Silk coating as a novel delivery system and reversible adhesive for stiffening and shaping flexible probes

Cinzia Metallo1*, Barry A. Trimmer²

¹Neuroscience Program, Sackler School of Biomedical Sciences, Tufts University School of Medicine, Boston, MA and ²Biology Department, Tufts University, Medford, MA, USA

* Corresponding author: Cinzia Metallo, Tufts Advanced Technology Laboratory, 200 Boston Avenue, Suite 2600, Medford, MA 02155, Email: cinzia.metallo@tufts.edu, Phone: (617) 627-0900, Fax: (617) 627-0909

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Abbreviations used: EMG, electromyography; UV, ultraviolet; M. sexta, Manduca sexta

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Abstract The performance of any implantable electrode depends not only on its recording or stimulation capabilities but also on its position relative to the target site. Electrode displacement during or after implantation represents a major issue as it might result in tissue damage or incorrect recording or stimulation location, complicating the interpretation of experimental data. Although thin-film electrode arrays have overcome some of the main limitations of more traditional, stiffer probes, their intrinsic flexibility and unilateral contacts represent a new challenge: they tend to bend during insertion and are difficult to implant simultaneously while maintaining a specific relative position. Here, we present a method that addresses all these issues using a coating of silk fibroin, a versatile protein derived from silkworm cocoons. The method is demonstrated by acquiring electromyographic (EMG) recordings in *Manduca sexta*, a soft-bodied animal that exemplifies the issues of electrode implantationion in delicate and deformable tissues.

Keywords: silk, thin films, coatings, flexible probes, electrophysiology, fluorescent microspheres

INTRODUCTION

Technological advancements in microelectronics and micro-fabrication techniques have led to the development of increasingly small implantable electrodes with a progressively larger number of recording and stimulating sites, significantly enhancing signal selectivity [1,2]. However, the overall performance of implantable probes is highly dependent on two additional factors: accurate placement and ease of insertion [3]. To guide implantation, different types of stereotaxic methods have been developed over the years [4,5,6]. Unfortunately, in many animal species, stereotaxic placement cannot be properly carried out because of the lack of a reliable reference frame or because the spatial relationship between body landmarks and internal tissues is too variable [7,4]. Furthermore, although post-mortem dissections are used to verify the location of implanted probes in the vast majority of in vivo animal studies, electrode displacement might occur during the dissection phase, especially when the tissue that is being dissected is soft. As a result, experimental data can be misinterpreted. Electrode migration during *in vivo* experiments is another key issue, not only because it might cause tissue damage and trigger an immune-response [8], but also because changes in the electrode position might result in incorrect recording or stimulation locations [3,4]. This problem is exacerbated in small animals, where a minimal migration of an implanted probe can significantly compromise data interpretation. Recently, the use of flexible polymers as substrates to fabricate implantable probes has led to the development of flexible thin-film electrode arrays able to reduce tissue injuries as well as electrode displacement during dynamic conditions in *in vivo* experiments [2,9,10,11]. However, their intrinsic flexibility complicates implantation as they are more prone to buckle and bend during insertion than traditional, stiffer probes [12,13]. Different methods to add stiffeners or rigid structures to flexible electrodes to facilitate implantation have been proposed [14,15]. Nevertheless, these methods involve the removal of the stiffener after implantation, resulting in potentially dangerous probe displacement and tissue damage during the removal procedure. Furthermore, temporary stiffening alone [16] might not be sufficient to precisely place probes on determined target locations. A specific temporary shape or curvature might also be required to select the most appropriate angle of insertion and let the electrode advance through a tissue with minimal damage. Shape memory alloy-based electrodes have been proposed [17] but they are difficult to assemble and operate due to their temperature-dependent properties.

An additional drawback of thin-film electrode arrays is the fact that standard fabrication techniques do not easily allow the patterning of recording (or stimulating) sites on both sides of the probe. Usually, only one side of thin-film devices is provided with contacts, allowing recording (or stimulation) from one side of the implant alone [10,3]. As a consequence, paired or bundled electrodes might be required. However, implanting multiple thin-films probes simultaneously while maintaining their relative position is challenging as they might bend and follow different paths during insertion.

Here, we report a method that addresses all the above issues by using thin films of silk fibroin, a versatile natural protein derived from

silkworm cocoons that has been extensively used for clinical and biomedical applications such as sutures, neural implants and scaffolds [18,19,20]. Silk fibroin has a unique combination of mechanical and physical properties: it is biocompatible [21,22], it can be deposited in conformal films of different thicknesses [23], and it fully degrades with a tunable degradation rate when it comes in contact with an aqueous solution [24,25]. Whereas a previously proposed silk-based method focused solely on the possible use of silk as stiffener to aid neural implants [16], the multifunctional method presented here not only extends the use of silk films to electromyographic (EMG) recordings in a vast range of animal models, but it also details the use of silk to achieve three additional goals: (1) to mark the location of a probe in vivo by delivering a fluorescent dye at specific times; (2) to temporarily stiffen and shape flexible, thin-film electrode arrays; and (3) to temporarily glue together electrodes for simultaneous implantation. EMG recordings were acquired using flexible thin-film probes in the larval stage of the soft-bodied animal Manduca sexta (M. sexta, caterpillars) [26] because these animals exemplify the problems of flexible electrode placement. In fact, not only caterpillars are highly deformable and have very few rigid body parts that can be used as reference or anchor points during insertion, but their soft tissue composition also results in the probes being easily displaced during post-mortem dissections as well as being more susceptible to movement during in vivo experiments.

MATERIALS AND METHODS

Electrode arrays

The thin-film electrode arrays used here were fabricated as previously described [11]. They consist of a chrome/gold conductive layer sand-wiched between two flexible insulating layers of parylene C. They are 20 μ m thick and the layout of the array (*i.e.* the position of the recording electrodes and the inter-electrode distances) was specifically designed to match the anatomical features of *M. sexta* muscles.

Animals

Second day fifth instar *M. sexta* larvae (caterpillars) were used in all the experiments described here. Animals were kept on a fixed 17:7 hour light-dark cycle at 27°C and fed an artificial diet [27].

Pure silk solution

The silk solution was prepared as previously described [28]. Briefly, Japanese *Bombyx mori* silk cocoons were cut and placed into boiling $0.02 \text{ M} \text{ Na}_2\text{CO}_3$ (Sigma Aldrich) for 30 min to degum the sericin component and isolate the silk fibroin protein. The isolated silk fibroin was then washed three times for 20 min in deionized water and let dry for 24 h. The dried silk was then dissolved in 9.3 M LiBr (Sigma Aldrich) at 60°C for three hours, dialyzed for three days to remove excess ions, and finally centrifuged at 10,000 revolutions per minute (rpm) to remove any suspended silk fibroin or debris. Deionized water was added to achieve a final protein concentration of 5% w/v.

Fluorescent silk suspension

To prepare the fluorescent silk suspension, 10 mg of ultraviolet fluorescing polyethylene microspheres (Cospheric, 365 nm UV, 27-32 μ m, water suspendable) were added to 1 ml of pure silk solution and

gently mixed by pipetting until the microspheres appeared to be uniformly distributed. By using a micro-pipette, 5 μ l of the silk suspension were applied as a thin layer on the back of the electrode arrays and let air-dry for 30 min at room temperature.

Water annealing

Since dried silk films start to dissolve as soon as they come in contact with an aqueous solution such as the caterpillar hemolymph, water annealing was performed to slow down the silk dissolution rate [29, 30]. Longer annealing times correspond to an increase in beta sheet crystallization, which in turn corresponds to longer silk degradation rates [29]. To measure the annealing times required to induce specific silk dissolution rates and release the fluorescent microsphere, a number of silk-coated devices were annealed in a water-filled vacuum chamber under a 25 inHg vacuum for different periods of time and let dry for 60 min. The coated devices were then placed in Miyazaki saline [31], which resembles the caterpillar hemolymph, and the time it took the silk to fully dissolve and completely release the microspheres from the devices was measured.

In vitro testing

Caterpillar reduced preparations were obtained as previously described [11] by making an incision along the body of an ice-chilled caterpillar, pinning down the caterpillar body with the ventral side up in Miyazaki saline [31], and removing the gut and the fat body covering the muscles. The exposed musculature was shone with a UV flashlight (LEDwholesalers Inc., Nichia 365 nm, 5 LED) to verify the location of the florescent microspheres released from implanted devices. Since caterpillar muscles are clearly visible under a microscope, the reduced preparation was used for three different purposes: (1) to slide silk coated arrays in-between muscle layers and test the ability of the silk films to efficiently release microspheres and mark the position of the array; (2) to verify the location of the microspheres after each *in vivo* experiment; and (3) to test the recording capabilities of two electrodes temporarily "silk-glued" together.

In vivo testing

Silk-coated devices were implanted in ice-chilled caterpillars by making an incision on the cuticle close to the target muscle and gently feeding the probe into the cut. To keep the electrode in place, a small drop of rubber cement (Elmer's Products Inc.) was distributed along the cut. By sealing the cut with rubber cement, it was also possible to limit hemolymph seepage and prevent the hemolymph from interfering with signal acquisition. A silver wire was then inserted through the horn of the animal to act as a ground wire. After each in vivo experiment, the location of the microspheres was determined using the reduced preparation described above. To verify that the fluorescent microspheres had not migrated from the implanted position, the probe was kept in place during the dissection phase and then gently removed to confirm that all the microspheres had been released. EMG signals were recorded using PowerLab data acquisition units (AD Instruments, ML4856 PowerLab 26T), amplified at 10,000 \times , high pass filtered at 10 Hz, and low-pass filtered at 10 kHz. To identify single signal waveforms within the recorded EMG traces, threshold analysis was performed using the data analysis software DataView (www.st-andrews.ac.uk/~wjh/dataview/).



Figure 1. Fluorescent silk films mark the position of implanted probes *in vitro*. **A.** A device whose back was coated with a thin layer of fluorescent silk suspension is placed in-between two layers of muscles in a caterpillar reduced preparation under UV light. The fibers of a muscle in the top layer is highlighted in gray, the contour of the electrode array in black, and the circular recording sites in orange. The coated side of the device is facing downwards, the recording electrodes are facing upwards. **B.** The same reduced preparation as (A) but without the sketched muscle fibers and array. The green fluorescent microspheres are clearly visible under UV light. **C.** When the silk film has fully dissolved and the device is removed, the fluorescent microspheres are left behind in between the two layers of muscles, marking the position of the electrode array.



Figure 2. Silk-coated probes. A. A device with a silk film on its back surface is shown under visible light. The fluorescent microspheres embedded in the silk film appear as yellow-white clusters of dots. **B.** Under UV light, the microspheres fluoresce and become clearly visible. **C.** After an *in vivo* muscle recording experiment, the silk coating has completely dissolved and the microspheres are no longer visible on the surface of the probe either under visible light or UV light (D). The scale bar in all the figures is 0.2 mm.

RESULTS AND DISCUSSION

Silk films to mark probe position

The ability of the silk films to efficiently release the fluorescent microspheres embedded in them and, consequently, mark the position of the probe relative to target muscles was first tested *in vitro*. Since, in a caterpillar reduced preparation, single muscle fibers are clearly visible without the need of molecular markers, silk-coated electrode arrays can be easily slid in-between specific muscles. **Figure 1A** shows a silk-coated electrode array placed underneath a ventral muscle whose fibers are sketched in grey. The position of the recording sites of the array are marked as orange circles and the overall shape of the probe is highlighted in black. In **Figure 1B**, the sketched muscle fibers and the sketched electrode array were removed to show the reduced preparation under UV light. The vertical white filaments that are now visible are *M. sexta* muscle fibers. The silk-coated area of the probe that is visible underneath the muscle in **Figure 1B** is 3.5 ± 0.2 mm. As soon as the silk film began to dissolve, the microspheres started to slowly be released.

After waiting a time proportional to the annealing time to ensure that the silk film was fully dissolved, the device was removed (**Fig. 1C**). The fluorescent microspheres were left underneath the muscle fibers, clearly marking the position once occupied by the probe. The triangular shape of the probe's tip was also roughly preserved.

A silk coated device was also implanted *in vivo* in the ventral side of a caterpillar. **Figure 2** shows the device before and after an *in vivo* experiment, under visible light (**Fig. 2A-C**) and under ultraviolet (UV) light (**Fig. 2B, D**). A more uniform density of fluorescent microspheres within a silk film can be obtained by lengthening the mixing step that yields the fluorescent silk suspension. After the silk film was fully dissolved, the microsphere were no longer present on the device surface (**Fig. 2C-D**). In this particular experiment, the thickness of the silk film was $6 \pm 0.5 \mu m$ and its area was $3.4 \pm 0.2 mm$. Films of different thicknesses and areas can be obtained by varying the amount of silk suspension used for the coating. Since silk is an insulating material, the probe was coated on the reverse side to leave the electrical contact sites exposed and allow recordings to start immediately after implantation.



Water annealing was used to delay the release of the fluorescent microspheres until after the probe had been properly implanted. In general, for silk films with a thickness in the range of 6 to 10 μ m, a 1 minute water annealing results in a dissolution time of 2 to 4 min, while a 10 min water annealing results in a dissolution time of approximately 6 min. Since short annealing times (< 10 min) yield slightly variable dissolution rates, annealing times of at least 15 min are necessary to obtain more precise rates of microsphere release. Silk dissolution rates after different annealing times have been previously reported and range from minutes to days [23,29,30].

In a separate set of experiments, several coated and water annealed probes were gently rubbed against M. sexta muscles and soft tissues to quantify any possible microsphere leak prior to the start of the silk dissolution phase. No early microsphere release was observed. Furthermore, it is not necessary to prepare the silk films immediately before an experiment. Probes can be coated and then stored for days or weeks in airtight containers without altering the dissolving properties of silk-it is important that the containers are airtight as prolonged exposure to air would lead to an increase in beta sheet crystallization, resulting in slightly longer silk dissolution times. After recording EMG signals for two hours, the animal was dissected to locate the microspheres and confirm the identity of the monitored muscles. Figure 3A-C shows a reduced preparation under visible and UV light. In Figure 3A, the fibers of the target oblique muscle are highlighted in gray. The ganglion (diamond shape), the nerve cord (solid line) and the dorsal nerve (dotted line) are also highlighted to provide a frame of reference. In Figure 3B, the sketched muscle fibers were removed to show the reduced preparation under visible light. Figure 3C shows the same reduced preparation under UV light. Although some microspheres were misplaced during the dissection phase (red arrows), the location of the probe during the in

vivo experiment could be easily deduced by looking at the microspheres confined around specific muscle fibers. In fact, when a coated electrode comes in direct contact with a muscle fiber, the microspheres tend to remain trapped around it, as shown in the inset of Figure 3C for one of the fibers of the oblique muscle represented in Figure 3A. In this particular case, since the silk coating had been applied on the back of the probe, and since M. sexta muscles are arranged in only two main layers, it was possible to deduce that the electrode was recording from the muscle positioned directly above the oblique muscle marked by the florescent microspheres. By incorporating additional fluorophores or crosslinking agents for myosin or actin, a more accurate muscle staining can be obtained and the location of the probe can be more easily determined. Crosslinking agents would also minimize possible microsphere migration during long-term experiments and during post-mortem dissections. Furthermore, a UV light source brighter than the UV flashlight used here would help determine the location of the microspheres with higher precision.

To monitor any changes in the position of the probe during *in vivo* experiments, two silk coatings containing microspheres of different colors can be subjected to two different total annealing times. In this way, the microspheres would be released from the two films at different times, effectively marking the position of the probe with different colors at two different time points. By varying single annealing times, the release of the microspheres can be induced minutes, hours or even days after implantation. By applying the two coatings on two different locations on the same side of the device, any increase in the overall thickness of the silk film-probe system can also be avoided. Alternatively, one of the silk films could be engineered to release specific drugs [32] at a predetermined time to assess the efficiency of a treatment on different target locations before and after the drug is delivered.



Figure 3. Fluorescent silk films mark the position of implanted probes in vivo. A. A caterpillar reduced preparation under visible light after an *in vivo* muscle recording experiment. The red arrows indicate some visible clusters of microspheres. The position of the target oblique muscle is highlighted in gray (white arrow) as well as the ganglion (diamond shape), the nerve cord (vertical solid line) and the dorsal nerve (dotted line). **B.** The same reduced preparation as (A) but without the sketched fibers, nerves and ganglion. **C.** Under UV light, all the microspheres become visible. While some microspheres were displaced during the dissection phase (red arrows), the majority of the microspheres were trapped around muscle fibers. The inset shows an enlarged representation of the area around the muscle fiber marked with a white arrow. Most microspheres are visible on top of the muscle fiber; however, because of the semi-transparency of *M. sexta* muscles, some microspheres can also be seen underneath the fiber, on a different focal plane. The horizontal scale bar in all the figures is 1 mm.



Figure 4. Silk as stiffening agent. A. When the tip of a flexible electrode is coated with silk, it becomes stiffer and cannot bend (**B**, red arrow). The rest of the (uncoated) shank retains its flexibility. **C.** Silk coatings can be used to induce a curvature or (**D**) to create other shapes that might facilitate insertion through a tissue. The horizontal scale bar is 3 mm in (A) and (B) and 1mm in (C) and (D).





Silk as temporary stiffening and shaping agent

After drying, silk coatings alter the mechanical properties of flexible probes. Probe stiffening as a function of silk thickness and as a function of time has been previously characterized [16]. Figure 4A and 4B visually illustrates the difference in the stiffness of a probe before and after coating its tip with silk. To facilitate insertion, a short-term increase in the overall stiffness of flexible probes can be induced by varying the coating thickness or by depositing successive silk layers. As the silk film dissolves, the probe regains its intrinsic flexibility and its intrinsic ability to conform to tissues, minimizing tissue damage and reducing motion artifacts. Different parts of a probe can be coated with silk films of different thicknesses and different dissolution rates to allow specific portions of the device to transition back to their flexible state at determined times during the implantation process and thus facilitate insertion and placement. If a silk film of a certain thickness needs to deliver specific markers but the resulting stiffness is not required, it is possible to condition the silk film to be more flexible by subjecting it to a glycerol treatment [33].

Silk coatings can also be used to give a probe a particular curvature or shape that might facilitate insertion. To do that, it is sufficient to place the flexible probe on a mold of any shape and coat it with silk. As the silk dries, the probe will shape itself to the mold. Figure 4C shows a probe with a curved tip, which significantly simplifies electrode placement in the dorsal side of M. sexta. An s-shaped probe is shown in Figure 4D. If an extremely flat probe is needed, a thin layer of silk can be applied on both sides of the device to ensure that the probe does not curl up as it dries. No significant differences were observed in the EMG signals recorded with a device before coating its electrical contact sites with a silk film (Fig. 5A) and after the applied silk film has completely dissolved (Fig. 5C). Single waveforms (n = 45, grey) and their average (black) from each channel of Figure 5A and 5C are shown in Figure 5B and 5D, respectively. Although the device was reinserted after being coated with silk, the amplitude and the shape of the waveforms are remarkably similar before applying the silk coating and after the silk film is fully dissolved.

Silk as reversible adhesive

Silk can also be used as a temporary and reversible adhesive to hold two devices together during insertion and allow for their simultaneous implantation, requiring only a single incision. A drop of silk between the back sides of two thin probes is enough to hold them together. As silk dries, it effectively acts as glue (**Fig. 6A-B**). After implantation and as soon as the silk film dissolves, the devices are released from one another, still maintaining their reciprocal position. In most cases, it is not necessary to perform water annealing



before implantation because the exposed area of the silk film between the two probes is so small that silk degradation is delayed for several minutes. However, water annealing can be performed to slow down the silk dissolution rate even further and hold the devices together for longer times. To facilitate implantation, the silk film can be deposited between the two probes with different thicknesses and thus can also act as a stiffener. During the silk drying phase, it is also possible to give to the paired devices a specific insertion curvature or shape. Furthermore, devices can be temporarily glued together with particular configurations. For example, if the goal is to record from two overlapping muscles that are slightly shifted from one another, the two probes can be glued together with the recording sites also shifted from one another to reflect the relative positions of the two muscles, as illustrated in **Figure 6C**. Using this method, EMG signals were recorded simultaneously from two overlapping *M. sexta* muscles (red and yellow muscles, **Fig. 6C**). The two muscles are largely coactive but their unique, individual activities can be clearly discriminated (**Fig. 6D**). The close proximity of the two probes does not alter their individual recording capabilities as no crosstalk between them was observed (**Fig. 6E**).



CONCLUSIONS

Appropriate placement of implantable probes considerably improves their recording or stimulation capabilities. To aid implantation and facilitate insertion of flexible probes, silk fibroin films can be used as a multifunctional method to (1) deliver markers and determine the location of implanted probes, (2) induce short-term probe stiffness, (3) act as temporary adhesive to hold probes together, and (4) induce short-term probe shapes or curvatures. By using these four functions in combination or alone and by varying parameters such as silk dissolution rate and film thickness, it is possible to engineer silk coatings with different properties to meet a vast range of implantation requirements. Because of its intrinsic multifunctionality, the silk coating method presented here is not limited to soft animals such as M. sexta. Indeed, silk films can be used for implants and subcutaneous recordings in a vast range of animal models - from annelids to mice- any time flexible probes are required but are also difficult to position and control with precision. Furthermore, this method has broad applicability and is not confined to neural or electromyographic probes. For instance, it could be easily implemented in the case of thin-film LEDs for optogenetics or flexible films with integrated electronics.

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