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Silk fibroin film from non-mulberry tropical tasar silkworms: A novel substrate for in vitro fibroblast culture

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Abstract

The silk protein fibroin, isolated from the cocoon of the domesticated mulberry silkworm, *Bombyx mori*, is used extensively in biomaterial design and in cell and tissue culture. We report here for the first time the potential application of fibroin obtained from the cocoon of non-mulberry tropical silkworm, *Antheraea mylitta*, as a substrate for in vitro cell culture. The mechanical strength of *A. mylitta* silk fibers indicates a stronger thread composition. The contact angle of *A. mylitta* fibroin films suggests that it has lower hydrophilicity and lower solubility in organic solvents compared to *B. mori* fibroin films. Retention of a secondary structure of fibroin in both *A. mylitta* and *B. mori* films is confirmed by Fourier transform infrared analysis. The adherence, growth and proliferation patterns of feline fibroblast cells on *A. mylitta* fibroin films suggest that this kind of film has a greater ability to support cell growth than *B. mori* fibroin films and is comparable to that of control. This study demonstrates that, as well as being non-toxic to dermal fibroblast cells, non-mulberry fibroin might be a useful alternative substrate to the more common *B. mori* fibroin for a variety of biomedical applications.

Keywords: Silk fibroin; Adhesion; Biocompatibility; Contact angle; FTIR

1. Introduction

Developments in the field of tissue engineering have accelerated the demand for biomaterials that are biodegradable, biocompatible and have suitable mechanical properties [1,2]. The surface properties of biomaterials (such as the combination of physical and mechanical strength, flexibility and chemical resistance) help to determine their performance in a biological environment [3].

Silk fibers have a long medical history as surgical sutures [4]. Silk proteins have been used in a wide variety of applications in the medical field, especially as scaffolds to promote cell growth, drug delivery systems, replacements for connective tissue, and antioxidants [5]. The fibrous protein collagen is also well known as a standard biomaterial for tissue engineering and biomedical uses [6,7]. Mulberry silk, produced by *Bombyx mori*, a domesticated silkworm, has been exploited for use in surgery and tissue engineering. In general, *B. mori* silk cocoons are composed of a fibrous protein fibroin core (72–81%) and a surrounding glue protein, sericin (19–28%) [8–10]. The major biomedical applications of silk revolve around fibroin, which is a hydrophobic protein with a molecular mass of around 400 kDa [11,12]. The *B. mori* fibroin has been utilized for osteoblast, fibroblast, hepatocyte and keratinocyte adherence and growth in vitro [13–16], and as an alternative to collagen in surgery, mainly in the form of sutures [17].

Fibroins from mulberry and non-mulberry silkworms are structurally distinguishable. X-ray diffraction patterns show that the mulberry silk fibroin from *B. mori* contains a -G-X-G-X- (G, glycine; X, alanine, or serine) repeat structure of β(1) type, whereas non-mulberry silk fibroin, generally produced by wild or semi-domesticated Saturniid silkworms, namely *Antheraea* sp. (wild) and *Philosamia ricini*, contain polyalanine repeat sequences of β(3a) type) [18,19]. *Antheraea mylitta*, a tropical

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non-mulberry tasar silkworm, has the greatest capacity for silk production of all the silk-spinning insects [20]. Fibroin isolated from the silk gland of *A. mylitta* has a molecular mass of 395 kDa and is a homodimeric protein, each monomer approximately 197 kDa [21]. The wall of *A. mylitta* cocoons, collected from the wild habitats, are found to be much tougher [22], and fibroin, the major constituent, makes a significant contribution to the toughness of the cocoon. Though the properties of *A. mylitta* fibroin point towards its potential use as a biomaterial, little is known about its physicochemical properties and biomedical usefulness.

This paper reports the mechanical and surface properties of fibroin film from the non-mulberry tropical silkworm *A. mylitta* to determine the suitability of non-mulberry fibroin as a potential substratum for supporting cell adhesion and proliferation.

2. Materials and methods

2.1. Materials

Fresh cocoons of the non-mulberry tropical tasar silkworm *A. mylitta* and the mulberry silkworm *B. mori* were obtained from the Midnapore district, West Bengal, India. Fine chemicals and fluorescent dyes were purchased from Sigma (USA); cell culture grade chemicals were from HiMedia Chemical Laboratories (India); and fetal bovine serum was from Invitrogen (USA).

2.2. Protein extraction

The silk protein fibroin was isolated from mulberry and non-mulberry silk cocoons following a standard extraction procedure [23]. Briefly, cocoons were cut into small pieces (approximately 1 cm × 1 cm) and boiled in an aqueous solution of 0.02 M Na_2CO_3 (10% w/v) for 30 min to remove sericin, washed thoroughly in distilled water and dried for 1 h at 50 °C, dissolved in 9.3 M lithium bromide for 12 h at room temperature before the insoluble material was removed by centrifugation. The supernatant was dialyzed extensively for 3 days against frequent changes of deionized water. The protein concentration was measured using the Bradford standard assay procedure [24].

2.3. Fibroin film preparation

The silk protein solutions were concentrated to 0.8 mg ml^{-1} using Centricon YM-30 (Millipore, USA). Around 0.5 ml of protein samples were cast gently on top of the polystyrene films (Haldia Petrochemicals, India) and air-dried under a laminar flow for 12–14 h [25]. The protein films were peeled off and dried in a desiccator for 12 h. Films with a thickness between 0.04 and 0.07 mm (estimated using a Micro Hardness tester fitted with a CCD camera, Leco) were used for further analysis.

2.4. Surface characterization of silk fibroin fibers and films

2.4.1. Scanning electron microscopy

Scanning electron microscopy (SEM) images of silk fibroin films and fibers were obtained after gold sputtering using a JEOL JSM-5800 scanning electron microscope with incident electron beam energy of 1 keV and a working distance of 6 mm. The roughness of the films was estimated using the surface profilometer of a Universal Micro Hardness tester (Taylor Hobson, Model Surtonic Telesharp Unit) with a Gaussian filter of 0.8 mm.

2.4.2. Dynamic contact angle test

The advancing and receding contact angles of fibroin films of both *B. mori* and *A. mylitta* were determined using the Dynamic Wilhelmy method (DCAT 11, Dataphysics) in water as well as in an organic solvent, *n*-heptane, to study the comparative solubility.

2.5. Physicochemical characterizations of silk fibroin fibers and films

2.5.1. Mechanical properties

As a basis for mechanical testing, tests for failure strength and tenacity of fibers were conducted on a Hounsfield H25kS universal testing machine (Hounsfield, Surrey, UK) fitted with a 50 kN load cell according to the ASTMD 638 at a relative humidity ranging between 75% and 82%. The tenacity of different fibers was observed and reported as an average of at least three replicates. Data were analyzed using QMAT 3.1 software.

2.5.2. Fourier transform infrared spectroscopy

Fourier transform infrared (FTIR) analyses of fibroin protein and liquid was carried out using an FTIR spectrometer (Thermo Nicolet Corporation NEXUS-870) with a resolution of 2 cm^{-1} for both *A. mylitta* and *B. mori* fibroin films. Omnic software was used to study the amide I region of the proteins. Band narrowing was done with a full-width at half-height of 18 cm^{-1} and a resolution enhancement factor of 2. For the protein in aqueous solution, the spectrum of the water was subtracted and was analyzed.

2.5.3. Circular dichroism spectroscopy

The circular dichroism (CD) studies of fibroin were performed on a JASCO J-810 spectropolarimeter using a 0.1 cm path length quartz cell at 20 °C. The ultraviolet spectrum was collected at a protein concentration of 4 μM with a step resolution of 0.5 nm, a time constant of 1 s, a sensitivity of 10 mdeg, a scan speed of 50 nm min^{-1} and a spectral bandwidth of 2 nm. In order to reduce random error and noise each spectrum was an average of three scans in the range 400–190 nm. The background spectra were acquired from water used for dialysis of the protein samples. The spectra were corrected for the baseline. The percentages of α-helix, β sheets, turns and random coils

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