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Surface Modification of Spider Silk Particles to Direct Biomolecular Corona Formation

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KEYWORDS: recombinant spider silk; spider silk hybrid proteins; proteomics analysis; fibrinogen; drug delivery

ABSTRACT

In recent years, spider silk–based materials have attracted attention due to their biocompatibility, processability, and biodegradability. For their potential use in biomaterial applications, i.e. as drug delivery systems and implant coatings for tissue regeneration, it is vital to understand the interactions between the silk biomaterial surface and the biological environment. Like most polymeric carrier systems, spider silk material surfaces can adsorb proteins when in contact with blood, resulting in formation of a biomolecular corona. Here, we assessed the effect of surface net charge of materials made of recombinant spider silk on the biomolecular corona composition. In-depth proteomic analysis of the biomolecular corona revealed that positively charged spider silk materials surfaces interacted predominantly with fibrinogen–based proteins. This fibrinogen enrichment correlated with blood clotting observed for both positively charged spider silk films and particles. In contrast, negative surface charges prevented blood clotting. Genetic engineering allows the fine-tuning of surface properties of the spider silk particles providing a whole set of recombinant spider silk proteins with different charges or peptide-tags to be used for, for example, drug delivery or cell docking, and several of these were analyzed concerning the composition of their biomolecular corona. Taken together this study demonstrates how the surface net-charge of recombinant spider silk surfaces affects the composition of the biomolecular corona, which in turn affects macroscopic effects such as fibrin formation and blood clotting.
INTRODUCTION

Biomaterials exposed to complex biological fluids, such as blood, interact with a variety of different proteins, sugars, and lipids, and thus a layer forms on their surfaces, known as the biomolecular corona.\textsuperscript{1-6} The biomolecular corona is a two-component system consisting of an inner layer, often referred to as the “hard” biomolecular corona, and an outer layer of associated proteins, the so-called “soft” biomolecular corona.\textsuperscript{7-11} The presence of the biomolecular corona changes the synthetic identity of biomaterials, including their surface chemistry and topography, to a biological identity.\textsuperscript{12-13} Biological identity means the composition, \textit{i.e.} the type and amount of adsorbed proteins.\textsuperscript{13} This biological identity, in turn, influences the physiological response \textit{in vivo}, such as cytotoxicity and cellular association.\textsuperscript{1,9-11,13-16} Extensive efforts have been made to understand the correlation between the material design, and the biomolecular corona composition, however, there is still limited knowledge about how the presence of adsorbed proteins affects interactions between biomaterials and their environment.

The surface net charge of a material has a profound impact on the composition of the biomolecular corona. It is well known, that the abundance of proteins is higher on charged surfaces, compared to charge neutral materials.\textsuperscript{12-14} Furthermore, proteins with a isoelectric point (pI) > 5.5 preferably adsorb on negatively charged surfaces, while proteins with a pI < 5.5 favor positively charged surfaces.\textsuperscript{15} Extensive efforts have been directed towards evaluating the impact of such material design parameter on the protein fingerprint.\textsuperscript{16-20} Identifying proteins that are significant in mediating physiological responses may assist in understanding the correlation between material design and its physiological performance.
Biopolymers play an increasingly important role as biomaterials for medical applications, e.g. in tissue engineering and drug delivery. Materials made from spider silk proteins are promising candidates for a variety of biomedical applications since they are largely biocompatible, non-toxic and show reduced / no immunoreactivity.\textsuperscript{21} Importantly, there is no need for removal of spider silk based biomaterials after implantation, as once the biomaterial is in contact with the body, the spider silk material decomposes over time (from weeks to months) \textit{in vivo} into non-toxic degradation products (i.e. amino acids).\textsuperscript{22-24}

The recombinant production of spider silk proteins allows precise genetic engineering. Depending on the area of application, diverse morphologies, such as fibers\textsuperscript{25}, non-woven meshes\textsuperscript{26}, hydrogels\textsuperscript{27}, films\textsuperscript{28} and particles\textsuperscript{29} can be produced. Particles produced from recombinant spider silk are especially interesting for use in therapeutic and diagnostic applications. They have been previously shown to be suitable drug delivery vehicles for low molecular weight model drug substances\textsuperscript{30} as well as protein delivery,\textsuperscript{31} for coating of magnetic particles intended for the use in diagnostic imaging,\textsuperscript{32} or as a carrier system for a new vaccination strategy if antigenic peptides are incorporated by genetic engineering\textsuperscript{33}. Silk films can be used in tissue engineering, wound healing applications or as coatings of implants. While cell attachment on eADF4(C16) spider silk films is weak, it can be significantly enhanced by implementation of, e.g. an integrin-recognition RGD sequence as a tag onto the eADF4(C16) protein.\textsuperscript{34} Films prepared from polycationic spider silk eADF4(κ16) have successfully been employed for specific interaction with cardiomyocytes.\textsuperscript{35} Moreover, spider silk coatings of catheters revealed their potential as biomedical coatings to shield unfavorable attachment of cells, and strikingly, spider silk coatings of silicone implants have been shown to improve their biocompatibility \textit{in vivo} by reducing the risk of unwanted side effects such as fibrosis.\textsuperscript{36}
In spite of the broad field of applications of spider silk–derived materials and their successful evaluation for in vivo applications, little is known about interactions of spider silk materials with plasma proteins. In this work, we investigated the impact of spider silk material surface charges on the biomolecular corona formed upon exposure to human whole blood using a genetically engineered spider silk platform. Combining in-depth proteomics analyses with imaging microscopy, we demonstrated that positively charged particles and surfaces attracted a broad variety of fibrinogen–based proteins. Understanding the consequences of material properties, such as surface charge on the composition of the biomolecular corona and hence the physiological responses is crucial for the improvement of the biocompatibility of biomaterials.

RESULTS AND DISCUSSION

Spider Silk Variants with Different Net Charges

Materials made of different eADF4 variants were used (Scheme 1) to unveil the influence of a materials’ surface net charge and the contribution of (functional) peptide-tags on the interaction with blood proteins. eADF4(C16) is derived from the highly repetitive Araneus diadematus fibroin 4 core sequence and contains one negatively charged amino acid (glutamic acid) per amino acid sequence block, the so-called C–module. The 16 repeat units of module C in eADF4(C16) result in a negatively charged polyanionic protein, hence C16–based materials exhibit a negative surface net charge.\textsuperscript{37} Besides unmodified eADF4(C16), four variants thereof were synthesized to investigate the effect of different tags and charge distributions (Scheme 1). eADF4(C16) was carboxyl-terminally modified eADF4(C16) fused with a polyanionic octa-glutamic-acid tag E\textsubscript{8}G (eADF4(C16)E\textsubscript{8}G). Furthermore, the RGD motif (eADF4(C16)RGD)\textsuperscript{34} or a polycationic octa-
arginine tag R₈G (eADF4(C16)R₈G) ³⁸, and the polycationic variant eADF4(κ16) ³⁹ comprising the positively charged amino acid residue lysine instead of glutamic acid in each of the 16 κ modules, were studied (Scheme 1), as well as a blend of negatively charged eADF4(C16) and positively charged eADF4(κ16). The major focus of this study was on the influence of spider silk particle surface charge or surface-presented peptide tags on binding of proteins from human blood. An overview over selected properties of particles from the assessed spider silk variants is given in Table 1.

**Scheme 1:** Schematic illustration of the different spider silk variants used in this study. Polyanionic eADF4(C16) ³⁷ consists of 16 repeats of a repetitive amino acid motif (module C) derived from the repetitive core domain of the natural silk protein ADF4. Genetic engineering, i.e., exchanging the glutamic acid (E) in module C to lysine (K) gives rise to the cationic κ module and multimerization yields the polycationic
eADF4(κ16)³⁹. Optionally, further tags have been added to eADF4(C16) by means of genetic engineering to generate eADF4(C16)RGD ³⁴, eDF4(C16)R₈G ³⁸ or eADF4(C16)E₈G (this work).

Zeta-potential data of the spider silk particles confirmed the charges of particles made of the individual spider silk variants (Table 1). The surface net charge of eADF4(C16) particles yielded a zeta potential of -27 ± 1 mV. The negatively charged E₈G-tag further decreased the zeta potential to -32 ± 2 mV. Fusion of a positively charged octa-arginine resulted in a zeta potential of -21 ± 1 mV. The RGD–tag contributed one negatively as well as one positively charged amino acid residue resulting in a similar surface charge than the unmodified eADF4(C16). In contrast, the positive charges arising from the 16 κ modules resulted in a zeta potential of +15 ± 1 mV. The blend of eADF4(C16) and eADF4(κ16) in particles revealed a near-to-neutral surface charge. The heterogeneity of the examined silk types with various surface charges and differing surface-exposed peptide sequences allowed a detailed study on how spider silk particle surface characteristics relate to the formation and composition of a biomolecular corona.

Table 1. Charge distributions, molecular weight (MW), and zeta potential (ζ) data of the individual spider silk variants. Charges including those originating from N– and C– termini, and those originating from amino acid residues being charged at physiological conditions (pH 7.4). Note, that all spider silk proteins comprise an amino-terminal T7 tag, that contributes one positive charge. Theoretical pl and MW of the individual spider silk proteins were calculated using ProtParam. Zeta potential data of silk particles were taken from Schierling et al.³⁸ measured in 1/11 PBS except for variant eADF4(C16)E₈G. Data for eDF4(C16/κ16) blend particles are taken from Elsner et al.⁴⁰ in 10 mM KCl, pH 5.5.

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<th>Number of positively and negatively charged amino acid residues</th>
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<th>Theoretical pI</th>
<th>ζ [mV]</th>
<th>MW [Da]</th>
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<td>-15</td>
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n/a, Not applicable
The silk materials were used to analyze how different surface net charges affect the physiological response of the spider silk materials upon contact with human whole blood. (Figure 1). SEM imaging and proteomic analyses were performed allowing correlation of the composition of the biomolecular coronae to blood clotting effects.

![Proteomic analyses revealed that different proteins were attracted upon incubation in human whole blood based on the surface net charge of spider silk particles. Negatively charged particles predominantly attracted complement and immunoglobulins, whereby positively charged particles adsorbed mainly fibrinogen–based proteins.](image)

**Figure 1.** Proteomic analyses revealed that different proteins were attracted upon incubation in human whole blood based on the surface net charge of spider silk particles. Negatively charged particles predominantly attracted complement and immunoglobulins, whereby positively charged particles adsorbed mainly fibrinogen–based proteins.

**Surface Charge Correlated with Blood Clotting.** To begin with, interactions of negatively (eADF4(C16)) and positively (eADF4(κ16)) charged spider silk materials were investigated upon contact with freshly drawn human blood (Figure 2). Therefore, 2D spider silk films and microparticles were prepared and exposed to human blood. Scanning electron microscopy (SEM) and digital images of both planar films and particles manufactured from the negatively charged eADF4(C16) showed macroscopic blood compatibility, i.e. no signs of blood clotting or fibrin formation (Figure 2a–c). Some erythrocytes however, changed their shape from completely round
to cells with a frayed outline which occurred as a result of sample preparation.\textsuperscript{41} In contrast, the presence of the positively charged eADF4(κ16) films and particles in blood resulted in fibrin formation and blood clotting (Figure 2d–f). One basis for blood-clotting is thrombin-based fibrinogen cleavage yielding insoluble fibrin fibers, forming a network leading to blood coagulation.\textsuperscript{42-44}

\textbf{Figure 2.} Scanning Electron microscopy (SEM= (a, b, d, e) and digital (c, f) images of spider silk films (a, d) and particles (b, e) prepared from negatively (eADF4(C16), a–c) and positively (eADF4(κ16), d–f) charged spider silk materials following incubation in whole human blood. The scale bars are 10 µm.

\textbf{Fibrinogen–based Proteins Predominantly Adsorb on Positively Charged Spider Silk Particles.} For in-depth proteomic and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analyses of the biomolecular coronae formed on the surface of spider silk particles, the study was extended to further spider silk variants with modified surface net charge of with
functional peptide tags (e.g. RGD-tag) (Table 1). SDS-PAGE of proteins eluted off the spider silk particles showed slight differences concerning the identified proteins in the coronae amongst negatively (eADF4(C16), eADF4(C16)E₈G, eADF4(C16)RGD, eADF4(C16)R₈G) and close-to-neutral charged (eADF4(C16/κ16) blend) particles, but more severe differences in comparison to positively charged (eADF4(κ16)) particles (Figure 3a). Low molecular weight proteins adsorbed as predominant species on negatively and close-to-neutral charged spider silk particles, whereas the positively charged particles additionally adsorbed high and medium molecular weight proteins. Mass spectrometry was performed based on a recently published method to identify these proteins. Three replicates of each spider silk particle type were examined following incubation in human whole blood. The protein composition of whole human blood alone was determined as well, to analyze enrichment of specific proteins in the respective biomolecular corona in comparison to the incubation media. A normalized spectral abundance factor (NSAF), describing the relative abundance of each protein in a complex protein solution, was determined next. For mass spectrometry sample preparation, red blood cell (RBC) lysis was performed following incubation in whole blood, leading to the detection of additional intracellular proteins. As intracellular proteins are not present in the plasma and therefore can cause wrong interpretations of the proteomics data, all intracellular proteins were not used within the “raw data” for further analyses. This data set is referred to as the “processed protein abundance” data set. The relative abundance of proteins detected within the biomolecular corona of the different spider silk particles after incubation in human whole blood was compared using Pearson correlation (Figure 3b). Pearson correlation revealed that the biomolecular coronae of the negatively and close-to-neutral charged particles differed from those of the positively surface charged particles. Furthermore, the negatively and neutrally charged particles both exhibited coronae that were similar to the
composition of human whole blood, whereas the composition of eADF4(κ16) differed significantly therefrom.

**Figure 3.** Cropped Coomassie blue-stained SDS-PAGE gel (a) of proteins identified in the biomolecular coronae of spider silk particles eADF4(C16), eADF4(C16)E₈G, eADF4(C16)RGD, eADF4(C16)R₈G, eADF4(C16/κ16) blend, eADF4(κ16) following incubation in human whole blood. A reference band associated with particular molecular weights (left) and a sample of the incubation media alone is shown for comparison. Pearson correlation analysis (b) of the relative abundance (NSAF) of identified secreted proteins in the biomolecular coronae of spider silk particles. Data are presented as averages across all replicates. Lighter colours correlate with more similar biomolecular coronae.

The abundance of individual corona proteins was investigated. Therefore, a raw expression analysis (Figure 4a) and an enrichment analysis (Figure 4b) were performed. The raw expression analysis lists proteins based on the relative abundance identified in each biomolecular corona, whereby the enrichment analysis identified proteins that were over-represented in a particular coronae in comparison to the incubation media.
Figure 4. Raw expression analysis (a) of proteins identified in the coronae of negatively (eADF4(C16), eADF4(C16)E₈G, eADF4(C16)RGD, eADF4(C16)R₈G), close-to-neutral (eADF4(C16/x16) blend) and positively (eADF4(x16)) charged spider silk particles after incubation in human blood. The top 50 proteins are displayed in accordance with their maximum enrichment across all examined samples. The order is from top to bottom. Enrichment analysis (b) of proteins identified in the coronae of the analysed spider silk particles following incubation in human blood. Proteins not found in blood were excluded. Lighter colours indicate a high abundance on a particle surface when compared to the incubation media.

Serum albumin was identified as the main species in the raw expression analysis for all spider silk particles due to its high abundance in human plasma. For the negatively surface charged spider silk particles, as well as for the close-to-neutral charged particles, lactotransferrin, protein S100-A8, protein S100-A9, Ig kappa chain C region, complement C1q, and complement C3 were...
adsorbed with a high preference. Similar adsorption trends were obtained from the enrichment analysis: Lactotransferrin and complement C3 were identified as main proteins in the coronae of negatively and close-to-neutral charged particles. Transferrins, complement, and immunoglobulins are generally implicated in complement and inflammation responses. The adsorption to particles has been shown to promote phagocytic uptake. In contrast, both analyses identified fibrinogen–based proteins (fibrinogen beta chain, fibrinogen gamma chain, and fibrinogen alpha chain) as most abundant proteins in the coronae of the positively charged eADF4(κ16) particles. The average molecular weight of human fibrinogen is ~ 340 kDa. The observed bands in the SDS-PAGE gel (Figure 3) may be indicative for the high abundance of the different fibrinogen chains (α, β and γ), which were subsequently confirmed from the quantitative proteomics analysis. Fibrin promotes blood clotting by forming bridges between blood platelets through binding to surface membrane fibrinogen receptors. Furthermore, in the presence of thrombin and blood clotting factors, fibrinogen forms fibrin strands which develop into an extensive interconnected fibrin network. These networks are the basis for the formation of mature fibrin clots as seen in Figure 2. Similar adsorption trends were found by Sakulkhu et al. investigating the in vivo biomolecular corona of differently charged superparamagnetic nanoparticles.

CONCLUSION

We have presented an investigation on how the surface net charge of spider silk materials affect the composition of the biomolecular corona upon contact with human whole blood. Our data allowed the direct correlation of proteomics data to clotting. Fibrinogen–based proteins were detected to be the dominant species of adsorbed proteins on positively charged eADF4(κ16) spider
silk particles. In contrast, negatively charged spider silk particles consisting of either eADF4(C16), eADF4(C16)E₈G, eADF4(C16)RGD, or eADF4(C16)R₈G, and the close-to-neutral charged (eADF4(C16/ĸ16) blend particles adsorbed predominantly lactotransferrin and complement C3. Surface charge plays the most prominent role in biomolecular corona formation, while peptide tags exposed on a particle’s surface had only a minor influence on corona formation. We hypothesize that the high abundance of fibrinogen–based proteins in the biomolecular coronae of eADF4(ĸ16) particles triggered blood clotting and fibrin formation on particles as well as on planar silk–coated surfaces. Our work assists in understanding influencing variables of polymeric particles such as surface charge and peptide tags on the composition of the biomolecular corona upon contact with blood. It may allow to specifically tailor polymer- and especially spider silk-based materials to individual biomedical applications using genetic engineering.

EXPERIMENTAL SECTION

Materials. All chemicals were of analytic grade. They were used without further purification. High-purity water (Milli-Q water) was obtained from an inline Millipore RiOs/Origin water purification system (Millipore Corporation, Massachusetts, USA) or Milli-Q-system (Billerica, MA, USA) with a resistivity of >18.2 MΩ cm. Biomolecular corona characterization was done using Dulbecco's phosphate-buffered saline (DPBS), ammonium bicarbonate (NH₄HCO₃, 99.0%), acetonitrile (ACN) (99.5%), DL-Dithiothreitol (DTT, 99.0%), iodoacetamide (IAA), trypsin from porcine pancreas (proteomics grade), trifluoroacetic acid (TFA, 99.0%), and formic acid (FA, 95.0%) all purchased from Aldrich (Missouri, USA). BioRad 4-20% Mini-PROTEAN TGX Stain-Free protein Bis-Tris gels and 10 × Tris/Glycine/sodium dodecyl sulfate running buffer were purchased from BioRad (California, USA). LDS sample buffer (4×), NuPAGE sample reducing agent (10×), SeeBlue pre-stained protein standard, and SimplyBlue safe stain were purchased from
Thermo Fisher (Scoresby VIC 3179, Australia). Lysis of red blood cells (RBCs) was carried out using Pharm Lyse buffer from BD Bioscience (Victoria, Australia). eADF4(C16) was purchased from AMSilk GmbH (Planegg/München, Germany). Formic acid (99.0%), methanol (p.a.), ethanol (p.a.), formaldehyde and glutaraldehyde were purchased from Sigma Aldrich (Sigma Aldrich, München, Germany). All other chemicals and solvents were purchased from Roth (Karlsruhe, Germany) in analytical grade. They were used without further purification. Cover borosilicate glass were purchased from VWR (Ø = 19 mm, Karlsruhe, Germany). Polished (111) silicon wafers were purchased from CrysTec GmbH (Berlin, Germany).

**Genetic design of eADF4(C16)E₈G.** The genetically engineered spider silk proteins used in this study are all variants of eADF4(C16), the genetic design of which has been described by Huemmerich et al. 37. Shortly, a consensus sequence of the repetitive core domain of the spidroin ADF4 of the European garden spider *Araneus diadematus* has been derived (protein sequence of module C: GSS AAAAAAAA SGPGGY GPENQ GPSGPGGYGPGGP), back-translated into a nucleotide sequence and repeated 16 times. Additionally, a nucleotide sequence encoding a T7 tag (peptide sequence: MASMTGGQQMGR, spacer: GS) has been fused to the 5’ end for detection purposes. 37 The cloning cassette developed by the authors allows the addition of tags to the recombinant proteins using a seamless cloning strategy. Here, a new silk variant, eADF4(C16)E₈G, has been created. In analogy to the tag fusion strategy presented for the recombinant silk protein eADF4(C16)RGD by Wohlrab et al. 34, two synthetic oligonucleotides were annealed (E₈ tag fwd – GATCCATGGGCGAAGAAGAGGAAGGAAGGGCTAATGAA and E₈ tag rev – AGCTTTTCATTAGCCCTCTTTCTCTTTCTTTCTTTCCGCCCCATG), encoding the E₈G peptide with the oligonucleotide design generating sticky ends mimicking a *Bam*HI and *Hind*III restriction site, suitable for integration into a pCS cloning vector allowing
fusion with the eADF4(C16) gene and further sub-cloning into the pET expression vector. Successful cloning was verified by sequencing.

**Recombinant Spider Silk Protein Production**

Spider silk proteins were produced recombinantly in *E. coli* and purified as described previously. Shortly, bacterial cells were transformed with a pET vector containing the respective gene constructs. Gene expression was induced by addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to the fermentation broth; cells were lysed by ultrasonication, cell debris removed by centrifugation and the heat-stable silk proteins separated by heat denaturation of bacterial proteins. Proteins were precipitated using ammonium sulfate and lyophilized for storage.

**Particle Production and Characterization.** Particles were produced according to a previously described method. Spider silk proteins were dissolved in guanidinium thiocyanate, dialyzed against Tris buffer (pH 7.5), and spider silk protein concentrations adjusted to 0.5 mg mL⁻¹ prior to particle formation, which was induced by the addition of potassium phosphate at a final concentration of 1 M (pH 7.5). eADF4(C16/κ16) blends were obtained by mixing eADF4(C16) and eADF4(κ16) protein solutions in a one-to-one ratio before particle production. The particles were washed thoroughly and re-dispersed in water until further use. Size and zeta-potential measurements of the particles were undertaken using a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) equipped with a He-Ne ion laser (λ = 633 nm). Particle size and size distribution were measured using dynamic light scattering. The particles' electrophoretic mobility was measured in 1/11 PBS and their zeta potential calculated from the electrophoretic mobility according to the theory of Smoluchowsky.
**Spider Silk Film Formation.** Cover borosilicate glass was cleaned using piranha cleaning (the glass covers were immersed in a 3:1 ratio of sulphuric acid (H$_2$SO$_4$) and 30% hydrogen peroxide for 15 min at RT). After piranha cleaning, the cover glass was rinsed with Milli-Q water, 100% ethanol (EtOH) and air dried. eADF4(C16) and eADF4(κ16) were dissolved in formic acid at desired concentrations (1 mg cm$^{-2}$) and were overhead shaken for 30 min. Afterward, the silk solutions were centrifuged to remove aggregates (14 437 g, RT, 15 min). The silk solutions were cast on cover borosilicate glass at 1 mg cm$^{-2}$ on a balanced plate and dried overnight at ambient conditions. For post-treatment, the samples were placed in a methanol-saturated atmosphere in a desiccator overnight.

**Incubation of Spider Silk Materials in Human Whole Blood.** Blood was collected from a healthy human volunteer and stored in sodium heparin vacuettees (Greiner Bio-One). Informed consent was obtained in compliance with the University of Melbourne Human ethics approval 1443420 and the Australian National Health and Medical Research Council Statement on Ethical Conduct in Human Research or ethics approval BASEC No. PB_2016-00816 from the local ethics committee, St. Gallen, Switzerland.

Spider silk particles (10 µL of 1 mg mL$^{-1}$ spider silk particles dispersed in DPBS) were immersed in human whole blood (500 µL) for 1 h at 37 °C (Eppendorf Thermomixer Comfort, Germany). RBC lysis was performed using Pharm Lyse buffer at 20× blood volume and topped up to 4 mL using 1× PBS before pelleting the white blood cells (WBC) and particles (500 g, 40 min). Unbound and loosely bound proteins were removed by washing the particles and WBCs followed by multiple centrifugation (300 g, 3 min) and resuspension steps (DPBS, 3×), followed by washing steps without redispersion (in DPBS (3×)) to remove the WBCs.
For SEM analysis, recombinant spider silk particles were re-suspended in water at a concentration of 1 mg mL\(^{-1}\), and 100 µL of this suspension was cast on a 0.5 cm\(^2\) silica wafer surface and air-dried. After immobilization of the particles to the silica wafer surface, the samples were sterilized with 70% EtOH for 2 min in sterile 12-well plates and air-dried. Spider silk films were prepared as depicted above and then placed in well plates. Blood was collected into S-monovettes with a heparin concentration of 0.43 IU/ml and used within 1 h after withdrawal. 500 µL per sample of the blood was transferred into well plates on top of the substrate and incubated with the immobilized spider silk particles or films (10 rpm, RT, 35 min.). After incubation, the blood was removed from the samples, and the samples were fixed with Karnovsky fixing solution and dehydrated with EtOH (50%, 70 %, 80 %, 90 %, and 100 %), dried overnight and sputtered with 10 nm gold prior to SEM analysis.

**SDS-PAGE and Sample Preparation** was done as published previously.\(^{10}\)

**Mass Spectrometry, Protein Identification and Proteomics Data Processing.** Sample preparation, protein identification via liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), protein identification and data processing were carried out as described recently.\(^{10,74}\) Briefly, in gel digested samples were analyzed by LC-MS/MS using Q-Exactive plus mass spectrometer (Thermo Scientific). The spectrometer was equipped with a nanoflow reversed-phase-HPLC (Ultimate 3000 RSLC, Dionex). The nano-LC system had an Acclaim Pepmap nano-trap column (Dionex – C18, 100 Å, 75 µm × 2 cm) and an Acclaim Pepmap RSLC analytical column (Dionex – C18, 100 Å, 75 µm × 50 cm). All spectra were measured in positive mode using full scan MS spectra scanning from m/z 375-1400 at 70000 resolution with AGC target of \(3e^6\) with maximum accumulation time of 50 ms. Lockmass of 445.120024 was used. The 15 most intense peptide ions with charge states ≥2-5 were isolated with isolation window of
1.2 m/z and fragmented with normalized collision energy of 30 at 17500 resolution. Data were analyzed using the PatternLab for Proteomics 4.0 suite including the Comet search engine. Searches and filtering of peptide spectrum matches and protein quantification were performed as published previously.

ASSOCIATED CONTENT

none

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Author Contributions

The design of the experiments was performed by A.C.G.W., H.M.H., S.L., F.C., and T.S.. Preparation of the paper was performed by A.C.G.W, H.M.H. and S.L. H.M.H. and S.L. prepared and characterized the spider silk particles. A.C.G.W performed characterization via SDS-PAGE and all mass spectrometry studies and the respective data analysis. S.L produced the spider silk films and performed the blood coagulation assays. Q.A.B. performed statistical analysis of the blood assay data. M.F. assisted with analysis of the mass spectrometry data. C.-S.A. performed mass spectrometry experiments and protein identification. T.S. and F.C. supervised the studies and edited the paper.
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REFERENCES


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figure 1

434x286mm (150 x 150 DPI)
figure 2

607x388mm (150 x 150 DPI)
figure 3

269x142mm (150 x 150 DPI)
**Figure 4**

683x537mm (150 x 150 DPI)
scheme 1

ADF4

\[ \text{consensus sequence} \]

\[ \text{module C} \rightarrow \text{genetic engineering} \]

\[ \text{module } \kappa \rightarrow \text{multimerisation} \]

\[ + \text{optional tag modification} \]

\[ \text{eADF4(C16)} \]

\[ \text{eADF4(C16)E_8G} \]

\[ \text{eADF4(C16)RGD} \]

\[ \text{eADF4(C16)R_8G} \]

\[ \text{eADF4(\kappa 16)} \]
Recombinant spider silk production → Particle engineering → Incubation in blood

TOC

462x210mm (150 x 150 DPI)
Minerva Access is the Institutional Repository of The University of Melbourne

Author/s:
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