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The development of a novel wound healing material, silk-elastin sponge

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ABSTRACT

Silk-elastin is a recombinant protein polymer with repeating units of silk and elastin blocks. This novel wound healing promoting material has the ability to self-assemble from a liquid to a gel. We have already reported that an aqueous solution of silk-elastin has the potential to accelerate wound healing; however, there are several problems in applying silk-elastin in the clinical setting. To solve these problems, we developed a silk-elastin sponge that is easy to use in the clinical setting. In the present study, we examined whether the wound healing effect of the silk-elastin sponge is equal to the aqueous solution of silk-elastin *in vivo*. The granulation tissue formation promoting effect of the silk-elastin sponge was equal to that of the aqueous solution the silk-elastin, as after application to the wound surface, the sponge was absorbed and dissolved by the exudate. At body temperature the silk-elastin then formed temperature gel. The silk-elastin gel that was obtained contained abundant cytokines from the exudate. We believe that silk-elastin sponge can be applied to various wounds that are difficult to treat with the aqueous solution.

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KEYWORDS

Silk-elastin; wound healing; cytokine; granulated tissue

1. Introduction

Silk-elastin is produced by recombinant DNA technology using the genes involved in the production of silkworm fibroin and human elastin to generate peptide repeats of silk fibroin (GAGAGS) and elastin-like (GVGVVP) units, respectively [1]. Combining silk fibroin and elastin-like units in various ratios and sequences enables the production of various biomaterials with diverse material properties. Previous reports on silk-elastin have shown that the solubility, material strength, immunogenicity and *in vivo* degradation profile of silk-elastin can be controlled by varying the composition and sequence of the units [2, 3]. In another report, protein fibers with a high tensile strength and high deformability were able to be fabricated by combining silk- and elastin-derived sequences into a single silk-elastin protein polymer [4]. The silk-elastin shows the biocompatibility and high elasticity of human elastin, combined with the mechanical and tensile strength of silk fibroin in a molecular structure

that is not naturally present in one molecule [5]. At concentrations of $\geq 4\%$ (w/v) or higher, water-soluble silk-elastin at room temperature can form a hydrogel at body temperature [6]. Before its application, the silk-elastin is in a liquid state; however, it can solidify to form a hydrogel at body temperature [7]. Thus, when silk-elastin is applied to a wound in an aqueous condition, it automatically forms a silk-elastin hydrogel, which covers the wound and maintains a moist condition without inflammation. This property of self-gelation is useful for use in wound covering. In addition to the gel property, we previously reported that silk-elastin has the potential to promote the migration of fibroblasts and macrophages, and the production of fibroblast collagen [8]. Silk-elastin has a unique property of suppressing the adhesion of cells without suppressing their apoptosis [9]. The electrospinning of a silk-elastin-based tissue scaffold resulted in a scaffold with excellent mechanical properties and biocompatibility [10]. The biocompatibility of silk-elastin was experimentally confirmed by intradermal injection to guinea pigs [5]. Furthermore, the medical and pharmaceutical applications of silk-elastin in tissue engineering and drug delivery systems have been investigated. [11–13].

On the other hand, the administration of silk-elastin as an aqueous solution in the clinical setting was associated with the following problems: (1) it took a long time to perform and it was troublesome to prepare the solution before use; and (2) it was difficult to fill large wounds with the liquid.

In order to solve the above problems, we created a silk-elastin sponge. After administration, the silk-elastin sponge is absorbed and dissolved by the exudate. The silk-elastin then forms a gel at body temperature. In this study, we describe the development of the silk-elastin sponge and investigated whether it was converted to the gel form after application, and whether the silk-elastin sponge had an effect on wound healing.

2. Materials and methods

2.1. The preparation of silk-elastin sponge

Silk-elastin is composed of four silk fibroin-like blocks, seven elastin-like blocks, and one modified elastin block containing a lysine (K) substitution (MDPVVLQRRDWENPGVTQLNRLAAHPPFASDPMGAGSGAGAGS [(GVGVP)4 GKGVP (GVGVP)3 (GAGAGS)4]12 (GVGVP)4 GKGVP (GVGVP)3 (GAGAGS)2 GAGAMDPGRYQDLRSHHHHHH) [1]. The silk-elastin was supplied by Sanyo Chemical Co., Ltd., Kyoto, Japan.

We prepared ultrapure water and ultrapure water solutions containing silk-elastin at concentrations of 12.5, 25, 50, and 100 mg/cm³ and casted each solution to each formwork to obtain silk-elastin sponges with a final thickness of 5, 1.25, and 2.5 mm. We then subjected the casted formworks to freezing, primary drying, and secondary drying using a freeze dryer (Nihon Techno Service Co., Ltd.) and thereby obtained six types of silk-elastin sponges (A–F in Table 1). The sponge structures were visualized using a scanning electron microscope (Quanta 250 FEG; FEI company [please describe the company location]) (Figure 1).

We evaluated the solubility and gelling performance of the silk-elastin sponges as follows: 50 μ l of ultrapure water was put on petri dishes to form water spots and then silk-elastin sponges of 8 mm in diameter were placed on the spots. The silk-elastin sponges absorbed the water and the sponges were dissolved into water and formed a hydrogel (Figure 2). The

Table 1. Silk-elastin sponge.

No.	A	B	C	D	E	F
Density (mg/cm ³)	12.5	25	50	100	25	25
Thickness (mm)	5	5	5	5	1.25	2.5
Gelation time (min)	240	200	–	–	300	240
Pore size (μm) (N = 10)	40.7 ± 11.2	26.0 ± 8.5	23.4 ± 7.4	22.2 ± 6.5	27.8 ± 7.1	27.8 ± 7.5
Energy to Failure (J/m ³) (N = 6)	14.8 ± 2.1	28.3 ± 4.5	455.6 ± 13.0	496.8 ± 46.3	27.4 ± 6.2	28.1 ± 1.2

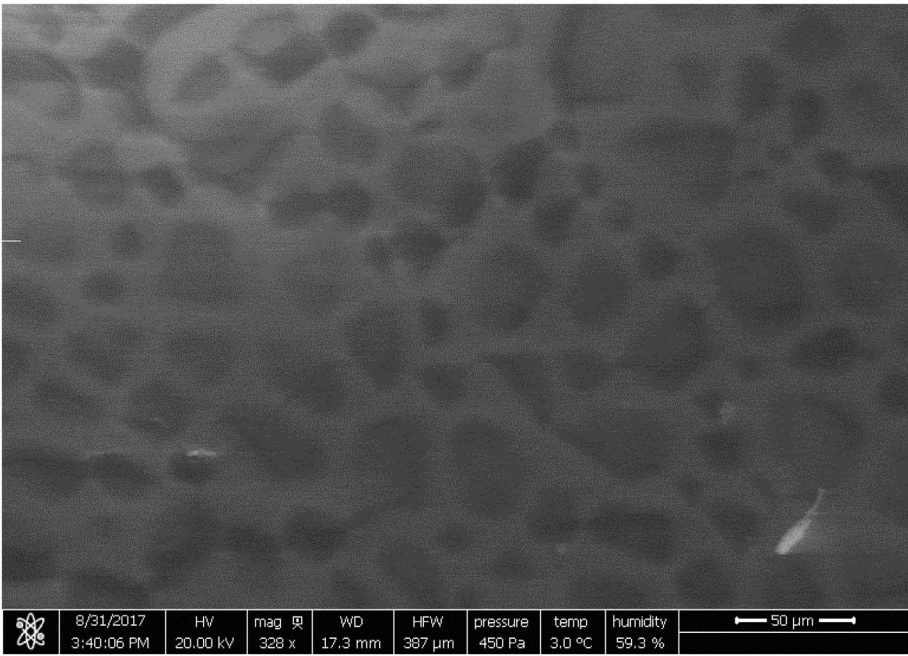


Figure 1. SEM picture of silk-elastin sponge.
Note: Silk-elastin sponge E (density, 25 mg/cm³; thickness, 1.25 mm).

time course from dissolution to gelation (when the aqueous solution of silk-elastin was not dripping from the overturned dishes) was defined as the gelation time.

We measured the pore size of the silk-elastin sponge using digital microscope DSX 510 (OLYMPUS). To evaluate the breaking strength of each silk-elastin sponge, we measured the energy to failure using a CREEP METER RHEONER II RE2-3300513 (YAMADEN). The measurement conditions were as follows: cylinder type with a plunger diameter of 8 mm, measurement speed: 0.1 mm/s, contact area: 8 mm, number of load cells: 2, measured under 20 N.

From the measured stress-strain curve, the integral value until reaching the yield point was taken as the energy to failure and expressed using the following formula:

$$E_n = \int_0^{\epsilon_f} P d\epsilon$$

where E_n is the energy to failure, ϵ_f the breaking strain, P the stress, and ϵ the strain.

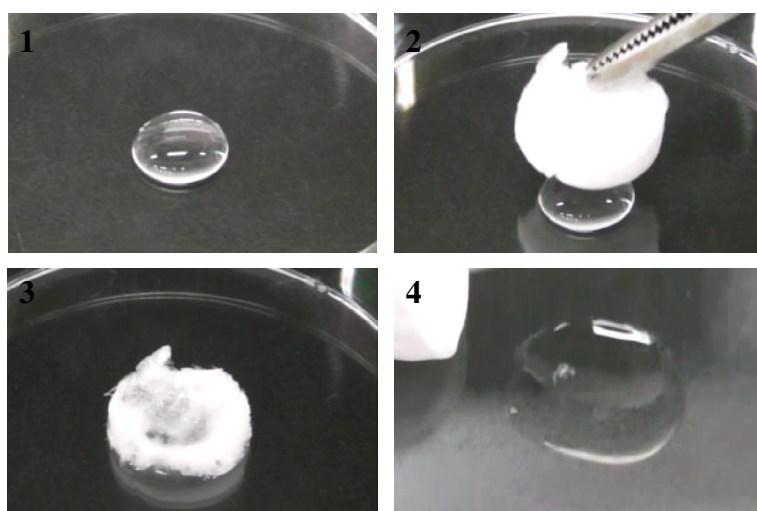


Figure 2. The dissolution and gelation of the silk-elastin sponge.

Notes: The silk-elastin sponge was rapidly dissolved by water (wound exudate). (1) Water was placed on top of the plate. (2) The silk-elastin sponge was placed on the water. (3) The silk-elastin sponge dissolved. (4) The silk-elastin self-assembled into a gel.

2.2. Animals and operations

The animals were maintained at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. The number of animals used in this study was kept to a minimum, and all possible efforts were made to reduce their suffering in compliance with the protocols established by the Animal Research Committee of Kyoto University.

2.3. The application of silk-elastin sponges on full-thickness skin defects in guinea pigs

Seven-week-old healthy female guinea pigs (std: Hartley) (Japan SLC, Inc.) were anesthetized, and depilated. After cleansing, full-thickness wounds were created (8 mm in diameter) on the backs of the guinea pigs. After hemostasis and drying, silk-elastin sponges or silk-elastin solution (20 wt% aq.) were applied to the wounds, which were then covered with polyurethane film (Figure 3). The control wounds were covered with polyurethane film alone. Thereafter, the wound area was covered with gauze, which was fixed to the skin around the wound areas with a nylon thread ($N = 4-8$). The guinea pigs were sacrificed on the 5th day of the treatment and skin samples from the wounds were taken for histological studies.

2.4. Cytokine assay

The silk-elastin sponge E (density 25 mg/cm³, thickness 1.25 mm) was implanted into the full-thickness wounds of guinea pigs. At 24 h after implantation, the silk-elastin sponges were collected. The extract was prepared by adding 100 μ l of D-PBS to the silk-elastin sponge. As a control, a full-thickness wound, which was only covered with a polyurethane film for 24 h, was rinsed with 100 μ l of D-PBS. The rinsed D-PBS was collected. We

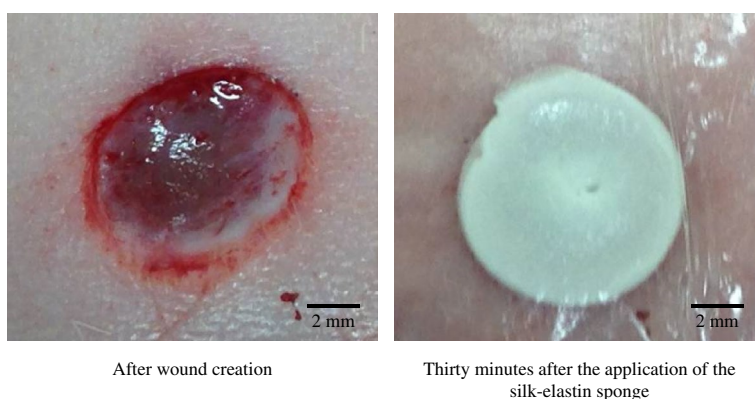


Figure 3. The application of silk-elastin sponge on full-thickness skin defects.

measured the concentrations of cytokines (bFGF, TNF α , and IL-1 β) using enzyme-linked immunosorbent assay (ELISA) kits ($N = 3$).

2.5. The application of silk-elastin sponges on pressure sores in diabetic mice

The 9-week-old male BKS.Cg- β Leprdb/ β Leprdb/Jcl (db/db) mice were obtained from CLEA (Tokyo, Japan). All of the mice had their backs and abdomens shaved and depilated under anesthesia with diethyl ether (Wako Pure Chemical Industries, Osaka, Japan) and were then positioned on experimental tables. In our previous study, we developed a pressure induced ulcer model using diabetic mice and a pneumatic compressor (Kawai et al., 2005) [14]. In this study, 4 h of prolonged pressure (two pressure sessions of 2 h in length with a 2-h interval between pressure sessions; 500 g/cm²) was loaded onto the area above the femoral trochanters of the mice, using a pneumatically driven compressor for 2 consecutive days (Earth Man AC-20 OL, Takagi Co. Ltd., Niigata, Japan). The air pressure was regulated using a precision regulator, which provided a constant pressure level. Five days after the completion of pressure loading, the area of necrosis was clearly demarcated. After hemostasis and drying, silk-elastin sponge B (density 25 mg/cm³, thickness 5 mm, net amount of silk-elastin 6.3 mg) or silk-elastin solution (20% [w/v] aq. net amount of silk-elastin 6.3 mg) was implanted into the resulting wounds. Polyurethane film was then placed on the resulting wounds. Thereafter, each wound area was covered with gauze, which was fixed to the skin around the wound areas with a nylon thread ($N = 8-9$). The diabetic mice were sacrificed on the 14th day of the treatment, skin samples were taken from the wound areas for histological studies.

2.6. Histological evaluation

The tissue specimens were fixed with 4 wt% paraformaldehyde in PBS at 4 °C for 24 h and embedded in paraffin to prepare histological sections. The 4 μ m-thick sections were stained with hematoxylin and eosin. The area of granulated tissue was measured in histological sections under a light microscope.

2.7. Statistical analysis

The data are shown as the mean $\pm$ standard deviation. The results were compared using the Tukey–Kramer paired comparison test or Student's *t*-test, as appropriate. *P* values of < 0.05 were considered to indicate statistical significance.

3. Results

3.1. The physical and chemical characteristics of silk-elastin sponge

We measured the gelation time of each silk-elastin sponge and confirmed that silk-elastin sponges A, B, E, and F formed gels, while silk-elastin sponges C and D were only partially dissolved. A previous study showed that the gelation time of silk-elastin aqueous solution at 37 °C was between 200 and 300 min (Table 1) [5]. A correlation between the pore size of the silk-elastin sponge and density was observed. However, when the breaking strength was C or D, it was roughly 16 times that of B.

3.2. The application of silk-elastin sponges to full-thickness skin defects in guinea pigs

Images of representative histological sections are shown in Figure 4. We investigated the effect of the density of the silk-elastin sponge on the area of granulation tissue formation. The areas of granulation tissue in the histological sections of the 12.5 and 25 mg/cm³ silk-elastin sponges were almost the same as the areas that were treated with the aqueous solution of silk-elastin. In contrast, the areas of granulation tissue that formed when the 50 and 100 mg/cm³ silk-elastin sponges were used were significantly smaller in comparison to the areas that were treated with the silk-elastin solution by about 30% (Figure 5). The areas of granulation tissue with the silk-elastin sponges 50 and 100 mg/cm³ in size were not significantly different from the control group.

We also investigated the effect of the thickness of a silk-elastin sponge with a density of 25 mg/cm³ on the area of granulation tissue formation. There was no significant difference in the areas of granulation between the groups in which silk-elastin sponges of 0.25–5 mm were used and the silk-elastin solution group (Figure 6). The areas of granulation in each of the silk-elastin sponges and solution groups were significantly larger in comparison to the control group. The depth of the wounds on the backs of guinea pigs was 1.6 mm.

3.3. Cytokine assay

In the cytokine assay the amounts of bFGF, TNF α , and IL-1 β in wounds covered with the silk-elastin sponge were significantly larger in comparison to control (Figure 7). Specifically, the silk-elastin sponge contained 3.2 times the bFGF, 20 times the TNF α , and 3 times the IL-1 β of the control.

3.4. The application of silk-elastin sponges on pressure sores in diabetic mice

We evaluated the performance of the silk-elastin sponge with a Stage IV pressure-induced ulcer model using diabetic mice (Figure 8). The silk-elastin sponge had the same effect

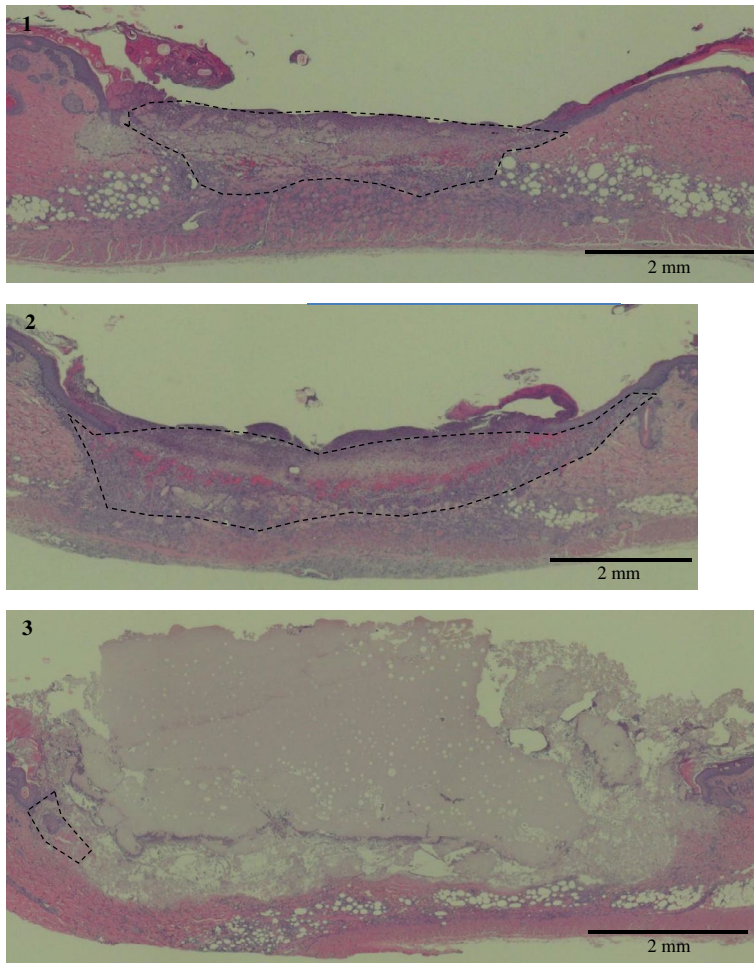


Figure 4. Histological sections obtained five days after implantation.

Notes: The following materials were applied: (1) silk-elastin solution, (2) silk-elastin sponge E (density, 25 mg/cm³; thickness, 1.25 mm), (3) silk-elastin sponge D (density, 100 mg/cm³; thickness, 5 mm). Hematoxylin and eosin staining (original magnification $\times 100$). The area of granulation tissue is surrounded by a broken line.

in the pressure sore model as the silk-elastin solution. Both the silk-elastin sponges and silk-elastin solution significantly accelerated granulation by 3.5-fold in the pressure sores compared to the control (Figure 9).

4. Discussion

We successfully fabricated sponge-like silk-elastin from an aqueous solution of silk-elastin in which the distinctive feature of thermosensitive hydro-gelation was maintained. The density and thickness of the silk-elastin sponge are flexible. Sponges with high density generally had a high energy to failure and were only partially dissolved. The gelation time was not measured. Poor gelation might have inhibited the start of wound healing and caused delayed granulation. The use of the silk-elastin sponge resolved the two above-mentioned issues that occurred when silk-elastin solution was used to promote wound healing – specifically,

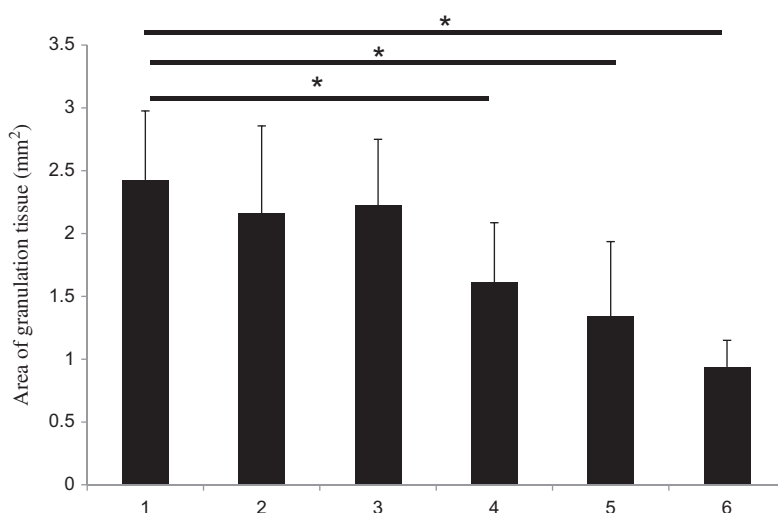


Figure 5. The evaluation of the granulation tissue (Density).

Notes: (1) Silk-elastin solution, (2) Silk-elastin sponge A (density, 12.5 mg/cm³; thickness, 5 mm), (3) Silk-elastin sponge B (density, 25 mg/cm³; thickness, 5 mm), (4) Silk-elastin sponge C (density, 50 mg/cm³; thickness, 5 mm), (5) Silk-elastin sponge D (density, 100 mg/cm³; thickness, 5 mm), (6) Control.

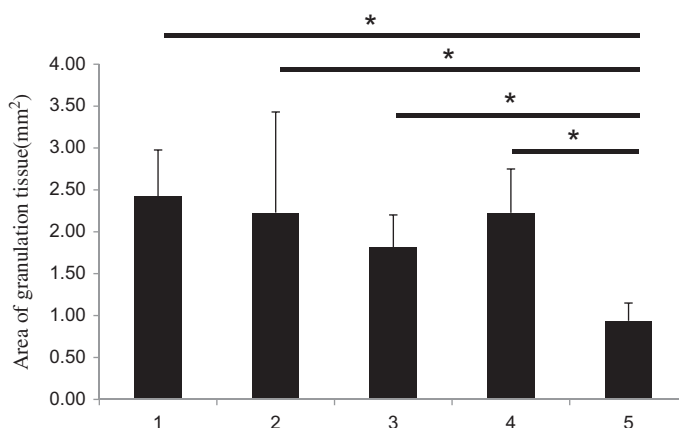


Figure 6. The evaluation of the granulation tissue (Thickness).

Notes: (1) Silk-elastin solution, (2) Silk-elastin sponge E (density 25 mg/cm³; thickness, 1.25 mm), (3) Silk-elastin sponge F (density, 25 mg/cm³; thickness, 2.5 mm), (4) Silk-elastin sponge C (density, 25 mg/cm³; thickness, 5 mm), (5) Control.

the time taken to apply the solution and the troublesome nature of its preparation, and the difficulty associated with filling large wounds with the liquid (Figure 9).

In fact, applying the silk-elastin sponge to the back of the guinea pig was much easier than administering the silk-elastin aqueous solution. In the future, we will evaluate the handling properties of silk-elastin through clinical trials.

This study showed that the silk-elastin sponge had the same wound healing properties as the silk-elastin aqueous solution. We inferred that the sponge-like form had no influence on the silk-elastin property with regard to the migration of fibroblasts and macrophages. Ozaki et al. showed that the cell migration property of silk-elastin was due to chemotaxis

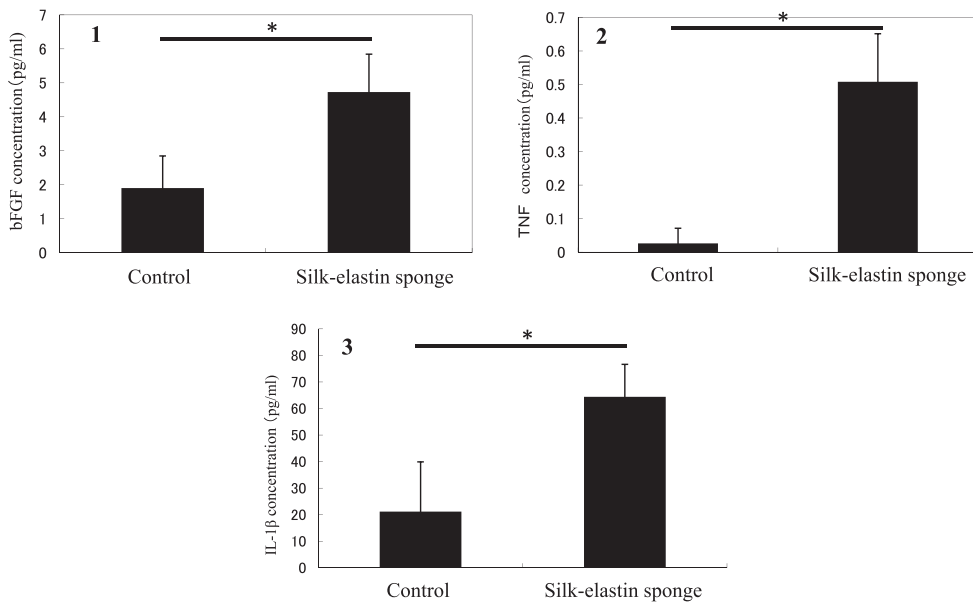


Figure 7. The measurement of cytokines in silk-elastin gel.

Notes: (1) bFGF, (2) TNF α , (3) IL-1 β . The silk-elastin sponge used was sponge E (density, 25 mg/cm³; thickness, 1.25 mm). In the control group, only polyurethane film was applied to the wound.

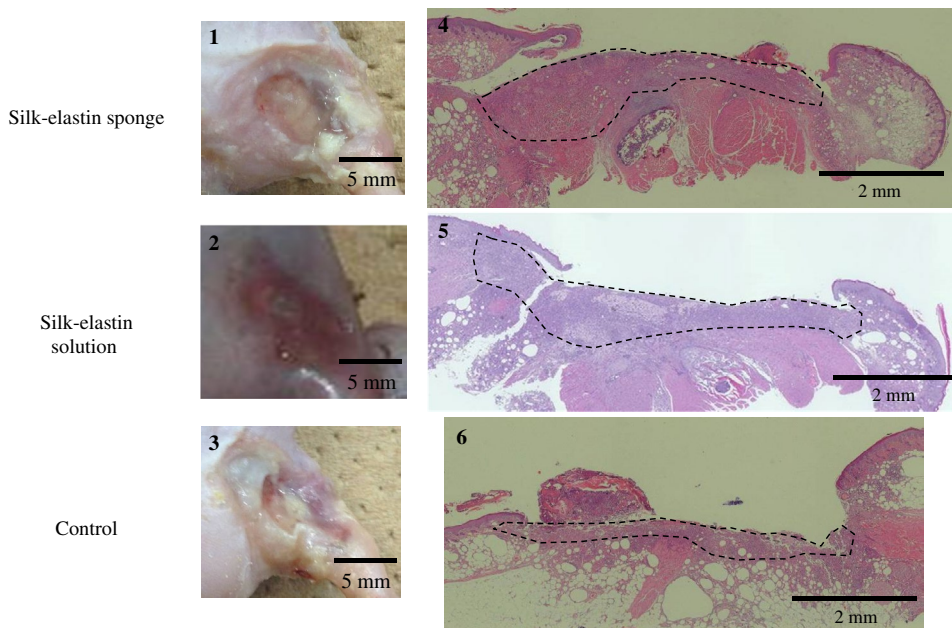


Figure 8. Macroscopic views of the wound surface and histological sections obtained 14 days after the implantation of Silk-elastin.

Notes: Macroscopic views of the wound surface in the (1) Silk-elastin sponge B (density, 25 mg/cm³; thickness, 5 mm), (2) Silk-elastin solution and (3) Control groups. Histological sections of the (4) Silk-elastin sponge C (density, 25 mg/cm³; thickness, 5 mm), (5) Silk-elastin solution and (6) Control groups (Hematoxylin and eosin staining). The area of granulation tissue is surrounded by a broken line.

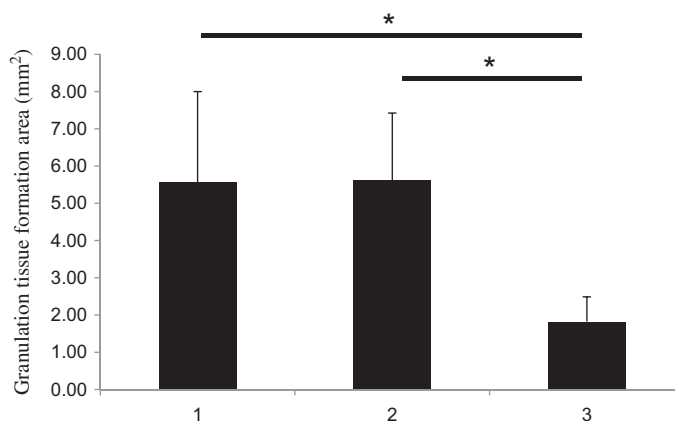


Figure 9. The evaluation of the granulation tissue in diabetic mice.

Notes: (1) Silk-elastin solution (2) Silk-elastin sponge B (density 25 mg/cm³; thickness, 5 mm), (3) Control.

[8]. Therefore, we believe that silk-elastin, which becomes a gel on the wound surface, exerts cell migration through diffusion via the disintegration or decomposition of silk-elastin.

On the other hand, we found that silk-elastin sponge with a high density of silk-elastin had no effect on the formation of granulation tissue, possibly because high-density silk-elastin was not fully dissolved in the exudate after it was applied to the wound surface; this would have resulted in defective hydrogel formation leading to an imperfect wet environment (which is important for cellular proliferation).

The silk-elastin sponge successfully kept cytokines contained in the exudate because the sponge was absorbed by the exudate to form a hydrogel. We confirmed that the hydrogel promoted cellular proliferation (data not shown), which suggested that cytokines were active in the hydrogels. Our preliminary study showed that the silk-elastin aqueous solution maintained bFGF levels similar to the results shown in Figure 7(1). The concentration of bFGF was 3.8 ± 0.6 pg/ml, which was significantly higher than in the control group. Considering that the silk-elastin sponge contains active cytokines, the silk-elastin sponge would be expected to have the same performance as the aqueous silk-elastin solution (when used to promote wound healing).

Silk-elastin sponge of 12.5 or 25 mg/cm³ in density could be easily (and perfectly) applied to wound areas, to promote stable wound healing. At present, we are considering a clinical trial, which will be performed in the near future to confirm the safety and the clinical effects of this material, which was easy-to-use and which had excellent results in the promotion of wound healing in various types of wounds.

5. Conclusion

The silk-elastin sponge accelerated wound healing similarly to the silk-elastin solution.

It seems easier to use in the clinical setting than the silk-elastin solution. We believe that silk-elastin sponge can be applied to various wounds that are difficult to treat with the aqueous solution. In the future, we will evaluate the handling properties of silk-elastin through clinical trials.

Disclosure statement

No potential conflict of interest was reported by the authors.

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