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Impact of processing parameters on the haemocompatibility of *Bombyx mori* silk films

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Abstract

Silk has traditionally been used for surgical sutures due to its lasting strength and durability; however, the use of purified silk proteins as a scaffold material for vascular tissue engineering goes beyond traditional use and requires application-orientated biocompatibility testing. For this study, a library of *Bombyx mori* silk films was generated and exposed to various solvents and treatment conditions to reflect current silk processing techniques. The films, along with clinically relevant reference materials, were exposed to human whole blood to determine silk blood compatibility. All substrates showed an initial inflammatory response comparable to polylactide-co-glycolide (PLGA), and a low to moderate haemostasis response similar to polytetrafluoroethylene (PTFE) substrates. In particular, samples that were water annealed at 25 °C for 6 h demonstrated the best blood compatibility based on haemostasis parameters (e.g. platelet decay, thrombin-antithrombin complex, platelet factor 4, granulocytes-platelet conjugates) and inflammatory parameters (e.g. C3b, C5a, CD11b, surface-associated leukocytes). Multiple factors such as treatment temperature and solvent influenced the biological response, though no single physical parameter such as β -sheet content, isoelectric point or contact angle accurately predicted blood compatibility. These findings, when combined with prior *in vivo* data on silk, support a viable future for silk-based vascular grafts.

Keywords

Silk; Blood compatibility; PLGA; *Bombyx mori*

1. Introduction

Despite the development of synthetic polymers, silk exhibits high toughness (160 MJm^{-3}), strength (1 GPa) and extensibility ($0.18 \text{ e}_{\text{max}}$) that is currently not matched by any synthetic fibre [1]. Silk is approved for human use for a number of indications including use as a

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suture material [2]; a medical application of silk that dates back many centuries [1]. Rapid advancements in cell biology and biotechnology are providing new and exciting avenues in the field of tissue engineering and regenerative medicine. Many studies have used silk-based materials for in vitro vascular tissue-engineering applications and propose its wider use (reviewed in e.g. [3–5]), though few studies have set out to translate those findings into *in vivo* studies (e.g. [6–8]). Recently, the use of silk for blood vessel engineering [4], small vascular grafts [7,9], pericardial repair [10,11] and stent coating for sustained drug release [12,13] have been reported; however, none of these studies examine the haemocompatibility of silk substrates in great detail.

A number of studies reported rapid blood coagulation when exposed to soluble silk fibroin and silk-collagen scaffolds [14,15] and reduced clotting for functionalised silk substrates for example silk blends with either heparin, ferulic acid or chemical modification of silk such as sulphation or grafting of S-carboxymethyl keratin [14–17]. Qualitative methods suggested higher serum protein adsorption and fibrin polymerisation for silk fibres when compared to methanol treated silk films [18,19]. These observations were partially geometry dependent, although process-induced structural differences have also been attributed to differential blood compatibility [20]. All reported studies to date assessed blood compatibility by determining single parameters (for example clotting time or fibrin(ogen) adsorption) and/or lacked relevant reference materials [14–21]. Biomaterial-associated thrombosis is a complex cascade that is best studied by examining a multitude of factors that closely reflect the inflammatory and haemostasis response both at the cellular and humoral level [21,22]. In this study, we set out to determine silk haemocompatibility by (1) using whole human blood, (2) quantifying cellular functions in conjunction with humoral response (3) using clinically relevant reference materials and (4) minimising factors such as a blood/air interface, blood sedimentation or endotoxin contamination that confound measurements. We generated a library of silk films that was exposed to various solvents and treatment conditions to reflect current silk processing techniques; additionally, we studied each one for blood compatibility.

2. Materials and methods

2.1. Polylactide-co-glycolide (PLGA) and silk film preparation

A *Bombyx mori* fibroin solution was prepared as described previously [23]. Briefly, cocoons were cut into ~25 mm² pieces, boiled for 30 min in an aqueous solution of 25 mM NaCO₃ and then rinsed in ddH₂O to remove sericin proteins. Extracted silk fibroin was subsequently air dried and dissolved in 9.3 M LiBr solution at 60 °C for 4 h, yielding a 20 wt% solution. This solution was dialysed against ddH₂O (molecular weight cut off 3500) for 48 h to remove the LiBr salt. The resulting aqueous silk fibroin solution was cleared by centrifugation and diluted with ddH₂O to ~6 wt%. Next, 7 ml of this solution was cast into 9-cm diameter dishes and allowed to dry under an airflow of 0.46 m/s. To generate model substrates with varying amounts of crystallinity (β -sheets), the dried films were treated with (1) 100% methanol for 6 h (designated as Silk-MeOH), (2) 70% ethanol for 6 h (designated as Silk-EtOH), (3) water vapour annealing at 25 °C for 6 h (designated as Silk-25 °C), (4) 37 °C for 12 h (designated as Silk-37 °C), and (5) 20 min steam autoclaving at 121 °C (designated as Silk-Autoclaved). Further details regarding the water vapour annealing process has been described in detail elsewhere [24]. In addition to aqueous-based casting, silk films were cast from hexafluoroisopropanol (HFIP). These samples were prepared by snap freezing the aqueous silk solution, freeze-drying the sample and subsequently re-dissolving the lyophilised silk fibroin in HFIP at ~7 wt%. The films were cast and the HFIP allowed to evaporate. Next, we annealed the films with water vapour at 25 °C for 6 h to induce crystallinity (designated as Silk-HFIP) or exposed the films to 98% formic acid (Silk-Formic acid) and allowed the solvent to evaporate. PLGA reference samples were prepared

(Mw 50,000 to 70,000 g/mol and a lactide to glycolide ratio of 85 to 15, Sigma–Aldrich, St. Louis USA) by dissolving the polymer in acetone and adding 300 μ l of the 5 wt% PLGA solution to plasma treated 25-mm glass cover slips and allowing the samples to dry. For electrokinetic measurements, silicon dioxide sample wafers were plasma treated and spin coated with a 6 wt% aqueous silk fibroin solution and allowed to air dry. To induce β -sheet formation, the wafers were treated with MeOH or water vapour at 25 °C as detailed above.

2.2. Film characterisation by contact angle measurements, Fourier transform infrared spectroscopy (FTIR) analysis and streaming potential/streaming current electrokinetic measurements

We analyzed the silk samples with a Jasco FT/IR-6200 spectrometer equipped with a horizontal MIRacle attenuated total reflectance attachment (Ge crystal, Pike Technologies, Madison USA) by acquiring 128 scans and fourier transforming them with the Genzel-Happ apodisation function to generate spectra with a 4 cm^{-1} resolution ranging from 400 to 4000 cm^{-1} . Fourier self-deconvolution was conducted to assign the secondary structure of silk from the spectra by first identifying the amide I region (1595–1705 cm^{-1}) using Opus 5.0 software (Brucker Optics, Billerica USA) and subsequently assigning the following bands: 1610 – 1625 cm^{-1} and 1696 – 1704 cm^{-1} as β -sheet structure, 1640–1650 cm^{-1} as random coil structure, 1650 – 1660 cm^{-1} as α -helical bands and 1660 – 1695 cm^{-1} as β -turns. We have previously reported the details for this procedure elsewhere [25]. The wettability of all substrates was determined by employing static contact angle measurements using degassed, ultrapurified water with a camera system coupled to a computer-assisted contour analysis program (OCA 30 Dataphysics, Filderstadt Germany). Electrokinetic measurements were performed on silk coated silicon wafers using the streaming potential/streaming current technique to determine the isoelectric point and the pH dependent electrokinetic potential of the samples in electrolyte solutions with a custom built set-up described elsewhere [26]. Briefly, a 1 mM KCl electrolyte solution was used to conduct streaming current/streaming potential pH titrations starting at an alkaline pH. These data are reported as streaming current versus pressure gradient (dIs/dp) to follow the charge formation at the silk surface.

2.3. Haemocompatibility testing of silk and PLGA films

Freshly drawn heparin anticoagulated (1.7 IU/ml) human whole blood was obtained as described previously [27]. Briefly, we ensured that donors had not taken nonsteroidal antiinflammatory drugs over the past 10 days or were on any other medication that could interfere with the blood coagulation cascade. Blood was drawn from 2 AB0-compatible healthy male/female volunteers and pooled for each study. When the study was repeated, 2 different blood donors were used and a total of 5–6 film samples were analysed. We used 2 ml of blood that was exposed for 2 h to 6.2 cm^2 sample surface using in house designed incubation chambers that were kept at 37 °C under constant revolution avoiding air contact and blood sedimentation. After the 2 h exposure time to whole blood, the amount of complement C3b (reactivity for subdomain C3c, Dako, Hamburg Germany) was determined on the surface by standard immunochemical methods. To quantify soluble thrombotic components of the blood after the 2 h exposure time, blood samples were mixed with the indicated stabilizer to determine thrombin-antithrombin (TAT) complex (Dade Behring, Marburg Germany), platelet factor 4 (PF4) (Haemochrom Diagnostica, Essen Germany) and the complement fragment C5a (DRG Instruments, Marburg Germany). The samples were centrifuged and the plasma stored at –80 °C until analysis. Blood cell counts were performed using a clinical cell counter (Beckmann Coulter AcTdiff, Krefeld Germany). Flow cytometry (BD Biosciences FACScalibur, Franklin Lakes USA) was used to determine the level of leukocyte activation and leukocyte-platelet conjugate formation by staining cells with phycoerythrin CD11b (clone ICRF44, Biozol, Eching Germany), peridinin-chlorophyll protein cyanine 5.5 conjugated CD41a and allophycocyanin conjugated CD14 (Clone

M5E2, BD Pharmingen, Franklin Lakes USA) antibodies at a 4 μ l to 20 μ l dilution of antibody to blood using a lyse-no-wash protocol. We determined granulocytes and monocytes by their characteristic forward-side scatter profile. Monocytes were specifically identified by expression of CD14. We performed flow cytometric analysis using the median intensity of the CD11b signal and CD41a positive events in the granulocyte and monocyte population, respectively. To quantitatively assess cell surface population on the films after the 2 h incubation period, samples were carefully washed with PBS to remove nonadherent cells, fixed with 2 wt% paraformaldehyde and permeabilised with 0.2% saponin and stained with 0.5 mg/ml DAPI. Samples were imaged with a 20 $\times$ objective magnification and at least 7 fields of view were acquired for image analysis as detailed elsewhere [22]. For high resolution images, the samples were fixed with 2 wt% glutaraldehyde in PBS, dehydrated with increasing concentrations of ethanol, critical point dried, gold sputtered and analysed by scanning electron microscopy (FEI-Philips, Eindhoven The Netherlands). All studies were approved by the ethics board of the Medical Faculty at the Technical University Dresden in collaboration with the Leibniz Institute for Polymer Research Dresden and TU Dresden and complied with institution and international guidelines.

2.4. Endotoxin release of silk and PLGA films

To exclude endotoxin contamination as a reason for inflammatory response, we measured endotoxin release from all samples using a chromogenic limulus amoebocyte lysate assay (Chromo-LAL, Pyroquant Diagnostik, Mörfelden-Walldorf Germany), as described previously [28].

2.5. Statistical analysis

Data were analysed using GraphPad Instat 3.06 (GraphPad Software, La Jolla USA). Sample pairs were analysed with the Student's *t*-test. Multiple samples were evaluated by one-way analysis of variance (ANOVA) followed by Bonferroni or Dunnett's post hoc tests to evaluate the statistical differences ($P < 0.05$) among all samples or between samples and controls, respectively. All error bars were standard deviation (SD).

3. Results

3.1. Physicochemical characterisation of the films

The goal was to generate a library of silk films with a graded degree of β -sheet content using water annealing and organic solvents. Deconvolution of FTIR spectra of amide I (1600–1700 cm^{-1}) and amide II (1450–1600 cm^{-1}) regions showed that increasing the processing temperature from 25 $^{\circ}\text{C}$ to 37 $^{\circ}\text{C}$ and 121 $^{\circ}\text{C}$ during water annealing increased the β -sheet content from 35% to 42%, and then to 56%, respectively (Fig. 1a and Table 1). Untreated silk films had a β -sheet content of $\sim$ 13% and showed no characteristic amide I peak at the wavenumber 1600 to 1640 cm^{-1} , which is the main absorbance region for the β -sheet crystal. Silk samples treated with ethanol, methanol and formic acid had β -sheet contents between 36%, 54% and 46%, respectively, that appeared at 1626 cm^{-1} and was accompanied by a decrease in random coil (1640–1649 cm^{-1}) and the α -helix peak (centred at 1650 cm^{-1}) (Table 1). We have previously described the mechanical properties of these substrates [24]. Next, the wettability of the films was determined using static contact angle measurements (Fig. 1b). Contact angles for Silk-HFIP and Silk-EtOH were 56 $^{\circ}$ and 64 $^{\circ}$, respectively, and were thus significantly lower than the reference material PLGA that had a contact angle of 74 $^{\circ}$ (Fig. 1b). For Silk-Formic acid, Silk-MeOH and Silk-36 $^{\circ}\text{C}$ the average contact angles were higher than for PLGA though not statistically significant. Only Silk-Autoclaved and Silk-25 $^{\circ}\text{C}$ samples, with a contact angle of 84 $^{\circ}$ and 82 $^{\circ}$, respectively, were significantly higher than for the PLGA reference material. Besides studying the wettability of the samples the charge characteristics of Silk-25 $^{\circ}\text{C}$ and Silk-MeOH (Fig. 1b) were also

determined that showed the most consistent differences in thrombogenic response. These substrates were stable under the high shear stress experienced in this dynamic set-up because repeated measurements gave consistent results and silk coated wafers retained their typical light diffraction patterns after electrokinetic analysis. For Silk-25 °C materials an isoelectric point of pH 3.5 was determined indicating an acidic net charge of the sample surface, probably resulting from an excess exposure of acidic amino acid side chains. In contrast, Silk-MeOH exhibited a significantly ($P = 0.05$) elevated isoelectric point of 4.4, reflecting a more alkaline behaviour (Fig. 1c). For the given electrolyte system, surfaces without dissociating functionalities show isoelectric point values of about pH 4.1 due to the preferential adsorption of hydroxide ions [30]. After structural characterisation of the silk films, the inflammatory and haemostasis parameters of the silk substrates was determined using human whole blood.

3.2. Inflammatory response to silk substrates

First, the amount of endotoxin contamination of the silk and reference materials was determined. All substrates released less than 1 EU/cm². This is low enough to enable the discrimination between complement driven inflammatory responses to implant materials [28]. Complement activation, typically on the alternative pathway, is a frequent response to foreign materials. We therefore examined activation levels by measuring the surface bound C3b, which initiates the alternative complement pathway and serves as a dominant ligand for leukocyte adhesion. Surface-associated C3b was highest for Silk-MeOH samples and exceeded levels seen for the reference material PLGA (Fig. 2b). Besides the high C3b levels observed for Silk-MeOH and PLGA, all other substrates had lower levels of complement activation (Fig. 2b).

The plasma concentration of the more downstream complement fragment C5a was measured. All tested materials induced C5a levels that were similar to PLGA, though Silk-MeOH induced the highest levels of all tested substrates (Fig. 2a). Leukocyte adhesion is a cellular component of the inflammatory response and the number of leukocytes at the surface were therefore counted (data not shown, qualitative data Fig. 4), in addition to leukocyte depletion from whole blood samples (Fig. 2c). Although PLGA showed substantial C3b and C5a activation when compared to polytetrafluoroethylene (PTFE), comparatively little leukocyte adhesion to the substrate was observed, and this was similar for Silk-25 °C (qualitative data Fig. 4). All other silk samples showed a high degree of leukocyte depletion from whole blood samples (Fig. 2c) that correlated with the measurements of surface-associated leukocytes. In addition to leukocyte adhesion, the extent of leukocyte activation was determined by monitoring the expression of CD11b in granulocytes and monocytes by flow cytometry after their 2 h exposure to substrates (Fig. 2d). Lipopolysaccharide (LPS) was used as a positive control that induced significant CD11b expression in leukocytes. In line with C5a expression, surface-associated leukocytes and leukocyte depletion from whole blood samples, PLGA samples showed the lowest CD11b activation that was similar to the response seen for Silk-25 °C. Substantially higher values were observed for all other silk samples, in particular for Silk-EtOH and Silk-MeOH (Fig. 2d). In addition to determining key regulators of the complement system, we also determined key haemostasis parameters.

3.3. Haemostasis response to silk substrates

Thrombin formation, measured as thrombin-antithrombin (TAT) complex, serves as a good indicator for the plasmatic coagulation activation [21]. The highest levels of TAT were measured for Silk-37 °C (295 ng/ml) and PLGA (270 ng/ml) samples, whereas all other silk samples had lower levels of activation with minimal TAT concentration observed for Silk-25 °C (137 ng/ml) (Fig. 3a). Typical TAT values determined for the negative control

PTFE and the positive control glass were 140 ng/ml and 6800 ng/ml, respectively. Thrombin is major activator of platelets by activating their protease activated PAR-1 and PAR-4 receptors [29]. Therefore, the extent of platelet activation was determined by measuring the release of PF4 from α -granules. All substrates showed low levels of PF4 release with Silk-37 °C being the only exception, where PF4 values were above the levels seen for glass substrates (520 U/ml). PF4 levels for Silk-37 °C were significantly higher than those measured for the other silk substrates (Fig. 3b). Most of the studied substrates had low PF4 levels that were similar to PTFE levels (200 U/ml). In addition to PF4, the extent of granulocyte-platelet conjugates was also determined, which is a major indicator for platelet, but not leukocyte, activation [30] (Fig. 3c). In the LPS control sample ~100% of leukocytes were present as conjugates with platelets, followed by Silk-37 °C (60%), Silk-MeOH (49%) and similar levels for the remaining samples (25%–35%). Silk-37 °C induced significant levels of granulocyte-platelet conjugates when compared to Silk-25 °C or Silk-Formic acid samples. PTFE and Silk-25 °C induced a comparable level of platelet conjugates. In addition to determining the extent of granulocyte-platelet conjugates, the depletion of platelets from whole blood samples was assessed. Similar trends were observed as for the granulocyte-platelet conjugates where most platelets were depleted from whole blood samples for the Silk-37 °C films and subsequently less for the other silk samples (Fig. 3d). Scanning electron microscopy analysis of substrates showed a consistently high number of adherent platelets for Silk-MeOH substrates in addition to abundant leukocytes that were also frequently observed on Silk-EtOH, Silk-HFIP, Silk-Formic acid but less abundant on Silk-25 °C and PLGA (Fig. 4 and Supplementary Fig. 1). For Silk-EtOH and Silk-Formic acid occasional fibrin deposits were present. Although we observed inherent donor variability for these scanning electron microscopy studies, the overall trends were consistent throughout.

4. Discussion

Silk is used in the clinic as a suture material and for soft tissue repair [2]. However, the use of purified silk proteins as a scaffold material for vascular tissue engineering goes beyond its traditional use because it exposes large amounts of purified silk protein to the blood stream. This new biointerface for silk requires application-orientated biocompatibility studies because only load bearing applications are currently applications for silk-based materials in the clinic (i.e. no blood contact).

Currently there are a number of strategies to generate vascular grafts using biomaterials [31]. The first one relies on the use of synthetic polymeric materials such as expanded PTFE (GoreTex) and polyesters (Dacron) (and in the past nylon, 40 polypropylene, polyacrylonitrile and silicon rubber [32]) that serve as vascular prostheses. PTFE grafts have been used in millions of patients and still dominate clinical practice. Vascular prostheses should minimise intimal hyperplasia to retain vascular patency paired with low thrombogenicity by avoiding blood platelet adhesion [32]. The second strategy employs biomaterials such as silk [7,9], decellularized vascular grafts [33,34] and chimeric materials (for example PTFE coated with collagen and heparin [35], or polyester coated with silk [8] (reviewed in [31]). These substrates can be seeded *in vitro* with endothelial cells and/or support the adhesion and growth of endothelial cells *in vivo* to yield haemocompatible surfaces. In addition to lumen endothelialisation, minimising intimal hyperplasia is a major challenge for small diameter vessels (<6 mm) [31,36].

Despite the common use of silk in biomedical engineering applications there are limited data on the blood compatibility of silk. We therefore set out to examine the haemocompatibility of processed silk films using human whole blood. The use of whole blood is the best *in vitro* set-up available to date to evaluate overall blood compatibility because studies focussing

only on either platelet activation or coagulation activation may miss activating cross-talk between these pathways [22]. For the purpose of this study, a number of blood compatibility parameters were measured that can be broadly classified as (1) haemostasis parameters (e.g. platelet decay, TAT, PF4, granulocytes-platelet conjugates) and (2) inflammatory parameters (e.g. C3b, C5a, CD11b, surface-associated leukocytes) [21,37]. A number of reference materials were also included, such as glass (strong activator of hemostasis/inflammation), PTFE (the polymer of the commonly used GoreTex grafts) and PLGA (used as coating material for drug eluting stents in humans) to allow the direct comparison to silk. The inflammatory response of human whole blood to silk substrates was comparable to PLGA across a number of inflammatory markers such as the complement activation with C3b and C5a formation and leukocyte activation observed as CD11b expression (Fig. 2). The only exception to this trend was Silk-MeOH which demonstrated the highest inflammatory levels, in particular for complement factors C5a and C3b. High silk crystallinity leads to reduced molecular mobility of the $(A)_n(AG)_n$ repeats and has previously shown to result in a higher contact angle [20] and a material with a greater stiffness [24]. Electrokinetic measurements for Silk-MeOH and Silk-25 °C also suggested that the higher crystal content and reduced chain mobility for Silk-MeOH samples leads to a differing exposure of dissociable groups on amino acid side chains (Fig. 1). The more flexible Silk-25 °C had a significantly lower isoelectric point indicating a more acidic surface charge than Silk-MeOH, which showed an excess of basic amino acid side chains. However, samples with a comparable secondary structure to Silk-MeOH (e.g. Silk-Autoclaved) showed substantially lower inflammatory response indicating that the chosen annealing process impacts blood compatibility per se. Across all inflammatory parameters, Silk-25 °C and Silk-Autoclaved showed the most favourable profile suggesting that optimised aqueous processing is best suited to minimise inflammation.

Thrombin formation, measured as thrombin-antithrombin (TAT) complex, was used as an indication for plasmatic coagulation activation [21]. Overall, all substrates induced a low to moderate plasmatic coagulation with very low levels for Silk-25 °C, which were identical to PTFE (Fig. 3a). Thrombin is a strong platelet activator; therefore, PF4 levels and granulocyte-platelet conjugates were also quantified. With the exception of Silk-37 °C, all substrates had a lower activation than the positive control glass (520 U/ml) and similar levels to PTFE (200 U/ml) (Fig. 3b). The low platelet activation for Silk-25 °C was also reflected by granulocyte-platelet conjugates that were comparable to PTFE. Based on platelet activation and thrombin formation, Silk-25 °C samples appear to be an excellent silk-based substrate to minimise platelet adhesion and activation.

Overall, only a limited number of studies have used silk-based vascular grafts. Recent studies observed an excellent clinical response in dogs and rats that surpassed current therapies that used synthetic polymers such as PTFE and collagen coated polyester grafts [7,8]. In particular, silk grafts (ethanol cross linked) promoted the ingrowth of endothelial cells and the formation of a medial layer and vasa vasorum, whereas such structures were absent for PTFE grafts that frequently showed a thrombogenic response [7]. It appears that silk, in particular, promotes the ingrowth of endothelial cells that result in robust grafts with a low haemostasis and inflammatory response [7,8]. However, a study from the 1980s using Chinese pure silk with excellent mechanical properties reported a significant thrombogenic response in sheep [6]. The use of such silk materials is known to induce an inflammatory response in humans [38] due to the presence of the antigenic protein sericin in the fibre that was quantitatively removed using our sample processing [39]. We have confirmed the possibility that sericin can impact haemocompatibility by conducting preliminary studies to show that silk films containing sericin increased the inflammatory and the haemostasis response (data not shown).

5. Conclusion

A library of silk substrates was prepared that were processed to reflect current treatment regimes for the induction of β -sheets. Silk processing had a significant impact on haemostasis and inflammatory response *in vitro*. Multiple factors such as treatment temperature and solvent influenced the biological responses. However, single parameters such as β -sheet content, isoelectric point or contact angle were poor predictors for blood compatibility. Samples that were water annealed at 25 °C for 6 h showed the best blood compatibility, based on haemostasis and inflammatory markers, similar to PTFE.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Impact of processing parameters on the physicochemical characterisation of silk films. Silk films were either water annealed at 25 °C (Silk-25 °C), 37 °C (Silk-37 °C) 121 °C (Silk-Autoclaved) or treated with hexafluoroisopropanol (Silk-HFIP), formic acid (Silk-Formic acid), ethanol (Silk-EtOH) or methanol (Silk-MeOH) to induce β -sheets. (A) FTIR spectra of treated silk films, (a) Untreated silk, (b) Silk-Autoclaved, (c) Silk-37 °C, (d) Silk-25 °C, (e) Silk-MeOH, (f) Silk-EtOH, (g) Silk-Formic acid, (h) Silk-HFIP, line (A) indicates turn, (R) random coil and (B) β -sheet. (Typical data sets shown). (B) Static contact angle measurements of silk films and polyglycolide-*co*-lactide (PLGA). Vertical lines of the Box plots depict the median and error bars represent the SD. (Statistical differences between PLGA and silk films were determined using one-way ANOVA and Dunnett's multiple comparison *post hoc* test * $P < 0.05$; *** $P < 0.001$; $\pm$ SD; $n = 6$). (C) Streaming current versus pressure gradient of the silk films as a function of the solution pH (1 mM KCl). The intersection of the vertical line with the curves depicts the isoelectric point of the respective silk film ($\pm$ SD $n = 3$).

Fig. 2.

Inflammatory response of whole human blood to silk substrates. All samples were incubated with whole blood for 2 h and the inflammatory response was measured. (A) Complement fragment C5a in plasma. (B) Surface-associated complement fragment C3b. (C) Percentage leukocyte reduction from whole blood samples when compared to initial values. (D) Activation of granulocytes and monocytes by monitoring the expression level of CD11b. Dotted lines indicated the typical response to PTFE, dashed lines the response to glass. (Statistical differences between PLGA and silk films were determined using one-way ANOVA and Dunnett's multiple comparison post hoc test, $*P < 0.05$; $***P < 0.001$; $\pm$ SD; $n = 3$). Figure legends are detailed in Fig. 1.

Fig. 3.

Haemostasis response of whole human blood to silk substrates. All samples were incubated with whole blood for 2 h and the haemostasis response was measured. (A) Thrombin-antithrombin (TAT) complex formation was determined in the plasma and served as a marker for thrombin activation/plasmatic coagulation. (B) Plasma levels of platelet factor 4 (PF4) served as an indicator for platelet activation. (C) The amount of granulocytes in conjugation with platelets serves as functional indicator for platelet activation. (D) Percentage platelet reduction from whole blood samples when compared to initial values. Dotted lines indicated the typical response to PTFE, dashed lines the response to glass. (Statistical differences between PLGA and silk films were determined using one-way ANOVA and Dunnett's multiple comparison post hoc test, * $P < 0.05$; *** $P < 0.001$; $\pm$ SD; $n = 3$). Figure legends are detailed in Fig. 1.

Fig. 4. Qualitative assessment of silk substrates exposed to whole human blood. Scanning electron micrographs and fluorescent images (inset) of substrates after a 2 h incubation with blood. Fluorescent images depict DAPI stained nuclei of adherent leukocytes (scale bar 65 μ m). In PLGA and Silk-MeOH scanning electron microscopy images examples of leukocytes (L) and platelets (P) are marked. Silk-Formic acid and Silk-EtOH samples shows local fibrin deposits.

Table 1

Secondary structure of processed silk. Samples were analysed by FTIR analysis and deconvoluted as detailed in the materials and methods section.

Secondary structure (%) ^a	Silk-HFIP	Silk-Formic acid	Silk-EtOH	Silk-MeOH	Silk-25 °C	Silk-37 °C	Silk-autoclaved
β-sheets	24.1	45.7	36.2	53.8	35.1	42.2	56.3
Random coils and α-helix	45.7	16.8	26.8	19.2	24.1	19.5	16.2
Side chains	4.4	6.8	3.3	4.9	2.9	2.2	2.9
β-turns	25.8	30.7	33.7	22.1	37.9	36.1	24.6

^aData are representative values (±2%).