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Re-engineering native silk fiber spinning by *Bombyx mori*

Nereus Patel[1], Gary G. Leisk[2], David L. Kaplan[1] and Fiorenzo Omenetto[1]

[1]Department of Biomedical Engineering; [2]Department of Mechanical Engineering, Tufts University, Medford, Massachusetts 02155, USA.

ABSTRACT

Bombyx mori (*B. mori*) silkworms have been used for thousands of years to generate silk fibers for the textile industry, based on the native process of spinning oval cocoons. Here we sought to modify the normal cocoon formation process by altering the spinning environment of the worms during the mounting (spinning) stage. The goal was to prevent traditional cocoon formation by removing steep gradients that are normally required to anchor and initiate the process, and manipulate fiber extrusion from and deposition by the worm in order to foster the biological spinning towards alternative material formats. Results were achieved based on direct observation of the worm's innate behavior and environmental constraint required to spin a cocoon. Multiple artificial surface geometries and materials were used to establish platforms onto which the silkworms were placed and allowed to naturally spin silk into non-cocoon formats but yet new material architectures. Fiber alignment via the direct deposition of silk from a worm while moving over gaps or discontinuities in the surfaces was utilized to attain anisotropic spun fiber mats. Three dimensional silk mats were also constructed by placing the worms on pyramid structures with a gentle gradient. The methods employed demonstrate the versatility in utilizing natural spinning, in this case silkworm silk fibers, by modulating the environment in order to generate new material structures. This biologically-based biomanufacturing approach suggests new ways to think about materials fabrication, the formation of composite structures and the use of green methods for these goals.

KEYWORDS: *Bombyx mori*, silkworm, silk, fiber, scaffold, biomanufacturing

INTRODUCTION

Silk was reportedly first discovered approximately five thousand years ago when a cocoon fell into the teacup of the Chinese princess Xi Lingshi [1]. Since then, *Bombyx mori* silkworms have been domesticated and remains as the only insect dependent on humans for reproduction and survival [1, 2]. Silk fibers have since maintained an important connection with humans over this time due to their high economic value in trade and textiles. In addition, silk has been used as a biomaterial for centuries, dating back to ancient India and China, where the fiber was used in wound treatments or sutures [3]. The utility of silk fibers for medical materials arises from their excellent mechanical properties, biological compatibility and useful handling/knotting features [4-6]. Recently, silk has also been explored as a biomaterial by solubilizing fibers back into silk solution, followed by transformation into various material formats including fibers, films, foams, micro- and nano-particles, and three dimensional scaffolds [4, 5].

The lifecycle of the silkworm (Figure 1) is well studied [7, 8]. After a period of dormancy, a silkworm egg hatches to give rise to a silkworm larva which grows for approximately a month during which it undergoes five stages of growth known as instars [8]. The larva sheds its skin when progressing from one instar stage to the next as this allows the worm to grow in size. Once the silkworm reaches its fourth and fifth instar stages,

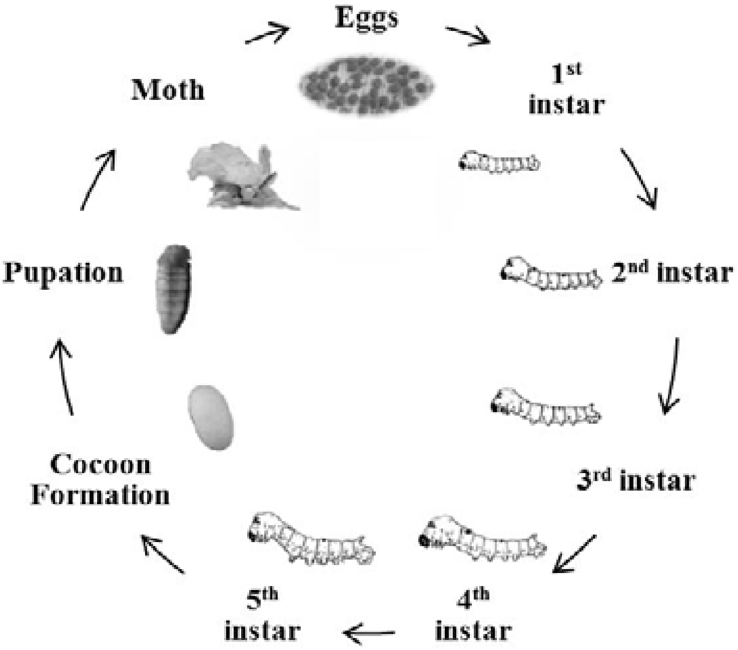

Figure 1. Life cycle of a *Bombyx mori* silkworm (Adapted from [8]).

it begins to eat ferociously as during these two stages the majority of silk protein dope is produced and stored in the silk gland [9]. Once the worm is ready to spin the stored silk it stops eating and searches for a space to build and secure its cocoon, and subsequently spins a cocoon of silk over a period of one to three days until all the silk is expelled from its glands. The overall size of the *B. mori* dictates the length of time for which the worm spins the fiber and the size of the cocoon, with larger worms spinning for longer periods and building larger cocoons as they contain more silk dope in their glands than do smaller worms. The larva then undergoes metamorphosis, typically for one to two weeks, where it changes to a pupa and finally to a moth. The moth proceeds to secrete an enzyme, serrapeptase [10], which partially dissolves the cocoon allowing the moth to more easily break through the cocoon. The spinning process explained above, which covers 1 to 3 days, was the focal point of the present study. During this process, the silkworm larva uses several anchor points to tether multiple fibers in place to construct and secure a housing within which to safeguard the cocoon to be spun. The silk is spun continuously out of the mouth via modified salivary glands, laid down in a figure eight pattern, following the motion of the worm's head [11, 12].

Previous work by Bressner [13] showed that *B. mori* requires a minimum vertical height to build its housing and cocoon. If this minimum height cannot be attained the worm extrudes silk fiber onto a flat surface while still moving its head in a figure eight pattern. Bressner also proved that introducing materials such as dyes and antibiotics into the worm's diet can result in the absorption of the substance into the native silk dope and presence of it in the extruded fiber.

The goal of the present work was to alter the spinning environment around the worm, including the geometry, surfaces and anchor points, to correlate these changed conditions to the changes in architecture of the spun materials. Various convex and concave shapes were used to investigate how the spinning pattern of *B. mori* could be manipulated to obtain anisotropic scaffolds. The long-term goal is to utilize this biological manufacturing approach to explore the range and options for generating new, bioactive materials. The silkworm serves as a useful model system due to the robust mechanical properties of silk fibers, the green, all aqueous, ambient environmental process, and the large quantities of silk spun from individual worms.

METHODS

Rearing silkworms

B. mori silkworms were purchased in their first to second instar stages from Costal Silkworms (Jacksonville, FL), along with pre-made mulberry chow. Initial information regarding the rearing of silkworms and optimal rearing conditions was obtained from various silkworm retailer websites, care sheets, and sericulture blogs to promote healthy growth of the larvae. The worms were distributed by size into petri dishes which were 15 cm in diameter and 2.5 cm in height, with no more than ten worms in one petri dish (Figure 2A). The petri dishes were kept slightly open to prevent the buildup of condensation inside, as this can lead to mold growth. The silkworms were kept at a temperature of 23 °C to 27 °C, and at 60% to 70% humidity [8, 14-16]. A lamp was switched on every morning, and switched off every night to preserve the diurnal cycle of the worms.

Spinning platform designs

In brief, previous work [13, 17, 18] has shown that the geometrical surroundings of a silkworm determine the final construct spun by it. For example, a worm left in a petri dish will usually tether its housing and build its cocoon by anchoring fibers to the top, bottom, and side of the dish. Furthermore, if the covered dish is tall compared to the length of a silkworm in its fifth instar (typically between 7.62 cm and 8.89 cm) the constructed cocoon may not be anchored to the top cover of the dish and will result in the production of a standard cocoon observed in nature. However, if there is insufficient space in the dish, the worm will attach its cocoon to the bottom and the cover of the petri resulting in a truncated cocoon with flat rather than concave sides on parts of the cocoon. If no vertical constraint or attachment point is provided, the silkworm will extrude the silk dope as a fiber onto a flat surface; the silkworm must expel all its silk before it metamorphosizes into a pupa.

Using the above information, the silkworm was placed on two dimensional platforms (Figure 2B and Figure 3A-C) and allowed to spin silk thereby preventing it from building a cocoon and encouraging the construction of two dimensional mats. The innate behavior of the silkworm to continuously search for the required three dimensions to build its cocoon resulted in the worm crawling around and depositing fiber on the platform as it searched for anchor points for its housing. Various materials such as cast acrylic, etched cast acrylic, and Teflon were used to build these stages in order to investigate how variations in surface roughness and stickiness affected the worm's spinning behavior and fiber deposition.

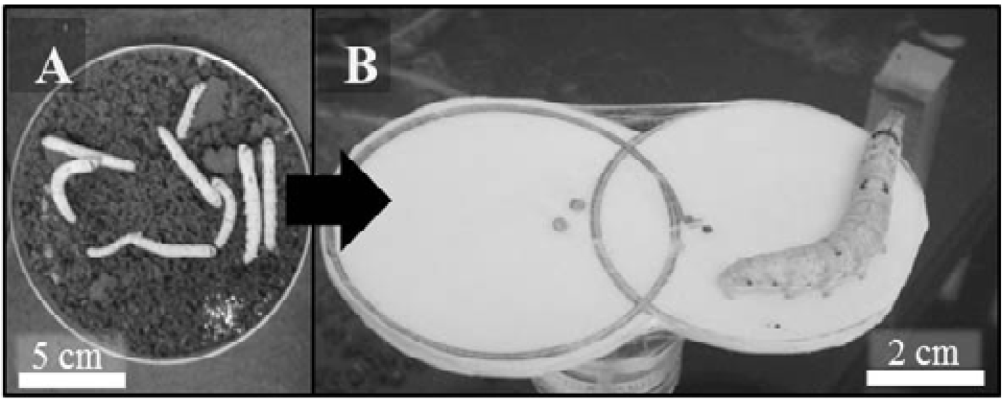

Figure 2. (A) *Bombyx mori* silkworms reared in petri dishes having a diameter = 15 cm and height = 2.5 cm. Silkworms were fed mulberry chow that was grated into each dish. (B) Worm spinning on a 'Figure 8' platform of Teflon.

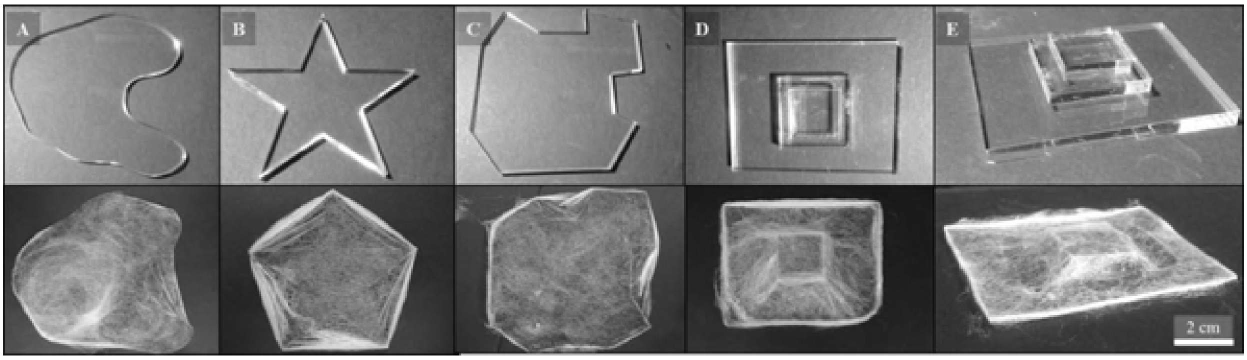

Figure 3. Various shapes studied to modify the spinning patterns of *B. mori*. The top row shows stages that were designed and the bottom row shows the silk mats obtained. (A) Series of splines cut from acrylic (length = 12.7 cm, width = 12.7 cm) (B) Star cut from acrylic (length = 9.78 cm, width = 9.78 cm), (C) Irregularly shaped polygon cut from acrylic (length = 12.7 cm, width = 12.7 cm), (D) Top view of a rectangular pyramid made from acrylic (length = 8.89 cm, width = 6.98 cm, height = 1.91 cm), (E) Side view of a rectangular pyramid made from acrylic (length = 8.89 cm, width = 6.98 cm, height = 1.91 cm).

Various platform designs were created in Solidworks (Solidworks Corporation, Waltham, MA), including squares, circles, and polygons. These designs were imported into Engrave Lab Pro 8 (Roland DGA Corporation, Hamamatsu, Japan), and the platforms were either cut or etched onto acrylic (McMaster Carr, Robbinsville, NJ) using a laser cutter (Speedy 300, Trotec Laser Inc., Canton, MI), and housed on posts approximately 7.62 cm high. Three dimensional tapered cones were also constructed using a three dimensional printer (Dimension Elite, Stratasys, Eden Prairie, MN) in order to investigate the preferences of *B. mori* for spinning. Various platforms were designed including regular and irregular shapes and sizes (Figure 3) including shapes with patterns or gaps, etched acrylic surfaces, Teflon mats, and shapes cut from wire mesh. Three dimensional platforms were also created by gluing multiple pieces of acrylic on top of each other to provide three dimensional geometries for spinning while simultaneously removing the third dimension required by the worm for attachment points during spinning. All platforms were attached to posts which were approximately 7.62 cm tall. The initial platforms were designed based on our prior studies [13] wherein 7.62 cm by 7.62 cm Teflon squares, large acrylic sheets approximately 30.48 cm by 30.48 cm, and petri dishes 10 cm in diameter were used to obtain two dimensional silk mats. Therefore, the first platforms used were 7.62 cm by 7.62 cm Teflon or acrylic squares and 7.62 cm diameter circles to observe whether surface stickiness affected the spinning behavior of *B. mori*. To investigate whether or not a silkworm would conform to geometrical restraints, regular polygons such as stars and triangles were cut from acrylic, as were irregular polygons and ellipses (Figure 3). A preference for surface roughness and change in depth were explored by etching various patterns into acrylic. The influence of a shallow incline was explored by sticking multiple squares of different sizes on top of each other (Figure 3D, E) to see if the change in height would trigger spinning a cocoon or if a three dimensional mat could be biomanufactured.

Spinning silk scaffolds

Once a worm showed signs that it was ready to spin silk it was placed on one of the platforms (Figure 2B) and allowed to spin until it began its metamorphosis into a pupa. If the worm fell off it was immediately placed back onto the platform. If the worm was able to crawl to a wall and begin spinning a cocoon, the cocoon was carefully opened and the worm was placed back onto its original platform to confine the spinning to the geometry prescribed with that platform.

RESULTS

Silk mats

Geometries with concave contours, such as a polygon with a gap between two sides (Figure 3A, C), or a star (Figure 3B), had aligned fibers distributed along the concave edges. On the other hand, worms deposited fibers along straight or convex edges allowing the mats to take the shape of the platforms themselves. If two different platforms such as two 7.62 cm by 7.62 cm platforms were placed in close proximity of each other (e.g., the distance between the platforms was approximately equal to the length of the worm) the gap between the platforms was covered mostly with aligned silk fibers, similar to the case where concave shapes where covered with aligned fibers (Figure 2B, Figure 3A-C). Three dimensional silk mats were obtained using pyramid platforms (Figure 3D, E). Aligned fibers were observed around the edges of the topmost (smallest square) and bottom (largest rectangle) acrylic pieces of the pyramid, and also connecting the corners of the top and bottom pieces of acrylic (Figure 3D, E). The presence of a small incline between the top and bottom acrylic pieces prevented alignment of fibers around any other acrylic pieces, though Figure 3E does show some aligned fibers connecting the corners of the top and bottom pieces indicating that the worms were able to detect the incline and attempted to create some tethers for the housing and cocoons. However, due to the absence of a steep incline and the expulsion of all the silk from their glands, the worms were unable to construct the necessary housing for a cocoon and as a result no cocoons were produced when using this pyramidal geometry.

Some variability was also seen between mats of similar geometry and size with respect to the thickness of the silk mats. For example, one worm spun a square mat on a 7.62 cm by 7.62 cm Teflon square, while a second worm of similar size spun a mat that did not cover the entire square and was thicker on one end compared to the other (Figure 4). It was also observed that the worms were able to tether silk fibers better on acrylic surfaces rather than Teflon surfaces since mats on Teflon platforms often slipped from at least one edge of the geometry producing mats that were partially curled inward at one end.

Additional unusual features were also observed. For example, using platforms etched using the laser cutter produced a reflective surface over the etched part of the mats (Figure 5). While the intensity of the reflected light varied, the reflective property was observed in all mats that were spun on etched surfaces.

Worms in tapered cones produced cocoons at various points along the cones, with no preference for the gap required to build a cocoon (Figure 6), as one cocoon was spun further inside the cone (towards the narrow part of the cone) while another cocoon was generated further outward near the wider part of the cone, even though both cones were of the same size and shape. However, the space in which a cocoon was constructed determined the size and thickness of the cocoon. For example, a cocoon spun in a wide cross-sectional area was larger in width, height and diameter than a cocoon spun in a smaller cross-sectional area of the cone. However, the former cocoon was thinner than the latter one.

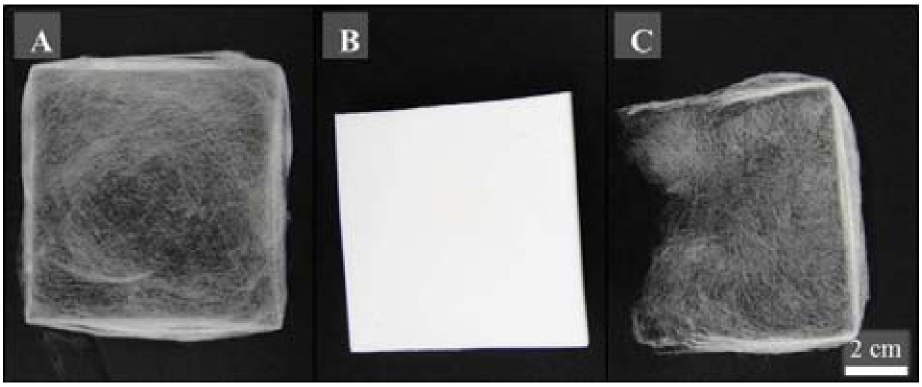

Figure 4. A Teflon square (B) (length = 7.62 cm, width = 7.62 cm) was used to obtain a two dimensional mat; one worm made a complete square (A) while a second worm completed only part of the geometry (C).

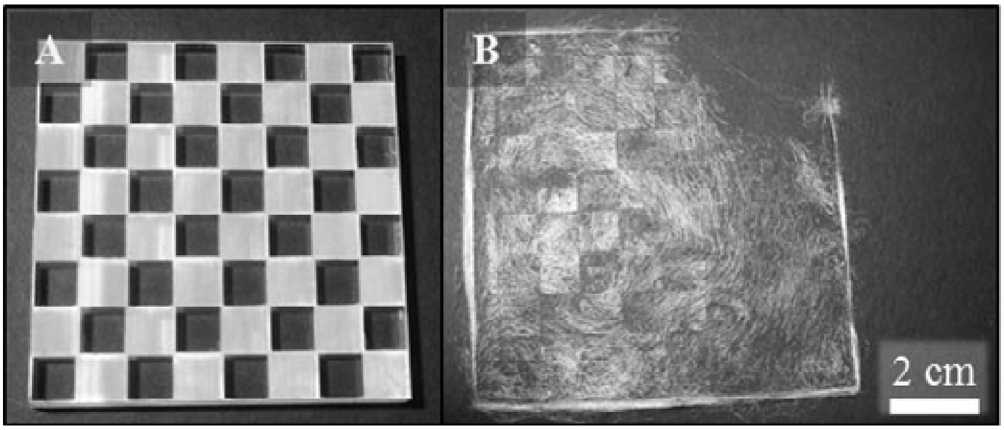

Figure 5. Scaffolds spun on etched acrylic surfaces (A) produced patterns of reflective materials (B).

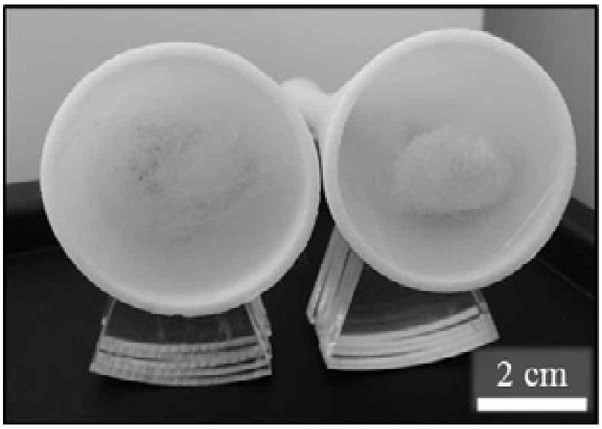

Figure 6. Cocoons spun in tapered cones (inner diameter = 4.57 cm, outer diameter = 5.08 cm). No preference was observed for the location of spinning with respect to overall gap size.

DISCUSSION

The concept of using living organisms to generate or biomanufacture structures is inherent in nature. However, generally, as this process has been understood and exploited in domestication, the goal has been to generate uniformity in outcomes to foster scale up and commercial viability. The canonical example of this process is sericulture and the silk industry, where through over more than 5,000 years of domestication, the silkworm process has been optimized to generate uniformly shaped oval cocoon structures that form the basis for a longstanding and very successful textile industry. The goal in the present study was to redirect this process, and instead of uniformity in architectural outcomes, to start to gain insight into the variables that can be utilized to change the morphology of the traditional oval cocoons. Toward this goal, changing substrate material, shapes and gaps was used to begin to learn about the plasticity of the natural silk spinning process and the types of structures that can be generated.

During the fourth and fifth instar stages *B. mori* produces and stores the silk dope in its glands and this results in rapid expansion of the worm size, both in length and diameter [9]. This was observed during the process of growing and cultivating silkworms and when they are ready to spin cocoons their appetite is reduced or absent. The worms initially search for anchor points in order to begin building a cocoon. To achieve this goal a silkworm continuously crawls around, periodically lifting its head and part of its body to detect higher structures to locate anchor points in order to tether fibers which will help support and hold the cocoon in place as the worm spins [12]. During this process the worms leave a trail of silk fiber in the petri dish. Removing the third dimension, the height, forced the silkworms to spin two dimensional mats as opposed to three dimensional cocoons. The elimination of the third dimension also resulted in the worms continuously wandering in search of elevated surfaces to anchor cocoons, regardless of whether or not they had already begun extruding silk. As a result of the need to search for anchor points, worms placed on platforms often fell off the stage and continued to crawl and extrude silk. This led to incomplete two dimensional mats, or thinner mats (Figure 4). Silkworms also preferred to deposit silk while on top of an already extruded fiber. Worms would often attempt to lay fiber on the edge of platforms with a greater amount of silk and try to build the mat beyond the geometry, a feature noticed more on Teflon platforms as opposed to acrylic platforms. This was done to support the worm's weight as it attempted to crawl beyond the stage in search of anchor points. This insight also suggests further options to generate more complex architectures and more robust materials, via sequential spinning processes, where the initial mats provide the template for subsequent rounds of spinning by additional worms.

Constructs obtained from geometries that contained gaps, holes, or any type of discontinuities had aligned fibers across these features (Figure 3A, B, C). These aligned fibers were also seen in cases where a larva would build a scaffold beyond the boundary of a given platform, thereby extending the platform (as seen on the right side of the mat in Figure 4C). This may represent an attempt by the worm to extend the surface in search of elevated anchor points for cocoon construction. The deposition of aligned fibers was explored using various geometries such as stars, irregularly shaped polygons, donuts, and joined platforms. In all cases, the worms filled the gaps by connecting two points across the space with aligned fibers, thereby producing scaffolds with high fiber alignment. The larvae would then continue to lay down more fiber in a random orientation across this aligned region to support its weight as it crawled across the gap. This trend was also observed in three dimensional space, when worms placed in an incubator crawled out of their housing in search of anchor points and laid numerous fibers across two walls (Figure 7). This is likely an innate behavior of the worms to build their cocoons in mulberry trees, and could be used to construct silk mats.

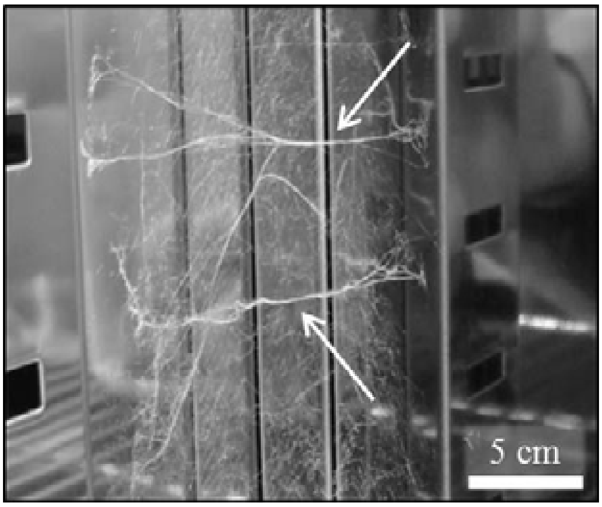

Figure 7. Silkworms connected two adjacent walls of an incubator with robust, aligned fibers (indicated by the white arrows) in order to crawl from one wall to the other in search of anchor points for cocoons.

Another interesting observation was made when obtaining two dimensional scaffolds from etched acrylic surfaces. While no fiber alignment was observed, parts of the scaffolds were optically reflective (Figure 5B). This trend was noted on scaffolds spun on etched acrylic surfaces, regardless of the depth of the etch or of the pattern etched on the surface. The change in depth on the surface appears to have altered the material properties of the deposited fiber, particularly over the etched regions as fiber over these areas appear to reflect light more than fiber on non-etched surfaces.

If the worm is provided with three dimensions, such as a pyramid, a three dimensional scaffold or mat can be obtained. When spinning on a pyramid, the worm tends not to find the required anchor points because even though it may sense that three dimensions are present, the gradient from the highest to lowest points on the platform is not steep enough. As a result, the worm tends to extrude and lay silk fiber down as if on a two dimensional platform, but the overall scaffold takes the shape of the three dimensional pyramid. This also leads to the presence of some aligned fibers (Figure 3D, E), where fibers connecting the top of the pyramid to the corners of the lowest part of the platform are aligned.

Previous literature [13, 19-24] has shown that direct consumption of various materials by *B. mori* and spiders results in the transfer of these substances to the silk dope and fiber. Asakura *et al*. [23] fed silkworms an isotopically labelled diet to label and study silk structure *in situ* and after spinning.

Similarly, Holland *et al.* [22] fed adult female *Nephila clavipes* spiders a ^{13}C rich MEM5550 solution and showed that select proteins, namely glycine, alanine, glutamine, and serine, could be marked in fibers extruded from the spiders, and that these four amino acids made up the bulk of the dragline silk. Such investigations provide a potential means by which to study how the *B. mori* alters the silk dope as the worm ages, and as the native dope passes through the gland. Tansil *et al.* [21] showed that xenobiotics, mixed with silkworm food could be transferred to the *B. mori's* gland and end up in the fibers with no observable harm to the animal. Furthermore, the authors showed that different chemicals were readily absorbed by different parts of the worm's body. For example, Rhodamine B was readily absorbed by the silk gland but a majority of acridine orange was taken up by the worm's fat rather than the gland. Thus, the type of substance being consumed by the worm, hydrophilic versus hydrophobic, had an effect on the quantity transferred across the gland epithelium and into the native silk solution within the gland. This was also demonstrated by Nisal *et al.* [24] who observed that the concentration of six dyes in *B. mori* cocoons varied from dye to dye. It has been suggested that an optimum balance of hydrophobicity/hydrophilicity in the administered xenobiotic impacts this process, including interactions with various ions to influence diffusion of the substance across the gland epithelium [21, 24]. Tansil *et al.* also showed that materials consumed by first generation *B. mori* can be transferred to the next generation, as was evident by the presence of Rhodamine B in the offspring of the moths. Similarly, Bressner [13] demonstrated that antibiotics such as rifampicin were absorbed through the silk gland epithelium and into the dope, and was present in the resulting scaffolds spun by the worms. He also showed that increasing the dose of the antibiotic in the food resulted in more of the drug present in the scaffold. Thus there is evidence to suggest that therapeutic substances such as drugs and nanoparticles can be incorporated into silkworm feed and result in the formation of bioactive or therapeutic silk fibers and scaffolds. The use of appropriate surfactants to maintain the required hydrophobic and hydrophilic balance can also encourage the absorption of a substance by the gland thereby increasing its concentration in the silk fibroin.

Combining the ability to incorporate additional substances into the silk via feeding, with the alteration of the silkworm spinning to create two dimensional mats can result in the formation of functionalized scaffolds. For example, adding gold nanoparticles to silkworm chow can produce fibers with gold nanoparticles embedded in the silk. Sutures with gold nanoparticles could be vibrated with an external energy source, thereby providing localized therapy through heating such as to control the functions of the fiber and attached muscle. Alternatively, if the site of the fiber becomes infected, the nanoparticles could be heated with a laser to kill the infection, thereby eliminating the need for strong antibiotics or additional surgeries. Incorporating dyes into the silkworm's feed can also produce pre-colored cocoons, which can have useful commercial importance to the textile industry as the cost of extrinsically dying the fiber and the resulting environmental costs may decrease.

In summary:

1. Removing steep gradients prevents silkworm spinning cocoons to induce fiber deposition in the x, y plane only, promoting the deposition of two dimensional mats.
2. The presence of gaps or concave shapes fostered fiber deposition across the gaps generating two dimensional mats with aligned fibers (Figure 3A, B, C).
3. Pyramid geometries with shallow gradients prevented spinning cocoons despite the presence of three dimensions, resulting in three dimensional silk mats containing aligned fibers between the highest and lowest points of the geometry (Figure 3D, E).
4. Etched acrylic surfaces led to the production of two dimensional mats with optically reflective surfaces (Figure 5) suggesting the difference in height between the etched and unetched surfaces impacted material properties of the deposited fibers.
5. The opportunity to manipulate the local environment to generate new architectures for silkworm materials provides a useful biomanufacturing approach to explore organism-based production of materials.

CONCLUSION

B. mori silkworms have been domesticated over thousands of years to spin cocoons for protection and for silk textile production. In the present study this spinning behavior was modified by preventing the silkworm from forming the housing or guidelines needed to secure the cocoon. As a result, the silk fiber spinning process was altered to generate two dimensional silk mats as opposed to 3D oval cocoons. Furthermore, providing discontinuities in the spinning platform forced the silkworm to construct scaffolds with aligned fibers, thereby producing anisotropic scaffolds. Three dimensional scaffolds, with some fiber alignment, were also obtained by using three dimensional platforms in the form of pyramids. Overall, these observations provide a novel approach towards biomanufacturing, whereby a natural spinning process can be guided into new material formats by altering the local environment. Since the worms have remarkable capacity for spinning robust protein fibers, with continuous strands around 1,000 meters, this approach provides a unique way to guide biological materials production while avoiding intermediate polymer storage, processing and then deposition methods. Such biomanufacturing approaches suggest new ways to generate useful materials for a range of needs in the future, from medicine and the environment, to artistic and educational.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no conflicts of interest regarding the work.

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