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Biocompatibility and biodegradability of spider egg sac silk

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Abstract Spider egg sac silk (SpESS) were enzymatically cleaned and their biodegradation in vivo and in vitro, as well as their biocompatibility were studied. Proteinase K treatment diminished the tenacity and the strain of the SpESS fibers in proportion to the enzyme concentration. Fibers treated with trypsin were not significantly affected. Tensile properties of Vicryl®, SpESS and of silkworm (*Bombyx mori*) silk fibers (SWS) were measured after incubation in phosphate buffered saline (PBS) at 37 °C up to 12 weeks. Biodegradation of SpESS and SWS was insignificant compared to Vicryl®. Five milligram SpESS fibers from laboratory grown spiders (*Araneus diadematus*) were treated with proteinases before sterilization and subcutaneously implanted in Wistar rats. After 1, 4 and 7 weeks the immunological reaction was compared to untreated SpESS and polyglactin (Vicryl®) control samples. SpESS samples treated with trypsin only or in combination with a Proteinase K treatment induced less inflammatory reactions than untreated silk fibers. The enzymatical cleaning could diminish the tensile properties, but enhanced the biocompatibility of the SpESS fibers rendering them appropriate for use in biomaterial application where the slow biodegradability is an advantage.

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1 Introduction

The interest for silk, and spider silk in particular, in the field of biomaterial research has grown in the past decades. Nevertheless spider silk threads have never been used in commercial products. The main reason is that spiders are hard to breed due to their cannibalistic nature [1]. Two of the up to seven kinds of silk fibers a spider can secrete are easy to obtain: dragline and egg sac silk. Grown in a laboratory, the dragline silk can be drawn from a fixed spider on a daily base [2]. Egg sacs are produced at the end of their life. In both kinds of fibers, the beta-pleated sheet containing crystalline regions embedded in a helical amorphous phase [3, 4], lead to a strong and deformable fiber, competitive with high-performance synthetic fibers like Kevlar or nylon [5]. The protein nature of the fiber is an interesting property for biomaterials. The promising mechanical characteristics are explaining the race to understand the correlation between the sequence, the protein structure and the fiber properties, to create spider silk artificially. Biotechnological methods have made it possible to produce the

spider silk proteins or spider silk-like proteins in bacteria [6], in insect cells [7], in plants like tobacco and tomato-plants [8] and in goats [9]. Spinning these proteins into high-performance fibers remains a challenge [10]. Material scientists propose applications such as bullet proof vests, parachute chords, fishing lines, etc. [5]. Silkworm silk has been used as suture material for a century and it is believed that silkworm silk and spider silk could be a good base material for textile fabrics in tissue engineering applications. Spider dragline silks are tougher and finer than their egg sac silks; the latter do not lose their toughness in aqueous environments [11]. Their mechanical properties are especially useful where high mechanical loads or tensile forces are required as in potential bio-engineered cartilage [12, 13], meniscus, ligaments [14] or bone [15]. A resistant single strand thread could also be used to guide vascular and nerve cells.

If spider silk would be used as a biomaterial, the in vivo immune response of the silk should be tested [16] and compared to a known biomaterial. In this study the *Araneus diadematus* spider egg sac silk (SpESS) was used because of the high abundance of the spider-species, the high amount of silk in one egg sac compared to other species and the mechanical properties of the fibers [17, 18]. The samples were enzymatically cleaned and tensile tests were used to clarify whether this cleaning had an influence on the mechanical properties. Cleaned and uncleaned fibers were then implanted in white Wistar rats. The local reaction was compared to polyglactin using the methods described in ISO 10993-6 [19]. As for some biomedical applications biodegradation is desired, the surface corrosion after implantation was investigated and in vitro tests were performed to compare the rates of the decrease of the mechanical properties of spider egg sac silk, silkworm silk (SWS) and polyglactin in a physiological environment. SWS and polyglactin are both widely applied in sutures, in Mersilk® and Vicryl®, respectively.

The biocompatibility of *Bombyx mori* silkworm silk (SWS) sutures has already been compared to other materials in vivo and in vitro [20–24]. Biocompatibility and allergy problems with SWS have been attributed to the coating sericin glue-like protein [25]. The SWS filament is a composite material formed by two fibroin filaments surrounded by a cementing layer of sericin. This sericin layer is removed during the degumming-step in the silk processing, by boiling in an aqueous solution containing soap or alkali, but this removal can also be done using proteases [26]. Recent research revealed the hypothesis of a coating on spider egg sac silk fibers (SpESS) [27, 28] but the production of sericin by spiders is never observed.

2 Material and methods

2.1 Preparation of spider egg sac silk samples

The silk fibers were obtained from *Araneus diadematus* spiders caught in nature during the summer and grown in the laboratory for several months by feeding flies. They each produce one egg sac in October/November just before they die. The eggs were removed manually from the egg sac using a pair of tweezers. Five milligram flocs were sampled for in vivo biocompatibility tests and 5 cm long threads were removed to test their resistance to tensile forces.

2.2 Influence of enzymatic treatment on tenacity and strain of the fibers

To test the influence of an enzymatical treatment on the mechanical properties of the SpESS-fibers, tensile strength was measured on the threads of three different egg sacs. The threads from these three egg sacs were divided in eight parts; four parts were treated with trypsin (bovine Pancreas, 12.400 U/mg, Sigma-Aldrich, Bornem, Belgium) and four with Proteinase K (*Tritrachium album*, 47 U/mg, Sigma-Aldrich). Both enzymes were used at four concentrations (1, 0.1, 0.01 and 0.001 mg/mL) in phosphate buffered saline (PBS, GIBCO Invitrogen Co, Merelbeke, Belgium) for 4 h at 55 °C. Tests were done in 25-fold (600 tests in total).

The tensile strength measurements were performed with an automatic single fiber strength tester (FAVIMAT, Textechno, Mönchengladbach, Germany). This instrument first measures the linear density (dtex), which is a textile term proportional to the average diameter (1 dtex = 0.1 tex = 0.1 g/km). The measurement of the linear density is based on the vibration method at constant fiber tension and fiber length and variable stimulating frequency. Subsequently, the fiber is elongated at a constant increasing strain (% strain) while the corresponding Force (cN) is measured, until the fiber breaks. This allows determining the tenacity (cN/tex), which is the specific force, calculated as the ratio of force to linear density. This is a parameter often used to evaluate fiber strength independent of fiber diameter. Because of the high variability of the linear density of spider silk, these relative values are required in order to allow comparisons.

All tests were done on 20 mm fiber length, at an extension speed of 20 mm/min, in a controlled climate condition of 20 ± 2 °C and a relative humidity of 65 ± 2%.

2.3 Subcutaneous implantation of SpESS samples in rats

Due to the fibrous shape of the material, it was impossible to make test strips of a defined size according to ISO-norm 10993-6 [14]. Therefore it was chosen to implant flocs of a definite weight. Twenty-seven egg sac silk flocs of 5 mg were taken (Fig. 1). Nine of them were sterilized in an autoclave (121 °C, 1 bar, and 20 min). Eighteen egg sacs were enzymatically cleaned to remove possible coating proteins or accidentally contaminating proteins. Nine of these were cleaned with trypsin (bovine Pancreas, 12.400 U/mg, Sigma-Aldrich) and subsequently with Proteinase K (*Tritrachium album*, 47 U/mg, Sigma-Aldrich) both 1 mg/mL for 4 h at 55 °C in PBS. The other nine were cleaned with 1 mg trypsin/mL PBS for 4 h. After the enzymatic cleaning the samples were extensively rinsed with distilled water and sterilized by autoclaving.

The control samples were nine flocs of polyglactin (Vicryl®, Ethicon, Sommerville, NJ, USA) with the same weight as the spider egg sac silk flocs and comparable shape, also sterilized by autoclaving.

The chosen laboratory animals were male, Wistar Hsd/Brl/Han rats (Harlan, Horst, The Netherlands) and weighed ±140 g before implantation. The rats were anaesthetized intraperitoneally with a mixture of ketamine (Ketalar®, 0.09 mg/g), Atropine (0.0001 mg/g) and Xylazine (Rompun®, 0.0075 mg/g). After shaving the back of the rat, a midline stab incision of approximately 1 cm length was made 7 cm below ear-level. A dorsal, subcutaneous pouch was made 1.5 cm paravertebrally. The sample was placed left of the incision. A 5 mg polyglactin sample was also implanted in nine rats on the heterolateral side of the incision. The skin was sutured with one stitch of polyglactin 910 (polyglactin™ 1, Ethicon) suture.

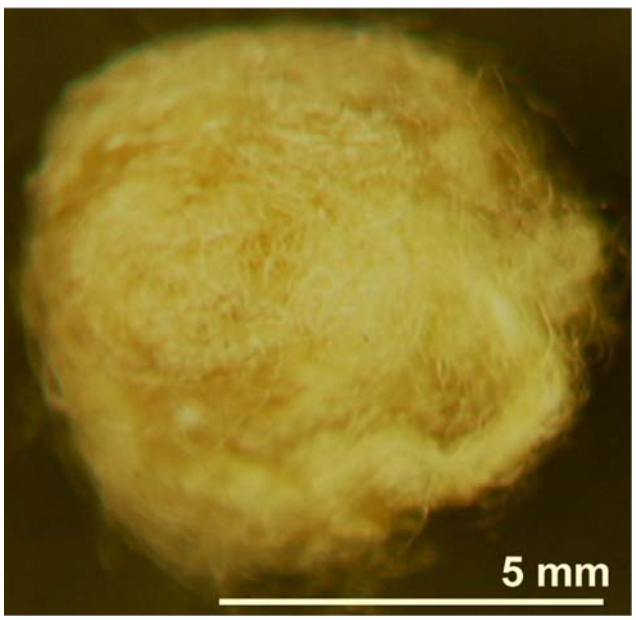

Fig. 1 Five milligram of untreated spider egg sac silk from *Araneus diadematus* for implantation

For the four implant types, after 1, 4 and 7 weeks the rats were euthanized with cis-Atracurium besylate (Nimbex®, 2 mg/mL) after sedation with natriumpentobarbital (Nembutal, 25–35 mg/kg). Preliminary tests showed us that the inflammatory reaction towards SpESS subsided after 12 weeks; therefore comparative tests were only performed up to 7 weeks. The implants and surrounding tissue were sampled in 5% phosphate buffered formaldehyde. The whole procedure was evaluated and approved by the ethical committee of the Ghent University hospital (ECP 02/28).

2.4 Macroscopic and histopathological analysis of the immunological reaction

The rats were checked weekly for local inflammation: the occurrence of heat, redness and swelling. Upon retrieving the implant, the surrounding tissue was inspected for abscedation. The samples were embedded in paraffin and cut into several 5 μm slices with a microtome. The samples were deparaffinized with xylene and rehydrated with ethanol before staining with haematoxylin and eosin, and examined under a light-microscope. According to ISO 10993-6, the different slices were semi-quantatively scored for granulocytes, giant cells and fibrosis. Per implant in each of the three experimental animals, a score was given between 0 and 3 on three implant-areas of 0.25 mm^2 for these parameters. A score of 0, 1, 2 and 3 stood respectively for 0–25, 25–75, 75–200 and >200 granulocytes/0.25 mm^2, 0, 1–5, 5–20 and >20 giant cells/0.25 mm^2 and 0, 0–10, 10–40 and >40% of area that was characterized by the predominance of fibroblasts and fibrotic tissue. Granulocyte infiltration was absent in fibrotic areas. The scoring was performed blind to the nature of the implant and to the time of implantation.

2.5 In vivo and in vitro biodegradation of the fibers

A scanning electron microscope (JEOL JSM-5600 LV SEM) was used to look at the surface of the fibers to evaluate the in vivo surface biodegradation after different implantation periods.

To test in vitro biodegradation, SpESS, SWS and Vicryl® fibers were put in phosphate buffered saline (PBS) and stored in the dark at 37 °C. After 0, 1, 2, 4, 8 and 12 weeks in PBS, the fibers were dried at room temperature for 2–3 days. The mechanical properties were measured with the Favimat single fiber tester. SWS fibers were

uncleaned as comparators as these fibers are in use for sutures in surgical practice (Mersilk®, Ethicon)

2.6 Statistics

On all data, SPSS 12.0 for windows was used to perform statistic analysis with a significance-level of 0.05. On the results of the mechanical tests of the fibers treated with different concentrations of trypsin and Proteinase K, a 2 factor ANOVA-test was performed. The egg sac was taken as the random factor and the concentration of the enzymes as the fixed factor. A one way ANOVA (post-HOC Tukey) comparison was made on the results of the tensile strength tests on the in vitro biodegraded fibers. The number of weeks the fibers were kept in the PBS-solution was taken as the fixed factor. The scores ranging from 0 to 3 given for granulocytes, giant cells and fibrosis spotted on the H/E-stained slides were evaluated for the different fiber types per implantation time. A one-way ANOVA Bonferroni test was used with the fiber type as a factor.

3 Results

3.1 Influence of enzymatic treatment on tenacity and strain of the fibers

The linear density, tenacity and strain of the untreated control samples were respectively 0.95 dtex, 2.04 cN/dtex and 32.86% (Fig. 2). Treatment with trypsin did not significantly affect the above-mentioned values ($p > 0.05$, Fig. 2). Treatment with proteinase K left the linear density unaffected (Fig. 2a), but dose-dependently affected the tenacity and strain significantly. When compared with control values, the tenacity of the fibers was reduced by 22.06, 33.33 and 71.57% with 0.001, 0.01 and 0.1 mg/mL of Proteinase K respectively (Fig. 2b). Strain values were reduced by 12.75, 66.74 and 93.88% with 0.001, 0.01 and 0.1 mg/mL of Proteinase K respectively (Fig 2c). The highest concentrations of proteinase K rendered the threads so brittle that mounting the fibers in the clamps became impossible for 1 mg/mL and only three tests could be performed for 0.1 mg/mL of the enzyme.

3.2 Macroscopic analysis of the inflammatory reaction

No obvious outward inflammatory signs could be detected on six animals 1 week after implantation of the untreated spider egg sac silk. Two rats showed a swollen tissue around the implantation site. Another of these animals showed remarkable swelling and abcedation in the area of implantation. No inflammatory reactions at all were seen

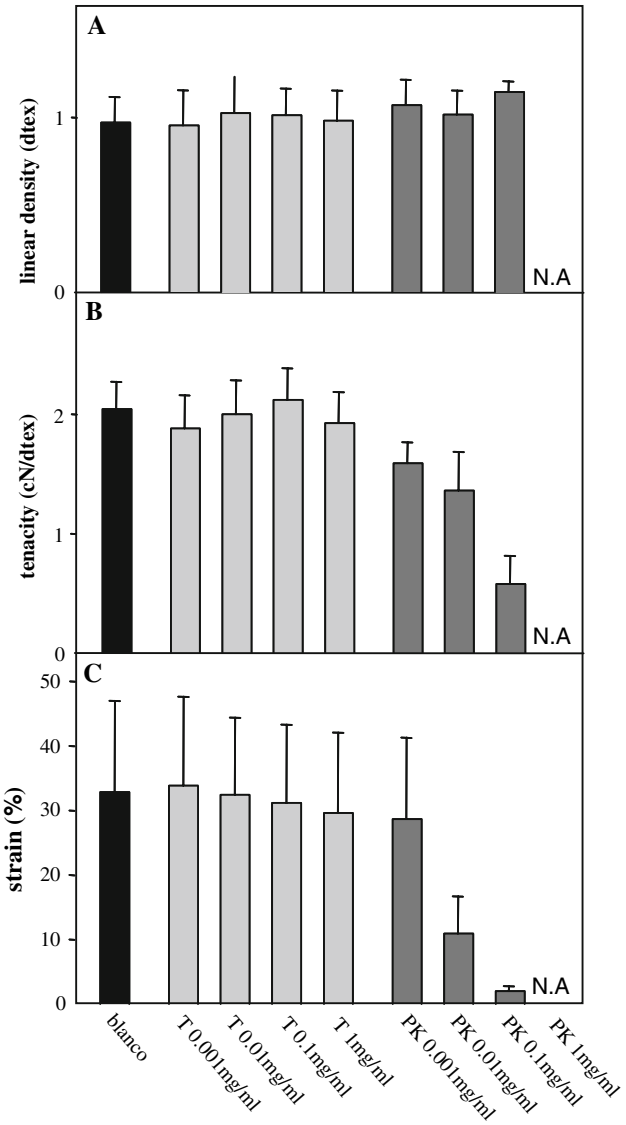

Fig. 2 Influence of trypsin (T) and proteinase K (PK) on mechanical properties of the SpESS fibers. Enzymes were used at 0.001, 0.01, 0.1 and 1 mg/mL for 4 h at 55 °C. (**a**) linear density (dtex) (**b**) tenacity (cN/dtex) and (**c**) strain (%). Mean values and 1 SD are given; *N*: number of tests was 75; NA: not applicable

around the implant areas with enzymatically treated SpESS and Vicryl® (SpESS: Fig. 3).

3.3 Histological analysis of the inflammatory reaction towards the silk fibers

All implanted flocs appeared to be surrounded by a layer of fibrotic tissue (Fig. 3b), the thickness of which gradually increased with time. Inflammatory reactions towards the silk fibers were investigated for each implant type in three animals after 1, 4, and 7 weeks of implantation. Microscopical slices were scored for granulocytes, giant cells and fibrosis (Table 1, Fig. 4).

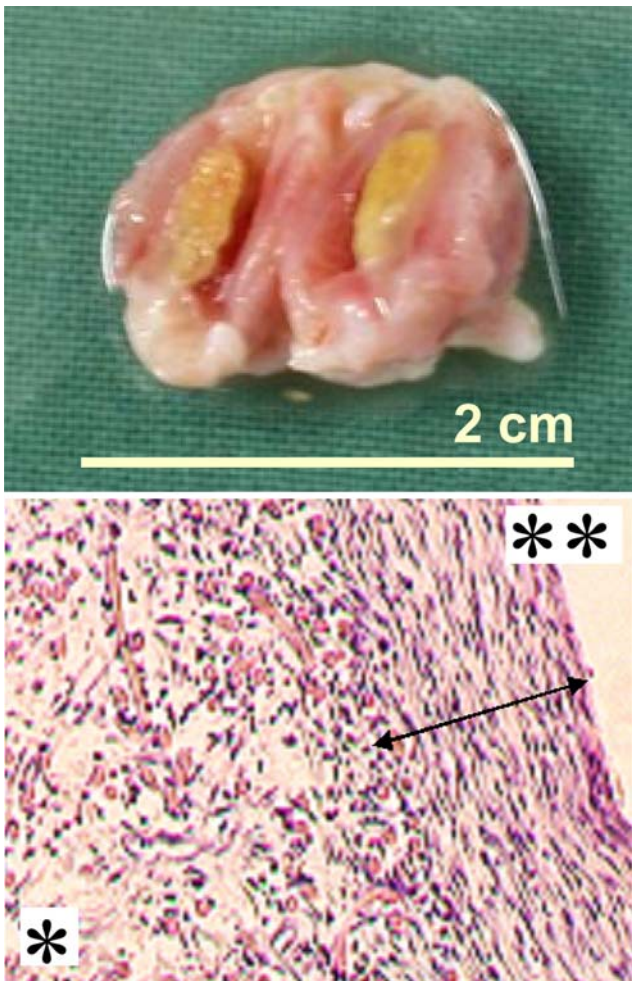

Fig. 3 (**a**) Five milligram of untreated spider egg sac silk in encapsulating tissue as retrieved after 3 months of implantation in Wistar rats. The implant was cross-sectioned and unfolded. (**b**) Histological section showing the implant (*) surrounded with a layer of fibrotic tissue (**). ↔: 475 μm

Untreated egg sac silk implants invariably provoked a severe inflammatory reaction with massive leukocyte infiltration (2.89 ± 0.33) that sustained for 7 weeks (1.89 ± 0.60) (Fig. 4a). Scarce giant cells participated in the clearance of the implanted foreign material. Fibrosis was absent during the first week but became apparent after 4–7 weeks (Table 1).

Implantation of the control Vicryl® samples caused less pronounced granulocyte infiltration (1.67 ± 0.71) during the first week after implantation. This inflammatory infiltrate disappeared after 7 weeks. One week after implantation, significantly more giant cells and reactive fibrotic tissue were present in and around the Vicryl® implants when compared with the untreated egg sac silk (Fig. 4b). This reaction towards biologically inert implanted fibers became more pronounced after 4 and 7 weeks of implantation.

Giant cell infiltration and fibrotic response in both enzyme treated SpESS samples (trypsin/Proteinase K treated: SpESStrypK: Fig. 4c; and trypsin treated SpESS: SpESStry: Fig. 4d) and in Vicryl® did not differ significantly at week 1 (giant cells in SpESStrypK, SpESStry and Vicryl®: 1.00 ± 0.50, 0.89 ± 0.33 and 1.00 ± 0.50, respectively. Fibrosis in SpESStrypK, SpESStry and Vicryl®: 0.89 ± 0.60, 0.67 ± 0.50 and 1.11 ± 0.33, respectively). At week 4 a similar picture was observed (Fig. 4b–d).

At the week 7 timepoint the scores for inflammatory response in the SpESS samples showed a tendency to decrease. The values for granulocyte infiltration however were significantly higher than in the Vicryl® control samples (SpESStrypK and SpESStry: 1.22 ± 0.44; Vicryl®: 0.33 ± 0.50; $p = 0.004$). Giant cell responses in enzymatically treated SpESS and Vicryl® samples were not statistically different. Fibrosis in and around the samples was less pronounced in the treated SpESS than in the Vicryl® tissues (SpESStrypK and SpESStry: 1.56 ± 0.73; Vicryl®: 2.44 ± 0.53: $p = 0.048$; Fig. 4b–d).

3.4 In vivo and in vitro biodegradation

No distinct differences with the unimplanted SpESS fibers could be observed on the microscopic and SEM-pictures of SpESS fibers retrieved after the different implantation-periods in vivo. The typical smooth surface with grooves parallel to the axis was present in all samples (results not shown). Microscopy showed clearly visible cracks on the Vicryl® fibers from week 1 on (Fig 4b), these Vicryl® fibers could not be retrieved and isolated for SEM as were the SpESS fibers.

The in vitro biodegradation tests showed a significant difference between the silk fibers (SWS and SpESS) and Vicryl® fibers (Fig. 5). Tenacity and strain of the Vicryl® fibers decreased significantly after week 2 and after week 4 ($p < 0.001$). After 8 weeks in PBS the Vicryl® fibers lost all tensile properties and after 12 weeks they were fully dissolved. The measurements on Vicryl® could therefore only be performed up to week 4. Tenacity and strain of the SpESS and SWS fibers did not change during 12 weeks in the PBS solution. At all time points, SpESS fibers showed higher strain and lower tenacity values than the SWS fibers.

4 Discussion

As stated in the introduction, it was expected that cleaning SpESS would improve biocompatibility. The results of this investigation show clearly that enzymatic treatment can improve the biocompatibility of SpESS. A severe acute reaction occurred when untreated SpESS samples were implanted. Although there was a high amount of

Table 1 Average ± 1SD scores for granulocytes, giant cells and fibrosis in the different implanted samples after 1, 4 and 7 weeks

Scores	Week 1 Average ± 1 SD	Week 4 Average ± 1 SD	Week 7 Average ± 1 SD
Untreated spider egg sac silk			
Granulocytes	2.89 ± 0.33	2.44 ± 0.53	1.89 ± 0.60
Giant cells	0.11 ± 0.33	0.44 ± 0.53	0.67 ± 0.50
Fibrosis	0.00 ± 0.00	0.56 ± 0.53	0.89 ± 0.60
Spider egg sac silk treated with Proteinase K 4 h and trypsin 4 h, 1 mg/mL			
Granulocytes	1.78 ± 0.67	1.33 ± 0.58	1.22 ± 0.44
Giant cells	1.00 ± 0.50	1.78 ± 0.58	1.89 ± 0.60
Fibrosis	0.89 ± 0.60	1.00 ± 0.58	1.56 ± 0.73
Spider egg sac silk treated with trypsin 4 h, 1 mg/mL			
Granulocytes	1.89 ± 0.33	1.44 ± 0.53	1.22 ± 0.44
Giant cells	0.89 ± 0.33	1.22 ± 0.44	1.44 ± 0.53
Fibrosis	0.67 ± 0.50	1.44 ± 0.53	1.56 ± 0.53
Vicryl			
Granulocytes	1.67 ± 0.71	1.22 ± 0.58	0.33 ± 0.50
Giant cells	1.00 ± 0.50	1.67 ± 0.50	1.78 ± 0.44
Fibrosis	1.11 ± 0.33	1.78 ± 0.58	2.44 ± 0.53

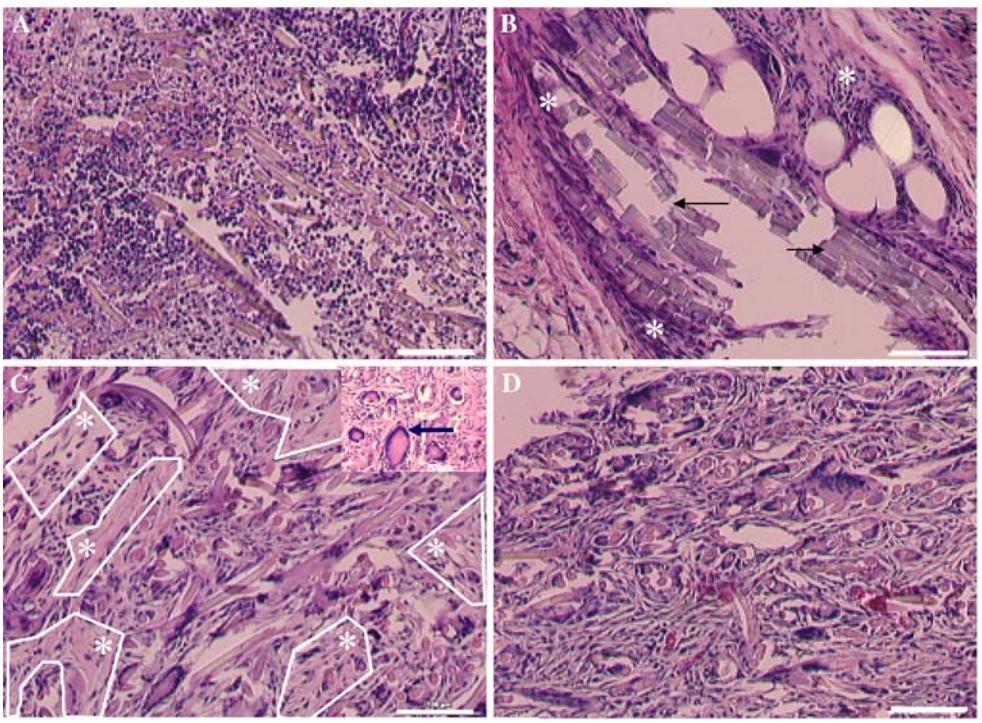

Fig. 4 Hematoxyline and eosine coloured rat tissue sections, after 4 weeks of implantation: (**a**) Untreated SpESS showing massive leukocyte infiltration. (**b**) Vicryl® implant: rare leucocyte and giant cell invasion, marked fibrosis (*). The Vicryl® fibers are broken (→), illustrating the biodegradation. (**c**) SpESS consecutively treated with trypsin and proteinase K for 4 h with 1 mg/mL; abundant giant cells (insert, →) and marked fibrotic tissue (*—marked areas). (**d**) SpESS treated with 1 mg/mL trypsin for 4 h, showing similar microscopic features as in **c**. Scale bar: 100 μm

granulocytes present in the first weeks, this inflammatory reaction subsided after 4 and 7 weeks and giant cell and fibrosis formation showed the start of healing. The evaluation of the implants shows large differences between the treated and untreated spider egg sac silk fibers, but no significant difference between the Proteinase K/trypsin and the trypsin-only treatment in the inflammatory response. When treated SpESS fibers or Vicryl® was implanted,

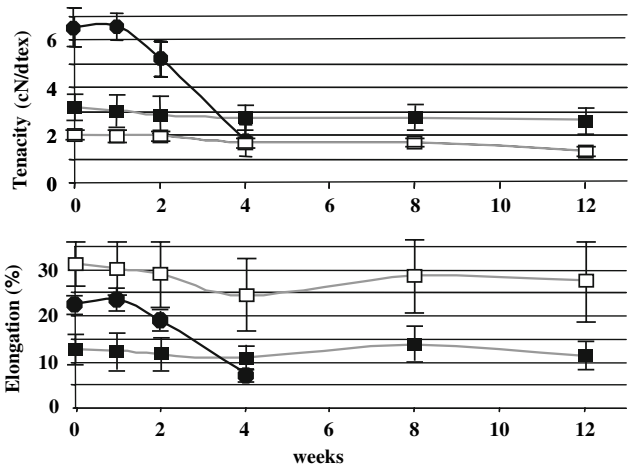

Fig. 5 Tenacity (cN/dtex) and elongation (%) of untreated SpESS (■), SWS (□) and Vicryl® (●) after 0, 1, 2, 4, 8 and 12 weeks of biodegradation in PBS in the dark at 37 °C

neutrophil and eosinophil infiltration was much less pronounced and giant cells—characteristic of a foreign body reaction—and fibrosis were observed earlier and in larger amounts in between the fibers. The enzymatic treatment resulted in fibers provoking comparable reaction in the first weeks as the widely accepted polyglactin biomaterial. After 7 weeks there was a significant difference between the Vicryl® and all the SpESS fibers, which could be explained due to higher rate of biodegradation of Vicryl®. Whether the removed coating was produced by the spiders themselves or consisted of external particles attached to the surface of the silk fibers is not clarified. In this research only two enzymes were tested, the specific enzyme trypsin and Proteinase K having a broad spectrum of action. The enzymatic treatment presented here is meant to improve the biocompatibility of the silk (measured by the inflammatory reaction) without having a detrimental effect on the mechanical properties of the fibers.

The results from the tensile tests showed that enzymes used on silk fibers can affect the mechanical properties of the fiber. The enzymes are probably cutting in the easily accessible helices, which are responsible for the elasticity of the silk fiber. The impenetrable β-sheets are more protected to enzymatic cuts; as a consequence the tenacity was only diminished by an early breakage. The tensile tests reveal that proteinase K significantly damaged the fiber in proportion to its concentration. Trypsin, which did not change the mechanical properties significantly, can be used to improve the biocompatibility of the SpESS threads in contrast to proteinase K. As the linear density of the fibers did not diminish in proportion to the enzyme concentration, the removed part could not have been a thick coating.

Next to the biocompatibility the rate of biodegradability is important for biomaterial applications. Just as silkworm silk, spider egg sac silk appears to be either undegradable or at least to biodegrade very slowly in vivo, in contrast to the spider dragline silk [11, 25, 29]. These in vivo tests did not quantify the rate of biodegradation because the remaining silk fibers could not be recovered for weighing without losing some implanted material nor without leaving some rat-tissue on it. The in vitro biodegradation tests showed a huge difference between Vicryl® and the silk fibers. The tensile properties of Vicryl® fibers decreased significantly after 2 weeks and after 2 months the fiber was fully degraded. It could be hypothesised that the biodegradation rate will be faster in vivo, due to enzymatic and mechanical effects. Both SpESS and SWS fibers retained their tensile properties up to 12 weeks in vitro and in vivo SpESS did not seem to biodegrade to the same extend as Vicryl®. The difference in biodegradation might explain the difference in inflammatory signals after 7 weeks of implantation between Vicryl® and the SpESS-fibers. As most material is already disappeared or diffused, the reaction is consequently decreased. Slow biodegradation rate can be a benefit for some applications, where slow recovery needs a long-lasting biomaterial, for example in cartilage and meniscus tissue engineering. Slow biodegradation rate will give the cells time to migrate in the scaffold and to regrow the tissue.

To acquire a sufficiently biocompatible fiber it is necessary to treat the egg sac silk from *Araneus diadematus* in an enzyme bath. Reckoning with the influence of the enzymes on the mechanical properties of the fibers, it can be concluded that trypsin treatment is permitted and is sufficient to make the silk fibers as biocompatible as polyglactin. This simple treatment makes the strong and flexible protein fiber ready to be used in many biomedical applications, where the slow biodegradability can be useful.

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