BIODEGRADABLE HYDROGELS FROM SILK SERICIN & GELATIN: DEVELOPMENT AND CHARACTERIZATION FOR MEDICAL APPLICATIONS

A DISSERTATION SUBMITTED

BY

DEEPTHI M. NAIR

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

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DECLARATION

I, **Deepthi. M. Nair**, hereby declare that I had personally carried out the work depicted in the dissertation entitled '**Biodegradable hydrogels from silk sericin: Development and characterization for medical applications'** under the direct supervision of **Dr. Roy Joseph,** Scientist F, Polymer Processing Laboratory, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Trivandrum, Kerala, India. External help sought are acknowledged.

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CERTIFICATE

This is to certify that the dissertation entitled **"Biodegradable hydrogels from silk sericin: Development and characterization for medical applications"** submitted by **Deepthi. M. Nair** has been carried out in partial fulfilment for the Degree of Master of Philosophy in Biomedical Technology to be awarded by this Institute. The entire work was done by her under my supervision and guidance at Polymer Processing Laboratory, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology (SCTIMST), Trivandrum-695012.

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LIST OF ABBREVIATIONS

PBS	Phosphate Buffered Saline
MES	Morpholino Ethyl Succinate
FBS	Foetal Bovine calf Serum
D.H2O	Distilled water
DMEM	Dulbecco's Minimal Essential Medium
SEM	Scanning Electron Microscope
ESEM	Environmental Scanning Electron Microscope
SGH	Sericin- Gelatin Hydrogel
XRD	X-Ray Diffraction
TGA	Thermo gravimetric Analysis
DMA	Dynamic Mechanical Analysis
HLECs	Human corneal limbal epithelial cells
ATE	Adipose tissue engineering
hADSCs	Human derived adipose stem cells
IPN	Interpenetrating polymer network
Phema	Poly hydroxyl ethyl methacrylate
TEWL	Trans epidermal water loss
UTM	Universal Testing Machine
µ-CT	Micro computed tomography
DPBS	Dulbecco' s Phosphate Buffered Saline

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SYNOPSIS

Silk protein, sericin has a long history of being discarded as a waste material in the textile industry by a process called degumming. But nowadays, sericin has become a hot topic in the area of tissue engineering and regenerative medicine. It is a hydrophilic protein with large amount of OH groups. Exploration of the potential applications of sericin has just begun. Sericin has got wide applications in various industrial sectors. In the cosmetic industry, it is being used as a moisturizer since it resembles natural moisturizing factor (NMR). In addition, it has the property of inhibiting tyrosinase enzyme, which is responsible for melanin biosynthesis. Also, it is a natural ingredient for the food industry because its consumption enhances the bioavailability of Zinc, Iron, Magnesium and Calcium. Sericin plays an important role in wound healing, by promoting collagen synthesis. It possesses antimicrobial property and accelerates mammalian cell growth during serum deprivation. Moreover, studies are going on the basis of transforming sericin into a biomaterial by cross-linking with natural as well as synthetic polymers.

Without a doubt, gelatin has an important role in the area of biomaterial science. It has being widely used in a variety of forms since it is a biodegradable, biocompatible natural polymer. Hydrogels are three dimensional polymeric networks with high water holding capacity. They can be cross-linked either by physical or chemical methods. They are extremely useful for a variety of applications in pharmaceutical as well as medical industry. They are capable of retaining large amounts of water, possess soft and rubbery consistency and

provide a moist environment. These features make them closely resemble with living tissues. Since it has a low interfacial tension, its adsorption with proteins in the body fluid is negligible. Also, the cross linked network contributes high porosity, which is required for cell proliferation and attachment. An effort is put forth in the present study to develop a hydrogel from silk protein sericin and gelatin without any chemical cross-linkers. Owing to this background information, it can be hypothesized that:

Cross-linking two biopolymers, sericin and gelatin, would produce a biodegradable and biocompatible hydrogel system having sufficient hydrophilicity, strength, and handling characteristics so that they can be used for biomedical applications.

The objectives adopted to illustrate this hypothesis are:

- Chemical Characterization of sericin and gelatin
- Fabrication of hydrogel by optimising reaction conditions
- Characterization of hydrogel by chemical and thermal methods, mechanical testing, morphological studies and porosity measurements.
- Cytocompatibility evaluation by dermal fibroblast monolayered culture

Sericin and gelatin were chemically characterized by spectroscopic, thermal and diffraction techniques. Reaction conditions for hydrogel development were optimized by varying process parameters. Hydrogel was characterized by evaluating its morphology, thermal stability, degradation behaviour, haemolytic property, 3D micro architecture and biological properties.

Surface morphology and 3D micro architecture was evaluated by using scanning electron microscopy and Micro Computer Tomography techniques. The hydrogel was found to possess interconnected porous network with an average pore diameter of 23.5μm. Mechanical testing of hydrogel showed sufficient strength of the material. Biological characterization of the hydrogel revealed its cytocompatibility with limited ability for cell attachment.

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND

Hydrogels are three dimensional materials with the ability to absorb large amount of water, retaining its structural integrity. The history of hydrogels began in 1960, when Wichterle and Lim introduced the use of hydrophilic networks of cross-linked poly(2-hydroxyethyl methacrylate) (pHEMA,) as soft contact lens material (Wichterle, 1960). Hydrogels have become a hot topic during the last few decades, as indicated by the increasing number of papers on hydrogel-based materials published from 1995 onwards. Among the large variety of definitions, the most acceptable one for hydrogel is referred to as hydrogels are water-swollen, cross-linked polymeric structures containing: (1) covalent bonds produced by the reaction of one or more co monomers, (2) physical cross-links due to chain entanglements, (3) association bonds including hydrogen bonds or strong van der Waals interactions between chains, or (4) crystallites bringing together two or more macromolecular chains (Peppas, 2000).

Silk worm, Bombyx Mori is gaining a much important space in the area of biomedical technology because of its two powerful protein members Sericin and Fibroin. Fibroin is widely used in textiles, industrial and medical applications (Panilaitis, 2003). Sericin is a glue-like protein that holds fibroin fibres together. For centuries, it has been discarded as a waste material by a process called degumming in textile industry. Exposing this unutilised sericin to environment poses serious threat due to high oxygen demand for its degradation (Aramwit,

2012). Polymers of natural origin always hold a better position for use as a biomaterial. Since they possess compatibility with the living tissues, favourable cell-material interaction, non toxic degradation products, minimal immunological responses and adverse reactions to a greater extent, biomaterial from bio-polymers, particularly proteins, is highly relevant. Current study focuses on the fabrication of a hydrogel by cross-linking sericin with gelatin.

Sericin is a polypeptide composed of 18 amino acids with high content of serine and aspartate (Takasu, 2002). The molecular weight of sericin depends on the method of its extraction from silkworm cocoons. Ethanol precipitation of sericin yields a molecular weight of 800 Da (Yoko, 2002). It is a hydrophilic macromolecule with high content of hydroxyl and carboxyl groups (Patel, 2011). Structural analysis of sericin gene revealed two closely related mRNAs of length 11.0 and 9.6 kilobases. A strong homologous region in the sericin and fibroingenes at their corresponding 5' flanking sequences was identified**.** (Harumasa *et al*. 1982). Sericin is soluble in hot water and insoluble in cold water; however, it is easily hydrolyzed. In this process, the long protein breaks down into smaller molecules which are easy to solubilise in hot water (Gulrajani, 1988). Sericin is highly advantageous because of its special properties like oxidation resistance, antibacterial and UV resistance. It absorbs and release moisture easily (Mondal, 2007). Reports are available on the diverse biological activities of sericin, such as anti-oxidation (Kato, 1998), anti-bacterium (Sasaki,2000) anti-coagulation (Takeuchi, 2005) and its ability to promote cell growth and differentiation (Miyazaki,2004). Sericin is reported to be composed of three polypeptides, sericin A, M and P with amino acids like serine, aspartic acid,

glutamic acid and glycine in large proportions (Yoko, 2014). In the field of regenerative medicine, owing to its biodegradability, easy availability, and hydrophilicity with many polar side groups, sericin is mostly copolymerized, cross-linked, or blended with other polymers to form various scaffolds in order to help obtain improved properties for relevant biomedical applications, such as skin regeneration (Wang, 2014).

Sericin is widely utilised in cosmetic industry. Excessive Trans epidermal water loss (TEWL) is one of the causes of dry skin and skin moisturizers have been used to overcome it. The silk sericin has resemblance with the natural moisturizing factor (NMR) (Patel, 2011). Moreover, it is being regarded as a valuable natural ingredient for food industry, since it increases the bioavailability of Zn, Fe, Mg and Ca (Masahiro *et al*, 2000).

Acidic or basic hydrolysis of collagen yields a biodegradable protein, Gelatin, involving breakage of the collagen's triple helix structure into random coils (Bouhadir, 2001). Gelatin is composed of a variety of amino acids forming a linear polymer with molecular weight varying between 15,000-250,000 Daltons. The sol state of gelatin transforms into the gel state upon cooling of aqueous solution of gelatin, which involves a partial rearrangement of the gelatin structure from random coil into triple helix (Bigi, 2004). Gelatin is widely used in biomedical applications, for example in tissue engineering, wound dressing, gene therapy, and drug delivery due to its high biocompatibility and biodegradability. Gelatin will easily form hydrogel in water at room temperature, but is fragile.

Gelatin is tremendously utilised in the field of biomaterials by cross-linking with synthetic or natural polymers. Sufficient mechanical properties may be attained by modifying primary amino acid residues present in gelatin like lysine, hydroxylysine, histidine, and arginine. There are two types of gelatin available: Type A and B. Type A gelatin is obtained from porcine skin by acidic treatment while Type B gelatin from bovine skin by alkaline treatment. Both types of gelatin are rich in glycine followed by proline and arginine. Gelatin is able to form physical and chemical gels. Physical gel is formed by merely cooling gelatine solution to room temperature. Chemical gels are formed with the help of a cross-linker. A cross-linker forms covalent links C-N with the amine groups of gelatin coils (lysine, hydroxylysine, histidine).

The main goal of the study is to fabricate sericin- gelatin cross-linked hydrogel. High serine content in sericin makes it highly hydrophilic. Also the richness of acidic amino acids in sericin and basic amino acids in gelatin is being targeted here. If the carboxyl group in sericin is made available to crosslink with the amino groups in gelatin, it can form a covalently cross linked network. Reports are available on this by using chemical crosslinkers like formaldehyde, glutaraldehydes (Jayakrishnan and Jameela 1996), glyceraldehyde, hydrogen peroxide, benzene, sulphonic acid, guanidine hydrochloride and genipin (Huang et al. 1999). But here, the crosslinking is aimed to achieve without the involvement of any chemical cross linkers, thus eliminating the possibility any kind of toxicities.

Hydrogels are widely accepted as a scaffold which supports effective cell growth and proliferation because of their high porosity that leads to high permeability of oxygen, nutrients, and metabolites. At the same time hydrogels can protect the cells from the host immune system and high molecular weight complexes like immunoglobulin (Li, 2006; Auslander, 2012). Furthermore, the softness and flexibility rendered by hydrogels reduces the mechanical stress and friction on cells and also on adjacent tissue upon transplantation (Wilson, 2013). Not only that, the highly hydrophilic nature of hydrogels lead to high water content, providing a tissue like environment to the attached cells (Billiet, 2012). On the basis of these advantages, it is expected that the hydrogel resulting from the cross linking of sericin and gelatin can offer possibilities for future studies in tissue engineering. Indeed, gelatin and sericin are already known as a favourable material for tissue engineering and regenerative medicine. However, the combination of both these proteins without any cross-linkers is not yet studied. In the present work, we attempt to fabricate hydrogel from sericin and gelatin by optimising the reaction conditions.

1.2. REVIEW OF LITERATURE

As designed by the mother nature, the silk spinnerets of Bombyx mori produce a delicate twin thread of silk and surround it with a protective cover of sericin during the spinning procedure. This principle is now being imitated and perfected to provide an innovative active ingredient for the pharma industry. For centuries ago, silk has been a scientific curiosity. Silk fibers have been used in the form of sutures. Recently silk solutions have become an ideal candidate in the field of biomaterials such as gels, sponges and films, for medical applications. Surface properties of silk can be tuned through chemical modification of amino acid side chains so as to immobilize cellular growth factors. Silks with precise features can be developed through molecular engineering of silk sequences: for example cell recognition or mineralization. The mode of processing and the subsequent content of β sheet crystallinity will in turn relate to the degradability of silk biomaterials. Different silk biomaterials have been a successful substratum for the growth of several primary cells and cell lines so as to demonstrate a range of biological outcomes. Also they proved to be biocompatible *in vitro* and *in vivo*. Silk scaffolds have been a flourishing nominee in wound healing and in tissue engineering of bone, cartilage, tendon and ligament tissues (Vepari, 2007).

1.2.1 Silk protein sericin

Silk is mainly composed of two types of proteins, silk fibroin and sericin. Sericin contributes about 20-30 per cent of total cocoon weight. It is a biopolymer with high serine content (32%) and 18 amino acids, including essential amino acids. Isolation of sericin from silk thread can be done by different modes. Sericin's

characteristic features like solubility, molecular weight, and gelling properties are influenced by the method of isolation. Its main role is to envelop fibroin. Sericin is widely applied in pharmaceuticals and cosmetics such as, wound healing, bioadhesive moisturizing, antiwrinkle and antiaging (Padamwar, 2004).

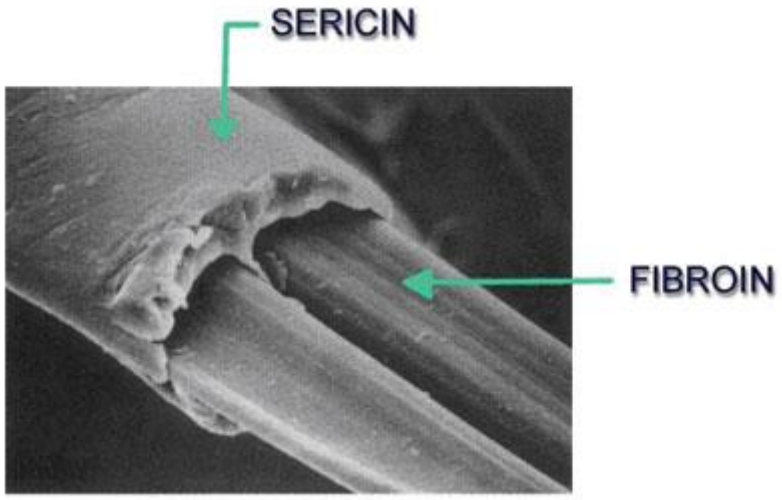


Figure 1: Sericin and fibroin

It is sericin that makes the fibres hard and tough. On the other hand removal of it results in a soft and lustrous fibre. The occurrence of sericin is mainly in its amorphous random coil and to a lesser extent, in a β-sheet organized structure. As a result of repeated moisture absorption and mechanical stretching, random coil structure simply converts to β-sheet structure (Voegeli, 1993). Based on solubility pattern, sericin can be grouped into three - sericin A, sericin B, and sericin C. The hot water insoluble outermost layer is fraction A. It is composed of 17.2% of nitrogen and amino acids like, serine, threonine, glycine, and aspartic acid. The middle layer is fraction B and acid hydrolysis of it yields amino acid of sericin A, in addition to tryptophan. It is composed of 16.8 % of nitrogen. The hot water insoluble, adjoining to fibroin C is the innermost layer. It can be separated from

fibroin by treating with hot dilute acid or alkali. Its acid hydrolysis yields proline besides amino acids of sericin B. It also contains sulphur and 16.6 % of nitrogen (Shaw, 1951).

Sericin has been classified into various species derived from its relative solubilities. Many other researchers have also nominated various fractions of sericin with respect to their dissolution behaviour like sericin A and B, or sericin I, II, III, and IV, or S1, S2, S3, S4, and S5, and as α, β, and γ modification (Komatsu,1996). Soluble form of sericin always attains a random coil molecular conformation. But the β-sheet structure is more difficult to dissolve. The more crystalline structure resulting from repeated moisture absorption contributes to reduced solubility. Moisture absorption increases crystallinity by making molecular aggregation structure denser. Three layer structure of sericin was reported by γ-ray study. The outer layer contained some fibre direction filaments, middle layer exhibits cross-fibre direction filaments, and the inner layer shows longitudinal filaments (Wang, 1985). Casting temperature also influences the structure of sericin. As the casting temperature lowers, sericin molecules attain more β-sheet structure rather than random coil.

1.2.2 Availability

The cultivated silkworm, Bombyx mori Linn., is a lepidopteran molecular model. Now a days, it is being considered as an important economic insect that can serve as an ideal molecular genetic resource so as to resolve a broad range of biological troubles. B. Mori, in its final stage of larval development, produces a bulk amount

of silk proteins. The middle silk gland is the storehouse of these proteins. At the end of the fifth instar, these are expelled through the anterior duct and spinneret. Sericulture is now in the path of technological development as silkworm serve as a bio factory for the production of useful protein using the silk gland. Owing to this background, silkworm can be classified as a value added biomaterial for medical application, silk protein fibroin and sericin as a biomaterial and other seri -by products (Mondal, 2007).

1.2.3 Properties

Sericin is insoluble in cold water, however, it is easily hydrolyzed. In this process, the long protein breaks down into smaller molecules which are easy to solubilise in hot water (Gulrajani, 1988). Sericin is highly advantageous because of its special properties like oxidation resistant, antibacterial, UV resistant and absorbs and release moisture easily, inhibitory activity of tyrosine and kinase, etc. (Mondal, 2007).

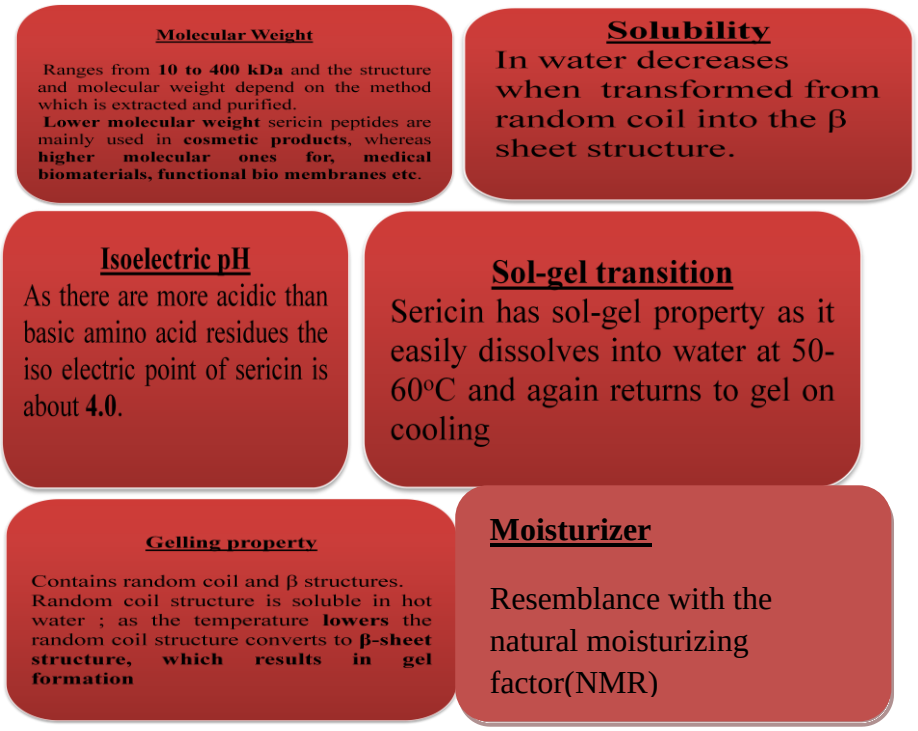


Figure 2: Properties of sericin

1.2.4 Structure of sericin

Amino acid residues with short side chains comprises the drying inducible crystallized form of sericin, located in the middle silk gland and is folded into the globular matrix made of stretches with longer side-chains, crystallizing less readily. On drying, it becomes film of non-oriented crystal structure. Stretching, drying and swelling in water causes this film to attain oriented fiber structure. Though, the orientation is unstable by hot water treatment, there lies a reversible relationship between the orientation and non-orientation (Komatsu, 1975). There was no much clear cut difference in crystalline structure obtained from wild silks and that is obtained from B. mori. One noted difference is that the wild silk sericin is comparatively insoluble. This is attributed mainly due to chemical interaction between silk sericin and inorganic minor components or tannins

present within wild silk (Tsukada, 1983). Sericin with random coil structure was found to be extracted from the liquid silk and fresh cocoon shells of a silkworm hope with 5- 10 % b-structure and no α-helix (Lizuka,1969).

Also, in aqueous solution, sericin exhibits both random coil and β- structure, but lacks α-helix (Komatsu, 1975). The relationship between solubility and molecular conformation is confirmed by studying sericin from both the liquid silk in the silk gland and the cocoon filament (Komatsu, 1982). Till now, it is evident that occurrence of sericin is mainly in its random coil or β-structure. Also it is believed that β structure is intrinsic to liquid silk. Later, a circular dichroism spectrum showed that sericin extracted from liquid silk for 45 minutes with water contains a small fraction (10%) of α-helix. During the dissolution of the liquid silk in water, part of the sericin IV become a white suspension due to the coexisting cocoon yarn wax but does not coagulate and the β structure was originally present in the liquid silk. β -structure sericin is more insoluble than random coil sericin (Komatsu 1980). Though, these reports are not in conformity with reports of Tsukada (1983).

The hygroscopic conditions favour the molecular transition of sericin from its random coil to β - structure (Komatsu, 1980). Ishikawa and Hirabayashi (1968) reported that sericins have a cross β structure in a water solution of sericin containing 50% dioxane (Iizuka, 1969) or methanol (Ishikawa et al., 1987)

Komatsu (1975) believed that the sericin fraction closest to fibroin in the cocoon filament has molecular chains also arranged in cross β structure, rather than having the main axis of crystallites oriented at right angle to the fiber axis. The intra- molecular bonds of sericin having random coils are broken by the absorption

of water molecules and the folded structure becomes unfolded into an extended structure and is transformed into a β structure. β Structure is more stable regarding energy. Part of the sericin thus transformed into β structure is fixed by its new intermolecular hydrogen bonds and remains crystallized even when the water molecules are removed by drying. In a new cycle of water absorption, the crystalline structure already formed, remains unaffected, whereas, a further fraction of random coils state crystallizes into a β structure thereby increasing the portion of β structure and promotes crystallization. Heating during moisture absorption activates the thermal motions of segments and accelerates transformation. Thus the sericin gets modified in the direction of difficult solubility due to repeated moisture absorption and loss.

According to scientific data, sericin of cocoon shell fall under two proportions: (1) α-sericin and (2) β-sericin. Outer layer of shell is composed of α - Sericin and β -sericin in the inner layer. α -sericin contains lesser C and H and somewhat more N and O than the β -sericin (Bose et al., 1989). α -sericin is more soluble in the boiling water than β - sericin. Sadov et al., (1987) have drawn a fact that native sericin is mixture of two substances, Sericin A and Sericin B. Degumming of silk results in the isolation of sericin in aqueous solution. It is not a single chemical substance rather a mixture of at least two substances. There are scientific descriptions that the sericin layers are formed from the outside to inside in the order of I, II, III to cover the fibroin on the cocoon thread. These three fractions of sericin were found to have different solubilities and named them sericin fractions I, II, and III. The order was based on the ease of hot water dissolution and their

ratios were approximately 40:40:20. Sericin I was found to be amorphous in nature whereas the other fractions as crystalline. Komatsu (1975) observed a fourth fraction of sericin which was much harder to dissolve as sericin IV. This fraction was reported to have higher specific gravity and crystallinity.

Robson (1985) reported that sericin may be separated into sericin I, II, III and IV by their different solubilities in hot water and assessing the degree of solubility by UV absorption. The greatest sericin content is present in the outer layer of cocoon whereas the least sericin proportion is present in the innermost layer of a cocoon.

1.2.5 Amino acid composition

The total amount of hydroxy amino acids in sericin is 45.8 per cent. There are 42.3 per cent of polar amino acid and 12.2 per cent of nonpolar amino acid residues (Padamwar, 2004). Sericin of mulberry wild silkworm, Bombyx morimandarina, is characterized by the same kind of amino acids as the domesticated silkworm, B. mori (Yamada, 1978). On the other hand, the wild silkworm sericin is composed of serine, proline, methoinine, glucosamine, galactosamine and histidine in lower amount and throeonine, glutamic acid, cystine and phenyl amine in higher amount. The inner layer of cocoons was found to be rich in threonine, galactosamine and glucosamine than the outer layer. Moreover, the sericin extracted from the floss showed high contents of serine, glycine, valine and tyrosine but low contents in threonine, aspartic acid, alanine, cystine, leucine, glucosamine, galactosamine, lysine and histidine as compared with the sericin of cocoon layer.

More non polar amino acid and less polar amino acids were observed in the wild silkworm sericin when compared to that of the domesticated silkworm sericin. It was showed that the amino acid composition of sericin extracted from the cocoon is species specific. Structural analysis of sericin gene revealed two closely related mRNAs of length 11.0 and 9.6 kilobases. A strong homologous region in the sericin and fibroingenes at their corresponding 5' flanking sequences was identified (Harumasa *et al*., 1982). The co-efficient of pattern-similarity in amino acid composition was high between the sericin of wild silkworm and domesticated silkworms, while sericin of the wild silkworm such as Antheraea or Phylosamia showed significantly low similarity.

1.2.6 Applications in the market

Sericin is an excellent member in the cosmetic area that possesses moisture absorption and preservative ability. The hydrophilicity of sericin renders it to absorb water 50 times high than that of glycerine. Also it was shown that sericin has a capability to inhibit tyrosine kinase activity, which is involved in the production of skin melanin. Applying it on the skin will result in softness and smoothness and makes the hair soft and flexible. It is also an ideal candidate in shaping of hair. Sericin is a less irritant moisturizer and is gentle on a delicate skin. Sericin cream is available in the market with wound healing properties, since it is essential in collagen synthesis. Dermal and corneal wound healing have been tried by many researchers using sericin.

Figure 3: Applications of sericin

Reports on sericin about its biological functions are expanding day by day. It is already known that sericin support excellent growth and proliferation of mammalian cells. Also it is being suggested as a valuable ingredient for the food industry since sericin consumption has shown to enhance the bioavailability of Zn, Fe Mg and Ca. Sericin possess a supreme ability to prevent cell death and promote cellular growth after acute serum deprivation (Patel, 2011). Moreover, its role as a biodegradable, biocompatible functional biomaterial begins to get explored nowadays.

1.2.6.1 Biodegradable materials

Environment - friendly biodegradable polymers can be produced by blending sericin with other resins (Annamaria et al., 1998). The Polyurethane foams incorporating sericin are said to have excellent moisture absorbing and desorbing properties (Minoura et al., 1995). Polymer films, foams, molding resins, and fibers

containing sericin (0.01-50% w/w) can be produce by reacting a composition comprising a polyol, tolylene, di- isocyanet, di-butyltin di-laurate (catalyst) and trichloromonofluoromethane (a blowing agent) in the presence of sericin. The moister absorption/desorption rate of the sericin containing polyurethane form was found to be two-to five fold greater than that of control. Other procedures have also been reported for producing sericin-containing polyurethane with excellent mechanical and thermal properties (Hatakeyama, 1996).

1.2.6.2 Membrane materials

Membrane based separations (e.g., reverse osmosis, dialysis, ultra filtration and micro filtration) are used in process such as desalination of water, production of extremely pure water, the bioprocessing industry and some chemical processes. Pure sericin not easily made into membranes, but membranes of sericin cross-linked, blended, or copolymerized with other substance are made readily, because sericin contains large amount of amino acid with neutral polar functional groups. Sericin and fibroin can be used to make membranes for use in separation processes. The in solubilized silk fibroin membrane could be used to separate the mixture of water and alcohol (Chisti, 1998). Mizoguchi et al. (1991) describe a cross- linked thin film made of sericin for use as a separating membrane for water and ethanol. Sericin containing membranes are quite hydrophilic. Acrylonitrile used in making certain synthetic polymers can be copolymerized with sericin to prepare a protein containing synthetic polymer film for separating water from organics (Yamada and Fuwa 1994; Yamada et al., 1993).

1.2.6.3 Functional biomaterials

Nakajima (1994) has found that sericin film located on lay of liquid crystal can uniformly orient the liquid crystal molecules to provide distortion-free high-quality crystal displays. Sericin- coated film is used on the surface of refrigeration equipment because of its anti frosting action (Tanaka and Mizuno, 2001). Use of the coated sericin film is an effective anti frosting method that can be widely applied to refrigerators, deep freezers and refrigerated trucks and ships. Moreover use of the coated film on roads and roof can prevent frost damage. Sericin protein can be coated on surfaces of various durable materials to enhance functionality (Li, 1996). Sericin can be used in preparation of art pigments and for surface protection of articles. The material coated the sericin have excellent weatherability, good permeability and do not warp on drying. Sericin blends with water-soluble polymers, especially with polyvinyl alcohol (PVA). A blended hydrogel made of sericin and fibroin and PVA is said to have excellent moisture absorbing and desorbing properties and elasticity (Yoshii et al., 2000). The hydrogel can be used as a soil conditioner and in medical materials and wound dressing.

1.2.6.4 Medical biomaterials

Silkworm silk fibers have been the primary silk- like material used in biomedical applications particularly as sutures. During decades of use, silk fibres have proven to be effective in many clinical applications. At the same time, some biological responses to the protein have raised questions about biocompatibility. Tasubouchi (1999a) developed a silk fibroin- based wound dressing that could accelerate

healing and could be peeled off without damaging the newly formed skin. The non-crystalline fibroin film of the wound dressing had a water content of 3-16% and a thickness of 10-100 μm. Subsequently, the wound dressing was made with a mixture of both fibroin and sericin (Tsubouchi, 1999b). 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. Film made of sericin and fibroin has excellent oxygen permeability and is similar to human cornea in its functional properties. It hoped that the sericin- fibroin blended film could be used to form article corneas (Murase, 1994). A novel mucoadhesive polymer has been prepared by template polymerization of acrylic acid in the presence of silk sericin (Ahn et al., 2001). Silk protein can be made into a biomaterial with anticoagulant properties, by a sulfonation treatment of sericin and fibroin (Tamada, 1997).

Kato et al. (1998) provided the first evidence of antioxidant action of the silk protein by showing that sericin suppressed in vitro lipid peroxidation. Furthermore, sericin also found to inhibit tyrosinase activity. These results suggested that sericin is the valuable natural ingredient for food and cosmetics. The biopolymer sericin has a strong affinity to keratin. Excessive transepidermal water loss (TEWL) is one of the causes of dry skin and skin moisturizers have been used to overcome it. The silk sericin has resemblance with the natural

moisturizing factor (NMR). Sericin gel is prepared by using sericin solution with pluronic and carbopol as a stabilizer to prevent water loss from the upper layer of the skin. It forms a moisturizing, semi-occlusive, protective, antiwrinkle film on the skin surface imparting an immediate, long lasting, smooth, silky feeling (Padamwar et al., 2005). The configuration of sericin is very close to the one of human beings. That is why sericin can naturally saturate into skin and revitalize cells. It is discovered that sericin can restrain the functions of active-oxygen (major factor of aging), which brings wrinkles and dark spots.

The use of oxygen-permeable membranes from silk fibroin and silk sericin, containing about 60% water for contact lens, artificial skin, etc. The other uses of sericin includes, as a soil conditioner, coagulant for purification of waste waters, hygroscopic moisture-releasing polyurethane foams and their manufacture for furniture and interior materials, as additives for health foods to prevent colon cancers, medical composites of sericin, additives to rice cooking, fabric care compositions, light and sunscreen compositions, foam-forming aerosol shaving gels, sericin-coated powders for cosmetics, as dermatitis inhibitor, as wound protection film, nail cosmetics, and chewing gums (Gulrajani, 2005). Fibroin has been explored as a biomedicine for various applications.

Sericin and fibroin have been recently explored in the field of drug delivery Systems. Silk protein sericin, suppress DMBA-TPA induced mouse skin tumor genesis by reducing oxidative stress, inflammatory responses and endogenous tumor promoter TNF-alpha (Zhaorigetu et al., 2003).

1.2.7 HYDROGEL

Hydrogels are, physically or chemically cross linked three dimensional polymer networks with an interesting ability to absorb aqueous solutions. The hydrophilicity of the material is the underlying reason for this capability. These products are a hot topic in the field of biomaterial research. They can be of different types based on many criteria. Since hydrogels possess low interfacial tension, they show negligible tendency to adsorb proteins from body fluids (Gupta, 2002). Literature on this particular area is expanding day by day.

1.2.7.1 Preparation

Hydrogel is defined as polymer networks having hydrophilic properties. Mostly these are prepared from hydrophilic monomers. However, occasionally, hydrogels are prepared from hydrophobic monomers in order to regulate the properties for specific applications. Generally, hydrogels can be made from synthetic or natural polymers. When compared to natural polymers, synthetic are hydrophobic and chemically stronger. They possess a slow degradation rate owing to their mechanical strength and provide the durability as well. Through an optimal design, these two opposite features can be balanced (Tabata, 2009). When the natural polymers have suitable functional groups or have been functionalized with radically polymerizable groups, it is applicable for the preparation of natural polymer hydrogels also (Shantha, 2002).

In the most precise sense, a hydrogel is regarded as a hydrophilic polymeric network cross-linked in some fashion resulting in an elastic structure. In this way, any method that results in the formation of a cross-linked polymer can be used to

produce a hydrogel. Some common methods are copolymerization / cross-linking free-radical polymerization by reacting hydrophilic monomers with multifunctional cross-linkers. Similarly, water-soluble linear polymers of both natural and synthetic origin are cross-linked to form hydrogels in a number of ways:

1) Linking polymer chains via chemical reaction.

2) Using ionizing radiation to generate main-chain free radicals which can recombine as cross-link junctions.

3) Physical interactions such as entanglements, electrostatics, and crystallite formation.

In most cases, the three integral parts of the hydrogel preparation are monomer, initiator, and cross-linker. Diluents like water or other aqueous solutions can be applied to control the heat of polymerization and the final hydrogel properties. Then, the hydrogel mass needs to be washed to remove impurities left from the preparation process. These include non-reacted monomer, initiators, cross-linkers, and unwanted products produced via side reactions. Usually polar monomers serve as the starting material for hydrogels. They can be classified based on the starting material into natural polymer hydrogels, synthetic polymer hydrogels, and combination of the two classes. Many preparative methods are there like, graft polymerization, cross-linking polymerization, networks formation of water-soluble polymer, and radiation cross-linking, etc.. The polymerization techniques include bulk polymerisation, suspension polymerisation/cross-linking, suspension

polymerisation/inverse suspension polymerisation, grafting to a support, polymerisation by irradiation (Enas, 2013).

1.2.7.2 Classification of hydrogels

- **Classification based on source**

Hydrogels can be classified into two groups based on their natural or synthetic origins (Wen, 2013).

- **Classification according to polymeric composition**

The method of preparation leads to formations of some important classes of hydrogels. These can be exemplified by the following:

a) Homopolymeric hydrogels are referred to polymer network derived from a single species of monomer, which is a basic structural unit comprising of any polymer network (Takashi, 2007). Homopolymers may have cross-linked skeletal structure depending on the nature of the monomer and polymerization technique.

(b) Copolymeric hydrogels are comprised of two or more different monomer species with at least one hydrophilic component, arranged in a random, block or alternating configuration along the chain of the polymer network (Yang, 2002)

(c) Multipolymer Interpenetrating polymeric hydrogel (IPN), an important class of hydrogels, is made of two independent cross-linked synthetic and/or natural polymer component, contained in a network form. In semi-IPN hydrogel, one component is a cross-linked polymer and other component is a non-cross-linked polymer (Maolin, 2000; Hacker, 2011).

- **Classification based on configuration**

The classification of hydrogels depends on their physical structure and chemical composition can be classified as follows:

(a) Amorphous (non-crystalline).
(b) Semicrystalline: A complex mixture of amorphous and crystalline phases.
(c) Crystalline.

- **Classification based on type of cross-linking**

Hydrogels can be divided into two categories based on the chemical or physical nature of the cross-link junctions. Chemically cross-linked networks have permanent junctions, while physical networks have transient junctions that arise from either polymer chain entanglements or physical interactions such as ionic interactions, hydrogen bonds, or hydrophobic interactions (Hacker, 2011).

- **Classification based on physical appearance**

Hydrogels appearance as matrix, film, or microsphere depends on the technique of polymerization involved in the preparation process.

- **Classification according to network electrical charge**

Hydrogels may be categorized into four groups on the basis of presence or absence of electrical charge located on the cross-linked chains:

(a) Nonionic (neutral).

(b) Ionic (including anionic or cationic).

(c) Amphoteric electrolyte (ampholytic) containing both acidic and basic groups.

(d) Zwitterionic (polybetaines) containing both anionic and cationic groups in each structural repeating unit.

Hydrogel-forming natural polymers include proteins such as collagen and gelatine and polysaccharides such as starch, alginate, and agarose. Synthetic polymers that form hydrogels are traditionally prepared using chemical polymerization methods.

- **Hydrogel product sensitive to environmental conditions**

As mentioned, hydrogels as three-dimensional cross-linked hydrophilic polymer networks are capable of swelling or de-swelling reversibly in water and retaining large volume of liquid in swollen state. Hydrogels can be designed with controllable responses as to shrink or expand with changes in external environmental conditions. They may perform dramatic volume transition in response to a variety of physical and chemical stimuli, where the physical stimuli include temperature, electric or magnetic field, light, pressure, and sound, while the chemical stimuli include pH, solvent composition, ionic strength, and molecular species.

The extent of swelling or de-swelling in response to the changes in the external environment of the hydrogel could be so drastic that the phenomenon is referred to as volume collapse or phase transition (Jinsub, 2010). Synthetic hydrogels have been a field of extensive research for the past four decades, and it still remains a very active area of research today.

1.2.8 Natural polymer hydrogels

Hydrogels may be of natural polymer based; for example polysaccharides and proteins. These natural macromolecules are repetition of glycosidic and amino acid monomers. Since hydrogels are characterized of its softness and rubbery consistence, they resemble closely with the living tissues. Even if they shows minimal tendency to adsorb serum proteins, natural polymers like collagen,

gelatine and their derivatives often possess affinity towards serum fibronectin. Interestingly, studies on this fact provides the potential of these hydrogels to function as ideal scaffolds that support cell growth for tissue engineering purposes and as dug delivery vehicles (van Vlierberghe, 2011).

Hydrogels of natural polymers, especially polysaccharides, are in general, non-toxic and biodegradable. Considerable research and technical work have been reported. Hyaluronic acid, chitosan, chondroitin sulphate, cellulose derivatives, alginate are the excellent members under this category. The chemical modification of starch or modified starch via vinyl graft copolymerization constitutes the most important fields for improving the properties of starch and enlarging the range of its utilization. The starch graft-copolymer such as starch-g-polystyrene, starch-g-polyvinyl alcohol, starch-g-methacrylonitrile, and starch-g-acrylonitrile have been produced by generating free radicals on the surface of the starch granules followed by copolymerization of these free radicals with the respective vinyl monomers. These copolymers have also limited biodegradability because of the presence of a non-biodegradable part of the polymer (Doyle, 2006).

It has been reported that the synthesis of hydrogels by modification of natural polymers (for example, biocatalytic) has been used for preparation of sugar-containing poly(acrylate) hydrogels. These authors found that by the introduction of small quantities of agar, they were able to eliminate the relative brittleness of the polyacrylamide hydrogels and reduce the formation of undesirable fine particles during wet milling. Raju et al. (2001) grafted acrylonitrile onto cassava starch. These authors investigated the effects of the reactant concentrations and duration of the polymerization.

1.2.8.1 Protein hydrogels

Protein is the natural macromolecule with amino acid monomers. In the area of biopolymer based hydrogels, protein is an ideal candidate. Collagen, gelatin, elastin, fibroin and sericin are the prominent members under this category. Hydrogels based on these are excellent tools in the field of tissue engineering. To date, hydrogels from these are useful for bone tissue engineering, skin tissue engineering, blood vessel regeneration and ocular tissue engineering purposes. Porous collagen scaffolds have been made by freeze drying and stereolithographic methods. They can be used for bone application by mixing with calcium phosphates. Also, it can be blended with synthetic polymers like, poly(lactic acid) (Gong, 2007; Yang, 2009) poly(glycolic acid), (Wen, 2007; Kawazoe, 2009) or poly(caprolactone) as well as (modified) glycosaminoglycans (Ohyabu, 2009; Haugh, 2009; Tierney, 2009) such as (photocross- linkable) hyaluronic acid forming semi-IPNs.

Gelatin, a hydrolysed product of collagen is a crucial member in this field. It can be chemically / enzymatically cross-linked so as to get a stable hydrogel product. Porous scaffolds from this can be made by freeze drying and phase separation methods. It can also be blended with poly(L-lactic acid), (Lazzeri, 2007) polyurethanes, (Wei, 2008) and PCL (Rim, 2009).

Elastin constitutes the greater part of elastic, thus mechanically active tissues including tendon, blood vessels, and elastic cartilage (Krishna, 2010). Matriderm

is a commercially available dermal substitute composed of elastin and collagen described frequently in literature (Keck, 2009; Kolokythas, 2008).

Silk fibroin synthesized by the silkworm Bombyx mori, is a natural protein (Cao, 2009). Its primary structure mainly consists of glycine, alanine, and serine (Sundar, 2010). The protein is an ideal candidate of biomaterial science and drug delivery from which, films, (Lawrence, 2010; Nogueira, 2010) nanofibers, (Wang, 2009; Wang *et al.*, 2009; Jin, 2004; Meinel, 2009) scaffolds, (She, 2009) membranes (Ghassemifar, 2010) gels, (Gong, 2010; Yucel, 2010) and powders (Yucel, 2010; Tao, 2010) can be developed (Sundar, 2010). Fan et al. have developed porous gelatin-based hybrid scaffolds for ligament tissue engineering. In addition to composite scaffolds with other proteins (e.g., collagen, fibroin has also been combined with GAGs including hyaluronan (Ren, 2009; Garcia, 2009). The scaffolds developed were suitable to support mesenchymal stem cell adhesion (Garcia, 2009). Porous fibroin scaffolds can be made by freeze drying and leaching out porogens. In addition to freeze-drying, (Mandal, 2009; Mauney, 2007) leaching out of porogens (Makaya, 2009).

1.2.9 SERICIN AS A BIOMATERIAL

Tissue engineering provides combinations of cells and biomaterials, and/or drugs, genes & gene products that may be designed and fabricated in the form of biological substitutes. An important element of this field is biomaterial design. This involves incorporation of physical, chemical and biological cues in order to lead cells into functional tissues through the process of migration, adhesion and differentiation. Mostly, biomaterials should possess a degradation rate matching

with the new tissue formation so that cells will be capable to deposit new extracellular matrix (ECM) and regenerate functional tissue. These biomaterials must be able to support mechanically, in accordance with the level of functional tissue development as well. On the whole, for a material to be called as a biomaterial, it must be biocompatible and elicit little to no host immune response.

In this context, silk have been investigated as biomaterial due to the successful use of silk fibers from *B. mori* as suture material for centuries (Moy, 1991). Silks obtained from different species and within a species shows some functional differences as a result of the changes in primary amino acid sequence, processing, and the impact of environmental factors (Vollrath, 2001). Silks are a unique family of structural proteins having biocompatibility, degradability, mechanical superiority. This ultra class of proteins offer a wide range of properties, open to aqueous or organic solvent processing and can undergo chemical modification in order to go well with a wide range of biomedical applications.

For the treatment of superficial wounds, genipin cross-linked sericin/poly(vinyl alcohol) (PVA) films were proved to be an efficient 2 dimensional wound dressing agent. The property of silk sericin in enhancing collagen production and resultant epithelialisation in wounds is applied in this system. Moreover excellent attachment of fibroblast and keratinocytes on sericin also contribute to the excellence in using sericin as a wound dressing material. Complete investigation of the physical and biological properties of the system revealed increased surface density, tensile strength, and percentage of elongation, but decreased percentage

of light transmission, water vapor transmission rate, and water swelling, compared to the non-cross-linked films, thus promoting the formation of a more rigid molecular structure by minimising the mobility of molecular chain. The release of sericin from film was in a sustained manner. Desirable percentage of viability could be shown by L929 mouse fibroblast or HaCat keratinocyte cells when cultured on the silk sericin / PVA films cross-linked with 0.075 and 0.1%w/v genipin. Safety test conducted in this study renders the system a completely biocompatible one (Siritientong, 2013).

A 3D tissue engineered sericin construct has been developed as an alternative to autografts, allografts and xenografts. In the fabricated matrix co-culture of fibroblast and keratinocytes were used. Keratinocytes were grown on the upper surface and fibroblasts on the lower surface. Freeze drying was employed in the fabrication method and genipin was the crosslinker resulting in a porous matrix. The characterisation of the matrix showed a good growth of co-cultured fibroblasts and keratinocytes *in vitro* on 3D sericin matrices. This construct was aimed to deliver at the target wound site where it could promote the wound closure and gradually accelerate healing mechanism. This 3D tissue-engineered skin model is a potential source of living skin equivalents for grafting *in vivo* (Sunita, 2013).

Sericin is a highly hydrophilic protein family acting as the glue in *Bombyx mori* silk. In order to apply sericin as a wound dressing, a novel sericin film named gel film was prepared by a simple process without using any chemical modifications. Sericin solution was gelled with ethanol into a sheet shape and then dried. Infrared

analysis revealed that the sericin gel film contained water-stable beta-sheet networks formed in the gelation step. This structural feature rendered the gel film morphologically stable against swelling and gave it good handling properties in the wet state. The sericin gel film rapidly absorbed water, equilibrating at a water content of about 80%, and exhibited elastic deformation up to a strain of about 25% in the wet state. A culture of mouse fibroblasts on the gel film indicated that it had low cell adhesion properties and no cytotoxicity. These characteristics of sericin gel film suggest its potential as a wound dressing (Teramoto *et al.*, 2008).

A study was conducted by Theodora (2014) to investigate the synergetic effect of racemic flavanone Naringenin (NRG) and the protein sericin as TNF-α blockers. Sericin micro particles were fabricated by using spray-drying method, which was loaded with naringenin. Characterization in terms of morphology and particle size distribution, and encapsulation efficiency was done, which were correlated well with the motto of the study. This particular study provides the proof of concept that sericin-based microspheres loaded with TNF-α-blockers could contribute to the down regulation of the cytokine and represents the starting point for the development of new topical formulations for the treatment of middle-stage psoriasis. It is to be drawn from this work that sericin may improve skin barrier function, thus preventing water loss from the upper layer of the skin (Theodora, 2014).

Silk fibers have been the primary silk- like material used in biomedical applications particularly as sutures. During decades of use, silk fibres have proven

to be effective in many clinical applications. Subsequently, the wound dressing was made with a mixture of both fibroin and sericin (Tsubouchi, 1999b). 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.

In the area of cartilage tissue engineering, bio hybrids consisting of biodegradable scaffolds with cultured cells is a recent development. In a study aimed at developing a 3D porous scaffold based on natural compounds, collagen-sericin scaffold supporting human adipose –derived stem cells (ASCs) was fabricated and characterized. It was already proved that Hyaluronic acid and chondroitin sulphate are the most potent prochondrogenic factors involved in the biomaterial design for cartilage tissue engineering application. The fabricated scaffolds were of good porosity, high water absorption capacity; non-denatured triple helical structure of collagen. These features make them perfect for cartilage tissue engineering. Cytotoxic assays also were found to be positive (Sorina, 2013).

Role of sericin in ocular tissue engineering is in its path of exploration. In a study conducted under this field, investigated attachment and growth in vitro of human corneal limbal epithelial cells (HLECs) on sericin-based membranes. Primary cell cultures from two donors were established in serum supplemented media in the presence of murine feeder cells. Membranes made of sericin and sericin-fibroin blends were assessed in terms of cell growth and attachment. The results

demonstrated that B. mori sericin-based materials were mechanically weak compared to sericin-fibroin blend. However, the attachment of human corneal limbal epithelial cells to sericin or sericin-rich blends was far superior to that to fibroin or fibroin-rich blends, with no evidence of any cytopathologic response. This is a strong recommendation that sericin is an apt material that could serve as a scaffold in ocular surface regeneration (Traian, 2013).

The role of sericin as an active participant in tissue engineering field becomes a hot topic. Fabrication of a novel 3D sericin / gelatine scaffold and 2D films supports this fact. The fabricated matrices were characterized and optimized biophysically and biologically. The results were in accordance with the aim of the study. It was showed that the matrices were cytocompatible and biocompatible. They showed excellent characteristics like uniform pore distribution, improved compressive strength, high swell ability and overall high porosity, the critical parameters for tissue engineering and biomedical applications. This work also provides a new dimension to silk-protein sericin in the form of novel blended 3-D scaffolds for efficient and effective use as biomaterial (Biman, 2009).

Limitations in the current clinical strategies for adipose tissue engineering (ATE) strongly recommend the application of a 3D cell-scaffold construct. In this respect, a 3D scaffold made of 60% collagen and 40% collagen preceded with human adipose derived stem cells (hADSCs). These scaffolds were well characterized and optimized for the specific application. Interestingly, it was shown that incorporation of the sticky protein sericin along with collagen

enhanced the adhesion and proliferation rate of seeded cells, making it a biocompatible marix. Sericin showed an astonishing ability of stimulating PPARγ2 over expression, triggering a consequent up regulated expression profile of FAS, aP2 and perilipin adipogenic markers. In addition to this, sericin is well known by its property to stimulate collagen synthesis. Moreover, higher rate of adipogenesis was correlated with the presence of sericin in the cellular environment. These remarkable features makes the fabricated matrix out of it as a strong candidate for soft tissue reconstruction and wound healing applications. However, *in vivo* implantation of these constructs should be addressed in further work during ATE applications in order to confirm the reproducibility of these data and to validate safety claims (Sorina, 2013).

Antibacterial property of sericin was examined by growing *Micrococcus leutus* bacteria by modified agar diffusion method. A zone of inhibition of (9-12) mm in diameter for 10 & 20 mg/ml concentration was observed. Also, the antibacterial efficiency was found to be increased with increasing concentration of sericin. This study provides an insight to use sericin for medical applications after isolation and identification of some pathogenic bacteria like *Pseudomonas aeruginosa, Staphyllococcus aureous Escherchia coli* to produce medical bandages, mouth wash, antibacterial soaps & tooth paste. In this context, more research has to be explored (Khalid, 2010).

Sericin based nano biomaterials was reported. Nano sized sericin was obtained using urea treatment by a simple and cost effective method. Nano sericin obtained

was tested for many applications like hydrogel, biopolymers, biomaterials, and antioxidant and for other antimicrobial applications. Moisture absorbing and releasing property, inherent gelling property rapid gelation on addition of cross-linkers make it an ideal candidate for wound dressing application and as soil conditioner. The developed nano sericin is suitable for various applications like moisturizer in cosmetics, as scaffold, replacing binding material in perfume sticks and in the production of antimicrobial products (Pushpa, 2013).

1.2.9.1 Sericin based hydrogel

Silk sericin is a precious protein resource obtained from silk worm, the queen of textiles. Usually, it is considered as a waste product and discarded by a process called degumming in the textile industry. Studies have proved that sericin is a valuable biomaterial that can be made into gels, membrane, foam, fiber and other materials. Hydrogels based on sericin is a remarkable output of such studies. These sericin hydrogels are ideal for drug delivery applications and tissue engineering. Hydrogel matrix produced from sericin shows excellent cell homing ability, which makes them suitable for wound healing application.

In situ semi interpenetrating on-mulberry tropical tasar silk sericin/polyacrylamide hydrophilic network showed cell homing ability and rapid gelation. They successfully entrapped sericin physically within the 3D hydrophilic structure of polyacrylamide. The injectable composite material developed through such a relatively simple technique possessed favourable properties required for *in situ* cell scaffolding and tissue regeneration. The characteristics of the matrix like the gelation time, hydrogel micro-architecture, swelling, and equilibrium water

content, compressive strength and in vitro degradation were found to be influenced by sericin concentration. Another remarkable achievement of this work is that no chemical cross linkers are involved in this (Kundu, 2012).

Sericin hydrogels were also used as drug vehicle. Interpenetrating polymer networks (IPNs) composed of silk sericin (SS) and poly (N-isopropylacrylamide) (PNIPAAm) were prepared by the simultaneous-IPN method. The miscibility of the two was confirmed by the single Tg found in thermograms. The interaction between two members which favoured miscibility was deduced from FTIR. The release profile of a model drug BSA was studied. It showed a burst release within first 12 hours. This IPN based on sericin is being modulated as an ideal drug carrier (Zuo, 2006).

A photo luminescent, injectable hydrogel had developed from sericin via covalent cross linking method with glutaraldehyde for delivery of cells and drugs. The fabricated hydrogel system was showed to acquire marvellous cell adhesive capability, guiding efficient cell attachment, proliferation and long term survival of various types of cells. Moreover, the system was able to release bioactive agents in a sustained manner. In addition to that, the sericin hydrogel could exhibit good elasticity, high porosity and pH dependent degradation dynamics which make it suitable to function as a delivery vehicle for cells and therapeutics. Since the system is injectable, it allows minimal invasive approaches for implantation. The photoluminescence property enables bioimaging and *in vivo* tracking. Among the three types of cell types tried, HEK293, C2C12, HaCaT, HEK293 exhibited

cluster type growth on hydrogel surface, whereas the other cell types did not show this particular pattern. This demonstrates that sericin hydrogel influences spatial distribution of cells (Wang, 2014).

1.3 HYPOTHESIS

- *Cross-linking two biopolymers, sericin and gelatin, would produce a biodegradable and biocompatible hydrogel system having sufficient hydrophilicity, strength, and handling characteristics so that they can be used for biomedical applications.*

1.4 OBJECTIVES

- Fabrication of hydrogel by optimising reaction conditions
- To carry out physico-chemical Characterization of Hydrogel
- To perform biological evaluation of Hydrogel

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS AND EQUIPMENTS

The materials and equipments used are listed in table below:

Table 1 : List of Materials used

Sl. No	Name of Chemical	Grade	Source
1	Gelatin Type A(from Porcine skin	Laboratory Grade	Sigma Aldrich, USA
2	Sericin		Central Silk Board,Bangalore
3	Sodium chloride	GR	Merck, Germany
4	Potassium dihydrogen phosphate	GR	Merck, Germany
5	Disodium hydrogen phosphate	GR	Merck, Germany
6	Morpholino Ethyl Succinate	98%	Alfa Aesar, Britain
7	Iscove's modified Dulbecco's Minimal Essential Medium-F12	Cell Culture grade	Invitrogen, USA
8	Antibiotic-antimycotic solution	Cell Culture grade	Invitrogen, USA
9	Foetal Bovine Calf Serum	Cell Culture grade	Invitrogen, USA
10	Trypsin-EDTA	Cell Culture grade	Invitrogen, USA
11	Trypan Blue	Cell Culture grade	Sigma Aldrich, USA
12	Formaldehyde	Laboratory Grade	Merck, Germany
13	Texas red –	Cell	Molecular

	Phalloidin	culture Grade	Probes, USA
14	Picogreen	Cell culture Grade	Molecular Probes, USA
15	Hoechst	Cell culture Grade	Molecular Probes, USA

Table 2 : List of Equipments used

Sl. No	Name of Equipment	Model	Make
1	FTIR Spectrometer	FT/IR-6300	JASCO,Japan
2	Micro Computed Tomography	µCT-40	Scanco Medical AG,Switserland
3	Universal Testing Machine	Instron 3345	Instron,USA
4	Phase contrast microscope	DMIRB	Leica, Germany
5	CO2 Incubator	Thermo Thermo electron Ltd. USA	Thermo Thermo electron Ltd. USA
6	Lyophilizer	Alpha 1-4 LD.	Christ, Germany
7	Hot air Oven	Pyrodevices-114	Pyrodevices,USA
8	Bacteriological Incubator	Labline-314	Labline,USA
19	X-Ray Diffractometr	Bruker D8	Bruker, Germany
110	Environmental Scanning Electron Microscope	Quanta 200	FEI, Netherlands

11	Improved Neubauer Counting chamber	Bromma S16126	BROMMA, Sweden
12	Dynamic Mechanical Analyser	Tritec 2000B UK	Triton Technology, Ltd., United Kingdom
13	Thermo gravimetric Analyser	SDT-2960	TA instruments,USA

2.2 METHODS

2.2.1 RAW MATERIAL CHARACTERIZATION

2.2.1.1 FTIR Spectroscopy:

Fourier Transform Infrared (FTIR) spectra of raw materials (sericin and gelatin) were recorded using a FTIR spectrophotometer (model 6300, Jasco, Japan). Samples in the powder form was mixed with KBr and made into thin transparent disks using a hydraulic hand press by applying a pressure of about 10-ton for about 3 minutes. KBr without the samples was used as the control.

After warming up the instrument for 20 minutes, background measurements were made with control KBr pellet. CO_2 and moisture peak removal was made effective after this recording. The sample disks were monitored after background measurements using the "Spectra manager" software. Spectra were collected in the range 400-4000 cm^{-1} using at least 16 scans at a resolution of 4 cm^{-1}. The obtained FTIR spectra were processed for baseline corrections, smoothening and labelling of peaks using the software.

2.2.1.2 X-Ray Diffraction analysis

X-Ray Diffraction (XRD) data of the powdered samples were analyzed with a Bruker D8 Advance diffractometer with Cu Kα radiation at a scan speed of 4^0/min over 10-90^0.

2.2.1.3 Thermal Analysis

Thermal stability of the sample was studied using thermogravimetry (TGA) based on the method given in ASTME-1131-19 by using a SDT-2960 model thremogravimetric analyser (M/s. TA instruments, USA). An amount of 50 mg of

powdered sample was heated at a rate of 10^0C/min in an aluminum crucible from 30 to 500 ^{0}C in a dynamic inert nitrogen atmosphere. Continuous recording of sample temperature, sample weight and heat flow were made.

2.2.1.4 Dynamic Mechanical Analysis

DMA was done in tensile mode with a Tritec 2000B DMA instrument (Triton Technology, Ltd., United Kingdom) on a rectangular gelatin sample of 4-mm width, 20-mm length and 0.2-mm thickness. A frequency of 1 Hz with an amplitude of dynamic deformation of 50μm was applied in the temperature-ramp experiments. A heating rate of 1^0C/min was used over a temperature range from -100^0C to 90^0C

2.2.2 FABRICATION OF SERICIN –GELATIN HYDROGEL

Hydrogels of sericin and gelatin (SGH) were fabricated by mixing solutions of both gelatin and sericin at approximate temperatures and later drying in an incubator. Silk protein, sericin was dissolved in hot water at 60^0C and gelatin in distilled water at 40^0C. Then the two solutions were mixed at different reaction conditions. Reaction mixture was then incubated at 37^0C for 10-60 minutes for gelation. The process of fabrication of hydrogel was optimized by varying the parameters such as solution concentration, temperature, reaction time and incubation.

2.2 .3 CHARACTERIZATION OF HYDROGEL

2.2.3.1 FTIR Spectroscopy

FTIR characterization of the hydrogel was done using the method mentioned above.

2.2.3.2 XRD Analysis

XRD data of the powdered sample was done using the method reported above.

2.2.3.3 Thermal Analysis

Thermal stability of the hydrogel was analysed by using TGA and DSC.

2.2.3.4 Dynamic Mechanical Analysis

Dynamic Mechanical Analysis (DMA) of the materials were performed in tensile mode with a Tritec 2000B DMA instrument (Triton Technology, Ltd., United Kingdom) on a rectangular sample of 4 mm width, 20 mm length and 0.2 mm thickness. A frequency of 1 Hz with an amplitude of dynamic deformation of 50µm was applied in the temperature-ramp experiments. A heating rate of 1^0C/min was used over a temperature range from -100^0C to 90^0C.

2.2.3.5 Swelling studies

The dynamic swelling of the hydrogel was analysed in alkaline (PBS), acidic (MES) and neutral (distilled water) media. Dried circular disk samples (2cm diameter × 1 mm thickness) were pre-weighed and immersed in three media at 37°C for 24 h. At different time points, hydrogels were wiped with Whatmann No.1 filter paper to remove surface water and swollen weight was determined using an analytical balance.

Degree of swelling (Q) was calculated by the formula

$$\mathbf{Q = (Ws-Wd)/Wd}$$

$$\mathbf{Percentage\ Swelling = [(Ws-Wd)/Wd] \times 100}$$

$$\mathbf{EWC = [(Ws-Wd)/Ws] \times 100}$$

Where Wd and Ws are the dry weight and swollen weight of hydrogels, respectively.

2.2.3.6 Mechanical Properties

A Universal testing machine (Instron, model 3345, USA) equipped with a 10 N load cell was used to determine mechanical properties of hydrogel. Rectangular samples of size 4cm length, 1cm width and 1mm thickness were used for the tests. The samples were tested at a crosshead speed of 10 mm/min at 25^0C. Dry samples and wet samples swelled in acidic (MES), alkaline (PBS) and neutral (Distilled water) pH conditions were tested. Samples were pre-conditioned by keeping them for 24 hours in the room temperature where test is to be conducted. Samples were tested at a crosshead speed of 1mm/min (Figure 4). Tensile properties such as tensile strength, Young's modulus and elongation at break were determined.

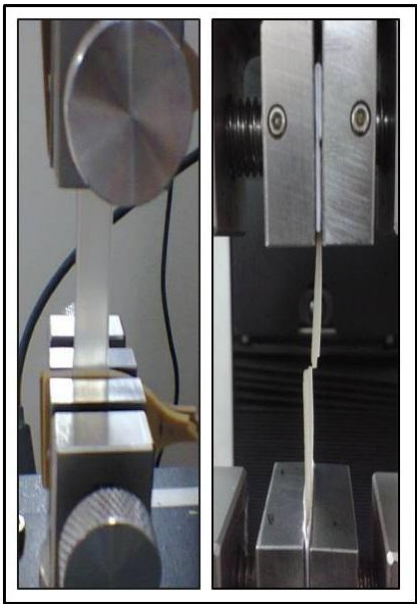


Figure 4: Tensile testing of hydrogel materials

2.2.3.7 Surface Morphology

The microstructure of the hydrogel sample was investigated using a scanning electron microscope (SEM; Hitachi S2400, Japan). Briefly, after fabrication the hydrogels were immersed in de-ionized water and washed thrice to remove the un-reacted components, if any. The resultant gels were lyophilized and dried. The samples were gold coated and examined under vacuum under SEM with a working voltage of 25 KV. The images were taken at different magnifications. The pore sizes within the hydrogels were measured using Image J 1.40 software.

2.2.3.8 Micro Computed Tomography Analysis

Lyophilised hydrogel samples were subjected to micro computed tomography (μCT). The samples were placed in the sample holder (PMMA tube) for detecting the X-Ray attenuation and scanning was performed with 45KV X-ray energy, 177μA intensity and 10μm resolution (2D slice thickness). Two-dimensional reconstructions was done using Cone beam algorithm. Region of interest in the 2D slices were contoured and 3D evaluation was performed by setting appropriate threshold value of X-ray attenuation to actual 3D image of the sample.

2.2.3.9 Degradation studies

Hydrogel samples were fabricated as described previously. Degradation of hydrogel in alkaline (PBS), acidic (MES) and neutral (distilled water) media was studied for 3 weeks. Pre-weighed hydrogel samples in the form of circular disk (2 cm diameter × 1mm thickness) of average weight 0.1g were immersed in 5ml PBS, MES and distilled water along with 0.1% Sodium azide solution and incubated at 37^0C. The weight loss of the sample was determined using an

analytical balance in seven days interval, which indicates hydrolytic degradation of the samples.

2.2.3.10 Hemolysis

Hemocompatibility test of hydrogel systems was carried out broadly on the basis of ISO 10993-4:2002 (E) 'Selection of tests for interaction of materials with blood'. The test is mainly aimed at finding the extent of hemolysis caused by the sample. Blood from healthy human volunteer was collected into the anticoagulant, citrate-phosphate-dextrose-adenine [CPD-A]. Samples kept in PBS were taken out and placed in polystyrene plates. Blood (2 mL) was added to each sample; 1 mL blood was taken immediately for initial analysis and another 1 mL blood was incubated with the samples for 30 min under agitation at 70±5 rpm using an Environ shaker (Labline Instruments Inc., Melrose Park, USA) thermostated at 35±2°C. Four empty polystyrene culture dishes were exposed with blood as reference. The total hemoglobin in the whole blood samples was measured using automatic hematology analyzer (Sysmex-K 4500, Sysmex Corporation, Japan). The free hemoglobin liberated into the plasma after exposure to materials was measured using diode array spectrophotometer (HP 8453; Hewlett-Packard GmbH, Germany) and the hemolysis (%) was calculated using the formula

$$\% \text{ Hemolysis} = \frac{\text{Free Hb}}{\text{Total Hb}} \times 100$$

2.2.4 BIOLOGICAL EVALUATION OF HYDROGEL

2.2.4.1 Isolation and culture of human skin dermal fibroblasts

Human skin dermal fibroblast was received from Thrombosis Research Unit, SCTIMST, Trivandrum.

2.2.4.2 Hydrogel conditioning

Lyophilised washed hydrogels were rehydrated for 24 hours in PBS and conditioned in serum free medium for 6 days. Medium was changed on alternative days.

2.2.4.3 Seeding

Scaffolds were analyzed for their cell attachment and proliferation. Primary fibroblasts after second passage were used for hydrogel wound dressing seeding. Scaffolds were statically seeded with a cell density of 5000 cells/cm^2 and allowed to attach. Unattached cells were removed after 12 hours and replenished with fresh medium.

2.2.4.4 Cytotoxicity by direct contact Method

Primary fibroblasts were seeded on to tissue culture treated polystyrene surfaces and allowed to form a complete monolayer. Circular hydrogel discs were placed on top of the cell monolayer and fed with complete medium. Efforts were taken to avoid the floating of the hydrogel. Medium was replenished on every alternative day. After 48 hours of culture, scaffolds were removed and cell monolayer integrity was observed through phase contrast microscope.

2.2.4.5 Cell viability Assay

Trypan blue exclusion assay was used to quantify the cell viability in the presence of hydrogel. The cells cultured in the presence of hydrogel for 72 hrs as mentioned above were harvested by trypsinization after removing the gel from the dish. The cell pellet was then re-suspended in lmL sterile PBS. Trypan Blue stain was diluted into 1:9 ratio with DPBS and mixed 90µL stain to 10µL cell suspension. About 20µL was taken into the Neubauer counting chamber and counted using 10 X magnification of microscope. Cells seeded in culture wells served as control.

2.2.4.6 Biochemical evaluation for Cell Proliferation- Pico green

The proliferation of human dermal fibroblasts on day 3 and day 6 days in culture was determined using Picogreen® dsDNA Quantitation reagent (Molecular probes). For this, the cell-seeded materials (n=6) were washed with PBS twice and kept in -80°C until analysis. Frozen cell samples were thawed for 20 min on ice and lysed with 1% Triton X-1 00 *(300* µl) for 20 min with sonication for 10 min. The lysate (10 µl) then mixed with Picogreen in Tris-EDTA buffer (190 µl) for 5 min and the intensity of fluorescence was measured with a multifunction microplate reader (HIDEX Chameleon) at an excitation and emission wavelength of 485/ 535 nm. Relative fluorescence units were correlated with cell number using a calibration line constructed from cell suspensions with increasing concentrations of cell numbers.

2.2.4.7 Two dimensional cell attachment

Scaffolds were statistically seeded with a cell density of 5000 cells/cm^2 and allowed to attach for 72 hours**.** The scaffold was then observed under Environmental scanning electron microscope (ESEM). Material alone served as the control.

2.2.4.8 Cell attachment assay

Scaffolds were statically seeded with a cell density of 5000 cells /cm_2 and allowed to attach for 48 hours. The scaffolds were then fixed with 3. 7% formaldehyde for 20 minutes and then permeabilised with 0.2% triton X 100. Texas red conjugated antibodies against actin cytoskeleton were added and kept at 4 0 C for overnight. Scaffolds were counter stained with Hoechst. Washed thoroughly and observed through an inverted fluorescent microscopy.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Raw Material Characterization

3.1.1 FTIR Spectroscopy

FTIR spectra of gelatin and sericin are shown in Figures 5 & 6 respectively.

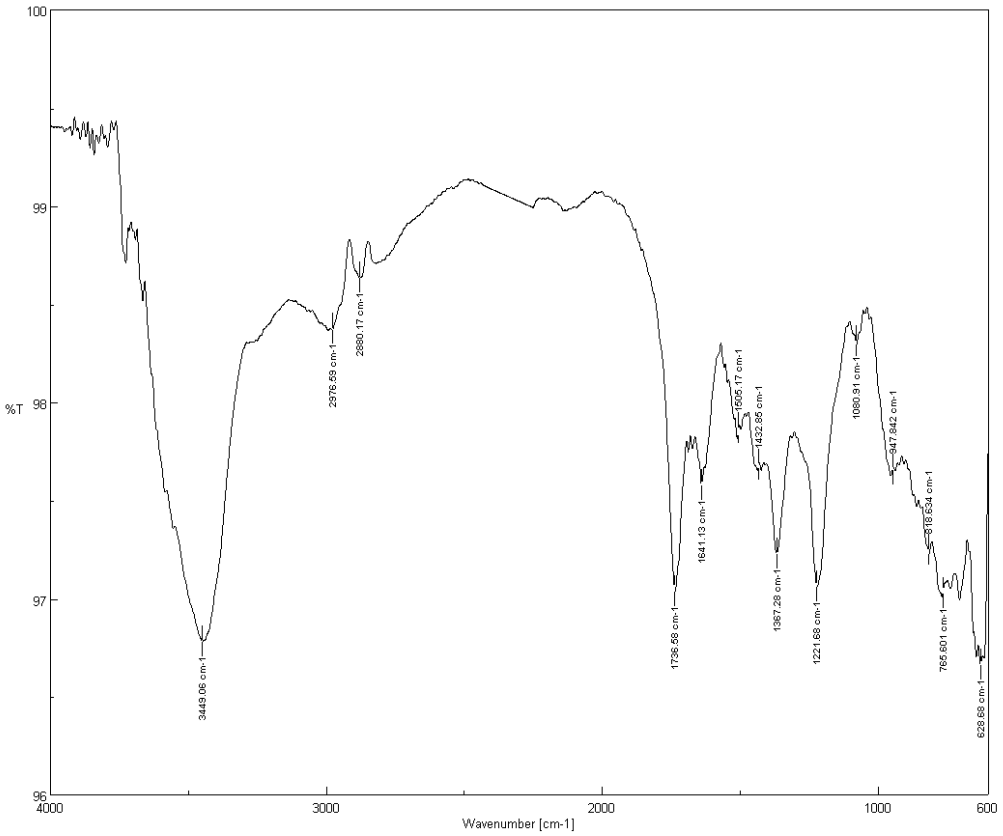


Figure 5 : FTIR Spectrum of Gelatin

Gelatin shows its characteristic peaks of N-H stretching at 3449 cm^{-1}, C-H stretching at 2976 and 2880 cm^{-1}, Amide I band at 1736 cm^{-1}, Amide II band at 1551 cm^{-1}, C-H deformation at 1432 cm^{-1}, and Amide III band at 1221 cm^{-1} (Saarai, 2012).

In sericin, the polypeptide and protein repeat units produce multiple characteristic infrared absorption bands, including amide I, II, and III. Amide I (1600–1690 cm^{-1}) mainly arises from the C=O stretching vibrations of the peptide linkages. Amide II (1480–1575 cm^{-1}) derives from N-H bending and the C-N stretching vibration (Turbiani, 2000). Amide III (1229–1301 cm^{-1}) primarily represents C-N stretching vibration linked to in-plane N-H bending vibration. Amide I is most suitable for analysing sericin secondary structures. The peak at 1632 cm^{-1} is the characteristic peak for β sheet (Teramoto, 2003, 2005). The spectrum shows characteristic amide A band at 3080 cm^{-1} and amide I and II peaks at 1648 and 1518 cm^{-1}. There is a further signature peak for sericin at 1443 cm^{-1} and a much enhanced absorbance at 1066 cm^{-1} (Turbiani, 2000). The prominence of these features ascribed to the relatively high content of carboxylic acid side chains of sericin.

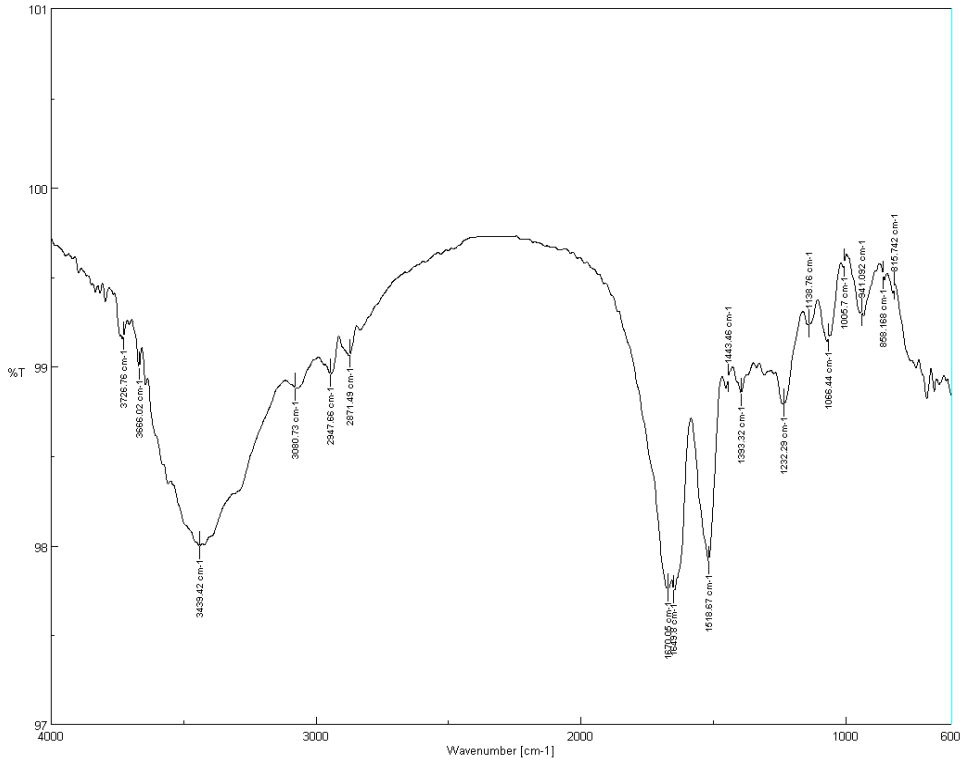


Figure 6 : FTIR Spectrum of Sericin

3.1. 2 X-Ray Diffraction

XRD pattern of gelatin is obtained as shown in Fig 7: The XRD spectrum of gelatin shows its characteristic diffraction peak at $2\theta = 20^0$ (Kunal, 2007).

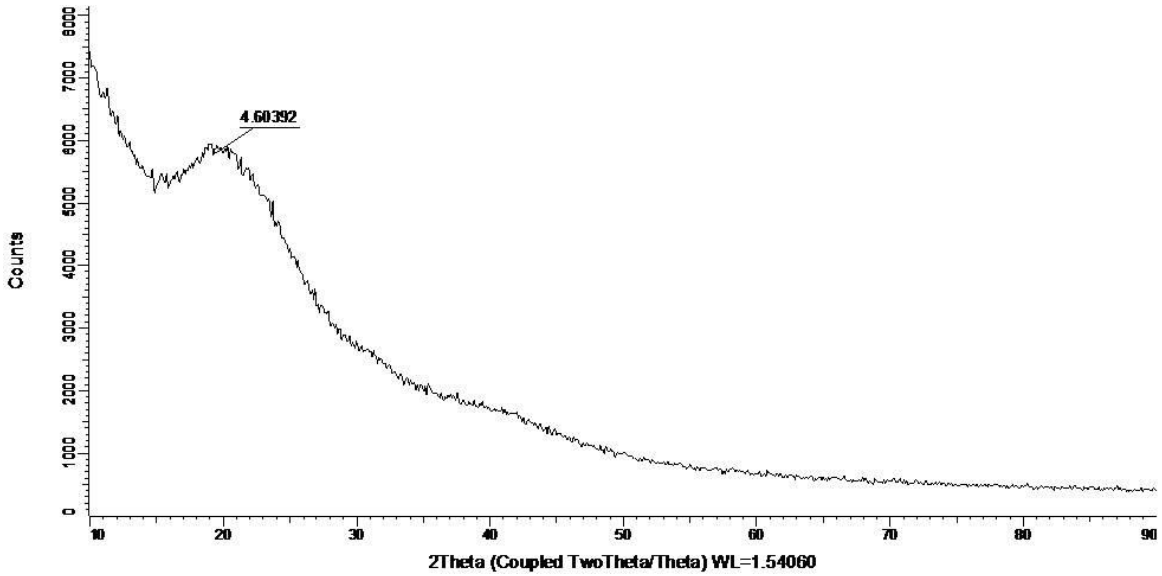


Figure 7 : XRD Spectrum of gelatin

XRD pattern of sericin is shown in Fig 8:

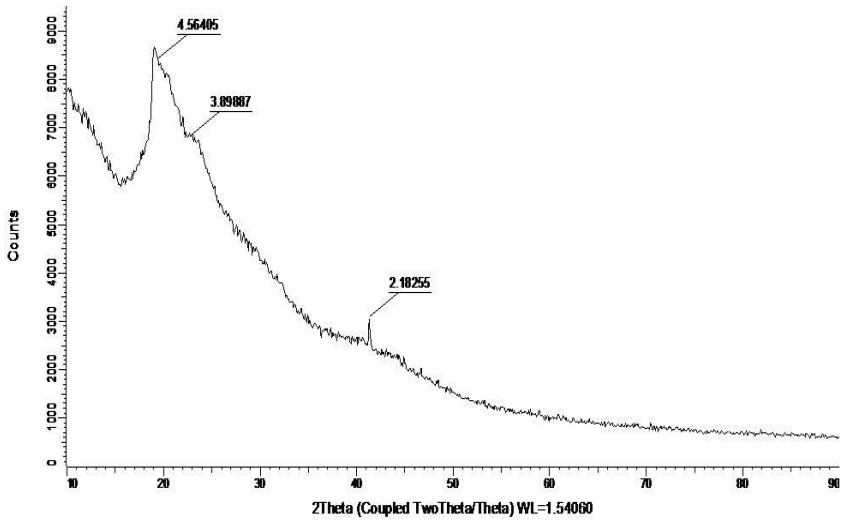


Figure 8 : XRD Spectrum of Sericin

The spectrum reveals that Sericin exhibit a strong diffraction peak at 2θ =21, 24 and 42 (Hajim, 2003). These are the characteristic diffraction peaks of high molecular weight sericin, indicating its crystalline nature.

3.1.3 Thermal Analysis

Thermal stability of sericin and gelatin was studied by TGA and DSC. TGA curves of sericin and gelatin are given in Fig: 9 (A, B).

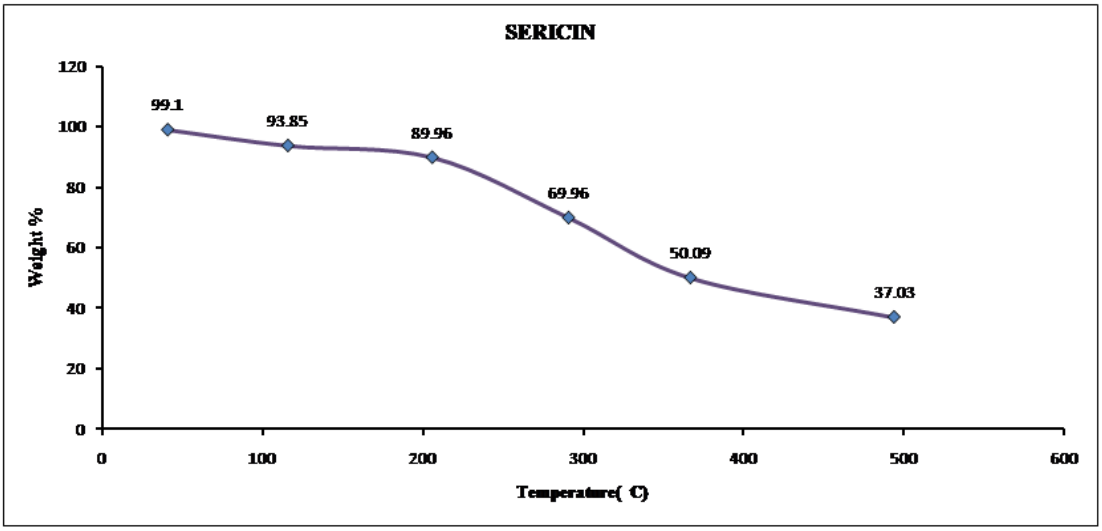


Figure 9: A) TGA curve of sericin

The thermal degradation of sericin found to occur in 4 stages, characterized by evident mass loss rates. The initial weight loss below 116^0 C is attributed to the evaporation of water, and is followed by nearly constant weight from 116^0 C to 206^0 C. The low mass loss observed during this stage is attributed to the loss of low temperature volatile species. In the next stage, from 206 to 291^0 C, weight reached 69%. It is followed by the third stage, where weight reached 50 % at 367^0 C. The fourth stage is characterized by a steep mass loss from 50 to 37%. The

evident mass losses from second to fourth stage is attributed to the breakdown of amino acid side chains as well as the cleavage of peptide linkages (Kweon, 2000, Freddi, 200

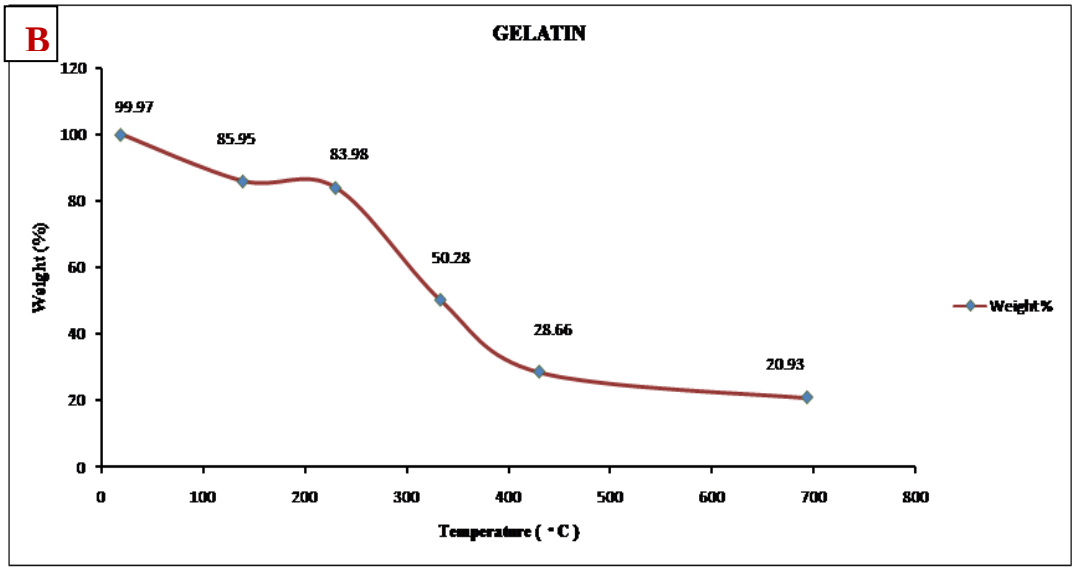


Figure 9: B) TGA curve of gelatin

The TGA curve of gelatin shows that it degrades much faster than sericin on heating: The degradation pattern can be divided into 4 regions, characterized by evident mass loss rates. The initial weight loss below 139^0 C due the loss of volatiles is followed by nearly constant weight from 139^0 C to 230^0 C. From 230^0 C to 333^0 C, a steep in mass loss was observed and the mass retained was only 50 %. At 430^0C, mass reached 28.66%. In the last stage, from 430 to 693^0 C mass was losing slowly and reached 20%. Initial steep in weight can be due to the breakdown of large numbers of weak bond like van der waals and hydrophobic interactions present. As the temperature advances, breakdown of more strong bonds like peptide bonds and amino acid side chains are apparent (Kweon, 1997).

3.1.4 Dynamic Mechanical Analysis

DMA was carried out to find the thermo mechanical properties of gelatin dried film.The variation in the storage modulus (E') and tan δ with temperature is given in Figure 10. Tg of gelatin was found to be at 18^0C.

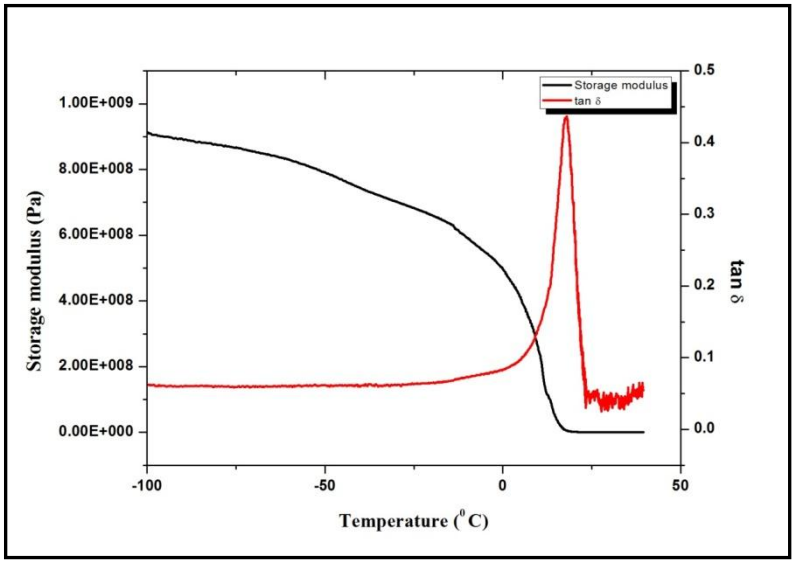


Figure 10: Temperature dependence of storage modulus and tan delta of Gelatin

3.2 Characterization of Sericin-Gelatin hydrogel

3.2.1 Process Optimization

The optimum conditions for preparing hydrogel from Sricin and Gelatin were obtained by choosing parameters such as solution concentration, dissolution temperature, reaction time, incubation time etc. The conditions used and results obtained are given in the table 3. Fig 11 : shows hydrogel casted in different molds.

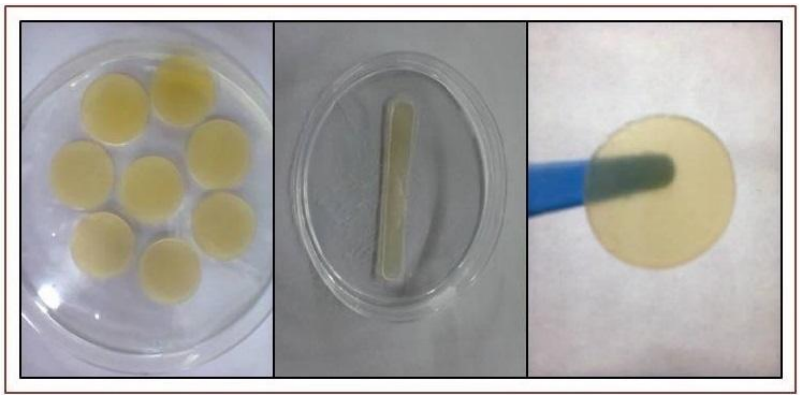


Figure 11: Hydrogel fabricated in different shapes

Table 3. Process parameters used for the preparation of hydrogel

Sl. No	Solution concentration % (w/v)		Dissolution temperature (^{o}C)		Reaction temp. (^{0}C) & Time (Min)		Incubation time (Min)	Gel formation (Yes/No)
	Sericin	Gelatin	Sericin	Gelatin	Temp	Time		
1	10	5	60	40	37	5	5	**No**
2	5	10	60	40	40	10	10	**No**
3	10	10	60	40	45	15	15	**No**
4	10	10	60	40	50	20	20	**No**
5	10	10	60	40	55	20	25	**No**
6	10	10	60	40	60	20	30	**No**
7	10	10	60	40	65	20	30	**No**
8	10	10	60	40	70	20	30	**No**
9	10	10	60	40	75	20	30	**No**
10	10	10	60	40	80	20	30	**No**
11	**10**	**10**	**60**	**40**	**85**	**20**	**30**	**Yes**

Gel formation was observed within 30 minutes of incubation at 37^0C. Gel formation was noted by checking the flow behaviour of the reaction mixture. When the gel was formed reaction mixture would stop flowing due to gravity. Figure 12 shows reaction mixture before and after gelation.

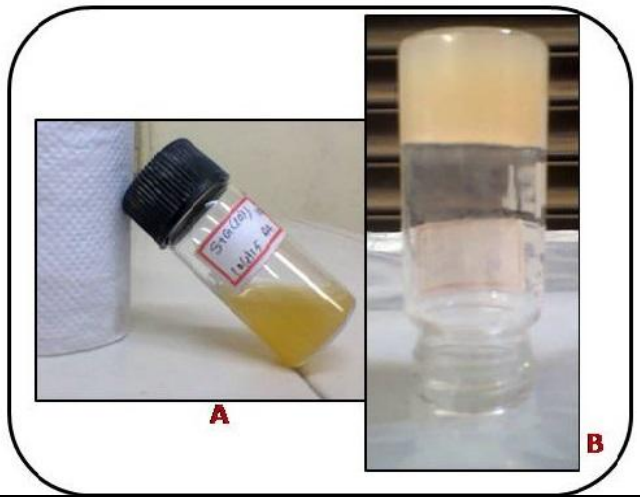


Figure 12: (A) Reaction mixture before gelation, (B) After gelation

3.3 Characterization of Sericin-Gelatin hydrogel

3.3.1 FTIR Spectroscopy

The FTIR spectrum of gelatin shows the characteristic absorption bands of its protein structure .The bands at 1640 and 1505 cm^{-1} are assigned to the N-H stretching vibration peaks for amide I and amide II respectively. Sericin has a broad amide A band (3080 cm^{-1}) amide I and amide II peaks at 1648 and 1518 cm^{-1}. There is a further signature peak for sericin at 1443 cm^{-1} and a much enhanced absorbance at 1066 cm^{-1}. Also, peaks in the range of (1320 cm^{-1}-1000 cm^{-1}) are apparent in sericin (1240 cm^{-1},1134 cm^{-1}), gelatine (1240 cm^{-1}) and SGB (1229 cm^{-1},1067 cm^{-1}) spectrum. The prominence of these features may be ascribed to

the relatively high content of carboxylic acid and alkyl hydroxyl containing amino acid side chain in sericin. In the case of hydrogel, all those signature peaks of sericin and gelatin in the fingerprint region was found to be significantly reduced (1230 cm^{-1} only), indicating the unavailability of these functional groups. But in the spectrum of sericin-gelatin blend, fingerprint regions of sericin and gelatin remained as such to an extent. N-H bend was observed at 1649 cm^{-1} in sericin and 1641 cm^{-1} in gelatin. In SGH, a peak was observed at 1659 cm^{-1}, inferring a shift in N-H bend as a result of crosslinking. The peak at 1518 cm^{-1} in sericin reveals Amide II band. Gelatin also exhibits Amide II at 1505 cm^{-1}. In SGH, a shift was observed at this peak towards 1482 cm^{-1}. Moreover, such a peak is absent in the case of SGB. The significant reduction of peaks corresponding to C-O stretch of acids and N-H bend of amines in SGH suggest the unavailability these functional groups. Owing to these observations, it can be said that a crosslinked hydrogel network was formed as a result of the interactions of reactive groups in sericin and gelatin. Table 4 shows the peaks obtained for Sericin, gelatin, hydrogel and a blend of gelatin and hydrogel.

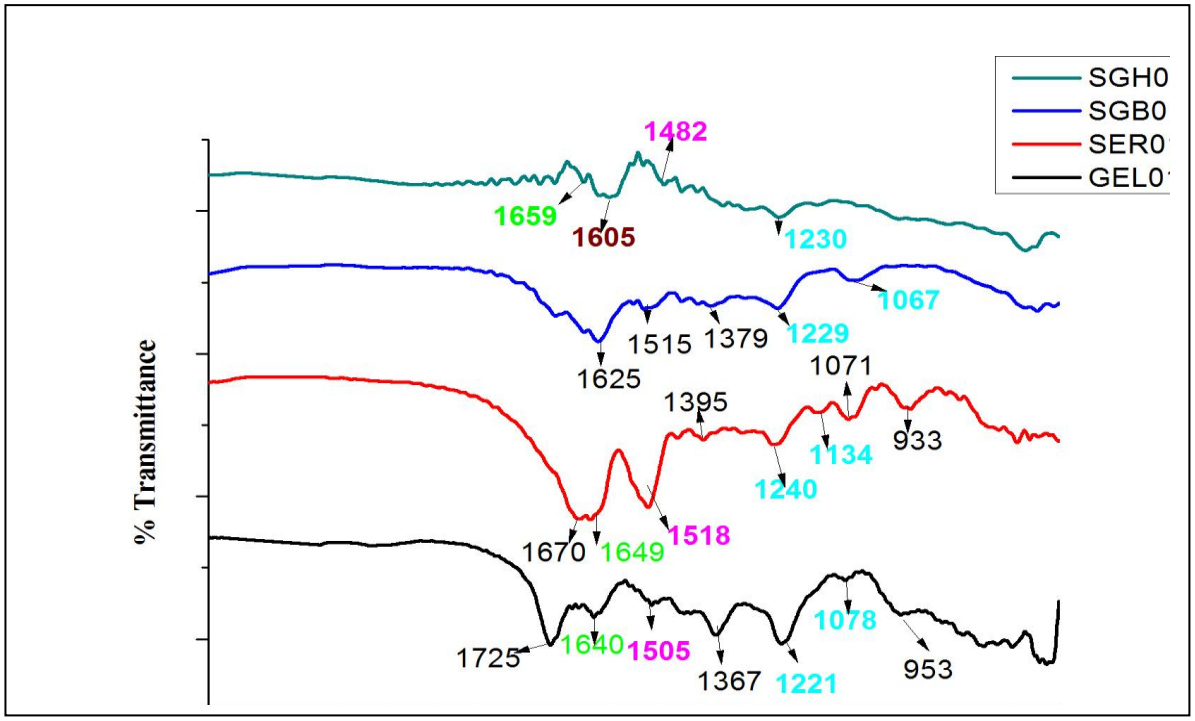


Figure 13 : FTIR spectrum of sericin, gelatin , sericin –gelatin blend (SGB) and hydrogel (SGH)

Table 4: Peak assignments of hydrogel along with the gelatine, sericin and their blend.

Sericin	Gelatin	Sericin-Gelatin blend	Sericin-Gelatin hydrogel	Peak assignment
	1736.58	1724.05	1730.8	C=O stretch of COOH
1670.05		1667.41		C=O Stretching of Amide I
1649.8	1641.13	1630.52	1659	N-H bend of primary amines
			1605	Amide I
1518.67	1505.17		1482.7	Amide II
1443.46	1432.85	1443.46	1447.31	C-H bend of Alkane
1393.32			1407.64	C-C stretch of aromatics
	1367.28	1378.85	1353.78	C-H rock of alkane
1232.29	1221.68	1229.4	1226.5	C-N and N-H Stretch Amide III
1138.76			1138.76	C-H wag of alkyl halides
1066.44	1080.91			C-N stretch of amines
1005.7				C-O stretch of acid
941.092	947.842			C-O stretch of acid
858.868				C-O stretch of acid
	847.842			C-O stretch of acid

3.3.2 X-Ray Diffraction

XRD patterns of sericin, gelatin and SGH are given Figure 14. Crystallinity of the hydrogel was analysed using X-ray diffraction. Diffraction peaks at 2θ = 21, 24 and 42 confirms the crystalline nature of high molecular weight sericin (Hajim, 2003). Gelatin exhibited characteristic peak at 2θ = 20 (Kunal, 2007). In the case of hydrogel, peak intensity was found to be reduced indicating the partial loss of crystallinity owing to the three dimensional network formation.

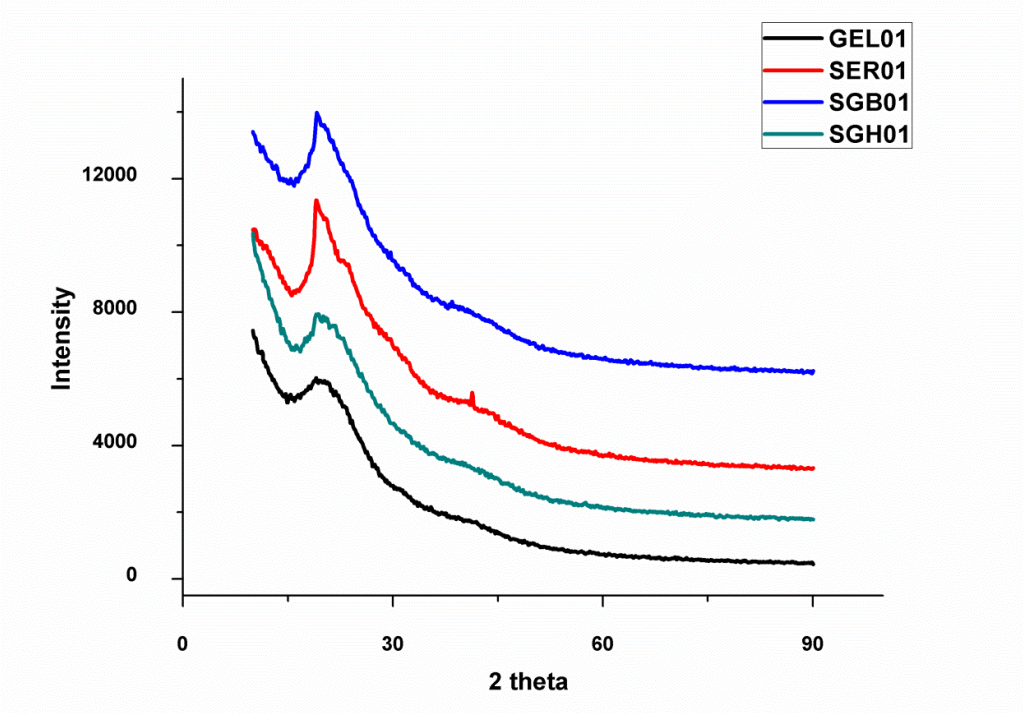


Figure 14 : XRD pattern of sericin, gelatin SGB and SGH

3.3.3 Thermal Analysis of Hydrogel.

Thermal analysis of hydrogel was studied using TGA and DSC. Fig 15 : shows the TGA curves of hydrogel

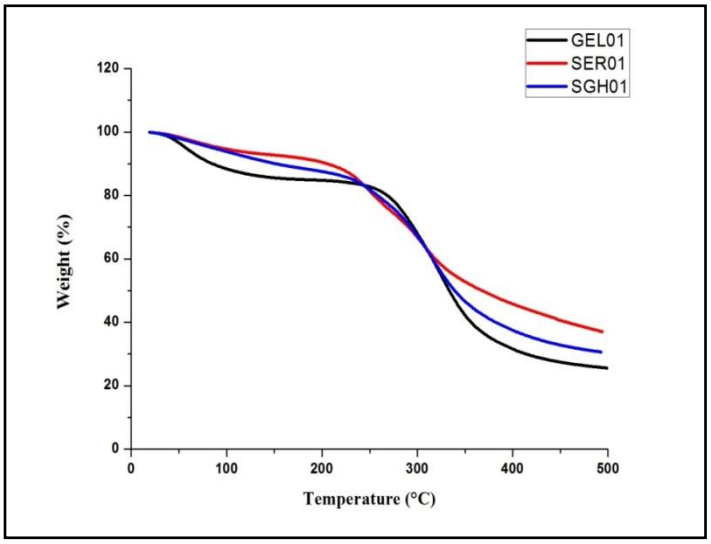


Figure 15: TGA curves of gelatin, hydrogel and sericin

The degradation of hydrogel is characterized by two stages of mass loss. The apparent mass loss began at 229^0. This indicates that the thermal stability of hydrogel found to be increased compared to that of sericin and gelatin individually. Both sericin and hydrogel showed 30% weight at 493^0C. The initial weight loss below 229^0C corresponds to dehydration from the sample. At 229 ^{0}C, weight becomes 85%. Further loss was observed from 85 to 50% in the first stage, ie at 339^0C. This may be attributed to the cleavage of weak or labile bond present in the hydrogel and loss of structural integrity. In the second stage, reason behind the weight loss can be suggested as decomposition of more thermally stable structures which were produced from cross linking reactions during heating. The acquired thermal stability of hydrogel relates to the significant covalent cross linking between the two polypeptides sericin and gelatin.

3.3.4 Dynamic Mechanical Analysis

DMA was carried out to find the thermo mechanical properties of hydrogel. The variation in the storage modulus (E') with temperature is given in Figure 22. It shows the variation of tan δ with increasing temperature. The narrow tan δ peak, that is, the glass transition temperature (Tg), appeared at two points 18 ^{0}C and 50 ^{0}C. Transition at 18^{0}C corresponds to the Tg of gelatin and that at 50^{0}C corresponds to that of sericin. It can be inferred from the data that the hydrogel material will soft and flexible to handle at room temperature. The appearance of two Tg may be due to the low crosslinking between functional groups. More experiments need to be done on the basis of crosslinking degree.

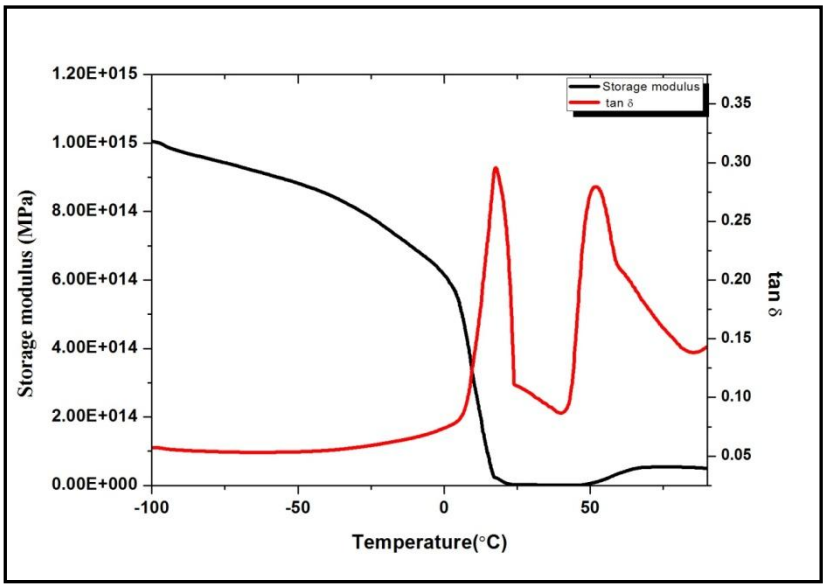


Figure 16 : Temperature dependence of E' and tan δ for SGH

3.3.5 Swelling studies

Swelling properties of a hydrogel indicate the ability to absorb water, which is crucial as it affects many aspects of a hydrogel, such as diffusion of encapsulated bioactive agents (Akturk,2011) (Smart,2005). To characterize the swelling

behavior of this sericin hydrogel, the swelling process of the dried hydrogel scaffolds in three conditions: acidic, neutral and alkaline were examined for 12 hrs. Hydrogel samples were immersed in media adjusted to different pH: PBS (7.4) ,MES(5.00) and distilled water (7.00) at 37 ^{0}C. Equilibrium swelling of hydrogel was shown in Figure 25.Hydrogel immersed in PBS reached equilibrium swelling fastly compared to others. In both MES and D.H2O, an inconsistent manner of swelling was observed upro 4 hrs. During this period, a decrease in swollen weight was also observed, which might be due to the release of unreacted components present in the hydrogel. In both the cases, swelling attained equilibrium from 5th hour onwards. In all the cases, Equilibrium water content (EWC) lies in the range of 70-74%.Swelling was found to be affected by the pH of medium. The swelling percentage was found to be highest in MES (295 %) (Figure 26). Swelling percentage was 271 and 241 in PBS and distilled water respectively.

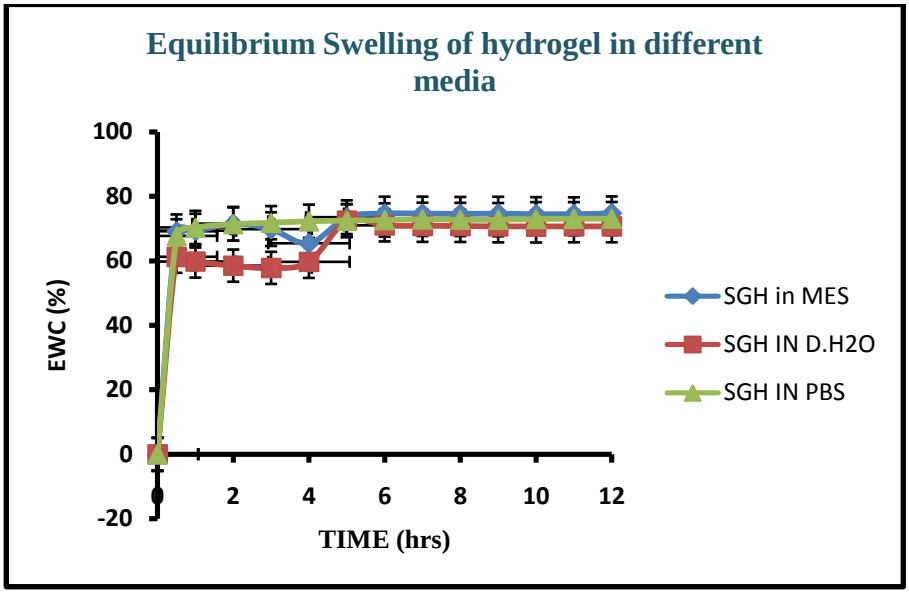


Figure 17 : Swelling behaviour of hydrogel in different media

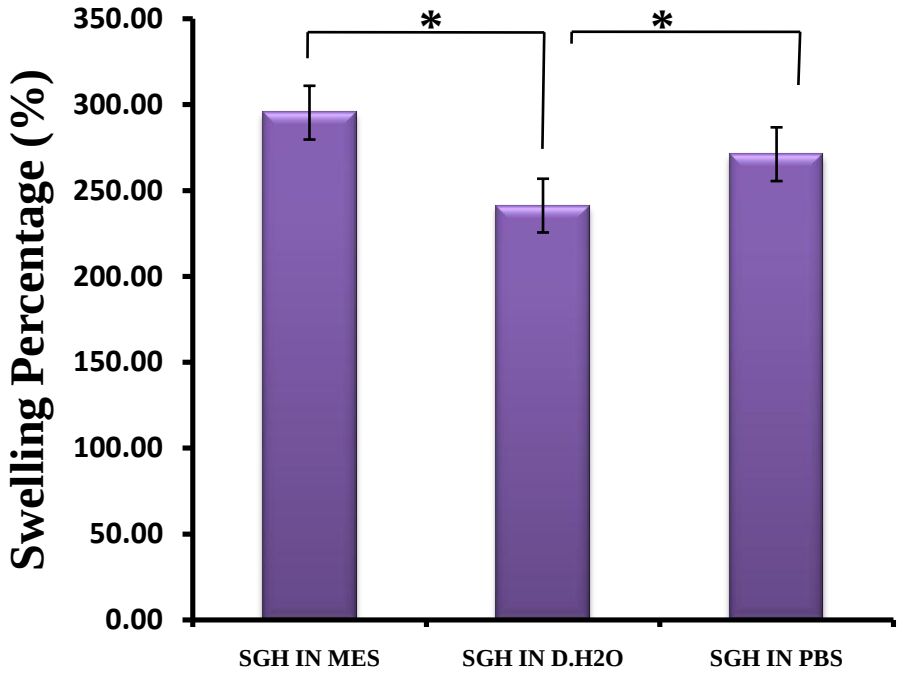
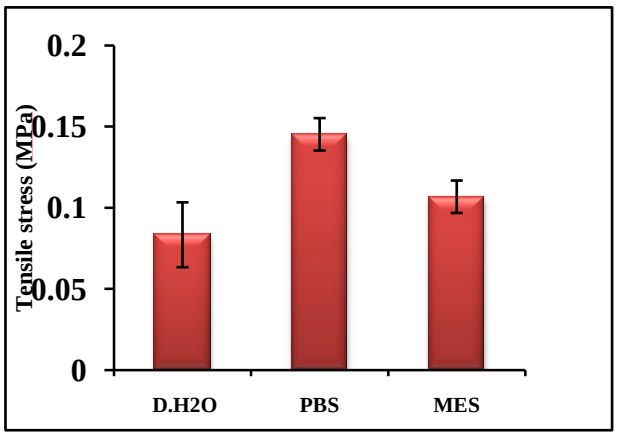


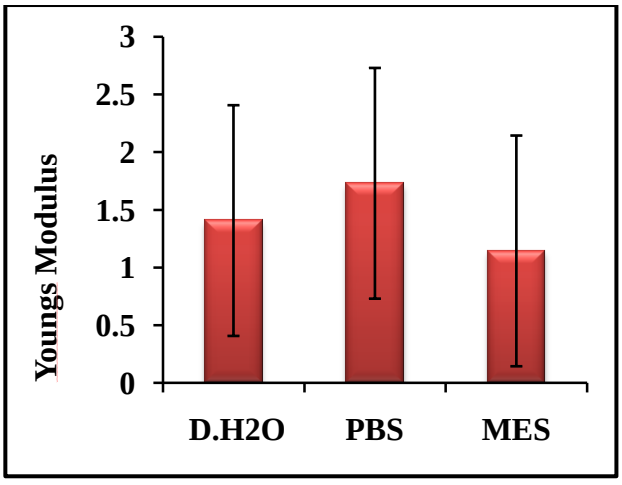
Figure 18: Effect of medium on percentage swelling

3.3.6 Mechanical Properties

Mechanical properties of a hydrogel are influenced by its porosity, which is an important feature of a 3D hydrogel (Annabi et al., 2010). Tensile properties of hydrogel in dry state were also evaluated. In its dry state, SGH possess tensile strength of 29.58 MPa, which is a sufficiently high for several applications. Table 5 shows the tensile properties of dried hydrogel sample (SGH). In the swollen state, hydrogels are mechanically weak (Rethikala, 2015). At the same time, for a material to be used for wound dressing purpose, it should possess mechanical strength comparable with that of normal skin. Tensile characteristics of the sericin-gelatin hydrogel are shown in (Figures 19-21) where hydrogel immersed in PBS showed highest tensile strength (145 ± 10 kPa), Young's modulus (1.73 ±

0.22 MPa) and Elongation at break % (35±13%). Fig 22: (a,b,c) represents sample stress strain curves of sericin-gelatin hydrogel in PBS, MES and distilled water. The result reveals that the hydrogel posses acceptable strength and elasticity in biological pH, rather than acidic and neutral conditions, which is required. Also, it has sufficient handling strength. Moreover, it is to be noted that the hydrogel is mechanically stable in an altered pH condition.





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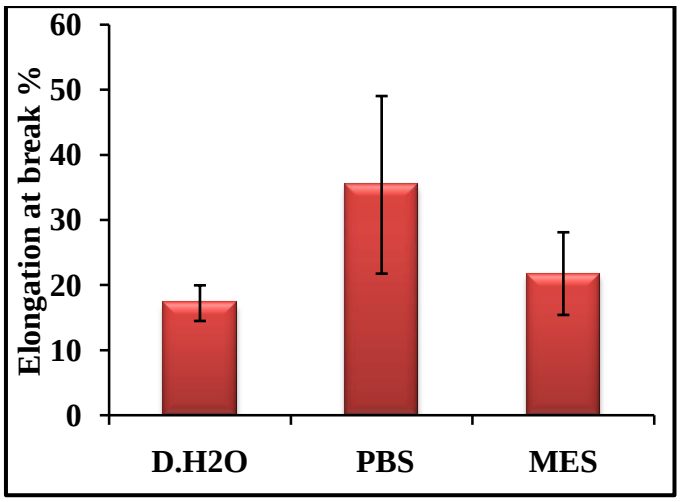


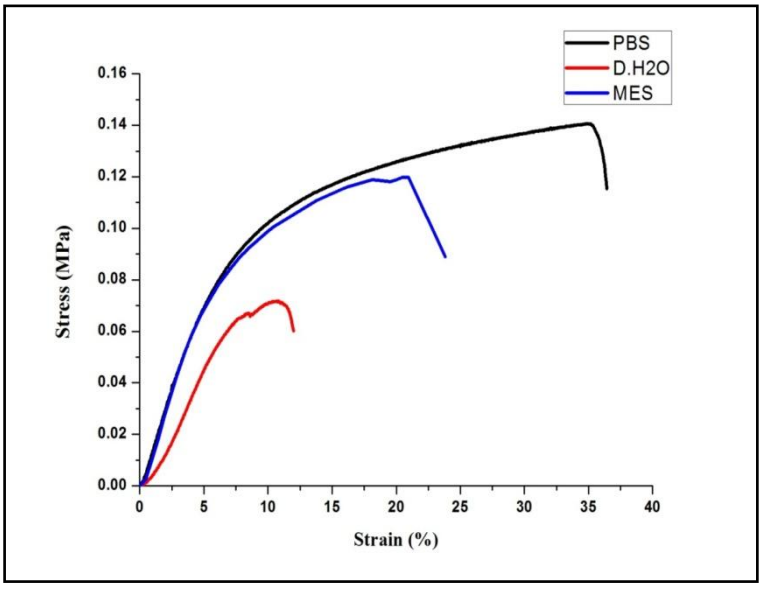


Figure 22: Stress- strain curves of hydrogels immersed in different media: (a) PBS (b) MES (c) Distilled water

Table 5 : Mechanical properties of dried SGH sample

Tensile strength (MPa)	Youngs Modulus (MPa)	Elongation at break (%)
29.58 ± 3.5	722.70 ± 0.25	7.0 ± 0.0

3.3.7 Surface Morphology

Porosity is an important characteristic of an ideal scaffold. Cell proliferation and survival is favoured by porous matrices. It also provides micro-environment for retention and release of bioactive molecules (Wang, 2009). Fig 16-A) and B) represents SEM analysis of the hydrogel surface in higher and lower magnification. Interconnected porous network was observed at the sample surface. The average pore diameter was found to be 29.4±8µm, which is comparable with that of skin (optimal pore size, 20~125 µm (Agrawal, 2001). Figure 17: shows the frequency distribution of pores present in the hydrogel. The interconnected porous matrix of the sample was attributed to the appropriate cross-linking between sericin and gelatine in forming a cross-linked 3D hydrogel network, which can promote cellular attachment in a well mannered way.

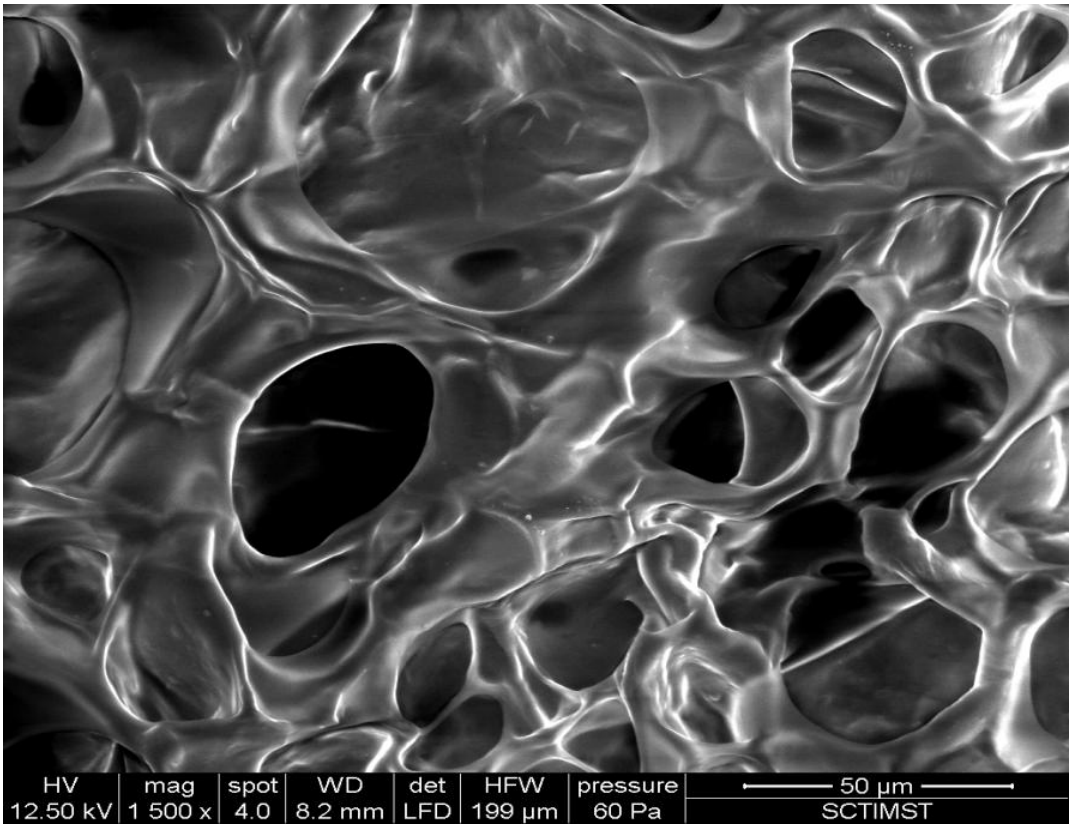


Figure 23: SEM images of lyophilised hydrogel

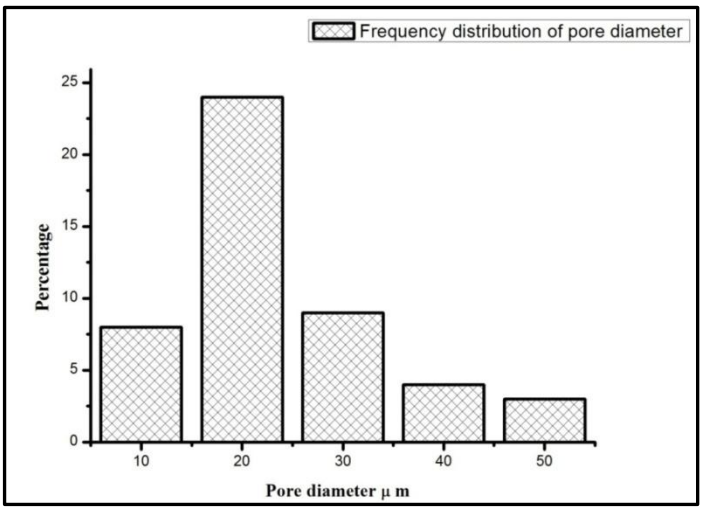


Figure 24: Frequency distribution of pore diameter

3.3.8 Micro Computed Tomography Analysis (µCT)

Porosity and 3D micro architecture of SGH lyophilised sample was evaluated using Micro Computed Tomography (µ- CT). 3D morphometric analysis was carried out for SGH sample washed in PBS and then lyophilised. Sample thickness distribution and porosity distribution (Fig 23) were determined. The average pore size was found to be 66µm. The pore size distribution curve is shown in Fig 24: The pore size 84µm showed maximum contribution of 28.20 % porosity. The porosity examination of sample showed 76.53 %. Table 6 shows porosity volume percentage. Higher porosity was observed for the hydrogel sample. These results suggest that the hydrogel possess highly porous network with abundant pores of size 84 µm.

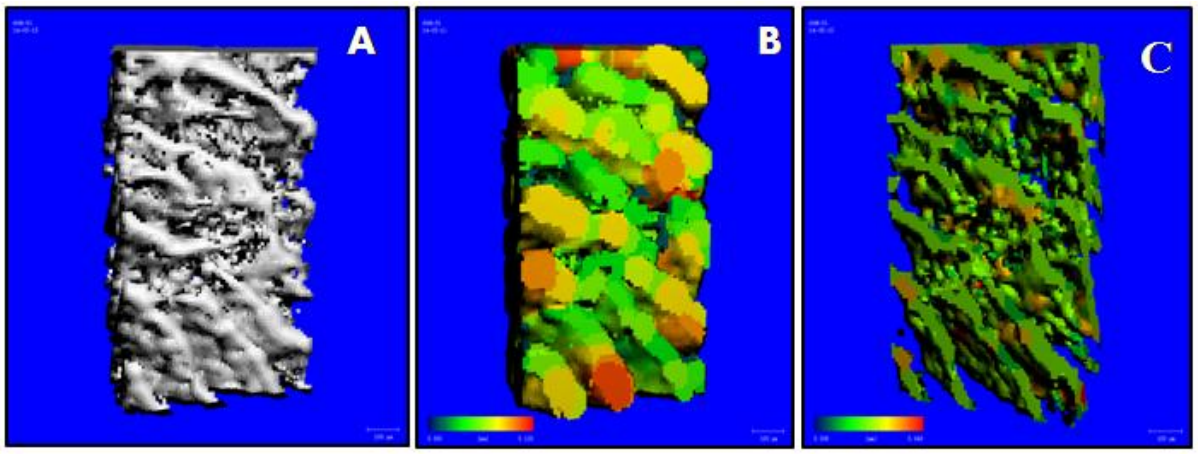


Figure 25 : A) 3D morphology image B) Pore size distribution image, C) Thickness distribution image

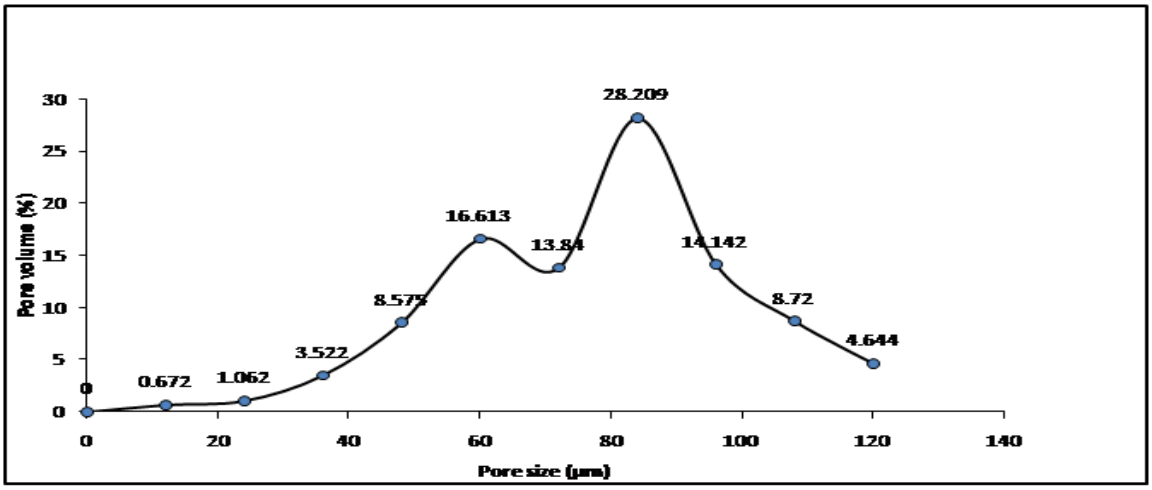


Figure 26 : Pore size distribution of SGH washed in PBS

Table 6: Porosity volume percentage of SGH sample

Micro CT parameter	SGH sample
Total volume (mm^3)	0.2598
Scaffold volume (mm^3)	0.0610
Scaffold volume/Total volume (mm^3)	0.2347
Porosity volume (%)	76.53

3.3.9 Degradation studies

The degradation of SGH in PBS, MES and D.H2O along with 0.1% sodium azide solution at 37^0C was investigated and reported as %mass remaining after 21 days [Fig.:28]. In PBS, 50 % weight loss was observed after 7 days. The degradation was rapid in PBS, compared to MES nad D.H2O. But later on, degradation rate in PBS was found to be lower. After 21 days, 60% weight loss was observed for PBS and 43 % and 83 % for MES and D.H2O respectively. This confirms that the pH of the medium do affect degradation rate.

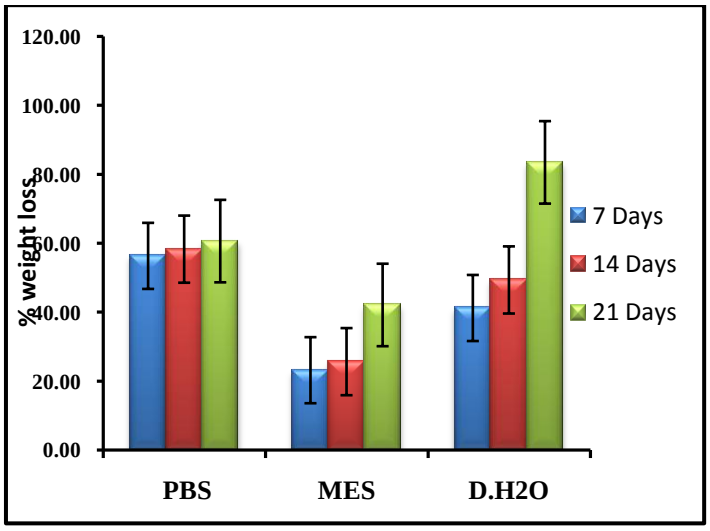


Figure 27 : Weight loss (%) pattern of hydrogel in PBS, MES and D.H2O due to degradation over a period of 3 weeks

3.3.10 Hemolysis

Hemolysis estimations have been used as a simple and reliable technique for estimating blood compatibility of materials (Lee, 2004). The percentage hemolysis of the samples was evaluated to determine the hemolytic property of the material. As per ISO 10993- 4:2002 (E), for material to be non-hemolytic, the percentage

hemolysis should be less than 0.1%. In this study, empty polystyrene dishes were used as a reference generating negligible hemolysis <0.1%. The hydrogel sample tested displayed less than 0.1% hemolysis (Figure 28

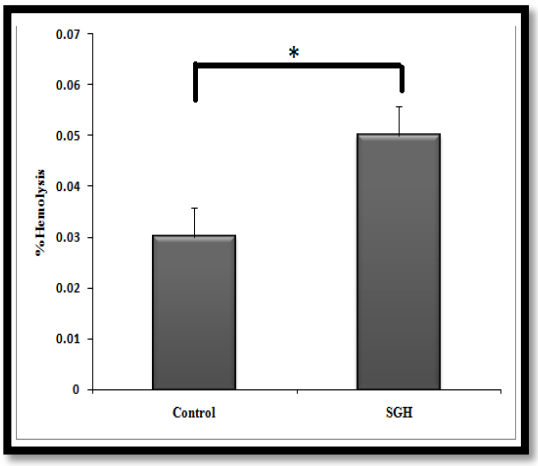


Figure 28: Percentage hemolysis of SGH with control

3.4 Biological Evaluation

Cytocompatibilty of the hydrogel sample was tested using human dermal fibroblast Cells received from Thrombosis Research Unit, SCTIMST. The cells were maintained in DMEM F12 medium containing Foetal Bovine Serum (FBS, 5%) and antibiotic antimycotic solution (1 %). Discs ofhydrogel samples with 8mm diameter and 2mm thickness were used for Cytocompatability evaluation.

3.4.1 Cytocompatibility Evaluation

Cytocompatability to human skin fibroblast was studied by direct contact method. The lyophilised hydrogel discs were used after sterilisation with 70% ethanol with final wash for three hour in PBS. These sterilized samples were placed on monolayer culture and kept in the incubator for two time periods: 3 days and 6 days.. The cells were then observed under phase contrast microscope showing

excellent proliferation. As the sample was transparent, outgrowth of cells from the surface of sample was apparent. No dead cells were observed, indicating the cytocompatibility of the sample. Figure 29 shows the phase contrast image of the cell morphology.

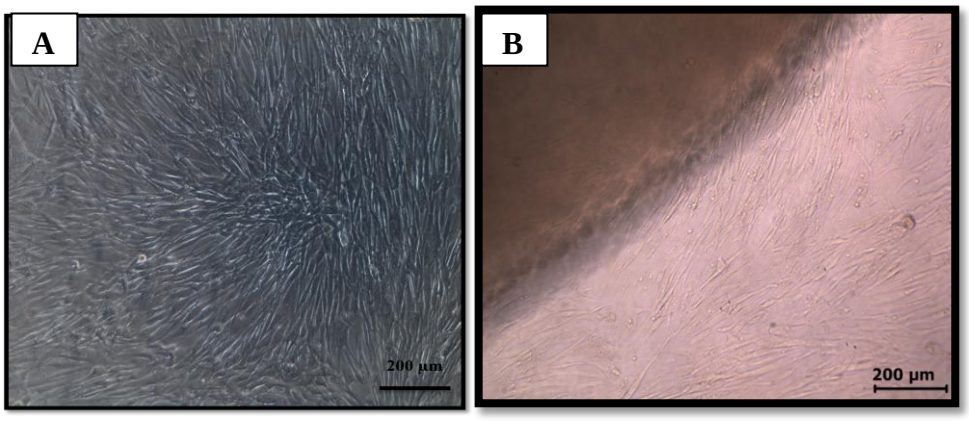


Figure 29 : Phase contrast microscopy images showing cytocompatibility of hydrogel **`A) Control fibroblast cells B) Sericin –Gelatin Hydrogel**

3.4.2 Cell viability Assay

Percentage viability of cells grown on the scaffold was determined by preliminary viability assay trypan blue dye exclusion method by counting cells in Neubauer counting chamber within 5 min of treating the harvested cells with Trypan Blue. Figure 30 shows the % viability of cells (Mean±SD) in control and test. It was found that the number of dead cells and live cells in both control (cells seeded in well plate) and test (cells seeded on material), indicating cyto compatibility of the hydrogel.

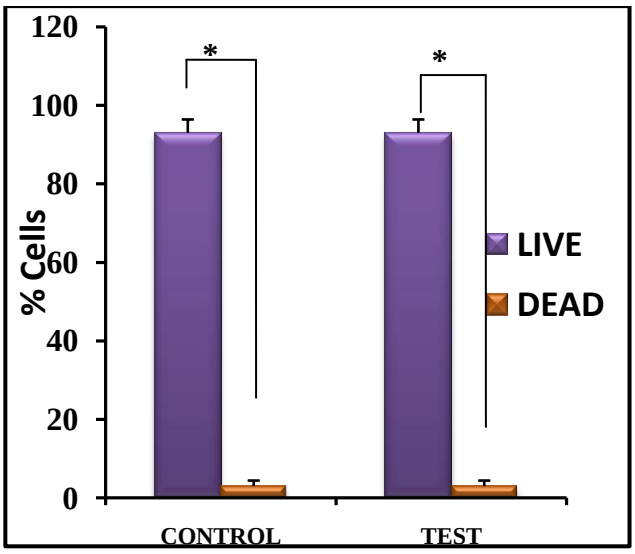


Figure 30 : Graphical representation of trypan blue exclusion assay

3.4.3 Biochemical evaluation for Cell Proliferation- Pico green

The assay was done for 3 days & 6 days. It was found that cell number increased tremendously after 6 dyas, compared to that of 3 days (Figure 31). This indicates that the cell can proliferate in an excellent manner in vicinity of the material, owing to the cytocompatibility of the material.

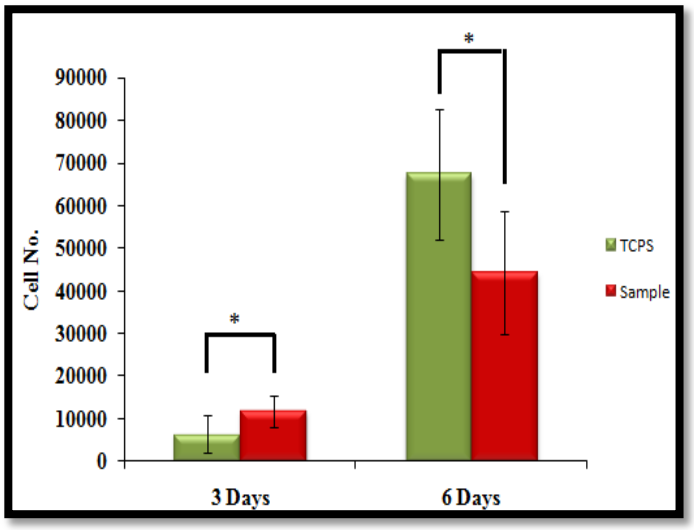


Figure 31 : Quantitative data on cell proliferation after 3 days & 6 days

3.4.4 Two dimensional Cell Attachment

Scaffolds were statistically seeded with a cell density of 5000 cells/cm^2 and observed under ESEM after 3 days of incubation to analyse the attachment ability of the scaffold. From the Figure 32 showing ESEM images of scaffold, it can be said that it possess poor cell adherence ability. Scaffold without cell served as the control.

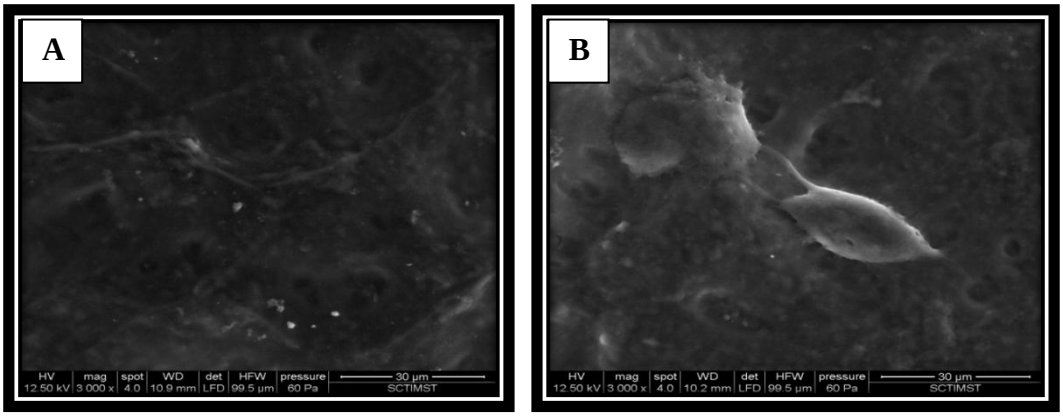
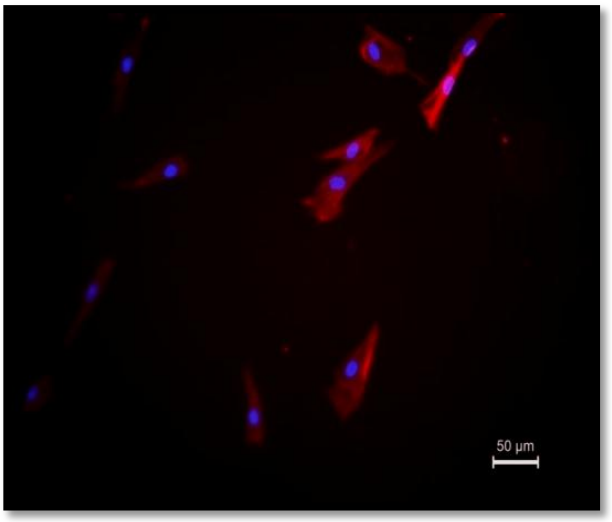


Figure 32 : ESEM images of cell seeded scaffold

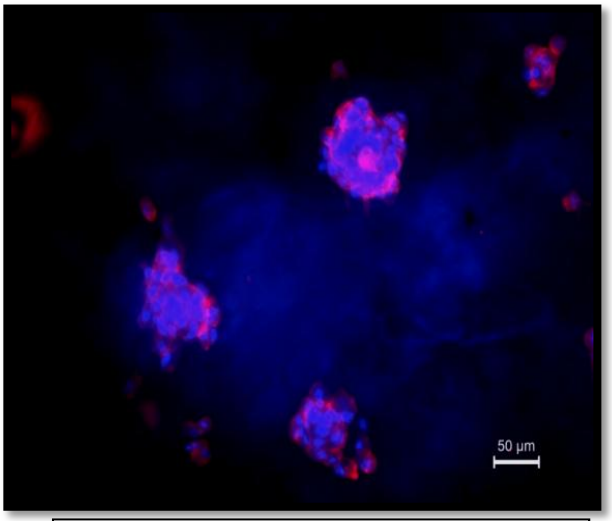
A : Control (Material alone) **B) Cell on hydrogel**

3.4.5 Cell attachment assay

Scaffolds were statically seeded with a cell density of 5000 cells /cm2 and allowed to attach for 48 hours. The scaffolds were then fixed with 3. 7% formaldehyde for 20 minutes and then permeabilised with 0.2% triton X 100. Texas red conjugated antibodies against actin cytoskeleton were added and kept at 4 0 C for overnight. Scaffolds were counter stained with Hoechst. Washed thoroughly and observed through an inverted fluorescent microscopy



A) Control fibroblast



B) Fibroblast on the hydrogel

Figure 33 : Rhodamine phalloidin (F-Actin) & Hoechst (Nucleus) staining on control fibroblast cells and hydrogel

In the control fibroblast cells, they maintained the spindle morphology. In the case of cells seeded on the hydrogel sample, the cells were found to form spheroids, indicating poor attachment on the material. When the material is not suitable for adhesion, cells will show tendency to adhere themselves. This confirms the poor adhesion on the material.

CHAPTER 4

SUMMARY AND CONCLUSION

Biopolymers are a versatile class of materials with widespread applications in the area of tissue engineering and regenerative medicine. Members under this class were found to have thermo-responsive solubility behaviour. This opens perspectives in developing gels with water retaining capacity, defined as hydrogel. Hydrogels serve as an important class of biomaterials. Silk protein sericin is an emerging candidate in the biomedical field. It has a long history of being discarded as a waste material. Natural polymers are always given a prominence for use as a biomaterial, owing to its biocompatibility. Gelatin, a product derived from collagen is being explored widely in this area.

In the current study, an effort was put forth to develop a hydrogel from silk protein sericin and gelatin. The sericin-gelatin hydrogel in the present investigation uses a simple and cost effective fabrication technique without any chemical cross-linkers. The fabricated hydrogel was characterized chemically, mechanically and biologically. The raw materials were characterized by spectroscopic, thermal and mechanical methods.

The parameters for hydrogel formation were optimized. The fabricated hydrogel was also characterized spectroscopically and compared with that of raw materials. Thermal stability of the hydrogel was also done, which was found to be increased

when compared to raw materials. Swelling, degradation and mechanical studies were done in hydrogel immersed in different media PBS (slightly alkaline), MES (acidic) and distilled water (neutral). Swelling and degradation was found to be affected by the pH of the medium. Tensile properties of hydrogel were the highest when it was swelled in PBS. Dynamic mechanical analysis showed two glass transitions temperatures corresponding to sericin and gelatin.

Two dimensional surface morphology of the hydrogel was revealed by Scanning Electron microscope, with a porous network with an average pore diameter comparable with that of human skin. Poor adhesion was confirmed by Rhodamine phalloiidn & Hoechst staining. Further, three dimensional micro architecture was done by Micro CT, showing a continuous and uniform pore size distribution with a porosity volume of 76.53.

Hemocompatibility of the hydrogel (0.05%) was revealed by percent hemolysis method. Biological evaluation was carried out by cytocompatibility studies using human dermal fibroblasts. Phase contrast images of the scaffold seeded with cells revealed no cytotoxicity and excellent proliferation. ESEM images of scaffold with cells showed poor cell adhesion. Preliminary evaluation of cytotoxicity was quantified by trypan blue exclusion method, which revealed 90 % viability of cells seeded on scaffold.

From the results obtained, it can be concluded that the sericin –gelatin hydrogel possess good swelling, mechanical and thermal stability, adequate porosity and cytocompatibility with poor cell adhesion, to use as a wound dressing material.

The present study can be considered as a pioneer work, which has to be explored further in terms of biological evaluation for use as a potential wound dressing material. This study involves a simple and novel method of developing hydrogel from two bio polymers without any chemical cross-linkers. Incorporation of growth factors and /or drugs and the release studies could be done in the future. As literature supports the fact that sericin possess anti microbial activity, studies could be extended in terms of sericin release from hydrogel and subsequent anti microbial activity. Moreover, the present study is an attempt to explore the utility and value of Sericin, a waste material as green material.

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APPENDIX

REAGENTS USED:

1. Dulbecco's Phosphate buffered saline (DPBS):

NaCl- 8g

KC1-0.2g

KH2P04- 0.2g

Na2HP04- 1.7g

DI Water-1000 mL

pH -7.4

2. Fibroblast culture medium (50mL):

DMEM-F12- 45mL

Foetal Bovine Calf Serum (FBS)- 5mL

Antibiotic-antimycotic solution - 500µL

3. 3.7% Formaldehyde (l0 mL):

Formaldehyde (37%)- lmL

DPBS-9mL