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Antimicrobial coating of spider silk to prevent bacterial attachment on silk surgical sutures

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ABSTRACT

Microbial infections from post-surgery or other medical-related procedure is a serious health problem. Nowadays, the research is focused on the development of new drug-free materials with antibacterial properties to prevent or minimize the risk of infections. Spider silk is known for its unique biomechanical properties allied with biocompatibility. Recombinant DNA technology allows to bioengineering spider silk with antimicrobial peptides (AMP). Thus, our goal was to bioengineered spider silk proteins with AMP (6mer-HNP1) as an antibacterial drug-free coating for commercial silk sutures (Perma-Hand®) for decreasing bacterial infections. Perma-Hand® sutures were coated with 6mer-HNP1 by dip coating. In vitro tests, using human fetal lung fibroblasts (MRC5), showed that coated sutures sustained cell viability, and also, the contact with red blood cells (RBCs) demonstrate blood compatibility. Also, the coatings inhibited significantly the adherence and formation of biofilm, where sutures coated with 6mer-HNP1 produced a 1.5 log reduction of Methicillin-Resistant *Staphylococcus aureus* (MRSA) and a 2 log reduction of *Escherichia coli* (*E. coli*) compared to the uncoated Perma-Hand® suture. The mechanical properties of Perma-Hand® sutures were not affected by the presence of bioengineered spider silk proteins. Thus, the present work demonstrated that using spider silk drug-free coatings it is possible to improve the antibacterial properties of the commercial sutures. Furthermore, a new class of drug-free sutures for reducing post-implantation infections can be developed.

Statement of Significance

Microbial infections from post-surgery or other medical-related procedure is a serious health problem. Developing new drug-free materials with antibacterial properties is an approach to prevent or minimize the risk of infections. Spider silk is known for its unique biomechanical properties allied with biocompatibility. Recombinant DNA technology allow to bioengineering spider silk with antimicrobial peptides (AMP). Our goal is bioengineered spider silk proteins with AMP as an antibacterial coating for silk sutures. The coatings showed exceptional antibacterial properties and maintained intrinsic mechanical features. In vitro studies showed a positive effect of the coated sutures on the cell behavior. With this new drug-free bioengineered spider silk coating is possible to develop a new class of drug-free sutures for reducing post-implantation infections.

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1. Introduction

Nosocomial infections are one of the main causes of patient mortality and morbidity after extensive surgery or other types of healthcare practices [1]. The European Centre for Disease Control

estimates that 4.1 million people acquired a healthcare-associated infection every year in the European Union [2]. More than 20% of nosocomial infections are related to surgical site infections (SSI) [3,4]. These SSI represent a heavy economic burden to the healthcare systems with an estimated cost of €325 per day in Europe or €22,401 per infection in the United States [5,6] due to additional post-operative surgery and longer patient hospitalization [7].

Surgical sutures are one of the most susceptible materials related to the occurrence of SSI [8,9]. The main function of sutures is to hold and connect tissue edges together after an injury or surgery, promoting wound healing while restricting contamination that can lead to infections. Nevertheless, sutures can harbor or introduce bacteria to enable proliferation, leading to the formation of biofilms and related infections that become problematic to treat [10–12].

Silk fibers from *Bombyx mori*, a natural polymer, have been used as sutures for centuries because of their high strength, especially knot-pull tensile strength, excellent physical and handling properties, as well as biocompatibility [13]. Silk sutures are used in general soft tissue closure and/or ligation, such as ocular, neural, or cardiovascular surgery [14]. Also, due to the percutaneous nature of sutures as a conduit from the environment to the tissue, they can be employed to enhance better healing for tendon/ligament repairs, for example [15]. However, one of its major drawback is related to silk sutures scarce antimicrobial properties, being necessary to treated them with antimicrobial agents in order to render them with antimicrobial properties. In fact, braided and multi-filamentous silk sutures can be more susceptible to bacterial attachment, and consequently, to the formation of biofilms [16,17]. For preventing and overcoming the formation of bacterial biofilms, braided suture materials with antibacterial properties were envisaged. Moreover, antimicrobial coating should not negatively alter suture properties, such as tenacity, knot strength, surface friction, or biocompatibility characteristics. Silk sutures have been coated with different antibiotics, such as tetracycline [18], gentamicin [19], levofloxacin [17,20], among others, to prevent bacterial adherence and the formation of biofilms. Other antibacterial agents, like chitosan [21,22] or silver ions [23] have also shown a similar effect in preventing bacterial colonization of medical materials. Furthermore, the increasing of microbes resistant to antibiotics or antibacterial agents like triclosan, has pushed current research to look for alternative substances with antimicrobial properties to prevent SSI and at the same time to reduce the administration or use of antibiotics [24].

Antimicrobial peptides derived from the human innate immune system offers a new prospective source of antimicrobial candidates with extended clinical lifetimes [25]. However, due to the labile nature of peptides and potential toxicity concerns of most antimicrobial peptides, their period of action can be reduced, with lower protein stability or inefficient delivery of the antimicrobial peptide to the target site, hindering their clinical development [26]. Human Neutrophil Defensin-1 (HNP1) is an α-defensin that belongs to a large a subfamily of cationic antimicrobial peptides with known activity against a broad range of microorganisms, with preference against Gram-positive bacteria [27–29]. The antimicrobial potential of other α-defensins has also been reported. For instance, HNP4 is more active against Gram-negative bacteria than to Gram-positive bacteria [30,31]; while HNP2 and HNP3 have predominant antibacterial activity against *Staphylococcus aureus* or *Escherichia coli* [30]. In fact, HNP1 or other similar antimicrobial peptides may represent an alternative to antibiotic agents due to their low toxicity against mammalian cells, promoting the development of protective immune responses, and thus functioning as adjuvants [32]. Dabirian et al. [32] reported that the infectivity of *Leishmania* spp. on cells was significantly reduced in the presence of recombinant HNP-1. Also, Sharma et al. [33] reported the thera-

peutic potential of HNP1 against *Mycobacterium tuberculosis* in in vivo studies.

Currently, new strategies have been explored for delivering antibacterial peptides, for example using functionalized gold nanoparticles to deliver antimicrobial peptides to the wound site infections [34]. In the last years, bioengineered spider silk proteins have been widely explored as novel biopolymers. Spider silk are natural protein polymer with unique chemical and physical properties, excellent biocompatibility, absent or minimal immunogenicity, and controllable biodegradability, making them suitable for different applications in the biomedical field [35–37]. Through recombinant DNA technology, we can design and functionalized spider silk chimeric proteins with antimicrobial peptides (AMP) [38,39,34]. Our purpose is to engineer chimeric proteins with antimicrobial properties by recombinant DNA technology and use them as a coating for commercial silk surgical sutures in order to prevent or reduce SSI. In this work the importance of designing spider silk-based protein functionalized with antimicrobial domains instead of using AMP only, relies on the fact that the recombinant protein (6mer-HNP1) retain the antimicrobial properties of HNP1 plus the β-sheet formation capability even after adding the antimicrobial domains. The maintenance of the capacity to form β-sheet is important, since these physical cross-links are responsible for the exceptional mechanical properties, stability and slow degradability of silk [38,39]. Furthermore, due to HNP1 peptide nature, these biomolecules are more susceptible to proteolytic digestion [40]. Therefore, their combination with spider silk proteins may represent an advantage by decreasing proteolytic digestion [41] and by allowing for more to prolong effectiveness over time.

To achieve this goal, commercial silk sutures (Perma-Hand®) were coated either with the spider silk protein alone (6mer-control) or with the spider silk protein with antimicrobial peptide (6mer-HNP1-treatment group). The mechanical properties as well as cytotoxic/hemolytic behavior of the novel coated sutures and antibacterial properties against Methicillin-Resistant *S. aureus* (MRSA, Gram-positive) and *E. coli* (Gram-negative) were investigated.

2. Material and methods

2.1. Production of the bioengineered spider silk proteins 6mer-HNP1 and 6mer

The bioengineered spider silk protein 6mer consists of six repeats of the consensus sequence motifs derived from the major ampullate dragline silk I protein from *Nephila clavipes* (MaSp1; Accession number P19837) [39]. As for the bioengineered spider silk protein 6mer-HNP1, the DNA encoding the antimicrobial peptide HNP1 was inserted in the pET30 vector already containing the recombinant spider silk protein 6mer [39]. The production of the bioengineered proteins 6mer-HNP1 and 6mer was made in *E. coli* and the purification of these proteins were performed as previously described [39]. Briefly, overnight bacteria cultures were grown in 100 mL Luria-Bertani (LB) broth medium supplemented with 50 µg/ml kanamycin at 37 °C and used to inoculate 1 L of HyperBroth (0107; Athens Enzyme Systems Protein Expression) supplemented with 50 µg/ml kanamycin at 37 °C with agitation (250 rpm) until an OD600 of 0.7–0.9 was reached. Protein expression was induced by adding isopropyl β-D-thiogalactoside (IPTG) at a final concentration of 1 mM for 4 h, and harvest by centrifugation at 8000 rpm for 20 min at 4 °C. The cell pellet was placed at −20 °C for 12 h, thawed in ice for 30 min, and then resuspended overnight in a denaturing buffer (100 mM NaH_2PO_4, 10 mM Tris HCl, 8 M urea, pH 8.0) at 4 °C. Lysate supernatant was recovered by centrifugation at 8500 rpm for 30 min at 4 °C, removing cell debris and the

lysate was then added to a 50% Ni-NTA slurry in a ratio 1:4 (Ni-NTA: lysate), and mixed gently overnight at 4 °C. The supernatant/Ni-NTA resin mixture was added onto a glass Econo-column (Biorad), and washed with denaturing buffer (see above) at pHs 8 and 6.3. The recombinant proteins were eluted with denaturing buffer at pH 5.8 and 4.5 and then analyzed by SDS-PAGE to confirm protein purification. Proteins were dialyzed into cellulose ester snakeskin membranes with a 100-500 Da molecular weight cutoff (Spectra/Por Biotech, 131054), for one day, on 100 mM sodium phosphate buffer (pH 5.5), to maintain a steady pH and avoid protein precipitation. Afterward, extensive dialysis against water was followed for three days to remove all salts. Finally, the dialyzed proteins were lyophilized. The purity of the recombinant proteins, 6mer and 6mer-HNP1, was confirmed by SDS-PAGE using a Bis-Tris 4–12% gels (NuPAge, Invitrogen) and staining with Coomassie blue.

2.2. Materials

Commercial, non-absorbable, multifilament silk sutures (black, size 3/0 USP; Perma-Hand®, Ethicon, USA) was used for the preparation of the antibacterial sutures, and also used as a control. Absorbable multifilament polyglactin 910 sutures containing triclosan antibacterial agent (violet, size 3/0 USP; VicrylPlus® Antibacterial, Ethicon, USA) were used as a control in the antibacterial tests.

2.3. Preparation of sutures coated with bioengineered proteins

Prior to coating, the Perma-Hand® sutures were soaked in 5 mL of a 0.1 N NaOH solution for 2 h, followed by washing three times with sterile deionized H_2O to remove wax coatings or impurities present in the sutures. The sutures were coated with 6mer or 6mer-HNP1 proteins using dip-coating technique, which is a well-established method for different materials and applications. Moreover, it has been demonstrated that this process is facile and effective as a surface treatment of fibrous materials, like silk sutures, including dip-coating in a solution containing a synthetic polymer or other antimicrobial compounds, which can be immobilized on the suture surface [16]. Two different protein solutions were used: (i) 2% of the 6mer and (ii) 2% of 6mer-HNP1. The concentration of protein solution used (2%) was chosen based on a previously published study [39]. Briefly, the protein 6mer or 6mer-HNP1 (1 mg) were dissolved in 5 mL of ultrapure water and uncoated sutures (20 cm) were dipped for 120 s, followed by a drying period of 120 s at room temperature. This process was repeated four times for obtaining uniform coating along the suture length. After the drying process, the coated sutures were placed into a 70% methanol solution for 2 h, to allow the formation of β-sheet, thus rending the silk coating water insoluble.

The diameter and weight of the coated and uncoated Perma-Hand® sutures were measured at five different positions along the length of the suture. The average diameter of each type of suture was calculated using an electronic thickness gauge and expressed in mm. The weight of each type of suture was determined using a precision electronic balance (TB2150, Denver Instruments, Germany) and expressed in mg. The weight concentration of the protein coating on the sutures was also calculated as the amount of protein coating normalized per length of suture (mg/cm), according to Obermeier et al. [8].

2.4. Characterization of the coated sutures

The surface of the Perma-Hand® suture uncoated and coated with 6mer and 6mer-HNP1 proteins were examined by High-resolution field emission scanning electron microscope (SEM, Aur-iga Compact, Zeiss, Germany). Prior to any SEM observations, all sutures types were sputter-coated with palladium by ion sputtering (EM ACE600, Leica, Germany).

The tensile strength of the coated and uncoated Perma-Hand® sutures were assessed by the straight-pull and knot-pull uniaxial tests, using an Instron 5540 Universal Machine (USA) with a load cell of 1 kN. The straight-pull tensile tests were carried out at a crosshead speed of 2 mm/min and a gauge length of 10 mm until rupture, and the maximum tensile strength and strain values at rupture were determined. The knot-pull tensile test was performed using a simple knot, according to the European Pharmacopoeia standard [42] (01/2005:0324 standard; minimum required for U.S. Pharmacopeia (USP) [43] 3-0 non-absorbable sutures are 9.41 N or 20.9 GPa). Briefly, a simple knot was formed by placing one end of a suture held in the right hand over the other end held in the left hand, passing one end over the suture and through the loop so formed and pulling the knot tight. The length of suture between the clamps was 12.5 cm and the knot was midway between the clamps and driven at a constant rate until rupture, as mentioned above.

The tensile modulus was estimated from the initial slope of the stress-strain curve using the linear regression method. Five specimens per condition were tested and the results are expressed as means ± standard deviation.

2.5. In vitro degradation of the coated sutures

In vitro degradation of the coated and uncoated Perma-Hand® sutures was assessed in the presence and absence of protease XIV from *Streptomyces griseus* (Sigma). Pre-weighed coated and uncoated sutures (10 mm in length) were individually immersed in 5 mL of phosphate-buffered saline (PBS) solution containing 1 U/mL protease XIV for 0, 1, 4, and 7 days at 37 °C. The enzymatic solution was changed every 2 days. A control, in PBS only, was also performed. After each degradation period, the specimen were rinsed with distilled water, dried overnight at 37 °C and weighed.

The percentage of weight loss of each specimen was determined according to the following equation:

$$\% \text{ Weight loss} = (\text{mi} - \text{mf})/\text{mi} \times 100\%$$

where mi indicates the initial dry weight and mf the dry weight at different time points (n = 4). The results are expressed as means ± standard deviation.

2.6. Antibacterial properties

The antibacterial properties of the Perma-Hand® suture coated either with 6mer or 6mer-HNP1 proteins were assessed against MRSA and *E. coli*. Prior to the antibacterial tests, coated sutures (10 mm in length) were sterilized using a 70% ethanol solution for 2 h, washed three times in sterile PBS (pH 7.4) and dried overnight in a flow chamber. Sterile commercial Perma-Hand® sutures and VicrylPlus® Antibacterial suture were used as controls. Bacterial cultures were grown in LB broth medium at 37 °C overnight with agitation (150 rpm). Cells were centrifuged at 9000 rpm for 5 min at 4 °C, and the bacterial pellet washed twice with sterile PBS (pH 7.4). Bacterial cells were re-suspended in sterile PBS (pH 7.4) and adjusted to an optical density of 0.3 (λ = 610 nm). Coated sutures and controls were immersed in 1 mL of LB medium inoculated with 3.1×10^6 or 2.1×10^6 of *E. coli* and MRSA, respectively, and incubated at 37 °C for 24 h. Afterwards each suture was washed and placed in 1 mL of PBS (pH 7.4) and sonicated for 20 s with 5 s impulses to remove the attached bacteria on the sutures. Then, serial dilutions were made and spread onto LB agar plates and incubated overnight at 37 °C. The adherent viable bacteria to

the sutures were evaluated as log reduction of the colony forming units (CFU mL^{-1}). Each experiment was performed in triplicate.

The viability, adherence and formation of bacterial biofilms on the coated sutures and controls were further analyzed by Live/Dead staining and SEM as previously described [39]. Briefly, after 24 h incubation, each suture was washed twice with sterile PBS (pH 7.4) and stained with 1 mL solution of a Live/Dead® Bacterial Viability Kit (Molecular probes, L7007, Invitrogen) for 15 min at room temperature. Afterwards, sutures were washed three times with sterile PBS (pH 7.4) to remove excess dye. Live bacteria were stained green using SYTO 9 and Dead bacteria were stained red using PI and visualized using a Fluorescence Microscope (Imager Z1m, Zeiss), under 40× magnification. Four random images were acquired using a digital camera (AxioCam MRm5, Zeiss) and analysed by ImageJ (NIH, Maryland, USA). For SEM, sutures were fixed with 4% glutaraldehyde for 60 min in the dark at room temperature. Suture samples and control materials were dehydrated in a series of ethanol-water solutions with increasing ethanol concentration twice (50, 70, 80, 90, and 100%, v/v) and left to dry overnight prior to coating with palladium by ion sputtering.

2.7. Cytotoxicity

The biological behavior of the coated sutures with 6mer or 6mer-HNP1 fusion proteins was assessed using a cell line from human fetal lung fibroblasts (MRC5). Sterile commercial Perma-Hand® sutures were also used as a control material. Coated suture materials were sterilized as described in Section 2.6. Prior to cell seeding, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% penicillin-streptomycin (v/v) at 37 °C with 5% CO_2 under a humidified environment. After reaching 70% confluence, cells were trypsinized (0.25% trypsin/EDTA solution) from the culture flask. The sterile coated sutures and controls were cultured in 24-well plates with 100 μL of a cell suspension containing approximately 40,000 cells and incubated for 4 h at 37 °C with 5% CO_2 in a humidified environment. After incubation, 900 μL of DMEM medium was added and the cultures were incubated for 1 and 3 days under the same conditions as mentioned above. MRC5 cells cultured onto tissue culture polystyrene (TCPS) with DMEM were used as a negative control. MRC5 metabolic activity was determined by Alamar Blue assay (BUF012B, Bio-Rad), according to the manufacturer instructions. Briefly, DMEM medium was removed from each well after the specific incubation time. Cells were washed twice with sterile PBS (pH 7.4) and 1 mL of an Alamar Blue solution (1/10 in DMEM; v/v) was added directly to each well and incubated for 4 h at 37 °C with 5% CO_2. The metabolic activity was recorded by fluorescence at an excitation wavelength of 570 nm and emission at 585 nm. Data was normalized by DNA content to account for variation in cell number. Samples without cells were used as controls.

MRC5 cell proliferation was also evaluated by dsDNA picogreen quantification (P11496, Invitrogen), according to the manufacturer's instructions. Briefly, after each incubation time, DMEM medium was removed from each well and cells were washed twice with sterile PBS (pH 7.4). Then, 1 mL of ultrapure water was added to each well to induce complete membrane lysis, incubated at 37 °C for 1 h and stored at −80 °C until further analysis. After thawing, samples were analyzed for DNA content and measured at an excitation wavelength of 485/20 nm and at an emission wavelength of 528/20 nm. DNA content was calculated according to a standard curve. The experiments were run in triplicate.

2.8. Evaluation of the hemolytic activity of the coated sutures

In the case of materials used as blood contacting devices, blood compatibility should be assessed to establish safety. The red blood cell damage was assessed when exposed to the coated and uncoated Perma-Hand® sutures as well as reference silk sutures. The coated and uncoated Perma-Hand® sutures, as well as the reference materials (medical steel, silicone and rubber), were cut into 0.5 cm^2 pieces and sterilized as described in Section 2.6. The hemolytic activity of all materials was evaluated using the Haemoscan Hemolytic Assay [44] (HaemoScan bv, Groningen, Netherlands) according to the manufacturer's protocol. This method is adequate to evaluate the hemocompatibility of biomaterials and medical devices according to the international standard ISO 10993-4:2009. First, the commercial supplied whole blood of human origin (Haemoscan Hemolytic Assay kit component tested and confirmed negative for HbsAg, HCV, HIV I/II, HTLVI/II and Treponema) was centrifuged at 3000 rpm for 10 min to isolate red blood cells (RBCs), discarding plasma and white blood cells. RBCs (1 mL) were diluted in 9 mL of Tris buffer solution (TBS; 10 mM Tris buffer, pH = 7.0, 150 mM NaCl), and diluted by a factor of 40 as a RBC stock suspension [45]. All materials were washed three times with sterile dH2O distilled water, followed by rinsing with physiological saline solution (150 mM of NaCl) three times before incubation with the RBCs. Then, all the materials were individually immersed in a tube containing 120 μL of a working solution of 10^7 of RBC/mL (corresponding to 0.25% of the red blood cells stock solution) in TBS buffer (150 μL), and incubated in a water bath at 37 °C for 24 h. Afterward, all materials were removed from the tube and the fluid medium was centrifuged at 1000 rpm for 5 min. The supernatant liquid (30 μL) was removed and diluted with 100 μL of TBS buffer. RBCs in TBS buffer without any materials was used as negative control and 0.1% Triton X-100 in TBS buffer as a positive control. Hemolytic activity was calculated spectrophotometrically at OD 450, 425 and 380 nm, according to the Harboe method [46]. Hemoglobin concentration was quantified against a standard curve.

2.9. Statistical analysis

All the quantitative results were obtained from triplicate samples. In the case of the mechanical tests, four replicates were analyzed per each treatment. Data are reported as mean ± standard deviation and tested for normality. For statistical analysis, a one-way ANOVA test was performed using GraphPad Prism 6.0 software. Means were compared using the Tukey post hoc test for multiple comparisons when a significant F-value was obtained ($P < 0.05$). In the degradation tests, the weight loss of Perma-Hand® sutures coated and uncoated were compared by unpaired t-test with an interval of confidence of 95%.

3. Results and discussion

3.1. Bioengineered spider silk proteins 6mer-HNP1 and 6mer production

The colonization and biofilm formation by bacteria pathogens on surgical suture is currently one of the most serious health-related problems often leading to SSI [47]. Synthetic absorbable surgical sutures coated with antibacterial compounds (i.e. Vicryl-Plus® Antibacterial suture) were developed [12] aiming to reduce post-operatory surgical site infections. However, so far, in the case of non-absorbable surgical sutures like Perma-Hand® silk suture, there is no commercialized silk suture materials with antibacterial properties. Moreover, the exhaustive use of antimicrobial compounds to prevent or fight microbial infections led to an increase of multi-resistant pathogens [48], making it urgent to develop new drug-free antibacterial coatings for surgical silk sutures surfaces as real alternatives to antibacterial compounds.

In the present study, we explored the use of a bioengineered spider silk protein with antibacterial properties, 6mer-HNP1, as new an antibacterial drug-free coating for Perma-Hand® silk suture. Perma-Hand® silk sutures coated with 6mer or uncoated were used as controls. Fig. 1 shows the successful expression and purification of the fusion proteins 6mer-HNP1 and 6mer analyzed by SDS-PAGE. The molecular weight of 6mer and 6mer-HNP1 were determined as 27 kDa and 30 kDa, respectively. Protein sequencing enabled the confirmation of N- and C-terminal of the proteins, especially the insertion of the HNP1 gene in the spider silk 6mer protein.

3.2. Characterization of the coated sutures

The bioengineered protein 6mer-HNP1 already demonstrated broad antimicrobial activity against well-known pathogens when used in solution or in films combined with silk fibroin [39]. Herein, the bioengineered spider silk proteins, 6mer-HNP1 and 6mer, were applied as coatings on the braided silk sutures using a dip-coating approach. After dip coating process, weight concentration of the coating on the sutures was calculated. In the sutures coated with 6mer protein, the weight concentration on the coated suture with 6mer was 11.0 ± 0.3 µg/cm and in the coated suture with 6mer-HNP1 of 10.8 ± 0.5 µg/cm. The surface morphology of the sutures uncoated and coated with 6mer or 6mer-HNP1 were observed by SEM (Fig. 2).

A typical braided structure of the multifilament silk sutures was observed in all type of sutures, coated with 6mer (Fig. 2A) or 6mer-HNP1 (Fig. 2B) or uncoated sutures (Fig. 2C). In the coated sutures, a non-uniform distribution of the bioengineered proteins (6mer and 6mer-HNP1) along the multifilament sutures was observed (Fig. 2A, 2B). These non-uniform coating is probably due to the formation of protein aggregation in the last dipping layer. In fact, spider silk protein self-assembly procedure in aqueous solutions can result in the formation of nanofibril structures due to the self-assembly features of the proteins. Spider silks retain its intrinsically unstructured conformation in aqueous solution but can self-assemble into nanofibrils depending on the solution conditions [49,50]. Nevertheless, the application of spider silk proteins as a coating did not affect the morphological structure of the silk braided sutures.
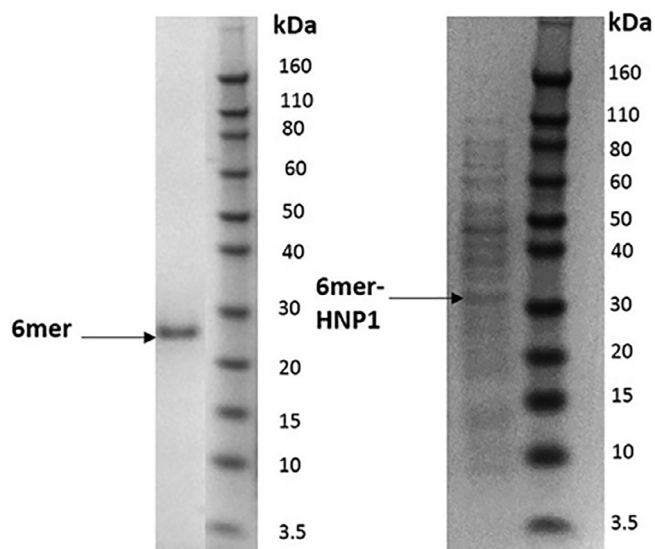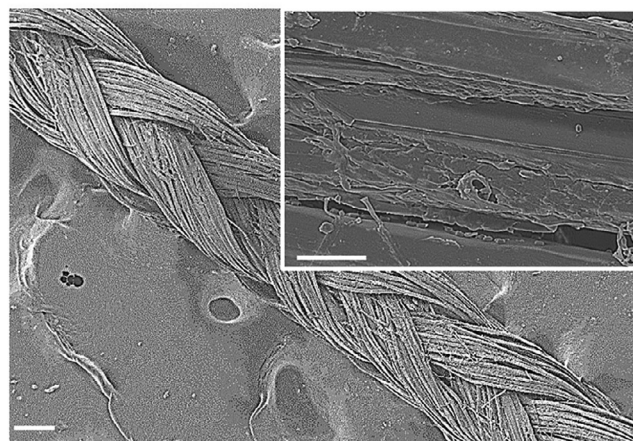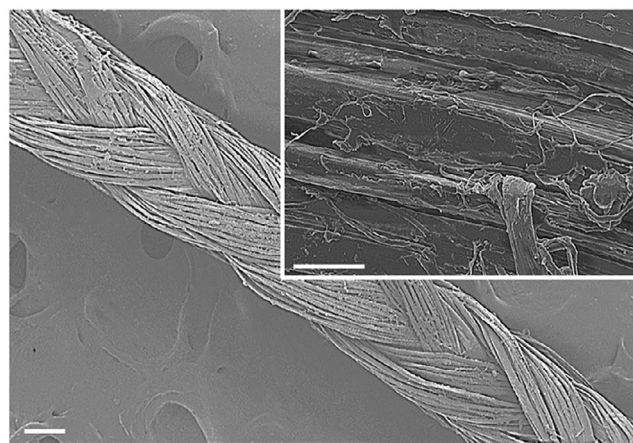

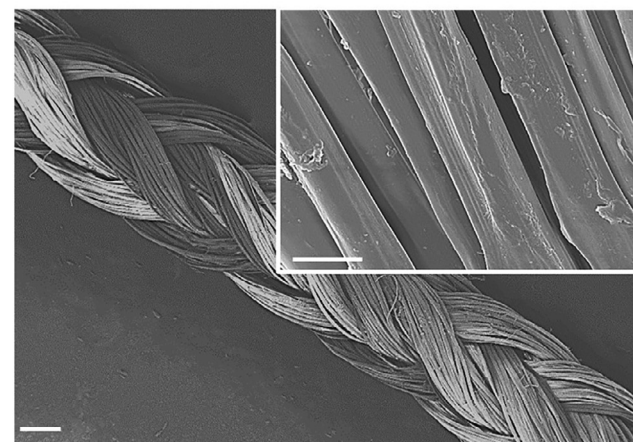**Fig. 2.** SEM micrographs of Perma-Hand® suture coated with 6mer and 6mer-HNP1, and uncoated suture. The scale bar corresponds to 10 µm. Insets are magnified images of SEM images (scale bar corresponds to 1 µm).

Fig. 1. SDS-PAGE gel electrophoresis demonstrating the expression of 6mer and 6mer-HNP1, with molecular weight around 27 kDa and 30 kDa, respectively.

The diameter and weight of the sutures coated with 6mer or 6mer-HNP1 were significantly higher (P < 0.010) when compared to the uncoated sutures (Table 1). These are due to the application of different layers of the protein coatings. Since the number of coating cycles may influence suture diameter or weight [16], the mechanical properties of the developed coated sutures were

Table 1
Diameter and weight of Perma-Hand® sutures coated with 6mer and 6mr-HNP1, and uncoated Perma-Hand® suture.

Suture	Diameter (mm)	Weight (mg)
Perma-Hand® suture coated with 6mer	0.31 ± 0.04 b	0.52 ± 0.04 b
Perma-Hand® suture coated with 6mer-HNP1	0.30 ± 0.02 b	0.47 ± 0.05 b
Commercial Perma-Hand® suture	0.23 ± 0.03 a	0.30 ± 0.01 a

Means ± SD with different letters in the same column differed significantly according to Tukey's Multiple Range test at P < 0.05.

assessed under tensile conditions, by straight-pull and knot-pull tensile test.

In the straight-pull test, the maximum tensile strength of the sutures coated with 6mer or 6mer-HNP1 (37 ± 6 MPa and 39 ± 7 MPa, respectively) showed no significant differences (P > 0.05) when compared to the uncoated sutures (47 ± 9 MPa) (Table 2). Likewise, the strain at the breaking point of the coated sutures with 6mer or 6mer-HNP1 (22 ± 2%, 22 ± 1%, respectively) remains similar (P < 0.05) to the uncoated sutures (24 ± 1%) (Table 2). These results demonstrated that the presence of bioengineered protein coating on commercialized silk sutures have no effect on suture maximum tensile strength and breaking point.

The tensile modulus of the sutures coated with 6mer was significantly lower (P < 0.050) when compared to the uncoated sutures (12 ± 2 GPa) or coated with 6mer-HNP1 (12 ± 1 GPa, respectively) (Table 2). This difference could be due to the insertion of specific domains into the spider silk segment, which can interfere with the ability of silk to form β-strands, leading in a change in assembly and thus, in mechanical properties [51]. The strength of bioengineered spider silk proteins reside in the interactions between β-sheet regions as polyAla, responsible for its higher tensile strength [52,53]. Although β-sheet form weak hydrogen bonds, post-treatment with organic solvents (like methanol) can provide extra tensile strength to the spider silk motifs. The higher stiffness on sutures coated with 6mer-HNP1 compared to sutures coated with 6mer could be due to the hydrogen bonds between spider silk chain segments and HNP1 domain. In our previous study [39], higher β-strands of the protein 6mer-HNP1 were observed compared to the protein 6mer, because of an increase in the number hydrogen bonds in the 6mer-HNP1 proteins. These differences are due to the insertion of HNP1 domain in the 6mer, resulting in an increase of hydrogen bonds between spider silk and HNP1 and thus, the sutures coated with 6mer-HNP1 were stiffer [51,54]. Despite the fact that the sutures coated with 6mer-HNP1 showed a significantly higher diameter than the uncoated sutures, their tensile properties were not affect by the coating.

In the knot-pull test, the maximum tensile strength of the sutures uncoated and coated with 6mer or 6mer-HNP1 showed no significant differences (P > 0.05) (Table 2). Concerning the tensile modulus and strain at break point on the knot-pull tests, no significant differences (P > 0.05) between the coated and uncoated

sutures were also observed (Table 2). For instance, the tensile modulus of the sutures coated with 6mer was 4 ± 1 GPa, 4 ± 1 GPa for 6mer-HNP1 and for uncoated ones were 5 ± 1 GPa; whereas, the strain at break point was 14 ± 1%, 16 ± 1%, and 18 ± 2%, respectively. From the knot-pull tests, it is important to emphasize that the strength values of the coated sutures with 6mer or 6mer-HNP1 (40.5 or 43.1 GPa, respectively) were higher compared with the values (31.3 GPa or 14.1 N) required by the USP 29-NF 24 for non-absorbable sutures with a USP size of 2-0 (or Gauge size of 3). Overall it can be said that 6mer-HNP1 coating does not affect the mechanical properties (straight-pull and knot-pull tests) of silk sutures, whereas sutures coated with 6mer show a lesser performance when compared to the commercial sutures. Therefore, the new protein coating (6mer-HNP1) do not compromise the function of the material, maintaining the desirable tensile and knot strength.

3.3. In vitro degradation

The degradation behavior of silk biomaterials is a very important feature for medical applications in vivo [55], since the structure and morphology of silk sutures or coatings depend on the crystallinity of the β-sheets structures [56]. In the present study, the in vitro degradation of the sutures coated with 6mer or 6mer-HNP1 and uncoated sutures were evaluated in PBS with and without protease XIV (Fig. 3).

In the presence of protease XIV, no significant differences (P > 0.05) were observed between the coated and uncoated sutures after 1 day of incubation (Fig. 3A). The weight loss of the sutures coated with 6mer was 25 ± 3% %, sutures coated with 6mer-HNP1 was 29 ± 1%, and uncoated sutures was 20 ± 8% (Fig. 3A). After 4 days of incubation in the presence of protease XIV, sutures coated with 6mer-HNP1 showed a significant (P < 0.050) higher weight loss profile (42 ± 8%) when compared to the sutures coated with 6mer or uncoated sutures (23 ± 7% and 23 ± 9%, respectively; Fig. 3A). However, after 7 days in the presence of protease XIV no substantial differences (P > 0.05) were observed in the degradation profile of sutures uncoated and coated with 6mer or 6mer-HNP1 (Fig. 3A). The weight loss of suture coated with 6mer and 6mer-HNP1 was 25 ± 20% and 41 ± 11%, respectively, whereas the uncoated Perma-Hand® sutures had a weight loss of 38 ± 14% (Fig. 3A). Overall, the in vitro degradation behavior of suture coated with 6mer-HNP1 seems to follow the same pattern as the uncoated sutures, in the presence of protease XIV, suggesting that their degradation profile could be related to the erosion of the suture surface and not from the bulk degradation of the suture material itself. Commercial Perma-Hand® sutures are coated with wax or silicone to prevent fraying [56] and fast in vitro degradation. Suture coated with 6mer show a fast degradation of the 6mer coating after 1 day in the presence of protease XIV. However, after day 4, the degradation profile seems to have reach a limit, possibly due to limited availability of coating to the enzyme's active site. Spider silk coatings have a high content in β-sheet that confers protection

Table 2
Mechanical properties of Perma-Hand® sutures coated with 6mer and 6mr-HNP1, and uncoated Perma-Hand® suture.

Test type	Suture	Maximum tensile strength (GPa)	Breaking strain (%)	Tensile modulus (GPa)
Straight-pull test	Perma-Hand® suture coated with 6mer	37 ± 6 a	22 ± 2 a	7 ± 1 a
	Perma-Hand® suture coated with 6mer-HNP1	39 ± 7 a	22 ± 1 a	12 ± 2 b
	Commercial Perma-Hand® suture	47 ± 9 a	24 ± 1 a	12 ± 1 b
Knot-pull test	Perma-Hand® suture coated with 6mer	41 ± 7 a	14 ± 1 a	4 ± 1 a
	Perma-Hand® suture coated with 6mer-HNP1	43 ± 4 a	16 ± 1 a	4 ± 1 a
	Commercial Perma-Hand® suture	42 ± 8 a	18 ± 2 a	5 ± 1 a

Means ± SD with different letters in the same column differed significantly according to Tukey's Multiple Range test at P < 0.05.
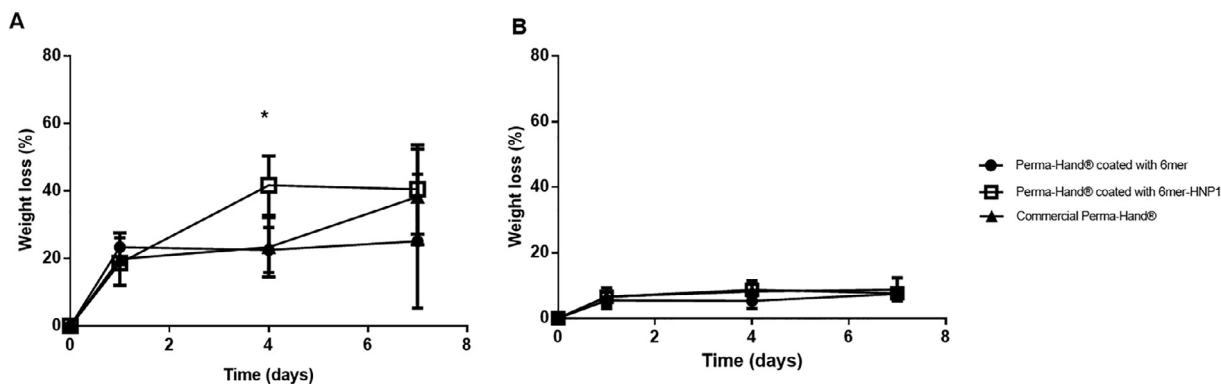
Fig. 3. A. Enzymatic degradation behavior of Perma-Hand® suture coated with 6mer, 6mer-HNP1 and uncoated suture until 7 days in the presence of protease XIV at 37 °C; B. Degradation behavior of Perma-Hand® suture coated with 6mer, 6mer-HNP1 and uncoated suture until 7 days in PBS solution (pH 7.4) at 37 °C.

against in vitro degradation [57], but they can are still be susceptible to enzymatic degradation leading to differences in material weight and affecting their strength [58]. In addition, the presence of antibacterial spider silk coatings on silk surgical sutures had no significant effect on the biodegradability of the suture itself, maintaining enough structural integrity on the suture throughout the degradation study.

As for sutures in PBS and devoid of enzymes, the weight loss of the sutures coated with 6mer ($8 \pm 2\%$), 6mer-HNP1 ($8 \pm 1\%$), and uncoated sutures ($9 \pm 5\%$) did not change significantly after 7 days of incubation (Fig. 3B). Results suggest that both coated and uncoated sutures showed a similar degradation pattern in PBS.

3.4. Antibacterial properties

Human-derived antimicrobial peptides, like HNP1, have gained great attention as a novel class of antimicrobial compounds due to their broad-spectrum activity against Gram-positive, Gram-negative, fungi or virus, as well as their effectiveness against multi-resistant pathogenic microorganisms like MRSA [59]. Unlike antibiotics, the development of microbial resistant strains to these peptides remains a rare phenomenon since they target specific features on the microbial cellular membrane [25]. Moreover, these

antimicrobial peptides play an important role in human innate and adaptive immune response, conferring a high degree of plasticity but slower response to any attack [25,60]. The braided nature of the silk sutures can work as a suitable surface for the adherence of bacteria and inducing the formation of biofilms, in which creates a niche for infections that can result in SSI [23,59].

Herein, the viability and adherence of MRSA and *E. coli* on the surface of the Perma-Hand® sutures coated with 6mer and 6mer-HNP1 were assessed after 24 h growth (Fig. 4). VicrylPlus®, an antibacterial suture, and uncoated Perma-Hand® were used as reference material since there are no commercially available antibacterial silk sutures. Bacterial log reductions were calculated referred to uncoated Perma-Hand® suture.

Perma-Hand® sutures coated with 6mer-HNP1 showed a significant ($P < 0.50$) inhibitory effect against MRSA and *E. coli* bacteria when compared to sutures coated with 6mer or uncoated sutures (Fig. 4). As can be observed, Perma-Hand® sutures coated with 6mer-HNP1 demonstrated a significant ($P < 0.001$) 1.5 log reduction of MRSA and a 2 log reduction against *E. coli* when compared to the uncoated Perma-Hand® suture. In contract, Perma-Hand® sutures coated with 6mer show a 0.5 log reduction of MRSA and a 1log reduction of *E. coli*. VicrylPlus® shows a log reduction of 2 against the tested bacteria. (Fig. 4). Previous study [23] reported
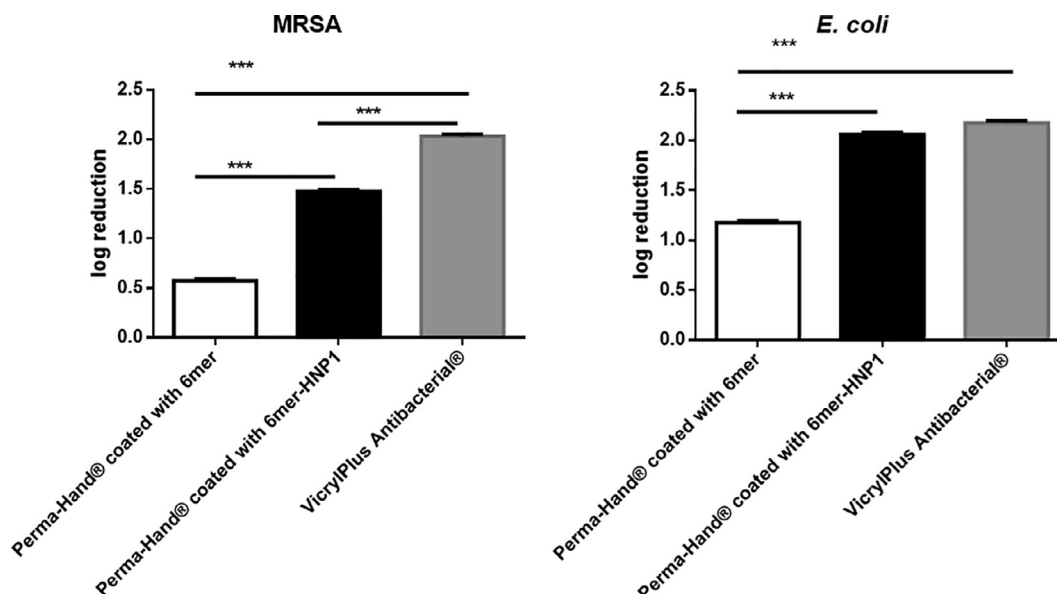
Fig. 4. Log reduction of MRSA and *E. coli* adherence to the Perma-Hand® sutures coated with 6mer and 6mer-HNP1 at 37 °C. VicrylPlus® was used as reference material. Bacterial log reductions were calculated referred to uncoated Perma-Hand® suture.

that the incorporation of antimicrobial agents such as silver ions, as silk suture coatings showed an inhibitory effect against *S. aureus*. Similarly, Dhas et al. [61] demonstrated that silk sutures coated with a biobased silver nanocolloid polymer showed an inhibitory effect against *Pseudomonas aeruginosa* and *S. aureus* growth. In our study, a significant reduction in the adherence of MRSA and *E. coli* onto the surface of the silk sutures coated with 6mer-HNP1 was observed after 24 h of incubation (Fig. 4), suggesting that 6mer-HNP1 coating also shows an anti-adhesive potential.

The adherence and formation of biofilm of MRSA and *E. coli* on all type of sutures was observed by SEM and fluorescence microscopy (Fig. 5). MRSA and *E. coli* were able to adhere onto the surface of Perma-Hand® sutures coated with 6mer, leading to the formation of bacterial biofilm after 24 h incubation (Fig. 5A). The same trend was observed with the uncoated Perma-Hand® sutures (Fig. 5A). Regarding the Perma-Hand® sutures coated with 6mer-HNP1, the adherence and proliferation of MRSA and *E. coli* decreased, while the formation of biofilm was not observed 24 h incubation (Fig. 5A). The bacterial adhesion and formation of biofilm on the surface of VicrylPlus® antibacterial sutures exhibited

the same inhibitory activity that of the Perma-Hand® sutures coated with 6mer-HNP1 (Fig. 5A). Gómez-Alonso et al. [62] reported that VicrylPlus® antibacterial sutures demonstrate an inhibitory behavior against *S. aureus* and *E. coli*, which are in line with the results obtained in the present work. The outcome from this antibacterial assessment suggests that Perma-Hand® sutures coated with 6mer-HNP1 exhibit the similar antibacterial behavior when compared to VicrylPlus® antibacterial sutures. Moreover, the presence of 6mer-HNP1 coating on Perma-Hand® sutures induced a reduction on bacteria adhesion on suture surface. Thus, the application of the bioengineered spider silk protein, 6mer-HNP1, as an antibacterial coating could envisage a new class of drug-free sutures, as a promising alternative to replacing the use of sutures with antibiotics.

3.5. In vitro response

Regarding the cell metabolic activity, no significant differences ($P > 0.05$) was observed between all sutures (Fig. 6A). After 1 day of culture, no significant differences ($P > 0.05$) in cell proliferation
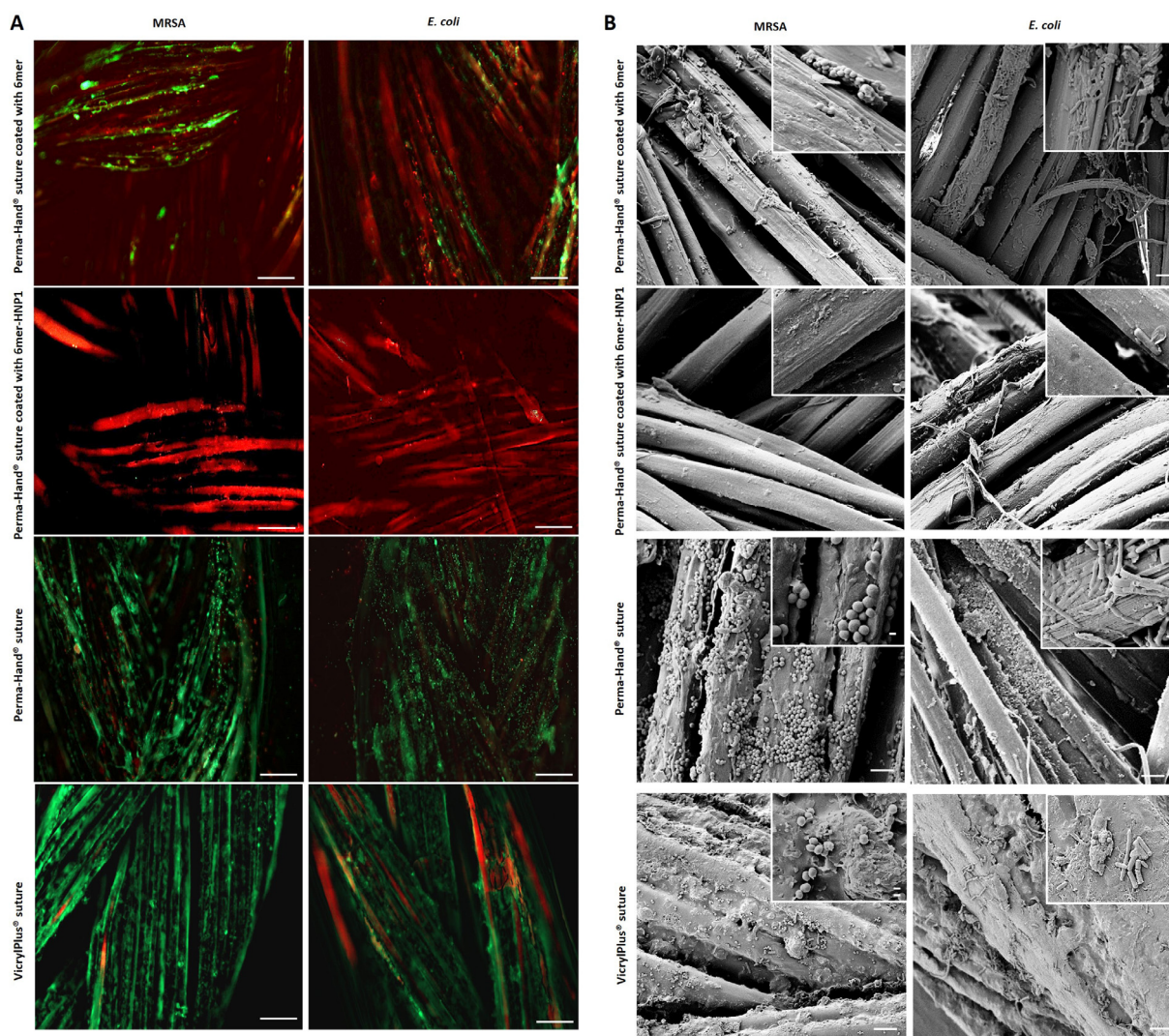
Fig. 5. Viability of MRSA and *E. coli* on Perma-Hand® suture coated with 6mer and 6mer-HNP1, as well as uncoated Perma-Hand® suture. VicrylPlus® suture was used as a reference material. (A) Representative images of MRSA and *E. coli* adherence and biofilm formation. Live bacteria were stained green using SYTO 9 and Dead bacteria were stained red using PI. The scale bar correspond to 50 μm. (B) SEM micrographs of the adherence and formation of biofilm of MRSA and *E. coli* on Perma-Hand® suture coated with 6mer and 6mer-HNP1, as well as uncoated Perma-Hand® suture. VicrylPlus® suture was used as a reference material. The scale bar corresponds to 10 μm. Insets are magnified images of SEM images (scale bar corresponds to 1 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
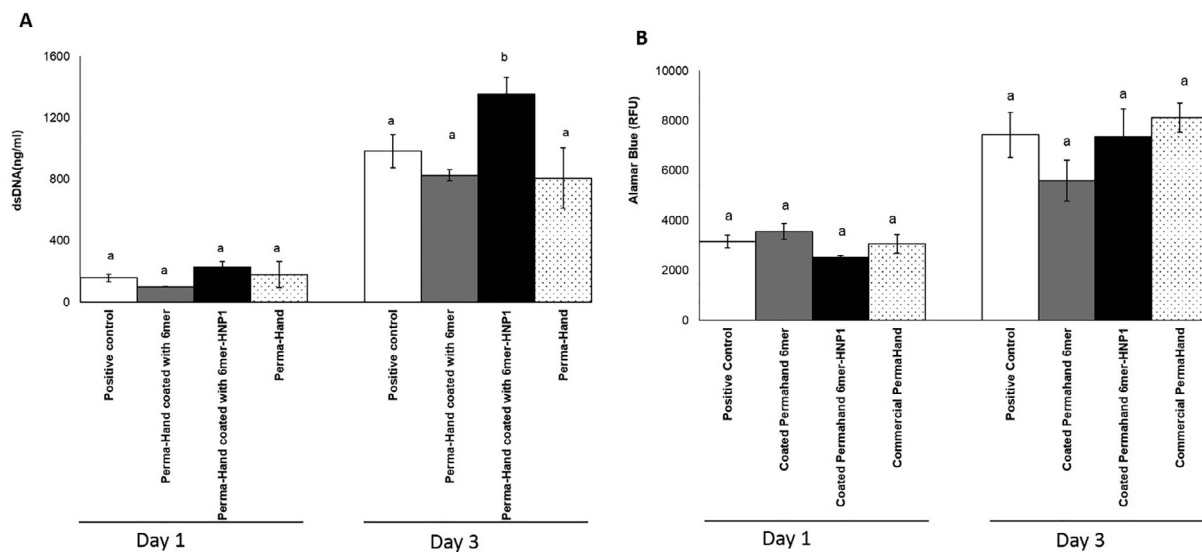
Fig. 6. MRC5 cellular viability/proliferation (Alamar Blue assay) (A) and double strand DNA (dsDNA) quantification assay (B) cultured on the Perma-Hand® sutures coated with 6mer and 6mer-HNP1 fusion proteins after 1 day and 3 days in DMEM medium. Tissue culture plastic was used as a positive control. Columns with different letters differed significantly according to Tukey Multiple Range test at P < 0.05.

were observed between the sutures coated with 6mer or 6mer-HNP1 and the uncoated sutures (Fig. 6B). Nevertheless, after 3 days of culture, the sutures coated with 6mer-HNP1 showed a significantly higher cell proliferation (P < 0.05) than the other sutures.

Furthermore, when cell viability/proliferation results for all sutures were compared with the positive control, no significant differences were observed (P < 0.05) (Fig. 6).

Biocompatibility studies showed that Perma-Hand® sutures either coated with 6mer or 6mer-HNP1 support cell viability and proliferation (Fig. 6). These results further support the positive cellular response of the 6mer and 6mer-HNP1 fusion proteins when used as suture coatings together with antibacterial properties.

3.6. Hemolytic activity

The hemolytic activity of the sutures (either coated with 6mer or 6mer-HNP1, or uncoated) showed a lower significant hemolytic activity (P < 0.05) in contrast to the reference materials (silicone, rubber, and steel) (Fig. 7). Comparing with uncoated Perma-Hand® sutures as reference material, no significant differences (P > 0.05) were observed.

The hemolytic behavior is a common complication affecting the compatibility of medical devices when in contact with blood, which can lead to organ failure [63]. The interaction of implementable materials, like sutures with blood, can result in the release of hemoglobin [44], influenced by the surface of the material [64]. Spider silk proteins are known for their low immunogenicity response in vivo and good biocompatibility, making them suitable for numerous medical applications [65,66]. Likewise, HNP1 as well as analog proteins without cysteine, had reduced activity lysing human red blood cells, possibly due to the high hydrophobicity of HNP1, thus suggesting that HNP1 has no hemolytic activity when in solution [67]. Moreover, HNP1 prevented hemolysis by three cytolysins studied [68], thus suggesting that HNP1 also has no hemolytic activity. In the present study, the bioengineered spider silk protein 6mer-HNP1 showed no hemolytic activity when applied as coatings for silk sutures, as well as the bioengineered protein 6mer, suggesting that they present good blood compatibility.
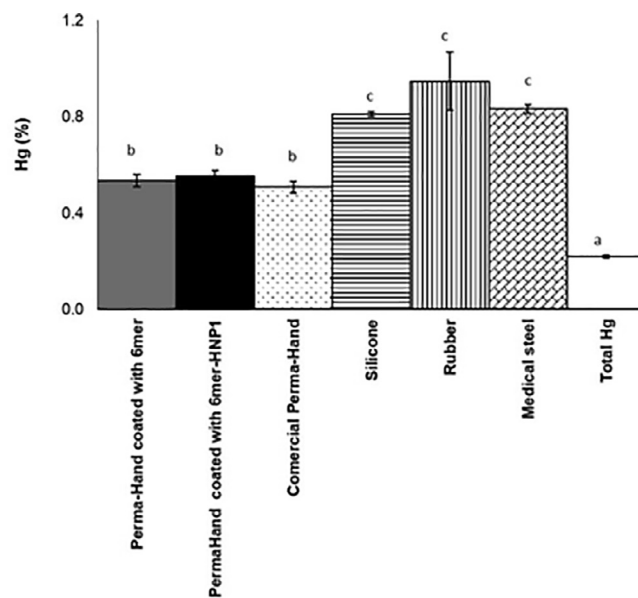
Fig. 7. Haemolysis (%) of Perma-Hand® sutures coated with 6mer and 6mer-HNP1 fusion proteins, and commercial Perma-Hand® sutures. The reference materials, silicone, rubber, and medical steel, were used positive hemolysis reference controls. Total Hg (Total hemoglobin) represent the total lysed hemoglobin without any material and was used as negative control.

4. Conclusions

In this study, we have demonstrated the positive effect of using spider silk proteins functionalized with antimicrobial peptide (6mer-HNP1) as a coating for silk sutures preventing/eliminating or substantially reducing the extent of bacterial attachment and biofilm formation on their surfaces. The coated sutures retained tensile and knot strength, maintaining silk suture inherent performance, surface morphology and in vitro biodegradability. In addition, significant inhibition of MRSA and *E. coli* adhesion was observed as well as good cytotoxicity and hemolytic properties.

The importance of this in vitro study relies on the engineering new spider silk polymer proteins with antimicrobial peptides to prevent SSI, and at the same time avoiding the utilization of antimicrobial agents or antibiotics. This new antibacterial suture coating for silk sutures represents a potentially important option for antibiotic-free sutures. Nevertheless, further studies are necessary to explore the inflammatory response as well as mechanical behavior of these new antibacterial coatings in vivo, to fully address the potential of this approach for sutures or other medical devices.

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