CHAPTER - 5

DISCUSSIONS

Two indigenous species of non-mulberry silkworms viz. Muga and Eri which produces different types of silk were considered for the study. The protein from the glands and fibers were extracted and redesigned into biomaterials in the kind of films and microparticles. The biomaterials were analyzed for morphological properties and physicochemical properties by different analytical instruments. On the applications part, the biomaterials were tested for biocompatibility by using them as substrate to grow fibroblast cells and biodegradability was tested by treating the samples with enzymes present in the human body. The detailed discussions correlated with the results and compared with the extensively studied biomaterials of B. mori silk are presented in the following pages.

The silk fiber proteins are synthesized in the silk gland cells of the silkworm and are formed by stretching of the liquid silk through the spinneret with the movement of the head of the silkworm to form the cocoon. Prior to spinning the silk are stored in liquid form in the glands. The posterior silk gland contains the fibroin and the middle silk gland contains the sericin. Fibroin while coming out from the gland gets coated with sericin, which acts like an adhesive to join the fibroin fibers together. The SDS PAGE of liquid fibroin from Muga silk in reducing conditions showed a prominent band at around 250 kDa. According to Kar et al. (2013) the estimated molecular weight of Muga fibroin
in the non-reducing condition is approximately 500 kDa which is a homodimer of 250 kDa polypeptides. It is reported that fibroin of *B. mori* is composed of two chains, one of 350 kDa and the other of 25 kDa Yamada *et al.* (2001). But in the current study as the SDS- PAGE was run in reducing conditions the 250 kDa band was prominent. However, two distinct bands of fibroin were observed in Eri, around 160 and 50 kDa in the reducing condition. Inoue *et al.*, (2003) reported that fibroin from *S. ricini* contains a single band of 330 kDa in non reducing conditions and the same is broken by the disulfide bond in reducing condition to give two 160 kDa bands. However, the 50 kDa band found in the study has not been reported elsewhere, which may indicate low molecular weight fibroin in Eri silk.

SDS- PAGE showed a prominent band at 66 kDa for sericin from Muga and Eri which are in conformity with the earlier report (Ahmed *et al.* 2004). A band at 100kda was also observed which has not been reported elsewhere. A smear was found in the high molecular weight range from 150- 200kda. The smear in the high molecular weight may be due to presence of fibroin impurities in the samples as during extraction of sericin from the middle silk gland, some amount of fibroin may get mixed as there is no proper demarcation between posterior and middle silk gland. Another smear was found in the region of 50- 36kda which indicates low molecular weight sericin in the cocoons of Muga and Eri. High molecular weight sericin bands of 200kda and more than 200kda has been reported in another non-mulberry silkworm, *Antheraea mylitta*. It has been reported that high molecular weight sericin increases the strength of the fiber while the
lower molecular weight sericin protects the pupa from various environmental stresses (Dash et al., 2007).

The surface morphology of the biomaterials was studied by SEM. For the purpose of better understanding, fibers of Muga and Eri were also studied and compared with $B. \text{ mori}$ fibers and films. The external structure of the silk fibers observed in Muga and Eri samples was very fine and smooth reflecting the complicated and refined natural appearance which is responsible for some special features like luster and color (Minagawa, 2000). The surface of the Muga fiber was found to have striations with groovy structures, which may be the genetic Characterization of the non-mulberry silks as similar striations were reported for the wild silkworm Antheraea yamamai silk by Matsumura, (1980). These striations may account for the uneven structure of the $A. \text{ assamensis}$ silk. In the case of Eri silk fiber, the external structure is close to $B. \text{ mori}$ fibers with smooth surface which is more or less even. The surface of the Muga fiber was rough compared to the surface of Eri and $B. \text{ mori}$ fibers. The rough surfaces may also be due to the presence of crystals of calcium oxalate. As reported by Freddi et al., (1994) that Muga fiber contains crystals of calcium oxalate.

When thin films were prepared from the liquid silk by addition of distilled water and regulating the concentration of the protein, some morphological differences were observed in relation to the fibers. The film surface of Muga and Eri was uneven with undulating structures and roughness. However, $B. \text{ mori}$ film surface looked smooth and even than the other films. On higher magnification, aggregation of secondarily accumulated microfibril was observed in the rough surfaces. At the same time,
aggregation of some ball-like globular particles of fibroin and formation of fibril of relatively smaller size were observed. The nano-filaments inside globules spread out from the center and were surrounded by random nano-filaments. The random nano-filaments were entangled with each other and linked the globules together, to form a stable network structure. Globular fibroin structure of 2 μm diameter and filamentous structures have been seen in all the samples. The globules and filamentous structures are said to be due to the less ordered random coil conformations of the films (Lu et al., 2010).

Regenerated silk fibroin solution from Muga and Eri were prepared by dissolution of the fibers. Dissolving the Muga fiber was very difficult as it remains integrated in most solvents. Lithium thiocyanate was used to dissolve the Muga and Eri fibers. By this process 60-70% of Muga gets dissolved in solution, however more than 95% Eri fiber gets dissolved at the same condition. The surface morphology of the films fabricated from regenerated silk solutions of Muga and Eri silks were found to be smooth than their counterparts. The roughness and unevenness was not found in these films which may be due to the fact that during the dissolution process molecular reorientation and reorganization has occurred which gave an ordered conformation when cast as a film in contrast to the liquid films which was cast as a raw form obtained from the silk glands. Moreover the filamentous and globular structures were found in the films forming the base of the films. Lu et al., (2010) prepared silk films from B mori and made them water insoluble, the SEM images showed that the silk films prepared by different methods were composed of nano-filaments, while no specific nano-structures...
were found in soluble film. Nano-filaments formed after methanol or water treatment and these assembled to form globules about 200–1000 nm in diameter. These results suggest that nano-filaments inside and outside the globules might have different secondary structures. Putthanarat et al. (2002) reported that four major classes, including particles, grains, nanofibrils, and an irregular morphology, existed in fibroin films. Lv et al. (2005) added to the above findings that drying temperature plays an important role in the formation of filamentous and globular structures. Increase in the temperature favours formation of β sheet structures and vice versa. Acharya et al. (2009) studied fibroin films of B. mori and non-mulberry silk Attacus atlas and reported the non-mulberry film to be coarser than the B. mori film which is similar to the results obtained in this study as Muga and Eri film were coarser than the B. mori film.

Recently, biomaterials derived from composites are gaining prominence and finding new applications in the biomedical field, mainly because of the improved properties and structures. With this view in mind, composites from sericin and starch were prepared and characterized. Sericin is a waste product of the degumming process in silk industries and water containing starch is also discarded during preparation of rice in the households. These composites are expected to find new applications in the clinical field, cosmetic industries and also as food supplements.

In the sericin starch blend (SS) films the native granular structure of sericin has been seen sparsely while those of rice starch have lost completely. The surface of SS film without any cross linker was observed to be rough, porous and uneven in texture. However, distinct formation of nano-scale fibrils, with diameters ≤ 2 μm forming a
network structures was observed. These may be attributed to the high-scale spinning of the solubilized mass during the preparation of film-forming solution (Xian et al., 2011). On heat treatment neither gelatinized starch, which are seen as non-uniform clusters throughout the scanning area exhibit such fiber formation properties, nor sericin (Mali et al., 2004; Zhang et al., 2012). However, starch has its intrinsic plasticizing properties on certain materials and sericin forms composites only after addition of cross linkers (Mandal et al., 2009). It may be inferred from this observation that starch exerts its plasticizing effect on sericin and due to the high temperature spinning during film fabrication; the observed nanofibrils were formed. The network of fibrils were however not observed in the cross linked samples of sericin and starch with glycerol and PEG which indicate superior intimation of sericin and starch due to application of the cross linkers. SSG film had a nano scale granular appearance while SSP film exhibiting a better uniformity is demonstrative of better cross linking property of PEG in the sericin starch system as evident from the SEM images.

Microparticles from Muga, Eri and B. mori fibers were fabricated by wet milling method and the morphology was determined from images of SEM and TEM. SEM results showed that the different types of silk fibroin microparticles were porous in nature, spherical in shape and the morphology was uneven and rough. The microparticles were not transparent and hollow as evident from the figures. Rajkhowa et al., (2010) fabricated silk fibroin microparticles using B. mori and found similar results. In biomedical applications, microparticles have created considerable interest for its application as drug delivery carriers for the delivery of large molecular weight protein
and peptides in health care treatments (Mandal et al., 2001; Salerno et al., 2014). The size of the microparticles was determined by SEM. Results showed that the size of the microparticles from all the silk samples were around 4-5 μm. The results are in agreement with microparticles fabricated from B. mori previously by the same method (Rajkhowa et al., 2009; Bhradwaj et al., 2015).

Accordingly, the microparticles were analyzed by TEM for confirmation of the morphological structures. It was seen that the fibroin microparticles were almost similar as observed under SEM. The microparticles were rough in morphology with nearly spherical shape and equal in size distribution without much apparent aggregation or adhesion. The structural stability of the microparticles was also assessed by SEM, microparticles incubated for 15 days in PBS did not show any marked difference in size, morphology and distribution when compared with day 1 samples. The significance of the results lies in the fact that the microparticles will not undergo any physical change in normal cell culture conditions when applied for biomedical uses in drug delivery until the purpose is solved.

Study of the solubility and wetting properties of the films by Dynamic contact angle tests showed that the fibroin film surfaces were hydrophobic in nature and immiscible in organic solvents like n-heptane. The contact angles of Muga and Eri films were found to be more than that of B. mori films prepared from gland fibroin. Previous reports on B. mori films also showed lower contact angles compared to other fibroin films (Kundu et al., 2008; Acharya et al., 2009). Accordingly, contact angles of films prepared from fibers were similar to that of liquid films showing hydrophobic surface
properties. Mikos et al., (2001) emphasized that the hydrophobic property of the fibroin films may be advantageous for application of pharmacological agents to the biomaterials during fabrication in solvent casting/particulate leaching procedures in organic solvents. Hoffman et al., (2006) reported that the methanol treated silk films of B. mori showed a significantly higher contact angle than for untreated films. Contact angles remained stable for methanol treated films whereas a significant lower value was observed for untreated films. This may be due to the fact that, methanol treatment induced a shift to higher amounts of crystalline β-sheets structures. Therefore, it is necessary to treat the films with alcohol before applying as a cell culture substrate, so that the films become insoluble.

FTIR spectroscopy technique helps to study the molecular conformation and crystalline structure of silk protein since the position and intensity of amide bands are sensitive to molecular conformation of proteins. The absorption bands of amide I, amide II, amide III, amide IV and amide V are mainly responsible for the molecular conformation which may be either α-helix or random coil or β-sheet structure. In general, in the silk protein, the amide I mode associated with the β-sheet conformation occurs in the range of 1618–1640 cm\(^{-1}\), the random coil conformations give bands in the range of 1640–1650 cm\(^{-1}\) and the α-helical conformation results in bands between 1650 cm\(^{-1}\) and 1660 cm\(^{-1}\) (Tsukada, 1989; Freddi et al., 1997; Kweon et al., 2000).

Amide I absorption primarily represents the C=O stretching vibrations of the amide group. The amide C=O bonds involved in different secondary structures absorbs infrared radiation of different wave numbers. This occurs because the frequency of
vibration of C=O in the protein backbone changes due to the influence of hydrogen bonds between N-H and C=O, which are dependent on conformations adopted by protein chains. Therefore, the major secondary structure contained in the protein can be estimated from the amide I peak position.

In the spectra of Muga and Eri fibers, the amide I peak was found at 1620 cm\(^{-1}\). This peak has been assigned to \(\beta\)-sheet conformation in \(B.\) mori by Chen et al., (2007). Additionally, the amide II and III peaks for sample Muga fiber at 1518 cm\(^{-1}\) and 1235 cm\(^{-1}\), respectively, have been assigned to \(\beta\)-sheet secondary structures by Freddi et al. (1997). In case of Eri fiber, the amide II peak shifts to 1520 cm\(^{-1}\) representing crystalline \(\beta\)-sheet conformation along with the peak at 1235 cm\(^{-1}\). Earlier report reveals that in Muga fiber both \(\alpha\)-helical and \(\beta\)-sheet conformations are present and inter-conversion occurs in them due to stress (Devi et al., 2011).

The films from the liquid proteins from Muga Eri and \(B.\) mori showed highly amorphous structures. The peak at 1643 cm\(^{-1}\) of Muga film is assigned to random coils/extended chain conformations as previously reported by Tretinnikov and Tamada, (2001) and Teramoto and Miyazawa, (2005) and the 1650 cm\(^{-1}\) peak of Eri film to random coil conformation. Arondo et al., (1993) has assigned the 1650 cm\(^{-1}\) peak to \(\alpha\)-helical structures. Additional peaks at 1540 cm\(^{-1}\) (Amide II) is assigned to random coil conformations (Um et al., 2001; Wang et al., 2005) and the peak at 1546 cm\(^{-1}\) in Eri film is due to the alpha helical conformation (Li et al., 2003). The amide III band at 1240 cm\(^{-1}\) found in the present study in Muga and Eri liquid silk film has been attributed to random coil conformations in the films (Um et al., 2001; Wang et al., 2005). The amide
I absorption peak of Muga and Eri fibroin film treated with 70 % alcohol was at 1625 cm$^{-1}$ and amide II was found at 1520 cm$^{-1}$ indicating β-sheet (silk II) structure (Hu et al., 2006).

It was observed that the films from Muga and Eri are composed of mainly random coiled conformation and α-helical structures of proteins, which on alcohol treatment shifted to peak positions signifying crystalline β sheet structures. It was reported that α phases are present in the liquid silk extracted from the silk gland of S. cynthia ricini (Asakura et al., 1999; van Beek et al., 1999) and random coil conformation in regenerated and gland protein B. mori fibroin films (Kundu et al., 2008). For subsequent use of these films as biomaterials, a more ordered crystalline structure is required which provides stability to the films (Kundu et al., 2008), and this has been achieved by treatment with methanol which is known to induce formation of crystalline beta-sheets (Tsukada et al., 1994; Chen et al., 2001a; 2001b). Therefore, the conformations of the fibroin films changed from amorphous to crystalline forms after treatment with alcohol.

The films prepared from fibers of Muga, Eri and B. mori showed similar conformations to the films from liquid silk. All the films showed similar peaks at around 1652 cm$^{-1}$ (amide I), 1540 cm$^{-1}$ (amide II) and 1237 cm$^{-1}$ (amide III). The peak at around 1652 cm$^{-1}$ can be attributed to random coil or α helical conformation, in both the cases it is the amorphous condition without any organized structure and composition. The 1540 peak also depicts the random coil conformation along with the amide III peak at 1237 cm$^{-1}$. 

133
The SS blending shows the amide I peak at 1630 cm\(^{-1}\). After composite formation, the intensity of the Amide I band gradually increased indicating increase in the amount of β-sheet structures. Earlier studies suggested native sericin containing both random coils and β-sheets representing amorphous and crystalline regions respectively (Tsukada et al., 1981; Teramoto et al., 2006; Dash et al., 2007). In case of starch, the broad band at 3336 cm\(^{-1}\) is attributed to the vibrational stretches of the hydroxyl groups. It shifts to 3389 cm\(^{-1}\) after composite formation. The band at 2925 cm\(^{-1}\) stands for the characteristic C-H stretching vibration.

The major band at 1022 cm\(^{-1}\) is sensitive to changes in starch conformation (Van et al., 1995). The bands at 1047 and 1022 cm\(^{-1}\) are linked with the crystallinity and amorphousness of starch, respectively (Sevenou et al., 2002). There was a clear increase in the intensity of the band at 1022 cm\(^{-1}\) indicating crystallinity of the samples, after cross linking with glycerol and PEG. It can be inferred that application of cross linkers increased the stability and formed a more ordered structure which indicates the potentiality of the composites to go further in the experiments. The absorption at 1636 cm\(^{-1}\) represents H\(_2\)O bending vibration (Zhang et al., 2007). The peak at 1083 cm\(^{-1}\) and 866 cm\(^{-1}\) are attributed to the anhydro glucose ring C-O stretch and the C-H stretch of residual carbon (Yu et al., 2009). The C-O-C skeletal vibration is represented by the medium to weak peak at 578 cm\(^{-1}\) (Thygesen et al., 2003).

Teramoto et al., 2005 studying sericin hydrogels found the amide I peak at around 1618 cm\(^{-1}\) for sericin hydrogels indicating β-sheet conformation and a peak at 1647 cm\(^{-1}\) for sericin water solutions indicating random coil structures. Dash et al.,
(2009) reported that liquid sericin from *A. mylitta* showed random coil conformation with peak at 1655 cm$^{-1}$ but after alcohol treatment the peak shifted to 1630 cm$^{-1}$ indicating ordered crystalline β-sheet conformation.

The microparticles fabricated from Muga, Eri and *B. mori* fibers showed crystalline β sheet structures with prominent peaks at the range of 1622–1629 cm$^{-1}$ for amide I, 1517–1518 cm$^{-1}$ for Amide II region and 1232–1238 cm$^{-1}$ for Amide III region. From the FT-IR analysis it is clear that films from liquid silk and fibers were amorphous in nature and changed their structure to crystalline form after alcohol treatment. The composite films showed increase in crystalline structures after addition of the cross linkers. As the microparticles were fabricated from degummed fibers without intervention of any chemicals, they retained the same structure of the original fibers.

Secondary structures of the biomaterials were also studied by XRD analysis. Three types of structures are designated for silk *viz.* Silk I, Silk II, and Silk III. Silk I refer to the water soluble structure existing within the silkworm gland before spinning. Silk II is the insoluble extended β-sheet conformation which forms after spinning of silk fibers. Silk III is an unstable structure observed at the water–air interface (Valluzzi *et al.*, 1996; 1997). In this study, degummed Muga fiber showed prominent peaks at 16.78$^0$, 20.28$^0$ and Eri fiber showed peaks at 16.78$^0$, 20.18$^0$. The peaks of Muga and Eri correspond to Silk II conformations denoting crystalline beta-sheet structures. Similar peaks were obtained in XRD studies of *B. mori* fibers by Zhou *et al.* (2000). Feng *et al.*, (2007) reported that main silk II diffraction peaks of silk fibroin are present at about 2θ = 18.9 and 20.7$^0$. 

135
Devi et al., (2011) reported that in Muga fiber both α and β structures co-exist and inter-conversion occurs between them due to stress. Like B. mori, extensibility of Muga may be due to movement of the β-phase and the rigidity may be accounted for by the α-phase. Chain mobility is a common phenomenon observed in silk fibroin and is well supported by the XRD pattern. Lucas et al., (1955) showed that upon stretching, the molecular segments in the amorphous region will flow more easily if they contain more bulky side groups of amino acids. The gradual changes of silk from α-phase to β-phase were also observed by the XRD pattern in tasar silk fibroin by Freddi et al., (1997). However, Freddi et al. (1994) stated that the XRD pattern of the A. assamensis silk is typical β-phase, with (Ala) n- repeats in the polypeptide.

In case of films prepared from liquid silk of Muga and Eri film, the first peak was found at 11.68° confirming Silk I structure and around 22.31° evident of Silk II structure. The main diffraction peaks of Silk I present at around 2θ = 12.2 and 28.2° which represents silk I structure, and pure silk fibroin film is reported to have a characteristic peak at 2θ ~ 12.28, but in this study a negligible shift was found from 12.2 to 11.68°. In a study by Tao et al. (2007) involving X-ray diffractogram of fibroin film from A. pernyi silk, the structure of the films were similar to Muga and Eri films, representing α-helical conformation.

Lu et al. (2010) reported in that in the film of B. mori the soluble silk fibroin showed an amorphous state, characterized by the presence of a broad peak in the 2θ scattering angle range from 10.5° to 25°. Water-insoluble films exhibited crystalline structure. The results indicate that both silk I and silk II structures formed after water
annealing in the process of film fabrication. At first the films remained soluble with amorphous nature but gradually changed their crystal structure from silk I to silk II during the slow drying, water annealing and methanol annealing processes. The whole process of film fabrication involves water treatment whether it is liquid silk or dissolution of fibroin. The findings of this study indicate that water has a significant influence on the formation of silk I structure.

Similar results were reported by Lv et al., (2005) exhibiting the copresence of α helix and β sheet structures in fibroin films. Drying temperature also played a pivotal role in the structural conformations i.e α-helix form was transformed to an antiparallel β sheet with increasing drying temperature, and the α-helix almost disappeared when the drying temperature was raised above 60°C. Tsukada et al., (1994) reported that methanol treated fibroin films of B. mori, shows typical X-ray diffractograms of the β-sheet crystalline structure, since methanol is a crystallization inducing solvent. It was inferred that amorphous silk fibroin is crystallized to β-sheet during processing of silk fibroin in the gland of B. mori (Kundu et al., 2008). From the study it is evident that the FT-IR and XRD reports confirmed the crystalline nature of the degummed fibers and amorphous nature of the films. The XRD spectra of films prepared from regenerated solutions and liquid silk are similar in nature.

The structure of the SS were also analyzed by XRD. The high amylose rice starch exhibited A-type X-ray diffraction pattern with distinct peaks at 2θ = 15.1°, 17.1°, 18.0° and 23.0°. During film fabrication involving hot mixing above gelatinization temperature, relative crystallinity of starch is lost with simultaneous loss of the native
crystalline conformation (Shih et al., 2007). Native sericin showed peaks at $2\theta = 12^0$, $19.7^0$ and $21.2^0$ as earlier reported by Miyake et al., (2003). Sericin mainly exhibits two diffraction peaks, around $19.2^0$ and a major peak at $23.2^0$ (Nagura et al., 2001; Tao et al., 2005). Native sericin is known to contain both random and β-sheets representing amorphous and crystalline regions, respectively (Tsukada and Bertholon, 1981; Teramoto et al., 2006; Dash et al., 2007). After composite formations the films exhibited higher crystallinity with altered crystalline patterns. Blend films containing PEG showed stronger peak at $2\theta= 19.7^0$ compared to films containing glycerol and native sericin and starch indicative of higher amount of crystalline pattern. In addition minor peaks at $15^0$ and $24.3^0$ indicates retention of the native A-type and formation of retrograded B-type starch crystallites after the film fabrication involving heat treatment (Mahanta et al., 1989). On crosslinking with glycerol and PEG, the relative crystallinity is further increased because of formation of newer intermolecular H-bonds. This occurs mainly due to transformation of amorphous α-helical structures of sericin to crystalline β-sheets, well indicated by the intensity of the peak at $19.7^0$ in conformity with earlier report(Miyake et al., 2003).

According to Nayak et al., 2012 sericin films cross linked with glutaraldehyde XRD spectra showed two peaks at $19.3^0$ (2θ) and $22.4^0$ (2θ) corresponding to β-sheet crystalline structure. Peaks at $19.3^0$ (2θ) and $22.4^0$ (2θ) were observed in crosslinked sericin. It was reported that the crystallinity increases with gluteraldehyde crosslinking and alcohol treatment. This was attributed to the covalent bond formation and alcohol treatment that induce transition of the random coil to β-sheet formation due to
intermolecular hydrogen bonding (Teramoto et al., 2005; 2007). The β-sheet crystalline structure observed in the glutaraldehyde-treated membrane was probably induced by the crosslinking as relevant to the current study. Sericin is amorphous in nature and crosslinking with other agents results in the chemical modification of proteins that reinforce chemically, joining of two or more molecules by a covalent bond, making compact structures resulting in increased integrity and stabilization of protein.

Therefore the results of the secondary structure analysis of the fibroin and sericin films specifically confirm the structure of the films in native state and after treatment with other chemicals and cross linking agents. This secondary structure analysis will be the backbone of the further studies which involves specific application of these biomaterials in various fields.

The thermal properties of the biomaterials were studied with the help of DSC and TGA. The thermal behavior in fibers of wild silkworms including Muga and Eri were reported by Talukdar, (2012), which stated that in DSC thermograms an endothermic peak is observed below 100 °C in all the samples. This peak is attributed to evaporation of water. There were two minor and broad endothermic peaks at 219.6-227.2 °C and 292.3-301.5 °C, together with a major endothermic peak at 353.0-364.4 °C which has been attributed to the decomposition of the fibroin with oriented β-conformation which are in conformity with earlier report on non-B. mori silks including A. assamensis as well as tasar silks (Ishikawa et al., 1972; Tsukada, 1988; Freddi et al., 1995; Tsukada et al., 1998; Das et al., 2010; Reddy et al., 2011). In case of tasar silks, Nagura et al., (1978) explained that most thermal events occurring at above 200 °C can be attributed to
the molecular motion of the fibroin chains not only in the amorphous but also in the crystalline regions.

The thermal analysis study in Muga, Eri, and B. mori fibers showed that the first endothermic peak was at 65.5°C, 77.5°C, and 64.8°C, respectively, which are due to loss of bound water as moisture. Two minor and broad endothermic transitions appeared above the glass transition temperature at 230 and 300°C in Muga and Eri fibers which can be related to the degradation of amorphous silk (Nakamura et al., 1986), and the major endothermic peaks at 364°C for Muga, 356.4°C for Eri, and 267°C for B. mori are attributed to the thermal degradation of silk fibers with beta-sheets. Similar results were reported for A. pernyi by Tsukada, (1988). From the studies it can be inferred that the non-mulberry fibers degraded at a higher temperature than the B. mori silk fibers, and highest thermo tolerance was recorded for Muga fiber.

In the films from liquid silk, DSC data reveals that the first endothermic peak below 100°C in all the samples is due to the evaporation of bound water. The films contained higher amount of bound water than the fibers because of their higher amount of amorphous region as moisture can bind to silk through hydrogen bonds in the amorphous region. Two major endotherm-exotherm transitions above the glass transition temperature Tg (190–200°C) (Nakamura et al., 1986) reported for fibroin, were seen in the temperature range of 220–226°C in Muga film and 208–223°C in Eri film. These transitions are reported due to the conversion of the amorphous films to crystallized ones during the scans (Tsukada et al., 1992; Kweon et al., 2001). The glass transition and subsequent crystallization provide clear evidence of large amount of amorphous
structure in the films. The films started to degrade around temperature 350°C. It is noteworthy that the films decomposed at a lower temperature than the fibers may be because of its amorphous nature and crystals that form during the thermal transition are not as strong as those available in a fiber. On the contrary, *B. mori* film degraded at a much lower temperature of 264°C indicating that films from non-mulberry sources have higher thermal stability.

Lu *et al.*, (2010) reported that the films from *B. mori* silk treated with methanol showed an endothermic peak at around 100°C, a non-isothermal crystallization peak at around 213°C and a degradation peak at 257°C. The endothermic peak at around 100°C was due to the evaporation of bound water and indicated that silk fibroin interacted with water. The middle peak is due to unstable non-crystal structures which transformed to β-sheet. Finally after the appearance of the crystallization peak the film started to degrade, with an endothermal peak at around 257°C.

The films from regenerated fibers, the first endothermic peak was found around 85.4°C, which is lower than the liquid silk films. This may be accounted for by the aqueous system in which the films are extracted and fabricated may result in the final containment of bound water and the final degradation peak of the films were found at about 355°C. An endo- exo transitions for the films were clearly seen around 216-224°C. The sericin starch blend films were found to have low melting temperatures compared to fibroin films, although addition of cross linkers increased the melting temperature to some extent, while native sericin starch film exhibited the first loss of moisture peak at 48.2°C and the degradation temperature of 312°C, SSG showed the
first peak at 64.3\(^{\circ}\)C and degradation peak at 310\(^{\circ}\)C while, SSP showed first peak at 73.7\(^{\circ}\)C and degradation at 332\(^{\circ}\)C. Application of PEG and glycerol on sericin starch films as cross linkers enhanced the thermal properties, and PEG treated film showed higher stability than the glycerol. It indicates the higher bonding of the PEG on the film.

The DSC of fibroin microparticles showed the first endothermic peak at 80-100\(^{\circ}\)C for all the samples attributed to the loss of bound water and the second in the range of 360-365\(^{\circ}\)C, for Muga and Eri, which is the final degradation of fibroin. In B. mori microparticle the degradation peak was at 320\(^{\circ}\)C. It can be inferred that the thermal stability of non-mulberry silk remained same in spite of fabrication of biomaterials like film, microparticles with varied processes.

The weight loss of the biomaterials in relation to temperature and time was quantified by Thermogravimetric analysis (TGA). In the fibers, the initial weight loss was around 96.4\(^{\circ}\)C due to less water containment. The second weight loss took place in the range of 245–386\(^{\circ}\)C which is due to the gradual decomposition of the proteins. In case of the films fabricated from liquid silk, the initial weight loss in the films at 138.5\(^{\circ}\)C is due to the loss of moisture. The second weight loss took place in the range of 235–377\(^{\circ}\)C associated with the breakdown of fibroin. The first degradation peak was higher in films compared to fibers because of more amount of bound water. It was reported that silk fibroin cast from aqueous solutions contains bound water even after they are dried (Kundu et al., 2008). The TGA results for films fabricate from fibers were more or less similar to the liquid films, with negligible shift of the peaks. First weight
loss of the films was found around 131°C and the second major weight loss around 250°C to 384°C.

Kundu et al., (2008) studied the TGA thermogram of films from liquid silk of B mori and reported that the initial weight loss of films was at 149°C due to loss of moisture. The second weight loss took place in the temperature ranged from 218 to 330°C. This is associated with the breakdown of side chain groups of amino acid residues as well as the cleavage of peptide bonds. Kar et al., (2013) reported that fibroin film of Muga silk exhibited an endothermic peak at 371°C representing the degradation and disorientation of the protein molecule and concluded that the thermal stability of the non-mulberry liquid fibroin is much more than that of the B. mori silk fibroin. This suggests a greater chemical stability of the non-mulberry liquid fibroin as evident from this study.

Thermal stability was also studied in the SS films. The first major weight loss for all the samples occurred at around 262°C and the values were similar for all three composite films. However after that the weight loss was progressively higher and the second major weight loss started at 310°C. The weight loss percentage for SS film was more followed by glycerol and PEG cross linked films. It was reported that the former peak signifies the molecular mobility and melting induced thermally, while the latter indicates thermal decomposition (Mandal et al., 2011). The observed degradation temperature corresponded to an endothermic peak of sericin powder of A. mylitta at 311°C that were reported by Mandal et al. (2011) and in B. mori at 210°C reported by
Aramwit et al., (2010). Lamoolphak et al. (2008) stated that the thermal stability of sericin may be influenced by the use of chemicals during the extraction process.

In Muga and Eri silk fibroin microparticles, the first weight loss was found at around 100–105 °C and the second weight loss at around 350–360 °C denoting the similar two step breakdown method. However, in B. mori fibroin microparticles the first weight loss peak was also observed at around 100–105 °C but the second weight loss peak was observed at 266 °C, which again demonstrates the superior thermal stability of the Muga and Eri microparticles over the B. mori microparticles.

It is well documented that the materials of natural origin are superior and noteworthy targets for further innovation in biomedical field, which solely rely on the development of different forms of natural polymeric biomaterials (Shrestha et al., 2014; Chan et al., 2014; Prata and Grosso, 2015). The advantage and superiority of natural polymeric biomaterials in biomedical applications is that they show better compatibility with the native extracellular matrix (ECM) (Prata and Grosso, 2015; Stoppel et al., 2015; Bhardwaj et al., 2015). The structure and form of the biomaterial is important in selecting the type of application to be used, silk fibroin-based biomaterials have been fabricated and utilized for a broad range of biomedical applications in different forms such as films, fibers, nanoparticles, microparticles, hydrogels and scaffolds (Omenetto and Kaplan, 2010; Bhardwaj and Kundu, 2010; 2011; 2012).

Among these, recently, micro and nano sized silk particles have received much interest for their size, efficiency and applicability for drugs and growth factors for
therapeutics and tissue engineering applications (Wang et al., 2010; Pritchard and Kaplan, 2011; Shchepelina et al., 2011; Luo et al., 2013). Currently, there is a range of various methods for fabrication of silk fibroin microparticles such as emulsion polymerization, phase separation, and solvent evaporation/ extraction and others for various biomedical applications (Yeo et al., 2003; Hino et al., 2003; Hofmann et al., 2006; Wang et al., 2007; Wenk et al., 2008; Lammel et al., 2010; Luo et al., 2013).

Silk biomaterial especially from the B. mori silk has been used in different applications like clinical purposes; cosmetics should have good properties in bulk as well as on the surface. The biomaterial surface first comes into contact with the living tissues when the biomaterial is planted in the body. The initial response of the body to the biomaterial depends on its surface properties. Surface properties like surface topography, contact angle and surface charge influence the biocompatibility. The biomaterial should be able to mimic the living tissues to be replaced possessing good tensile and mechanical characteristics. Both the biocompatibility and the mechanical properties of biomaterials play pivotal roles in biomaterial applications (Kundu et al., 2008).

Therefore, any biomaterial proposed for such applications needs to be characterized and checked the biocompatibility for various purposes. Biomaterials prepared in this study have been tried using cell lines. Mouse fibroblast L929 cell lines were used to study the biocompatibility of the films and microparticles. Cells were grown on the surfaces of the films using them as substrates, in standard media and conditions. After monitoring for 1, 3 and 5 days, it was seen that the cells proliferated
and spread well on this surfaces. The attachment of the cells to the matrix surface was promising and the cell morphology remained intact as in control. These results are in accordance with the reports on *B. mori* films by Kundu *et al.*, (2008).

It was observed that when the cells are plated onto artificial surfaces, the cells first attach themselves to the surface; slowly they attain their normal morphology. After ascertaining the cell adhesive property of the matrix, the signalling pathways to cell spreading is elicited. The early stages of cell spreading are biochemically and biophysically involved in regulating the cell functions (Cavalcanti *et al.*, 2006). Factors like matrix properties, such as the presence of integrin binding sites, affect cell attachment and its spreading on the artificial surfaces. The cell morphology particularly the spreading of the cells, is an important indicator of healthy conditions. Proper spreading indicates that the supplied matrix is biocompatible. Either more or too little spreading of cells causes deformed cell morphology, which indicates the poor health of the cells.

The fabricated microparticles were treated with mammalian cells (L929) in *in vitro* conditions in different concentrations, to check the biocompatibility. It was found that L929 mouse fibroblast cell adhered to the microparticles and also spread properly. Confluence of the cell was attained within 5 days of incubation which signify that the cells proliferated very well on these surfaces.

The growths of cells on the non- mulberry fibroin films were found to be significantly promising. These results are in agreement with the earlier comparative
studies conducted between non- B. mori and B. mori matrices (Acharya et al., 2008; Talukdar et al., 2011). The cells showed a similar matrix response pattern irrespective of initial cell seeding density. The enhanced cell attachment, spreading and proliferation on non-B. mori fibroin matrices may be due to the presence of RGD sequences as reported earlier by many authors (Sezutsu et al., 2000; Datta et al., 2000; Hwang et al., 2001). The results are in agreement with earlier works on non-B. mori fibroin (Acharya et al., 2008; Wang et al., 2008; Mandal et al., 2008; Talukdar et al., 2011). The cell culture results on Muga and Eri fibroin films indicate that the matrix is biocompatible.

Determination of apoptosis and live cells by acridine orange and ethidium bromide double staining method was carried out on cells grown on fibroin films as substrate and also on microparticles treated cells. Acridine orange is taken up by both live and dead cells and emits green coloured fluorescence when intercalated into double stranded DNA. Ethidium bromide is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. After growing the cells for 48 hours the staining was done and observed under fluorescent microscope. From the images it can be seen that the cells were viable with negligible amount of dead cells when grown in the surfaces and with the microparticles. Very less number of cells emitted orange fluorescence confirming the presence of live cells with green fluorescence when compare with the control. The results are in correlation with the phase contrast images confirming the biocompatibility of the cells on the surfaces and with the microparticles.

MTT assay is a quantitative colorimetric assay for study of mammalian cell survival and cell proliferation. It is an indirect method for assessing cell growth and
proliferation, since mitochondria oxidize the MTT solution, giving a typical blue–violet end-product, O.D. value of 540 nm can be quantified to cell number. In the current study involving fibroin films the MTT results shows that the biocompatibility of the silk film obtained from both the sources is found to possess more or less similar non-toxic behavior. The viability of the cells grown on the film of Muga silk was highest followed by Eri and B. mori films which was statistically significant (p<0.05).

MTT assay was carried out in different concentrations of the microparticles viz. 50, 100 and 200 μg ml\(^{-1}\). The concentration of microparticle (100 μg ml\(^{-1}\)) was found to be most appropriate with maximum cell growth and proliferation in all the samples. The cell viability results probably show that the microparticles are easily encapsulated by the cells and thus demonstrate the nontoxic nature of microparticles. Similar to the films, the non-mulberry fiber microparticles showed higher rate of cell viability compared to the B. mori microparticles.

The cell morphology, particularly the spreading of the cell is an important indicator of healthy cells and is biochemically and biophysically involved in regulating the cellular functions. There are many factors, mostly matrix properties such as the presence of integrin binding sites, which affects the cell spreading (Cavalcanti et al., 2006). After cell seeding, cells first attach themselves to the adhesive surfaces without immediately attaining their normal morphology, followed by spreading of cells after ascertaining the cell adhesive properties (Kar et al., 2013). Optimal cell spreading is crucial for the maintenance of cellular morphology. Non-B. mori silk fibroin microparticles showed significantly better cell spreading in \textit{in vitro} conditions. Thus, the
result shows good growth and spreading of cells incubated with non-B. mori microparticles, which indicates the non-toxic and compatible nature of microparticles. Therefore, non- mulberry silk fibroin microparticles with cell binding motifs maintained the better cell viability and proliferation which are in conformity with other reports where silk fibroin scaffolds and films were utilized (Mandal and Kundu, 2008; Mandal et al., 2010; Bhardwaj and Kundu, 2012).

Biodegradability is also an important aspect in utilization of biomaterials in clinical purposes. Human cells are exposed to a range of proteolytic enzymes which degrade the proteins in different ways to replace old cells and in defense mechanisms against foreign pathogens. In this context when fabricated biomaterials are replaced in place of original tissues it must be able to tolerate the proteolytic enzymes without getting degraded until the purpose is served. This has been termed as biodegradability in terms of biomedical applications of biomaterials. Here, the films from fibroin and sericin were exposed to two types of proteolytic enzymes and their degradation monitored against time. It was found that very negligible weight loss occurred in the films in presence of the enzymes which signifies that the films are not easily biodegradable and can withstand the enzymes in the human body. The significance of the results depicting the biomaterials to be compatible with proteolytic enzymes is well supported by works done in a similar manner in B. mori biomaterials (Lu et al., 2010). It was also reported that silk films containing a higher silk I content degrade more rapidly in comparison with those with a higher silk II content, and the degradation time can be controlled by changing the secondary structure of the silk films.
The liquid silk film, fiber film and microparticles of two non mulberry silk may be considered as potential biomaterial by virtue of its good mechanical strength, water stability, favorable secondary structure conformations, thermal properties, surface roughness, biocompatibility and biodegradability as these characteristics are essential for clinical and tissue engineering applications. Previous work on cell growth on biomaterials reported that attachment of the cells to Muga fibroin films was similar to that of *A. mylitta* fibroin films. This study confirmed that *S. ricini* fibroin film also supported cell growth and attachment comparable to *A. assamensis* films. The matrices of Muga and Eri responded similarly to the control tissue culture plate in terms of cell adhesion properties. The biodegradability of the films was also found to be negligible when treated with enzymes, so when the films will be put in in vivo condition in human body, it can withstand the proteolytic degradations and aid in mimicking the tissue to be replaced.

Microparticles fabricated from Muga and Eri also were found to be biocompatible and biodegradable. Cell growth and proliferation was significant in presence of the microparticles, and the optimum concentration for cell growth was found to be 100μg ml⁻¹. Interestingly, the thermal properties and cell viability was found to be more in non-mulberry biomaterials than the *B. mori* ones.

This study will broaden the field of non-mulberry silk’s biomaterials applications in the clinical and biomedical aspects. Further the study needs to be focused on specifically targeted applications using different cell lines and environments. The avenues of composites are also opened up with this study. Blending and crosslinking
different polymers with the non-mulberry composites could be better biomaterials with enhanced properties. This may lead to the use of eco-friendly components, indirectly benefiting the waste management issue. Top of all, the fascinating non-mulberry silk, the precious bioresource which are the part of the life of people of N.E Region will get momentum for exploration in many more empirical ways.