

RESEARCH ARTICLE

A Leishmania secretion system for the expression of major ampullate spidroin mimics

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Abstract

Spider major ampullate silk fibers have been shown to display a unique combination of relatively high fracture strength and toughness compared to other fibers and show potential for tissue engineering scaffolds. While it is not possible to mass produce native spider silks, the potential ability to produce fibers from recombinant spider silk fibers could allow for an increased innovation rate within tissue engineering and regenerative medicine. In this pilot study, we improved upon a prior fabrication route by both changing the expression host and additives to the fiber pulling precursor solution to improve the performance of fibers. The new expression host for producing spidroin protein mimics, protozoan parasite *Leishmania tarentolae*, has numerous advantages including a relatively low cost of culture, rapid growth rate and a tractable secretion pathway. Tensile testing of hand pulled fibers produced from these spidroin-like proteins demonstrated that additives could significantly modify the fiber's mechanical and/or antimicrobial properties. Cross-linking the proteins with glutaraldehyde before fiber pulling resulted in a relative increase in tensile strength and decrease in ductility. The addition of ampicillin into the spinning solution resulted in the fibers being able to inhibit bacterial growth.

Introduction

Silk is a natural composite fiber containing protein at its core [1] and has been used in a wide array of applications from traditional textiles to medical devices. While most of the silk used within commercial applications has been traditionally collected from silkworms, other naturally produced silk fibers, such as those produced by spiders, have been shown to have relatively superior and unique mechanical properties.

Spiders can actually produce up to seven different silk fiber types that are each specialized for specific applications within their natural environment [2]. This variety in mechanophysical properties makes spider silk fibers attractive for new biomaterials development [3]. Dragline silk, which makes up the reels within orb weaver webs, is the most characterized spider fiber and has been shown to display specific tensile characteristics that are higher than the properties of steels and other man-made materials [4,5].

The core of a dragline fiber is composed almost exclusively of the major ampullate spidroins 1 and 2 (MaSp1 and MaSp2) that self-assemble into a fiber [6,7]. Spidroins remain concentrated in solution within the duct before being drawn through the spinneret as a solid fiber. It has been postulated that the N-terminal and C-terminal domain regions (NTD and CTD, respectively) of MaSP1 and MaSP2 aid in maintaining the solubility and in regulating the self-assembly process while the central block repeat units, that are flanked by the NTD and CTD, contribute largely to the overall strength of the molecule [2,8,9].

A limiting factor to realizing commercial products made from native spider silk has been the intractability of harvesting silk directly from spiders [10]. One method to incorporate spider silks into commercial products is to fabricate fibers from recombinantly-produced spidroins or spidroin-like proteins. This approach, however, is dependent upon successful cloning of the highly-repetitive central block repeat coding regions and many researchers have addressed this complication by assembling multiple copies of synthetic block repeat domains [9,11]. These block repeat domains, alone and in various combinations with NTD and/or CTD, have been expressed in heterologous prokaryotic and eukaryotic expression systems including, but not limited to, bacteria [12,13], yeast [14], insect cell lines [15,16], and plants [9,17,18,19], albeit with varying levels of success. Most heterologous expression systems, however, have been plagued by low expression levels for a variety of reasons. These include instability of cloned repetitive nucleic acid sequences (rearrangements/deletions, [20]), translational pausing [21,22], depletion of amino acid and/or tRNA pools (due to highly-repetitive protein sequences, [23]), and low solubility [24,25].

Leishmania are single-celled eukaryotic insect vector parasites that naturally secrete various proteins and protein polymers/gels including a high molecular weight phosphoproteoglycan that accumulates in the insect midgut and is the vehicle for parasite transmission to the animal host [26,27]. Another particularly abundant secreted protein is an invertase [28] that has been reported to contribute to the availability of metabolizable sugars from plant-derived polysaccharides present in the sandfly midgut [29]. It has been demonstrated that invertase secretion is directed by an N-terminal signal sequence (SS, [28]), that the signal sequence functions efficiently in secretion of recombinant invertase, and that the SS is absent from the mature protein [28]. Previous work has also shown that the free-living insect vector stage (promastigote) grows rapidly in a simple synthetic medium to high density [30]. Because of the availability of a characterized protein secretion system, relatively simple culture conditions, and the inability of *L. tarentola* to infect mammals (the natural host is the gecko), we chose to explore the use of Leishmania as a possible new recombinant expression system for spidroin mimics.

Materials and methods

Leishmania strain and vectors

For the purposes of this study, we used *Leishmania tarentolae* which is a species that infects reptiles such as geckos [31]. Recombinant major ampullate spidroins 1 and 2 (rMaSp1 and rMaSp2) coding regions were designed for expression in the Leishmania vector *pKSNEO*. A 6-histidine (His₆) tag was placed on the C-terminal end of the deduced protein sequence and a secretion signal from *L. mexicana* invertase was added to the N-terminal end of the deduced

protein sequence. These allowed for secretion and affinity purification of the recombinant protein. Secretion expression of the mini spidroin mimics was chosen for several reasons. First, spidroin proteins are natively secreted into the silk gland. Second, secretion simplifies protein purification. And third, the accumulation of a non-native protein inside the *Leishmania* cells, that could have serious deleterious consequences, was avoided. The *Leishmania* used in this expression study were *Leishmania tarentolae* J101 (Jena Bioscience). The nomenclature follows Clayton et al. [32]. Plasmid *pKSNEO* was chosen as the expression vector for secretory spidroin mimics based on the previous successful use of *pKSNEO* for recombinant *Leishmania* secretory protein expression [28,33,34]. The shuttle vector *pUC19* (New England Biolabs) was also used in this study to aid in the molecular cloning process.

Routine cell line maintenance

Leishmania cell lines were routinely passaged in M199 media (Sigma cat #M2520-1L) supplemented with fetal bovine serum (FBS) at a 10% (v/v) final concentration, adenosine, pen/strep, folic acid, hemin, glutamax, and bicarbonate. From a max density culture ~10⁸ cells/mL, 100–200 µL of culture was passaged into 5 mL of media with or without appropriate drug selection.

Transformation/transfection of bacteria and *Leishmania*

Electroporation methodologies were used to introduce nucleic acids into both *E. coli* [35] for cloning purposes and into *Leishmania* [28] to generate spidroin mimic cell lines. *E. coli* cells were plated on LB+ampicillin plates (100 µg/mL ampicillin) for selection and colonies were screened by mini-plasmid prep isolation [36], restriction enzyme digestion and sequencing. To establish *Leishmania* cell lines, the drug G418 was added to the M199+10%FBS (fetal bovine serum) growth media at 10 µg/mL initially. Cell lines were passaged routinely and the drug concentration was increased to 50 µg/mL gradually over a one-month period.

MaSp1 and MaSp2 mimic constructs. The *Nephila clavipes* NTD and CTD sequences used for MaSp1 and MaSp2 expression constructs were previously isolated and described [9,37]. Generation of synthetic MaSp1 and MaSp2 consensus block repeat domains and the assembly of NTD-R#-CTD units (where R# symbolized the number of block repeats) has also been described elsewhere [9,37]. A His₆ tract followed by an SpeI site was added to the end of the CTD by polymerase chain reaction (PCR). A secretory signal sequence (SS) domain encoded by the *L. mexicana* invertase gene (amino acids 1–22,) was amplified by PCR (primers detailed in Table 1) using *pKSNeo::LmexINV-HA* [28] as a template and cloned upstream of the NTD. The entire SS-NTD-R8-CTD-His₆ units for MaSp1 and MaSp2 were independently cloned into *pKSNEO* as SpeI fragments. This is diagrammatically shown in Fig 1 with the utilized restriction enzymes indicated. Expression and secretion of recombinant protein (including removal of the signal peptide) should result in an N-terminal K-S-R-T-P-G upstream of the native mature MaSp1 or MaSp2 NTD sequence [28].

Protein production

To produce protein for fiber production, 500 mL of M199+1% FBS and 50 µg/mL G418 was seeded with 5 mL of culture at max density in a 2 L shake flask. After one week of growth at

Table 1. Primers used in the construction of plasmids containing *L. mexicana* invertase secretion signal. The underlined DNA sequences indicate the addition of restriction sites to the primer for use in the molecular cloning process.

Primer Name	Sequence (5' to 3')
F LmexINVSS	GGATCCACTAGTATGCGCCGCGGGGTCATTCTGC
R LmexINVSS	GTCGACACTAGTGCGCCGCTCTAGACTTTACAAGGGCGCCTGC

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Fig 1. Construct diagram. A diagram highlighting the major landmarks for the construction of *pKSNEO* MaSps. Between the restriction sites *SpeI* and *XbaI* a *Leishmania* secretion signal was devised (Green labeled box). Between the restriction sites *XbaI* and *AgeI*, the N-terminal domain (NTD) was inserted (Blue labeled box). Between the restriction sites *AgeI* and *NgoMIV*, the repeat domain region was inserted (Purple labeled box). Between the *NgoMIV* and *XhoI* restriction sites, the C-terminal domain (CTD) was inserted (Orange labeled box). Lastly, between the restriction sites *XhoI* and *NotI/SpeI* a *His₆* tag was inserted (Red labeled box).

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26°C and 150 rpm, cell cultures were centrifuged at 3500 rpm (~2000 xg) for 15 minutes to pellet the cells. The liquid supernatant was then run through a column containing 1 mL of Ni beads (Roche Product# 05893682001) by gravity flow, the column was washed and bound protein eluted with elution buffer as directed by Roche protocol. Aliquots of elutions were kept for SDS-PAGE analysis and the remainder of each elution could be dialyzed against 5 mM ammonium bicarbonate. The samples, dialyzed or not, were then frozen at -80°C and subsequently lyophilized to powder (0.08 mBarr, -40°C; Labconco FreeZone 2.5).

Protein detection using Coomassie and western blot techniques

Protein samples were electrophoresed on 10–12% SDS-polyacrylamide gels and either stained with 0.1% Coomassie Blue R250 or transferred to PVDF membrane.

Spidroin mimic proteins were detected using a 1° rabbit anti-NTD antibody [9], an AP-conjugated 2° goat anti-rabbit/mouse antibody and Lumi-Phos WB alkaline phosphatase solution (Pierce, Cat. No. #34150) for visualization with a Fujifilm LAS-1000plus imager.

Hand-pulled fiber production

Lyophilized protein powder (~0.25–0.5 g) was then dissolved in 2 mL of a spin solution (80 mM urea, 0.5 mM Tris, 5 mM NaH₂PO₄, 10 mM NaCl, pH 5)[38]. Gellan gum solution (0.5% in water) was kept at 55°C to maintain a liquid state. To pull fibers, 10 to 20 µL of protein solution was placed on Parafilm next to 100 to 200 µL of 0.5% gellan gum solution. Then, using forceps, the gum was collided with the protein droplet and fibers were pulled out from the interface. Pulled fibers were allowed to dry at RT on wooden applicator sticks (Fisher Scientific).

Hand-pulled fiber production using glutaraldehyde as a cross-linking agent

Protein powder (~0.25–0.5 g) was added to a 10% glutaraldehyde solution (100–200 µL) and allowed to react at RT for a few hours to overnight. After the incubation period, 10 µL of the solution was added to 50 µL of spin solution and fibers were pulled as described above using Parafilm.

Hand-pulled fiber production with ampicillin addition

To add ampicillin to the fiber production methodologies described above, ampicillin was added to the 0.5% gellan gum solution at 10 mg/mL.

Mechanical property characterization

The mechanical properties of the fibers were determined using tensile tests with a Bruker CETR UMT2 system. Specimens were sectioned from larger fibers using scissors and then mounted onto 9 cm² paper chads with a 1 cm² opening in the center using superglue such that the initial length of the fibers were 1 cm. [39]. Immediately prior to testing, the paper chad was cut to leave the fiber as the sole connection between the top and bottom of the chad. The engineering stress, engineering strain, Young's modulus and toughness for each fiber were calculated to take into consideration the average specimen diameter.

Before the tensile tests were performed, the general shape, diameter and evenness of the fibers was characterized (Nikon SMZ1500 with a Q Imaging MicroPublisher 3.3 RTV camera, 11.25 X magnification). After tensile testing, the fracture surfaces were imaged using both optical and electron microscopy to inform the analysis of the tensile data with observational break point phenotypes.

Electron microscopy of pulled fibers

To more accurately assess the fiber surface and breakpoint morphologies, electron microscopy was used. The fibers were attached to a chad at a 45 degree angle such that the break point could be visualized by SEM without a need to tilt. Once attached the samples were coated with platinum by sputter coating for 5 minutes (~5–7 nm thick coat). SEM was performed on an SF4800 High Resolution Scanning Electron Microscope (Hitachi) at the Clemson University Electron Microscopy Facility. Magnification was performed up to 2000X for the images taken.

Growth inhibition assays

Two assays were used to test the ability of the fibers produced with ampicillin-supplemented gellan gum to inhibit growth of bacteria cells. In one, LB liquid medium was inoculated with a single colony of *E. coli* JM109 and was split into two tubes. A fiber produced in the presence of ampicillin was added to one tube and a fiber produced in the absence of ampicillin was added to the other tube (control). After overnight incubation at 37°C (~200 rpm), cultures were observed for bacterial growth. The second inhibition assay used LB solid medium onto which *E. coli* JM109 cells were spread. After the plates were spread, fibers produced in the presence or absence of ampicillin were placed on the plates. After overnight incubation at 37°C, the plates were evaluated for growth.

Results

Creation of *Leishmania tarentolae* pKSNEO::SS-MaSpR8-His₆ cell lines

The first goal of this study was to create a cell line of *Leishmania tarentolae* capable of secreting mini-spidroin protein mimics. Two expression cassettes were assembled in pKSNEO, one each for MaSp1 and MaSp2, containing eight copies of the respective block repeat domain, as described in the Materials and Methods section (Fig 1). The N-terminus of the protein coding region was modified to include a secretory signal domain from the *Leishmania mexicana* invertase [28] to enable secretion of the spidroin mimics into the culture medium.

Extraction/purification of recombinant R8 mini-spidroins from culture

Successful secretion of mini-spidroin proteins would enable us to put our protein of interest in the culture media liquid. To reduce the amount of unwanted protein within the culture, the media was supplemented with 1% (v/v) FBS instead of the typical 10% added to most routine *Leishmania* promastigote cultures. To purify the protein, the full 500 mL volume of centrifuged culture supernatant was passed over a single Ni column (1 mL bed volume). After washing, the protein was eluted in a final volume of 5 mLs. Subsequently, the protein eluate was optionally dialyzed and then lyophilized to produce a white powder. Fig 2A shows Coomassie Blue results of a typical purification of recombinant spidroin proteins and indicates the relative amounts of total protein present in collected fractions during the protein purification. As the protein is purified on the column, multimers were detected (lanes 4 and 5). Immuno-detection of protein in the culture supernatant from *L. tarentolae* cells transfected with the *pKSNEO* SS: MaSpR8:His₆ constructs by anti-NTD western blotting after nickel ion: His₆ tag affinity purification demonstrated proper secretion (Fig 2B).

Morphology and mechanical properties of the hand-pulled fibers

Fibers (Fig 3) were successfully produced using interfacial polyion complexation based on Meier and Welland (2007)[40]. The different treatments used for fiber pulling are summarized in Table 2. It was determined that straight fibers could be produced if the fibers were dried with attachment points at the top and bottom. If allowed to dry while hanging with only one attachment point, the fiber would twist and curl (Fig 3B). Optical analysis of the individual hand-pulled fibers showed that the average diameters ranged from 20–150 μm among the fibers. The variation along a single fiber was much smaller, suggesting that the variance in fiber diameter could be strongly influenced by variability in manual pulling style and speed for each fiber.

The tensile results for each sample type can be used to compare the influence of each processing variation on fiber mechanical properties (Fig 4). The number of specimens varied between each fiber type since specimens showing evidence of ‘slip’ from the superglue during testing were excluded. In general, fibers made from the MaSp1 and MaSp2 proteins (Fig 4A

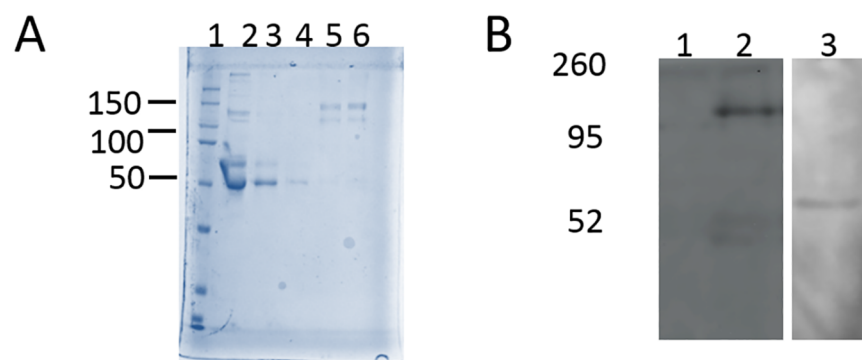


Fig 2. Coomassie Blue and western blot detection of recombinant spidroin mimics. A, Coomassie Blue-stained 10% SDS-PAGE gel analysis of rMaSp2R8 purification steps. Lane 1, molecular weight markers (kDa); Lane 2, culture supernatant; Lane 3, flow through; Lane 4, pooled washes; Lane 5, pooled elutions; Lane 6, column matrix after purification; B, Immuno-detection of rMaSp1R8 and rMaSp2R8 using MaSp NTD-specific antibody. Lane 1, *Leishmania* cells without the addition of any of the *pKSNEO* MaSp vectors; Lane 2, *Leishmania* cells which express protein from the *pKSNEO* MaSp1R8 vector system; Lane 3, *Leishmania* cells which express protein from the *pKSNEO* MaSp2R8 vector system. Molecular weight markers are shown to left (kDa).

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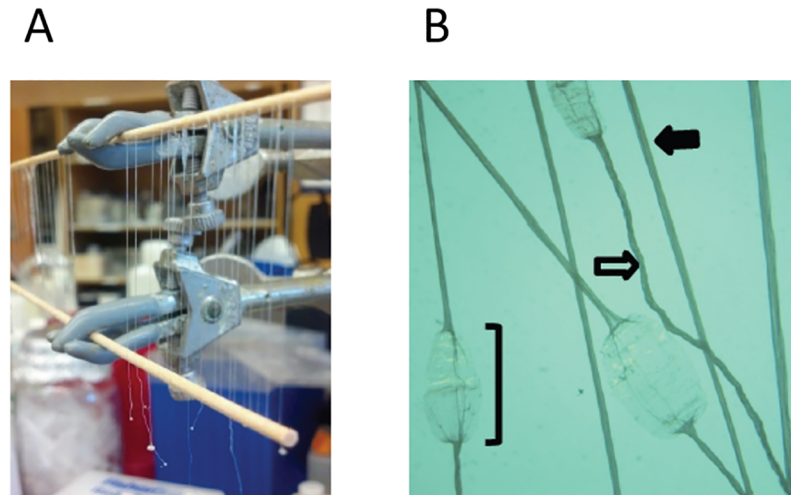


Fig 3. Hand-pulled fiber production. A, a photo of recombinant MaSp fibers drying when attached at both ends; B, a stereoscope image indicating the basic morphology of fibers produced. The solid arrow indicates a fiber that was attached at both ends when dried. The open arrow indicates a fiber that was only attached at one end and allowed to dry while suspended. The bracket indicates an attachment point where a fiber was dried and flattened due to surface adherence.

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and 4B), displayed much greater elongation prior to failure than equivalent fibers made with the crosslinking agent glutaraldehyde or with ampicillin (Fig 4C–4F). Table 3 quantifies this difference and the other tensile parameters (Young’s modulus, breaking stress, breaking strain and toughness).

Fracture surface characterization

The break points from the tensile tests were observed under a microscope. The breakpoint morphology varied in appearance under stereoscope microscopy indicating that fibers had different breaking mechanisms. Electron microscopy revealed a clean/smooth break for the glutaraldehyde-treated samples and a torn break for the untreated samples (Figs 5 and 6). Fig 5 contains representative SEM images of break points for the rMaSp1R8 fibers produced while Fig 6 contains representative SEM images of break points for the rMaSp2R8 fibers. The smooth breaks are consistent with the proposed hierarchical structure of spidroin proteins in fibers [41]. The clean break morphology of the glutaraldehyde samples indicates that these fibers were much more brittle than the untreated fibers. Additionally, it has been observed that the rMaSp1R8 fibers tend to form a fiber shaped like a celery stalk (C-shaped) while the rMaSp2R8 fibers tend to be round in shape. Salt crystals could be observed on the outside and

Table 2. Summary of spidroin-like fibers produced. This table shows the naming scheme used within this manuscript. Check marks indicate the presence of gellan layering and additives in the fiber pulling process.

Abbreviated Fiber Name	Gellan layering	Glutaraldehyde pretreated	Ampicillin addition to gellan
MaSp1R8 Only	✓		
MaSp2R8 Only	✓		
MaSp1R8 & Glut	✓	✓	
MaSp2R8 & Glut	✓	✓	
MaSp1R8 & Amp	✓		✓
MaSp2R8 & Amp	✓		✓

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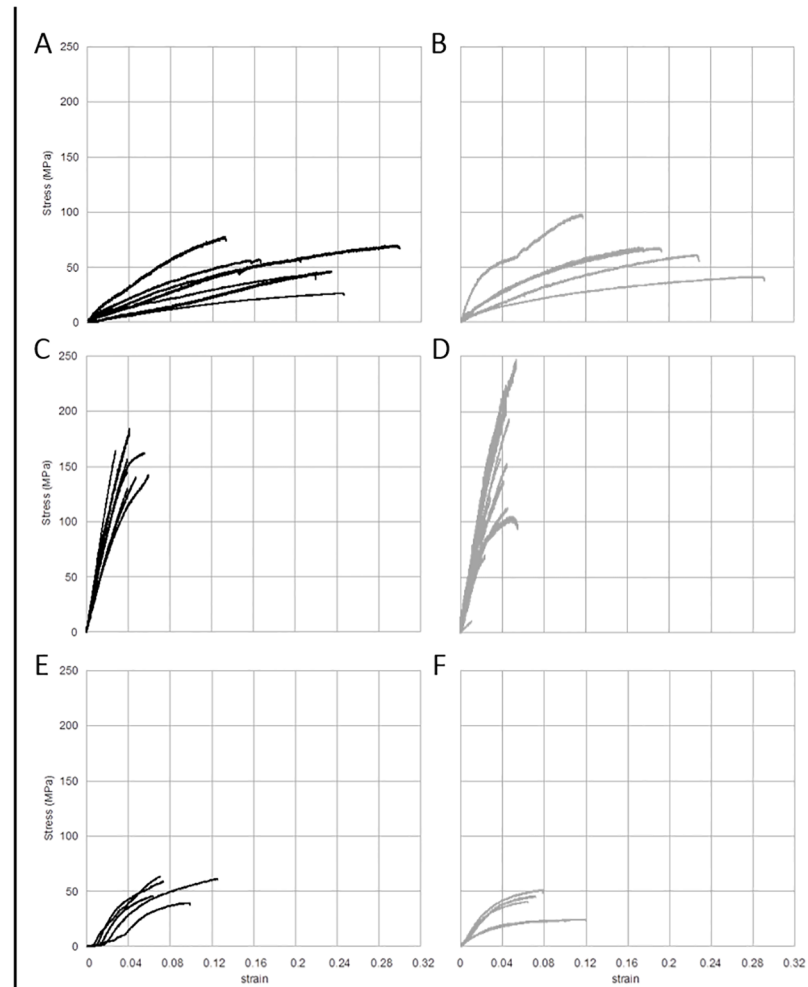


Fig 4. Stress/Strain curves for MaSp1R8 and MaSp2R8 hand-pulled fibers. Stress/Strain curves produced from tensile measurement of hand-pulled fibers. A, Sp1R8 only; B, Sp2R8 only; C, Sp1R8 plus glutaraldehyde; D, Sp2R8 plus glutaraldehyde; E, Sp1R8 plus ampicillin; F, Sp2R8 plus ampicillin. Individual lines in each panel represent replicates.

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inside of many of the samples in which dialysis was not performed and in the ampicillin treated fibers (Figs 5 and 6).

Inhibition of bacterial growth with ampicillin-enhanced fibers

Two *in vitro* approaches, one using solid medium, one using liquid, were devised to assess whether the ampicillin-enhanced recombinant silk fiber mimics could inhibit growth of bacteria. Both approaches would determine whether the antibiotic remained active as a component of the fiber and if the antibiotic would be able to disperse from the fiber. In the first approach, spidroin fiber containing ampicillin was placed on an LB agar plate spread with *E. coli* bacteria. The LB agar plate approach showed a definitive zone of inhibition around the ampicillin-enhanced fibers when compared to control fibers (Fig 7A and 7B). A similar result was found using liquid culture inoculated with *E. coli*. A single fiber proved very effective in inhibiting bacterial growth (Fig 7C).

Table 3. Tensile testing of spidroin-like fibers. Mean values for tensile properties and the corresponding standard deviations (STDEV) were recorded. Units for each parameter in the table are described in the column headers. Data for *N. clavipes* major ampullate (MA) silk comes from Gosline et al. [5].

Sample	Sample Number	Young's Modulus (MPa)		Breaking Stress (MPa)		Breaking Strain (unitless)		Toughness (J/cm ³)	
		mean	STDEV	mean	STDEV	mean	STDEV	mean	STDEV
Sp1R8 Only	7	572	339	53	17	0.21	0.05	6.63	2.89
Sp1R8 & Amp	5	1465	671	53	11	0.08	0.02	2.56	1.15
Sp1R8 & Glut	9	4587	854	148	21	0.04	0.01	3.45	1.35
Sp2R8 Only	5	1220	853	67	21	0.20	0.07	8.07	0.55
Sp2R8 & Amp	5	1067	342	37	13	0.09	0.02	2.15	0.40
Sp2R8 & Glut	14	4500	1347	142	59	0.04	0.01	3.35	1.79
<i>N. clavipes</i> MA Silk		22000		1300		0.12		80	

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Discussion

It is extremely difficult to get large quantities of spider silks for any commercial application. Spiders do not adapt well to captivity. In taking a molecular approach, the problems of spider feeding and cannibalism are eliminated yet, new challenges arise. Spider silk genetic sequences are difficult to clone using general molecular biology techniques due to their size and repeat units. As a result, we and other groups have designed mini spidroin mimics of varying composition and size. We incorporate all three of the dragline silk domains in our design: NTD, Block Repeat Region, and CTD.

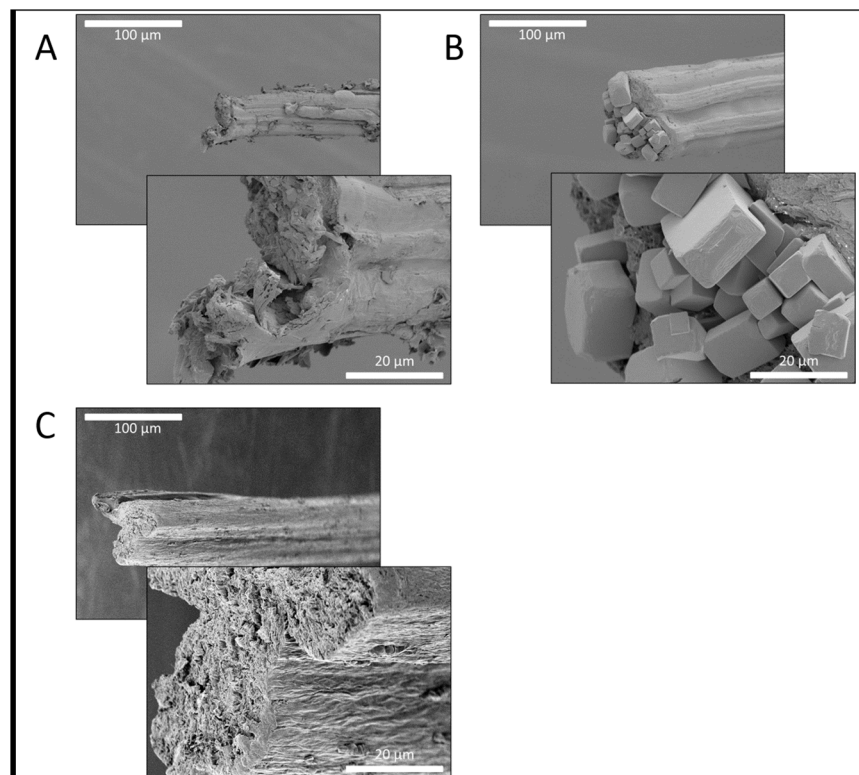


Fig 5. SEM break point observations of rMaSp1R8 fibers. Panels A, rMaSp1R8 fiber; Panels B, rMaSp1R8 fiber pretreated with ampicillin before fiber pulling; Panels C, rMaSp1R8 fiber pretreated with glutaraldehyde before fiber pulling. Scale bars show 100 µm or 20 µm lengths.

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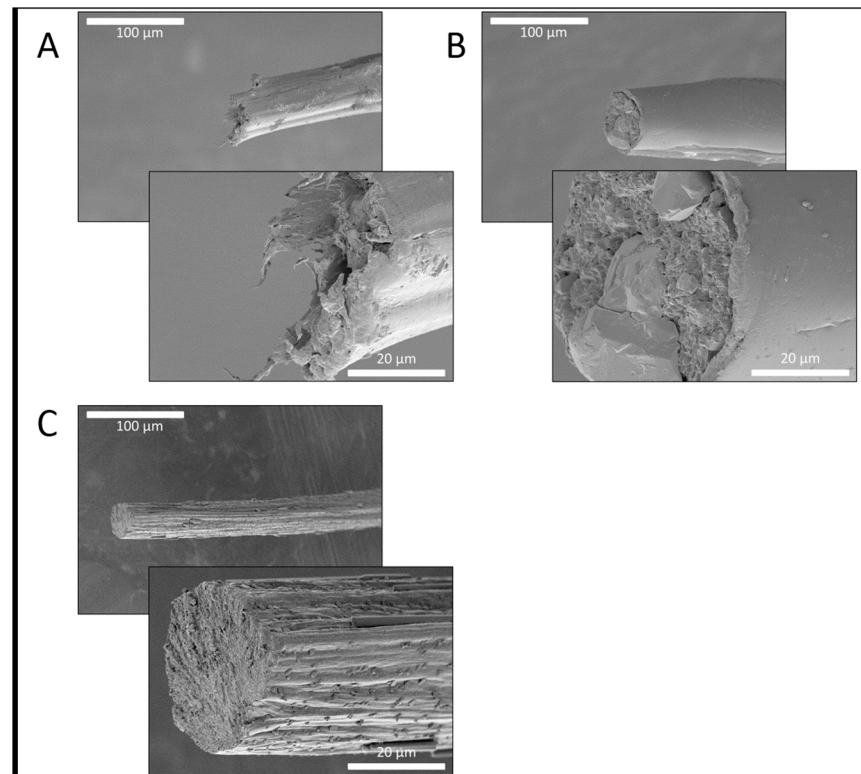


Fig 6. SEM break point observations of rMaSp2R8 fibers. Panels A, rMaSp2R8 fiber; Panels B, rMaSp2R8 fiber pretreated with ampicillin before fiber pulling; Panels C, rMaSp2R8 fiber pretreated with glutaraldehyde before fiber pulling. Scale bars show 100 μm or 20 μm lengths.

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In nature, species of *Leishmania* cause Leishmaniasis, a global disease manifested as cutaneous, mucocutaneous and visceral diseases in humans and other animals [42]. It is spread by the bite of parasite-infected sand flies. During the life cycle of the parasite, it is alternately present in two hostile microenvironments, the sand fly midgut (insect host compartment, nutrient-poor, pH \sim 9.0, [43]) and macrophage phagolysosomes (animal host compartment, nutrient-rich, pH \sim 5.0–6.0, [44]). Promastigotes, the insect vector stage, reside and divide within the midgut of the sand fly and so are dependent on the feeding practices of the sand fly for nutrients. Nutrients are abundant immediately after a blood meal (about every seven days) between which the fly relies on plant-derived sugar meals. These complex carbohydrates (e.g. sucrose) must be broken down into simpler sugars for energy utilization and the promastigotes secrete large quantities of an invertase that facilitates this process [28]. Promastigotes also secrete abundant amounts of phosphoproteoglycans that eventually accumulate to form a gelatinous plug that closes off the midgut. In order to properly feed, the sand fly must expel the infectious plug, resulting in the introduction of parasites into the bite wound [45,46]. The ability of *Leishmania* parasites to secrete large quantities of various proteins, the availability of a characterized secretion system [28], and the wide range of acceptable growth conditions, make *Leishmania* an ideal candidate for large-scale spider silk protein production.

Leishmania tarentolae was chosen as the expression system due to its fast growth, high cell density and simple media formulation. In this way, the rMaSp1 and rMaSp2 can be grown at RT to 26°C and secreted into the medium similar to natural conditions of silk production by spiders. Secretion also simplified protein preparation/purification. After a simple

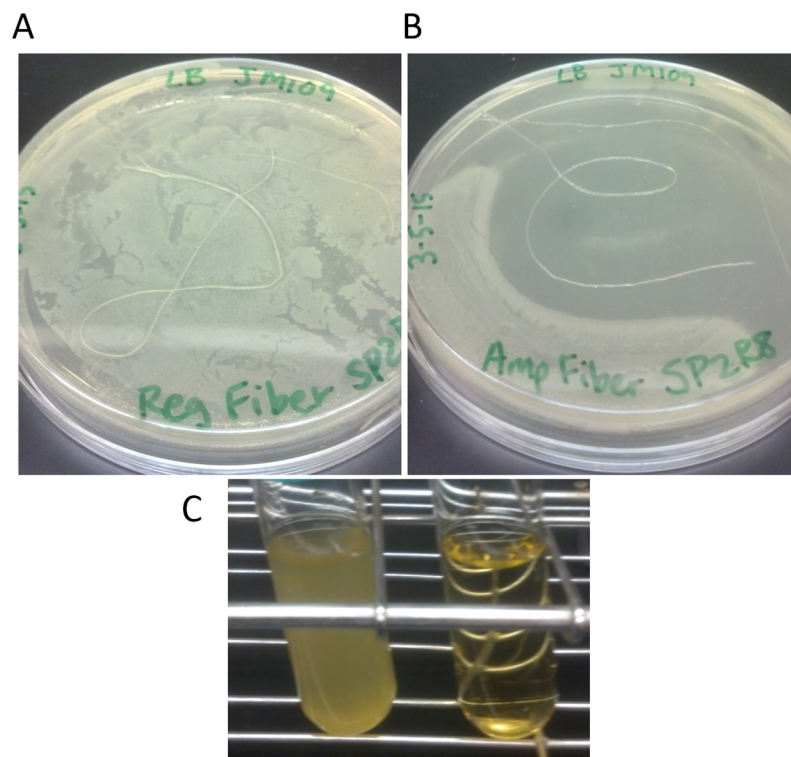


Fig 7. Bacterial growth inhibition assays. Inhibition zone assay with Leishmania derived fibers treated with or without ampicillin. Ampicillin was added to gellan gum solution at 10 mg/mL prior to pulling fibers. Fibers were allowed to air dry for several hours and then placed on LB-agar plates spread with *E. coli* bacteria. A, an inhibition plate assay using a recombinant MaSp2R8 fiber; B, an inhibition plate assay using a recombinant MaSp2R8 fiber which was treated with ampicillin during the fiber production stage; C, liquid culture showing inhibition of bacterial growth when grown in the presence of a recombinant MaSp2R8 fiber treated with ampicillin during the production process.

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centrifugation step, the culture media is allowed to pass through a nickel ion affinity column to purify the recombinant protein. The protein was prepared for fiber production with dialysis and lyophilization clean up steps to yield powdered recombinant protein.

Our hand-pulling technique proved effective at producing fibers. However, the recombinant fibers were significantly thicker than native fibers. Tensile measurements of each of the produced fibers confirmed our initial hypothesis that addition of glutaraldehyde or ampicillin would alter the fiber properties.

Glutaraldehyde has been shown to act as a non-zero-length cross-linking agent that can alter the mechanical properties of a variety of protein-based biomaterials [47,48] and remains one of the most widely used cross-linking agents due to availability and practicality [49]. In studies with gelatin membranes, glutaraldehyde crosslinking enhanced mechanical stability and decreased sensitivity to *in* enzymatic degradation [48]. Similar enhancement of mechanical stability and resistance to enzymatic degradation by glutaraldehyde treatment have been found with amniotic membranes [47]. However, glutaraldehyde treatment of biopolymers typically compromises their biocompatibility as evidenced by decreases in cell proliferation and elevated expression of IL-6 in cytokine bioassays [47,48].

For this study, glutaraldehyde addition increased both initial stiffness (Young's modulus) and strength (breaking stress). The toughness of the fiber, however, decreased by at least 50%

due to a reduction in extensibility (breaking strain). Reduced breaking strain is also consistent with the increase observed in initial stiffness. SEM revealed that the break points of the fibers containing glutaraldehyde were extremely smooth compared to the protein alone fibers (compare Figs 5A with 5C and 6A with 6C), indicative of a strong yet brittle fiber.

Ampicillin addition gave the recombinant spidroin fibers the ability to inhibit bacterial growth (Fig 7) but decreased the overall toughness due to a reduction in strength and/or extensibility. This reduction in toughness may be explained by a build-up of crystalline structures, presumably ampicillin and other salts, leading to the creation of weak points in the fiber (Figs 5B and 6B).

So far, the results have been promising. One major concern with this system is protein yield for commercial scale up applications. Currently, we are investigating the use of bioreactors as a way to increase yield through controlled cellular proliferation. Additionally, more controlled methods for fiber production may lead to more controlled widths and lengths. Such methods of future investigation will include electrospinning and alcohol coagulation baths [50,51]. It is our intent to achieve fiber thickness closer to native spider silk (2–4 μm); however, larger and smaller widths could still yield fibers suitable for certain applications. We are also developing expression constructs containing increased numbers of the block repeat. These extended fiber mimics will be interesting to test and a set of extended repeat unit lengths should give rise to a range of tensile properties to choose from for biomedical, textile, and novel uses.

This study not only demonstrates that *Leishmania tarentolae* is a viable option for the production of recombinant spidroin protein mimics for fiber assembly but once purified, the addition of small molecules to the fiber pulling process can yield an array of fibers with varying properties for future beneficial products.

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