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Silk gland sericin protein membranes: Fabrication and characterization for potential biotechnological applications

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ABSTRACT

This study describes the potential use of silk gland sericin protein as a biocompatible natural biopolymer in its native form. The membranes were fabricated using native silk sericin protein extracted from middle silk gland of *Antheraea mylitta*, a non-mulberry tropical tasar silkworm without using any cross-linking agent. The fabricated membranes were biophysically characterized and optimized for cell culture. Silk sericin protein extracted from gland contained higher amount of β-sheets, which increased upon treatment with ethanol as observed by FTIR and XRD. The membranes did show robustness, good mechanical strength and high temperature stability. Cytocompatibility of the membranes was evaluated by MTT assay and cell cycle analysis using feline fibroblast cells. Morphology of growing cells was assessed by confocal microscopy that indicated normal spreading and proliferation on the silk sericin membranes. The membranes showed low inflammatory response as observed assaying TNF α release. This study reveals the potential of native silk sericin protein from silk gland as biocompatible biopolymer for potential biomedical applications.

1. Introduction

Silk—a natural polymer produced by Lepidopteron insects of the family Bombycidae and Saturniidae has been widely used in textile industries due to its lusture and mechanical properties. The silk fibres are composed of a fibrous core protein fibroin with sericin surrounding it (Dash et al., 2007; Magoshi et al., 1996). Fibroin is hydrophobic in nature while sericin is hydrophilic. Silk is produced by the epithelial cells and stored in the lumen of specialized silk glands of matured 5th instar larvae before being secreted from the silk gland (Gamo et al., 1977).

While manufacturing silk, sericin is removed as waste during the degumming process. This study however finds an interesting application (Freddi et al., 2003; Zhang, 2002; Kundu et al., 2008). In this study *Antheraea mylitta* (of Saturniidae family) is used as the silk sericin source as compared to the extensively studied *B. mori* (of Bombycidae family) (Mandal and Kundu, 2008a,b; Altman et al., 2003). Molecular weight of sericin protein ranges from 24 to 400 kDa with predominant amino acid group's serine (40%), glycine (16%), glutamic acid, aspartic acid, threonine and tyrosine (Gamo et al., 1977; Takasu et al., 2002; Tokutake, 1980). Thus it consists of polar side chain made of hydroxyl, carboxyl and amino groups that enable easy cross-linking, copolymerization, and blending with other polymers to form improved biodegradable materials (Cho et al., 2003; Ahn et al., 2001; Nagura et al., 2001). In addition to *B. mori*, reports suggest the presence of sericin in other wild non-mulberry silkworm cocoons like *Philosamia ricini*, *A. mylitta* and *Cricula trifenestrata* (Dash et al., 2007; Yamada and Tsubouchi, 2001). Our previous studies reported sericin of molecular weight 70, <200 and >200 kDa from the cocoons of *A. mylitta* having a similar secondary structure but differing biochemically and immunologically from that of *B. mori* (Dash et al., 2007).

Non-textile utilities of sericin includes skin care, food, antioxidant and anti-apoptotic, tumour suppression, anticoagulant and wound healers (Dash et al., 2008a,b; Zhang, 2002; Sasaki et al., 2000a,b,c; Kato et al., 1998; Zhaorigetu et al., 2001, 2003; Kundu et al., 2008). Whereas, some reports suggest sericin matrices providing enhanced attachment and growth of cells without any cytotoxic effects (Minoura et al., 1995; Tsubouchi et al., 2005). Sericin is also known to accelerate proliferation of various cell lines including mammalian and hybridoma as an additive in serum free media (Sasaki et al., 2005; Ogawa et al., 2004). Sericin elicits immune responses depending on its physical association with fibroin and not when completely separated from fibres (Panilaitis et al., 2003). Further *in vivo* experiments verified sericin peptides without any immunogenicity and hence effectively used for various biomedical applications (Zhang et al., 2006a,b).

Sericin with such high potential has been always neglected in the field of tissue engineering due to its weak structural proper-

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ties and high water solubility. Sericins are known to form fragile films and 3D structures that are difficult to fabricate because of its gelatinous nature and wide range of molecular weights (Marcelino et al., 2007). Also during its extraction from cocoons the harsh treatment seems to make the protein denature that eventually leads to inferior biophysical properties. Due to this reason researchers have blended sericin with other natural/synthetic polymers to fabricate 2D and 3D matrices (Ahn et al., 2001; Wu et al., 2006; Tao et al., 2005).

Hope-sericin produced from genetically modified silkworm *B. mori* (Teramoto et al., 2005; Teramoto and Miyazawa, 2005) possesses superior biophysical properties in comparison to cocoon extracted sericin (Tsukada and Bertholon, 1981; Teramoto et al., 2006). Studies reveal that Hope-sericin consists of random coiled and β-sheet rich structures (Tsukada and Bertholon, 1981), this perhaps results in superior mechanical strength (Teramoto et al., 2006). Whereas these β-sheets are heat and alkali labile and contribute to weak biophysical properties of sericin when degummed in the usual process (Teramoto et al., 2006, 2007).

In this study we have investigated the possibility of using gland silk sericin in its native state for fabrication of 2D membranes with improved biophysical properties to be used as bio-based material. This method not only excludes the harsh process of silk degumming but also avoids usage of toxic chemicals for both extraction and cross-linking. This study is intended to promote the usage of native undegraded silk sericin protein for the fabrication of non-mulberry sericin 2D films for various biomedical applications.

2. Materials and methods

2.1. Materials

Protein molecular weight marker (Amersham, UK), fetal bovine serum (FBS), DMEM medium, trypsin–EDTA, penicillin–streptomycin (Gibco BRL, USA), thiazolyl blue (MTT), FITC-Phalloidin, lipopolysaccharide (LPS) from *Escherichia coli* (Sigma, USA), Hoechst 33342 (Invitrogen, USA), tumour necrosis factor alpha determination kit (GE Healthcare, USA), polyethylene glycol 6000 (SRL, Mumbai, India), cellulose dialysis tubing 3500 Da (Pierce, USA), tissue culture grade polystyrene plastic flasks and plates (Tarsons, India).

2.2. Isolation and SDS-PAGE of silk sericin

Fifth instar larvae of *A. mylitta* were collected from local farms. Sericin protein was obtained from its middle silk gland (MSG) (Takasu et al., 2002). MSGs were removed and washed in deionized water. The glands were then squeezed into a beaker containing deionized water and stirred for 30 min. The resultant solution was then centrifuged and the supernatant was used as the silk sericin protein solution. Protein concentration was determined by Bradford assay at 595 nm using a PerkinElmer UV-VIS spectrophotometer. Sericin thus obtained was electrophoresed under reducing condition on an 8% SDS polyacrylamide gel. The gel was stained with Coomassie brilliant blue R 250 after electrophoresis. Molecular weights were determined using a molecular weight marker.

2.3. Fabrication of silk sericin membranes

Aqueous solution of 2 wt% sericin obtained from the MSG was poured on teflon-coated dishes and left overnight to dry at a temperature of 37 °C. Sericin membranes were peeled off using fine forceps and treated with 70% (v/v) ethanol to induce insolubility and crystallinity. The membranes were then used for subsequent investigations.

2.4. Fourier transformed infrared spectroscopy

Infrared spectra were collected using a FTIR spectrometer, Thermo Nicolet Nexus 870 at a resolution of 4 cm^{-1}. FTIR spectra of treated and untreated sericin membranes measuring 10 mm × 5 mm were scanned. The measurements were taken in a range of 500–2000 cm^{-1} with a scanning frequency of 64 times.

2.5. Wide-angle X-ray scattering

X-ray diffraction curve of both treated and untreated sericin membranes was measured by Philips PW 1710 diffractometer. Cu Kα was used to investigate changes in crystallinity. Diffraction range (2θ) was between 10–50° at a scan speed 3°/min.

2.6. Thermal analysis

Differential scanning calorimetry of both treated and untreated sericin films were performed by Pyris Diamond DSC (PerkinElmer). All the measurements were taken in an inert atmosphere of nitrogen and a temperature ranging from 200 to 500 °C. The heating rate was 10 °C/min.

2.7. Scanning electron microscopy

Surface morphology of treated and untreated sericin membranes was observed by a scanning electron microscope (JEOL JSM-5800) at a voltage of 20 kV. Surface of specimen was gold sputter coated before analysis.

2.8. Atomic force microscopy

Surface analysis of treated and untreated sericin membranes were performed in non-contact mode by Veeco CP II, USA with tips mounted on cantilevers with spring constant of 40 N/m (as specified by manufacturer). The membranes were coated on glass surface and air-dried. Measurements were done in air at room temperature with a scan area of 40 μm × 40 μm.

2.9. Mechanical testing

Mechanical properties i.e. tensile strength and percentage elongation at break of sericin membranes (both treated and untreated) were measured using Universal Testing Machine, Housfield-H25KS equipped with a 100 N load cell at 25 °C and 80–85% relative humidity. Membranes fabricated of 2 wt% sericin were cut into dumble shape of 25 mm length and 10 mm width at the centre. They were then examined using a crosshead speed of 1 mm/min with a gauge length of 10 mm in between clamps.

2.10. Cell attachment and proliferation studies

2.10.1. Maintenance of fibroblast cell line

AH927 feline fibroblast cells were cultured in complete DMEM medium containing 10% fetal bovine serum, L-glutamine and penicillin–streptomycin and incubated at 37 °C in an atmosphere of 5% CO_2. The culture medium was changed every third day. The fibroblast cells were harvested and sub cultured at a confluence of 60–70% using 0.25% trypsin and 0.02% EDTA.

2.10.2. MTT assay

Cell viability assessment was carried out using MTT assay (Ng et al., 2005). MTT substrate (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrasodium bromide), which is reduced by living cells to a dark-blue formazan requires active mitochondria and is thus an accurate measure for mitochondrial activity and cell viability.