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Recombinant spider silk as matrices for cell culture

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ABSTRACT

The recombinant miniature spider silk protein, 4RepCT, was used to fabricate film, foam, fiber and mesh matrices of different dimensionality, microstructure and nanotopography. These matrices were evaluated regarding their suitability for cell culturing. Human primary fibroblasts attached to and grew well on all matrix types, also in the absence of serum proteins or other animal-derived additives. The highest cell counts were obtained on matrices combining film and fiber/mesh. The cells showed an elongated shape that followed the structure of the matrices and exhibited prominent actin filaments. Moreover, the fibroblasts produced, secreted and deposited collagen type I onto the matrices. These results, together with findings of the matrices being mechanically robust, hold promise not only for *in vitro* cell culturing, but also for tissue engineering applications.

1. Introduction

The ideal matrix for cell culturing should enable cell attachment, migration, proliferation and differentiation. Moreover, it should allow cell-cell-interactions, provide structural support and have versatile processing options to alter structure and morphology according to cell or tissue type [1]. If intended for clinical applications, e.g. tissue engineering, the matrix also needs to be compatible with the host immune system and biodegradable at a rate that allow for new tissue to form [2].

Traditionally, two dimensional (2D) systems, e.g. micro-well plates, tissue culture flasks and Petri dishes, have been used in cell culturing [3]. However, culturing under these conditions forces the cells to adjust to a flat, rigid surface, which can lead to generation of cells that do not maintain their physiological phenotype [3–5]. In order to increase cell attachment, tissue culture plates can be coated with a thin layer of bioactive molecules, e.g., poly-lysine, laminin or fibronectin. However, studies have shown that not only the biochemical but also the mechanical properties of the microenvironment can modulate adhesion, growth and differentiation of cells [6–9]. Moreover, cells growing in their natural *in vivo* milieu are connected to other cells, structures and molecules in a complex three dimensional (3D) fibrous network. A 3D matrix can therefore offer a more realistic environment for cell culturing [10] also providing larger flexibility in applications. *In vivo*, cells are embedded in considerably different 3D microenvironments depending on the tissue. Therefore, a large variety of matrices and matrix formats are probably needed in order to imitate different *in vivo* conditions.

Polymeric matrices used for cell culturing and tissue engineering can be divided into two subtypes; i.e. synthetic polymers (e.g. poly glycolic acid, poly lactic acid and poly ethylene glycol) and naturally derived polymers (e.g. collagen, fibrin, Matrigel™, and silk) [2,3]. The synthetic polymers offer great flexibility in the design of composition and structure for specific needs, but generally suffer from poor bioactivity and requirement of harsh polymerization conditions [3]. Naturally derived polymers, on the other hand, generally have better biomimetic properties, but as they are often isolated from tissues or tumour cell lines (e.g. collagen and Matrigel), their undefined composition reduces the degree of experimental control [3]. Moreover, there is a risk of contamination with infectious agents, which could potentially affect experimental results or cause disease transmission [11,12]. Given the above, there is a need for defined culture systems of non-animal origin, where uncontrolled external factors are largely avoided. This is particularly important if these systems are to be employed for clinical applications. Recently, three groups reported fully defined 2D coatings (recombinant laminin, peptide-acrylate and a synthetic

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polymer) that support long-term culture of human embryonic stem cells [13–15]. Whether these coatings can be transferred to stable 3D systems remains to be investigated.

Silk is a group of natural polymers which are well known for their mechanical properties and have been employed for centuries as suture material [1]. Silk is produced by e.g. silk moth larvae and spiders. Spider silk is stronger than silk from the conventionally used silkworm *Bombyx mori*, and also lacks the immunogenic coat protein sericin [16]. In contrast to the silkworm, spiders are territorial and therefore difficult to farm. Major efforts have therefore been directed to the production of recombinant spider silks. Several factors, including the long, repetitive and aggregation-prone sequence of spider silk proteins, complicate recombinant production of soluble protein and controlled polymerization [17,18]. However, a recombinant miniature spider silk protein, 4RepCT, can be produced at comparatively high levels in soluble form when fused to a soluble protein partner [19]. Moreover, the purified 4RepCT spontaneously forms fibers in physiological buffer, without the need for spinning into coagulation baths. There are a few reports published on cytocompatibility [20–25] and on biocompatibility *in vivo* [26,27] of recombinantly produced spider silk. These reports suggest that recombinant spider silk is an interesting material for cell culture and also, since it appears compatible with living tissue, for tissue engineering purposes [17,28]. However, although the possibility to manufacture different 3D structures is one of the benefits of the material, studies of the effect of different matrix morphologies on cultured cells are lacking.

The recombinant spider silk produced herein (4RepCT) is of non-animal origin, easily sterilized [22], biodegradable and well tolerated *in vivo* [27] and can form different structures. In this paper, the attachment, growth and extracellular matrix (ECM) production of human primary fibroblasts on films, foams, fibers and meshes made of recombinant spider silk are investigated.

2. Materials and methods

2.1. Fabrication of cell culture matrices

The recombinant miniature spider silk protein 4RepCT (Fig. 1) was produced in *Escherichia coli* as described previously [29]. In initial experiments, a procedure for depletion of Lipopolysaccharides (LPS) was also included, as described by Hedhammar and co-workers [22]. After purification, the protein solution was filter sterilized (0.22 μm) and concentrated to 1 or 3 mg/mL by centrifugal filtration (Amicon Ultra, Millipore) before preparation of matrices. Protein solutions of 1 mg/mL were allowed to self-assemble into fibers overnight. The fibers were then sterilized through autoclaving for 15 min at 121 °C in distilled water, at 2.8 bar, a process which does not affect the chemical stability, mechanical or structural properties of the fibers [22]. The fibers (dry weight 50 μg/well) were either used without further processing or chopped into a mesh. As control, a fibrous mesh (dry weight 50 μg/well) was prepared from degummed silk from the silkworm *B. mori*. Protein solutions of 3 mg/mL (50 μg protein/well), were used to cast film or were foamed before applied to the wells. In addition, hybrid matrices were prepared, where wet fibers or meshes were applied on top of pre-formed film, which resulted in partly integration upon drying. All matrices were allowed to dry over night at room temperature under sterile conditions, then washed twice with sterile PBS and pre-incubated with complete cell culture medium for 1 h at 37 °C with 5% CO_2 before cell seeding. The matrices were prepared in 96-well hydrophobic cell culture polystyrene plates treated for cells in suspension (Sarstedt), to prevent attachment to the plastic surface, or on cell culture chamber glass slides (LabTek). The same hydrophobic wells without matrices were used as negative control (HP), while tissue culture treated plates (TCT) (Sarstedt) were used as positive control. For microscopic studies, chamber glass slides without matrix were used as positive control. For analysis of the microstructure of the matrices, an inverted Nikon Eclipse Ti light microscope equipped with differential interference contrast was used at 20× magnification. The matrices were also applied on stubs with carbon tape and vacuum coated with gold before analysed with scanning electron microscopy (SEM) using a LEO 1530 with field emission columns (Zeiss) and an acceleration voltage of 2 kV.

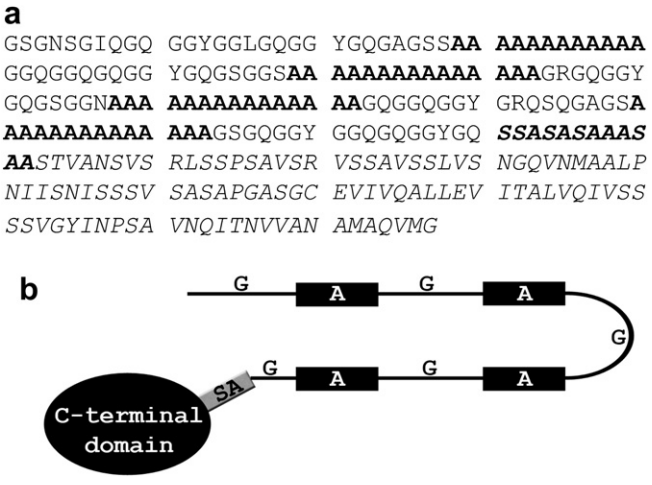

Fig. 1. The recombinant spider silk protein 4RepCT. (a) The amino acid sequence of 4RepCT. Poly-alanine stretches are shown in bold, the serine and alanine rich stretch is shown in bold italics, and the non-repetitive C-terminal domain is shown in italics. (b) Schematic presentation of 4RepCT. Lines indicated with 'G' represent glycine rich segments, black boxes marked 'A' represents poly-alanine stretches, the grey box marked with 'SA' represents a serine and alanine rich stretch, and the black oval represents the non-repetitive globular C-terminal domain.

2.2. Cell culture

Primary human dermal fibroblasts of neonatal origin, HDFn (ECACC) were cultured in Dulbecco's modified Eagle's medium nutrient mixture F12 HAM (Sigma) supplemented with 5% foetal bovine serum (Gibco), Penicillin and Streptomycin (National Veterinary Institute, SVA). For serum-free conditions, normal primary human dermal fibroblasts from juvenile foreskin, NHDF (PromoCell) were cultured in PC-1 (Lonza), supplemented with L-glutamine (Swedish veterinary institute) and ascorbic acid (Sigma, 50 ug/mL). Cells were expanded for 7 days after thawing, and cell viability checked with trypan blue prior to seeding onto matrices at 3000 viable cells/cm^2 (HDFn), and 4000 viable cells/cm^2 (NHDF), as recommended by the manufacturers. In addition, a higher seeding density (15,000 cells/cm^2) was used for the HDFn to evaluate supportive capacity of the matrices for high cell numbers. After two weeks culture on the matrices, cells were harvested and reseeded onto glass slides and in TCT plates in order to examine cellular phenotype. All experiments were performed in passage 8–9 at 37 °C with 5% CO_2 and 95% humidity.

2.3. Cell viability analysis with alamar blue

Cell growth on 96 well plate matrices was monitored with Alamar Blue cell viability assay (Molecular Probes) every second day during the culture period. After 4 h incubation with Alamar blue diluted 1:10 in cell culture medium, fluorescence intensity of 100 uL supernatants from the cultures was measured with a fluorescence plate reader using excitation at 544 nm and emission at 595 nm (FarCyte, TECAN). Each matrix type was analysed in hexaplicates. Three and two independent experiments were performed for matrix type comparisons, serum free cultures and silk type comparisons respectively. The assay gives a measure of the number of living cells in each culture well. Fluorescence intensities were plotted over time to yield growth profiles of cells seeded on the different matrices.

2.4. Cellular stainings

Cells cultured on matrices in chamber slides were stained for either viable/dead cells, filamentous actin or collagen type I every third day during the culture period. The stained cells were analysed with a confocal Leica DM IRE2 laser scanning microscope (Leica Microsystems, Germany) using software Leica TCS SL (Leica Microsystems, Germany). Excitation at 488 nm and detection at 550–530 nm was used to monitor green fluorescence, whereas excitation at 543 nm and detection at 620–660 nm was used for red fluorescence. All images were captured using sequential scanning mode to eliminate overbleeding between signals. Fiber and foam structures were readily visible in all stainings with the exception of foam in the phalloidin staining. Therefore, the foam in the phalloidin stainings was detected in an additional separate channel (excitation at 543 nm, detection at 570–610 nm, shown in grey) with maximal gain, where the strong signals from the cells (white) could be separately deleted as outliers using image analysis software ImageJ. The green or red channels were not affected by this procedure. For Z-stack of phalloidin stainings (foam day 10), an inverted Zeiss LSM 510 confocal microscope was used (green fluorescence: excitation at 488 nm, detection at 505–530 nm, red fluorescence: excitation at 561 nm, detection at LP650, foam detection: excitation at 561 nm, detection at 530–600 nm). In total 28 scans with 3 μm interval were