 

# INFUSING SYNTHETICALLY SPUN SPIDER SILK WITH RIFAMPICIN

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## ABSTRACT

Antibacterial resistance is growing exponentially, teetering on a crisis that can only be solved with "out-of-the-box" solutions. The silk-based delivery systems seems to be a promising approach to combat this problem. This system includes particles and surfaces with grafted antibiotics; however, acquisition of raw silk protein for such applications poses a significant obstacle to clinical translation. Although natural silk is readily used in several biomedical applications, artificial production of fibers may be cost effective for customized applications. Hence, to produce fibers with tailored mechanical, electrical, and biochemical characteristics, the development of silk spinning systems that can mimic the complexity of *in vivo* silk spinning glands is necessary. To assess the feasibility of infusing an antibiotic directly into a silk fiber, natural and recombinant spider silk as well as natural silkworm silk were dissolved in an appropriate solvent. Both microfluidic and wet spinning techniques have been used to artificially respun the silk solutions. A modification of a standard Kirby Bauer zone of inhibition (i.e. disk diffusion) assay against *S. aureus* has been used to evaluate the efficacy of this method. Both silk fibers as well as rifampicin incorporated fibers were placed on *S. aureus* spread LB agar plates. While no zones of inhibition (ZOI) were seen for silk fibers without any drug infused, the ZOI for fibers containing the rifampicin ranged from 5 mm to 27 mm; depending on the length, diameter, and material of silk fibers. Our observations showed that the drug could successfully be integrated into fibers and could potentially be used as suture materials as a novel way to address and prevent antibiotic resistant infections.

**Keywords:** silkworm silk, spider silk, recombinant spider silk, Rifampicin, wet spinning, microfluidic spinning, drug delivery

## INTRODUCTION

Fast-growing antibiotic-resistant bacteria have become a widespread dilemma that has compromised the effectiveness of antibacterial medication for many years [1], [2]. Even though the mechanisms of resistance are relatively well understood, inappropriate prescribing and poor patient compliance remain the two main causes of the epidemic. In fact, based on studies, treatment indication, choice of agent, or duration of therapy are inaccurately prescribed 30-50 % of the time [3], [4]. Thus, discovering and developing either new antibiotics or improving the use of existing antibiotics, although a lengthy and non-lucrative pursuit, is no longer a luxury [5], [6]. Without creative strategies to successfully act against increasingly-resistant bacteria, debilitating and lethal diseases will continue to increase in frequency and scope [7].

Before the 1950s, the only method for drug delivery was an orally dissolving pill [11]. Since then, new methods have been developed containing features such as sustained release, time release, extended release, etc. The use of controlled drug release systems may offer a promising alternative to preserve the efficacy of our antibiotic arsenal and slow or prevent the evolution of antibiotic resistance. By regulating the release of a drug into an environment, the pharmacokinetics of drug release will be more accurate,

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potentially thwarting the possibility for bacterial resistance. Additionally, the local drug delivery has been used to reduce drug toxicity and improve therapeutic efficacy in many situations [8], [9], [10]. Unfortunately, the most common drug dissolution strategy remains today as it did before the 1950s, limiting the development of new bioengineered macromolecular drugs such as peptides, proteins, and nucleic acids that are unable to handle the harsh acidic environment of the human stomach [9].

Recently, nanoparticle-based drug delivery systems, often associated with targeting, have gained momentum as successful strategies for drug delivery [10]. Research into nanoparticle based drug delivery systems have investigated not only how to change the physiochemical and surface properties of the particle but also have grafted drug, imaging, or targeting moieties to the outside of the particle. Although nanoparticle-based methods have revolutionized drug delivery, self-regulated drug delivery systems are quite possibly the most significant and advanced development in drug delivery and offer real promise for multiple disease states. This delivery method incorporates a biological sensor to interrogate the cellular microenvironment and release its drug payload only under certain conditions. Given that this method can specifically differ in its frequency based upon the microenvironment need, this strategy may prevent the misuse of antibiotics commonly associated with bacterial resistance. Self-regulated delivery systems need not only be particle based; a time-released fiber could be implemented for the targeted, local delivery of an antibiotic to an infected area. Infusing a synthetically spun high-performance silk fiber with an antibiotic may prove to be such a strategy. Silk proteins may not only be genetically engineered for this approach but may also be processed into a variety of materials (e.g., particles, fibers, sheets, etc.).

Silk fibroin is an insoluble protein created by spiders, silkworms, and numerous other insects [12]. Due to its excellent biocompatibility, biodegradability, and low immunogenicity, silk-based drug delivery systems have recently received considerable attention [13], [14], [15]. In this release system, the therapeutic efficiency of various drugs, which are dissolved, dispersed, or encapsulated into the drug carrier, can be improved because of high binding capacity, controlled drug release properties, and mild preparation conditions [13]. It seems to be a promising system to deliver not only small molecule drugs but also biologics such as proteins and nucleic acid drugs. Therefore, this manuscript investigates the development of artificially spun silk infused with the rifampicin, a broad-spectrum antibiotic, during the spinning process.

## METHODS

In order to evaluate the broad utility of the antibiotic infusion into a fiber, three different silk and silk-like proteins have been used to create the solutions:

- Silkworm silk from *Bombyx mori* purchased from a commercial vendor.
- Natural dragline silk derived from the major ampullate gland of *Nephila clavipes* (*Golden Orb Weaver*) obtained through forcible silking.
- Recombinant major ampullate silk protein produced in *E. coli*.

***Natural spider silk solution*:** Major ampullate spider silk was forcibly drawn from *Nephila clavipes* under a dissecting microscope [16] and was directly dissolved in Hexafluoroisopropanol (HFIP) to create 8 % wt/vol silk solution [17]. In order to expedite the process and get more uniform solution, a 50 °C and 900 rpm thermomixer has been used.

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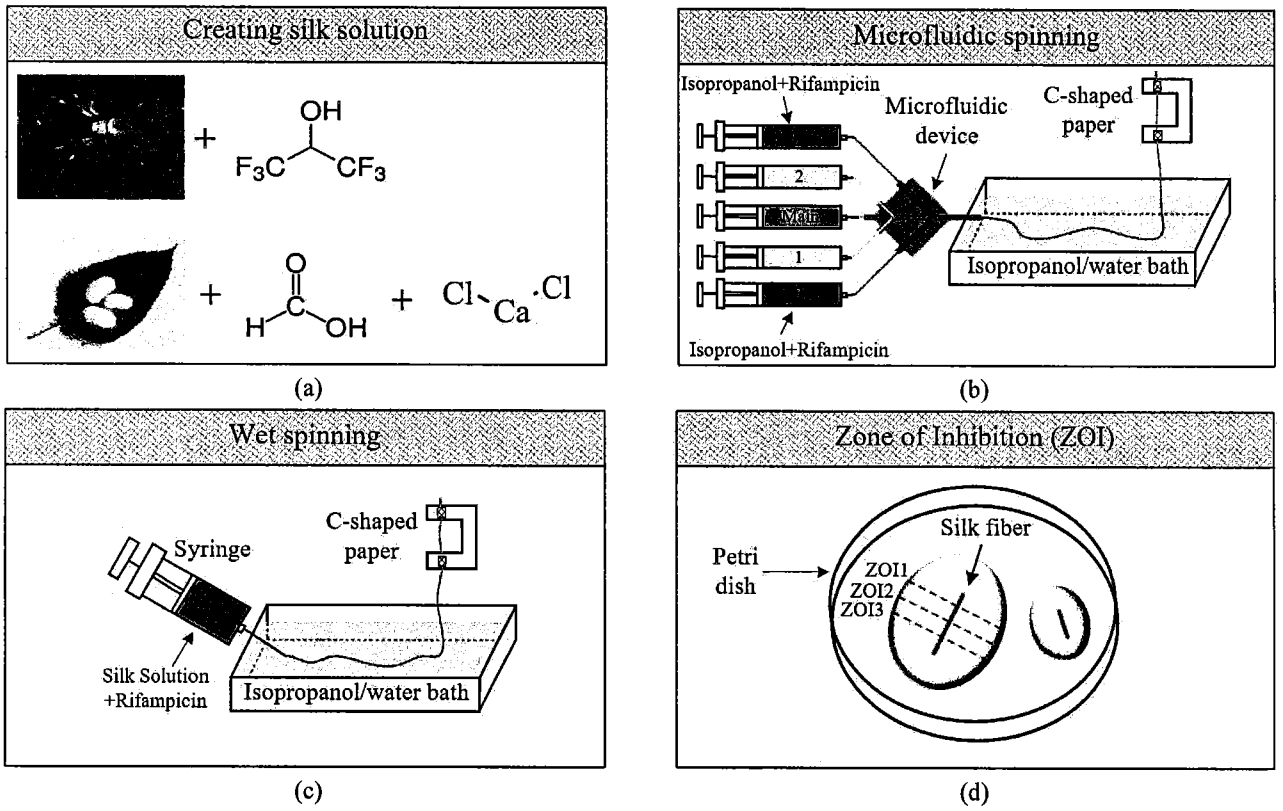


**Figure 1:** Investigating the inhibitory effects of rifampicin infused silk fibers on *S. Aureus*: (a) creating natural spider and silkworm solutions, (b) producing silk fibers using microfluidic spinning, (c) wet spinning technique, and (d) zone of inhibition (ZOI).

***Recombinant spider silk production in E. coli*:** Recombinant silk protein was produced in *E. coli* according to previously published report [18]. Solutions for spinning were made analogous to the natural silk solutions as described above.

***Silkworm silk solution*:** Silk from *Bombyx mori* was degummed by boiling in a 0.5 % $Na_2CO_3$ at 100 °C for 60 min to remove the outer sericin protein. The degummed silk was allowed to air dry for 24 hours and then directly dissolved into a Formic acid ($CH_2O_2$) with 8 % Calcium Chloride ($CaCl_2$) to achieve a concentration of 10 % of silk in the solution.

***Spinning:*** Both microfluidic and wet spinning techniques [19] have been used to artificially respin the fibers as illustrated in Figs. 1(b) and (c). Once the silk was uniformly dissolved in an appropriate solvent in order to create a "spin dope", rifampicin was infused into the fiber either through incorporation into the spin dope or through the side channels of microfluidic device. While for wet spinning, the rifampicin was directly added to the silk spin dope to achieve a 50 mg/mL concentration and the spin dope was spun into an Isopropanol/water bath as illustrated in Fig. 1 (c), 50 mg of rifampicin was dissolved in 10 mL of Isopropanol and vortexed for several minutes for microfluidic spinning technique. Subsequently, the solution was centrifuged and supernatant was collected to create the microfluidic infusion. The silk solution was pumped through the main syringe connected to the center channel of the 3D printed microfluidic device as illustrated in Fig. 1(b). While syringes 1 and 2 in Fig. 1(b) contained Isopropanol, the saturated rifampicin solution was pumped through syringes 3 and 4.

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The artificially spun silk was then collected onto C-shaped support paper and allowed to dry before assessment. After measuring the fiber diameters using brightfield microscopy on a Leica DMi8, the fibers were placed on the *S. aureus* spread agar plates to determine the growth inhibition.

***Kirby Bauer Zone of Inhibition Assay:*** In order to investigate the efficacy of the drug incorporated with the silk, a Kirby Bauer zone of inhibition assay was used. As shown in Figure 1 (d), small fibrils were placed on the *S. aureus* spread agar plate and the plate was incubated for 24 hours at 37 °C. Subsequently, the cleared zone (i.e., zone of inhibition) was recorded. Because the zones were elliptical in form, multiple measurements were taken across the length of the silk fiber and the average value was calculated as illustrated in Fig. 1(d). Silk fibrils without antibiotic were used as a negative control for inherent antibiotic activity of silk protein or incident activity from the synthetic spinning process. Zones of inhibition (ZOI) were normalized to the area of related fibers to obtain the normalized ZOI per unit area ($mm^2$).

## RESULTS

Rifampicin is a broad-spectrum antibiotic with a red orange color that, when incorporated into a synthetically spun silk fiber, allows for a visual confirmation of infusion. All fibers appeared to have a red orange color indicative of rifampicin incorporation, with thicker fibers having a deeper color. The precise amount of antibiotic loaded into each fiber was not assessed during this pilot study. Moreover, infusion into the fiber does not necessarily equate to bioactivity. Thus, the ability of the fiber to release rifampicin was assessed using a Kirby Bauer zone of inhibition study. *Staphylococcus aureus* (*S. aureus*) was spread on LB agar plates and spun silk fibrils were placed on it and allowed to incubate for several hours. Silk fibers with no infused antibiotic were used as a negative control. Regardless of the silk protein source, no zone of inhibition was seen for any of the negative controls (data not shown), while strong zone of inhibition was measured and recorded with all test samples.

Recombinant spider silk protein produced in *E. coli* was used to produce synthetic fibers by both microfluidic (rifampicin infused through the side channels) and wet spinning (rifampicin mixed into the spin dope) techniques and showed strong zones on inhibition in a preliminary study (data not shown). Importantly, although the recombinant spider silk samples were not microscopically analyzed, they appeared slightly thinner than silkworm samples and were brittle and inflexible, leading to difficulty maintaining full contact with the growth plate. Hence, the ZOI experiment was repeated for dissolved and synthetically respun natural spider and silkworm silk fibers of various lengths and diameters to observe the effects of these parameters on the bacterial inhibition. The zone of inhibition of wet spun fibers with incorporated rifampicin ranged from 17.46 mm to 24.21 mm for silkworm silk fibers while it reached up to 27.2 mm for spider silk fiber (data not shown). Nevertheless, these data are preliminary and must be repeated.

Due to the abundant raw materials, silkworm silk fibers were spun at variable diameters ranging from 33.65 μm to 88.36 μm. Five 1-cm long fibers with different diameters were placed on a lawn of *S. aureus* to observe the inhibitory effects (Figure 2). Not surprisingly, fibers with larger diameters appeared to be more effective drug carriers as indicated by the larger zones of inhibition, although the relationship is not linear, potentially due to the 3D nature of drug diffusion in an agar plate. Notably, these thicker fibers also have lower mechanical strength [19]. Alternatively, although possible, respinning dissolved natural spider silk proteins led to fibers of inconsistent lengths and diameters,

 

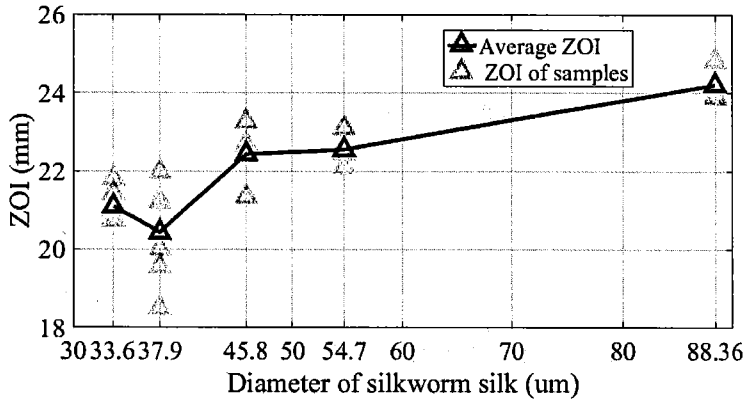


**Figure 2:** Zone of inhibitions (mm) versus dimeters of silkworm silk samples (μm).

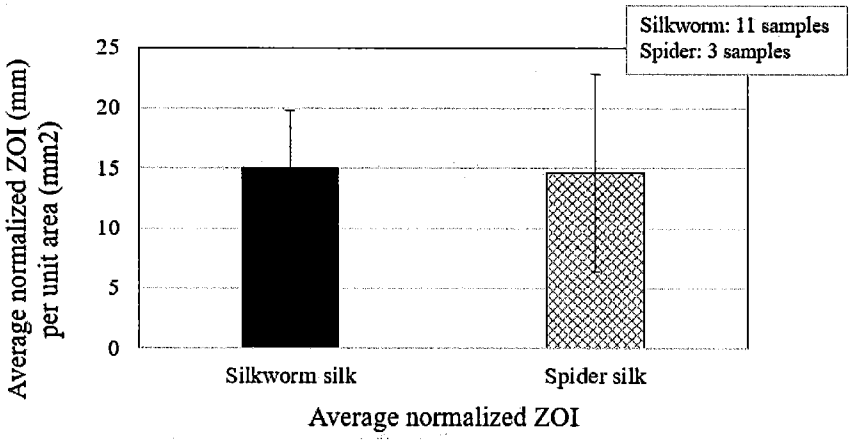


**Figure 3:** Average normalized ZOIs for both natural silkworm silk and natural spider silk are 15.04 mm/mm$^2$ and 14.66 mm/mm$^2$, respectively.

complicating comparison. Thus, in order to be able to compare the zone of inhibition of different silk fibers with varied lengths and diameters, each ZOI has been normalized using the following equation.

$$Normalized\ ZOI = \frac{ZOI}{\frac{\pi d^2}{2} + \pi d l} \quad \text{(eq. 1)}$$

where $ZOI$ is the average zone of inhibition, $l$ is the length of the silk fiber, and $d$ represents the diameter of the silk fiber.

After normalizing the ZOIs of both silkworm and spider silks, the average value was calculated. Fig. 3 compares the average normalized ZOIs per unit area (mm$^2$) from both antibiotic infused respun natural spider silk (n=3) and silkworm silk (n=11).

## DISCUSSION

Artificially-spun silk was developed using both microfluidic and wet spinning in order to mimic the general biological process of natural silk spinning by both a spider and silkworm [16]. Although wet spinning is the most common spinning technique and requires very little equipment or technical expertise, it is a very simplified and crude representation of the biological process of spinning.

 

However, since this study was not trying to replicate the mechanical properties of the fibers and was merely trying to provide a proof of concept for antibiotic infusion, wet spinning was appropriate.

Regardless of the diameter of the fiber produced, all rifampicin infused fibers were able to diffuse drug in a quantity sufficient to inhibit *S.aureus* growth over 24 hours. Not surprisingly, larger diameter fibers showed larger zones of inhibition, likely because of increased drug loading. This may also indicate that the antibiotic was truly integrated into the structure of the fiber and did not phase separate during spinning, acting instead as a fiber coating. If the drug acted as the fiber coating, then the amount of drug loaded would rely solely on the surface area and not the fiber volume. Importantly, the zone of inhibition did not have a linear relationship with the geometric properties of the fiber. Again, this is not surprising, since other disk diffusion based studies also indicate a lack of linear behavior [20]. It is hypothesized that this relationship may either reflect inconsistent drug loading or the three-dimensional nature of the bacterial agar plate, which allows diffusion through the thickness of the agar and not just of the surface of the agar. Based on these results, as a drug carrier, thicker fibers would be more desirable. Therefore, future trials could integrate a higher amount of antibiotic or different types of antibiotics in order to experiment with the versatility of the antibiotic-infused silk. Other applications of this integration of antibiotics into silk fibers could extend to a method of spinning termed integrated spinning, which would utilize a combination of wet spinning, microfluidic spinning, and electrospinning to more accurately mimic the biological process of silk spinning by both a spider and silkworm, effectively providing the pH gradients, ionic gradients, and mechanical fluid dynamics of the biological system.

## CONCLUSIONS

Due to the resistance of bacteria to antibiotics, a local controlled drug release system is likely an effective means to combat antibacterial resistance. The antibiotic rifampicin was integrated into respun natural spider silk and recombinant spider silk proteins as well as silkworm silk via both microfluidic and wet spinning techniques, revealing that the drug could be successfully integrated into the fibers as shown by zone of inhibition studies. The results are promising, and this study serves as a baseline for future work with microfluidics, including the possibility of using a voltage gradient to assist in the silk formation during a process termed integrated spinning. This application and many like it could open up the door to wide-scale uses of silk in biomimetic applications such as small diameter sutures for the purpose of combating antibacterial-resistant infections.

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