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Production, structure and in vitro degradation of electrospun honeybee silk nanofibers

Corinne R. Wittmer [a], Xiao Hu [a], Pierre-Chanel Gauthier [a], Sarah Weisman [b], David L. Kaplan [a], Tara D. Sutherland [b,*]

[a] Department of Biomedical Engineering, Tufts University, Medford, MA 02155, USA
[b] CSIRO Ecosystem Sciences, Clunies Ross St., Acton, ACT 2601, Australia

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ABSTRACT

Honeybees produce silken cocoons containing four related fibrous proteins. High levels of each of the honeybee silk proteins can be produced recombinantly by fermentation in *Escherichia coli*. In this study we have used electrospinning to fabricate a single recombinant honeybee silk protein, AmelF3, into nanofibers of around 200 nm diameter. Infrared spectroscopy found that the molecular structure of the nanofibers was predominantly coiled coil, essentially the same as native honeybee silk. Mats of the honeybee nanofibers were treated with methanol or by water annealing, which increased their β-sheet content and rendered them water insensitive. The insoluble mats were degraded by protease on a time scale of hours to days. The protease gradually released proteins from the solid state and these were subsequently rapidly degraded into small peptides without the accumulation of partial degradation products. Cell culture assays demonstrated that the mats allowed survival, attachment and proliferation of fibroblasts. These results indicate that honeybee silk proteins meet many prerequisites for use as a biomaterial.

1. Introduction

The field of tissue engineering has invested considerable efforts into the development of scaffolds that mimic the structure and biological functions of the extracellular matrix (ECM). The ECM is principally constructed from nanoscale fibers composed of the proteins collagen, elastin, fibronectin and laminin, as well as proteoglycans, glycosaminoglycans and hyaluronic acid. Much of the information guiding cells to their correct location, cell anchorage and cell proliferation is encoded in the protein sequences of the ECM. The native proteins of the ECM have therefore attracted much attention as tissue engineering scaffold materials. Collagen in particular has been of interest because it is non-toxic, produces a minimal immune response, promotes attachment and proliferation of a range of cells, is resorbed in the body and can be fabricated into different forms (reviewed in Ref. [1]). However, collagen that is regenerated from natural sources is variable in composition and behavior, and requires chemical treatments to generate suitable mechanical properties. In order to achieve high levels of reproducibility and control, the research community has developed alternative artificial scaffolds such as poly(ethylene glycol), poly(lactic acid), polycaprolactone, polyphophates, ceramics and metals. Although these materials provide suitable bulk and mechanical properties for a range of tissue engineering applications (reviewed in Ref. [2]), they generally lack chemical cues to promote good cell attachment and proliferation. The most widespread current strategy to improve cell attachment is the generation of composite scaffolds where naturally sourced ECM proteins, usually collagen, are used to coat scaffolds generated from synthetic materials.

A possible alternative material for construction of tissue scaffolds is the silk of honeybees. Honeybees spin silk threads that they use to structurally reinforce their soft waxen hives. The threads are strong and highly extensible (132 MPa breaking stress; 204% breaking strain [3]). The silks are composed of four small (approximately 32 kDa), non-repetitive proteins (AmelF1-4) that do not require post-translational modification [4]. The proteins can be expressed recombinantly in *Escherichia coli* at high yield [5] and potentially at large scale, with the AmelF3 protein being produced at the highest levels at over 2 g l^{-1} ferment [5]. Circular dichroism and dynamic light scattering studies of recombinant honeybee silk proteins show that they readily self-assemble into their native coiled-coil structure [6–8]. Solutions of all four recombinant proteins [5] or AmelF3 alone [6] can be fabricated into fibers with mechanical properties approaching those of the native silk [3]. Although the honeybee silk proteins are particularly rich in alanine (29–33%), they contain a wide variety of other amino acids, including 20–25% charged amino acids (Arg, Asp, Glu and Lys) and sub-

* Corresponding author. Tel.: +61 2 62464236.
E-mail address: tara.sutherland@csiro.au (T.D. Sutherland).

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stantial levels (4–8%) of leucine, asparagine, glutamine, threonine and valine [4]. The combination of a consistent protein production method, good mechanical properties and the chemical functionalities arising from the complex amino acid composition make honeybee silk unlike any other existing biomaterial and of considerable interest. Furthermore, the proteins have low constraint on their primary sequence [9], and therefore offer the potential to be engineered to encode signaling sequences from the ECM that direct specific cell adhesion.

Electrospinning is a technique that generates nanoscale fibers from charged solutions of concentrated polymers. As the topology of electrospun fibers resembles parts of the ECM, electrospun fibers of synthetic polymers and regenerated natural polymers have been thoroughly explored as scaffolds for tissue engineering applications (reviewed in Ref. [10]). In this work we electrospun nanoscale fibers from solutions of the most readily produced recombinant honeybee silk protein (AmelF3), and systematically described their structure, cell response and in vitro enzyme degradation, in order to determine the suitability of electrospun honeybee silk as a scaffold biomaterial.

2. Materials and methods

2.1. Preparation of honeybee silk protein

Honeybee silk protein AmelF3 was expressed into the inclusion bodies of *E. coli* as described by Weisman et al. [5]. Inclusion bodies were purified using BugBuster Master Mix (Novagen) according to the manufacturer's protocol and then the silk protein was solubilized in 3% sodium dodecyl sulfate (SDS) with a 2 h incubation at 60 °C. Detergent was extracted by the addition of KCl (300 mM final concentration), causing the precipitation of potassium dodecyl sulfate (KDS). The KDS precipitate was removed by centrifugation at 16,000*g* for 5 min and the solution was dialyzed against 20% PEG 8000 until the protein concentration reached around 12.5% (as calculated by weighing the protein in solution and then after drying). Concentrations of around 12.5% were the maximum that could be achieved when the protein was prepared in this manner, as solutions gelled at higher concentrations and were not suitable for electrospinning. Silk/polyethylene oxide (PEO) blends were prepared by adding the required amount of 5.0% (w/v) PEO (900,000 MW, Sigma) into the silk solution and gently stirring for 20 min.

2.2. Electrospinning

The silk/PEO solution was delivered through a 16G stainless steel capillary maintained at a high electric potential at a flow rate of 5–10 μl min^{-1} using a Sage syringe pump (Thermo Scientific). The collector was a grounded aluminum foil placed on a 10 cm diameter aluminum plate. Silk was collected directly on the aluminum foil except for the cell study, where it was collected on 9×9 mm^2 glass coverslips that were placed on top of the usual aluminum foil. The applied voltage, solution flow rate and distance from capillary to collector for each solution are described in Table 1. Electrospinning was conducted at room temperature (20–22 °C) and humidity levels of 16–17%.

2.3. Microscopy

Electrospun fibers were analyzed by scanning electron microscopy (SEM) and polarized optical microscopy. For SEM imaging, the silk mats were sputter-coated with platinum/palladium using a 208 HR Sputter Coater (Cressington) and examined using a Zeiss Ultra 55 field emission scanning electron microscope at 5 kV. Polarized optical microscopy of mats was performed on a phase contrast microscope with a TCS SP2 scanner (Leica Microsystems).

2.4. Fourier transform infrared (FTIR) spectroscopy

FTIR analysis of the electrospun mats were performed with a Jasco FT/IR-6200 spectrometer, equipped with a deuterated triglycine sulfate detector and a multiple reflection, horizontal MIRacle ATR attachment (Ge crystal, Pike Tech). The instrument was continuously purged with nitrogen gas to remove atmospheric water vapor. Each measurement incorporated 128 scans (wavenumber from 600 to 4000 cm^{-1}) that were Fourier transformed using a Genzel–Happ apodization function to yield spectra with a nominal resolution of 4 cm^{-1}. Secondary structure peak assignments were as described in Refs. [11–13]: absorption bands in the frequency range of 1610–1625 cm^{-1} represent the β-sheet structure; absorption bands around the frequencies of 1650, 1640 and 1630 cm^{-1} were ascribed to the coiled-coil fingerprint spectrum, and peaks above 1660 cm^{-1} were ascribed to β-turns.

2.5. Cell culture

Green fluorescent protein (GFP)-expressing rabbit corneal fibroblasts (rCFs) were generated after isolating primary stromal fibroblast cells from excised rabbit corneas (Pel-Freeze) as previously described [14,15] and cultured in rabbit fibroblast medium (rFB medium) consisting of Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 1% penicillin–streptomycin–fungizone, and maintained at 5% CO_2. The previously described lentivirus system [16] was used to generate the GFP-expressing line of rCFs (GFP-rCFs). The rCFs were transduced at a multiplicity of infection of 1, with 5 ml of virus-containing supernatant (100,000 virus particles ml^{-1}) added to 5×10^5 rCFs. An additional 5 ml of rFB medium and protamine sulfate (6 mg ml^{-1}) was added to enhance infection. The cells were incubated for 3 h with the lentivirus, then washed twice with PBS before adding rFB medium. Fluorescence microscopy and fluorescence-activated cell sorting analysis was used to evaluate the efficiency of GFP transduction. Stable GFP-rCFs were determined by the presence of fluorescence in cells over multiple cell passages. For seeding, GFP-rCFs were grown to confluence, detached from their substrates using 0.05% Trypsin (Gibco) and then replated at passage 10–12 (P10–12) onto prepared electrospun honeybee mats as described below.

Table 1
Conditions for electrospinning recombinant honeybee silk protein solutions.

Additive	Silk:PEO ratio	Fiber diameter (nm)	Voltage (kV)	Distance (cm)	Flow rate (ml min^{-1})	Fiber quality
1% PEO	12.5:1	207 ± 21*	9.8	15	0.015–0.008	Uniform diameter
0.7% PEO	18.7:1	314 ± 22*	10	15	0.015–0.008	Uniform diameter
0.5% PEO	25.0:1	150–300	9–12	15–20	0.02–0.008	Fibers uneven
0.4% PEO	31.3:1	<100 plus beads	9–12	15–20	0.02–0.008	Fibers beaded
None	–	–	10–20	10–20	0.02	No fibers obtained

* Standard deviation of 12 measurements.

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