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Production and Biocompatibility of Spider Silk Proteins in Goat Milk

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PRODUCTION AND BIOCOMPATIBILITY OF SPIDER SILK PROTEINS IN GOAT MILK

by

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of

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in

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ABSTRACT

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Due to its biocompatibility and impressive mechanical properties, spider silk has great potential for a variety of commercial applications, from biomaterials to textiles. Unfortunately, it is difficult and impractical to obtain native spider silk in sufficient quantities to fully investigate these applications. In consideration of this problem, recombinant spider silk proteins have been produced in a variety of hosts, including microbes, plants, silkworms, and goats. While these recombinant proteins have potential for meeting the demands associated with investigating spider silk's impressive properties more fully, each of the current production methods needs to be improved upon in one way or another. Currently, goats produce the highest consistent yields of recombinant proteins, but there is room for improvement in both production capacity and protein length. The first focus of this dissertation was to establish a goat cell line designed to increase the purity and quantity of recombinant proteins produced in goat milk by incorporating a spider silk gene that encodes for a histidine-tagged protein into the goat genome. Although multiple genomic integration techniques were investigated, the PiggyBac™ Transposon Vector System was ultimately used to successfully establish a

new “spider goat” cell line.

The second focus of this dissertation was to investigate the biocompatibility of materials made from the current goat-derived recombinant spider silk proteins. Prior to testing the biocompatibility of these proteins, a method was developed for destroying endotoxins in the proteins that had been introduced during milk collection and downstream processing. Two *in vivo* biocompatibility studies were conducted in rats by implanting two different silk materials that had been treated using the endotoxin destruction method established herein. In response to the low level of biocompatibility discovered during these studies, another *in vivo* study was conducted using materials made from goat-derived proteins that had been purified using reverse phase chromatography. It was determined based on results from this final study that goat-derived spider silk proteins are not biocompatible in their current state due to impurities in the proteins and that further work is needed to improve the protein purity.

(103 Pages)

PUBLIC ABSTRACT

Production and Biocompatibility of Spider Silk Proteins in Goat Milk

Richard E. Decker Jr.

Due to its strength, flexibility, and biocompatibility, spider silk is a highly appealing material for applications in the medical field. Unfortunately, natural spider silk is difficult to obtain in large quantities because spiders are territorial and cannibalistic, making them impractical to farm. Synthetic spider silk proteins produced by transgenic hosts such as bacteria and goats have made it possible to obtain the quantities of spider silk needed to study it more fully and to investigate its potential uses. The spider silk proteins produced in our laboratory do not have an optimal purification method to remove all of the non-biocompatible contaminants and have not previously been tested for their biocompatibility. The first focus of this dissertation was to create goat cells that can be used to create new goats. These new goats will produce proteins that can be purified more efficiently and more completely. The second focus of this dissertation was to perform biocompatibility tests on goat-derived spider silk proteins. Prior to performing any biocompatibility tests, a method was established for removing endotoxins – an impurity that causes an immune response in the body – from the proteins. This work has shed light on areas for improvement in the silk protein purification process and laid groundwork for the production of new goat-derived proteins. These steps will help make it possible for synthetic spider silk to progress further toward becoming a viable biomaterial.

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CHAPTER 1

INTRODUCTION

Spider Silk

Spider silk's biocompatibility and impressive mechanical properties make it an ideal material for many applications. From wound healing among the ancient Greeks to crosshairs in pre-World War II optical devices, spider silk has been used by mankind for centuries[1,2]. Orb-weaving spider silk is of particular interest because orb-weavers produce six different types of silk and one glue, composed of proteins ranging in size from 200-350 kDa (Figure 1-1)[3]. Each of these silks is unique in its molecular structure, function, and mechanical properties. The most studied spider silk is major ampullate silk, largely due to its high tensile strength and toughness[3]. Major ampullate silk is made up of two proteins, Major Ampullate Spidroin 1 (MaSp1) and Major Ampullate Spidroin 2 (MaSp2)[4]. Major ampullate silk is used in web construction and is also a lifeline for the spider. It is often referred to as dragline silk because a spider will leave, or drag, a trail of major ampullate silk when walking to catch itself if it falls. The extensibility and tensile strength of dragline silk fibers prevent the spider from falling too far or stopping too abruptly.

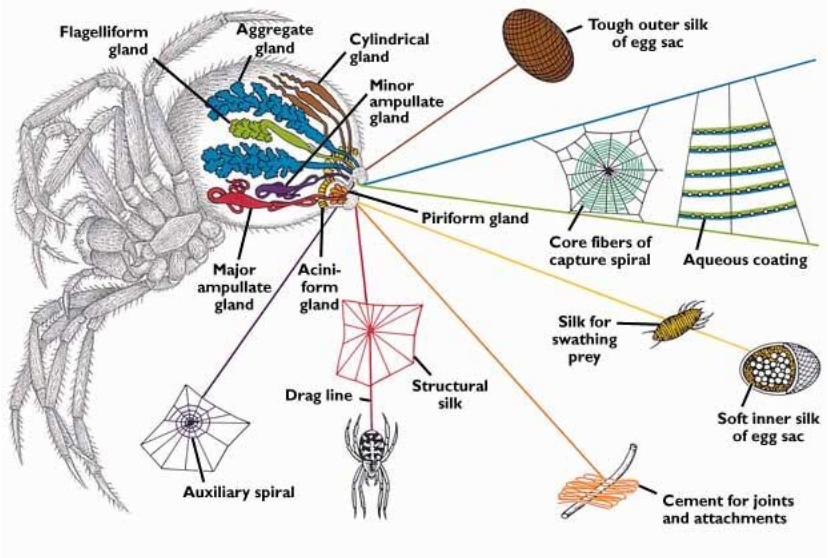

Figure 1-1: There are six different types of spider silk: aciniform (yellow), cylindrical or tubulliform (brown), flagelliform (green), major ampullate (red), minor ampullate (purple), and pyriform or piriform (orange), as well as one protein-glue, aggregate (blue). As shown (via color coding), each type of silk has a different use for the spider with mechanical properties that are specific to their role in survival, prey capture and/or reproduction[5] (Image reprinted with permission. Credit: Patricia J. Wynne).

While these mechanical properties are essential for the life of the spider, they also make spider silk a highly promising material for use in the biomedical field[6–8] and for a variety of other uses. Some of the proposed uses for spider silk include non-medical applications like parachute cords, tire linings, and high-performance sportswear, as well as medical applications such as replacement ligaments, tissue scaffolds, drug storage matrices, and drug delivery systems[9–11]. Although spider silk holds great promise, natural silk is very difficult and impractical to harvest because spiders are territorial and cannibalistic. Lack of silk material makes it nearly impossible to investigate most of silk's potential uses, let alone create any products at a marketable scale.

To obtain spider silk proteins in the quantities required to study silk for its many proposed applications, synthetic spider silk proteins are being produced in a variety of transgenic hosts (Figure 1-2)[7,12–16]. There are hurdles involved with creating materials

from recombinant silk proteins created in transgenic hosts, such as the difficulty of spinning fibers from the proteins and the introduction of contaminants into the materials during protein production and processing that can affect the biocompatibility of the materials. In spite of these hurdles, there are advantages to producing silk proteins using transgenic hosts beyond just increased quantities of protein. Another major advantage is that materials made from recombinant spider silk proteins can be tailored through post-production processing of the materials or genetic alterations to the proteins themselves to have increased extensibility or increased strength, further increasing the potential applications of spider silk.

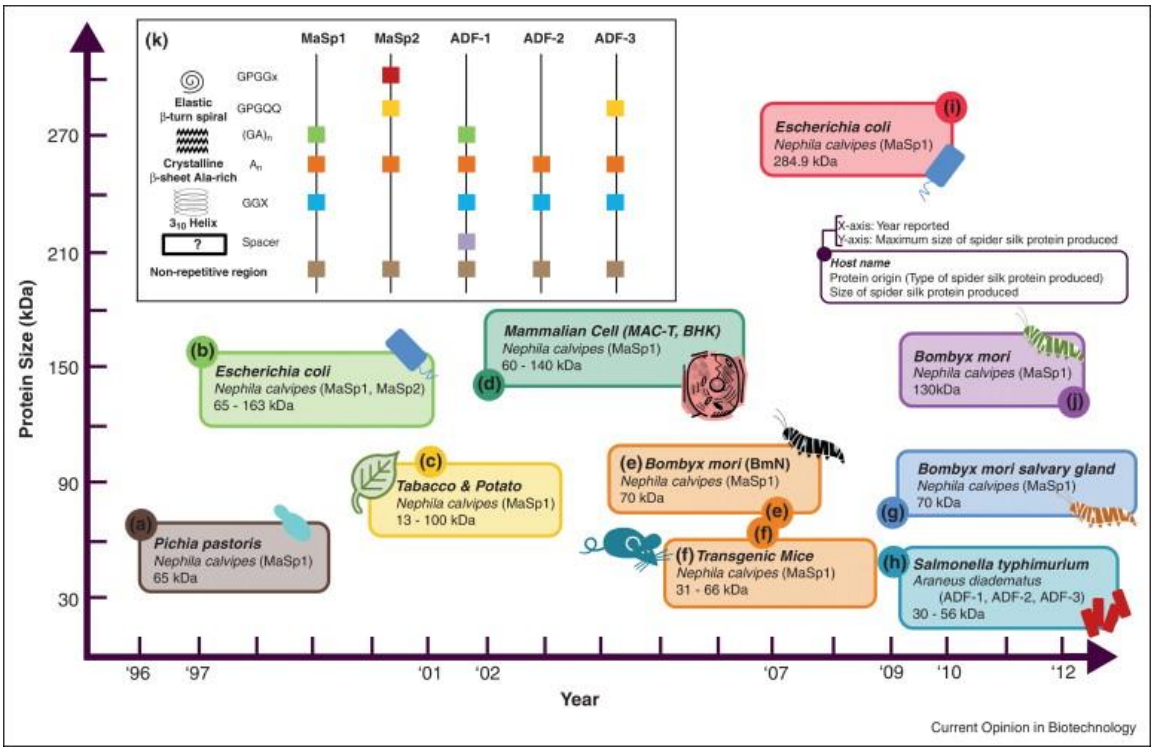

Figure 1-2. Brief history of recombinant spider silk protein production: **(a)** Production of synthetic spider dragline silk protein in *Pichia pastoris* in 1996[13], **(b)** synthetic spider dragline silk proteins and their production in *Escherichia coli* in 1997[17], **(c)** production of spider silk protein in tobacco and potato in 2001[12], **(d)** spider silk fibers spun from soluble recombinant silk produced in mammalian cells in 2002[18], **(e)** expression of EGFP-spider dragline silk fusion protein in BmN cells and larvae of silkworm, which showed that solubility is the primary limitation for the yield of spider silk protein, in 2007[19], **(f)** construct synthetic gene encoding artificial spider dragline silk protein and its expression in the milk of transgenic mice in 2007[20], **(g)** transgenic silkworms (*B. mori*) producing recombinant spider dragline silk in cocoons in 2009[21], **(h)** engineering the *Salmonella* type III secretion system to export spider silk monomers in 2009[22], **(i)** native-sized recombinant spider silk protein produced for the first time in metabolically engineered *E. coli* resulting in a Kevlar-strength fiber in 2010[23], and **(j)** transgenic silkworms transformed with chimeric silkworm–spider silk genes producing composite silk fibers with improved mechanical properties in 2012[24]. The organization of the important motifs in spider silk proteins, which is modified from Hayashi *et al.*[25] and Teule *et al.*[14]. The colored-squares indicate the modules contained in each silk protein. The proteins are: MaSp1 and MaSp2, major ampullate spidroin 1 and 2 from *Nephila clavipes*; ADF-1, ADF-2, and ADF-3, minor ampullate, putative cylindrical, and major ampullate, respectively, from *Araneus diadematus*[25] (Reprinted from Chung *et al.*[7] with permission).

Transgenic Hosts

Eukaryotic Cells

One potential method for producing recombinant silk proteins is through cultured cells. Our group has shown that spider gland cells may produce silk proteins when in culture, but because glandular cells do not proliferate we have been unable to successfully establish a glandular cell line (unpublished data). Insect cells, which should have similar biological and chemical properties to spider cells, have also been explored for spider silk production. While there are a limited number of studies relating to spider silk production in insect cells, spider silk-like proteins have been produced in both BmN *Bombyx mori* (BmN) cells and *Spodoptera frugiperda* (Sf9) cells[19,26]. One of the reports on silk proteins produced in BmN cells related sparse results, simply stating that the proteins were only about 70 kDa and "probably occupied 5% of the total cell protein"[19]. Vollrath *et al.* showed that silk could be produced in Sf9 cells that had the same chemical properties as natural silk, but the resulting fibers were too fragile to be tested mechanically[26]. While this might indicate that the proteins were not truly similar to those produced by spiders, the fragility was more likely due to poor fiber spinning techniques available at the time.

In 2002, Lazaris *et al.* successfully produced silk in cultured mammalian cells, including bovine mammary epithelial cells and baby hamster kidney (BHK) cells[18]. Like fibers produced from insect cell-derived proteins, the fibers produced from the protein created by bovine and BHK cells were also brittle. It was suggested that this could be due to a depletion of the aminoacyl-tRNA pool *in vitro* causing truncated proteins[18], although, similar to silk from insect cells, the brittleness of the fibers was more likely due to a lack

knowledge regarding spinning synthetic spider silk fibers available at the time (and the lack of proteins needed to improve that knowledge) leading to poor spinning methods. Unfortunately, regardless of whether silk produced in insect or mammalian cells would be similar to those in nature with more biomimetic spinning techniques, the cost associated with producing proteins in a cell line is much too high to consider on a commercial scale for a non-therapeutic protein.

Bacteria (*E. coli*)

Escherichia coli (*E. coli*) has been well studied and has a short doubling time, making it an attractive host for producing high quantities of silk proteins. Because of this, *E. coli* has been used to express multiple spider silk proteins[17,27]. In 2010, researchers claimed to have produced silk proteins in *E. coli* that are similar in size to native silk proteins[23], but no group has been able to reproduce these results. Because protein size is at least partially responsible for the strength of spider silk fibers, truncated proteins are detrimental to the strength of the resultant materials[23,28]. One of the proposed causes of the truncated proteins is that spider silk proteins are highly repetitive and contain large amounts of glycine, alanine, and proline[3,4,29]. *E. coli* have a limited tRNA pool and, therefore, do not produce enough of these amino acids to create full-length proteins and still support regular cell function. Although increased work on silk production in *E. coli* is leading to the production of larger proteins at increased quantities, other transgenic systems, such as plants, silkworms, and goats, have been investigated to avoid or minimize tRNA pool depletion issues.

Plants

There have been some notable achievements in spider silk production using plants. In theory, plants should be able to produce larger silk proteins without the addition of any tRNAs. Scheller *et al.* were able to produce silk-like proteins in both potato and tobacco plants in which there was no genetic instability caused by the recombination of repetitive proteins, a problem seen in some other transgenic systems[12,15]. The silk proteins comprised up to 2% (average >0.5%) of the total soluble protein when produced in the endoplasmic reticulum (ER) [12]. It is noteworthy that the silk proteins had a high resistance to heat, which made them easier to extract and purify by removing smaller proteins[12]. Proteins of up to 100 kDa were detectable in the plant tissue, which is comparable to, if not better than, products from other transgenic systems, but still much smaller than the natural protein size[12].

Our group is investigating production of spider silk proteins in alfalfa. These studies have led to transgenic plants that produce silk proteins predominately in their leaves. Transgenic alfalfa would be one of the most cost-effective systems for producing spider silk proteins for two major reasons: 1) alfalfa is a perennial plant, meaning transgenic fields could be established and then grown year after year and 2) the plant waste could potentially be used as feed for livestock or fuel for ethanol production, thus boosting the economic value of the crop as a whole. In spite of the accomplishments in developing plants that produce spider silk proteins and their great potential, the major hurdle preventing the use of plants for spider silk production at a commercially viable scale is the protein purification process. Using metal affinity chromatography, such as a His-tag purification system, is problematic because it cannot be scaled to the level

necessary to extract silk proteins on a large scale. Metal affinity chromatography is also problematic for purification from plants because chlorophyll is a charged protein that is extracted along with histidine-tagged silk proteins. Extracted chlorophyll leads to contamination, damaged metal affinity media and equipment, and lower yields of silk. While developing a method for extracting recombinant silk from alfalfa or other plants at a large scale would lead to one of the most scalable and economically sustainable methods for producing large quantities of spider silk proteins, purification from this system remains a significant challenge.

Silkworms

Through the use of targeted genomic integration, our laboratory group has also recently been able to create transgenic silkworms that produce up to 15% spider silk in combination with their native silk. One major advantage to using silkworms to produce spider silk is that they are a self-contained spinning apparatus, which removes the complications associated with trying to spin spider silk fibers from recombinant proteins with biomimetic equipment that will likely never be as good as natural spinning systems. Although the data is unavailable due to patent filing concerns, the fibers produced by transgenic silkworms have mechanical properties approaching those of native spider silk fibers. In spite of the benefits of using silkworms to produce spider silk, there are still some downfalls: 1) the silkworms still produce sericin, a protein glue that is immunological, which must be removed via multiple treatment steps before the silk can be used as a biomaterial, and 2) extensive processing of the fibers is required to make it possible to create non-fiber materials. In both cases, the processing required can lead to a reduction in the overall strength of the proteins.

Goats

Following the work done by Lazaris *et al.* to produce spider silk in mammalian cells[30], Nexia Biotechnologies, in cooperation with Dr. Randy Lewis, created transgenic goats that produce spider silk proteins in their milk (Nexia Biotechnologies, unpublished; 2002)[31]. Those goats' offspring are currently the best system for producing large amounts of transgenic spider silk proteins, albeit at a shorter size than native proteins (65 kDa). In spite of their shorter size, the large quantity of available protein has enabled improvements to be made in the spinning process, which has led to fibers that are mechanically more similar to native fibers[32], as well as the investigation of other silk-based materials such as thin films and hydrogels[33,34].

Transgenic goats are ideal animals to work with for the production of spider silk proteins in milk. Goats are relatively small, their waste is easy to dispose of, and they produce a reasonably large amount of protein-rich milk. The spider silk genes are heritable, so the herd size can be scaled up relatively easily through standard goat breeding. Although scalable purification is a concern for using goat-derived silk proteins for markets requiring excessive amounts of materials such as the textile industry, using the proteins for medical applications is economically favorable given the lower amount of silk that would be required and the high value of the materials compensating for more costly production when compared to alfalfa or *E. coli*[35]. Also, the FDA has previously approved the production of pharmaceuticals in goats' milk[36], so there is a standardized pathway for FDA approval. Goats are, therefore, currently the optimal method for obtaining transgenic silk for medical applications because they will allow for protein to

be produced at high enough quantities in a relatively short period of time and in an FDA approvable manner.

Dissertation Research Aims

Aim 1: Transgenic Goat Cell Line

To create the "spider goats" in use today, Nexia incorporated spider silk genes into a modified version of the pBC1 Milk Expression Vector system (Invitrogen, no longer commercially available). The pBC1 plasmid uses the goat β-casein promoter to produce a protein of interest in a host's milk. Not only is the pBC1 plasmid extremely large (22 kb) and difficult to modify due to its multiple cloning site containing a single restriction enzyme cut site, but it also utilizes random integration to incorporate its cargo into the host genome. Although many random integration systems can be used to effectively incorporate a gene of interest into a host, there are potential issues with random integration, beyond the obvious problem of not knowing ahead of time where the foreign gene will integrate into the host genome. It is possible that the gene of interest will be incorporated such that it will interrupt an existing gene, thus silencing production of a native protein, or that the gene of interest will be incorporated at a locus that will generate lower protein yields. Since the creation of the original "spider goats," there have been advances in genome editing that have led to the development of techniques that are superior to pBC1 for incorporating a foreign gene into a host genome. The first aim of this dissertation was to explore some of these improved techniques of genomic editing in order to establish a transgenic goat cell line to be used for somatic cell nuclear transfer to create goats that will produce histidine-tagged proteins from a codon optimized silk gene. Although this work is presented in Chapter 2 – CRISPR and PiggyBac Mediated

Development of a Transgenic Goat Cell Line, some background on the targeted gene editing techniques investigated is provided below.

TALENs

Researchers have known of transcription activator-like effectors (TALEs) for decades, but the key to understanding their specificity was deciphered less than a decade ago[37]. TALEs are produced by *Xanthomonas* bacteria and are composed of variable regions that recognize specific DNA sequences[38–40]. Following the elucidation of their specificity, TALEs became a major topic of interest. Cermak *et al.* developed a method to engineer transcription activator-like effector nucleases (TALENs) by combining the DNA fragments coding for naturally occurring TALEs with a mutated FokI endonuclease[39]. These engineered TALENs can be designed to create double-stranded breaks (DSBs) at nearly any region of the genome of nearly any host[39–41]. In 2012, Carlson *et al*. successfully used TALENs to facilitate a gene knockout in livestock, proving that engineered TALENs are a viable method for creating DSBs and genetic manipulation in large mammals[40]. One potential drawback of using TALENs is that each DSB requires a pair of TALEN proteins. This increases the load placed on the cells into which the TALENs are transfected and can decrease the transfection efficiency, especially if multiple cuts are needed. In spite of this, it has been shown that when two TALENs are used in tandem the efficiency of gene modification was similar to that of the predecessor to TALENs, Zinc Finger Nucleases (ZFNs), but without the unintended chromosomal rearrangements that are a common side effect when using ZFNs[40].

CRISPRs

The newest site-specific gene editing tool employed in this project is clustered regularly interspaced short palindromic repeats (CRISPRs). CRISPRs create CRISPR-associated (cas) proteins, which are RNA-guided nucleases that create targeted DSBs[42]. The most commonly used cas is Cas9, which has been shown to function in many different systems, including human cells[43,44], rice[45,46], hamsters[47,48], and mice[49]. Compared to other engineered site-specific nucleases such as zinc-finger nucleases and TALENs, CRISPRs are quicker, easier, and less expensive to create, in that they only require a standardized plasmid and a 17-22 bp double-stranded oligonucleotide matching the target sequence. There have been concerns regarding off-target recognition and cleavage by the CRISPR/Cas9 method due to the smaller size of the recognition sequence leading to off-target recognition and non-specific cleavage[43], but it has been shown that using shorter recognition sequences actually decreases the frequency of off-target cutting[50]. The low cost and ease of production, diverse targets and hosts, and high efficiency of CRISPR/Cas9 make it one of the most promising gene editing systems available. The impact the CRISPR/Cas9 system is having on research in biotechnology, agriculture, and gene therapy and other medical applications, such as providing a potential "cure" for HIV-1[51], is very impressive[52].

Aim 2: Endotoxin Removal and Biocompatibility Studies

Although native spider silk is well known as a biocompatible material, synthetic spider silk produced in our laboratory (both goat- and bacterially-derived) has not been tested for its biocompatibility. To bring synthetic spider silk into the medical field, biocompatibility tests are essential. Results of these tests can either be used to confirm

biocompatibility or elucidate areas of improvement in protein purification and processing to establish biocompatibility. As such, the second aim of this dissertation was to perform biocompatibility tests on materials made of goat-derived spider silk proteins.

Aim 2-1: Endotoxin Removal

One major concern when preparing to test the biocompatibility of any biomaterial is the presence of endotoxins. Endotoxins, also known as lipopolysaccharides (LPS), are small, heat stable toxins present in gram-negative bacteria that are released during growth and upon cell death. Endotoxins can illicit an immune response independent of the effects of the biomaterial itself, thus producing confounding results in biocompatibility studies[53]. Consequently, it is essential to ensure endotoxins are removed from or destroyed in any biomaterial prior to investigating its biocompatibility. The most common methods for endotoxin removal/destruction – dry heat (>180 °C) and caustic rinses (e.g. NaOH) – cannot be used on most proteins, including spider silk, as they denature and/or leave residual chemicals in the proteins[53]. In the case of spider silk, this is detrimental, as denaturation of the proteins leads to the loss of mechanical properties and negates one of the key reasons for using spider silk as a biomaterial. To avoid these issues, a method to destroy endotoxin in synthetic spider silk protein, without compromising its mechanical abilities, was developed and employed to prepare samples for *in vivo* biocompatibility tests. This method and its effectiveness are presented in Chapter 3 – Method for the Destruction of Endotoxin in Synthetic Spider Silk Proteins.

Aim 2-2: In vivo Biocompatibility Tests

Although *in vitro* tests were performed on our synthetic spider silk using Chinese hamster ovary cells and BHK cells (unpublished data), these tests did not provide much

useful information regarding the silk's biocompatibility beyond demonstrating that the silk does not appear to affect cell viability or have any cytological effects on the cells. To obtain impactful data on the biocompatibility of goat-derived spider silk, *in vivo* tests were conducted in rats using spider silk films and hydrogels that had been treated with our newly developed method for endotoxin destruction (Chapter 3). The details and results of those *in vivo* studies are presented in Chapter 4 – Investigation of the *In Vivo* Biocompatibility of Goat-derived Spider Silk Protein. Chapter 4 also includes information regarding an effort to redesign the processing and purification pathway of the goat-derived silk proteins in an attempt to reduce the inflammatory response seen in our initial studies that were likely caused by contaminants in the synthetic silk.

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CHAPTER 2

CRISPR AND PIGGYBAC MEDIATED DEVELOPMENT OF A TRANSGENIC GOAT CELL LINE

Chapter Preface

This chapter presents work completed and problems encountered relating to incorporating a spider silk protein gene into the goat and hamster genomes. Four methods of incorporating the spider silk gene were employed: transcription activator-like effector nucleases (TALENs), pBC1, clustered regularly interspaced palindromic repeats (CRISPRs) and CRISPR associated protein 9 (Cas9), and PiggyBac. TALENs and CRISPR/Cas9 were both used to produce targeted double-stranded breaks in the genome and induce homology directed recombination (HDR). PiggyBac and pBC1 are both random integration plasmid systems into which the spider silk gene was incorporated. The pBC1 Milk Expression Vector (Invitrogen, K270-01), which utilizes the goat β-casein promoter to facilitate protein production in milk, is no longer commercially available, but it was the original expression system used to create transgenic "spider goats," which is why its use was attempted early on in the project.

TALENs and pBC1 were used in the early stages of the project (first year, 2012-2013), but there were many difficulties associated with both systems, especially in regards to determining whether either system had worked successfully. Although repeated attempts were made to use the TALEN system to cleave the goat genome, no success was observed. It is possible that this was due to the nature of the cleavage being attempted – two cuts to remove a large portion of the genome (~11kb), requiring four TALEN plasmids (two per cut). It is also possible that cleavage (TALENs) and

incorporation (pBC1) was not detected due to low transfection, expression, and incorporation efficiencies. This, combined with a lack of selection markers to promote proliferation of successfully transfected/edited cells, would have made it nearly impossible to detect positive results.

Because the CRISPR/Cas9 system was developed in 2013, soon after the start of the project, the TALEN and pBC1 systems were abandoned in favor of this easier, less expensive, higher efficiency method. In light of this, the TALEN and pBC1 work will not be included in the following chapter, as it does not add any significant value to the work attempted and completed. Regardless, the researchers would like to acknowledge that the plasmid kit used for generation of TALENs in this project was a gift from Daniel Voytas and Adam Bogdanove (Addgene kit # 1000000024)[1].

Introduction

Genetic manipulation of organisms by humans has been conducted for millennia, from farmers selectively growing crops to breeders selectively breeding for particular traits in animals. Although genetic work has been conducted in a laboratory setting for nearly a century, much of this work has been conducted using random integration methods – a gene of interest is "forced" into the genome at a random or semi-random location. Using these random integration methods, the gene of interest is usually incorporated at multiple loci within the genome that can be difficult, if not impossible, to determine. Fortunately, genetic manipulation has become significantly more "programmable" and consistent over the last two decades thanks to the discovery of programmable nucleases such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced repeats (CRISPRs) and CRISPR associated proteins (Cas), with Cas9 being the most widely used Cas[2,3]. With the advent of these technologies, it has become possible to quickly, easily, and precisely make modifications to the genome of many different organisms. The applications for targeted genomic engineering range from studying diseases, particularly genetic diseases, to the amplification or addition of specific traits in food crops such as rice, to altering the genome of a mammal[4–8].

One interesting example of genetic manipulation is the production of spider silk in transgenic goats' milk[9]. Spider silk, particularly major ampullate or dragline silk, has gained a lot of interest as a potential biomaterial due to its strength and biocompatibility[10–12]. Due to complications with farming spiders efficiently at a large scale, synthetic spider silk has been investigated as a substitute for natural spider silk.

Although many hosts have been investigated to produce synthetic spider silk, such as bacteria[13,14], silkworms[15], yeast[16], and plants[17], the most consistently high yields of larger spider silk proteins are currently produced in transgenic goats. Unfortunately, the silk-coding genes have been randomly integrated into the goat genome using an outdated system (pBC1) without provision for affinity chromatography. Because of this, it is believed that the goats may not produce silk as efficiently as possible. It is also speculated that the current protein purification process not only leads to a loss of protein, but also increases the opportunity for contamination with endotoxin and other environmental contaminants. To address these issues, we sought to integrate a spider silk gene with a histidine tag at a targeted locus in the goat genome.

The main focus of this study was to replace one of the native milk protein coding genes in the goat genome, specifically the α_{S2}-casein gene, with a gene encoding one of the two proteins that make up dragline silk, specifically major ampullate spidroin 1 (MaSp1)[18]. We theorized that incorporating the MaSp1 gene in the genome such that it would be controlled using the native protein production, excretion, and packaging systems of the goat would improve the quality and increase the quantity of the spider silk protein being produced by the goat. To attempt this, the CRISPR/Cas9 system was employed to induce multiple double-stranded breaks (DSB) in the regions of the genome surrounding the α_{S2}-casein locus in order to incorporate the MaSp1 gene into the genome via homology directed recombination (HDR).

Although fully removing and replacing an existing gene via targeted DSB production and subsequent HDR was the ideal situation in this project, there are many complexities associated with the work that make the process extremely difficult to

complete. Therefore, it was concluded that, even if full gene replacement was not possible, randomly incorporating a MaSp1 gene with a histidine tag would still have an important impact on improving the overall amount and purity level of silk protein obtained. In light of this, the PiggyBac™ Transposon Vector System, a semi-random integration system, was used as a contingency plan for integrating silk genes with a histidine tag.

Due to some issues early on with knocking the silk into the goat genome, we also attempted to knock the same silk construct into the Syrian hamster (*Mesocricetus auratus*) genome as a pilot study to determine whether our method (using CRISPR/Cas9 and HDR to incorporate a large gene) would be feasible before continuing the work in the goat genome. Hamsters were chosen for their shorter gestational period and available secondary cell line – baby hamster kidney (BHK) cells. The BHK cell line is very hardy and easy to work with. Also, CRISPR gene editing in hamsters has been successfully accomplished by others previously[6,7,19].

Herein we present the hurdles, failures, and successes associated with integrating a histidine-tagged spider silk gene into the goat genome.

Materials and Methods

CRISPR/Cas9

CRISPR/Cas9 constructs (referred to herein as CRISPRs) were created by incorporating CRISPR guide RNA sequences (gRNAs) into the pX330-U6-Chimeric_BB-CBh-hSpCas9 plasmid backbone, referred to herein simply as pX330. pX330-U6-Chimeric_BB-CBh-hSpCas9 was a gift from Feng Zhang (Addgene plasmid # 42230)[20]. Guide RNAs were selected using the CRISPR Design tool at crispr.mit.edu

with NGG as the protospacer adjacent motif (PAM) sequence. Guides of 17-20 bp were prioritized. Potential off-target loci were determined by running the gRNAs in BLAST against the goat or hamster genome to find sites that matched the seed sequence (first ~10 bp) and included the PAM sequence. Hamster off-target scores were also assessed via the Benchling CRISPR design wizard tool. Based on these criteria, no off-target sites were predicted.

The efficiency of each CRISPR was determined using the GeneArt Genomic Cleavage Detection Kit (Life Technologies A24372). CRISPRs determined to have the highest efficiency were also tested together in pairs to determine whether their efficiency would persist when co-transfected. Based on the results of these efficiency tests, final gRNAs were selected – the highest efficiency gRNA sequences are shown in Table 2-1.

Table 2-1. Highest efficiency gRNAs incorporated into the pX330 plasmid to create CRISPR constructs. Duplexes of these sequences were created by IDTDNA with "sticky end" overhangs (not shown) to facilitate incorporation into pX330.

Target Genome	**Name**	**Sequence (5' – 3')**	**Notes**
Goat	CRISPR 2	TCTTTGATTATAGGTTTC	
	CRISPR 3	GAAACCTATAATCAAAGA	
	CRISPR 8	GTCTTAGGTTGAGTCCA	For distal end of gene
Hamster	CRISPR U2	GGCTTGAGTATCTCATTCTGG	
	CRISPR U5	AACTATTTAGCATTAACACG	For NHEJ work
	CRISPR D1	GTGATTTAACTGATCAACTTGG	For distal end of gene

PiggyBac Transposon

The PiggyBac™ Transposon Vector System (System Biosiences, PB513B-1) (referred to herein as PiggyBac), a semi-random integration method, was also used to incorporate a spider silk gene into the goat genome. In order to facilitate silk production in milk, the goat β-casein promoter was incorporated into PiggyBac along with the spider

silk gene. A Neomycin resistance cassette (neo cassette) was also incorporated into PiggyBac to allow for cell selection. Separate regulatory elements (promoter and stop codon) were incorporated for the neo cassette. A plasmid map of the final PiggyBac construct is shown in Figure 2-1.

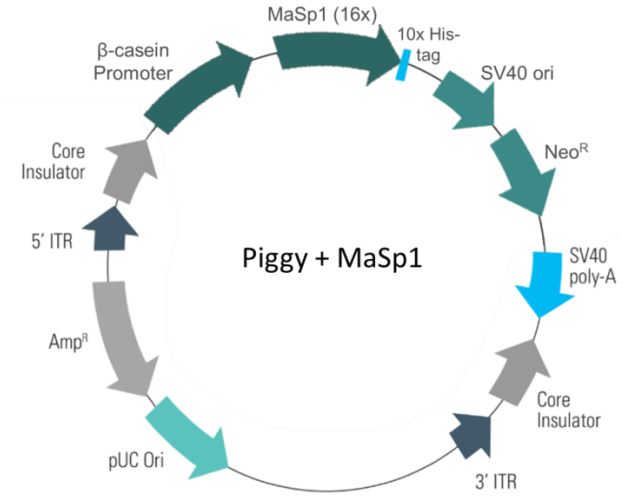


Figure 2-1. Plasmid map of final PiggyBac construct with incorporated promoter, silk coding sequence (MaSp1 (16x)), histidine tag, and neomycin resistance gene.

Silk Sequences and Homology Arms

MaSp1 and MaSp2 silk sequences used in this project were 16x repeats of sequences created by LifeTechnologies specifically for our research group. Each 16x repeat is 2046 bp long and encodes for an approximately 74 kDa protein. For pBC1 and PiggyBac studies, silk sequences were cloned into a custom designed insert containing a Kozak Consensus Sequence and milk secretion signaling sequence. For targeted integration studies, silk sequences were cloned between two homology arms (approximately 500 bp each) matching the flanking regions of the TALEN or CRISPR target sites in either the goat α_{S2}-casein or hamster β-casein genes to encourage silk

integration via homology directed repair (HDR). Primers for the production of these homology arms are shown in Table 2-2.

Table 2-2. Primers used for PCR and homology arm production.

Category	Name	Sequence (5' to 3')
Genomic Integration Check	Goat Silk Up F	GGGAGGCAGGCTTCATGTT
	Goat MaSp1 Up R	CCTGCACCACCGGTCATATG
	Goat UF3	GCCCAAATGAGCCTCCAC
	Goat UR4	TCTGTGACTCTCCTGAACTTT
	Ham Silk Int Up 2 F	GGTAGCCCATGGAGGAGTCTTTAGAC
	Hamster BUF1	AGGAGGCAGAGTATAGTTTGAC
	Hamster BUR1	GGATATGCTGAGTGATTCCTTTTG
	MaSp1 Ham Check	CCACCGGTCATATGGCCG
	MaSp2 Ham Check	GGACCGGTCATATGGCCG
	MaSp1 F1	TAGCCAGGGTGCCGGTCG
	MaSp1 R3	CCCTCCCAGACCACCCC
Hamster Homology Arm Production	Ham Arms UF	CAGCATACATCCAAAAGGCCAAAA
	Ham Arms UR	AATCCGGATGCAAAAGCAAGTGCCAC
	Ham Arms DF	AATCCGGAAAGGTCTAAGAGGATTTCCAGG
	Ham Arms DR	GAAGTCACGACCCACATGTTG
	Ham HA2F	AATCCGGATGTCCAAAGGGAAATTCAGTGG
	Ham HA2R	CTTCCCTCACTGCTGGAAATG
Goat Homology Arm Production	New Goat HA DF	AATCCGGATGAAGATGGACAAAAATACCACTTC
	New Goat HA DR	TTGTTGCTCTTTAGTCTCTCAGTCGTG
	New Goat HA UF	GCATTTCTGATGATTCTCCACAAG
	New Goat HA UR	TTTCCGGATGCAAGGGCAACGGCC

Cell Culture

For all goat studies, goat fetal fibroblast (gFF) cells, provided by Dr. Irina Polejaeva's research group at Utah State University, were used. For all hamster studies, BHK cells, provided by Zhongde Wang's research group at Utah State University, were used. All cells were cultured in high glucose Dulbecco's Modified Eagle Media (DMEM) (VWR VWRL0101-0500) with 10% (v/v) Fetal Bovine Serum (FBS) (GE Healthcare SH30071.03) and 1% (v/v) penicillin/streptomycin (Life Technologies 15140-122); non-essential amino acids (NEAA) were also added to a final concentration of 5% (v/v) for

BHK cells. Cells were grown with 5% CO_2 at 37 °C, with regular passaging. Cryopreserved cells were stored in complete media with an additional 10% FBS and 10% DMSO.

Transfection and Cell Selection

All transfections were conducted using the 4D-Nucleofector™ X Unit (Lonza AAF-1002X). For gFF cells, Lonza's Amaxa™ P3 Primary Cell 4D-Nucleofector™ X Kit was used (Lonza V4XP-3024). For BHK cells, Lonza's Amaxa™ SE Cell Line 4D-Nucleofector™ X Kit L was used (Lonza V4XC-1024). Based on preliminary cell viability and transfection efficiency experiments (data not shown) the EO-114 program was used for gFF cells. The CA-137 program was used for BHK cells as recommended on Lonza's website. Due to variance in cell viability levels post-transfection, the number of cells used between gFF and BHK transfections varied. Generally, $0.5x10^6 – 0.75x10^6$ gFF cells were used and $0.3x10^6$ BHK cells were used.

For PiggyBac experiments, a PiggyBac vector expressing green fluorescent protein (GFP) was used as both a positive transfection control and a negative Neomycin selection control. A PiggyBac vector containing a neo cassette was transfected as a positive antibiotic selection control. Non-transfected cells were plated as a negative antibiotic selection control. Cell selection was achieved by adding 900 µg/mL G418 Sulfate (VWR 97063-060) to the DMEM media starting at 48 hours post-transfection. Transfected cells were not harvested until complete cell death in negative control wells (typically ≥12 days).

Some cells were diluted and plated in a 96-well plate at ~200 cells/well to establish single cell colonies. Remaining cells were plated in 6-well plates. Cells grown

in 96-well plates with the highest probability of being single cell colonies (chosen based on visual inspection) were transferred to 12-well plates when they reached 50% confluence. Cells grown in 12-well plates were then harvested for analysis and cryopreservation at 50% confluence. Cells grown in 6-well plates were harvested at >90% confluence for analysis and cryopreservation.

Genomic DNA Extraction and PCR

Following transfection and cell selection, genomic DNA (gDNA) was extracted from cells using the Qiagen QIAamp DNA Mini Kit (Qiagen 51304). Genomic DNA was then evaluated via PCR using custom primers to detect for genetic changes in the form of base pair insertions or deletions (indels) in the case of CRISPR evaluation studies or to check for silk integration. Primers used in these PCRs are listed in Table 2-2. PCR bands were extracted using Promega's Wizard SV Gel and PCR Clean-Up System (Promega A9281). Samples were then sequenced via the Sanger method.

Results and Discussion

CRISPRs in Goats, Part I

Initially, there were concerns that the CRISPR/Cas9 system would not work as we were hoping, specifically to knockout and replace the α_{S2}-casein gene. Because many of the common cleavage detection kits in use now were not available during the initial stages of the project, the efficiency of the CRISPRs being used had not been properly evaluated. In spite of this, we attempted to incorporate spider silk anyway and then focused on looking for silk incorporation to determine whether the CRISPR and HDR method had worked. Unfortunately, this was not an ideal process to determine whether

the CRISPRs were working effectively, as it was dependent on the success of the silk integration. After numerous attempts to incorporate silk using a dual cutting CRISPR/Cas9 strategy, it was determined that it would be beneficial to conduct a proof of concept study in a hamster model.

CRISPRs in Hamsters

The CRISPR gRNAs used in hamster work were designed around the β-casein gene – the only known milk protein gene produced in hamster milk – and the CRISPRs with the highest cutting efficiency were selected. Genomic DNA of BHK cells transfected with CRISPRs targeting both ends of the β-casein gene was extracted 72 hours post-transfection. Analysis of the gDNA indicated that both CRISPRs were cutting when co-transfected. BHK cells were then transfected with CRISPRs and the plasmid containing homology arms and the spider silk gene. PCR analysis performed on the gDNA of these cells (also extracted 72 hours post-transfection) did not indicate successful incorporation of the spider silk gene. Because multiple iterations of the same experiment yielded the same results, CRISPRs and homology arms targeting/matching the flanking regions of the start codon and secretion signal of the β-casein gene were selected. Although HDR was the preferential repair mechanism for this study, the new "upstream" CRISPR was selected such that it would create a double-stranded break (DSB) in the middle of the homologous region of the genome and donor DNA, thus facilitating Obligate Ligation-Gated Recombination (ObLiGaRe), a method for site-specific gene insertions described by Maresca *et al*.[21]. Briefly, ObLiGaRe takes advantage of the NHEJ repair mechanism to ligate exogenous DNA into a specific locus in the genome by using a target specific genomic editing tool that recognizes both the

donor DNA and the target site in the genome[21]. The new CRISPRs designed to induce ObLiGaRe were evaluated for efficiency, and then co-transfected with the homology arm/silk construct. Genomic DNA was extracted after 72 hours and subjected to PCR analysis and subsequent sequencing. Both analyses showed that the CRISPRs had successfully produced indels, with the strongest sequence detected via sequencing showing spider silk gene integration (Figures 2-2 and 2-3). It is of note that the precise integration point of the 3' end of the gene could not be determined via PCR regardless of the primer pairs used. Due to the impracticality of producing "spider hamsters," these results were deemed sufficient to validate the feasibility of creating new spider goats using the CRISPR/Cas9 system (and to establish the skills of this researcher). Consequently, neither a transgenic cell line nor transgenic hamster pups were created.

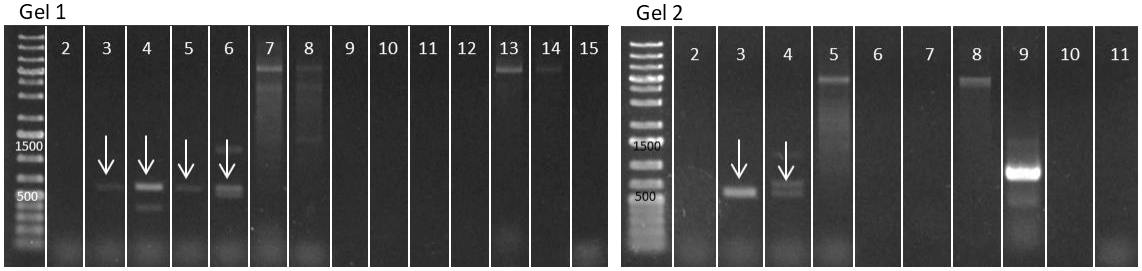
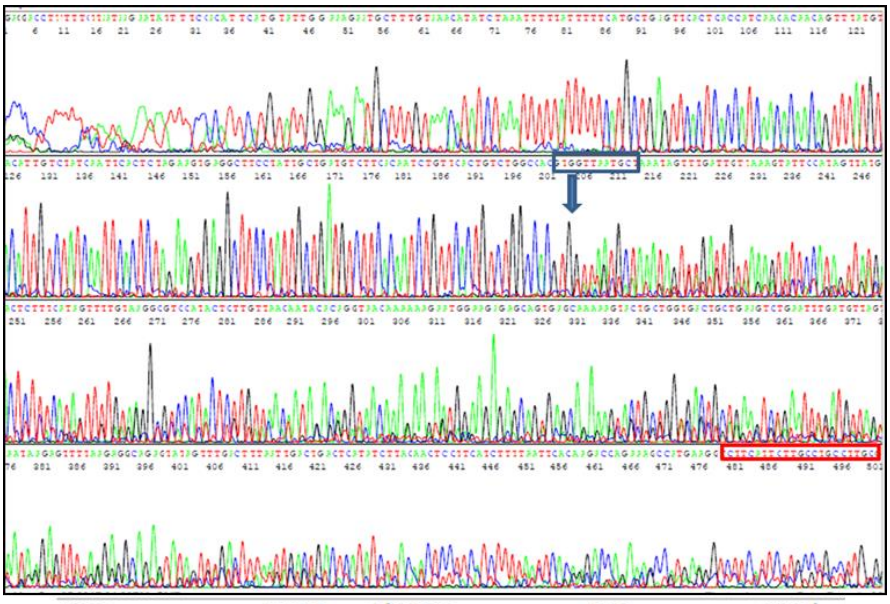


Figure 2-2. Gel electrophoresis analysis of PCR run on BHK gDNA. Primer pairs: (S1 or S2) Ham Silk Int Up 2F + MaSp1 (or MaSp2) Ham Check; (B) BUF1 + BUR1. Gel 1 – lane 2: BHK gDNA negative control (S1); lanes 3-6: cells transfected with MaSp1 (S1); lanes 7-8: MaSp1 plasmids (not gDNA) (S1); lane 9-14: DNA negative controls (same as 3-8, but no primers); lane 15: primer negative control (S1, no DNA). Gel 2 – lane 2: BHK gDNA negative control (S2); lanes 3-4: cells transfected with MaSp2 (S2); lane 5: MaSp2 plasmid (not gDNA) (S1); lane 6-8: DNA negative controls (same as 3-5, but no primers); lane 9: PCR positive control, BHK gDNA (B); lane 10: negative controls (no DNA or primers); lane 11: negative primer control (S2). White arrows indicate bands representing silk integration. Ladder: Thermo Scientific GeneRuler^{TM} 1kb Plus DNA Ladder.



Score	Expect	Identities	Gaps	Strand
950 bits(514)	0.0	524/528(99%)	3/528(0%)	Plus/Plus

```
Query  30   TTTTTACTAATAGAATAATTTCCACATTCATGTATT-GGAAAGAATGCTTTGTAAACATA  88
            ||||| |||||||| ||||||||||||||||||| |||||||||||||||||||||||
Sbjct  8    TTTTTTCTAATAGAA-AATTTCCACATTCATGTATTGGGAAAGAATGCTTTGTAAACATA  66

Query  89   TCTAAATTTTTATTTTTCATGCTGAGTTCACTCACCATCAACACAACAGTTTATGTACAT  148
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  67   TCTAAATTTTTATTTTTCATGCTGAGTTCACTCACCATCAACACAACAGTTTATGTACAT  126

Query  149  TGTCTATCAATTCACTCTAGAAGTGAGGCTTCCTATTGCTGATGTCTTCACAATCTGTTC  208
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  127  TGTCTATCAATTCACTCTAGAAGTGAGGCTTCCTATTGCTGATGTCTTCACAATCTGTTC  186

Query  209  ACTGTCTGGCCACGT-GTTAATGCTAAATAGTTTGATTGTTAAAGTATTCCATAGTTATG  267
            |||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187  ACTGTCTGGCCACGTGGTTAATGCTAAATAGTTTGATTGTTAAAGTATTCCATAGTTATG  246

Query  268  ACTCTTTCATAGTTTTGTAAGGCGTCCATACTCTTGTTAACAATAGACAGGTAACAAAGA  327
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  247  ACTCTTTCATAGTTTTGTAAGGCGTCCATACTCTTGTTAACAATAGACAGGTAACAAAGA  306

Query  328  AGAATGGAAGAGAGCAGTGAGCAGAAAGTACTGCTGGTGACTGCTGAAGTCTGAATTTGA  387
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  307  AGAATGGAAGAGAGCAGTGAGCAGAAAGTACTGCTGGTGACTGCTGAAGTCTGAATTTGA  366

Query  388  TGTTAGTAATAGGAGTTTTAGGAGGCAGAGTATAGTTTGACTTTAATTGACTGACTCATA  447
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  367  TGTTAGTAATAGGAGTTTTAGGAGGCAGAGTATAGTTTGACTTTAATTGACTGACTCATA  426

Query  448  TCTTACAACTCCTTCATCTTTTAATTCACAGGACCAGAAAGCCATGAAGGTCTTCATTCT  507
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  427  TCTTACAACTCCTTCATCTTTTAATTCACAGGACCAGAAAGCCATGAAGGTCTTCATTCT  486

Query  508  TGCCTGCCTTGTGGCACTTGCTTTTGCATCCGGCCATATGACCGGTGG  555
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487  TGCCTGCCTTGTGGCACTTGCTTTTGCATCCGGCCATATGACCGGTGG  534
```

Figure 2-3. (Top) Electropherogram produced by sequencing a PCR run on gDNA extracted from post-transfected BHK cells. Blue box and arrow indicate CRISPR/Cas9 target and cut site, respectively. Red box highlights spider silk sequence. (Bottom) BLAST® Alignment of expected sequence (Query) and strongest signal from sequencing (Sbjct).

CRISPRs in Goats, Part II

Following the success with BHK cells, CRISPR/Cas9 work was continued on gFF cells. The CRISPRs used previously were re-evaluated and changed as needed to improve

targeting efficiency in the goat genome. Similar to the hamster project, new CRISPRs and homology arms were also created for the flanking regions of the start codon and secretion signal of the α_{s2}-casein gene, as opposed to the entire gene. Although the CRISPR/Cas9 constructs consistently showed reasonable cutting efficiencies, spider silk could never be detected as having been incorporated into the genome. It is speculated that the silk could have been incorporated with the aid of selection pressure, but this avenue was not explored during the course of this project when using the CRISPR/Cas9 and HDR method. Another possibility for the lack of success in editing the goat genome is the recently discovered phenomenon in which the CRISPR/Cas9 complex did not detach from the site where it created a DSB, preventing cellular repair mechanisms from working properly[22]. It is also worth noting that the ObLiGaRe technique used to incorporate silk into the hamster genome was not investigated in goats due to concerns with the gRNAs targeting the α_{s2}-casein promoter region of the genome and the homology arms leading to indel creation in the promoter and upsetting the potential for protein production.

PiggyBac

The silk-containing PiggyBac vector, having been modified with a neo cassette and the goat β-casein promoter (Figure 2-1), was transfected into gFF cells. Following transfection (72 hours), GFP could be seen in >90% of the control cells under fluorescence microscopy. Genomic DNA was extracted from Neomycin selected cells 14-17 days post-transfection (depending on transfection batch and complete cell death in negative control wells) and analyzed via PCR for silk integration. As shown in Figure 2-4, all gDNA batches showed positive results for silk integration. These results, which

were confirmed via Sanger sequencing (Figure 2-5), indicated that both mixed population and single cell derived (or nearly single cell derived) cell lines containing the gene coding for the MaSp1 spider silk protein have been established.

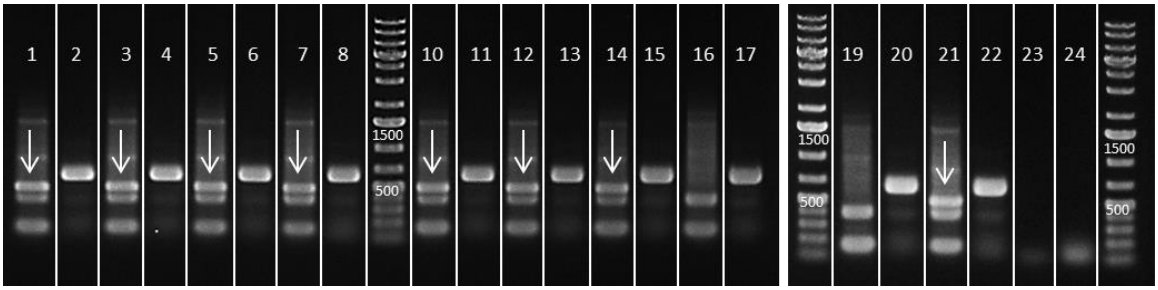


Figure 2-4. Gel electrophoresis analysis of PCR run on gDNA extracted from gFF cells. Lanes 1-8 and 10-15: PCR on cells transfected with Piggy+MaSp1; lanes 16 and 17: PCR on cells transfected with empty PiggyBac (negative integration control); lanes 19 and 20: PCR on wild type gFF cells (positive PCR control, secondary negative integration control); lanes 21 and 22: integration positive control; lanes 23 and 24: negative PCR controls (no gDNA). PCR reactions in lanes 1, 3, 5, 7, 10, 12, 14, 16, 19, 21, and 23 were all run with primer pair MaSp1 F1 and MaSp1 R3; all other PCR lanes were run with the UF2 and UR3 primer pair. White arrows indicate bands representing MaSp1 integration. Ladder: Thermo Scientific GeneRuler^{TM} 1kb Plus DNA Ladder.

Score	Expect	Identities	Gaps	Strand
835 bits(452)	0.0	455/456(99%)	1/456(0%)	Plus/Plus

```
Query  5697  GTGCTGGTGCAGCAGCAGCTGCCGCAGCAGCAGGCGGTGCAGGCCAAGGCGGATATGGCG  5756
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  12    GTGCTGGTGCAGCAGCAGCTGCCGCAGCAGCAGGCGGTGCAGGCCAAGGCGGATATGGCG  71

Query  5757  GACTGGGTTCACAGGGTGCAGGCCGTGGCGGTTTAGGTGGTCAAGGCGCAGGCGCTGCTG  5816
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  72    GACTGGGTTCACAGGGTGCAGGCCGTGGCGGTTTAGGTGGTCAAGGCGCAGGCGCTGCTG  131

Query  5817  CAGCCGCAGCGGCAGCAGCTGGCCAAGGTGGCTATGGTGGCTTAGGCTCACAGGGTGGCG  5876
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  132   CAGCCGCAGCGGCAGCAGCTGGCCAAGGTGGCTATGGTGGCTTAGGCTCACAGGGTGGCG  191

Query  5877  GTGCTGGACAGGGTGGATACGGTGGCCTTGGCAGTCAAGGTGCGGGTCGCGGTGGTTTAG  5936
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  192   GTGCTGGACAGGGTGGATACGGTGGCCTTGGCAGTCAAGGTGCGGGTCGCGGTGGTTTAG  251

Query  5937  GCGGTCAGGGTGCGGGTGCGGCTGCTGCAGCTGCGGCAGCGGGTGGTGCTGGGCAAGGCG  5996
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  252   GCGGTCAGGGTGCGGGTGCGGCTGCTGCAGCTGCGGCAGCGGGTGGTGCTGGGCAAGGCG  311

Query  5997  GTTACGGTGGATTAGGTAGCCAAGGTGCAGGACGCGGAGGTCTTGGTGGACAGGGTGCTG  6056
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  312   GTTACGGTGGATTAGGTAGCCAAGGTGCAGGACGCGGAGGTCTTGGTGGACAGGGTGCTG  371

Query  6057  GCGCTGCTGCGGCAGCAGCAGCCGCTGGGGGTGCTGGTCAAGGGGGTTATGGCGGTTTAG  6116
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  372   GCGCTGCTGCGGCAGCAGCAGCCGCTGGGGGTGCTGGTCAAGGGGGTTATGGCGGTTTAG  431

Query  6117  GATCTCAGGGTGCGGGACGGGGTGGTC-TGGGAGGG  6151
             ||||||||||||||||||||||||||| |||||||||
Sbjct  432   GATCTCAGGGTGCGGGACGGGGTGGTCCTGGGAGGG  467
```

Figure 2-5. BLAST® alignment comparison of expected sequence (Query) and sequencing results (Sbjct) for MaSp1 incorporation into the goat genome via PiggyBac.

Future Work

The most immediate future work will be to use the transgenic gFF cells for somatic cell nuclear transfer during the goat breeding season. Any kids produced from these cells can be tested for transgenesis. Kids testing positive can then be milked and silk protein can be extracted from the milk using affinity chromatography. The protein can then be tested and compared to current goat-derived silk proteins.

Because it is assumed that the biggest problem with introducing spider silk into the goat genome using the CRISPR/Cas9 system is the ability to select for positively transfected cells, we believe that it might still be possible to use the CRISPR/Cas9 system along with HDR or NHEJ to incorporate a spider silk gene into the goat genome at a targeted location, but further modifications to the protein coding sequence would be

necessary, i.e. incorporation of a selection marker. One possibility would be to include a strong GFP marker with the silk protein gene, as this could allow for identification of successful genomic integration as well as fluorescence activated cell sorting (FACS) to obtain only transgenic cells. Initially, FACS was attempted on gFF cells, as the pX330 backbone vector used in these experiments contains a GFP marker, but the fluorescence was not strong enough. Also, the presence of GFP in this case would not have indicated successful genomic integration, merely successful transfection, so FACS would not have been effective. By incorporating a stronger GFP coding cassette with the silk, the possibility of successful FACS would increase significantly, which would facilitate the establishment of a cell line with targeted silk integration.

Another possible selection method could be the incorporation of an antibiotic-resistance cassette into the silk/homology arm construct. It would be necessary to ensure that the antibiotic-resistance cassette was controlled with its own regulatory elements, similar to how the PiggyBac work was done, so the resistance gene would not be produced as part of the silk. Otherwise, the spider silk would include an antibiotic-resistance gene, which could cause significant problems when considering medical applications of the spider silk. The antibiotic-selection gene could also interfere with how the silk performed mechanically if it were to be produced with the silk. In spite of these concerns, with proper precautions antibiotic selection is a feasible approach to obtaining gFF cells with integrated spider silk.

One concern with this project regardless of the integration technique is the size of the resultant silk proteins. The mechanical properties of spider silk are dependent on the length of the silk proteins – the longer the protein, the stronger the material[23,24]. Full

length silk proteins range from 200 to 350 kDa[12,25], while the silk proteins produced as a result of this project will only be ~74 kDa. Although these proteins are not as long as we would like, they are comparable to the goat-derived proteins currently in use. Because of this, they can provide sufficient data for comparison between the current goat production system and the modified system established through this work. If the results of these comparisons are in favor of the new spider goats' proteins, larger silk-coding genes can be incorporated using the same methodology.

Conclusion

Although there is still work to be done, it is possible to create transgenic goat fetal fibroblasts with genes coding for histidine-tagged MaSp1 spider silk protein using the PiggyBac™ Transposon Vector System. These gFF cells will make it possible to create new and improved "spider goats" that will make proteins that are significantly easier to purify. This is an important step forward in the production of spider silk proteins for medical applications in that it may allow for the collection of larger volumes of protein at higher purity levels.

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CHAPTER 3

METHOD FOR THE DESTRUCTION OF ENDOTOXIN IN SYNTHETIC SPIDER SILK PROTEINS[1]

Abstract

Although synthetic spider silk has impressive potential as a biomaterial, endotoxin contamination of the spider silk proteins is a concern, regardless of the production method. The purpose of this research was to establish a standardized method to either remove or destroy the endotoxins present in synthetic spider silk proteins, such that the endotoxin level was consistently equal to or less than 0.25 EU/mL, the FDA limit for similar implant materials. Although dry heat is generally the preferred method for endotoxin destruction, heating the silk proteins to the necessary temperatures led to compromised mechanical properties in the resultant materials. In light of this, other endotoxin destruction methods were investigated, including caustic rinses and autoclaving. It was found that autoclaving synthetic spider silk protein dopes three times in a row consistently decreased the endotoxin level 10-20 fold, achieving levels at or below the desired level of 0.25 EU/mL. Products made from triple autoclaved silk dopes maintained mechanical properties comparable to products from untreated dopes while still maintaining low endotoxin levels. Triple autoclaving is an effective and scalable method for preparing synthetic spider silk proteins with endotoxin levels sufficiently low for use as biomaterials without compromising the mechanical properties of the materials.

[1] This chapter was published in Scientific Reports and was co-authored by Thomas I. Harris, Dylan R. Memmott, Christopher J. Peterson, Randolph V. Lewis, and Justin A. Jones.

Introduction

The biocompatibility and mechanical properties of spider dragline silk set it apart from most synthetic and natural materials as an ideal biomaterial. Dragline silk is made up of two proteins, MaSp1 and MaSp2[1,2], whose structures make the resultant fibers strong, extensible, and flexible.[3] With synthetic spider silk proteins, these properties can be harnessed into a variety of materials in addition to fibers, such as films, coatings, gels, and adhesives[4,5]. Synthetic spider silk materials (including fibers) can also be tailored to have increased strength and/or flexibility through mixing different ratios of the dragline proteins. Due to its versatility, synthetic spider silk has great potential for a variety of biomedical applications.

Although native spider silk is generally accepted as biocompatible[6,7], it is difficult to obtain in large quantities because spiders can only produce a limited amount of silk in a day and cannot be farmed efficiently due to their territorial and cannibalistic nature. In response to these issues, synthetic spider silk proteins have been produced in a variety of hosts[8,9], including yeast[10], bacteria[11,12], silkworms[13,14], tobacco plants[15], and goats[16]. While each of these expression systems has advantages and disadvantages for producing spider silk proteins, all are subject to contamination with environmental pyrogens, especially endotoxins, during production and processing.

Pyrogens are substances that produce a fever that can quickly become dangerously high. Endotoxins, one of the most prevalent pyrogens, are surface lipopolysaccharides (LPS) released from gram-negative bacteria that are heat stable to high temperatures[17]. As with all pyrogens, if endotoxins are present at high enough levels, they will induce a fever[18]. Because of this, endotoxin level testing and subsequent

removal or destruction is required for any implantable biomaterial[19,20]. Although endotoxins are produced by gram-negative bacteria, they can be found in most environments, particularly on a goat farm and in a laboratory environment[21].

The most common method for pyrogen destruction (depyrogenation) of materials such as packaging and medical devices is dry heat. Materials to be depyrogenated are treated at 250 °C for ≥30 min[17,22]. The temperature can be adjusted, but it cannot be lower than 180 °C, and at that temperature the samples must be treated for ≥3 hours[22]. Unfortunately, most proteins and other polymers cannot withstand these high temperatures. Although triple autoclaving has been proposed as a potential method for removing endotoxin, the effectiveness of this and other alternative methods of depyrogenation, including caustic rinses, is often debated[17].

Common methods for removing endotoxin from solutions, such as size-exclusion or ion-exchange chromatography, are effective for medical injectables and microbial-produced bioproducts such as small (<100 kDa), soluble proteins. Although spider silk can be solubilized under specific conditions, it is likely to solidify as the conditions change during the endotoxin removal processes. Ion-exchange chromatography often leads to an increase in salt concentrations, which are detrimental to the formation of spider silk materials[23], and would require additional washing steps for the silk proteins. Arguably the most significant problem with these methods of endotoxin removal is that they are most effective on smaller soluble proteins. The mechanical properties of spider silk materials are highly dependent on the size of the protein – larger (>100 kDa) is better[15,24,25]. Due to these issues, common methods for removing endotoxins cannot be used on silk proteins.

In light of this, a method to destroy or remove endotoxins from synthetic spider silk (or the materials produced from them) is needed to produce biocompatible and implantable materials. To our knowledge, there is no such method that has been reported in literature. To remedy this, we sought to determine the best method to either remove or destroy endotoxins present in synthetic spider silk proteins and synthetic spider silk protein-based materials while maintaining the valuable mechanical properties of the spider silk.

Results and Discussion

As can be seen in Table 3-1, it is possible to decrease the endotoxin levels of synthetic spider silk protein by autoclaving three times. Dry heating also decreased the endotoxin level (data not shown), but even at the lowest acceptable treatment temperature of 180 °C the recombinant spider silk's mechanical properties were compromised. Protein treated with dry heat made very poor films that could not be tested because they broke when handled. Similarly, dry heated fibers also became very brittle. This decrease in mechanical properties is likely due to the extreme dehydration and resultant degradation of the spider silk that occurs at high temperatures and/or pressures[23]. The dry heated protein also had significant discoloration (brownish-yellow or black), indicating that it had been charred. Due to the detrimental effects of dry heating on the mechanical properties of the samples, the dry heat treatment method was discarded. In contrast, the autoclaved protein samples maintained their color and the resultant films had mechanical properties similar to films made from untreated proteins (Table 3-2). It is also of interest that films maintained decreased endotoxin levels when they were made on endotoxin-free polydimethylsiloxane (PDMS) molds, but not when made on untreated PDMS molds

(Table A1-1, sample 15). This further confirms that autoclaving destroys the endotoxin in the silk proteins.

Table 3-1. Combined average endotoxin levels of all goat-derived spider silk proteins and films before and after treatments. Individual sample group averages are shown in Table A1-1. The R^2 of the endotoxin analysis kit standard curve was ≥0.989 for all experiments. Silk samples below 0.25 EU/mL are in bold. Standard deviations were calculated using STDEV.P in Microsoft Excel.

Sample Type	**Treatment**	**Results (EU/mL)**	**n**
Protein Powder	None	5.92 ± 0.07	2
Film	NaOH rinse + H_20 rinse	2.08 ± 0.11	2
Film	NaOH rinse + H_20 rinse	4.05 ± 0.42	2
Protein Powder	Doping	3.20 ± 1.71	16
Protein Powder	Doping + Autoclaving x3	**0.17 ± 0.09**	14
Films	Doping + Film	0.81 ± 0.36	4
Films	Doping + Autoclaving 3x + Film	**0.18 ± 0.07**	11

Only protein powder, dopes (protein solubilized in water using microwave irradiation to generate high heat and pressure; for details see Methods), and fibers were treated via autoclaving because films deformed (melted) when autoclaved and could not be tested. All film samples presented here were made from autoclaved dopes.

Although triple autoclaving did not cause fibers to become as brittle as dry heated fibers, which broke when handled, the autoclaved fibers still had a significant drop in mechanical properties. It was possible to handle most of the autoclaved fibers to prepare them for mechanical testing, but the samples broke before any meaningful data could be gathered during testing. Some samples were autoclaved immersed in water to test whether dehydration was the sole cause of the increased fragility, but the result was the same, indicating that the issue could be due to the combination of high temperature and high pressure over time.

Because early tests indicated that it would be necessary to eliminate endotoxins in synthetic spider silk fibers (data not shown), dry heat and autoclaving treatments were investigated. While it was found that autoclaving did decrease the endotoxin level in most fiber samples, the results were not as consistent as those of dopes and film samples.

Table 3-2. Data from tensile tests on films made from triple autoclaved and untreated synthetic spider silk dopes. Groups A, B, and C were made from different dopes of the same protein; all received the same 3x autoclave treatment. All MaSp1 sample groups were created from dopes made from the same goat-derived protein stock, but at different times. MaSp2 samples were made from dopes of bacterially-derived protein. Standard deviations were calculated using STDEV.P in Microsoft Excel.

Sample	**Group**	**Average Maximum Stress (MPa)**	**Average Maximum Strain (%)**	**Average Maximum Toughness (MJ/m^3)**	**n**
Unstretched MaSp1	Untreated	39.1 ± 21.2	1.8 ± 0.2	0.33 ± 0.2	6
	A	41.7 ± 3.8	1.7 ± 0.3	0.32 ± 0.08	4
	B	21.5 ± 7.1	1.6 ± 0.1	0.16 ± 0.04	2
	C	52.3 ± 6.1	1.9 ± 0.2	0.45 ± 0.08	4
Unstretched Resolubilized MaSp1	Not Centrifuged	73.9 ± 15.2	2.3 ± 0.3	0.82 ± 0.31	5
	Centrifuged	45.9 ± 10.2	2 ± 0.3	0.47 ± 0.14	3
Unstretched MaSp2	Untreated	189.1 ± 25	3.2 ± 0.4	3.06 ± 0.54	4
	Autoclaved	134.1 ± 29	2.5 ± 0.6	1.69 ± 0.82	4
Stretched MaSp2	Untreated	104.7 ± 32.3	57 ± 5	50.65 ± 10.55	4
	Autoclaved	79.2 ± 17.8	23 ± 11	15.24 ± 6.63	3

Unlike fibers, the mechanical properties of silk films were consistent between films made from untreated dopes and autoclaved dopes (Table 3-2). Generally, stretching

films increases the mechanical properties of spider silk films[5]. In this case, the average ultimate tensile stress of the films was decreased due to stretching, but the strain was greatly increased. It is possible that this was due to the degree of stretching used or due to the stretched samples being composed completely of MaSp2. Native spider dragline silk and many synthetic spider silk films contain at least some MaSp1, which contains additional strength producing crystalline motifs[3]. Although autoclaving has been shown to affect the structure of silkworm silk[26,27], Hedhammer *et al.* showed that one round of autoclaving spider silk did not affect the structure of the silk[28]. It is also very likely that any structural changes that may have occurred due to autoclaving are "reset" when the protein is redoped after autoclaving. This lack of structural difference not only explains the similar mechanical properties of the treated and untreated films, but is also beneficial when comparing treated and untreated proteins, as they are more analogous to each other.

The resolubilization "treatment" referenced in Table 3-2 consisted of triple autoclaving protein powder in water, then, following autoclaving, centrifuging the protein-water mixture, removing the water, and freezing the pellet, or simply freezing the autoclaved protein-water mixture. Both samples were then lyophilized and redoped. This was done to determine whether it would be possible to create stocks of endotoxin-free protein and whether it was better to remove the water previous to freezing and subsequent lyophilization or not. As can be seen in Table 3-2, the films made from dopes that received the resolubilization treatment had mechanical properties similar to films made from freshly treated dopes. This indicates that it is possible to create stocks of endotoxin-free synthetic spider silk that can be used at future times to make endotoxin-free materials. Unfortunately, the practicality of maintaining an endotoxin-free "common

stock" in a laboratory setting is that the stock will not stay endotoxin-free for very long. However, in certain conditions, such as a clean room, good laboratory practice, or good manufacturing practice, an endotoxin-free stock may be very practical and beneficial. Based on the "Not Centrifuged" and "Centrifuged" samples, autoclaving, freezing, and lyophilizing without removing the water from the dope may give better mechanical properties than centrifuging the dope and removing the water before freezing and lyophilizing.

Most of the samples tested in this experiment were from goat-derived spider silk protein. Ideally, synthetic spider silk will be produced predominately in *E. coli*. Hedhammar *et al.* were able to create fibers from small recombinant spider silk proteins produced in *E. coli* with low pyrogenicity by treating the bacteria with a combination of Tris, Ca^{2+}, and EDTA before cell lysis and subsequent silk extraction[28]. While this method of endotoxin removal is beneficial for a bacterial production system, it is still difficult to produce native size spider silk proteins in bacteria. Because of this, there is great benefit in developing a method of endotoxin removal/destruction that can be applied to synthetic silk proteins regardless of the production system. In this study, a batch of bacterially-derived spider silk protein was treated by triple autoclaving. Results indicate that the treatment method also effectively removes endotoxin in the bacterially-derived silk (Table 3-3). It is notable that autoclaving silk proteins is more easily scalable and likely less expensive than the endotoxin removal method developed by Hedhammar *et al.*, as autoclaving does not require any reagents or extra purification steps.

Table 3-3. Endotoxin levels of bacterially-derived protein and subsequent films that were either treated with autoclaving or untreated. Empty vials were vortexed with endotoxin-free water in them and the water was tested. The R^2 of the standard curve was 0.9913 for all tests. All samples had n=2 except B7, which had n=1 due to contaminants in the testing well that interfered with the absorbance reading. Silk samples below 0.25 EU/mL are in bold. Standard deviations were calculated using STDEV.P in Microsoft Excel.

Sample Type	**Sample**	**Treatment**	**Results (EU/mL)**
Empty vial	B1	None	0.05 ± 0.00
Empty vial	B2	Autoclave x3	0.06 ± 0.00
Powder	B3	None	2.28 ± 0.02
Film	B4	Doping + Film	2.20 ± 0.01
Film	B5	Doping + Film	2.29 ± 0.01
Powder	B6	None	2.25 ± 0.01
Film	B7	Doping + Autoclave x3 + Film	**0.185 ± 0**
Film	B8	Doping + Autoclave x3 + Film	**0.178 ± 0.01**

Conclusion

Treating synthetic spider silk protein dopes with three consecutive autoclave cycles is an effective method for reducing endotoxin levels. Autoclaving at the dope stage greatly reduces endotoxin levels without destroying the protein-based materials or compromising their mechanical properties, thus yielding the best combination of endotoxin level reduction and mechanical properties in the final products. This combination will allow synthetic spider silk research to progress to meaningful biocompatibility testing and, eventually, clinical studies without the concern of endotoxin as a confounding factor.

Methods

Preparation of Silk Samples

Most samples were made from goat-derived MaSp1 recombinant spider silk protein. Silk proteins were extracted from goat milk and formed into either films or fibers using the aqueous method described previously[5,29]. Briefly, silk proteins are removed from defatted goat milk via tangential flow filtration, precipitation, washing, and subsequent lyophilization. Silk protein solutions, or dopes, are then made by mixing the dry protein with water and microwaving the mixture in a tightly sealed vial in 5-10 s bursts to achieve a minimum temperature of 120 °C under high pressure to solvate. All dopes used in this study were 5% (w/v) protein (150 mg protein in 3 mL water). Dopes were then either poured onto PDMS molds to form films or spun into fibers using a custom "wet spinning" spin line[5,29].

Final samples were either a full film (30 mm x 6 mm x 50 μm), a six fiber bundle (~25 cm length, ~30 μm diameter), or just protein powder. Powder samples were prepared by vigorously vortexing 150 mg of protein powder in 3 mL of endotoxin-free water for >5 min. The mixture was then centrifuged at 4,185 x g for 10 min, after which 1 mL of the supernatant was removed and stored for each sample. Samples were also taken after doping following the same procedure used for the powders. For all sample sets, control samples were taken prior to any treatments (including doping) and negative controls (endotoxin-free water) were included.

Some samples were made from bacterially-derived MaSp2 recombinant spider silk protein. Silk proteins were produced in *Escherichia coli* and subsequently extracted

as previously described[30]. The proteins were then doped and samples were prepared as described above.

As a note, the doping process is sufficient to sterilize the proteins for cell culture work. Because of this, the doping process was tested for its effectiveness in destroying endotoxin. It was shown that any reduction in endotoxin levels caused by microwaving was insignificant (data not included), so other treatment methods were still required.

Endotoxin Removal/Destruction

Multiple methods of destroying endotoxins from samples were tried in this study: dry heat, caustic washes, water washes, and autoclaving. All equipment used for handling, storing, and preparing samples was depyrogenated via dry heat at 250 °C for at least 30 min.

The dry heat treatment involved heating samples to 250 °C for at least 30 min or 180 °C for at least 3 hours. During heating, all samples were placed in a loosely covered glass container. After heating, samples were covered and stored in a sterile PCR hood until ready for use. Treated samples were only handled with endotoxin-free equipment, as confirmed through the use of the preferred endotoxin detection kit used in these experiments (data not included).

Caustic washes were performed with 1 M sodium hydroxide (NaOH). Treatments were performed on dry spider silk samples by soaking in 40 mL of NaOH for 3 min. The NaOH was then removed via centrifugation at 4,185 x g and the samples were rinsed with endotoxin-free water three to five times, which was also removed via centrifugation. Samples were then stored in a small amount of endotoxin-free water until testing.

Autoclaving was performed on silk samples using a standard 20 min liquid cycle (121 °C, 15 psig, 1 min purge). The cycle was repeated three times. The door was opened for 1 min between each cycle to allow the autoclave pressure sensor to return to atmospheric pressure before proceeding.

Protein powder was mixed with water and then autoclaved in loosely capped bottles. After autoclaving, water was removed from powder samples via centrifugation at 4,185 x g and subsequent pipetting. Dopes were made as described above then transferred to a clean vial that was loosely capped for autoclaving. After autoclaving, the protein powder and dope samples were doped or redoped, respectively, before testing or casting films.

Film Stretching

Some films were stretched before mechanical testing (Table 3-2). Stretching is a common treatment performed on synthetic spider silk fibers and films to improve their mechanical properties[5,29]. In this study, films were stretched in an 80:20 isopropanol:water bath to three times their original length using a stretching apparatus developed by Tucker *et al.*[5]. Films were allowed to dry before being removed from the stretching apparatus and tested. The stretching was not performed under endotoxin-free conditions.

Resolubilization

To test whether it would be possible to make large stocks of endotoxin-free protein powder for future use without the silk losing its mechanical properties, two processes were tested on dopes that had been autoclaved three times: 1) the autoclaved

dope was centrifuged at 4,185 x g, the supernatant was removed, and the protein pellet was frozen; 2) the entire autoclaved dope was frozen. The frozen samples were then lyophilized, after which the protein was resolubilized in water. Films were then made using the standard procedure outlined above.

Endotoxin Level Analyses

The preferred kit to determine the endotoxin levels for these experiments was the Pierce Limulus Amebocyte Lysate (LAL) Chromogenic Endotoxin Quantitation Kit (Thermo Scientific Cat# 88282), which uses UV absorbance at 405-410 nm to determine endotoxin concentration. This kit has a working detection range of 0.1-1.0 EU/mL. Endotoxin levels above 1.0 EU/mL were extrapolated using an experimentally determined standard curve equation.

Because testing a solid piece of silk material interfered with the UV absorbance and confounded the endotoxin level readings, 1 mL of endotoxin-free water was added to samples after endotoxin destruction treatments. Sample/water mixtures were vigorously vortexed for >5 min to break apart the silk material and remove endotoxin from the silk and the container into the water; 50 µL of the water was then used for endotoxin testing.

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CHAPTER 4

INVESTIGATION OF THE *IN VIVO* BIOCOMPATIBILITY OF GOAT-DERIVED SPIDER SILK PROTEIN

Chapter Preface

The following chapter describes *in vivo* biocompatibility studies in which materials made from goat-derived spider silk proteins were implanted into rats. Although native spider silk is widely accepted as biocompatible, very little biocompatibility research has been done on recombinant spider silk proteins and none has been reported on goat-derived spider silk. Because the work presented in this chapter includes the first *in vivo* studies of goat-derived spider silk, it is highly significant and impactful in the synthetic spider silk research field.

Prior to the research described in this chapter, we performed some basic *in vitro* work on goat-derived spider silk to show that multiple cell types (BHK, CHO, and PC-12) could be grown on synthetic spider silk substrates (unpublished data), but these studies were merely exploratory. Because the data produced from this previous work have little scientific value beyond a basic assurance that cell viability is not affected when growing cells on silk-based materials, the studies and results are not included.

The work presented in this chapter shows that, in its current form, goat-derived spider silk is not biocompatible. A strong possibility for this unexpected negative result is that the proteins, which are produced in goat milk, are contaminated with casein proteins. Although the current protein purification method used to obtain the spider silk proteins includes filtration and heating to remove casein, even trace amounts could prove detrimental due to casein's high immunogenicity. If this is the case, it is anticipated that

proteins tagged with histidine and purified with affinity chromatography as described in Chapter 2 of this dissertation will show a higher likelihood for biocompatibility.

It is important to note that the synthetic spider silk materials used in these studies were susceptible to degradation by the rats' immune system, which likely explains much of the increased immunological response (e.g., increased numbers of macrophages at the implantation site). While this is a concern, longer term implants may be completely degraded and have little to no lasting effects on the host body. Although the studies presented here are essentially preliminary work to be followed up with longer term implantation studies using silk with a higher purity level, we feel that they provide a crucial step in the direction of using goat-derived spider silk materials for biomedical applications.

Introduction

Native spider silk, particularly major ampullate (dragline) silk, has long been known as a biocompatible material[1,2]. Dragline silk in particular has great potential in the biomedical field because of its biocompatibility and impressive mechanical properties[3]. Unfortunately, it is extremely difficult to obtain native spider silk in high quantities because of the difficulties associated with farming spiders due to their cannibalistic and territorial nature. To facilitate the use of spider silk in many different commercial applications, particularly biomedical applications, synthetic spider silk proteins have been created in multiple systems, including bacteria, silkworms, and goats[4–8]. Of these systems, the most consistently large quantities of silk proteins are produced in goat milk.

Although native spider silk and some recombinant spider silk proteins (rSSps) are known to be biocompatible[1,2], no *in vivo* biocompatibility studies of goat-derived rSSps have been reported to our knowledge. In order to determine the feasibility of using goat-derived rSSp materials for medical applications, it is essential that the silk's biocompatibility be determined. Below, we describe three *in vivo* studies in rats aimed at gathering preliminary data on the biocompatibility of two goat-derived spider silk products: thin films and hydrogels. Rats were euthanized after 1, 2, 4, or 6 weeks and tissue samples were collected to determine the localized immune response of the implanted material. The findings from these studies establish a baseline for the current biocompatibility level of goat-derived rSSps and highlight areas for improving that level to facilitate the use of these protein-based materials for biomedical applications.

Materials and Methods

Preparation of Implant Materials

Films

Silk films were composed of goat-derived Major Ampullate Spidroin 1 (MaSp1) – one of the two proteins that compose dragline silk – as described previously[9]. Briefly, the proteins were mixed in water to create a 5% (w/v) silk solution or dope. The dope was then heated in 5 – 10 s bursts under pressure to at least 120 °C. The dope was treated for endotoxin via triple autoclaving[10], then heated again before being poured onto polydimethylsiloxane (PDMS) molds that had been treated for endotoxin using dry heat (250 °C for 30 min). Resultant films were approximately 15 mm x 6 mm x 50 µm.

Thin polytetrafluoroethylene (PTFE) pieces were used as controls (Grainger, 30FZ37). The PTFE pieces were cut to match the films' surface area as closely as possible (approximately 15 mm x 6 mm x 25 µm) and then treated for endotoxin by either dry heating at 250 °C for 30 min or triple autoclaving. Pieces treated with both methods were used as controls.

Hydrogels

Silk hydrogels were either a 50/50 blend of MaSp1 and MaSp2, or pure MaSp1, depending on the study; hydrogel composition is noted for each study. Dopes were created and treated similarly to the dopes used to create films, but with the silk concentration being 20% (w/v) and the addition of 2% (v/v) propionic acid to aid solubility at this relatively high concentration. After autoclaving and reheating, dopes were cooled overnight in tightly sealed vials in a closed laminar flow hood. Hydrogel

discs measuring 3 mm thick and 6 mm in diameter were punched out of the solidified dope using metal punches that had been treated for endotoxin via dry heat (250 °C for 30 min). Hydrogels were placed in sterile petri dishes and left to soak in Dulbecco's phosphate buffered saline (DPBS) for 48-72 hours prior to implantation, with the DPBS being replaced after 24 hours. The pH of the DPBS was tested to ensure the propionic acid had leeched out to avoid a potential confounding factor.

Controls in the hydrogel study were made from Poly(ethylene Glycol) diacrylate (referred to herein as PEG) due to its biocompatibility and low immunological reponse[11,12]. 10% (w/v) PEG hydrogels were made in DPBS using PEG with an average MW of 10 kDa (Laysan Bio Inc, ACRL-PEG-ACRL-10K-5g). Photoinitiator (2-Hydroxy-4′-(2-hydroxyethoxy)-2-methylpropiophenone) (Sigma-Adlrich, 410896) was added to a final concentration of 0.25% (w/w), after which the mixture was vortexed vigorously until no particles were visible (30-60 s). Following mixing, the solution was exposed to UV light (365 nm) for 15 min to initiate crosslinking. PEG hydrogels were triple autoclaved to ensure endotoxin destruction and sterility, then allowed to sit overnight before being punched into implantable discs measuring approximately 3 mm thick and 6 mm in diameter. Discs were soaked in DPBS for 48-72 hours prior to implantation, with the DPBS being replaced after 24 hours.

Animal Studies

Wistar rats were obtained from Charles River Laboratories (Wilmington, MA). The rats were acclimatized for approximately 1 week prior to surgical procedures. All procedures were conducted in an AAALAC accredited Laboratory Animal Research Center at Utah State University. All procedures received prior approval by Utah State

University's Institutional Animal Care and Use Committee. Animals were anesthetized with isoflurane via inhalation. The dorsa of the animals were shaved and skin was disinfected with chlorhexidine and 70% isopropyl alcohol. Four 1 cm incisions were created approximately 2 cm apart on both sides of the back (8 incisions total), approximately 2 cm from the midline (Figure 4-1). Subcutaneous pockets were created using blunt dissection and implants were placed in the pockets. Following implantation, incisions were closed using 3-0 polyglactin suture (Vicryl®, Ethicon J460H). Buprenorphine (0.1 mg/kg) (PAR Pharmaceuticals) and enrofloxacin (5 mg/kg) (Baytril®) were administered intramuscularly before returning rats to their home cages. After the allotted time post-surgery, rats were euthanized via carbon dioxide asphyxiation. Sutures were removed after euthanization in the case of the 1 and 2 week groups or 14 days post-surgery in the case of the 4 and 6 week groups.

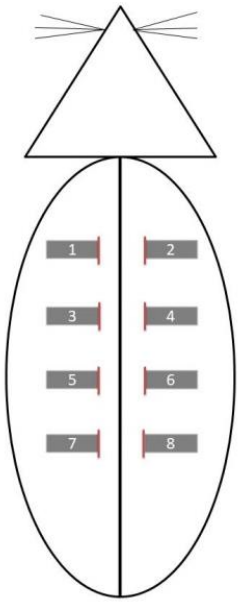

Figure 4-1. Graphical representation of incisions (red lines) and subcutaneous pockets (gray boxes) made in rats for implantation studies. Numbers indicate sample number and were assigned for ease of sample evaluation. Image is not to scale.

Film Study

The films study was conducted on 6 rats. Films and surrounding tissue were harvested 4 weeks post-implantation. The rats were shaved prior to tissue collection. Tissue samples were fixed for ≥24 hours in formalin before being taken for histopathological evaluation.

Hydrogel Study

The hydrogel study was conducted on 18 rats. Gels and surrounding tissue were harvested from 6 rats at 1 week, 6 rats at 2 weeks, and 6 rats at 6 weeks. The rats were shaved prior to tissue collection. Tissue samples were fixed for ≥24 hours in formalin before being taken for histopathological evaluation.

Histopathological Evaluation

Fixed tissue samples were sliced to approximately 3 mm thick, 1 cm deep, and 1.5 cm long in preparation for embedding in paraffin. Samples were then evaluated to produce a semi-quantitative score of local tissue response according to ISO 10993 Part-6 (2007)[13], and Muhamed *et al.*[14]. In each histology session, the following parameters were studied: number of inflammatory cells (neutrophils, plasma cells, lymphocytes, and macrophages), number of foreign body giant cells (FBGCs), severity of necrosis, extent of neovascularization, extent of fibrosis, and extent of fatty infiltration. The average semi-quantitative score was then calculated as [(subtotal-I x 2) + subtotal-II], wherein subtotal-I is the sum of scores for neutrophil, lymphocyte, plasma cell, macrophage, giant cell, and severity of necrosis and subtotal-II is the sum of the scores for neovascularization, fibrosis, and fatty infiltration. The difference of the score for the test material from the reference material was then designated as the "irritancy score."

A simpler, less quantitative analysis based on work by Nyska *et al.*[15], in which results were graded from 1 to 4 based on inflammation present (lymphocytes, neutrophils, macrophages) and fibrous capsule size, was also done: Grade I: slight reaction with a few inflammatory cells; Grade II: clear inflammatory reaction with one or two giant cells; Grade III: fibrous tissue with inflammatory cells, lymphocytes, and giant cells; and Grade IV: granuloma with encapsulated implants and clear foreign-body reaction.

Silk Clean Up

For one of the hydrogel implantation studies, potential contaminates were removed from goat-derived spider silk proteins using reverse phase chromatography (RPC). Proteins were solubilized in 4 M Urea at a 3% (w/v) concentration. The protein solutions were diluted to 2% (w/v) then run on an AKTA Avant with an XK 50 x 25 column with 200 ml of GE SOURCE 30RPC resin (GE Healthcare, 17-5120-02). Proteins were eluted using acetonitrile (Sigma-Aldrich 34851-4L), 0.1% (v/v) Pierce® Trifluoroacetic acid (ThermoFisher Scientific 28904) at a gradient of 0 – 50% over 10 column volumes. Fractions were collected at 45 mL intervals in 50 mL conical tubes (VWR 37001-468). Acetonitrile was allowed to evaporate in a chemical fume hood overnight before samples were concentrated by heating at 90 °C until samples had been reduced to 25% of their starting volume, which took approximately 12 hours. HPLC-grade acetone (Pharmco-Aaper 32900HPLC) was then added at a 3:1 ratio (acetone:sample); the mixture was shaken vigorously and then stored at -20 °C overnight to facilitate protein precipitation. Samples were then centrifuged at 4 °C at 3200 x g for 30 min in a Beckman Allegra™ 6KR centrifuge. The supernatant was analyzed via Western Blot to verify that all of the rSSp had been removed. The protein pellet was

frozen and lyophilized. The presence of silk was verified via Western Blot analysis. This silk protein is designated as RPC MaSp1 throughout this manuscript.

Western Blot

Western Blot analyses were conducted on RPC MaSp1 to determine which peaks contained silk. A 1 mL sample of each fraction was concentrated to 20 µL and diluted 1:1 in SABU. SDS-PAGE gels were loaded with 20 µL of the concentrated fraction/SABU solution and run at 110 V for 80 min. Gels were transferred to a nitrocellulose membrane overnight at 35 mA in a wet transfer apparatus. Western Blots were performed on the membranes with a custom made primary antibody produced in rabbits that targets the native C-term of major ampullate spider silk proteins used at a 1:1000 dilution. A donkey anti-rabbit Alkaline Phosphatase (AP) conjugated antibody (Rockland 611-705-127) was used as the secondary antibody at a 1:5000 dilution. The membranes were developed in 1-Step™ NBT/BCIP Substrate Solution (ThermoFisher Scientific 34042) for imaging.

For the RPC MaSp1 implantation study, blood was drawn prior to surgery and following euthanasia. Blood was allowed to sit for >30 min before being centrifuged at 2000 x g in a Beckman Coulter Microfuge®18 centrifuge. Serum was collected and aliquoted then stored at -20 °C. Serum samples were used as the primary antibody at a 1:2000 dilution with a rabbit anti-rat IgG AP conjugated secondary antibody (Sigma-Aldrich A6066) to determine whether the silk implanted had instigated an immune response, thus leading to the production of silk-specific antibodies in the rat. These Western Blots were run following the same protocol described above.

Results and Discussion

The first study conducted was the film implantation study. Upon visual inspection, the tissue showed similar reactivity to both silk and PTFE implants. The histopathology results initially only included the simpler grading scale (Table 4-1). Based on these results, the response of spider silk films was determined to be comparable to that of PTFE films, providing grounds to progress to a larger scale study. One concern with using films again was that we could not determine the implant site of many of the samples. In the case of the missing rSSp films, we suspect the films had been completely resorbed into the body, making it difficult to find the sample implantation site when excising the tissue. Consequently, it was determined that hydrogels would be a better material to test, as they are larger and it was anticipated that they would be more stable long-term under *in vivo* conditions.

Table 4-1. Average histology results from the film implantation study. Grading was on a whole number scale of 1 to 4: Grade I: slight reaction with a few inflammatory cells; Grade 2: clear inflammatory reaction with one or two giant cells; Grade 3: fibrous tissue with inflammatory cells, lymphocytes, and giant cells; and Grade 4: granuloma with encapsulated implants and clear foreign-body reaction. Standard deviations were calculated using Microsoft Excel STDEV.P.

Sample Type	Inflammation (lymphocytes, neutrophils, probably macrophages)	Fibrous capsule	Grade	n
Autoclaved PTFE	2 ± 0.447	4 ± 0.4	3 ± 0	10
Dry Heated PTFE	2 ± 0.433	3 ± 0.829	3 ± 0	4
Silk	2 ± 0.573	4 ± 0.442	3 ± 0	15

Contrary to the results we anticipated to see for the hydrogel study based on the results of the film study, it was very apparent when observing implant sites during necropsies that the silk hydrogels were not comparable to the PEG hydrogel controls. The

PEG gels were highly visible and the surrounding tissue did not show any obvious signs of inflammation (redness, bleeding, swelling, etc.) at any time points except for 1 week. Any inflammation at the 1 week time point was attributed to the incision, sutures, and surgical procedure in general. The silk hydrogels were not readily visible due to a blackish-red abscess surrounding each of the implants. Although the abscess surrounding the silk was not as visible at 1 week as at other time points, it was present (Figure 4-2A), especially when compared to the PEG tissue samples (Figure 4-2B). At 6 weeks the encapsulation of the silk material was very apparent, while the PEG remained free of encapsulation (Figure 4-2C). Histopathology results confirmed the visual inspection at both week 1 and week 6 (Table 4-2) – inflammation and fibrosis were significantly higher for silk samples. Although no pictures are available of samples at week 2, histopathology results are presented in Table 4-2 for comparison. As can be seen, the week 2 results are very similar to the week 1 results. None of the rats showed any signs of systemic infection post-surgery and the tissue inflammation was localized to the implant region.

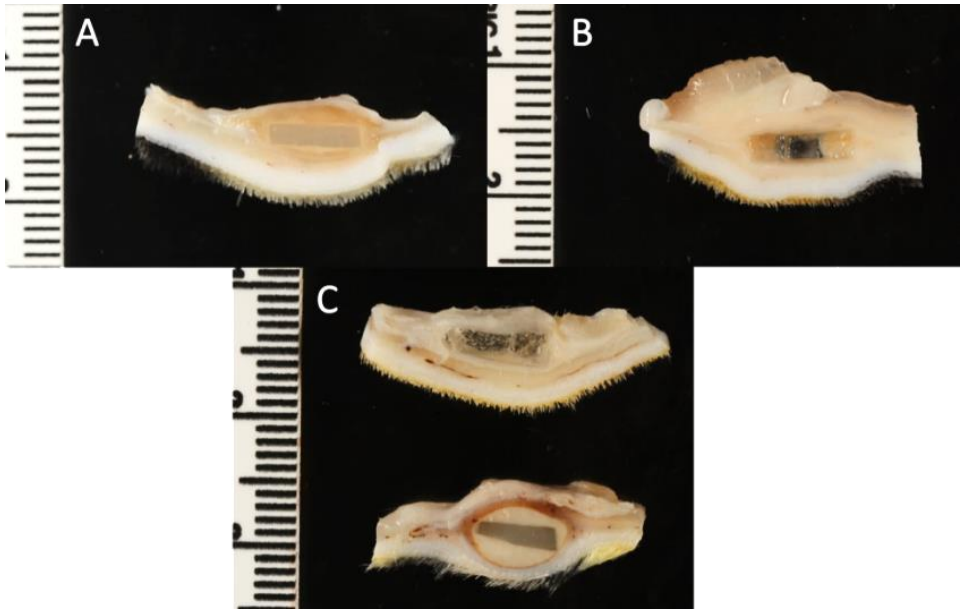

Figure 4-2. Tissue samples from rats implanted with silk and PEG hydrogels. A) Silk implant at 1 week. B) PEG implant at 1 week. C) PEG (top) and silk (bottom) implants at 6 weeks. A ruler with 1 mm increments was included with each image for scale.

Table 4-2. Average histopathology results for hydrogel implant study. Scores were rated from 1 to 4 in each category. Total score is equal to (Infl. Sub x2) + Fibro. Sub. Standard deviations were calculated using Microsoft Excel STDEV.P. Low n values for 6 week samples were due to the researchers only selecting a small sample group in light of the obvious encapsulation visible.

	Silk 1 Week	PEG 1 Week	Silk 2 Weeks	PEG 2 Weeks	Silk 6 Weeks	PEG 6 Weeks
Polymorphonuclear Cells	4 ± 0	0 ± 0.484	4 ± 0	1 ± 0.492	4 ± 0	0 ± 0
Lymphocytes	3 ± 0.2	2 ± 1	2 ± 0	1 ± 0.734	3 ± 0	0 ± 0
Plasma Cells	1 ± 0	0 ± 0.276	1 ± 0.196	0 ± 0	1 ± 0	0 ± 0
Macrophages	3 ± 0.2	2 ± 0.331	3 ± 0	2 ± 0.287	4 ± 0	1 ± 0
Giant Cells	0 ± 0.498	0 ± 0.611	1 ± 0.528	0 ± 0.516	1 ± 0	0 ± 0.471
Severity of Necrosis	4 ± 0.2	0 ± 0.276	4 ± 0	0 ± 0	4 ± 0	0 ± 0
Infl. Sub	**15 ± 0.64**	**5 ± 1.26**	**15 ± 0.632**	**4 ± 1.22**	**17 ± 0**	**1 ± 0.471**
Neovascularization	3 ± 0	1 ± 0.471	3 ± 0.196	2 ± 0.5	3 ± 0	0 ± 0
Fibrosis	4 ± 0	4 ± 0.599	4 ± 0	4 ± 0	4 ± 0	4 ± 0.471
Fatty Infiltrate	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Fibro. Sub	**7 ± 0**	**5 ± 0.789**	**7 ± 0.196**	**6 ± 0.5**	**7 ± 0**	**4 ± 0.471**
Total score	**37 ± 1.28**	**15 ± 2.67**	**37 ± 1.28**	**14 ± 2.68**	**41 ± 0**	**6 ± 0.471**
n	24	24	24	23	2	3

Following the hydrogel implantation study, more complex histology data for the film study samples was requested (Table 4-3). These data showed that, although the response due to silk and PTFE was indeed similar, the results had not actually been good. Because native spider silk is biocompatible, it was determined that the immune response was not actually due to the silk itself, but more likely due to contaminants present in the synthetic spider silk, such as casein, a highly immunologic milk protein. In order to investigate this, we sought to purify the silk using Reverse Phase Chromatography (RPC).

Table 4-3. Average histopathology results for film implants. Scores were rated from 1 to 4 in each category. Total score is equal to (Infl. Sub Score x2) + Fibro. Sub Score. Standard deviations were calculated using Microsoft Excel STDEV.P. Low n values for PTFE were due to tissue samples being harvested from incorrect implant locations.

	Silk	Dry Heated PTFE	Autoclaved PTFE
Polymorphonuclear Cells	1 ± 0.696	1 ± 0.471	1 ± 0.40
Lymphocytes	2 ± 0.848	1 ± 0	1 ± 0.748
Plasma Cells	0 ± 0.456	0 ± 0	0 ± 0
Macrophages	2 ± 0.478	2 ± 0.373	2 ± 0
Giant Cells	0 ± 0.322	0 ± 0	0 ± 0
Severity of Necrosis	1 ± 0.832	0 ± 0	0 ± 0.4
Infl. Sub Score	**7 ± 3.047**	**4 ± 0.687**	**4 ± 0.748**
Neovascularization	2 ± 0.730	1 ± 0.943	1 ± 0.4
Fibrosis	4 ± 0	4 ± 0.373	4 ± 0
Fatty Infiltrate	0 ± 0	0 ± 0	0 ± 0
Fibro. Sub Score	**6 ± 0.730**	**5 ± 1.067**	**5 ± 0.4**
Total score	**20 ± 6.608**	**14 ± 1.893**	**14 ± 1.744**
n	17	6	5

Spider silk dopes containing 2% spider silk in 4 M Urea were submitted to RPC as described in Materials and Methods. Peaks containing spider silk (as shown by Western Blot analysis) eluted in acetonitrile were collected. After acetone precipitation, centrifugation, and lyophilization, the silk protein (designated herein as RPC MaSp1) was

doped in nanopure water and treated for endotoxins via triple autoclaving. The protein was then redoped and hydrogels were made from it under sterile conditions. The RPC MaSp1 hydrogels, which were noticeably softer than non-RPC MaSp1 hydrogels, were implanted into 2 rats. A third rat was implanted with non-RPC MaSp1 as a positive control. PEG was again used as the negative control.

Two weeks post-implantation necropsies were performed on all 3 rats. After just 2 weeks, many of the RPC MaSp1 gels had been resorbed, either partially or completely. This was likely due to the decreased stiffness of the RPC MaSp1 gels, which made it much easier for the gels to be broken into very small pieces via mechanical forces, such as handling with forceps or pressure on the implantation site post-surgery. These small gel pieces were more accessible to the macrophages, making them easier to digest. A capsule was still visible around remaining silk implants, although it was less pronounced than those in the original hydrogel study and (visibly) the RPC MaSp1 implants were less encapsulated than the non-RPC MaSp1 hydrogels. Visual inspection (Figure 4-3) and histopathology results (Table 4-4) indicated that the inflammatory response was similar to the results seen in the first hydrogel study at 2 weeks (Table 4-2).

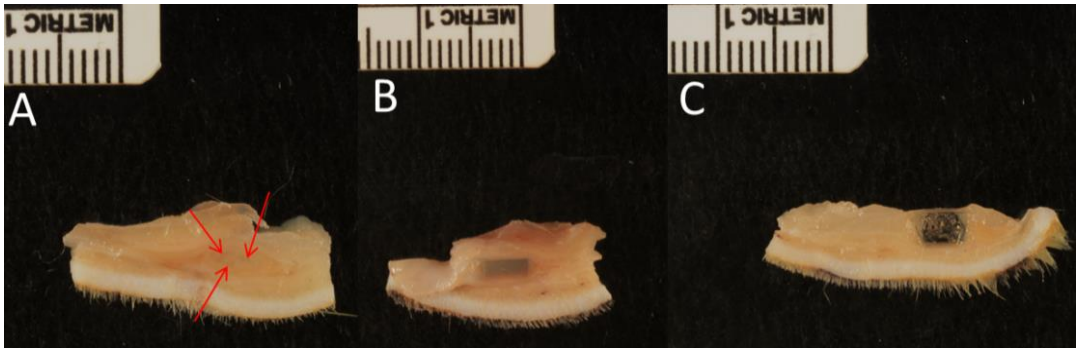

Figure 4-3. Tissue samples from rats implanted with silk and PEG hydrogels. Samples were collected 2 weeks post-implantation. A) RPC MaSp1 hydrogel; the gel is hard to see because it was broken in pieces during implantation – arrows have been added to indicate the 3 largest pieces of the gel. B) Silk hydrogel implant. C) PEG hydrogel implant. A ruler with 1 mm increments was included with each image for scale.

Table 4-4. Average histopathology results for hydrogel implant study. Scores were rated from 1 to 4 in each category. Total score = (Infl. Sub Score x2) + Fibro. Sub Score. Standard deviations were calculated using Microsoft Excel STDEV.P.

	RPC MaSp1	Silk	PEG
Polymorphonuclear Cells	1 ± 0.661	3 ± 0	1 ± 0.64
Lymphocytes	2 ± 0.331	2 ± 0	1 ± 0.64
Plasma Cells	0 ± 0.331	0 ± 0	0 ± 0
Macrophages	4 ± 0	3 ± 0	2 ± 0
Giant Cells	3 ± 0.696	1 ± 0	0 ± 0.64
Severity of Necrosis	0 ± 0.484	3 ± 0	0 ± 0.276
Infl. Sub Score	**10 ± 1.479**	**12 ± 0**	**5 ± 1.323**
Neovascularization	2 ± 0.331	3 ± 0	1 ± 0.493
Fibrosis	2 ± 1.936	4 ± 0	4 ± 0
Fatty Infiltrate	0 ± 0	0 ± 0	0 ± 0
Fibro. Sub Score	**3 ± 2.058**	**7 ± 0**	**5 ± 0.493**
Total score	**23 ± 4.885**	**31 ± 0**	**14 ± 2.9**
n	8	4	12

To determine whether the rats in the RPC MaSp1 study were creating antibodies to the spider silk, plasma was taken from the rats for use as a primary antibody against MaSp1 silk in a Western Blot analysis, with an anti-rat IgG secondary antibody. The band present in Figure 4-4 indicates that the rats were producing Immunoglobulin G in

response to the silk implant, implying that the silk hydrogel had elicited an immune response in the rats.

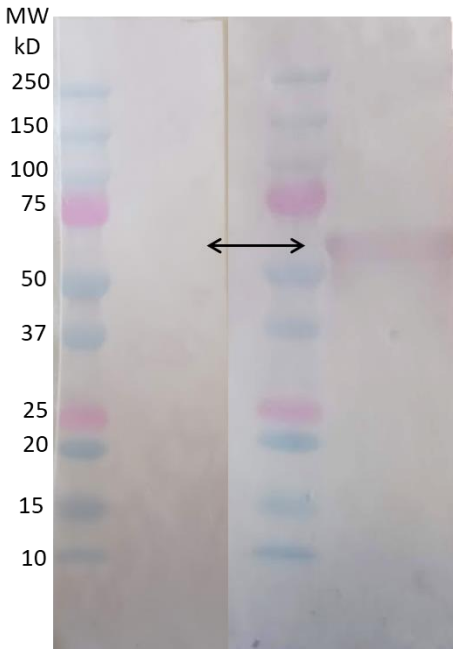

Figure 4-4. Western Blot analysis of rSSp using rat blood as the primary antibody. Left: blood drawn prior to implantation (pre-surgery). Right: blood drawn during necropsies (2 weeks post-surgery). Black arrow indicates 64 kDa where the MaSp1 band is present on the right, but missing on the left. The molecular weight standard was the Bio-Rad Dual Color Precision Plus Protein™ Standard.

Because of the genetic similarity between synthetic spider silk and native spider silk, it is more likely a contaminate present, possibly acting as an adjuvant, in the goat-derived transgenic spider silk proteins that is causing an immune response than the silk itself. Further clean-up of the silk and the material production conditions should lead to a decreased immune response. It is also likely that the immune response seen in the RPC MaSp1 hydrogel study was due to the rats' bodies responding as they would to nearly any foreign material: breaking it down to remove it from their system. We suspect that a much longer (≥6 weeks) time point would result in no visible immune response. If this is

the case, an early immune response is not only expected, but could actually be beneficial, as it would facilitate removal of the silk protein structure over time, which would be ideal for applications such as cell scaffolds and drug delivery.

Conclusion

At their current stage, goat-derived synthetic spider silk proteins are not sufficiently biocompatible due to contaminants in the proteins. Given the nature of the farm environment the proteins come from and, more significantly, the environment the proteins are purified in, as well as the purification process itself, these results are not all together unexpected. To further elucidate whether synthetic spider silk can be used as an effective biomaterial, more extensive work will need to be done. Further research into purifying and cleaning the silk proteins through RPC and other methods is still needed. It is highly possible that, over longer periods of time, the silk (especially the RPC MaSp1) will be completely degraded by the host, unlike the PTFE and PEG controls used in these experiments. This biodegradability would partially negate many of the concerns relating to the immediate inflammatory response the silk creates, especially because the response is localized, not systemic. Regardless, further work to investigate potential contaminates in the silk should provide much needed information about the negative response seen in these studies.

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CHAPTER 5

ENGINEERING SIGNIFICANCE

One of the key concepts in biological engineering is to harness the power of existing biological systems to develop innovative systems that either mimic or improve on naturally occurring ones, particularly with the goal of creating marketable products and/or improving human lives. A core principle in any field of engineering that readily applies to biological engineering is to design, build, and test. Both of these concepts were employed during the work described in this dissertation. As was discussed throughout, spider silk proteins have great potential for use in producing marketable materials, particularly biomaterials for medical applications, but the current quantities and purity level of spider silk are not sufficient for these to come to fruition.

In producing a new goat cell line to be used to create improved "spider goats," multiple iterations of designing, building, and testing were necessary. It was initially proposed to use targeted gene editing to replace a native goat milk protein gene with a spider silk protein gene such that it would be produced using the goat's native regulatory controls. Multiple gene editing systems were designed to target regions of the goat genome, but upon building and testing these systems, it was ultimately decided that, while it may be possible to incorporate silk in this way, the time, resources, and potential for failure (if the gene knockout proved fatal or the goats were unable to produce any milk), would likely not be compensated by the theoretical improvement of the silk proteins quality and quantity. Consequently, an effective and practical random integration method was designed and tested to facilitate the creation of new goats. As described in Chapter 2, this method was an effective way to create a goat cell line for use in creating

goats to produce silk proteins that can be purified more efficiently and more completely. This will allow for an overall increase in protein yield and purity, thus enabling research on spider silk as a biomaterial to move forward more effectively.

Although current recombinant spider silk proteins are being used to produce and evaluate a variety of materials, their biocompatibility had not been tested. Also, there was no established method for dealing with the issue of endotoxin contamination in the proteins that was likely to occur, especially in the bacterially-derived proteins. Lack of biocompatibility information and an endotoxin removal procedure were two major roadblocks to using these silk-based biomaterials for medical applications. In determining the best protocol for decreasing the endotoxins present in goat-derived silk to an FDA acceptable level, multiple methods were evaluated for their effectiveness as well as their impact on the mechanical properties of the silk materials. After building, testing, and redesigning protocols based on general techniques for endotoxin removal, a method was established to destroy endotoxins in spider silk proteins (regardless of the production source – goats or bacteria) without compromising the mechanical properties of the silk-based materials made from those proteins.

Following the endotoxin preparatory work, the biocompatibility tests described in Chapter 4 could be developed. The procedure for these tests was redesigned multiple times to determine the best materials to use to efficiently test the silk proteins. Initially, thin films were used, but it was determined that hydrogels would be a better testing material, as the films degraded quickly, making it difficult to find the implantation site. The control materials also had to be evaluated following initial studies to find a better material to match the hydrogels. Results from the film and hydrogel studies provided

feedback that could be used to redesign the silk purification process in an attempt to decrease the inflammatory response caused by silk-based materials. The results of these tests also re-emphasize the importance of creating new goats to produce proteins that can be purified more completely, as mentioned above. Through these *in vivo* studies, we have established a baseline for the biocompatibility of goat-derived silk proteins. This baseline gives us essential information regarding the future work needed to improve our recombinant silk protein purification process. For this future work, we suggest that characterization of the impurities present in our silk protein be completed to elucidate areas of improvement for the silk purification process. We have also established a standardized protocol for effectively testing silk-based materials regardless of its source (goats, plants, bacteria, or silkworms) that can be used for future biocompatibility evaluation.

CHAPTER 6

SUMMARY AND CONCLUSIONS

Recombinant spider silk proteins (rSSps) produced in transgenic hosts, particularly goats, hold great promise for use in the biomedical field. Increasing the amount and purity of these rSSps are two critical achievements needed to move protein-based materials from laboratory studies to clinic applications. The new "spider goat" cell line described in Chapter 2 will allow for production of more goat-derived proteins that can be purified more completely. Even with this improvement, endotoxin contamination is still a concern for these proteins as well as proteins acquired from other sources. The method established to remove endotoxins in rSSps described in Chapter 3, significantly increases the likelihood of creating medical-grade proteins.

Although the biocompatibility tests described in Chapter 4 did not yield the positive results we anticipated, the data obtained are valuable in that they have established a biocompatibility baseline for goat-derived rSSps. This baseline creates a starting point for future biocompatibility work – characterizing the contaminants present in the spider silk proteins and then determining the best method for removing those contaminants. This could be accomplished through the use of mass spectrometry, which could verify whether the immunological protein casein is present in our protein mixtures and provide information about whether or not other non-silk proteins are present.

More important than the biocompatibility baseline established through this work is the establishment of a standardized method for testing the biocompatibility of rSSps (whether from goats or another source). By having a standardized protocol, future studies can now be evaluated without the need of excessive background work and pilot studies

that could require the unnecessary use of more animal lives than needed. Although further work will be needed to improve the purity level of rSSps, the work completed for this dissertation has helped to establish a roadmap for that future work, moving goat-derived spider silk closer to biomedical applications.

APPENDICES

APPENDIX A

CHAPTER 3 SUPPLEMENTARY INFORMATION

Table A1-1. Endotoxin levels of goat-derived synthetic spider silk protein treated with various endotoxin destruction techniques. Although all protein came from the same stock, horizontal lines are used to separate different sample batches that were treated and tested at different times. The R^2 of the standard curve was ≥ 0.989 for all experiments. Silk samples below 0.25 EU/mL are in bold. Sample 9 had n=2 due to a negative absorbance on one of the sample replicates that was excluded from the average. Sample 15 was made on untreated PDMS. Standard deviations were calculated using STDEV.P in Microsoft Excel.

Sample Type	**Sample**	**Treatment**	**Endotoxin Level (EU/mL)**	**n**
Powder	1	None	5.02 ± 0.07	2
Film	2	NaOH rinse + H_20 rinse	2.08 ± 0.11	2
Film	3	NaOH rinse + H_20 rinse	4.05 ± 0.42	2
Powder	4	Doping	5.20 ± 0.24	3
Powder	5	Doping + Autoclave x3	0.307 ± 0.10	3
Film	6	Doping + Autoclave x3 + Film	**0.249 ± 0.08**	3
Powder	7	Doping	1.73 ± 0.10	3
Powder	8	Doping + Autoclave x3	**0.063 ± 0.01**	3
Film	9	Doping + Autoclave x3 + Film	**0.103 ± 0.01**	2
Powder	10	Doping	1.99 ± 0.05	3
Powder	11	Doping + Autoclave x3	**0.115 ± 0.00**	3
Film	12	Doping + Autoclave x3 + Film	**0.123 ± 0.06**	3
Powder	13	Doping	1.79 ± 0.13	3
Powder	14	Doping + Autoclave x3	**0.098 ± 0.01**	3
Film	15	Doping + Autoclave x3 + Film	1.62 ± 0.03	3

Powder	16	Doping	2.23 ± 0.02	2
Film	17	Doping + Film	1.17 ± 0.02	2
Film	18	Doping + Film	0.446 ± 0.02	2
Powder	19	Doping	2.28 ± 0.01	2
Powder	20	Doping + Autoclave x3	**0.136 ± 0.01**	2
Film	21	Doping + Autoclave x3 + Film	**0.202 ± 0.02**	2
Film	22	Doping + Autoclave x3 + Film	**0.214 ± 0.00**	2

APPENDIX B

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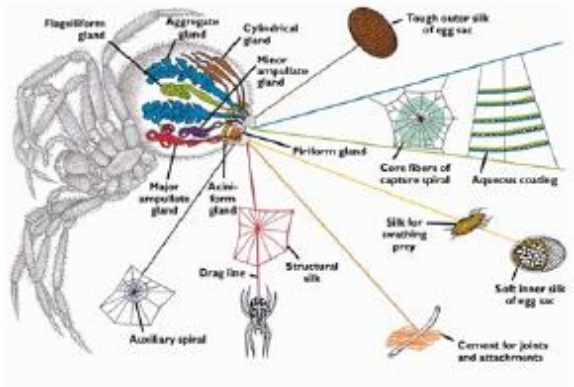

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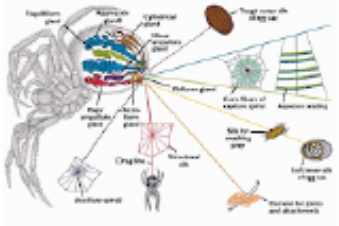

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Chapter 3 – Method for the Destruction of Endotoxin in Synthetic Spider Silk Proteins, published in Scientific Reports August 15, 2018.

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- Incorporated spider silk protein gene into goat and hamster genomes of goat fetal fibroblasts and BHK cells using CRISPR/Cas9 and PiggyBac. Verified genomic incorporation via PCR
- Designed and carried out *in vivo* biocompatibility studies of synthetic spider silk materials in 32 rats. Personally performed material implantation surgeries and necropsies
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Undergraduate Research Assistant, Metabolic Engineering, Utah State University — 2010 – 2012, Logan, UT

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Richard Decker, Celina Twichel, and Randy Lewis (2012). *Making Spider Goats with TALENs*. Institute of Biological Engineering Western Conference (poster presentation; 2nd place awardee).

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Justin A Jones, Thomas I Harris, Chauncey L Tucker, Kyle R Berg, Stacia Y Christy, Breton A Day, Danielle A Gaztambide, Nate JC Needham, Ashley L Ruben, Paula F Oliveira, **Richard E Decker**, and Randolph V Lewis (2015). *More than just fibers: an aqueous method for the production of innovative recombinant spider silk protein materials.* Biomacromolecules 16, 1418-1425.

Jia Zeng, **Richard Decker**, and Jixun Zhan (2012). *Biochemical Characterization of a Type III Polyketide Biosynthetic Gene Cluster from Streptomyces toxytricini.* Applied Biochemistry and Biotechnology 166, 1020-1033.