 CHAPTER 3

MATERIALS AND METHODS

Materials

Collection of Samples

Two silkworm species which are indigenous to the Northeastern part of India viz. Muga (*Antheraea assamensis* Helfer) and Eri (*Samia ricini* Donovan) of North east region of India were used in this study. Disease free layings (Dfl) were collected from Boko, Mangaldoi, and Sivasagar of Assam and reared at the food plants garden and rearing house of IASST’s campus to obtain healthy matured 5th instar silkworm and good quality cocoons. For convenience, *Antheraea assamensis* and *Samia ricini* will be referred to as Muga and Eri respectively from here on.

Chemicals

All the chemicals and reagents used in this study were procured from Sigma Aldrich and Merck. Double distilled mili Q water (Milipore, USA) was used for the experiments.
Methodology

Extraction and purification of raw silk from silk glands

Extraction of raw liquid silk protein fibroin was performed following the procedures of Shimizu et al., 1957 and Putthanarat et al., 2002. The mature fifth instar larvae of Muga and Eri were dissected and the whole paired silk gland was taken out and washed carefully without any pressure in double distilled. The posterior part was cut separately and washed in distilled water to remove any residual sericin and tissue debris. Immediately, they were cut into fine pieces and put into petri plates containing small amount of cold distilled very carefully and smoothly. After 10-15 minute, the swollen glandular tissues were removed by forceps from the petri plates, and the silk proteins remained in the petri plates. The proteins were collected and very gently shaken in dancing shaker (Tarsons) and left overnight in the cold (2–3°C). Processing and preservation of the protein is very hard, as it forms gel very easily, therefore the fibroin solutions are kept at low temperature overnight, the solution turns colourless and formation of gel is prevented. Next, the solution was centrifuged for 1 hour at 10,000g (Superspin-R), and the supernatant containing the fibroin was collected by decantation.

Similarly, sericin was extracted from the middle part of the silk gland. The middle part was cut into small pieces in cold distilled water in a petri plate. After 20-30 min the sericin comes out of the glandular tissues and dissolves in distilled water. The
swollen glandular tissues were taken out of the petri plate and the sericin solution was centrifuged (60 min, 10000 rpm) and the supernatant was collected.

![Flow chart showing extraction of fibroin and sericin from silk gland of fifth instar larvae](image)

**Fig 1. Flow chart showing extraction of fibroin and sericin from silk gland of fifth instar larvae**

**Molecular weight determination of silk proteins**

The molecular weight determination of liquid fibroin and sericin was done by SDS-PAGE in reducing condition following the method of Laemmli (1970), briefly 7.5% gel was used to resolve the samples and the samples were prepared by treating with SDS reducing buffer containing 5% mercaptoethanol and heating at 95°C for 5 min and adding bromophenol blue for tracking the gel while running. 20 μl of each sample was loaded into the stacking gel and running buffer added. The gel was run at 20-25 V/cm of gel length. After completion of running the gel was stained with Coomassie brilliant
blue for 1 hour in shaking plate and desatined with destaining solution for desired time till the bands became clearly visible.

**Preparation of films from gland silk**

The films from gland silk were prepared following the method described by Putthanarat et al. 2002 with a little variation. To determine the concentration of the silk solutions, 1ml of the silk solution was cast on a Teflon coated plate as substrate, at $25^0\text{C}$ and 65% relative humidity and dried under a laminar flow for 12–14 h. After complete drying the films were carefully peeled off by forceps and dried in room temperature by putting in desiccators for 12 h. The weight of the dried film was taken in a balance (Mettler Toledo, PB 153). The concentration of the film was determined as follows:

\[
\text{Concentration (\%)} = \frac{\text{Wt. of dried film (g)}}{\text{Volume of solution taken (ml)}} \times 100
\]

Further, the desired concentration of the film is achieved by adding required amount of distilled water to the protein solution. The protein concentration was adjusted to 1% and films were cast according to the above mentioned procedure on teflon coated plate as substrate, at $25^0\text{C}$ and 65% relative humidity and completely air-dried under a laminar flow for 12–14 h. After that the films were carefully peeled off with forceps and kept in dessicators to dry for further use. *B. mori* mature fifth instar larvae were also used for protein extraction and fabrication of films for comparative studies with Muga and Eri.
**Extraction and purification of fibroin and sericin protein from the cocoons**

**Degumming of cocoons**

Degumming of cocoons was done by using sodium carbonate using the standard method. 10 g cocoons of Muga were chopped into finer pieces and boiled in an aqueous solution of 1 litre 0.3 % Na₂CO₃ (pH 11) with material to liquid ratio (MLR) 1:100 for 1 hour at 90°C to remove sericin. After completion of degumming the fibers were thoroughly washed with tap water and rinsed with distilled water and kept at oven at 60°C overnight for drying.

Accordingly for Eri, 10g cocoons were cut and made into small pieces and boiled in an aqueous solution of 1 litre of 0.3 % Na₂CO₃ (pH 11) with material to liquid ratio (MLR) 1:100 for 30 min at 90°C to remove sericin. After degumming the fibers were washed thoroughly with tap water and rinsed with distilled water and kept at oven at 60°C overnight for drying.

**Preparation of fibroin solution**

The degummed fibers were than dissolved in lithium thiocyanate. 1 g of degummed and dried Muga fiber was weighed and dissolved in 20ml of 10M LiSCN solution and put in a thermomixer at 80°C for 3 hours at 750 rpm. For Eri fiber, 1g of fiber was dissolved in 20ml of 9M LiSCN and put in a thermomixer at 70°C for 2 hours at 750 rpm. The respective solutions were then dialysed against water at 4°C for 48 hours and pure fibroin solution was collected. The fibroin percentage was determined by
casting 1ml of fibroin solution onto Teflon coated plate and dried overnight as mentioned above. The concentration was adjusted to 1% by adding distilled water.

![Diagram showing extraction and purification of fibroin from cocoons]

**Fig. 2. Schematic diagram showing extraction and purification of fibroin from cocoons**

**Preparation of sericin solution**

Sericin form Muga and Eri cocoons were extracted by high temperature and pressure (autoclave) method. 10 g each of Muga and Eri cocoons were made into small pieces by cutting and dissolved in 1 litre of distilled water under universal autoclave (Equitron) condition (temperature 121°C and pressure 15 psi) for 20 min (MLR 1:100). After extraction the solution containing sericin was filtered and dried at room temperature until completely dried and the sericin powder weighed to determine the quantity of sericin.
Preparation of Starch solution

Starch was isolated from the rice by a method modified from the alkaline steeping method of Wang and Wang et. al. (2001). Briefly, the rice kernels were first moistened in excess water at 10°C and then wet ground in a mixer (Philips juicer mixer grinder HL1632) for 2 min. The crushed mass was then filtered through a standard sieve of size 100 µm and the filtrate was centrifuged (Hettich Zentrifugen EBA 21) at 2000 rpm for 4 min. The remaining pellet was resuspended in a 0.5 M solution of sodium thiosulfate (1:1 residue to solution) for 36 h, with continuous stirring to remove protein and then centrifuged at 3000 rpm for 5 min. The brown coloured protein layer formed at the surface of the white residue was carefully removed. The residue was then neutralised.
with 0.1 M hydrochloric acid and washed with distilled water two times. It was further washed with 50% ethanol two times to remove any ions. After collection, the material was dried under vacuum at 30°C, ground in a laboratory grain mill (Fritsch Pulverisette 14), passed through 75 µm sieves and stored at 4°C.

**Preparation of Fibroin Film from fibers**

Before casting the fibroin films from solutions, the concentration was adjusted to 1% by adding distilled water. Films were cast according to the above mentioned procedure on Teflon coated plate as the base, at 25°C and 65% relative humidity and air-dried under a laminar flow for 12–14 h. After that the films were carefully removed with forceps and kept in desiccators for further use.

The fibroin films prepared from liquid and fiber proteins were treated with 70% methanol for 2 hours to induce crystallinity and kept in dessicator for further use. *B. mori* fiber was also dissolved and film prepared by the above mentioned procedures for comparative studies with the non-mulberry counterparts.

**Preparation of films from the blending of sericin and starch**

The detailed procedure employed for fabrication of the film samples are given in Fig 4. Briefly, 5g each of sericin and starch powder were dissolved in 100ml of double distilled water at 95°C and stirred continuously for 30 min. The solution was then divided into three parts and 1ml each of glycerol and PEG were added to two of them and further stirred for 10 min. No cross linker was added to the third part. The concentration of respective solutions was determined by casting 1ml of solution onto
Teflon coated plates and taking the weight of the film after drying at 25°C and 65% relative humidity and air-dried in normal conditions under laminar flow for 12–14 h.

The final concentration of the solutions was made 1% by adding required amounts of distilled water. The films were prepared by casting 1.5 ml each of sericin+starch (SS), sericin+ starch+ glycerol (SSG) and sericin+ starch+ PEG (SSP) solutions onto Teflon coated plates and dried overnight at 37°C and 65% relative humidity. The films were carefully peeled off with a pair of forceps, dried in a desiccator and stored at 4°C for further analysis.

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**Fig. 4 Flow Chart showing the steps involved in preparation of thin films from sericin and starch**
Fabrication of microparticles from fibroin protein

Fibroin from Muga and Eri was used for fabrication of microparticles. Fibroin microparticles were fabricated using the wet milling procedure of Rajkhowa et al. (2008). For comparison B. mori fiber was also used in the study. Briefly, after degumming silk fibers were cut in a cutter mill (PulvErisette 19 from Fritsch, Germany) fitted with a 1 mm grid and operating at 2888 RPM. Wet milling of chopped leftovers was done in a stirred media mill (1S Attritor from Union Process, USA) with a stirring speed of 280 RPM. The media used for milling consisted of 20 kg yttrium doped zirconium oxide balls of 5 mm diameter and the time for wet milling was 6 hour for Muga and Eri, but 10 h for B. mori. The batch size used was 200 g of snippets and 2 L of deionized (DI) water with cold water circulation (approximately 18 °C) through the vessel jacket was used during milling. Finally, a laboratory spray dryer (B-290, Buchi Labortechnik AG) was used to obtain dry powders from the wet milled slurry. The conditions used during spray drying includes: inlet temperature, 130 °C; pump setting, 25% (18–20 mL/min); and aspirator setting, 100% (42.5 m3/h).

Characterizations of the biomaterials

The films and microparticles fabricated by the above procedures were characterized by the following methods.
Surface Characterization

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) images of different films were obtained by JEOL JSM-6360 scanning electron microscope. The samples were gold coated by sputtering using incident electron beam energy of 15 kV and 6mm working distance.

SEM analysis of silk fibroin microparticles was done and the concentration of the solution was made 0.1 mg/ml. Microparticles solutions were dried in vacuum and sputter coated with gold and analyzed with JEOL JSM-6360. For each type of silk fibroin microparticles analysis a minimum of 20 particles was examined.

Transmission electron microscopy (TEM)

Microparticles from Muga and Eri fabricated by wet milling method was analyzed by Transmission Electron microscope for proper understanding of the particles. JEOL JEM-2100 model (TEM) was used to study the morphology and size of the particles. The acceleration voltage of 200 kv was used. The concentration of the sample was 0.1 mg/mL and the preparation was done by putting 200μl of the microparticle solution onto carbon coated electron microscopy grids. The grid was air dried, which contained the sample microparticles in a dust-free clean environment at room temperature before examination under the microscope.
In vitro stability of silk fibroin microparticles

The non mulberry silk fibroin microparticles solution was prepared in PBS (pH-7.4) and the concentration adjusted to 0.1g/ml. The solution was incubated at 37°C for 1 to 15 days. After microparticle incubation for 14 days at room temperature, the samples were assessed by scanning electron microscopy and compared to the day 1 sample for structural and morphological changes.

Dynamic contact angle test

The fibroin films and blend films from sericin and starch were used to determine the advancing and receding contact angles using the Dynamic Wilhelmy method (DCAT 11, Dataphysics) in water as well as in heptane, to determine the comparative solubility of the films. The film samples were cut into small square pieces of size 10 mm by 40 mm and treated with ethanol, which imposes the transition from amorphous to crystalline structures. Then the films were attached to the holder before dipping into the respective solutions.

Physicochemical Characterization

Fourier transform infrared spectroscopy (FT-IR)

Fourier transform infrared (FTIR) analyses of the different film samples were performed using a Bruker, vector 22 FTIR spectrometer. To nullify the moisture effect the samples were made completely dry by keeping in desiccators. In the absorbance
mode, the spectra were obtained in the spectral region of 400–4000 cm\(^{-1}\). Each spectrum of the samples was a accumulated scan of 32 scans with a resolution of 4 cm.

Similarly, FTIR measurements for microparticles were made in the spectral region 400–4000 cm\(^{-1}\). For each measurement 32 scans were co-added and Fourier transformed at a resolution of 4 cm\(^{-1}\). All samples were measured by the absorbance method. All the spectra were recorded at room temperature and analyzed with Origin Version 6.0.

**X - Ray Diffraction (XRD)**

Wide-angle XRD diffractograms of the film different samples were measured by an X-ray diffractometer (PANalytical, X’PertPRO PW3040/60) using CuK\(\alpha\) radiation \((\lambda = 1.54 \text{ Å})\) in the 2\(\theta\) range of 5–40\(^{0}\). The voltage used was 40 kV and current of the X-ray source was 40 mA. The samples were done at room temperature. The film samples were prepared in the form of small pieces and pressed onto a conventional glass sample holder. Muga and Eri fibers were also done for comparison; fibers were chopped into small pieces and pressed into a die press using appropriate binder resulting in the form of a pellet. The pellet was put in the X-ray diffractometer and X-ray spectra were recorded at a scan step of size 0.1.

**Differential scanning calorimetry (DSC)**

Differential scanning calorimetry was performed to assess the thermal properties of the films and microparticles. DSC measurements of silk fibroin films and
microparticles were performed on a Perkin Elmer, DSC 6000. Dry nitrogen gas was used with a flow rate of 50 mL/min. The temperature range for the analysis was from 30 to 400 °C and the heating rate was 5° C/min.

**Thermogravimetric analyses (TGA)**

The weight loss percentage with increase of temperature of the samples containing fibroin films and microparticles were determined by Thermogravimetric measurements (TGA), using a TGA 4000 (Perkin Elmer, USA) system. The temperature range used for heating was from 30 to 400° C with a steep increase of 2° C min⁻¹ under an inert nitrogen gas atmosphere.

**Biocompatibility of films and microparticles**

**Maintenance of cell lines**

L929 mouse fibroblast cells were used in this study. Mouse fibroblast L929 cells were procured from the National Centre for Cell Science, Pune, India. The cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 2 mg ml⁻¹ sodium bicarbonate, 200 mM L-glutamine, and antibiotics of 100 µg ml⁻¹ penicillin/streptomycin. The cell culturing was done in 25 cm² flasks at 37° C in a humidified atmosphere of 5% CO₂. Confluency of the monolayers was disintegrated by treatment with sterile phosphate-buffered saline (PBS) and 0.05% trypsin/EDTA solution, and the replacement of the culture medium was done every 3 days.
**In vitro Cell culturing on films**

Silk gland protein fibroin (liquid silk) film, fibroin regenerated solution film were cut into circular shape of 1.2 cm diameter and cast on the bottom of 24-well tissue culture plate (BD Falcon) wells. The circular matrices/ films were sterilized and crystallized with 70% alcohol and then rinsed with sterile PBS extensively, three times. 0.2% BSA was used to block the uncoated wells surface by incubating the cells followed by washing three times with sterile PBS. Before culturing of cells, the protein coated wells were pre-wetted by immersion in DMEM for 12 h in the 37°C incubator. Cells were plated at a density of $1 \times 10^4$ cells per ml of DMEM after trypsinization, into the pre-wetted protein coated 24-well culture plates in triplicates. Culturing of the cells were done for 1, 3, and 5 days, cells were evaluated for changes in morphology with respect to a control (cells grown without materials) under inverted phase contrast microscope (Olympus CKX41) attached with an imaging camera. The images were captured using imaging software Optika vision-pro.

**In vitro cell culture using fibroin microparticles**

Silk fibroin microparticles were analyzed for biocompatibility by studying growth and proliferation of L929 fibroblast cells with the incorporation of the microparticles. The fibroblasts were seeded at a density of $1 \times 10^4$ cells/well in a tissue culture plate (24-well) with complete DMEM/F-12 media for 24 h. Fresh medium along with different concentration of fibroin microparticles was replaced with old medium.
Media containing only the cells, but without any microparticles incorporate served as control.

**Live dead analysis of cells by fluorescence imaging**

To analyze the viability of the cells when grown in films as substrate and with the microparticles, a live dead analysis was performed using fluorescence imaging. Two DNA binding dyes, acridine orange and ethidium bromide was used to stain the cells following the method of Zhang et al., (1998). The cells were grown in the films and microparticles and allowed to attain a confluency of 60-70%. After that cells were washed in cold PBS and then stained with a mixture of acridine orange (100 μg/ml) and ethidium bromide (100 μg/ml) at room temperature for 10min. After staining the cells were washed twice with 1X PBS and observed under a fluorescence microscope in blue filter (Olympus CKX41 with Optika Pro5 camera).

**Cell viability by MTT Assay for films and microparticles**

MTT assay was performed to assess the cell viability and cytocompatibility of the L929 cells when grown on the fibroin matrices and with the microparticles. MTT assay is carried out to measure mitochondrial cellular metabolism (viability) and number of viable cells. MTT assay is based on the capability of metabolically active fibroblast cells to reduce the yellow water-soluble tetrazolium salt (MTT) into purple formazan crystals using the mitochondrial enzyme succinate dehydrogenase (SDH). The number of viable cells is directly proportional to the intensity of purple colour formed. Mouse fibroblast cells (L929) were seeded at a density of (1 × 104 cells/well) into 24-well
plates and incubated with 5% CO$_2$ at 37$^\circ$C. After 24 hour of cell seeding, replacement of the culture media was done with fresh media containing microparticles with different concentrations (25, 50 and 100 μg/mL) and incubated for 48 h. After 48 h of incubation with microparticles, 100 μl of the MTT dye solution was added (5 mg/ml in PBS and filtered through a 0.2 μm filter before use, pH 7.4) in each well and the whole content was again incubated at 37$^\circ$C for 3h. After this the MTT solution was removed and 300 μl DMSO was added to each culture well. Incubation of the whole thing was done at room temperature for 30 min. After 30min, the cells were lysed and a homogenous colour was obtained. The solution was then centrifuged to remove the cell impurities for 2 min. The optical density (OD) was measured spectrophotometrically at 540 nm. Cells that were treated with MTT solution without films and microparticles was used as control. Films and microparticles treated with DMSO were used as blank. The % viability was calculated as follows.

\[
\% \text{ Viability} = \frac{\text{OD of test} - \text{OD of blank}}{\text{OD of control} - \text{OD of blank}} \times 100
\]

The cell viability of the cells on the tissue culture plate without the microparticles is considered to be 100% and treated as the control. With respect to the control, the relative cell viability of silk fibroin films and microparticles was calculated. For each experiment n = 6 samples were used and ± standard deviation calculated. To determine the statistical significance of the results, one way analysis of variance (ANOVA) was calculated.
Biodegradability of Fibroin and Sericin films by enzymatic methods

Degradability of the silk fibroin films prepared from gland silk and regenerated fiber solutions of Muga and Eri and the sericin starch blends were determined using two protease enzymes viz. collagenase type F and protease type XXI.

The films of weight 0.5g were made insoluble by treating with 70% ethanol and were incubated at 37°C in 40 ml of phosphate-buffered saline (PBS) containing 1mgml⁻¹ of collagenase type F and protease type XXI enzymes in different sets. To each solution an approximately equivalent mass (5 mg) of silk film was added. Solutions containing fresh enzyme was replaced and sample weight were collected daily. At designated time intervals the samples were thoroughly rinsed with distilled water and completely dried for taking the weight of the films. Samples immersed in PBS but without the presence of enzymes served as controls. The observation was carried on for 3 days.

Statistical Analysis

All data presented in the study were reported as mean ± standard deviation. For each experiment, sample size n = 6 were used and each experiment was run at least thrice. A single-factor analysis of variance (ANOVA) technique was used to determine the statistical significance of the results. SYSTAT 10.2 (Richmond, USA) was used to execute all statistical analyses. The different groups of samples that show p < 0.05 are considered to be statistically significant and those that show p < 0.01 are considered to be highly significant.