, and held in a recirculating tank in artificial seawater (34%, 10 °C) at the University of British Columbia and fed squid once per month to satiety (UBC Animal Care Protocol A02-0003). Slime was collected from anaesthetized hagfishes as described in Fudge et al (2003).

Dimensional stability, annealing and chemical cross-linking

Individual thread skeins were transferred in their native, condensed form into a dH2O-filled chamber using a sharpened toothpick. The threads were allowed about 10 min to unravel and were tied at both ends to vertical stainless steel rods (0.3 mm diameter). One rod was mounted in a stationary Plexiglas platform and the other was mounted on a movable platform attached to a (non-rotating) micrometer spindle. Threads were wrapped around the steel rods several times and carefully tied using square knots. Annealing was accomplished by allowing the threads to remain stretched in water at a given strain for a specific amount of time. Annealing times investigated were 1, 2, 10, 30, 60 and 2000 min. Threads were slackened and taut length was measured while in water at 1, 10, 30, 100, 1000 and 10 000 min. The effects of chemical cross-linking on thread tensile mechanics and dimensional stability were assessed by exposing threads to an 8% glutaraldehyde solution for 30 min. The cross-linking reaction was stopped by replacing the cross-linking solution with dH2O.

Effects of humidity

A series of dimensional stability tests was performed at 97% relative humidity. Threads were first isolated and mounted as above, and were then stretched to strain of 1.5 in water and held for 1 min. They were then transferred into air as described above. Following their isolation into air, threads were sealed in their chamber in the presence of a saturated potassium sulfate solution at 20 °C, which maintained a constant relative humidity of 97% (Rockland 1960). Thread length was measured at 1, 10, 30, 100, 1000 and 10 000 min.

Results

Draw transformation increases strength, reduces extensibility

The data presented in figure 2 show the properties of the native, hydrated slime threads, and dried slime threads in the unstretched and stretched states (multiple curves). Note that native slime threads are very soft, which allows them to stretch to more than double in length with little applied stress, but they show a dramatic strain-hardening beyond a strain of 1.0, which is likely caused by the onset of stretching the protein backbones within the constituent intermediate filaments and the formation of β-sheet crystals (Qin et al 2010, Fudge et al 2003). Drying dramatically changes the properties of slime threads, and slime threads that were stretched in water to a strain of 1.0 and then dried begin to show properties that resemble those of spider silk. Clearly, draw processing of
Table 1. Average diameter and material properties of control (native, unstretched, dry) slime threads \((n = 9)\) compared to threads stretched to a strain of 1.0 in water \((n = 7)\) and tested in air. Variability in tensile mechanics for the threads tested can be seen in figures 1 and 2. Asterisks denote a significant difference between control and stretched threads according to a \(t\)-test \((p < 0.05)\).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Diameter ((\mu m))</th>
<th>Stiffness (E_{int}) (GPa)</th>
<th>Breaking stress (MPa)</th>
<th>Breaking strain ((\Delta L/L_0))</th>
<th>Strain energy ((MJ m^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.27</td>
<td>8.91</td>
<td>467</td>
<td>1.20</td>
<td>306</td>
</tr>
<tr>
<td>Stretched</td>
<td>1.07</td>
<td>7.99</td>
<td>(*706)</td>
<td>(*0.36)</td>
<td>169</td>
</tr>
</tbody>
</table>

Table 2. Average material properties for slime threads cross-linked with 8% glutaraldehyde and tested dry at their native \((strain 0.0)\) \((n = 4)\) or stretched \((strain 1.5)\) \((n = 3)\) state.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Stiffness (E_{int}) (GPa)</th>
<th>Breaking stress (MPa)</th>
<th>Breaking strain ((\Delta L/L_0))</th>
<th>Strain energy ((MJ m^{-3}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glutaraldehyde, strain 0.0</td>
<td>3.6</td>
<td>800</td>
<td>0.45</td>
<td>200</td>
</tr>
<tr>
<td>Glutaraldehyde, strain 1.5</td>
<td>10</td>
<td>1200</td>
<td>0.3</td>
<td>160</td>
</tr>
</tbody>
</table>

Figure 2. Tensile stress–strain curve for six draw-processed hagfish threads. Threads were stretched to a strain of 1.0 in water and tested in air. Stress–strain curves for native slime threads tested in water and native threads tested in air are provided for comparison. Wet curve is from Fudge et al (2003) and dry native curve is a typical stress–strain curve from this study. The behaviour of dry native threads is in perfect agreement with the mechanics described in Fudge and Gosline (2004).

the hydrated threads made the dried threads stronger and less extensible than control (i.e. native, unstretched) dry threads (table 1). Breaking stress increased by about 50% on average, and extensibility was reduced to about 30% of the average value for control threads (table 1). The variability of breaking stress values was quite high, with some threads exhibiting values as low as 0.15. This variability was likely due to the difficulties of transferring the threads from water to air, which may have introduced additional strain in some threads. Stiffness values between control and stretched threads were not statistically different \((t\)-test, \(p \gg 0.05)\) (table 1).

Dimensional stability of draw-transformed and annealed slime threads

Threads stretched to a strain of 1.5 and annealed for 1 or 5 min in water exhibited some dimensional stability over the course of minutes to hours, but over the course of weeks, threads returned nearly completely to their pre-stretch length (figure 3). Threads held for annealing times of 30 min or greater exhibited greater dimensional stability, with the longest annealing times \((2000 \text{ min})\) leading to the greatest degree of dimensional stability, with thread length decreasing by only about 5% over the course of the experiment (figure 3).

Lower humidity improves dimensional stability

Threads annealed for 1 min in water and then held in air at 97% humidity exhibited far less change in length over time than threads in water. Over the course of 10 000 min, stretched threads decreased in length on average by about 10% (figure 4).

Chemical cross-linking effects on dimensional stability and mechanics

Threads that were cross-linked with glutaraldehyde in the stretched state exhibited superlative dimensional stability, with threads contracting by negligible amounts over the course of 10 000 min of immersion in water (figure 5(A)). Chemical cross-linking of native threads with glutaraldehyde created threads that are very tough (table 2), with stress–strain curves that resemble the curves for some of the draw-processed threads (figures 2, 5(B)). Cross-linking of draw-processed threads created threads that are stiffer, stronger and less extensible than threads that were cross-linked in the native state without a pre-stretch step (figure 5(C)).

Discussion

Draw-processed slime threads resemble spider dragline silk in their mechanics

Tensile tests in air of control threads obtained from \(M. glutinosa\) slime yielded stress–strain curves that were nearly identical to those from \(E. stoutii\) previously published in Fudge and Gosline (2004). This suggests that the material properties of the threads do not differ substantially among hagfish species, although more species will need to be tested to fully support this generalization.
Slime threads from *M. glutinosa* that were stretched to a strain of 1.0 in water and tested in air exhibited material properties that were similar to those of spider dragline silk (figure 6). The variability in strength and extensibility of threads that were draw-processed in this way can likely be attributed to variability in the amount of strain to which each thread was subjected. Some threads were likely inadvertently strained more during the isolation of the thread into air, and were therefore the least extensible and the strongest. Threads strained less were more extensible and weaker, but also tougher. These results suggest that the mechanical properties of the threads are tunable based on the magnitude of strain achieved during draw processing.

Processing can improve dimensional stability of slime threads

While draw processing led to stronger, silk-like threads, these fibres were not dimensionally stable in water over the course of weeks, eventually returning nearly completely to their pre-stretch length. This attribute would be far from acceptable for an industrial textile material, so we investigated ways in which this long-term dimensional instability could be abolished or at least mitigated. Holding threads in the stretched state for varying amounts of time revealed that an annealing step could be used to improve the dimensional stability of hagfish slime threads, with times exceeding 30 min giving the best results. Keeping the fibres in air at high humidity rather than in water reduced the rate and magnitude of thread contraction, suggesting that an excess of water is required for the recovery of the pre-stretch protein conformations in the thread. To take advantage of this effect, an industrial fibre based on the slime thread model would need to be coated after draw processing to prevent it from re-hydrating and changing length. Cross-linking adjacent lysine groups with glutaraldehyde was even more effective at preventing the threads from returning to their pre-stretch dimensions. The two intermediate filament proteins that make up hagfish slime threads (called α and γ) (Koch *et al* 1994, 1995) contain about 40 lysine residues in total, most of which occur in the central rod domain of these proteins. In light of this fact, it is not surprising that...
Figure 5. Covalent cross-linking with 8% glutaraldehyde had dramatic effects on thread stability and extensibility. (A) Threads cross-linked at a strain of 1.5 exhibited negligible changes in length over the course of 10 000 min (note the semi-log scale). Cross-linking increased the stiffness and reduced the extensibility of both native (unstretched) threads (B) and threads cross-linked at a strain of 1.5 (C).

glutaraldehyde is an effective cross-linker of slime threads. In contrast, spider silks contain little to no lysine and therefore improving their dimensional stability with aldehyde cross-linkers is not an option. Overall, these results suggest that a combination of annealing and light covalent cross-linking might be a prudent strategy for improving the dimensional stability of draw-processed fibres made from intermediate filaments.

The post-stretching dimensional stability of hagfish slime threads differs markedly from that of hard $\alpha$-keratins, a group of mammalian biomaterials that are reinforced with intermediate filaments and include fibres like wool and hair. Dimensional stability in wool and hair is well studied due to strong interest from the textile (wool) and cosmetic (hair) industries (Speakman 1929, Farnworth 1957, Feughelman et al 1959). Setting of hard $\alpha$-keratin fibres is known to require steam and/or the presence of disulfide reducing agents like $\beta$-mercaptoethanol. These agents are thought to disrupt bonds within the elastomeric network of high-sulphur proteins, or ‘matrix’ in which the intermediate filaments in $\alpha$-keratins are embedded, and also may break disulfide bonds within and between the intermediate filament proteins (Feughelman et al 1958, Arai et al 1996). The matrix resists not only circumferential swelling of the intermediate filaments in water, but is also responsible for their ability to recover completely from large tensile strains (Fudge and Gosline 2004) over short timescales (on the order of seconds). Slime threads lack a matrix and as a result exhibit mechanical properties (such as initial stiffness ($E_{int}$)) that are far more hydration sensitive, and they are also far slower to recover from post-yield deformations. Slime threads are also incapable of forming disulphide bonds, because the two slime thread proteins from Pacific hagfish ($E. stoutii$) possess only one cysteine residue between them. These differences between slime threads and hard $\alpha$-keratin fibres make it unlikely that lessons learned from studies of the permanent set of wool and hair will be useful for understanding dimensional stability in slime threads.

Future prospects

One of the obstacles to producing mass quantities of spider silks is that the genes for spidroins are massive, which precludes the expression of full-length genes in bacteria, one of the cheapest and most efficient protein expression systems available. Hagfish slime thread proteins are only about one fifth as large as spidroins and therefore may be amenable to
mass production in bacteria. The 10 nm filaments that make up slime threads belong to a diverse family of cytoskeletal filaments known as ‘intermediate filaments’. Recent work on intermediate filaments suggests that their mechanical properties are similar among filaments from a wide range of tissue types (Kreplak et al 2005). This suggests that there are many possible genes to choose from (70 in the human genome alone (Szeverenyi et al 2008)) when optimizing the expression of intermediate proteins in systems like bacteria. Some, like the type III intermediate filament protein vimentin, are considerably smaller than the two proteins that make up slime threads (α and γ) and have the additional advantage of being constructed from a single protein instead of two such as the heteropolymERIC filaments in slime threads.

Assuming that it is possible to express large quantities of intermediate filament proteins in cheap expression systems like bacteria, spinning those proteins into fibres with useful material properties will also be a challenge, as it has been for those working on artificial spider silks. The fact that intermediate filament proteins self-assemble into 10 nm wide nanofilaments in aqueous solutions may be an important asset of the intermediate filament model. In addition, the secondary structure of intermediate filament proteins is dominated by an α-helical rod domain, which very readily forms coiled coils with the proper polymerization partner. The introspective nature of hydrogen bonding in α-helices makes these proteins less likely to aggregate with other proteins than the far more gregarious spidroins, which have a strong tendency to form β-sheets and β-sheet crystals. The spinning process for artificial slime threads therefore only needs to align the α-helices and arrange the proteins into a coherent fibre. Once these conditions are met, subjecting the proteins to modest stresses that exceed the yield stress (about 3 MPa) (Fudge et al 2003) during draw processing should open up the α-helices and allow for the formation of β-sheets among adjacent proteins and the formation of a strong, tough protein fibre.

Future work will focus on a more detailed characterization of the effects of cross-linking and annealing on fibre mechanics and structure, both in native slime threads and those that are produced from recombinant or isolated protein. In addition, we are optimistic that recently applied in silico techniques for understanding the structure and mechanics of intermediate filaments and silks (Qin et al 2010, Keten et al 2010) will yield new insights into the behaviour of native and artificially spun slime threads and will assist in the design and optimization of novel protein-based materials with useful physical properties.

Conclusions

The most important result from this study is that hagfish slime threads can be draw processed in water to yield fibres that are similar to spider dragline silk in their material properties. Post-draw modifications such as annealing and covalent cross-linking can be used to further improve the material properties. These results suggest that hagfish slime threads may prove to be a useful model for biomimetic efforts to produce protein fibres that exhibit high performance and environmental sustainability.

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