CHAPTER 2

REVIEW OF LITERATURE

Origin of Silk

Silk is a coin with ancient and valuable embarked on its both sides. Sericulture first started in China and the products with its striking characteristics were responsible for many socio economic impacts in the world history (Bizhannia et al., 2008).

In 2640 BC, Lady Xi Ling Shi, queen of the Chinese Emperor Huang Ti, popularly called 'Yellow Emperor' was drinking tea while sitting below a mulberry tree, when a cocoon feel into her hot cup of coffee. The fibers came out of the cocoon in the hot tea. This instance is said to be the beginning of sericulture. With this discovery the textile industry started to flourish in China. They realized the value of this material and the whole process was kept secret from the world. Due to this secrecy and the magnificent quality of silk the ‘silk road’ was born. In this strategic marketing silk was exchanged for gold, silver and wool with the west.

In 550 AD the Chinese monopoly with silk ended as silk was smuggled out of China tactically, the whole world came to know about this mesmerizing fiber and how to cultivate it. Sericulture swept the whole world like a wild fire from the Chinese plains to the African deserts.
Silk is a polymeric natural fiber produced by certain insects, the main purpose of secreting silk is to build their cocoons and webs from protection from enemies and environment and also to prey and metamorphose. These fine fibers are long, delicate and have large natural brightness. High elasticity is found in silk fibers. Silk fibers absorb moisture to a high extent. From thousands of years ago, these excellent properties of the silk have caused people in different countries to rearing silkworm as well as a lot of scientists and researchers to study and develop this silk industry for preparing silk yarn, silk fabrics and silk dyeing economically and scientifically (Seidavi et al., 2006).

The primary use of silk is as textiles. Different ethnicity has developed textiles symbolic to their cultures and rituals. The most basic definition of ‘textile’ is a material that has been fabricated by some weaving processes. The word ‘textile’ has been derived from the Latin word ‘textere’, which means ‘to weave’. The term textile can also be applied to materials manufactured by the interweaving of yarn-like materials as well as some non-yarn based materials (Leene, 1972). The pioneers in the process of reeling silk yarn from cocoon are the Chinese. The Chinese word “Seres” is considered the first word for silk from which all the other words denoting silk in different languages (soie, seide, seta, seda, silke, serikon, etc.) are derived.

Many insects are known to produce silk, but the silks produced by the mulberry silkworm, B. mori along with a few others of the same genus are being commercial used in the silk industry. The silk produced by other insects, like spiders, has very limited applications in commercial purposes. Silk filament is extracted from the cocoons built by 'silkworms,' which are not considered, worms, but are silkworm pupae. It has wings
and the body is covered by hair. The female moth lays hundreds of eggs in that time. The eggs hatch into larvae which eat plant leaves and mature into pupa.

Over the centuries, the value of silk has highly increased as textile fiber. The unbeatable qualities of silk fibers contributing to the huge demands are strength, softness, elasticity, affinity for dyes, absorbency and adaptability to various forms of twisting and mechanical pressures continues to dominate the markets. As a textile fiber, the position of silk is very high and special. It is an extraordinary combination of beauty and strength. Compared to any other natural fiber, silk is soft, at the same time strong, supple, and lighter in weight. It is prized for combining lightness with warmth, sheerness with strength and delicacy with resiliency (Potter et al., 1967).

Competition from artificial fibers continues with development of new types of fibers with desired qualities but silk has sustained its own position in the fabrication of quality textiles and allied goods of the supreme quality. The unbeatable qualities of silk include its high tensile strength, lustrous nature and ability to chemically bind dyes. However, silk is still quite unaffordable to the ordinary consumers, because of the expensive and labour intensive work related to the initial growth and production and subsequent processing of the fiber (Miller, 1992).

Types of silk

The insects in the Order Lepidoptera are most productive silk producers. Relatively small amount of silk is produces by spiders compared to silkworms but it has long been recognized that spider silk exhibits unique combinations of strength, stiffness
and toughness which are unrivaled by either man-made or other natural fibers (Lucas et al., 1955; 1964; Zemlin, 1968; Denny, 1976; Work, 1976; Cunniff et al., 1994; Kaplan et al., 1994; Dunnaway et al., 1995; Kohler et al., 1995; Volrath et al., 1996).

Commercially used silks are derived from silk cocoons, the protective case that the larvae spin to protect themselves during morphogenesis from microbial degradation and predators (Zhao et al., 2005). Some of these types of silk have been used for centuries to produce textiles and in particular they include silk produced by moth larvae in the Family Saturniidae, Lasiocampidae and Thaumetopoeidae (Peilger, 1993).

Based on their habitat a generalized classification of silkworms are made viz. ‘domestic’ or cultivated varieties (e.g. B. mori) and the “wild” varieties (Kweon and Park, 2001). Cultivated silk is produced by a carefully controlled process in which the silkworm lives in an artificial and protected environment for the mere purpose of producing fibers in the form of cocoons. On the other hand, wild silk production is not under human control. Instead, these silkworms live on plants under natural conditions far from human habitat and feed on the leaves. On maturity the silkworms spin cocoons in the trees (Hollen and Saddler, 1973). The cocoon is exploited and used for production of silk fibers and yarns. A sub division of the wild varieties is “semi domesticated” silkworm, refers to silkworms whose half of the life cycle is indoor and half is outdoor. A classic example is the silkworm Antheraea assamensis, from cocoon stage to hatching of eggs the whole process is done indoor and after hatching the larvae are reared in natural conditions in the food plants.
Depending upon the food habit, the silks produced by the silkworms are divided into two large groups, mulberry and non-mulberry silks. The *B. mori* silkworm is monophagous i.e., it solely feeds on the leaves of mulberry plant (*Morus* spp.) while the non-mulberry silkworms feed on the leaves of a large range of plants and are subsequently called polyphagous. Most commercially cultivated silks are the product of mulberry silkworm, *B. mori* (Wingate and Mohler, 1984). There are 400-500 species of silk-producing moths in the world, but only 9 species are commercially cultivated. The domesticated mulberry silkmoth, *B. mori* covers 99% of the total world silk production (Dingle *et al*., 2005).

Silkworms are classified into different categories such as univoltine, bivoltine and multivoltine according to the number of generations they produce per year. Univoltine produces only one generation per year, while bivoltine produces two generations and multivoltine more than two generations in a year (Dingle *et al*., 2005). Franck, (2001) reported that bivoltine strains produce a larger quantity of thread per cocoon than the multivoltine strains. The quality of the thread is very good as it is even, lustrous and strong.

Silk produced by *B. mori* makes up 95% of the total raw silk produced in India and the remaining 5% is covered by „wild silks“. In India, 95% of „wild silk“ or „vanya silk“ is spun by two species of *Saturniidae*, i.e. *A. mylitta* and *A. assamensis* (Ojha and Pandey, 2004). Other silkmoth species in the family *Saturniidae* spin silks of economic importance like *Samia ricini* which produces the Eri silk known for its thermal properties and low cost of production.
Apart from these, other non mulberry silks are found in selected geographical demarcations and their products are utilized all over the world in varied degree depending upon the availability and its demand. It has been reported that wild silk fibers are different from commercial \textit{B. mori} silk fibers, possessing some unique properties such as high porosity (Kawahara, 1993) and a degree of natural colour. Protein composition and configuration of the wild silks is drastically different than silks spun by \textit{B. mori} (Zhou \textit{et al.}, 2000). Other properties that can be accounted are texture, colour and reflectance making them unique materials for unique textiles (Kuroda, 2000). Thangavelu (1991) also reported that saturniids \textit{A. assamensis}, \textit{A. mylitta} and \textit{Philosamia cynthia} shows genetic diversity and natural variations in the wild population indicating natural adaptation to specific niches. The distribution is basically dependent on availability of leaves of food plants (Chinnaswamy, 2001).

The silk produced by \textit{Antheraea pernyi}, is known as “tasar silk” and is the foremost wild silk in production and use all over the world. Other wild silks found in India include those produced by \textit{A. mylitta}, \textit{A. assamensis}, \textit{Antheraea roylei}, \textit{Antheraea proylei}, \textit{A. frithi}, \textit{S. ricini}, \textit{A. atlas} and \textit{C. trifenestrata}.

Sericulture has been an important source of livelihood resulting in independent income generation preventing unemployment since long (Sahay \textit{et al.}, 1997; Jayaram \textit{et al.}, 1998; Rani, 2007). Currently, India is the largest producer of wild silk, commercially exploiting, \textit{A. mylitta}, \textit{A. assamensis} and \textit{S. ricini} with wide range of products. Recently production has decreased due to low productivity of these silkworms resulting mainly because of habitat degradation and loss. Research effort is required to identify the
unexplored wild silkworm species and the eco-races that remained and to increase the viability of the currently utilized species (Thangavelu et al., 2002; Nurmalitasari and Kuroda 2002; Saratchandra and Singh 2002; Sharma and Sharma, 2006; Kakati and Chutia, 2009; Reddy, 2009).

The “Muga” silk produced by A. *assamensis* originates from India and is the most valued silk in the North Eastern part of India. Cocoons are large and exhibit a bright brown colour. The eggs hatch 3-5 times a year and their size is smaller than that of other wild silkworms. The family *Saturniidae*, which includes the silkworm *A. assamensis*, comprises various *Antheraea* species, work has been done by many workers reporting the chemical composition of the different silks (Lucas et al., 1955; 1960; Komatsu, 1980).

India is unique in having wide biodiversity of domesticated as also wild and partly domesticated silkworm species (Wardle, 1881; Watt, 1883). NE India is a treasure-house of silkworms producing and exploiting all the economically important varieties of natural silks viz. “Muga” (*A. assamensis*), “oak tasar” (*A. proylei*), “Eri” (*S. ricini*) and “pat” (*B. mori*) silk (Choudhury, 1970).

Northeast India has always intrigued researchers from around the world to work on its vast biodiversity and flora and fauna and the field of sericulture is also not lagging behind. Ample reports are available regarding the wild silkworms of this region. Seitz (1933) reported 19 species of wild sericigenous Lepidopterans from the entire NE India including Sikkim and Assam. Chowdhury, (1983) and Thangavelu, (1991) recorded 10
and 9 sericigenous species respectively from these regions. Kakati and Chutia, (2009) revealed the presence of 14 species belonging to 8 genera in Nagaland of NE region of India. These findings provide great scope and potentiality of research in these unexplored regions which may probably redefine the sericulture aspects of these regions.

The semi-domesticated “Muga” silkworm, *A. assamensis* Helfer (chromosome number, n = 15; Lepidoptera: *Saturniidae*) (Deodikar *et al.*, 1962) is an economically important insect which produces golden yellow hued silk. Its name is derived from the amber (brown) colour of the cocoon, which is called “Muga” in Assamese. It is indigenous to NE India and found mostly along the Brahmaputra valley of Assam (Choudhury, 1981; Rao, 1978). The larvae of Muga is polyphagous in nature, it feeds on leaves of woody trees like som (*Machilus bombycina* King), sualu (*Litsaea polyantha* A. Juss), *L. citrata* Roxy, *L. salicifolia* Roxy and many other host plants belonging to the family *Lauraceae*. It is multivoltine in nature having 5-6 generations in a year. “Muga” silk due to its high durability and quality has high value and demand in the national and international market (Freddi *et al.*, 1994).

*Samia ricini* (chromosome number, n = 14; Lepidoptera: *Saturniidae*) (Dederer, 1907; 1915; Deodikar and Thakar, 1958) is a multivoltine silkworm. It is also polyphagous in nature and feeds on the leaves of Kesseru (*Heteropanax fragrans* Seem.), Tapioca (*Manihot esculenta*), Papaya (*Carica papaya*), Jatropha (*Jatropha curcas*), Barpat (*Ailanthus grandis*) and Payam (*Evodia fraxinifolia*) (Singh and Das, 2006; Chakravorty and Neog 2006; Bhattacharya *et al.*, 2006; Choudhary, 2006). It produces
cocoons of two different colours, white or creamy and brick red. They lack the peduncle. Eri silk has its demand because of its thermal properties.

The income generated by the wild silk productions has led to the identification of additional wild silkworms especially those who can contribute to the fall in dependence of rural farmers on resources from the forests that border their farms. Wild silk production has been successful in providing ample income and employment in rural and undeveloped parts of India because of the unique qualities and diversified applications.

In addition, recent interest in finding novel and environmentally stable materials has stimulated research on silk types and the organisms that produce them (Craig and Riekel, 2002; Sutherland et al., 2009). Wild silk materials are being used in cosmetics industries, and as dietary additives and animal feeds (Reddy, 2008). It has also been reported of wild silks being used as diverse medical products and optical technologies (Iizuka, 2002; Altman et al., 2003; Kim et al., 2010). The unique microstructure, optical properties, molecular composition and mechanical properties of silk spun by the Saturniidae are being recognized for their potential for biomedical (Akai and Nagahima, 1999; Acharya et al., 2008) and industrial use (Dash et al., 2008; Mandal and Kundu, 2008; 2009).

**Composition of Silk**

Biochemically, silk is a protein fiber which is continuous in nature produced by some Lepidoptera insects such as silkworms, spiders, scorpions and mites to protect themselves from harsh environmental conditions and predators by forming cocoons. 
(Altman et al., 2003; Kadolph et al., 2002). The process of morphogenesis also occurs inside the cocoons, hence this account for a significance aspect of the insect life apart from obtaining the silk from the cocoons.

The chemistry of silk is made up of two components, sericin and fibroin. Fibroin is the core of the silk fiber which is insoluble in most solvents and forms the main component of the silk fiber, whereas sericin is the water-soluble proteinaceous glue. In terms of configuration, fibroin is a fibrous or crystalline protein with ordered structure and sericin is a non-fibrous or amorphous material with no definite structure (Freddi et al., 2003; Reddy and Krishnan, 2003; Jiang et al., 2006). The formation of cocoons takes place by the production of fibroin by the two spinneret present on each side of the head, and the circular movement of the head of the cocooning larvae. The cementing of the two fibroin fibers is done by sericin which acts like a glue.

Fibroin accounts for about 75% and sericin for about 25% of the total fiber weight (Freddi et al., 2003; Jiang et al., 2006). The percentage varies slightly in different genus and species. The pair of silk gland is divided into three parts- posterior, middle and anterior. Fibroin is secreted from the posterior part and contains three components: heavy-chain fibroin (H-fibroin), light-chain fibroin (L-fibroin) and fibrohexamarine/P25 (Zhou et al., 2000; Izzetoglu et al., 2009). Sericin is produced into the gland from cells of the middle division of the silk gland (Zurovec et al., 1998). Interestingly, the fibroin L-chain and fibrohexamarine/P25 are not found in Antheraea yamamai and A. mylitta silk fibroin (Tanaka and Mizuno, 2001; Datta et al., 2001). The amino acid composition
of fibroin of different silkworms have been studied and reported by many workers (Kadolph et al., 2002; Freddi et al., 1994; Yanagi et al., 2000).

Fibrils that make up the silk threads may be either compact fibrils or porous fibrils (Akai, 2000; Narumi et al., 1994). Silkmoths in the family Saturniidae mostly produces the porous fibers. Lepidoptera larvae in the family Bombyciidae, Thaumetopoidae and Lasiocampidae spin compact fibers. The contrasting feature of silk threads produced by B. mori, with Saturniidae species is that, they are not permeated with fine tubules. A single filament of silk produced by A. yamamai contains nearly 1000 fine tubules. The diameter of the tubules varies widely as does their number and position. Large amount of sericin covers the column of the filament and fills spaces between the fibrils that make up threads spun into the cocoon shell. Some Saturniidae species are also reported to spin porous cocoons.

Although silk fibers are composed of fibroin and sericin, their respective molecular weight varies according to the species. SDS-PAGE analysis of fibroin and sericin has been done and the results shows that fibroin from B. mori is composed of two chains, one of 350 kDa and the other of 25 kDa (Yamada et al., 2001) while the estimated molecular weight of A. assamensis fibroin in the non-reducing condition is approximately 500 kDa which is a homodimer of 250 kDa polypeptides (Kar et al., 2013). The A. mylitta silk gland protein fibroin is a dimer of approximately 197 kDa of total 395 kDa (Datta et al., 2001; Mandal and Kundu, 2008). B. mori sericin has been reported to contain three major protein components whose molecular weights are 250, 180 and 100 kda (Teramoto and Miyazawa, 2005). In case of A. mylitta peduncle three
characteristic bands, more than 200, 200 and 70 kDa were reported along with some other low molecular weight fractions below 50 kDa (Biraja et al., 2009). Similar observations were made for sericin extracted from cocoon (Dash et al., 2007; 2008). In case of sericin from Antheraea assamensis and Philosamia ricini a prominent band at 66 kda has been reported by Ahmed et al, (2004). A band at 100kda was also reported along with smear in the high molecular weight range from 150- 200kda. Another smear was reported in the region of 50- 36kda which indicates low molecular weight sericin in the cocoons of A. assamensis (Dutta et al., 2012). 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).

Biomaterials

Recently the critical agenda about the depletion of natural resources and the process of recycling has led the interest to biomaterials with renewable properties. The traditional composites were normally made of glass, carbon or aramid fibers but due to the increasing environmental consciousness and the legal procedures these composites are reinforced with some biodegradable materials. Research and development work on natural fibers, genetic engineering and the field of composite materials has led to the development of superior materials with global sustainability and the added feature of being designed to meet specific requirements. The environmental problems can be tackled with composites made of natural fibers as they are cheap and biodegradable. Biodegradable polymers also automatically solve the problem of waste-disposal. The
challenge in this area is to find probable applications for these composites which would be beneficial economically and environmentally (Mohanty et al., 2000).

Silk has been considered as a potential biomaterial and accordingly used since long. Silk fibers of the silkworm B. mori have been the primary material used in biomedical applications particularly as sutures. During decades of use, silk fibers have proven to be effective in many clinical applications. The biological responses of silk are comparable to most other commonly used biomaterials. A misconception was there regarding silk as non-degradable but it has been proved that silk, as a protein, is susceptible to proteolytic degradation and is slowly absorbed (Altman et al., 2003). Apart from sutures there has been considerable development in the field of biomaterials regarding silk. B.mori silk was only used most commonly in biomaterial applications but in recent years other silk with great potentiality have evolved and the field of biomaterial involving silk has emerged stronger and promising.

Silk is considered to have all the required properties to be utilized as a biomaterial. The high molecular weight peptide sequences in silk are either hydrophobic or hydrophilic (Chen et al., 2002). The advantage of the highly repetitive primary domains of silk fibroin is that they assemble into regular structures during biomaterials formation and mimics the properties of synthetic block copolymers (Wilson et al., 2000; Vollrath et al., 2001; Valluzzi et al., 2002; van Beek et al., 2002). The primary protein sequence of silk fibroin have evolved to a great extent according to the environmental needs, such as spinning underwater nets to trap preys to trap air for breathing which signifies the ability to form robust biomaterials, the main factor being the repetitive
protein blocks and the side chains containing amino acids like, glycine, serine, and alanine (Altman et al., 2003).

Crystalline β-sheet structures are formed during spinning of silk due to hydrogen bonding and hydrophobic interactions, which accounts for the tensile strength and toughness of the fiber (Guhrs et al., 2000; Altman et al., 2002; 2003; Shao et al., 2002). The fiber spinning process starts from highly concentrated silk solutions in the silk glands which is followed by extraction of water, changes in salt concentration and finally through the spinnerets by mechanical stress and chain alignment. This process has been successfully transferred into in vitro environments, providing the basis for the fabrication of silk biomaterials as implant materials (Jin and Kaplan, 2003). These silk-based biomaterials have created an individual niche in the field of biomaterial applications, due to its robust mechanical, morphological and interesting structural properties (Meinel et al., 2004a; 2004b; 2004c; 2005).

Types of Biomaterials

Natural polymeric biomaterials are widely studied and researched nowadays because of their potentiality of being noteworthy targets in the biomedical filed (Shrestha et al., 2014; Chan et al., 2014). Primarily, silk fibroin derived from B. mori silkworm; B. mori (B. mori) has been widely used as suitable matrices/substrates due to its high oxygen permeability, superior mechanical properties and excellent biocompatibility (Vepari and Kaplan, 2007; Reddy et al., 2011; Tao et al., 2012; Huang et al., 2014; Yucel et al., 2014; Partlow et al., 2014). Silk based biomaterials have been
fabricated in different forms such as films, fibers, nanoparticles, microparticles, hydrogels and scaffolds and utilized for a broad range of biomedical applications (Mandal et al., 2009; 2012; Mandal and Kundu, 2010; Bhardwaj et al., 2010; 2011a; 2011b; 2015; Omenetto and Kaplan, 2010).

Films

The applications of silk films in biomedical and biotechnological fields have been going on for more than a decade. Representative examples are the use of silk fibroin films as oxygen and drug permeable membranes (Minoura et al., 1990; Chen et al., 1994), support for enzyme immobilization (Miyairi et al., 1978; Demura and Asakura, 1989) and substrates for cell culture (Inoue et al., 1998; Higuchi et al., 2000). Tretinnikov, (2001) prepared fibroin films from silk of *B. mori* and studied the near-surface structure and the wettability of silk fibroin films cast from aqueous solutions on varying the casting temperature. He concluded that the casting temperature plays a very crucial role in the secondary structures of the films, with different structures on varying the temperature. Lv et al., (2005) studied the effect of drying temperature on fibroin films which was found to control the formation of α helix and β sheet conformations. It was reported that the rate of drying made the films soluble or insoluble. Silk fibroin films as a drug delivery carrier was investigated by Hofmann et al., (2006) where drug was entrapped into the films during processing. Films were successful in delivering the drugs due to its controllable level of crystallinity and the ability to process the biomaterial in biocompatible fashion under ambient conditions to avoid damage to labile compounds to be delivered.
Silk fibroin films prepared from the gland of mature fifth instar larvae were used as two dimensional matrix for tissue engineering by Kundu et al., (2008), the matrix acts as a substrate where the cells adhere and grow. The films made from liquid silk were found to be amorphous and hydrophilic but after alcohol treatment it became insoluble. Mouse fibroblast cells were grown and proliferated in these matrices which accounts for their biocompatibility.

Earlier silk fibers were used as surgical sutures (Halstead, 1913). In the medical field silk proteins have been useful in an array of applications, especially as scaffolds to promote cell growth, drug delivery systems, replacements for connective tissue, and antioxidants (Mori et al., 2000). The B. mori fibroin has been utilized for osteoblast, fibroblast, hepatocyte and keratinocyte adherence and growth in vitro (Chiarini et al., 2003; Gotoh et al., 2004; Min et al., 2004; Unger et al., 2004), and as an alternative to collagen in surgery, mainly in the form of sutures (Panilaitis et al., 2003).

Acharya et al., (2009) reported for the first time the use of fibroin films from a non mulberry silkworm Antheraea mylitta as a substrate for in vitro cell culture. The dissimilarities of fibroins from B. mori and non-mulberry silkworms have been depicted by X-ray diffraction patterns, which shows that the B. mori silk fibroin contains a -G-X-G-X- (G, glycine; X, alanine, or serine) repeat structure, whereas non- mulberry silk fibroin, generally produced by wild or semi-domesticated Saturniid silkworms, namely Antheraea sp. (wild) and Samia ricini, contain polyalanine repeat sequences (Kirimura et al., 1962; Lucas and Rudall, 1968). Antheraea mylitta, a tropical non-mulberry tasar silkworm, is the highest silk producer (Akai, 1998). The wall of A. mylitta cocoons,
collected from the wild habitats, are found to be much tougher (Shamitha and Rao, 1998), and fibroin, is the main constituent of the cocoon. After evaluating the physicochemical properties of A. mylitta fibroin its potential use as a biomaterial was established, and was found to be better in cell attachment than B mori substrates, which can be a probable substitute to the B. mori silk biomaterials.

Mandal et al., (2009) used silk fibroin as drug release vehicles in combination with gelatin. Multilayered films were fabricated using fibroin and gelatin and bioactive molecule was encapsulated in the thin polymer films, directed to the targets. This versatile and tunable property of fibroin/gelatin multilayer films makes them potential candidates for the controlled release of a wide variety of bioactive molecules. While the structural conformation of the biomaterial is the key to its applicability considerable work has been done to improve the secondary structures of the silk biomaterials. Qiang et al., (2010) prepared water insoluble fibroin films by controlling the slow drying of the B mori silk solutions. Alcohol treatment is not required in this type of films and also degrades at a faster rate than the conventional films. This type of films is the substitute for the biomaterials which require faster degradation in in vivo conditions. Amornsudthiwat et al., (2013) treated thai silk fibroin films with low energy plasma which resulted in better and faster cell adhesion without any significant change in surface topography and bulk chemistry.

During the processing of silk fibers, sericin is normally removed during the degumming process as waste product, although interesting new applications are coming up for it (Freddi et al., 2003). The highly hydrophilic property of sericin is because of
strong polar side chains such as hydroxyl, carboxyl and amino groups, which enables easy cross-linking, copolymerization and blending with other polymers to produce biodegradable materials with varied applications (Ahn et al., 2001; Nagura et al., 2001; Cho et al., 2003). Sericin has been reported to be an anticoagulating agent, an antiwrinkle agent and an antioxidant due to the presence of moisturizing factors and mixtures of high-serine amino acids (Kato et al., 1998; Miyazaki et al., 2004; Takeuchi et al., 2005). Sericin is reported to prevent apoptosis in cultured mouse cells (Baba et al., 1996; Sasaki et al., 2000). Its topical application suppresses tumor promotion and reduces oxidative stress (Zhaorigetu et al., 2003). Sericin enhances mammalian cell proliferation and attachment of human skin fibroblasts, and is used as a media supplement for serum-free culture though may prove to be toxic at higher concentrations (Terada et al., 2002; 2005; Tsubouchi et al., 2005).

Considerable work has been done in utilization of sericin in the field of biomaterials. Turbiani et al. (2011) developed sericin films with dimethylolurea as cross-linking agent and glycerol as plasticizer. It was inferred that sericin-based film properties are dependent on components used to form film, which can used to tailor the desired film flexibility. Miyake et al., (2003) developed sericin films which were water resistant and had high mechanical properties, the films were compared with crosslinked sericin films on moisture characteristics and structure. Teramoto et al., (2005) developed sericin hydrogels from hope sericin silkworms which cocoons contains exclusively sericin. The hydrogels were evaluated as potential biomaterials.
Teramoto et al., (2008) fabricated sericin films from B. mori sericin without any chemical modification and used as wound dressing as the films possessed no cytotoxicity. The non mulberry silks are also being exploited for biomaterials. Dash et al., (2009) prepared sericin films from the liquid silk of the silkworm Antheraea mylitta and used for cell growth and proliferation. The films prepared were without any crosslinking agents and showed good mechanical and structural properties. Similarly, biocompatible biopolymer from Antheraea mylitta cocoons in the form of films and scaffolds were fabricated and evaluated by Mandal et al., (2009). The biomaterials were found to have low immunogenicity and optimum for tissue engineering applications.

**Scaffolds**

The main purpose of 2-D and 3-D matrices in the form of films and scaffolds in biomedical field is to be used for drug delivery, grafts and matrices for immobilization (Wang et al., 2006; Vepari and Kaplan, 2007). The requirements for a ideal scaffold are ease of fabrication, biodegradability, surface chemistry, biocompatibility, mechanical strength and interconnecting pores depending on the application (Altman et al., 2003; Edwards et al., 2004; Mandal and Kundu, 2008). A suitable scaffold should be identical to the tissue it replaces (Mikos et al., 2000). Natural and synthetic biodegradable polymers, proteins, polysaccharides and glycosaminoglycans have been tested as scaffolds as the material plays a pivotal role.

Silk has been used as a model protein for scaffold fabrication due to its widespread versatility as a mechanically robust, biocompatible, tissue engineering
material. Mandal et al., (2009) fabricated scaffolds from *B mori* and *A mylitta* silk fibroin and used it as a substrate for osteogenic and adipogenic differentiation of rat bone marrow cells. Scaffolds of wild non-mulberry tropical tasar silk gland fibroin protein were also utilized as a substratum for osteogenic differentiation of human mesenchymal stem cells by Mandal et al., (2009). Silk fibroin scaffolds has also been used for drug delivery practices, Mandal et al., (2009) embedded drug loaded calcium alginate beads in fibroin scaffolds and targeted the scaffolds to the drug releasing sites.

Further, silk fibroin films are widely used as delivery vehicles for bioactive compounds such as horseradish peroxidase and lysozyme, heparin, paclitaxel, and clopidogrel to evaluate vascular cell responses for stent application (Hofmann et al., 2006; Wang et al., 2007a; 2007b; 2008). Sericin has also been used as a 3D scaffold for many biomedical applications. Mandal et al., (2009) fabricated sericin combined with gelatin scaffolds for cell growth and proliferation. Low immunogenicity of the matrices shows that it can be successfully used as a biopolymer graft material. Research is going on in all the aspects of using silk as biomaterials, including fabrication of the matrices without the use of any chemicals. Qiang et al., (2010) developed a green process without the use of any organic solvents or harsh processing conditions to prepare silk fibroin scaffolds, but stable in cell and tissue culture applications. The process involved lyophilization and water annealing to achieve the 3D scaffold.
Micro/ Nanoparticles

The nanoparticles of synthetic and natural origin have had a significant and promising part in the biomedical field. The continuous need of new materials have opened up the scope of silk protein derived nano and microparticles as biomaterials. Various types of nanoparticles like solid lipid particles, micelles, liposomes have been reported as nanodelivery systems to encapsulate the drugs and other biotechnology products (Muller et al., 2000; Torchilin, 2004; Azarmi et al., 2006; Liu et al., 2007). Protein based nanoparticles generally vary in size from 50 to 300nm (Azarmi et al., 2006). They are usually stable during storage and in in vivo conditions, non toxic and ease of fabrication (Soppimath et al., 2002; Balthasar et al., 2005). In the last two decades, many studies have focused on the development of drug-delivery systems to achieve controlled release and mechanisms to enable drug targeting to specific tissue (tumor) sites by utilization of microparticles and nanoparticles (Gregoriadis, 1988; 1995; Gobin et al., 2006; Cheema et al., 2007). Generally three different methods are utilized for the fabrication of protein based nanoparticles namely, emulsion formation, coacervation or desolvation, water-in-oil emulsion (Weber et al., 2000).

Silk proteins fibroin and sericin have been used as nanoparticles for drug delivery due to its biocompatibility and slow degradation process. Nanoparticles are very small enabling them to pass through small capillaries to the targeted sites. In the sites they release the drug encapsulated in them or the therapeutic molecule. Silk gland fibroin isolated from tropical tasar silkworm A. mylitta has been utilized as a promising biomaterial for tissue engineering (Mandal and Kundu, 2008). Mandal et al., (2010)
fabricated silk protein fibroin spherical nanoparticles from both B. mori and non-B. mori silk utilizing dimethyl sulfoxide as desolvating and studied the cellular uptake of these nanoparticles by cells as well as the potentiality as drug delivery systems (Kundu et al., 2010). Apart from this, silk fibroin protein powder has been used as an additive to cosmetics, food, and as biomaterials due to its excellent properties like moisture absorption friendly to the human skin (Xu et al., 2006). Zhang et al., (2007) prepared fibroin based nanoparticles from B. mori in organic solvents and characterized them for potential biomedical use. It was found that the crystalline structure of the particles were one fourth of the fiber. Yan et al., (2009) conjugated silk fibroin derived nanoparticles with insulin for studying the effect of bioconjugated drug delivery systems. It was found that when insulin was coupled covalently with silk nanoparticles, the resistance of the modified insulin to trypsin digestion and in vitro stability in human serum were greatly enhanced. Recently, Bharadwaj et al., (2015) fabricated silk fibroin microparticles from the non mulberryi silkworms viz. A. assamensis, A. mylitta and P. ricini. The process employed for fabrication was wet milling followed by spray drying techniques. The study revealed that non mulberry particles were better in terms of cell adhesion, spreading and viability of cells in in vitro conditions.

Sericin has also been utilized as nanoparticles in diverse biomedical applications. Mandal et al., (2009) used sericin derived from cocoons of A. mylitta to prepare self assembled nanoparticles blended with pluronic. The nanoparticles were able to carry both hydrophobic and hydrophilic drugs for targeted delivery. Hazeri et al., (2012) reported the production of sericin nanopowder by electrospraying method. The
electrospraying solution was prepared by dissolving the sericin sponge in dimethyl sulfoxide. The average particle size of the particles was 25 nm, which is by far smaller than the particles produced by other techniques. The electrosprayed sericin nanopowder consisted of small crystallites and exhibited a high moisture absorbance.

**Properties of biomaterials**

**Morphological properties**

**Scanning Electron Microscopy**

The surface properties of the biomaterials is of great significance as it is that portion of the biomaterial that comes in direct contact with the cells or the therapeutic agents to be delivered. Therefore the fabrication process of the material is crucial as it has direct affect on the surface. Lu et al., (2010), while working on water-insoluble silk films prepared by different methods found that, films were composed of nano-filaments in scanning electron microscopy. The nano-filaments formed only after water or methanol treatment. When water-insoluble silk films were prepared by slow drying more complicated nano-structures were found. The nano-filaments assembled to form globules and linked the adjacent globules together. It was inferred that the filaments and globules were made up of different secondary structures.

Acharya et al., (2009) while testing the surface roughness of films found that *B. mori* film surface was less rough (0.7μm) compared to *A. mylitta* surface (1.7 μm). *B. mori* fibroin films when treated with methanol changes its physicochemical properties with the formation of globular surface structures which are absent on surfaces from
water treated films inducing a shift to higher amounts of crystalline β-sheets structures which eventually affects the hydrophobicity of the films indicated by contact angle, as reported by Hoffmen et al., (2006).

**Contact Angle Measurements**

Contact angle measurements determine the hydrophobicity of the surface of biomaterials. Dynamic contact angle attributes towards the surface roughness of the films. Contact angle of fibroin films from *B. mori* liquid silk was found to increase after methanol treatment indicating more surface roughness (Kundu et al., 2008). Contact angle of *B. mori* and *A. mylitta* have been studied by Acharya et al., (2009) which shows that non-mulberry fibroin film is more hydrophobic compared to *B. mori* films.

Sericin films are normally prepared in combination with a different chemical which acts as a cross linking agent. Mandal et al., (2009) reported granular appearance of sericin gelatin films, from cocoons of *A. mylitta* with no significant effect in granularity and porosity on increasing the sericin content. The films appeared rigid and resembled the golden yellow colour of non mulberry cocoons. The granular appearance is supposed to aid in cell attachment and proliferation (Servoli et al., 2005). Liquid sericin extracted from the middle silk gland of *A. mylitta* fifth instar larvae has also been used as biomaterials by Dash et al., (2009). Scanning electron microscopic analysis of the films showed that untreated films were uniform and smooth in texture as compared to alcohol treated films. It was inferred that alcohol treatment induced crystallinity generating a stretching force, which lead to rougher surface with more surface area and
better adherence capability to the growing cells. Nayak et al., (2012) fabricated sericin films crosslinked with glutaraldehyde. Surface topography observed by SEM images of membranes showed a rough surface morphology. This roughness is assumed to be the result of the alcohol treatment used during the membrane fabrication process. The results correspond to the previous reports of rough uneven surfaces of sericin blended films.

**Secondary structures of biomaterials**

**FTIR**

Mechanical properties of silk derived biomaterials are influenced by secondary structures of fiber protein and gland protein. In general, kinds of secondary structures of protein are α-helix, β-sheet, β-turn and random coil. Moreover, they can be divided as amorphous (silk random coil or silk I), crystal (silk II or β-sheet) and interfacial (silk III) parts (Vepari and Kaplan, 2007). Crystallization can be induced by some chemical reagents such as alcohol (Jiang et al., 2006), salt (Vepari and Kaplan, 2007), and heat (Freddi et al., 1997).

FTIR is an established technique to study the conformation of proteins; the secondary structures of protein are indicated by the amide groups of the silk composition (Kweon et al., 2000). This works on the principle of infrared radiation, indicating the wavelengths of radiation absorbed by the different samples, which vary depending on the conformation of the protein chain (Bhardwaj et al., 2015). The amide I mode associated with the α-helical conformation occurs at 1650-1660 cm\(^{-1}\), the random coil conformations give bands in the range of 1640-1650 cm\(^{-1}\), and the β-sheet conformation
results in IR bands between 1620 and 1640 cm\(^{-1}\) (Byler and Susi, 1986; Surewicz and Mantsh, 1988; Haris and Chapman, 1995). Lu et al., (2010) assumed a broader range for the amide absorptions reporting that the infrared (IR) spectral region within 1700–1500 cm\(^{-1}\) is assigned to absorption by the peptide backbones of amide I (1700–1600 cm\(^{-1}\)) and amide II (1600–1500 cm\(^{-1}\)), which have been commonly used for the analysis of different secondary structures of silk fibroin. The peaks at 1610–1630 cm\(^{-1}\) (amide I) and 1510–1520 cm\(^{-1}\) (amide II) are characteristic of silk II secondary structure (Hu et al., 2006). Although there is some ambiguity regarding the random coil conformations where some researchers have suggested that the absorptions at 1648–1554 cm\(^{-1}\) (amide I) and 1535–1542 cm\(^{-1}\) are indicative of random coil structure (Um et al., 2001) and other studies have revealed that these peaks correspond to the silk I conformation (Wilson et al., 2000; Jin et al., 2005). Different authors have confirmed that the amide I band represents primarily the C=O stretching vibration of the amide group. The frequency of this vibration depends on the strength of hydrogen bonding between the C=O and N-H groups, which in turn is determined by the particular conformational structure of the protein backbone. In general, the amide I mode associated with the \(\alpha\)-helical conformation occurs at 1650-1660 cm\(^{-1}\), the random coil conformations give bands in the range of 1640-1650 cm\(^{-1}\), and the \(\beta\)-sheet conformation results in IR bands between 1620 and 1640 cm\(^{-1}\) (Byler and Susi, 1986; Surewicz and Mantsh, 1988; Haris and Chapman, 1995).

FTIR has been one of the most important and primary instrument used to study the conformation of fibroin and sericin (Mathur et al., 1997; Tretinnikov and Tamada,
Biomaterials derived from silk are first observed in the IR spectra for the possible secondary structures which play an important role in application of the biomaterial. Lv et al., (2004) while working with fibroin films varied the drying rate of the films and found that the transition from from the α/random coil to an antiparallel β-sheet form occurred by the increase in the drying temperature.

Hoffmen et al., (2006) studied the structural conformation of fibroin films before and after methanol treatment. FTIR structural analysis of methanol treated films showed an N–H bending vibration bond (amide II) intensity shift from 1540 to 1535 cm\(^{-1}\) when compared to native films. Similarly, methanol treatment resulted in additional shoulders at 1630 cm\(^{-1}\) (amide I) and 1265 cm\(^{-1}\) (amide III). The water treated silk films for the most part lack the peaks for secondary structure at 1695, 1627 and 1520 cm\(^{-1}\). The results showed that after methanol treatment the β sheet content of the films increased depicting a more profound crystalline conformation. Kundu et al., (2008) worked with fibroin films as biomaterials extracted directly from the silk gland of the silkworm B. mori. Native films from gland showed strong absorption bands at 1650 (amide I), 1540 (amide II), and 1240 cm\(^{-1}\) (amide III), attributed to the random coil conformation. On the contrary, gland films treated with methanol showed similar absorption bands at 1640 (amide I), 1540 (amide II), and 1240 cm\(^{-1}\) (amide III), assigned to the random coil conformation along with peaks at 1450 cm \(^{-1}\) (methanol group) which was absent in the untreated films and 1070 cm \(^{-1}\) (protein backbone stretching) compared to that of the 1170 cm \(^{-1}\) (C–O stretching vibrations) in the untreated films. The samples of B. mori cocoon fibroin films were also studied for comparison. Untreated films from
cocoon showed strong absorption bands at 1655 (amide I), 1540 (amide II), and 1235 cm\(^{-1}\) (amide III), which are attributed to the random coil conformation.

To fewer extents, non-mulberry silk like \textit{A. mylitta} has also been studied as potential biomaterials. Acharya et al., (2008) fabricated fibroin films from the above said silkworm. Secondary structure analysis by FTIR revealed that fibroin showed a presence of a strong peak at 1640 cm\(^{-1}\) in \textit{A. mylitta} and a peak at 1650 cm\(^{-1}\) in \textit{B. mori} fibroin both in solution and as film, which point towards retention of a native secondary structure (amide I) of protein. Certain differences in physical parameters were observed though both proteins are essentially hydrophobic in nature and dissolve only in chaotrope solvents. The presence of \(\beta\) sheet in \textit{A. mylitta} fibroin was confirmed by the prominent peak at 1640 cm\(^{-1}\), whereas in \textit{B. mori} fibroin the peak lies around 1650 cm\(^{-1}\), which indicates that there is a greater proportion of \(\beta\) helices possibly leading to a more hydrophilic fibroin component than \textit{A. mylitta} fibroin. Lu et al., (2010) studied the effect on structure by controlling the drying rate of fibroin films. It was observed that the amide I band for soluble silk film showed one strong peak at 1644 cm\(^{-1}\), corresponding to random coil. After methanol treatment the amide I band showed one strong peak at 1622 cm\(^{-1}\), with a shoulder at 1652 cm\(^{-1}\), while in the water-annealed sample one peak at 1652 cm\(^{-1}\) appeared, with a shoulder at 1622 cm\(^{-1}\). When formed by slow drying the silk film exhibited strong absorption at 1652 cm\(^{-1}\), with a minor shoulder at 1622 cm\(^{-1}\). The same trend in structural change was also found in the amide II region. It was concluded that the secondary structure of silk can be controlled by changing the processing utilized – slow drying, water annealing or methanol annealing.
Considerable research is being carried out for utilizing sericin as biomaterials as well. Films, hydrogels, powders from sericin have been reported and used as biomaterials. Similarly to fibroin, the secondary structure of sericin also needs to be confirmed, and FTIR is the most apt technique to do so. Teramoto et al., (2008) studied sericin films from B.mori. The amide I peak was observed at 1614 cm\(^{-1}\) attributed to intermolecular β sheet structure. Amide bonds form the polypeptide backbone and due to specific vibrational frequencies result in conformational changes in protein molecules. Dash et al., (2009) studied the conformational changes in sericin films fabricated from B. mori before and after alcohol treatments. Treatment with 70\% (v/v) ethanol caused changes in the secondary structure of native silk gland sericin. The peak at 1655 cm\(^{-1}\) in the amide I absorption which corresponds to random coil shifted towards 1630cm\(^{-1}\) on ethanol treatment indicating a transition from random coil to β-sheets. Similarly, on treatment with aqueous ethanol the peaks at 1530cm\(^{-1}\) in the amide II region and 700cm\(^{-1}\) peak in the amide V region showed abundance of β-sheets.

Nanoparticles and microparticles are the future armors after films and scaffolds. Focus is being given to these particles because they are considered more effective than films and scaffolds. Due to the size, they can be used in remarkable ways and in different parts of the body. Zhang et al., (2007) fabricated fibroin nanoparticles from B. mori, on comparative analysis of the secondary structures of the particles and fibroin films, FTIR peaks at 1654 cm\(^{-1}\), 1554 cm\(^{-1}\), 1242 cm\(^{-1}\) corresponding to α helix and random coil structures in films were observed, whereas the nanoparticles exhibited peaks at 1635 cm\(^{-1}\), 1520 cm\(^{-1}\), 1238 cm\(^{-1}\) corresponding to β sheet conformations. These
chemical shifts were similar to the fibroin films after methanol treatment as reported earlier by Zhang et al., (1998a; 1998b). Similar results were reported by Kundu et al., (2010) with nanoparticles made from B. mori fibers and from A. mylitta liquid silk. The chemical shift of the absorption bands to 1632 cm$^{-1}$ (amide I), 1521 cm$^{-1}$ (amide II), 1233 cm$^{-1}$ (amide III) attributed to the β-sheet structure in B. mori silk fibroin nanoparticles. The A. mylitta nanoparticles showed chemical shift of the absorption bands to 1673 cm$^{-1}$ (amide I), 1517 cm$^{-1}$ (amide II) and 1234 cm$^{-1}$ (amide III) assigned to the β-sheet structures. In a recent comparative study of microparticles by Bhardwaj et al., (2015) involving silk fibroin protein microparticles of A. assamensis, P. ricini, A. mylitta and B. mori, the characteristic vibration bands for Amide I were found in the region of 1622–1629 cm$^{-1}$ (C O stretching) and 1517–1519 cm$^{-1}$ for Amide II (secondary NH bending) and 1232–1239 cm$^{-1}$ for Amide III. The characteristic peaks signifies the β-sheet conformation and represents the silk-II structure, which confirms the retention of original microstructure of silk fibers in microparticles as reported earlier by other authors (Hu et al., 2006; Bhardwaj and Kundu, 2011).

**X-Ray Diffraction (XRD)**

The key to application of biomaterials in biomedical field is to properly determine the secondary structure of the biomaterials. XRD is a technique which helps in understanding the crystalline structure of the material (Um et al., 2001). Generally, there are three types of crystalline structure proposed for silk. The glandular state in liquid form present in the silk glands of larvae, prior to crystallization is called silk I. Silk II is the spun silk state, i.e. the hardened fiber which forms after the silkworm spins
the cocoon through its spinnerets which consists of the b-sheet secondary structure and silk III an air/water assembled interfacial silk, which is neither liquid or fibrous and is considered a helical structure (Kaplan et al., 1998; Motta et al., 2002; Jin and Kaplan, 2003). The XRD diffractogram gives peaks in 2θ corresponding to the conformations of the samples. It is reported that the main diffraction peaks of Silk I present at around 2θ = 12.2 and 28.2°, while Silk II present at about 2θ = 18.9 and 20.7° (Miniura et al., 1995).

Pure silk fibroin film has a characteristic peak at 2θ ≈ 12.2°, (Feng et al., 2007) which indicates Silk I structure. Methanol treated silk fibroin films, shows typical X-ray diffractograms of the β-sheet crystalline structure, since methanol is a crystallization inducing solvent (Tsukada et al., 1994).

XRD pattern for non-mulberry silk fiber have been done by Talukdar, (2013). It was reported that A. assamensis silk fiber showed two sharp adjacent peaks corresponding to 2θ = 16.8° and 20.20° and two broad minor peaks at 2θ = 33° and 38° corresponding to β-phase. Additional sharp peaks appearing at 2θ = 16.81° – 43.73° were associated with the α-phase. Similarly, the XRD pattern for P. ricini silk fiber showed two sharp adjacent peaks corresponding to 2θ = 16.8 and 20.10° which corresponded to α-phase. There was a broad minor peaks at 2θ = 33.6° corresponding to β-phase. Though most of the XRD studies are confined to the B. mori silks, the non-B. mori silks are also studied by a large number of workers. Li et al, (2003) studied the wide angle X-ray diffraction (WAXD) pattern of A. pernyi silk fiber and reported a typical-structure pattern, with major peaks at 16.7 and 20.3°. It is very interesting to note that although tasar, Muga and Eri are genetically different; the nature of the crystalline
structure is identical, which probably depends on the ratio of alanine/glycine as reported by Talukdar, (2013).

Considerable work on B. mori biomaterials have led to a proper understanding of the crystalline structures of biomaterials. Lv et al., (2004) prepared fibroin films and rendered them insoluble by controlling the drying rate. XRD curves of the regenerated fibroin films dried at different temperatures were found to be different. The films dried at 40 and 50°C could be characterized by the presence of three peaks at 12, 20.5, and 23.7°, corresponding to both α-helix and β-sheet crystalline structures indicating copresence of both structures. When the films were dried above 60°C, a new peak at 9.0°, which corresponded to the β-sheet crystalline structure, appeared, and the peak at 12° disappeared. The results indicated that α-helix form was transformed to an antiparallel β sheet with increasing drying temperature, and the α-helix crystalline almost disappeared when the drying temperature was raised above 60°C. Similarly it was reported by Devi et al., (2011) that in Muga fiber both α helix and β sheet structure coexist and inter-conversion between them occurs due to stress.

Hoffman et al., (2006) while working on fibroin films fabricated from B. mori for applications as drug delivery reported that, fibroin films showed silk I conformation in raw state but after alcohol treatment the silk II conformation was evident indicating crystalline β sheet structures. An interesting observation was made by Kundu et al., (2008) while working with B. mori fibroin films made from liquid silk that amorphous silk fibroin is crystallized to β-sheet during processing of silk fibroin in the gland of B. mori 5th instar larvae.
Wang et al., (2013) studied the effect of regeneration of liquid silk fibroin on its structure and Characterization and reported that the XRD diffraction peaks for silk II appear at 9.1°, 18.9° and 20.7°, among which 20.7° is the most important. The RSFM had a broad peak at 20.5°, which was attributed to the randomly coiled structure. Treatment with methanol resulted in a strong peak at 20.3° and minor peaks at 9.0° and 24.3°, which represented β-sheet crystalline structure, concluding that crystallinity is improved significantly when regenerated fibroin films are treated with alcohol.

Interesting reports have been published regarding sericin by different authors. Dash et al., (2007) reported that native sericin contains both random coils and β-sheets representing amorphous and crystalline regions respectively. While Miyake et al., (2003) studying high molecular weight sericin films found a diffraction peak near 2θ = 20°, and a shoulder peak at near 2θ = 12°, 28°, and 43°. Turbiani et al.,(2011) studied sericin films from *B. mori* cross linked with DMU and found that the intensity of the peak near 2θ = 20° was decreased considerably, it was concluded that crosslinking decreased the crystallinity of the films because of transformation of β-sheet and aggregated β-sheet structure to random coil due to intermolecular hydrogen bonding as reported by Teramoto *et al.*., (2007).

Dash et al., (2009) involved non-mulberry silk *A. mylitta* liquid sericin in preparing thin films. On XRD the films were found to be rich in β sheet structures showing a minor peak around 2θ = 19.2° and a comparatively major peak 23.2°, the results were in confirmation with earlier studies reported by Nagura et al., (2001); Tao et al., (2005). On further treatment with 70% (v/v) ethanol, sericin membranes showed a
sharp increase in peak intensity due to the formation of more crystalline β sheet structures.

Nayak et al., (2012) studied the effect of crosslinking sericin protein with glutaraldehyde to cast films, XRD spectra of sericin, showed two crystalline peaks at 19.3° (2θ) and 22.4° (2θ) corresponding to β-sheet crystalline spacing. The peak intensity increased in crosslinked sericin. It was reported that the crystallinity increases with gluteraldehyde crosslinking and alcohol treatment, which is 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.

Hazeri et al., (2012) fabricated sericin nanoparticles by electrospraying method and the characteristic XRD peak at 2θ= 20° was evident. It was inferred that the sericin produced by this method had a low crystallinity and its ordered parts were composed of very small crystallites known as β sheets.

**Thermal properties**

Thermal analysis is a useful tool for monitoring important processing parameters and end-use properties of fibers and biomaterials. In addition to other factors, the response of a fibrous polymer to a thermal treatment depends on its chemical architecture and microstructure. The thermal transitions may have significant effect on the processing conditions. These may also give directions for effective modifications in the biomaterials to help improve their performance, such as degradability, mechanical behaviour and handling. It has been reported earlier that the B. mori and non-B. mori
silks differ in thermal properties. Thermal degradation of domesticated silk, *B. mori* takes place in a single step, contrast from those of wild silks that take place in several steps (Kweon et al., 2000).

Studies on thermal behaviour of *B. mori* and non-mulberry silks have been extensively done by many scientists. Magoshi and Nakamura, (1975); Magoshi and Magoshi (1977) studied the DSC thermograms of silk fibroin of *B. mori* and *A. mylitta*. They observed one endothermic shift at 175°C, which they assigned to the glass transition (Tg), one exothermic peak at 212°C due to crystallization and a peak at 280°C related to degradation.

Similarly, Freddi et al., (1994) investigated the thermal properties of *A. assamensis* silk fibers using DSC. They reported two minor and broad endothermic transitions appeared at 230 and 300°C, followed by a prominent endothermic peak at 362°C. The latter was attributable to the thermal decomposition of silk fibers with β-conformation (Tsukada, 1988). The two minor endotherms occurring above glass transition temperature, Tg (190-200 °C) (Nakamura et al., 1986) was related to the molecular motion of the fibroin chains either in the amorphous and laterally ordered regions (Tsukada et al., 1992) or in the crystalline regions.

Tanaka et al., (2002) investigated the thermal properties of liquid silk from domestic and wild silkworms. Liquid silks obtained from the silk gland of the domesticated silkworm, *B. mori* and four wild silk-worms, *S. ricini*, *Dictyoploca japonica*, *A. pernyi* and *A. yamamai* were used. The DSC curves for the wild silk-worm
silks, showed clear exothermic peaks corresponding to a phase transition from the α-helix conformation to the β-form.

The thermal behavior of silk fibers and biomaterials are further investigated by measuring the expansion and contraction properties in the course of heating. Silk fibroin films cast from water solution contain bound water even after samples are dried in a vacuum oven. To remove the bound water, heat treatment at elevated temperatures is required. Bound water is lost from the film during heating, and the loss of mass is quantified using TGA. Bound water in the silk film acts as a plasticizer, and a lower glass transition temperature of the silk–water system. TGA analysis allows the water loss to be monitored quantitatively.

Das et al., (2000) while studying the dynamic thermal curves (TGA and DTGA) of silk fibers found that thermal decomposition took place in three main degradation steps, namely, initiation, propagation and carbonization. The TGA curves showed an initial small mass loss step around 100°C. In the second stage, the TGA curves showed a major weight loss, in the range of 350–450°C by decomposition of the protein in the fiber. In the third stage, in temperature range 570–760°C, most of the decomposition of proteins took place.

Talukdar et al., (1991) have studied B. mori, Muga and Eri silk fibers by DTA and TGA. The DTA curves exhibited endothermic peaks at about 93°C for all the three silk fibers, which were followed by an exothermic peak at around 312°C for B. mori and Muga and about 315°C for Eri. From the TGA analysis, it was found that B. mori exhibited maximum weight loss of about 52%, followed by Eri (49%) and Muga (37%).
Kundu et al., (2008) studied the effect of temperature on regenerated fibroin films from liquid silk of B. mori. Thermogravimetric curves of the regenerated SF films showed that the initial weight loss of films at 149°C is due to loss of moisture. The second weight loss took place in the temperature ranged from 218 to 330°C which was associated with the breakdown of side chain groups of amino acid residues as well as the cleavage of peptide bonds. The amorphous sample showed faster degradation curve, indicating lower thermal stability than other crystalline sample having a β-sheet structure.

Lu et al., (2010) studied the thermal properties of fibroin films from B. mori before and after inducing crystallization. The raw fibroin samples showed an endothermic peak at around 100°C, due to evaporation of water, a non-isothermal crystallization peak at around 213°C in which the non crystal structures converted into crystallized ones, and a degradation peak at 257°C in which the film started to degrade. When the films were treated with methanol the film crystallized to form β sheets. The endothermic peak at around 100 °C disappeared, implying that the silk film became hydrophobic. The crystallization peak also disappeared because of the formation of β-sheet after methanol treatment, prior to DSC scanning. The degradation peak increased to 260°C, indicating a greater thermal stability of methanol treated fibroin films.

Dash et al., (2009) while studying the thermal properties of liquid sericin films by DSC, inferred that the raw sericin membrane showed a melting point at 354.66°C. On 70% (v/v) ethanol treatment the peaks shifted to 357.26 °C with suggesting enhanced thermal stability. Increase in melting point and enthalpy suggests the requirement of
higher amount of energy for breaking the bonds compared to untreated samples. It was concluded that the oriented β-sheet aggregates form a fibrous structure of sericin, which is crystalline and insoluble in water previously reported by Teramoto et al., (2007). Nayak et al., (2012) studied the thermal properties of sericin films cross linked with glutaraldehyde from A. mylitta silkworms. It was reported that in the case of uncrosslinked sericin powder, an endothermic peak near 144°C and another near 309°C were observed. An endothermic peak near 148°C and another near 346°C were observed for the crosslinked sericin membrane. The former peak signifies the molecular mobility and melting induced thermally, while the latter indicates thermal decomposition as well reported by Mandal et al., (2011).

Endothermic degradation temperatures of sericin powder obtained from B. mori at high-temperature and high-pressure degumming were also reported to be at 210°C (Aramwit et al., 2010a). Lamoolphak et al., (2008) inferred that thermal stability of sericin is influenced by the use of chemicals during the extraction process. It is now clear that higher temperature shifts in the crosslinked sericin membrane suggest that the crosslinking of the sericin decreases the molecular motion and hence shows higher temperature for thermal decomposition.

Thermal properties of nanoparticles and microparticles are also being studied extensively as the matrices are slowly replaced by nanoparticles. A comparative study of B. mori and non-mulberry silk microparticles was reported by Bhardwaj et al., (2015). The thermal properties were evaluated by DSC and TGA inferring that DSC results of A. assamensis, P. ricini and A. mylitta microparticles showed the first endothermic peak at
around 105–109°C due to loss of moisture and the second in the range of 360–365°C, which represent decomposition of the protein molecule. In contrast, B. mori silk fibroin microparticles showed a similar moisture peak at 105–109 °C but decomposition peak was at a lower temperature of 300°C. The weight loss pattern of TGA analyses showed a similar two-step breakdown as DSC. In A. assamensis, P. ricini and A. mylitta silk fibroin microparticles, the first weight loss was found at around 100–105 °C and the second weight loss at around 350–360 °C. However, in B. mori silk 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. The enhanced thermal stability of the A. assamensis, P. ricini and A. mylitta fibroin microparticles than B. mori silk fibroin microparticles demonstrates a greater chemical stability of the non-B. mori fibroin based microparticles. These results are well supported by previous reports with films and fibers (Kar et al., 2013; Dutta et al., 2013).

Zhang et al., (2007) prepared fibroin nanoparticles from B. mori and studied the thermal properties. The low temperature endotherms at < 100°C were due to the evaporation of water. With increasing temperature, amorphous liquid silk showed a broad endothermic peak at 203°C, and subsequently an apparent exothermic peak at 232.1°C that was ascribed to crystallization into a β-sheet structure. In case of the nanoparticles the exothermic peak at 232.1°C was not present due to b-sheet structure itself. A glass transition was reported at 203°C in the nanoparticles. The degradation peak of the nanoparticles shifted to a lower temperature 288.7°C. It was concluded that
the decrease of the thermal stability of silk fibroin related apparently to the breakage of amide bonds and non-oriented b-sheet arrangement in silk fibroin molecules.

Although *B. mori* and non-*B. mori* silk varieties are natural protein fibers, their thermal behaviour is different under different thermal conditions and in different structural compositions. Hence the molecular degradation started early in *B. mori* varieties making the non-*B. mori* varieties thermally relatively more stable. The molecular architecture, molecular weight and the crystalline nature seemed to play a role in a distinctly different behaviour of non-*B. mori* silks.

**Applications of Biomaterials**

Apart from use as a textile material, the foremost important application of silk was as sutures for wound ligation. Silk has been used as sutures for centuries (Moy et al., 1991), over the past 100 years it has surpassed collagen as a natural suture. As the application of silk as sutures continued there were some problems related to allergic reaction to the silk causing a type I allergic reaction (Kurosaki et al., 1999). Wen et al., (1990) reported that the extracted sericin was responsible for the development of a T-cell mediated allergic response by skin testing of 64 children with asthma. Further biochemical analysis by Dewair et al., (1985) concluded that upregulated IgEs were produced in response to the sericin. Hence sericin was mainly considered as the main allergenic agent in silk and not the core fibroin fibers. If sericin is removed, the biological responses to the core fibroin fibers appear to be comparable to most other commonly used biomaterials. In the 1970s and early 80s black braided silk was the name
given to the silk fiber after removal of sericin and coated with materials like waxes and silicone to enhance the properties of silk.

With the dawn of new technologies and emergence of new problems, the inquisitive human mind rediscovered and reconsidered silk matrices as potentially useful biomaterials for a range of applications in clinical repairs and \textit{in vitro} as scaffolds for tissue engineering (Minoura et al., 1995). Altman et al., (2003) has summarized the pros and cons with the use of silks for biomedical applications-

Pros

Superiority of silks, over other natural fibers due to its novel mechanical properties.

Natural fiber with a long standing history of use in clinical applications

The ability to regenerate silks in aqueous solutions for subsequent formation of films and other material formats, which relates to specific needs.

Ease of chemical modification and surface decorations, such as adhesion sites or cytokines, due to the availability of amine and acid side chains on some of the amino acids

Molecular weight, crystallinity, solubility can be genetically tailored to meet specific needs.

Slow rates of degradation \textit{in vitro} and \textit{in vivo}, particularly useful in biodegradable scaffolds in drug deliveries and tissue engineering.
No known risk of bio-burden.

Cons

Adequate removal of contaminating sericin from silkworm silk to avoid biocompatibility problems

Slow degradation of crystalline (β-sheet) regions

Aborted proteolytic attack by macrophages and giant cells leading to encapsulation and the formation of a granuloma

In some cases, sensitization to silk fibroin caused an allergic response upon exposure to the biomaterial.

**In vitro cell culture on biomaterials**

The biomaterials play an important role in the field of tissue engineering by supporting the development of tissues in vitro prior to implantation in vivo. The biomaterial transduces environmental cues to cells seeded within or on the matrix. The matrix acts as the translator between the local environment (either in vitro or in vivo) and the developing tissue, aiding in the development of biologically viable functional tissue. The properties that the matrix must possess are cell attachment, spreading, growth and differentiation. It is considered very advantageous if the matrix degrades into biocompatible fragments or monomers capable of being metabolized by host cells, after the function is completed.
Silk fibroin and sericin are emerging as versatile materials for design of different kinds of biomaterials according to the tissue engineering needs, where mechanical performance and biological interactions are the main factors for successful implantation. Some of the tissues that are being mimicked are bone, ligaments, tendons, blood vessels and cartilage (Altman et al., 2003).

Mohanty et al., (2000) also stressed the fact that increasing environmental consciousness and demands of legislative authorities, the manufacture, use and removal of traditional composite structures, usually made of glass, carbon or aramid fibres being reinforced with epoxy, unsaturated polyester resins, polyurethanes, or phenolics, are being considered.

Wang et al., (2006) reported that like most biomaterials used in tissue engineering, silk was first evaluated for cellular responses such as attachment and proliferation on 2D film in tissue culture wells. Minoura et al. observed that films formed from native silkworm fibroin collected from glands of B. mori domestic silkworms and Antheraea pernyi wild silkworms were comparable to collagen films in terms of supporting attachment, spreading and proliferation of murine L-929 fibroblasts (Minoura et al., 1995a; 1995b).

Inouye et al., (1998) and Gotoh et al., (1998) later found that films formed from regenerated silk fibroin prepared by dissolving silkworm cocoon fibers in 9–9.5M LiBr supported the attachment and growth of human and animal cell lines. The authors attributed this cell attachment to the presence of positively charged residues like arginine
near the C-terminus of the nonrepetitive (hydrophilic) regions of the silk fibroin sequence, considering the surface of mammalian cells are predominantly negatively charged (Minoura et al., 1995a).

The effect of the RGD sequence on the attachment of mammalian cells to silk fibroins was confirmed by Sofia et al. and Chen et al. through surface modification experiments with human osteoblasts, fibroblasts and bone marrow derived stem cells (Sofia et al., 2001; Chen et al., 2003). It was concluded that the enhancement of cell binding due to coupling RGD on silk fibroin may result from a combination of specific interactions mediated by integrin interactions and increased hydrophilicity on the otherwise highly hydrophobic silk fibroin materials.

Interestingly, films formed from sericin, the glue-like coating protein, also supported the attachment and growth of murine L929 fibroblast cells (Minoura et al., 1995b) and human primary skin fibroblasts (Tsubouchi et al., 2005). Ogawa et al., (2004) and Terada et al., (2005) reported an interesting property of sericin as promoter of cell proliferation if used as a medium supplement.

In vitro studies by Chiarini et al., (2003) and Dalpra et al., (2003) have reported that dermal fibroblasts spread and proliferate on fibroin coatings and scaffolds without secretion of pro-inflammatory interleukins. Oral keratinocytes also proliferate on woven fibroin meshes as reported by Min et al., (2004), a form that is likely to be used for wound healing applications. Fibroin films (Sugihara et al., 2000) and fibroin-alginate
sponges (Roh et al., 2006) have been found to enhance skin wound healing in vivo compared to clinically used materials

The majority of the research carried out into the use of silk-based biomaterials for bone tissue engineering has been performed by the group led by D.L. Kaplan. Fibroin solution was used to form films, scaffolds or processed into 3-D porous scaffolds. Additionally, RGD functionality (Meinel et al., 2005) or growth factors (Karageorgiou et al., 2006; Kirker et al., 2007) were incorporated. Osteoblast-like cells were successfully grown on fibroin films coupled with RGD peptides and were found to produce mineralized matrix (Sofia et al., 2001). Osteogenesis has also been achieved with stem cells grown in vitro on thin fibroin films (Wang et al., 2005).

Ma et al., (2005, 2006) reported that sulphonated and heparinised silk fibroin films have suitable mechanical properties for use as artificial blood vessels. These studies demonstrated that these films have good anticoagulant activity and platelet response and support endothelial cell spreading and proliferation.

Hoffmen et al., (2006) investigated the pharmaceutical utility of silk fibroin materials for drug delivery. Dextrans of different molecular weights, as well as proteins, were physically entrapped into the drug delivery device during processing into films. It was concluded that, silk fibroin is an interesting polymer for drug delivery of polysaccharides and bioactive proteins due to the controllable level of crystallinity and the ability to process the biomaterial in biocompatible fashion under ambient conditions to avoid damage to labile compounds to be delivered.
Acharya et al., (2009) reported for the first time the potential application of fibroin obtained from the cocoon of non-B. mori tropical silkworm, *Antheraea mylitta*, as a substrate for *in vitro* cell culture. The adherence, growth and proliferation patterns of feline fibroblast cells on *A. mylitta* fibroin films suggested that this kind of film has a greater ability to support cell growth than *B. mori* fibroin films. This study demonstrated that, non-*B. mori* fibroin might be a useful alternative substrate to the more common *B. mori* fibroin for a variety of biomedical applications.

Mandal et al., (2009) investigated the 3D scaffolds based on embedding drug loaded calcium alginate beads within silk fibroin protein fabricated for controlled dual drug release. The results highlighted the versatile and tunable properties of calcium alginate embedded fibroin 3D scaffolds making them exciting candidate for the controlled release of a wide spectrum of bioactive molecules from a single delivery vehicle.

Mandal et al., (2009) studied the potentiality of 3D silk scaffolds fabricated from non-*B. mori*, *Antheraea mylitta* and *B. mori*, *B. mori* silk gland fibroin proteins as substrate for osteogenic and adipogenic differentiation of rat bone marrow cells (BMCs). Proliferation and spreading of fibroblasts and bone marrow cells on silk scaffolds were observed to be dependent on scaffold porosity. The results suggested the suitability of silk fibroin protein 3D scaffolds as natural biopolymer for potential bone and adipose tissue engineering applications.
Kundu et al., (2010) fabricated nanoparticles from silk fibroin solutions of domesticated B. mori and tropical tasar silkworm Antheraea mylitta and investigated in respect to its particle size, surface charge, stability and morphology along with its cellular uptake and release of growth factors. The nanoparticles were stable, spherical, negatively charged, and exhibited mostly β-sheet structure and did not impose any overt toxicity. Cellular uptake studies showed the accumulation of fluorescence isothiocyanate conjugated silk nanoparticles in the cytosol of murine squamous cell carcinoma cells. In vitro VEGF release from the nanoparticles showed a significantly sustained release over 3 weeks, signifying the potential application as a growth factor delivery system.

Pure sericin is not easily made into membranes, but membranes of sericin cross-linked, blended, or copolymerized with other substances are made readily. Because sericin contains a large amount of amino acids with neutral polar functional groups, sericin-containing membranes are quite hydrophilic. Sericin composite membranes are permselective for water in an aqueous-organic liquid mixture. Mizoguchi et al., (1991) described a cross-linked thin film made of sericin for use as a separating membrane for water and ethanol.

Miyairi et al., (1978) reported a cross-linked sericin film for enzyme immobilization with glutaraldehyde as the cross-linking agent. A membrane composed of sericin and fibroin is an effective substrate for the proliferation of adherent animal cells and can be used as a substitute for collagen.
Minoura et al., (1995) and Tsukada et al., (1999) investigated the attachment and growth of animal cells on films made of sericin and fibroin. Cell attachment and growth were dependent on maintaining a minimum of around 90% sericin in the composite membrane. Films of pure component proteins (i.e., fibroin or sericin) permitted cell attachment and growth comparable to that on collagen, a widely used substrate for mammalian cell culture.

Murase et al., (1994) reported that film made of sericin and fibroin had excellent oxygen permeability and is similar to human cornea in its functional properties. It is hoped that the sericin–fibroin blend film can be used to form artificial corneas.

It is now quiet clear that silks can be processed into many forms suitable for a variety of biomedical and tissue engineering applications. They can be modified by chemical treatment or used in combination with other materials in order to vary mechanical properties and surface chemistry. By these means, biomaterials appropriate to specific applications can be produced. Silk-based biomaterials are at least as biocompatible in terms of inflammatory response and ability to support cell proliferation as many materials currently in use. The degradation behaviour can be tailored, depending on the application, from a few days to many months. These materials are promising for use in wound healing and as tissue engineering scaffolds, particularly for the development of skeletal tissue. Other applications, such as the use of silk for nerve regeneration, are yet to be fully investigated.
The wide range of molecular structures, remarkable mechanical properties, morphology control, versatile processability and surface modification options make silk fibroin an attractive polymeric biomaterial for design, engineering and processing into scaffolds for applications in controlled drug delivery, guided tissue repair and functional tissue engineering. 3D porous or fiber silk fibroin scaffolds with surface morphology, useful mechanical features, biocompatibility, and ability to support cell adhesion, proliferation, and differentiation have expanded silk-based biomaterials as promising scaffolds for engineering a range of skeletal tissues like bone, ligament, and cartilage as well as connective tissues like skin. The generally slow rates of degradation of silk fibroin in vivo, coupled with the versatile control of structure, morphology and surface chemistry, offer a range of utility for this family of protein polymers in many needs in biomaterials and tissue engineering. To date most of the impact with silk-based biomaterials has been with only one source of silk, the fibroin from B. mori silkworm.

As new sources of silk proteins become available, such as from non-mulberry species and via genetic engineering, cloning and modification of primary silk sequence chemistries, the range of material properties can be generated and utilized for biomaterials can be expected to further broaden the options and lead to additional beneficial impact. Finally, hybrid or composite systems with other biopolymers offer novel options to match complex mechanical and biological functions with tissue-specific needs (Wang et al., 2006).