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Silk fibroin protein from mulberry and non-mulberry silkworms: cytotoxicity, biocompatibility and kinetics of L929 murine fibroblast adhesion

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Abstract Silks fibers and films fabricated from fibroin protein of domesticated mulberry silkworm cocoon have been traditionally utilized as sutures in surgery and recently as biomaterial films respectively. Here, we explore the possibility of application of silk fibroin protein from non-mulberry silkworm cocoon as a potential biomaterial aid. In terms of direct inflammatory potential, fibroin proteins from *Antheraea mylitta* and *Bombyx mori* are immunologically inert and invoke minimal immune response. Stimulation of murine peritoneal macrophages and RAW 264.7 murine macrophages by these fibroin proteins both in solution and in the form of films assayed in terms of nitric oxide and TNFα production showed comparable stimulation as in collagen. Kinetics of adhesion of L929 murine fibroblasts, for biocompatibility evaluation, monitored every 4 h from seeding and studied over a period of 24 h, reveal *A. mylitta* fibroin film to be a better substrate in terms of rapid and easier cellularization. Cell viability studies by MTT assay and flow cytometric analyses indicate the ability of fibroin matrices to support cell growth and proliferation comparable to collagen for long-term culture. This matrix may have potential to serve in those injuries where rapid cellularization is essential.

1 Introduction

The in vitro attachment and spreading of mammalian cells on surfaces is a rather complex and versatile process depending on cell type and extracellular matrix (ECM) molecules involved in the process, and strongly affected by culture conditions. Different molecular mechanisms responsible for cell attachment and spreading give valuable insight into the interactions of cells with the ECM components and surfaces [1]. While cell/ECM interactions are highly important in various physiological processes such as cell differentiation, tissue regeneration or cell migration in tumour metastasis [2], cell–substrate interactions mediated by ECM proteins also determine biocompatibility and potential use in biomedical applications [3].

Cell–substrate interactions are mediated by extracellular cues such as cytokines or growth factors that induce changes in the cytoskeleton, cell substrate adhesion, focal contact points, and the extracellular matrix [4]. A prospective biomaterial has to undergo extensive in vitro and in vivo testings and should ensure non-toxicity to the biological environment where it is being applied [5]. In addition, it should not elicit appreciable macrophage and cytokine response. Macrophages are involved in the host defense and play an important role in immune regulation. They induce generation of nitric oxide, a high level of which could lead to a rejection of a biomaterial during in vivo studies. It, therefore, becomes essential to characterize the material factors determining the host response and to design new strategies for minimizing both acute and chronic inflammatory responses against implanted biomaterials.

Silk fibroin, a major protein component of cocoon spun by silkworms, has found diverse applications in the biomedical field which can be attributed to its high tensile strength, controllable biodegradability, haemostatic properties, non-cytotoxicity, low antigenicity and non-inflammatory characteristics [6–9]. The major bulk of silk is produced by two different categories of silkworms: the domesticated mulberry silkworm; and the wild non-

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mulberry silkworm. Mulberry silk, mainly produced by *Bombyx mori*, has been exploited for its use in surgery and tissue engineering. In general, *Bombyx mori* silk cocoons are composed of 72–81% of a fibrous protein fibroin core and a surrounding 19–28% glue protein sericin [10–12]. The major biomedical applications of silk revolve around fibroin, which is a hydrophobic protein with a molecular mass of around 400 kDa [13]. *B. mori* fibroin has been utilized for osteoblast, fibroblast, hepatocyte and keratinocyte adherence and growth [14–17], substrates for enzyme immobilization [18] and has also been used as an alternative to collagen in tissue engineering and surgery [19].

Non-mulberry silk fibroin, generally produced by wild or semi-domesticated Saturniid silkworms, namely *Antheraea* sp. (wild) and *Philosamia ricini*, differ from the mulberry silk fibroin in various aspects [20, 21]. Among these silkworms, *Antheraea mylitta*, a tropical non-mulberry *tasar* silkworm, has the greatest capacity for silk production among all silk-spinning insects [22, 23]. 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 [24]. *A. mylitta* cocoons collected from the wild habitats are found to be much tougher [25], mainly ascribed to sericin, the hydrophilic protein component [26]. Fibroin, the major constituent, makes a significant contribution to the composition of the cocoon. Since very little is known about physicochemical properties and biomedical usefulness of *A. mylitta* fibroin, the major biomaterial applications of fibroin revolve around *B. mori* silk fibroin.

In this study, we have evaluated the macrophage responses elicited by non-mulberry silkworm *Antheraea mylitta* fibroin protein and biocompatibility of these fibroin films by adherence of L929 murine fibroblasts as well as compared them with mulberry silkworm *Bombyx mori* fibroin protein and collagen matrices. Fibroin and collagen matrices have also been subjected to a study on comparative kinetics of adhesion of L929 dermal fibroblasts to help in formulation of dermal coverings or matrices which support a high cellularization potential with dermal cells at the time of wound recovery.

2 Materials and methods

2.1 Materials

Fresh cocoons of the non-mulberry tropical tasar silkworm *Antheraea mylitta* and mulberry silkworm *Bombyx mori* were obtained from the Midnapore district of West Bengal state in India. Tasar and mulberry silk fibers were obtained from Central Tasar Research and Training Institute, Ranchi, Jharkhand, and Central Sericulture Research and Training Institute, Berhampore district, West Bengal, respectively. Sodium bicarbonate, sodium carbonate, thiazoyl blue (MTT), urea, sodium dodecyl sulphate, N-1 napthylethylenediamine, sulphanilamide and ortho phosphoric acid (Analar grade, SRL, India), lithium bromide and cell culture grade chemicals (HiMedia Chemical Laboratories, India), fetal bovine serum (Invitrogen, USA), cellulose dialysis tubing (12 kDa exclusion limit, Sigma), collagen from calf skin and lipopolysaccharide from *E. coli* (Sigma, USA), tissue culture grade polystyrene-coated plastic flasks and plates (Tarsons, India) and Centricon (30 kDa exclusion limit Millipore, USA) were used.

2.2 Protein extraction

Silk protein fibroin was isolated from mulberry and non-mulberry silk cocoons following a standard extraction procedure [27]. Briefly, cocoons were cut into small pieces of approximately 1 cm × 1 cm dimensions. They were boiled in an aqueous solution of 0.02 M Na_2CO_3 for half an hour (around 10% w/v of silk cocoons and Na_2CO_3) to remove all traces of sericin for complete degumming of the shells. The cocoon pieces were subsequently washed thoroughly in distilled water and dried briefly for 1 h at 50°C. Degummed shell pieces were dissolved in 9.3 M lithium bromide for 12 h at room temperature and then centrifuged to remove the insoluble material. The supernatant was dialyzed extensively for 3 days against deionized water with frequent changes. The protein concentration was then measured using Bradford standard assay procedure [28].

2.3 Fabrication of films

2.3.1 Fibroin films

For cell culture studies, the fibroin isolated from both *A. mylitta* and *B. mori* cocoons were passed through a filter with a pore size of 0.22 μm. These films were cast onto the surfaces of 12-well tissue culture plates. An equal number of wells containing *A. mylitta* fibroin, *B. mori* fibroin, and control wells with original polystyrene coating were utilized. As standardized in the case of AH927 fibroblast cells (unpublished observations); a concentration of 100 μg/ml of protein was considered optimum for studying L929 adhesion on silk matrices. The plates were kept under laminar flow for 6–8 h for drying, and the films were rendered insoluble by methanol treatment before being washed with phosphate buffered saline. They were blocked using 0.02% BSA in sterile phosphate buffered saline for 1 h, and the excess BSA was removed by washing three times in phosphate buffered saline. The film-coated culture plates were then used in cell culture studies.

2.3.2 Collagen films

Commercially available collagen from calf skin (Sigma, USA) was dissolved at a concentration of 100 μg/ml in 0.1% acetic acid. This solution was cast within the wells of a 12-well tissue culture plate, dried under laminar hood and treated thereafter in the same way as fibroin films described above.

2.3.3 Films for scanning electron microscopy

Collagen and different fibroin films (*A. mylitta* and *B. mori*) were cast on the surfaces of cover glasses kept within the wells of a 12-well tissue culture plate. These films were dried, blocked and treated using the same procedure as for the other films described above.

2.4 Peritoneal macrophage stimulation

Macrophages were isolated by peritoneal lavage of Swiss albino mice using 1 × PBS (pH 7.4). Approximately 1×10^6cells/ml was taken in RPMI medium and 180 μl of medium containing cells were seeded onto each well of a 96-well plate. 20 μl of protein was added to it at a concentration of 1 μg/μl. The cells were cultured for 24 h at 37°C in a humidified 5% CO_2 incubator. For estimation of nitric oxide production under the influence of different stimulants, the cell concentrations were selected on the basis of values of nitric oxide production that was within the range of the standard curve for Griess reagent.

2.4.1 Nitric oxide production using Griess reagent

Production of nitric oxide was estimated by measuring nitrite levels in the supernatant with the Griess reagent. Griess reagent (1:1 ratio of 0.1% of N-1 napthylethylenediamine and 1% sulphanilamide in 5% phosphoric acid) was added to 100 μl of sample cell supernatant and was incubated at room temperature for 10 min. Absorbance was measured at 550 nm. The absorbance values were converted to their corresponding concentration of nitric oxide produced using a standard curve for $NaNO_2$.

2.5 RAW 264.7 stimulation

Peritoneal macrophages could only be monitored for a day as they cannot be sustained in vitro culture medium for a longer period. Long-term culture studies were conducted with RAW 264.7, a murine macrophage cell line. In order to study RAW 264.7 macrophage activation by fibroin, two different experimental protocols were designed. For both short- and long-term culture of macrophages, fibroin was tested in solution as well as in the form of films along with collagen solution and films. LPS was added in both liquid and film experimental sets to act as positive controls [29].

RAW 264.7 murine macrophage cells were plated at a density of 2×10^5 cells per ml in a 12-well format for 24 h prior to stimulation. These macrophages were stimulated with the different fibroin and other preparations like collagen and lipopolysaccharide (LPS) (100 ng/ml) for 1 or 7 days as specified. Cell supernatants were collected and assayed for tumor necrosis factor alpha (TNFα) at the end of specified incubation time.

The different silk fibroin and collagen films were also tested in the 12-well format, where RAW 264.7 cells were plated at a density of 2×10^5 cells per film. Cells were plated and incubated for 1 and 7 days on films to estimate the TNFα production during long-term culture on these films. In both experimental protocols for RAW 264.7 macrophage activation study, only those cell concentrations were selected which gave TNFα production values within the range of the standard curve generated with recombinant TNFα.

2.5.1 Determination of tumor necrosis factor alpha (TNFα) release

Determination of TNFα production was carried out by sandwich ELISA carried out under the manufacturer's protocol (Amersham mouse TNFα Biotrak ELISA system, GE Healthcare, USA). The ELISA had a detection limit of 10 pg/ml. Briefly, cell supernatants were collected at specified time intervals and assayed for the production of TNFα. 96-well plates pre-coated with an antibody against mouse TNFα, acting as capture antibody, were taken and 50 μl of cell supernatants as well as recombinant mouse TNF α standards (50–2450 pg/ml) was added to different wells. 50 μl of a biotinylated second antibody against mouse TNFα was added to all the wells and the plate was incubated at room temperature for 2 h. After stringent washing using the wash buffer provided, streptavidin-HRP solution was added to the wells for binding with the bound biotin and the plate was incubated for 30 min. After another cycle of stringent washing, the wells were incubated for 30 min with 100 μl of TMB substrate in the dark. Thereafter, the reaction was stopped with 0.18 M sulphuric acid and absorbance was measured within 30 min at 450 nm using Multiskan microplate reader (Thermo Electron Corporation, USA). The TNFα release per well was calculated from the standard graph constructed with the recombinant TNFα standards, the average release and standard deviation for triplicates taken for each sample was determined.

2.6 L929 murine fibroblast cultivation

L929 murine fibroblast cell line (National Center for Cell Science, Pune) was used in this study. Cryopreserved vials

of L929 cells were revived using Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum, 1% UI/ml Streptomycin-Penicillin, and seeded on 25 cm^2 culture flasks. They were incubated at 37°C in an atmosphere containing 5% CO_2 where the culture medium was changed every 3 days. At confluence, the fibroblasts were harvested and sub cultivated in the same medium. For experiments, fibroblasts were used at passage 3 after revival from liquid nitrogen stock at approximately 10–12 days from revival. Depending upon the total surface area for cell attachment, different cell numbers of L929 cells were seeded for different experiments.

2.7 Kinetics of L929 adhesion on different matrices

2.7.1 Morphology of L929 cells at different time intervals

L929 fibroblasts were seeded at a density of 4×10^4 cells per film cast on cover glasses placed within the wells of a 24-well plate, after blocking and processing as described in the earlier sections. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. At intervals of 4–24 h from seeding; i.e. at 4, 8, 12, 16, 20 and 24 h; L929 cells on fibroin and collagen matrices were fixed with 2.5% glutaraldehyde in phosphate buffer and left for 12 h. They were next subjected to two washes in phosphate buffer, and dehydrated through alcohol series from 50% to absolute, each grade being 30 min. The cover glasses were then transferred to glass Petri plates and incubated in iso-amyl acetate for 5 min, wrapped in blotting paper and subjected to critical point drying in HCP-2 critical point dryer. Gold ion sputtering was carried out in a HITACHI E-101 ion sputterer maintained at 0.1–0.01 Torr vacuum for a uniform coating of 300–350 Å. Scanning electron microscopy was performed with a JEOL JSM-5800 scanning electron microscope. SEM results were obtained with incident electron beam energy of 1 keV and a working distance of 6 mm.

2.7.2 Viability of L929 cells at different time intervals

Cell viability was measured by 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay using MTT as a substrate. Briefly, 4×10^4 cells were seeded on different matrices: *A. mylitta* fibroin, *B. mori* fibroin and collagen within a 12-well plate. After 4, 8, 12,16, 20 and 24 h from seeding, supernatant of each well was replaced with equal volume of MTT (1:10 dilution in serum free medium; stock containing 4 mg/ml, SRL, India) and the plates incubated at 37°C for 4 h. At the end of the assay, the tissue culture plates were centrifuged at $2500 \times g$ for 5 min, supernatant was discarded and the blue formazan reaction product was dissolved by adding equal volume of DMSO. 200 µl was transferred to a 96-well plate and absorbance was measured at 595 nm using a Bio-Rad 550 spectrophotometric microplate reader.

For an estimation of long-term cell viability on different fibroin substrates, cells were seeded at a density of 1×10^5 cells per film cast within the wells of a 6-well plate. After 7 days of incubation, cell viability was tested as described above.

2.8 Flow cytometric analysis of cell viability

Flow cytometry permits simultaneous detection of various parameters like necrosis and apoptosis. In our study, this technique was used for quantification of cell viability. The intercalating blue-excited dye, propidium iodide (PI) was used in this study. PI is a vital stain frequently used to identify non-viable, dying, or necrotic cells, based on loss of membrane integrity which permits the stain to bind to nucleic acids in a cell.

L929 fibroblasts were seeded at a density of 2×10^5 cells per film cast within the wells of a 6-well plate, while uncoated tissue culture plates were taken as control. Samples were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 1 week. A high seeding density as well as greater incubation time on different substrates ensured a greater number of cells to be analysed by flow cytometry. After the incubation time elapsed, the cells were harvested in different tubes by scraping. The cell suspensions were washed twice with 1 × PBS (pH 7.4) by centrifugation and resuspended in a solution containing 50 µg/ml PI, RNase 0.1 mg/ml in 1 × PBS (pH 7.4). The suspension was incubated at room temperature for 30–40 min. The resulting suspension were analyzed with a flow cytometer (FACS Calibur, BD Biosciences) at a 560 nm band pass filter for red fluorescence of PI.

2.9 Statistical analysis

All data were expressed as mean ± standard deviation for $n = 6$ (MTT cell viability assays) and $n = 3$ (for flow cytometric analysis). Single factor analysis of variance (ANOVA) technique was used to assess statistical significance of results. Student's *t*-test was carried out at a level of 95% significance.

3 Results

3.1 Peritoneal macrophage stimulation assay using Griess reagent

Peritoneal macrophage stimulation of Swiss albino mice, studied using Griess reagent, by the fibroin proteins as well as collagen solution showed a very low nitric oxide production.

However, macrophage stimulation by lipopolysaccharide (LPS) was considerable as reflected in the high nitric oxide production (Fig. 1).

3.2 RAW 264.7 macrophage stimulation assay by Tumor necrosis factor alpha (TNFα) release

RAW 264.7 macrophage activation was studied in two different experimental protocols (Fig. 2a, b). Estimation of short- and long-term release of TNFα was carried out over a period of 1 and 7 days respectively using fibroin and collagen proteins in solution as well as in the form of films, with LPS as positive controls. It was seen that RAW 264.7 cells stimulated with collagen solution and fibroin solutions from both *A. mylitta* and *B. mori* cocoons elicited very low levels of TNFα release after 1 day of incubation while strongly responding to LPS stimulation ($P < 0.01$). After 7 days of culture, this trend was repeated and the fibroin solutions continued to show low stimulation comparable to collagen, while differing significantly with respect to level of response with LPS controls (Fig. 2a).

Similar trend was observed when fibroin and collagen were tested in the format of films in 12-well plates. *A. mylitta* and *B. mori* fibroins showed very low stimulation of RAW 264.7 macrophages, comparable to collagen, while LPS elicited a very high TNFα release (Fig. 2b). At both days 1 and 7, the pattern was repeated and showed a very close TNFα release response as seen in stimulation using fibroin and collagen solutions.

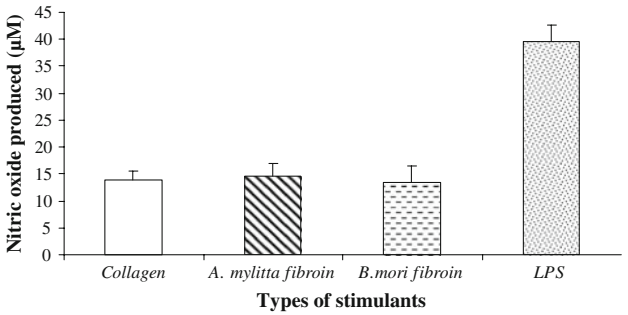

Fig. 1 Nitric oxide production by murine peritoneal macrophages. Peritoneal macrophages were stimulated with phosphate buffered saline (PBS), collagen solution; silk fibroin protein solutions from non-mulberry silkworm, *Antheraea mylitta* cocoon and mulberry silkworm, *Bombyx mori* cocoon; and lipopolysaccharide (LPS) from *Escherichia coli*

3.3 Kinetics of L929 adhesion on different matrices

3.3.1 Morphology of L929 at different time intervals

To assess the ease of adherence of L929 fibroblasts on different matrices, cells were monitored every 4 h from seeding. The morphology and the area of spreading of cells at different time points were noted (Fig. 3).Within 4 h, cells on *A. mylitta* fibroin matrix took on the regular spreading morphology of L929 cells while they remained rounded on collagen and *B. mori* fibroin matrices (Fig. 3a). The spreading area occupied by the cells at this stage on *A. mylitta* fibroin and collagen matrix was almost identical; while that on *B. mori* fibroin was lower (Fig. 3b). *A. mylitta* fibroin matrices showed a rapid pattern of cell adhesion and spreading than that seen on *B. mori* fibroin matrices. This rapid adherence and spreading was comparable to collagen

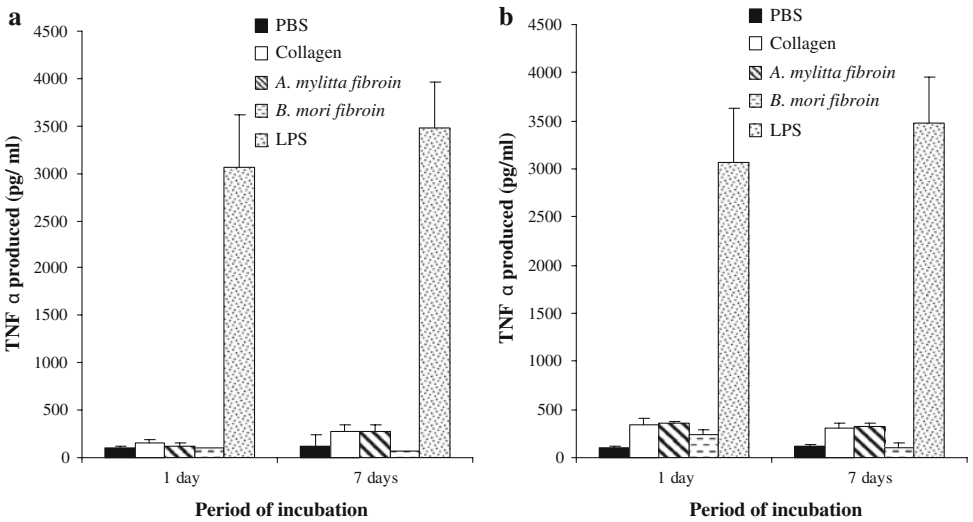

Fig. 2 (**a** and **b**). TNF α production by RAW 264.7 murine macrophages. The TNFα produced by the cells was estimated from a standard graph using recombinant TNFα standards. RAW 264.7 cells were stimulated with collagen; silk fibroin from non-mulberry silkworm, *Antheraea mylitta* cocoon and mulberry silkworm, *Bombyx mori* cocoon in (**a**) liquid form; and (**b**) in the form of films on which cells were seeded. Lipopolysaccharide from *E. coli* was added (100 ng/ml) to each experimental set as a positive stimulant. Error bars represent the standard error of the mean (n = 4)

matrices taken as control. After 12 h of seeding, fibroblasts on collagen and *B. mori* fibroin matrices started showing similar spindle-shaped morphology as those on *A. mylitta* fibroin matrix. However, even at this stage the spreading area occupied by cells on *A. mylitta* fibroin matrix was significantly higher ($P < 0.05$) as compared to the other matrices. After 20 h of seeding, collagen matrix showed similar spreading area as *A. mylitta* fibroin matrix while *B. mori* fibroin showed a much lower spreading area ($P < 0.05$). After 24 h, all matrices were equally cellularized.

3.3.2 Viability of L929 at different time intervals

Cell viability at different time intervals represents the active mitochondrial enzymes present in a cell capable of reducing MTT. It was seen that in the initial stages of cell adhesion, i.e. at 4 and 8 h, the number of viable cells on *A. mylitta* fibroin matrix was comparable to that on tissue culture polystyrene control (TCPS) which showed maximum number of viable cells (Fig. 4a). Moreover *A. mylitta* fibroin matrix showed significantly higher viability of seeded cells than collagen and *B. mori* fibroin matrices. After the initial adherence, cell viability on collagen and fibroin matrices remained more or less constant till 16 h though cell viability on TCPS kept increasing. After 24 h of seeding, it was seen that collagen and *A. mylitta* fibroin matrices showed comparable cell viability while *B. mori* showed significantly lower viability than *A. mylitta* fibroin ($F = 10.38, P < 0.05$). TCPS showed maximum number of viable cells and it was significantly higher than all other matrices chosen for the study.

Long term observations on the ability of these matrices to support cell viability and proliferation showed that after 7 days of incubation, *A. mylitta* fibroin and TCPS exhibited similar trend in supporting L929 proliferation (Fig. 4b). Collagen and *B. mori* fibroin matrix showed lower cell viability than TCPS control though this difference was not

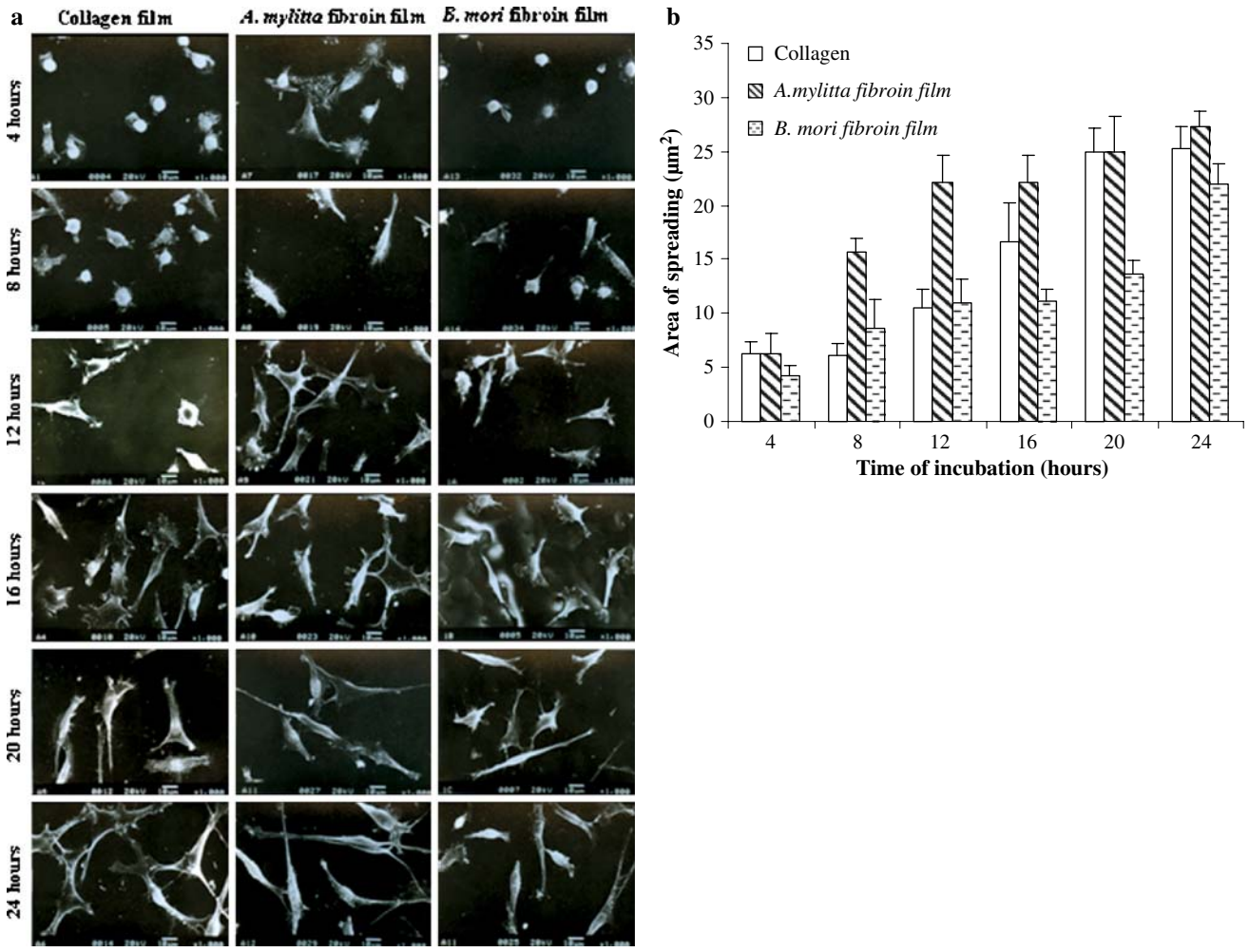

Fig. 3 (**a** and **b**) Kinetics of adhesion of L929 murine fibroblasts on different matrices. L929 cells were monitored at every 4 h-interval from seeding. (**a**) Scanning electron microscopy depicting morphology of cells seeded on collagen, *A. mylitta* (non-mulberry silkworm) fibroin and *B. mori* (mulberry silkworm) fibroin matrices, (**b**) Area of spreading of cells on different matrices at each time point of observation. Spreading area is represented as mean ± standard error of three fields studied for each type of matrix

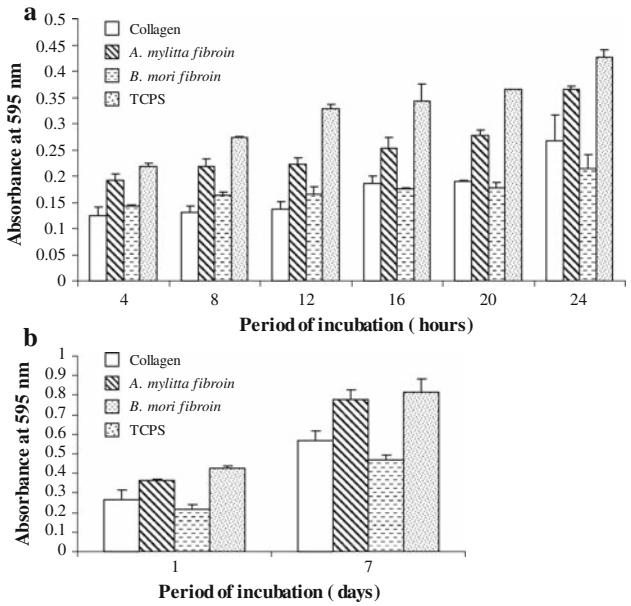

Fig. 4 (**a** and **b**). Viability of L929 murine fibroblasts on different matrices. MTT assay for cell viability was carried out for L929 cells seeded on collagen, *A. mylitta* fibroin, *B. mori* fibroin and tissue culture polystyrene (TCPS) matrices. Cell viability of L929 fibroblasts assessed (**a**) at every 4 h interval from seeding, till 24 h; (**b**) long-term (7-day) culture

significant. Therefore, it is assumed that on long-term culture these matrices are comparable in their ability to act as support matrices for fibroblasts.

3.4 Flow cytometric analysis

Using single staining of cells with propidium iodide, the damaged or non-viable cells on different substrates was estimated. As permeabilization of membrane was not carried out, it was assumed that propidium iodide would penetrate to the nucleic acids in those cells possessing damaged cell membranes or other dead non-viable cells. The histogram analysis of L929 cells on each matrix under study (Fig. 5) showed a population of stained cells when fluorescence intensity was plotted as a function of the population of cells. The pattern of distribution of cells on *A. mylitta* and *B. mori* fibroin matrices revealed two different populations of cells; one stained population close to the origin of the x-axis and a stained population progressing along the x-axis (Fig. 5b, c). In case of collagen film, the range of fluorescence intensity of stained cells (Fig. 5a) was comparable to that seen in the cells taken from fibroin matrices. The tissue culture polystyrene matrix showed slightly lower number of unstained cells (Fig. 5d).

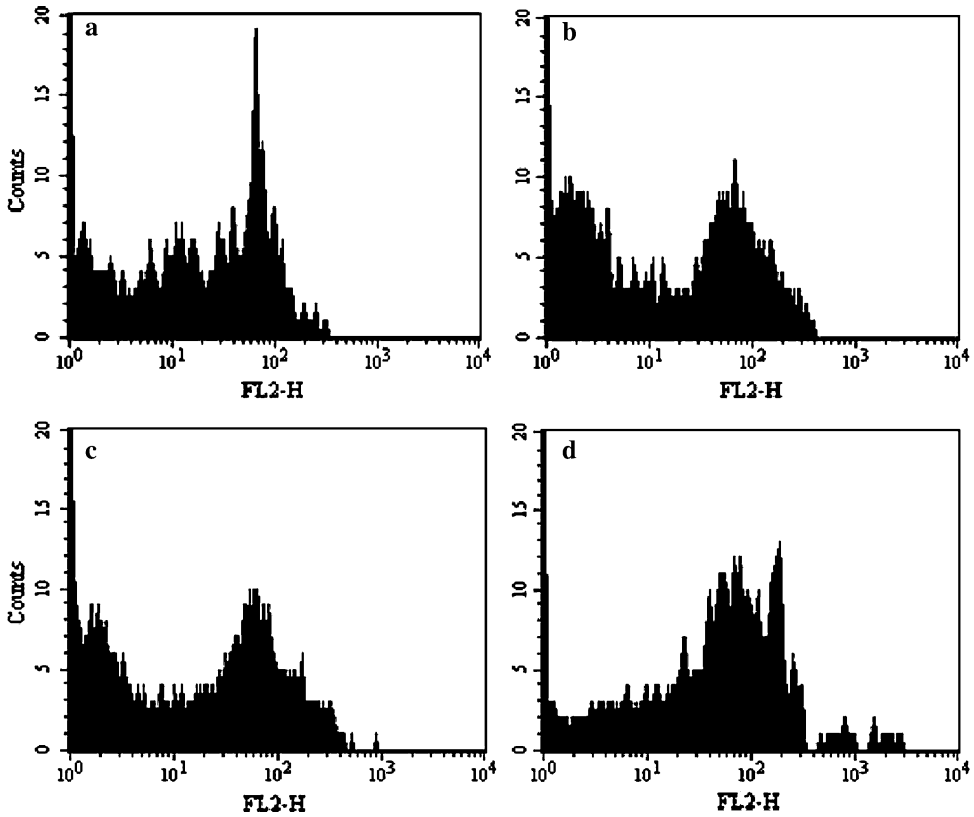

Fig. 5 (**a**–**d**). Flow cytometric analysis of L929 cells growing on different matrices, with non-viable cells labeled with propidium iodide (PI). A total of 5,000 cells were studied to analyze the fluorescence intensity of stained cells on each matrix. Histogram analysis of propidium iodide fluorescence intensity versus number of cells counted shows the number of cells stained with PI on different matrices; (**a**) Collagen, (**b**) *A. mylitta* fibroin, (**c**) *B. mori* fibroin, (**d**) tissue culture polystyrene

Table 1 The percentages of stained and unstained L929 fibroblasts cultured for 7 days on different matrices (n = 3)

Percentage of cells	Collagen film	*A. mylitta* fibroin film	*B. mori* fibroin film	Tissue culture polystyrene
Unstained viable cells	67.0	81.0	80.0	82.0
Stained non-viable cells	32.0	18.0	19.0	17.0

To further corroborate this data, percentage of unstained and stained cells on each matrix was calculated. The quantitative data (Table 1) showed a comparable number of cells on collagen, *A. mylitta* and *B. mori* fibroin matrices. Tissue culture polystyrene matrix showed a lower population of stained cells and more number of propidium iodide stained non-viable cells. However, this difference in cell number between the supporting matrices was not significant ($P > 0.05$).

4 Discussion

There is a continuous search for new biocompatible materials with better qualities that may serve the purpose of various tissue engineering applications. A significant issue to consider regarding the use of any biomaterial is the potential activation of the immune system. The innate immune system has an important role in determining the course of a potential immune response to any foreign substance in a host [19].

In this study, the innate immune response, specifically of macrophages to different fibroin solutions and films was studied. Collagen is very well recognized and important in the field of biomaterial engineering [30]. Therefore, it was used as a standard for our study and showed very low levels of macrophage stimulation. It was believed that particular wear debris of phagocytosable size generated from prosthetic materials was one of the main factors activating tissue macrophages [31, 32]. Moreover, internalization of particles is not required and it is suggested that the particle–cell membrane interaction, known as a 'frustrated phagocythosis', is sufficient to induce the cells' response [33, 34]. Therefore, the studied materials are added to the macrophage cultures in the form of solution, assuming that the response of macrophages would better reflect the complexity of in vivo conditions than if powders of particles of phagocytosable size are used [35]. Peritoneal macrophages were isolated from mice to study nitric acid production in response to silk solutions to mimic the in vivo conditions to the closest approximation. In the non-stimulated control (treated with PBS, pH 7.4) there was minimal nitric oxide production while that for lipopolysaccharide (LPS; positive control, 100 ng/ml) was very high (Fig. 1). The solutions under investigation, i.e. *A. mylitta* and *B. mori* fibroins, showed a higher response than unstimulated control, but were comparable to collagen with respect to generation of nitric oxide radical due to stimulation ($P > 0.05$, difference was not significant).

A murine macrophage cell line RAW 264.7 was monitored for stimulation by studying the level of release of tumor necrosis factor alpha (TNFα), a marker for acute and chronic inflammatory reaction, into the medium [36] over a period of 7 days under the influence of fibroin, collagen and LPS. After 1 day, *A. mylitta* and *B. mori* fibroins elicited a comparable TNFα generation to collagen while that for LPS was considerable (Fig. 2a). This trend was similar to that obtained in the stimulation of peritoneal macrophages. After maintaining the culture for 7 days with the different protein as well as LPS solutions, it was seen that RAW 264.7 elicited a very high TNFα secretion in LPS controls. There was not much variation in the levels of TNFα production in 1 and 7 days on stimulation with fibroins and collagen. A similar result was obtained by studying RAW 264.7 stimulation using nitric oxide assay by Griess reagent (data not shown). It could therefore be concluded that these protein solutions would not elicit significant macrophage response in short- as well as long-term culture [19].

Apart from the innate immune response generated against protein solutions, it is also necessary to evaluate the behaviour of films as these constitute a major form of execution of biomaterials. The essence of a film as a biomaterial lies in the study of the interaction between its surface and cells. On short-term (1 day) culture, it was observed that RAW 264.7 macrophages seeded on *A. mylitta* and *B. mori* fibroin films showed very low TNFα production (as compared to that generated by LPS treatment), comparable to collagen. Even after 7-day culture on different fibroin and collagen matrices, there was no appreciable TNFα response in *A. mylitta* fibroin, *B. mori* fibroin or collagen films (Fig. 2b). A film being a major niche of interaction between cells and biomaterial [37], *A. mylitta* and *B. mori* fibroin films invoked minimal immune response.

Kinetics of adhesion (short-term culture) was carried out between L929 murine fibroblast cells seeded on different matrices for assessment of biocompatibility. SEM images recorded for samples fixed every 4 h from the time of seeding, showed a variation in the initial spreading of cells. Within the initial 4 h of seeding, L929 cells on *A. mylitta* fibroin matrix showed a better spreading morphology while that on *B. mori* and collagen matrices, the cells showed a rounded morphology. This trend continued till the initial

12 h of adhesion (Fig. 3a). Thereafter, from 16 h of incubation, L929 cells seeded on all matrices showed spreading morphology, regular for fibroblasts. However, it was only after 24 h of incubation that the cells on collagen and *B. mori* fibroin matrices showed equivalent spreading to those on *A. mylitta* fibroin. This spreading morphology was reflected more or less accurately in spreading area of the cells where the cells on *A. mylitta* fibroin matrices always occupied a greater area on the film (Fig. 3b). There was a significant difference between area of spreading between *A. mylitta* fibroin; and collagen as well as *B. mori* fibroin films at 8, 12 and 16 h. Therefore, it could be assumed that in applications where a faster recovery of dermal fibroblast adhesion is essential, *A. mylitta* fibroin film would be more appropriate as a substratum.

For a quantitative assessment of cell adhesion and viability, MTT assay [38] was conducted every 4 h of seeding till 24 h. The ability of mitochondrial enzymes of viable cells on different matrices to reduce MTT substrate into blue formazan crystals was studied at 4 h intervals [39]. It was observed that *A. mylitta* fibroin films showed a higher ability to support viable cells than *B. mori* fibroin or collagen films from 4 to 24 h ($P < 0.05$). *A. mylitta* fibroin film supported higher cell viability than other films within 4 h; the viability on all films remained almost constant till 12 h of incubation while that on TCPS kept increasing. After 16 h of incubation, all the matrices show a gradual increase in supporting cell viability and after 24 h, *A. mylitta* shows comparable cell viability as TCPS and *B. mori* shows comparable viability to collagen matrix (Fig. 4a). The increased adherence, spreading and proliferation of cells on *A. mylitta* fibroin films can be attributed to the presence of the tripeptide Arg(R)-Gly (G)-Asp (D) [40], a recognition site for integrin-mediated cell adhesion [41–43]. RGD tripeptides are characteristic sequences of integrins and are known to enhance the cell adhesion property. Sequences lacking this tripeptide exhibit lower cell adhesion compared to sequences where RGD peptides are grafted or attached. As reported in the fibroin sequences of other wild *Antheraea* sp. silkworms, the fibroin gene fragments of *A. mylitta* code for numerous RGD peptides (***AY136274***). However, such a tripeptide was not found in the fibroin of the domestic silk worms [44] like *B. mori*. As cell proliferation was also higher in case of *Antheraea pernyi* than *B. mori* [44], we could hypothesize that the immunological response of fibroin from these two species, *A. pernyi* and *A. mylitta*, as well as other species under Genus *Antheraea* would be similar. Likewise, the response of other domesticated silkworms would also be similar to that observed in *B. mori*.

Establishment of a biomaterial requires a longer term evaluation; therefore, all the matrices were subjected to cell viability assays after 7 days of seeding. It was seen that though variations existed after 1 day of incubation, on long-term culture, most of these variations in the ability of different matrices to support L929 proliferation were removed. Though TCPS still showed highest cell proliferation and viability, *A. mylitta* fibroin film presented a comparable picture. *B. mori* fibroin as well as collagen showed lower proliferation and viability at 7 days (Fig. 4b) ($P < 0.05$).

Flow cytometric analysis of L929 cells stained with propidium iodide (PI) was carried out to ascertain the percentage of cells with/without perforated cell membranes (Table 1). The histogram plot of fluorescence intensity versus number of cells counted provides an estimation of the number of cells taking up a particular stain (Fig. 5) as PI can only enter through pores on the cell membranes. It was observed that *A. mylitta*, *B. mori* fibroin and collagen films show a similar histogram plot with two distinct populations representing PI unstained (towards origin of x-axis) and stained (progressing along x-axis) cells. However, the number of stained cells in TCPS control was greater than that in the other matrices [39, 45]. These cells could probably account for the recently permeabilized or ruptured cells which remain viable at the time of experiment. However, the difference in the number of viable cells in different matrices was not significant.

5 Conclusion

This study demonstrates that fibroin isolated from cocoons of both non-mulberry silkworm, *Antheraea mylitta* and mulberry silkworm *Bombyx mori* elicit very low macrophage stimulation. The innate immune system does not show any activation when fibroins are added in solution or are used as films. The kinetics of L929 murine fibroblast adhesion points towards the greater efficiency of adhesion and spreading of cells on *A. mylitta* fibroin. This may be an important factor in dermal dressings where a rapid cellularization is required. The matrices show comparable ability along with collagen to support long-term proliferation of cells. Flow cytometric analysis further corroborates our finding that *A. mylitta* fibroin is non-toxic to dermal fibroblasts and acts as a good supporting matrix for proliferating cells.

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