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Inhibition of Biofilm Formation on Orthopedic Implants Based on Spider Silk Coatings Increases Survival of *Galleria mellonella*

Supun Mohotti, Gopala K. Mannala, Hendrik Bargel, Volker Alt, and Thomas Scheibel**

The microbial repellence of some spider silk-based materials makes them interesting candidates for biomedical applications. This study investigates the microbial repellent properties of recombinant spider silk coatings on orthopedic metal implants, specifically targeting the prevention of biofilm-related implant infections caused by multidrug-resistant bacteria such as *Staphylococcus aureus*. Utilizing *Galleria mellonella* as an in vivo model, stainless steel and titanium implants coated with films made of three different recombinant spider silk proteins are analyzed concerning biofilm formation and its impact on animal survival. Amongst the tested spider silk variants, the polyanionic eADF4(C16) demonstrates superior bacterial-repellent properties and improved larval survivability. Scanning electron microscopy analysis reveals reduced bacterial presence on eADF4(C16)-coated wires compared to uncoated controls, correlating with survival data. Based on the results, the potential of recombinant spider silk coatings to enhance implant functionality and longevity is highlighted, presenting a novel solution to combat biofilm-related implant infections and address the growing threat of antimicrobial resistance. Furthermore, employing *Galleria mellonella* as an in vivo model underscores a commitment to ethical research practices in studying biofilm infections.

1. Introduction

Multidrug-resistant bacteria have been recognized as one of the most significant threats in health care.[1] The increasing prevalence of antibacterial resistance affects every facet of modern medicine, jeopardizing the efficacy of numerous medical treatments, including cancer care,[2] transplantation,[3] and surgical procedures.[4] *Staphylococcus aureus* is one of the most common, hazardous, and clinically important multidrug-resistant bacteria, largely because of its methicillin resistance.[5] In this context, *S. aureus* biofilm formation on implants can cause severe infections.

A particularly significant area for implantable medical devices is orthopedics, where materials such as stainless steel, cobalt–chromium, and titanium alloys are commonly used.[6] These materials have been instrumental in enhancing the quality of life for patients, offering durable and biocompatible options for implants supporting the restoration of function and mobility in individuals with musculoskeletal conditions.

The association of orthopedic metal implants with biofilm-related implant infections (BRII) reflects nowadays one of the primary challenges.[7] Once a biofilm has formed on the implant surface, effective treatment typically involves surgical removal and thorough local debridement.[8] This complex procedure poses significant risks, underscoring the difficulty of managing BRII and the critical need for preventive strategies and advanced treatment options in orthopedic care.[7] Therefore, it is necessary to search for new options to prevent the manifestation of biofilms on implant surfaces.

Natural silk fibers are produced by various arthropods, including Lepidopterans (silkworms), Arachnids (spiders), and

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Hymenopterans (ants, bees, and hornets),[9] and are based on structural proteins. Among different silk types, spider silk fibers stand out for their high toughness, attributed to a combination of elasticity and strength, making them an exceptional functional material.[10] Spider silk films further exhibit resistance to microbial decomposition across a range of climatic conditions. Additionally, several studies have demonstrated natural spider silk's inherent microbial repellent properties.[11]

However, providing spider silk, especially at large scale, is challenging due to the cannibalistic nature of most spider species, hampering the farming of spiders. Further, extraction of silk from spiders is complex, requiring either the meticulous harvesting of individual fibers, which poses a high risk of contamination, or the forceful removal of spidroins (the main structural proteins in spider silk) from the spider's silk gland after stunning or killing the spider. To address these limitations, recombinant production systems using various host organisms such as bacteria have been developed to generate spidroins in sufficient quantity and purity, enabling their use in material development for diverse biomedical and engineering applications.[12]

In this context, genetic engineering and novel processing technologies have grown silk's utility beyond that of natural fibers. Recombinant spider silk proteins can be functionalized and then be processed into scaffolds for tissue engineering,[13] coatings for medical devices,[14] or drug delivery systems[15] amongst other applications.

Recently, materials made of recombinant spider silk have also been tested concerning their microbial-repellent activity.[16] Kumari et al.[16] identified spider silk-based nanostructured coatings as well as hydrogels that effectively repel microbes while enhancing mammalian cell adhesion depending on the employed spider silk protein. The microbe-repellent properties of these coatings could be attributed to the characteristics of the protein structures at the nanoscale.[16] In a follow-up study, Sommer et al.[17] demonstrated the microbial-repellent activity of such spider silk coatings on silicon implant surfaces, specifically against opportunistic infectious microorganisms.[17]

Other studies further showed the prospects of using bioengineered spider silk proteins to combat infectious microbes. Franco et al.[18] introduced antibacterial coatings for commercial silk sutures based on bioengineered spider silk proteins combined with antimicrobial peptides. The coated sutures maintained cell and blood compatibility while significantly inhibiting the formation of biofilms, reducing the adherence of methicillin-resistant *S. aureus* and *Escherichia coli*. Further, they reported that the mechanical properties of the sutures remained unaffected. This approach has shown potential for creating drug-free sutures that help minimize infection risks after medical procedures.[18]

In this context, studying the process of biofilm formation is crucial, and for this purpose, both in vitro and in vivo models have been developed.[19] In recent years, there has been increased interest in models based on invertebrates, largely due to the ethical and animal welfare restrictions associated with using vertebrate models.[20] Invertebrate species such as *Drosophila melanogaster*,[21] *Caenorhabditis elegans*,[22] *and Galleria mellonella*[23] have been utilized as animal models to study biofilm formation, providing valuable insights while addressing ethical concerns in research.

Manala et al. (2021) developed an infection model using *G. mellonella* larvae as an efficient in vivo system to study biofilm-associated infections on stainless steel and titanium implants, offering a potential alternative to vertebrate animal experiments.[23] In the current study, we aimed to evaluate the repellent properties of spider silk-coated metal wires (K-wires) in the *G. mellonella* in vivo model by preincubating the artificial implants in bacterial suspensions, implanting them into the larvae, and monitoring the animal survival for 5 d at 37 °C. Finally, the biofilm formation on the implants in *G. mellonella* larvae was evaluated by analyzing explanted samples using scanning electron microscopy (SEM).

2. Results and Discussion

2.1. Suitable Spider Silk Coating Methods for Metallic Orthopedic Implants

Stainless steel and titanium implants are commonly used in orthopedic surgery to stabilize fractured bones. Here, we tested spider silk-coated stainless steel and titanium implants in *G. mellonella* larvae (**Figure 1**a,b) concerning their microbe repellence, which would increase the functionality and long life of such implants without biofilm formation. The metal wires (K-wires) used for this implantation procedure were 4–5 mm long and had a sharp tip (Figure 1f).

To identify the best possible spider silk coating procedure on the K-wires, the previously established polyanionic spider silk protein eADF4(C16), based on one of the major ampullate spidroins of the European garden spider *Araneus diadematus* (ADF4), was fluorescently labeled using an NHS-fluorescein as previously described.[1]] The labeled eADF4(C16) enabled efficient analysis of the surface characteristics of K-wires coated with the spider silk protein (Figure 1e). To evaluate the best-performing coating technique, several different coating methods were analyzed. Oxygen plasma treatment of the K-wires before coating increased the surface wettability, which generated an even coating compared to plasma-untreated K-wires. Therefore, the coating of oxygen plasma-treated K-wires was further evaluated.

Previously, polymeric materials were coated using dip coating.[14] Therefore, K-wires (0.8 mm in diameter and 4–5 mm in length) were dip coated first. To identify the most effective dip coating procedure, multiple rounds of dip coating were performed on plasma-treated K wires: plasma-treated K-wires dip coated once, twice, and three times. Fluorescence imaging revealed that increasing the number of dip-coating steps resulted in progressively homogenous silk coatings. Samples coated once had an uneven surface, while the samples coated two and three times exhibited more uniform coatings.

Additionally, spray coating was used for applying the silk proteins on the metal surfaces. Multiple rounds of spray coatings produced an even silk layer on the metal wires. Notably, combining three rounds of spray coating followed by one dip-coating step yielded the best results among all tested methods (Figure 1d), which were then further used to prepare the K-wires for the microbe repellence analysis.

For microbial repellence tests, K-wires were coated using the polyanionic eADF4(C16), the polycationic eADF4(κ16), and the neutrally charged eADF4(Ω16) differing in their net charge with otherwise almost identical properties (Figure 1c). Coatings were

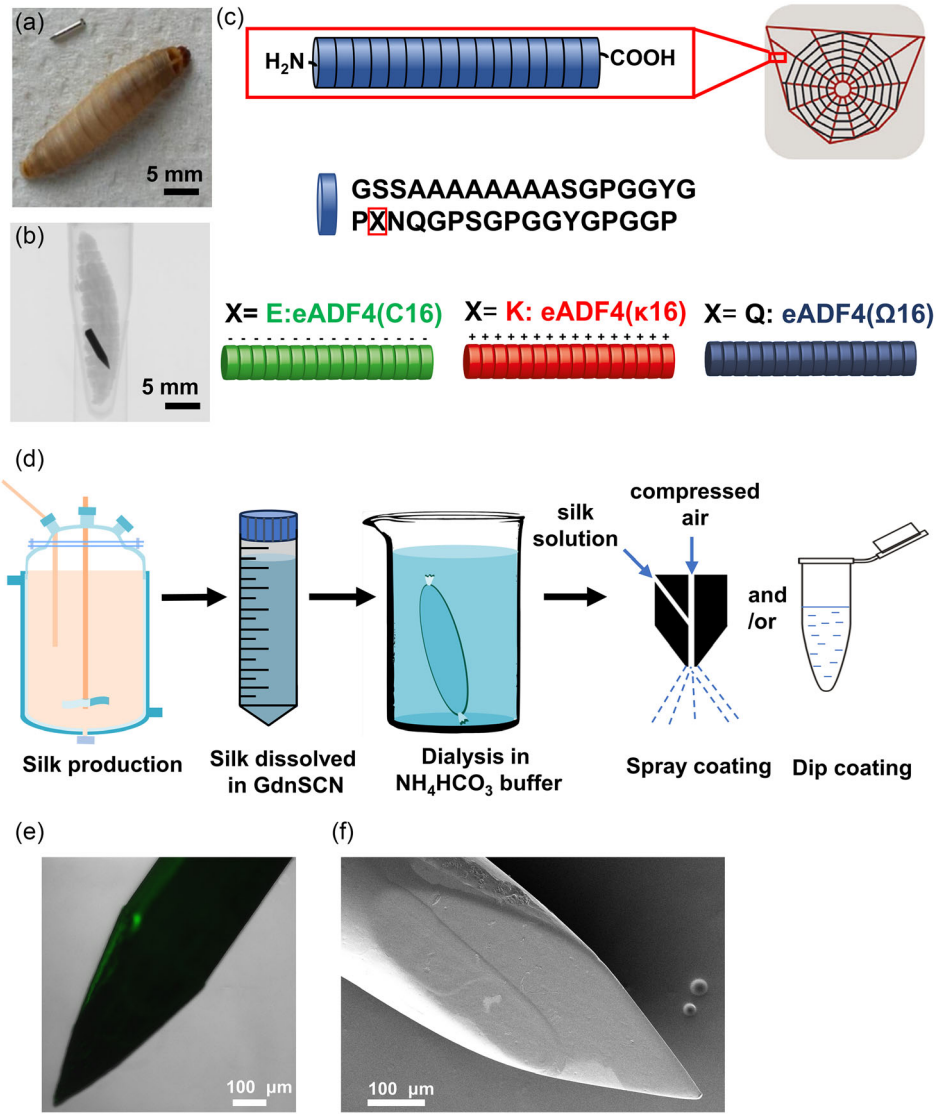

Figure 1. a) Stainless steel K-wire (K-wire S) implants with one sharp tip with a length of 4–5 and 0.8 mm in diameter next to a larva of *G. mellonella*. (Reproduced Creative Commons Attribution 4.0 International license.[23] Copyright 2021, The Authors. Published by ALTEX). b) Micro-computertomography cross-sectional imaging of a larva with an implanted K-wire S (Reproduced Creative Commons Attribution 4.0 International license.[23] Copyright 2021, The Authors. Published by ALTEX). c) The illustration shows 16 repeating units of a consensus sequence derived from the spider silk ADF4 (*A. diadematus* fibroin 4), along with the amino acid consensus sequence. In eADF4(C16), the amino acid residue at position X is glutamic acid as found in natural ADF4. In eADF4(κ16), all glutamic acid residues are substituted with lysine ones. In eADF4(Ω16), all glutamic acid residues are replaced with glutamine residues. d) Schematic diagram of the coating process: recombinant spider silk is produced in *E. coli*, purified, dissolved in Gdn SCN, dialyzed in NH_4HCO_3 buffer, and then used for spray coating and/or dip coating. e) Representative fluorescence microscopy image of a K-wire coated with fluorescein-labeled eADF4(C16). f) SEM image of a K-wire coated with eADF4(C16).

stabilized using methanol post-treatment to increase beta-sheet content in the proteins as previously published.[24] Increased beta-sheet content increased the stability of the coatings.[25]

2.2. In Vivo Examination of *S. Aureus* Preinfected Spider Silk-Coated K-Wires in *G. Mellonella*

To assess the effectiveness of the coatings, eADF4(C16), eADF4(κ16), and eADF4(Ω16) spider silk-coated K-wires made either of stainless steel (K-wire S) or of titanium (K-wire T) were used. Spider silk-coated K-wires were preinfected with *S. aureus*. Next, the preinfected K-wires were implanted into *G. mellonella* to assess the effectiveness of the spider silk coatings concerning microbial repellence. Survival rates of *G. mellonella* larvae were recorded over 5 days post-implantation. Uncoated K-wires with and without infection of *S. aureus* were used as controls. The implantation of coated and uncoated K-wires did not result in any observable adverse effects (with and without

infections of *S. aureus*), such as metal toxicity, impaired wound healing, or melanization at the implantation site.

Five days after the implantation of the positive control samples (without silk coating, preinfected), the survival dropped to 20% in larvae with K-wire T, while it dropped to 30% in those with K-wire S implants (**Figure 2**). Using eADF4(κ16) and eADF4(Ω16)-coated wires, the survival was 50% and 60% for K-wire S, and the survival numbers were slightly less for K-wire T implants. Among the tested coatings, those made of eADF4(C16) yielded the best survival numbers. eADF4(C16)-coated K-wire T implants yielded survival of 50% 2 days after implantation, and the numbers remained constant until the end of the experiment at day 5. For K-wire S implants coated with eADF4(C16), the survival was 60% and remained unchanged during the observation time of 5 days (Figure 2).

2.3. Analysis of Biofilm Formation on Spider Silk-Coated K-Wires after Explantation

To visualize putative biofilm formation, explanted K-wires were analyzed using SEM, and **Figure 3** shows representative images of the results obtained. A total of four K-wires was analyzed with a coating of each spider silk protein along with uncoated K-wires as controls. Tissue from *G. mellonella* was observed on all explanted K-wires, regardless of whether coated or uncoated. However, certain areas were not covered by tissue, allowing visualization of the underlying surface being either the spider silk surfaces on the coated K-wires or exposed metal on the uncoated controls.

The findings revealed that eADF4(C16)-coated samples showed fewer bacteria compared to eADF4(κ16) and eADF4(Ω16)-coated samples. Further, small bacterial agglomerations/colonies were observed on the uncoated control sample (Figure 3a2), but not on coated wires (Figure 3b2–d2). Regardless of the spider silk coating, bacterial agglomerates were present on the tissue-covered regions of all K-wires, including uncoated ones (Figure 3a3–d3).

The reduced bacterial presence on eADF4(C16)-coated areas refers to its microbe-repellent properties and aligns with the survival numbers. The eADF4(C16)'s bacterial-repellent properties are due to its nanostructuring, which inhibits bacterial adherence to its surface.[16] Engineered silk proteins offer a distinct approach to microbial-repellent functionality compared to conventional antimicrobial polymers. While traditional antimicrobial polymers often rely on embedded antimicrobial agents, charged functional groups, or chemical modifications, recombinant spider silk proteins achieve microbial resistance through their intrinsic structural properties.[16] The formation of

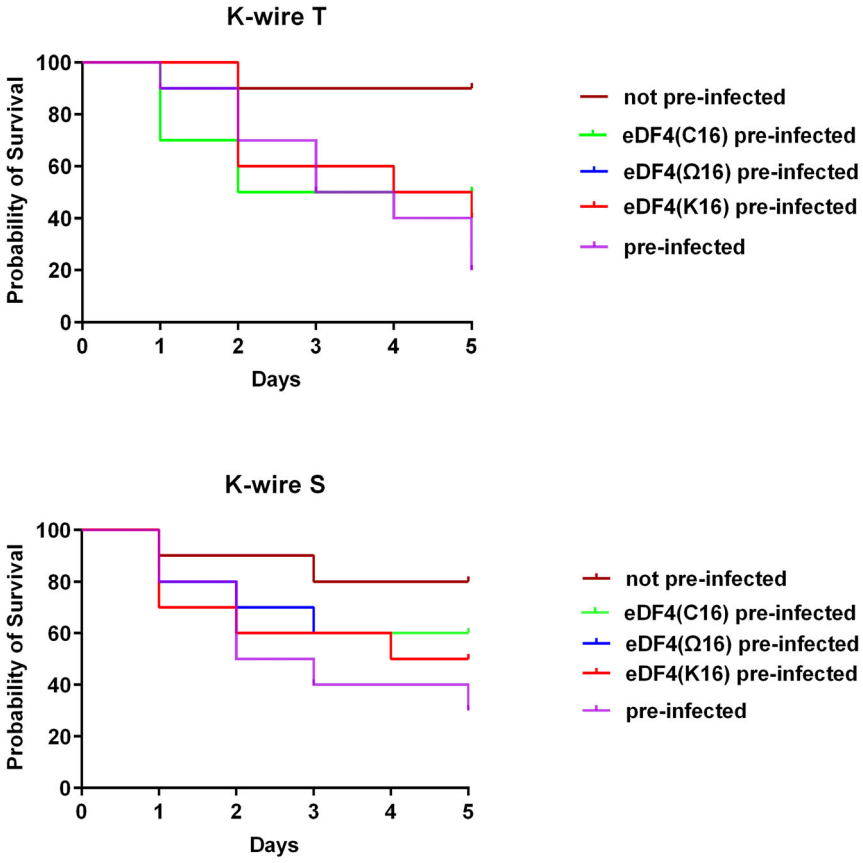

Figure 2. Survival of *G. mellonella* larvae after implantation with spider silk-coated K-wire T and K-wire S, with or without preinfection with *S. aureus* as indicated. For each group, 10 larvae were used ($n = 10$), the survival curves were plotted as Kaplan–Meier curves. For a meaningful statistical analysis, the sample numbers were too low. Uncoated wires (preinfected with *S. aureus*) were used as the positive control and without preinfection as the negative control.

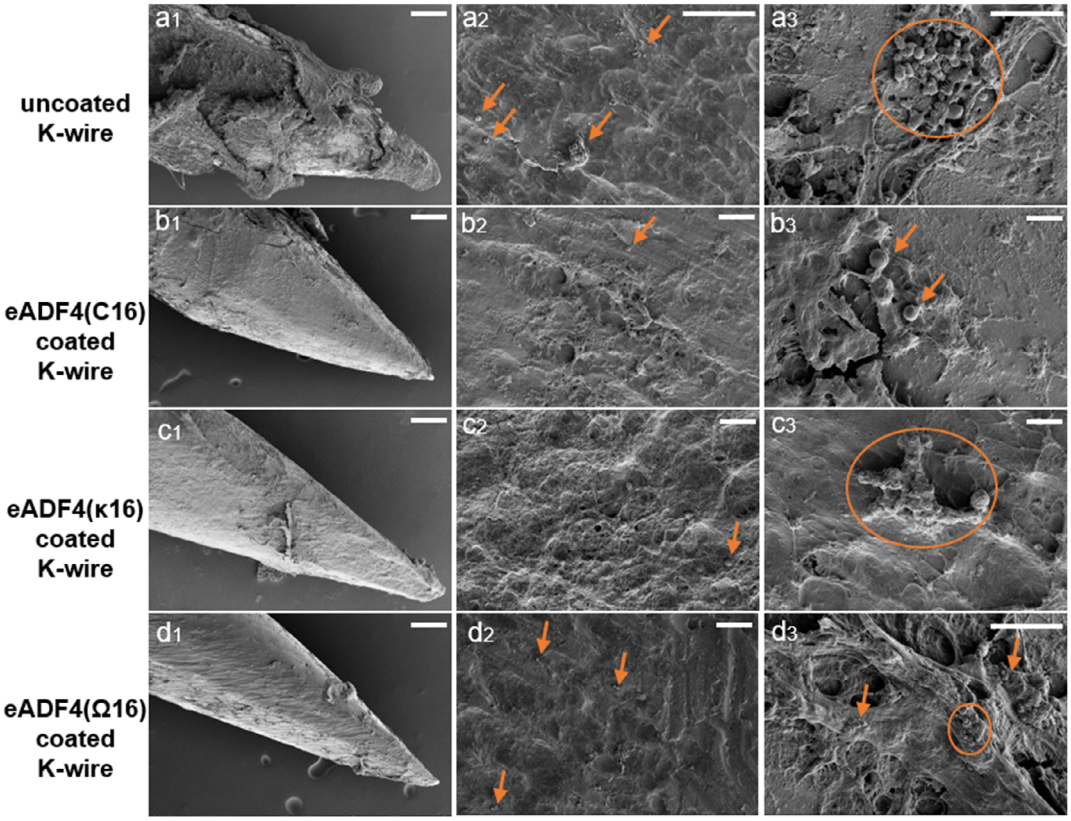

Figure 3. Representative SEM images depicting biofilm formation on uncoated a) and spider silk-coated (b–d) as indicated) K-wires explanted from *G. mellonella* after 5 days. The left column a1–d1) shows the tip regions of explanted K-wires (scale bars: 100 µm); the middle column a2–d2) displays implant metal surface regions free of *G. mellonella* tissue (scale bars: 10 µm); and the right column a3–d3) displays implant regions with adhering *G. mellonella* tissue (scale bars: 10 µm). Bacterial contaminations are highlighted by arrows in the case of single cells and by circles in the case of agglomerates/bacterial colonies.

hydrophobic patches, dictated by the protein's primary sequence, naturally prevents bacterial and fungal attachment without the need for additional antimicrobial components. This bioinspired, self-assembling material provides a biocompatible alternative to typically used antimicrobial coatings.

Our study using the *G. mellonella* in vivo model demonstrates that silk protein coatings on implants can prevent biofilm formation, thereby increasing larval survival in implant-associated infections. Given the growing challenge of antimicrobial resistance and the difficulty in eradicating biofilm-related infections, implant coatings inhibiting biofilm formation hold significant clinical potential. Further research, including preclinical studies in mice or rats and clinical trials, is necessary to advance the translation of silk protein coatings into medical applications.

3. Conclusion

The application of recombinant spider silk proteins as a coating for orthopedic metal implants has been explored, focusing on bacteria-repellent properties to inhibit biofilm formation induced by putative preinfections on implanted medical devices and to increase the survival numbers after implantation in *G. mellonella* used as a model organism. The research presents a significant opportunity to advance our understanding of BRII and to contribute to the ongoing battle against antimicrobial resistance. By employing *S. aureus,* which poses considerable challenges in clinical environments, this study validated the efficacy of spider silk coatings on metal implants significantly lowering the prevalence of BRII. The use of recombinant spider silk coatings offers a potential breakthrough in infection prevention and exemplifies a cutting-edge convergence of biotechnology and medical device engineering. Furthermore, the study's use of *G. mellonella* as an invertebrate in vivo model reflects a commitment to ethical research, providing a robust alternative to traditional vertebrate models. This approach facilitates the development of more ethical, efficient, and cost-effective methodologies for investigating biofilm infections, aligning with contemporary trends in biomedical research.

4. Experimental Section

Materials: Unless otherwise specified, all chemicals were obtained from Roth (Karlsruhe, Germany) and were of analytical grade. K-wires (15 mm length, 0.8 mm diameter,) made of stainless steel (K-wire S) or titanium (K-wire T) were purchased from Johnson & Johnson Medical GmbH, DePuy Synthes, Germany. eADF4(C16) was purchased from AMSilk GmbH (Planegg/München, Germany). 5-6-carboxy-fluorescein

succinimidyl ester (NHS-fluorescein) was purchased from Thermo Fischer Scientific (Darmstadt, Germany). Ethanol, methanol, and brain heart infusion media were purchased from VWR (Darmstadt, Germany). The water was obtained using a Merck Millipore system (Darmstadt, Germany).

Production of eADF4(κ16) and eADF4(Ω16): Production of eADF4(κ16) and eADF4(Ω16) was carried out in *E. coli* (BL21 gold) using a fed-batch fermentation method.[26] The recombinant spider silk protein eADF4(κ16), where all glutamic acid residues of eADF4(C16) were substituted with lysine ones, was purified as previously reported.[27] Similarly, eADF4(Ω16), in which all glutamic acid residues were replaced with glutamine ones, was purified using the previously described method.[26]

Preparation of Metal Implants for Coating: Sterile K-wires, 0.8 mm in diameter, were used as implant materials. The wires were cut into small segments of 4–5 mm in length (Figure 1a) using a cable cutter, and one end of each segment was sharpened. Before coating, the K-wires were washed with ethanol and air dried.

Coating Process Development Using Fluorescein-Labeled eADF4(C16): Due to the small dimensions of the K-wires (0.8 mm in diameter, 5 mm in length), a new coating process had to be established. For visualization along the process, eADF4(C16) spider silk proteins were labeled with NHS-Fluorescein as previously reported.[14] Fluorescein-labeled eADF4(C16) was dissolved in 6 M GdnSCN and then dialyzed overnight against 20 mM NH_4HCO_3 at pH 7.5, with the buffer being changed twice. The resulting protein solution was adjusted to a concentration of 10 mg mL^{-1}. Six different coating methods were then evaluated: three times dip coating of a plasma-untreated K-wire, one-time dip coating of a plasma-treated K-wire, two times dip coating of a plasma-treated K-wire, three times dip coating of a plasma-treated K-wire, three times spray coating of a plasma-treated K-wire, and three times spray coating of a plasma-treated K-wire followed by one-time dip coating.

The samples, cleaned with ethanol, were treated with oxygen plasma for 5 min at 100 W and 0.2 mbar (MiniFlecto, plasma technology, Herrenberg-Gültstein, Germany). For dip coating, the plasma-treated samples were directly immersed in the fluorescein-labeled eADF4(C16) solution (10 mg mL^{-1} in 20 mM NH_4HCO_3, pH 7.5) and then air dried. A custom-built device with a syringe pump was used for spray coating. The fluorescein-labeled eADF4(C16) solution (10 mg mL^{-1} in 20 mM NH_4HCO_3, pH 7.5) was loaded into a 3 mL syringe, which was set in the syringe pump at a flow rate of 1 mL min^{-1}. This setup was connected to a spray-coating device operating at 2 atm pressure, with the samples placed 12 cm away from the syringe nozzle. A 15 s spray coating was performed, followed by air drying. The coated K-wires underwent a post-treatment process, where they were placed in a desiccator saturated with methanol vapor for 1 day at room temperature. The coating was subsequently characterized using fluorescence microscopy.

Coating of K-Wires Using eADF4(C16), eADF4(κ16), and eADF4(Ω16): All proteins were denatured in 6 M GdnSCN and then dialyzed against 20 mM NH_4HCO_3 at pH 7.5 and room temperature, with the eADF4(κ16) dialysis specifically conducted at 4 °C due to its propensity to aggregate at room temperature. The method involving three rounds of spray coating followed by dip coating was identified as the most effective coating approach. After coating, the coated K-wires were subjected to post-treatment with methanol vapor as depicted above to further stabilize the coating.

Testing Silk-Coated K-Wires in G. Mellonella in vivo: The microbe repellance effect of spider silk was tested upon preinfection of silk-coated metallic implant materials. As infecting bacteria, *S. aureus* (EDCC 5055), was grown overnight at 37 °C in brain heart infusion media. The overnight-grown *S. aureus* cultures were subcultured (1:100) and grown at 37 °C until they reached an optical density of 1. The K-wires (K-wire T and K-wire S) coated with spider silk proteins were incubated for 30 min with 5×10^6 bacteria mL^{-1} at 37 °C, washed with phosphate-buffered saline (PBS) buffer, and implanted inside the larva. The implantation procedure involved sourcing *G. mellonella* larvae from Evergreen GmbH (Augsburg, Germany) and maintaining them on wheat germ at 30 °C. For the experiments, larvae in their final instar stage (400–450 mg) were used. The implants were inserted into the posterior region by piercing the cuticle with the sharp edge of the material and carefully positioning the K-wire for implantation.[23] Also, uncoated K-wire samples were subjected to the above incubation procedure. The larvae were incubated at 37 °C, and the survival of the larvae was observed over 5 days.

SEM Analysis: For SEM analysis, the implants were explanted from the larvae, washed twice in PBS to remove planktonic cells, and fixed with 1% sucrose and 2.5% glutaraldehyde for 1 day at 4 °C. Thereafter, samples were washed six times with PBS and dehydrated in an ethanol series (30, 50, 70, 80, and 96%) for 15 min each, then three times with 100% ethanol for 30 min. Samples were air dried, sputter coated with 1.3 nm platinum (Sputter Coater EM ACE600, Leica, Germany), and imaged at 2 kV accelerating voltage using the SE2-Detector in Standard Mode (Apreo VS, FEI/ThermoFisher Scientific, Germany).

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Conflict of Interest

TS is co-founder and consultant of AMSilk GmbH.

Author Contributions

Supun Mohotti: investigation (equal); writing—original draft (lead). **Gopala K. Mannala**: investigation (equal); writing—original draft (supporting). **Hendrik Bargel**: formal analysis (supporting); writing—review & editing (supporting). **Volker Alt**: conceptualization (equal); funding acquisition (supporting); supervision (supporting); writing—review & editing (supporting). **Thomas Scheibel**: conceptualization (equal); supervision (lead); funding acquisition (lead); writing—review & editing (lead). **Supun Mohotti** and **Gopala K. Mannala** contributed equally to this work and are co-first authors.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

bacterial repellences, dip coating, in vivo models, multidrug-resistant bacteria, spray coating, *Staphylococcus aureus*

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