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Research Article

Formulation and Evaluation of Nanosilver Loaded Silk Fibroin Topical Gel

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ABSTRACT

- The skin is providing barrier to the outside protecting the body from infection, radiation, and extremes of temperature.
- There are various types of wounds which can damage the skin including abrasions, lacerations, rupture injuries, punctures, and penetrating wounds.
- Many of the wounds are superficial requiring local first aid including cleansing and dressing.
- But some wounds are deeper and need medical attention to prevent infection and loss of function, due to damage to underlying structures like bone, muscle, tendon, arteries and nerves.
- The purpose of medical care for wounds is to prevent complications and function. While important, cosmetic results are not the primary consideration for wound repair.
- Wound healing, a common clinical entity contemporary to human beings, most often susceptible to microbial infection. The ultimate goal for wound healing is a speedy recovery with minimal scarring and maximal function.

Different approaches such as use of antimicrobials and cell growth promoting agents have been used for achieving the set goal. Silk fibroin, a cell growth promoter has been used as wound healing agent.

Out of the present antimicrobial agents silver nanoparticles exhibit broad spectrum of anti-microbial activity. The mono dispersed silver nanoparticles with narrow particle size distribution are more effective anti bacterial agents, because of high surface/volume fraction, so that large populations of silver atoms are in direct contact with the environment. Silver Nanoparticles are non toxic to human cell to the tune of 350µg/day.

INTRODUCTION

1.1 Wound

In medicine, a wound is a type of injury in which skin is torn, cut or punctured (an open wound), or in which blunt force trauma causes a contusion (a closed wound). In pathology, it refers to a sharp injury which damages the dermis of the skin[1] .

1.1.1 Classification

The wounds can be classified into two types as open wounds and closed wounds.

a. Open Wounds

An open wound is a type of injury in which skin is torn, cut or punctured and surrounding tissues are exposed. Most commonly open wounds are susceptible to infection from pathogens all around and should be cared for well. Open wounds can be classified according to the object that caused the wound. The most common causative organisms associated with open wound infections include *Streptococcus pyogenes*, *Staphylococcus aureus*/MRSA, Enterococci and *Pseudomonas aeruginosa* .

b. Closed Wounds

Closed wounds range from bruises or hematomas to crushing injuries. Different types of closed wounds have one thing in common: the skin remains intact and is not broken. If the skin is not broken, the risk of infection is lower than it is for an open wound. Closed wounds typically are caused by a bluned impact, which ruptures a blood vessel or capillary under the skin. The blood oozes out, but typically stop bleeding within about 30 seconds after the injury occurs. As body reabsorbs the blood, the skin shall discolor[1,2].

1.1.2. Risk factors

The risk factors involved in the wounds can be summarized as follows:-

a. Cellulittis:

Cellulitis is a diffuse inflammation of connective tissue with severe inflammation of dermal and subcutaneous layers of skin. Cellulitis is caused by a type of bacteria entering the skin, usually by the way of a cut, abrasion or break in the skin. This break does not need to be visible. Group A Staphylococcus and *Streptococcus* are the most common of these bacteria, and which are part of the normal flora of the skin but cause no actual infection while on the skin's outer surface. The typical symptom of

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cellulitis is an area which is red, hot, and tender[3].

b. Necrotizing subcutaneous Infection:

Necrotizing soft tissue infection is a rare but very severe type of bacterial infection that can destroy the skin, muscles, and underlying tissue. Necrotizing refers to something that causes tissue death. A very severe and usually deadly form of necrotizing soft tissue infection is due to *Streptococcus pyogenes*, which is sometimes called "flesh- eating bacteria."

Necrotizing soft tissue infection develops when the bacteria enters the body, usually through a minor cut. The bacteria begins to grown and releases harmful substances (toxins) that break down materials in the tissue, that rapidly spreads the bacteria, leads to widespread effects such as shock.

The first sign of infection may be a small, reddish, painful spot or inflammation on the skin. This quickly changes to a very painful bronze- or purple-colored patch that grows rapidly. The center become black and dies off. The skin may break open and fluid oozes out. The wound may quickly grow in less than an hour. Symptoms may include general ill feeling, sweating, nausea, dizziness, fever and finally shock. Without treatment, death may occur rapidly3,4.

c.Gas Gangrene:

Gas gangrene (also known as "Clostridial myonecrosis") is a bacterial infection that produces gas within tissues in gangrene. It is a lethal form of gangrene usually caused by *Clostridium perfringens* bacteria. It is a medical emergency. Gas gangrene is caused by exotoxin-producing Clostridial species (most often *Clostridium perfringens*, and *C novyi* but less commonly *C. septicum* or *C. ramnosum*), which are mostly found in soil but also found as normal gut flora, and other anaerobes (e.g. *Bacteroides* and anaerobic streptococci)[4].

Symptoms include:

- Air under the skin (subcutaneous emphysema)
- Blisters filled with brown-red fluid
- Drainage from the tissues, foul-smelling brown-red or bloody fluid (serosanguineous discharge)
- Increased heart rate (tachycardia)
- Moderate to high fever
- Ordinary to severe pain around a skin injury
- Pale skin color, later becoming dusky and changing to dark red or purple
- Swelling around a skin injury
- Sweating
- Vesicle formation, combining into large blisters
- Yellow color to the skin (jaundice)

1.1.3. Wound Healing

Wound healing, or wound repair, is the body's natural process of regenerating dermal and epidermal tissue. When an individual is wounded by a set of events takes place in a predictable fashion to repairs the damage. These events overlap in time and must be artificially categorized into separate steps: the inflammatory, proliferative, and remodeling phases (Some authors consider healing to take place in four stages, by splitting of different parts inflammation or proliferation into separate steps)[5].

In the inflammatory phase, bacteria and debris are phagocytized and removed. The factors are released that cause the migration and division of cells involved in the proliferative phase[5].

The proliferative phase is characterized by granulation tissue formation, collagen deposition, angiogenesis, epithelization, and wound contractions. In angiogenesis, new blood vessels grow from endothelial cells. In granulation tissue formation, fibroplasia, fibroblasts grow and forms a new, provisional extracellular matrix (ECM) by excreting collagen and fibronectin[6].

In epithelization epithelial cells crawl across the wound bed to cover it. In contraction, the wound has been made smaller by the action of myofibroblasts, which establishes a grip on the wound edges and contract themselves using a mechanism similar to that in smooth muscle cells. When these cells roles are close to complete, unnecessary cells undergo apoptosis. In the remodelling phase and maturation, collagen has remodelled and realigned along tension lines and cells which are no longer needed are removed by apoptosis[7,8].

In the remodelling phase and maturation, collagen is remodelled and realigned along tension lines and cells that are no longer needed are removed by apoptosis[9].

1.1.4. Approaches used in Wound Treatment

There are mainly two approaches used in wound healing. One is the use of anti-microbial agents and antiseptics and the other is the use of tissue or cell growth factors. Out of these two approaches cell growth factors would be a better approach as it accelerates the natural wound healing process, while the antiseptics and antimicrobial only kills the microbes affecting the wounds[9].

The advent and development of tissue engineering provides a new means of restoring tissues and organs. One of the challenges of tissue engineering is to establish a three-dimensional complex comprising cells and biomaterials. The three dimensional structure in which the cells acquire nutrition, exchange gas, and excrete waste is the physical foundation for the formation of new tissue and organs with shape and function. Such biomaterials often must be biodegradable. Therefore one of the prerequisite of practical tissue- engineering technology is to fabricate biodegradable material with superb biocompatibility. Silk fibroin is the material which is nontoxic, nonirritating, with good applicability. Moreover, it adheres well to fibroblast and epidermal cells. Tsubouchi et al., has reported the wound healing activity of silk fibroin Therefore, regenerated silk fibroin material is attractive for use as a biomaterial for wound healing. The silk fibres often have sericin

present on them. Removal of sericin is an important step in isolation of silk fibroin because sericin have problem with biocompatibility and hypersensitivity to skin [9,10] .

1.2 Silk

Silks are generally defined as protein polymers that are spun into fibers by some Lepidoptera larvae such as silkworms, spiders, scorpions and flies. Silk proteins have been produced within specialized glands after biosynthesis in epithelial cells, followed by secretion into lumen of these glands where the proteins are stored prior to spinning into fibers. Silks differ widely in the composition, structures and properties depending on its specific source. The most extensively characterized silks are from the domesticated silkworm, Bombyx mori, and from spiders (Nephila clavipes and Araneus diadematus). Many of the more evolutionarily advanced spiders synthesize different types of silks. Each of these different silks has a different amino acid composition and exhibits mechanical properties adapted their specific functions; reproduction as cocoon capsular structures, lining for prey capture, lifeline support (dragline), web construction and adhesion. Fibrous proteins present in silk render mechanical strength to the silk. Because of these impressive mechanical properties, this family of proteins provides an important set of material options in the fields of controlled release, biomaterials and scaffolds for tissue engineering. The relative environmental stability of these families of proteins, in comparison to globular proteins, in combination with their biocompatibility, unique mechanical properties, and options for genetic control to tailor sequence provides an important basis to exploit these natural proteins for biomedical applications[10].

Waste silk is a raw material of the most important branch of silk industry. The raw materials of this industry consist of production of continuous filament thread and waste collected during the reeling of raw material and subsequent stages of its processing[10]. Waste silk can classified as

a) Waste from the cocoon
b) Reeling waste
c) Thread waste

Silk wastes, which are produced during the silk processing, can be reutilized. It can be used as a coarse yarn and as a spun silk, which can be incorporated in natural rubber to achieve physicochemical properties. Silk being a protein in nature, the researchers find its relevance in pharmaceutical applications. To explore this, one needs to study the structural and chemical properties of silk.

1.2.1 Structure and Properties of Silk

Silk fibers are composed of two protein microfilaments (called as brins) embedded in a glue-like glycoprotein coating (Figure. 1.1A)[10]. The brins are fibroin filaments composed of bundles (of ca. 100nm in diameter) of nanofibrils (individually of ca. 5nm in diameter) which are preferentially aligned with the long axis of the fiber. The silk fibroin nanofibrils are composed of a complex of three proteinaceous components: a large protein, known as heavy chain (H-chain) fibroin (of ca.350k Da) that is linked to a second small protein, known as light chain (L-chain) fibroin (of ca.25 k Da) via disulfide bonds; and a third small glycoprotein, known as the P25 protein (of ca.30k Da) is associated via non-covalent hydrophobic interactions. The molar ratios of L-chain:H-chain:P25 are 6:6:1; the H-chain is hydrophobic and contains blocks of (Gly-Ala-Gly-Ala- Gly-Ser)n that are known to form anisotropic β- sheet-rich nanocrystals, whereas the L-chain is very hydrophilic and relatively elastic in nature, and the P25 protein is believed to play a role in maintaining the integrity of the complex [10].

Silkworm fibroin protein differs in composition depending upon source, extraction method and analysis. In general silks are insoluble in dilute acids and dilute alkalies, resistance to most proteolytic enzymes. It is hydrolyzed by conc. Sulfuric acid. Lithium bromide (LiBr) (9.3 M) solubilizes silk fiber, while specific mixture of acid solubilizes silk without decreasing molecular mass of the polymer. The mulberry silk, which is the domesticated Silk from B. mori. Contain 72-81% fibroin, 19-28% sericin and traces of lipids, pigment and carbohydrates. The trace components of Mulberry B.mori. Cocoon silk comprises between 2.5-3.7 % of total material[10,11].

Silk fibroin(SF) is composed of 18 kinds of amino acids, among them, the glyceine (Gly, 44.60 %(mol percent), the alanine (Ala,29.40%) and the serine (Ser, 12.10%) with simple side groups account for about 85%, that is about 4:3:1 in term of their molar ratios. They arranged in their relatively ordered chain segment according to certain sequences, most of these chains locate in the crystal region of SF. A hexapeptide repeat Ser-Gly-Ala-Gly-Ala-Gly (SGAGAG) was first reported as structural element. On the other hand, the tyrosine (Tyr, 5.17 %(mol percent), the phenylalanine (Phe, 0.63%) and the tryptophan (Try, 0.11%) with relatively large side-groups mostly locate in non-crystal region[11,12]

1.2.2 Therapeutic Applications of Silk Fibroin

The therapeutic applications of silk fibroin includes:-

a. Hypolipidemic Agent:

The amino acid composition of silk fibroin is comparatively simple; it is rich in Glycine, Serine, Alanine and Tyrosine. Considering the physiological functions of these amino acids and recent research focused on cholesterol lowering ability of Glycine and Serine has and thus it was proved that silk fibroin has hypolipidemic activity[12].

b. Antidote in Alcohol Toxicity:

Alanine promotes the metabolism of alcohol by liver thus useful in alcohol toxicity and fibroin is rich source of it [13].

c. Dementia:

Tyrosine is effective in preventing dementia and fibroin is rich source of its content [14].

d. Anticoagulant / Antithrombogenic:

The films of silk fibroin treated with 50% methanol for 15 min. and graft polymerized with ethyl methacylate and styrene by methane plasma initiation. Their antithrombogenicities were investigated by blood clotting method and platelet adhesion tests. Activity was improved by the surface grafting and varied with the fraction of polar component of surface tension to the total surface tension of film [14,15].

e. Wound Healing

Bioactive peptides with mol. wt. 300-5000 are obtained from silk fibroin hydrolysis. The peptides are said to have immune stimulant activity, antioxidant and anticancer promoting effects[15].

1.3. Role of Silk- Fibroin in Wound healing

Amino acids in silk fibroin play an important role in wound healing. Amino acid composition of different varieties of silk fibroin has been studied by Lucas et al. the main four varieties of Indian silks viz., Mulberry, Tusar, Eri, and Muga, have been investigated by Dhavalikar with reference to amino acid composition. In the case of Mulberry the composition due to glycine, alanine, and serine was found to be about 80%. The Amino acid composition in the case of Tasar, Eri, Muga revealed alanine as the major constituent with small quantities of glycine and serine. A portion of the amino acid sequence of silk fibroin produced by *Bombyx mori* has been identified[16]. The crystalline region of silk fibroin is composed mainly of the repetitive element Gly-Ala-Gly-Ala-Gly-Ser, and in amorphous domain of silk fibroin is found the amino-acid sequence Thr-Gly-Ser-Ser-Gly-Phe-Gly-Pro-Tyr-Val-Ala-Asp-Gly-Gly-Tyr-Ser-Arg-Arg-Glu-Gly-Tyr-Glu-Tyr-Ala-Trp-Ser-Ser-Lys-Ser-Asp-Phe-Glu- Thr. Alanine, Glycine, Serine are important amino acids for activating epidermal cell multiplication[17,18]. The silk fibroin acts as a natural wound healing agent at inflammatory phase, fibroplasia, and epithelization[19] as shown in the Figure1.2.

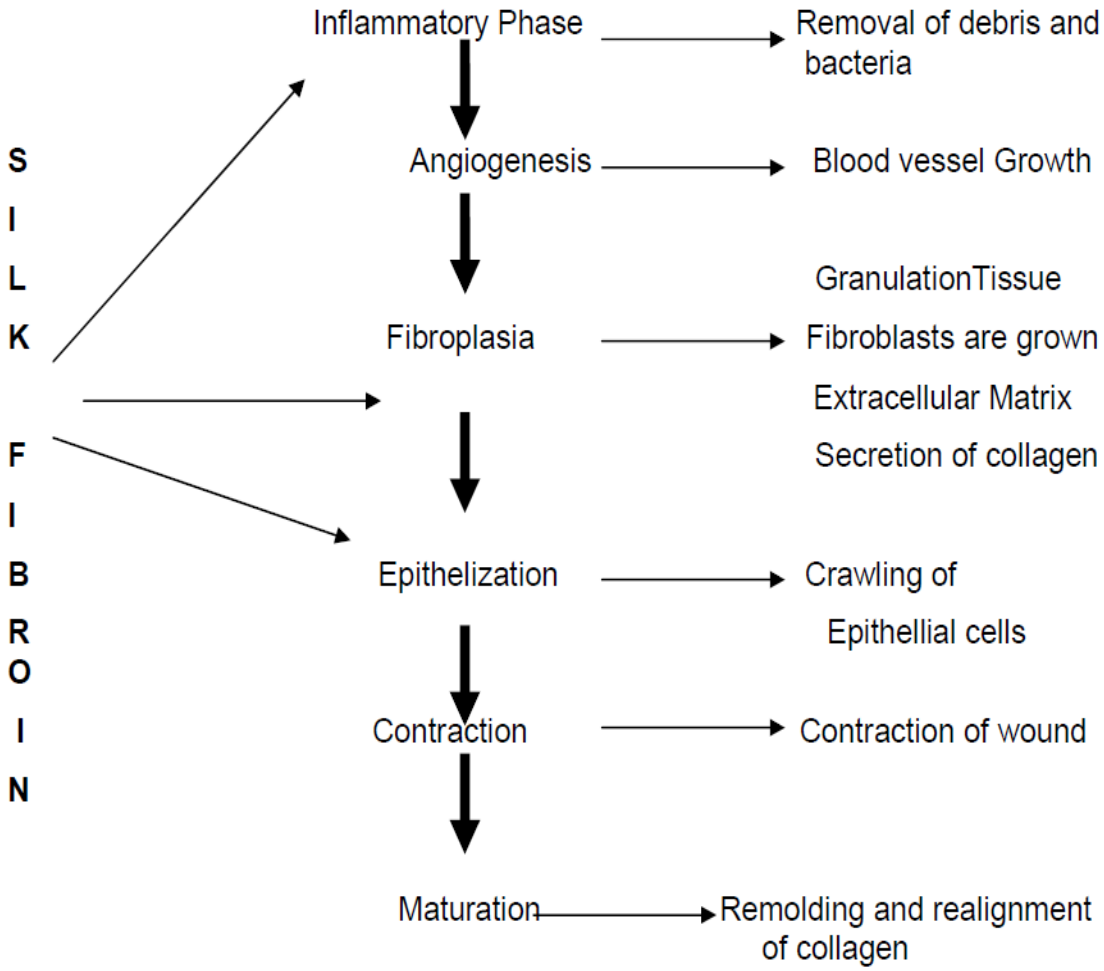


Figure 1.2: Role of Silk Fibroin in natural wound healing process

1.3.1 Advantages of Silk- Fibroin in Wound healing

Silk fibroin as a wound healing material has several advantages over other synthetic materials such as

a. Interact well with human cells in vitro are very well tolerated after implantation into animals in vivo.

b. Do not evoke any degree of foreign body response and do not induce the local production of pro-inflammatory the implants.

d. Favour the neo-angiogenic processes in a way cytokines.

c. Do not cause fibrous scarring around or inside that the regenerated tissue is also well vascularised and thereby better able to survive and properly function.

e. It is biocompatible, biodegradable, adherence to epidermal cells.

f. It is attractive material for biomedical applications because it is a fibrous protein that has high permeability to oxygen and water, relatively low thrombogenicity.

Therefore silk has been increasingly implemented in medical treatment and is preferred over other material for wound healing treatment[20].

1.3.2 Limitations of Silk- Fibroin in Wound healing

Silk fibroin is a cell growth promoting agent and itself does not possess any antimicrobial action. Open wounds are the most subject to infection from pathogens all around us and should be cared for well. These pathogens may impair with the wound healing process induced by silk fibroin. Thus there is need to develop a formulation which have cell growth promoting activity along with anti-microbial activity[21].

1.4 Role of Metal nanoparticles in health care

Upon reaching nanoscale, like other nanomaterials and primarily by virtue of immensely small size, silver particles exhibit remarkably unusual physicochemical properties and biological activities. Silver nanoparticles have shown bactericidal activity against various strains of bacteria including *Salmonella, Staphylococcus and Pseudomonas*[30]. Most importantly, silver attacks a broad range of targets in the microbes, so it is difficult for them to develop resistance against silver, because they would require developing a host of mutations to protect themselves [31]. The antibacterial activity of silver appears to be due to disruption of the cell wall leading to increased cell wall permeability and depletion of ATP of cell affecting its viability. The monodispersed silver nanoparticles with narrow particle size distribution are more effective antibacterial agents, because of the high surface/volume fraction so that a large proportion of silver atoms are in direct contact with their environment. Silver is known to be nontoxic to human cell to the tune of 350 µg/day[32,33].

1.4.1 Characterization of Metal nanoparticles

The monodispered metal nanoparticles can be characterized by following techniques:

1. UV-visible spectroscopy
2. X-ray diffractometry (XRD)
3. Transmission Electron Microscopy (TEM)

UV-visible spectroscopy is one of the most widely used techniques for structural characterization of metal nanoparticles. Silver nanoparticles show band at about 420 nm whereas gold nanoparticles at about 510 nm in UV visible sprectroscopy which is known as Surface Plasmon Resonance (SPR)[34].

Physical basis of SPR

According to Mie's theory, only a single SPR band is expected in the absorption spectra of spherical nanoparticles, whereas anisotropic particles may give rise to two or more SPR bands depending on the shape of particles. The number of SPR peaks increases as there symmetry of the nanoparticles decreases. Thus, disks, spherical nanoparticles and triangular nanoplates of Ag shows one, two and more peaks, respectively[37].

The X-ray diffraction patterns of silver shows characteristic peaks at 2θ =38°, 44° and 64°, which corresponds to {111}, {200} and {220} crystal planes. Presence of these crystal planes indicates face central cubic crystalline structure of silver and also ensures that silver metal is in zero valent state. The XRD pattern of silver nanoparticles can also be used to determine their particle size. This can be done by mathematical analysis of the Bragg's peaks using the Scherrer formula[38,39].

The TEM analysis helps to confirm the particle size of the nanoparticles. The electron diffraction pattern generated during TEM analysis gives an idea about the nature (e.g. crystalline or amorphous) of the particles. The crystalline particles shows presence of clearly split Debye-Scherrer rings. These rings represents {111}, {200} and {220} crystal planes of silver. Presence of these crystal planes indicates face central cubic crystalline structure of silver [40,41,42].

1.4.2 Methods for stabilizing colloids

An important feature in the production of metal nanoparticles is their ability to remain dispersed in liquids without sign of agglomeration and coagulation, since their activity is governed by monodispertsity and stability. Colloidal particles in a dispersion medium always show Brownian motion and hence collide with each other frequently[43,44]. The stability of colloids is thus determined by the interaction between the particles during such a collision. There are two basic interactions: one being attractive and other repulsive. When attraction dominates, the particles will adhere with each other and finally the entire dispersion may coalesce. When repulsion dominates, the system will be stable and remain in a dispersed state[44,45].

Because there are always strong, long-range attractive forces between similar colloidal particles, it is necessary to provide long range repulsion between the particles to

impart stability[46].

This repulsion should be at least as strong as the attractive force and comparable in range of the attractive interaction. Stability can be obtained by surrounding colloidal particles[46,47].

- With an electrical double layer (electrostatic or charge stabilization).
- With chemically attached polymeric molecules (i.e. adsorbed) (steric stabilization)[48].
- With free polymers into the dispersion medium (depletion stabilization).

1.5 Combination of Silk Fibroin with Silver nano particles

Considering the wound healing activity of silk fibroin and broad spectrum anti-microbial activity of silver nano particles, it is believed that the combination of Silk Fibroin with Silver nano particle may be highly beneficial in wound healing process since the combination could act on wounds by two ways:

- Cell growth promoting action of silk fibroin proteins
- Broad spectrum of anti-microbial action of Silver nano particles on the microbes affecting the wounds.

Therefore the current study focuses on preparation of silver nano loaded silk fibroin topical gel which may accelerate wound healing process[66].

Aim and Objectives:

The primary aim of the present study is to formulate Nanosilver loaded silk fibroin for for these reasons:

- There is need to develop a formulation having dual role (i.e. cell growth promoting activity along with antimicrobial activity).
- Moreover, microbes are also unlikely to develop resistance against silver nanoparticles. Silver nanoparticles exert anti-microbial activity by increasing the bacterial wall permeability leading to loss of cell contents.
- The nanoparticles of Silver are more effective antibacterial agents, because of high surface area.
- Amino acid compositions of silk fibroin have wound healing activity as they promote the epithelization.
- The combination of silk fibroin and silver nanoparticles may accelerate the wound healing process.

2. EXPERIMENTAL (With Result And Discussion)

2.1 Preparation of Silk Fibroin Dialyzate

For preparation of silk fibroin gel, preparation of silk fibroin dialyzate was primary and mandatory step. The preparation of dilyzate involves following steps-

2.1. 1 Preparation of purified fibers

2.1. 2 Solubilization of silk fibers to make silk solution

2.1. 3 Purification by dialysis

2.1.1 Preparation of Purified Fibers

Reeled silk waste was cut and boiled in water at 90-100^{0}C for 1 hr. It was then degummed by treatment with 300 ml of 0.5 % w/v sodium carbonate solution at 90^0-100^{0}C for 2hrs and washed with hot distilled water till the washings were free from alkali. After air drying, fibers were defatted by treatment with Pet. Ether (40-60) for 24 hours and were dried again.

2.1.2 Solubilization of Silk Fibers

Purified silk fibers thus obtained were dissolved in aqueous solution of Lithium Bromide (9.3M) with continuous stirring at 48-50^{0}C to obtain silk fibroin solutions with concentrations varying in the range of 1-7 %w/v.

2.1.3 Purification of Silk Fibroin Solution (SF)

A thick paste of dissolved silk, thus obtained was transferred in dialysis bag and dialyzed against distilled water with constant stirring for 3 days to remove traces of lithium. During this period distilled water was replaced with a fresh one after every 2-3 hours interval. Specification of dialysis tubing used were as follows-

Mol. wt. cut off: 12000 Daltons Avg. flat width: 1.6 inches, Diameter:1.inch

Capacity: approx. 150 ml/ft.

The dialyzate thus obtained was centrifuged at 4000 rpm for 20 mins. and was filtered through muslin cloth.

Figure 2.1: Optimization of Silk Fibroin and $AgNO_3$ Concentration A) 6% Silk Fibroin Solution, B) 6% Silk Fibroin with 10^{-4}M $AgNO_3$ solution, C) 6% Silk Fibroin with 10^{-3}M $AgNO_3$ solution and 6% Silk Fibroin with 10^{-2} M $AgNO_3$ solution.

Table 2.1: Characteristics of Silk fibroin solution

Concentration of Silk fibroin	Nature of Dialysate	pH of Dialysate	
		Before dialysis	After Dialysis
1-2%	Low viscous solution	2.5±0.23	5.4±0.15
3-6%	Moderate viscous solution	2.7±0.31	5.9±0.32
7%	Thick viscous solution	2.8.±0.12	6.3±0.16

Table 2.2 Optimization of Silk Fibroin and $AgNO_3$ Concentration

Batches	Conc. of silk fibroin	Conc. of Silver Nitrate	pH	Colour of colloidal solution	Inference	Conclusion
1.	1-6% w/v	10^{-2}M	7.5 - 8.5	Black coloured	Aggregation occurred	Failed
2.	1-3% w/v	10^{-3}M	7.5 - 8.5	Reddish brown coloured	Aggregation occurred	Failed
3.	1-3%w/v	10^{-4}M	7.5 - 8.5	Reddish brown coloured	Aggregation occurred	Failed
4.	4-6%w/v	10^{-3}M	7.5 - 8.5	Yellow coloured	Stable colloidal solution	Passed
5.	4-6%w/v	10^{-4}M	7.5 - 8.5	Yellow coloured	Stable colloidal solution	Passed
6.	7%w/v	10^{-3}M	7.5 - 8.5	No colour change	No Silver nano formation	Failed

Table 2.2 shows the batches planned to carry out the *in situ* reduction of silver nitrate (10^{-2} – 10^{-4}M) using silk fibroin solutions (1-7%w/v) From the results obtained batch 4 (as mentioned in Table 2.2) containing 6% SF with 10^{-3}M $AgNO_3$ at pH 7.5-8.5 was selected. The high amount of silk fibroin and silver nanoparticles present in batch 4 forced us to consider it for the further studies.

2.2 Synthesis of silver nanoparticles by *In-situ* reduction of Silk fibroin (NSF)

SF aqueous solution of different concentrations in the range of 1-7% w/v containing varied amount of $AgNO_3$ in the range of 10^{-2} -10^{-4}M was prepared. The prepared solutions were investigated for the effect of pH in the range of 4-8.5. The formation of silver nano particles was indicated by the appearance of pale yellow colloidal solution.

2.3. Spray Drying of Colloidal Solution

The colloidal solution synthesized using optimum concentration of SF and SF were then spray dried so as to obtain dry powder. Spray- drying was performed with a Mini Spray-dryer Labultima (Mumbai, India). The applied process parameters were: inlet temperature150^{0}C, Outlet temperature 100^{0}C, aspirator was 45 m^3/hr, feed rate 10 ml/min. The spray dried powders were recovered and stored at room temperature in desiccators, and analyzed by FTIR (Jasco, V 5300, and Japan) and XRDP.

2.4. Characterization of Nanosilver loaded silk fibroin solution (NSF)

The NSF obtained by *In-situ* reduction was subjected to following tests.

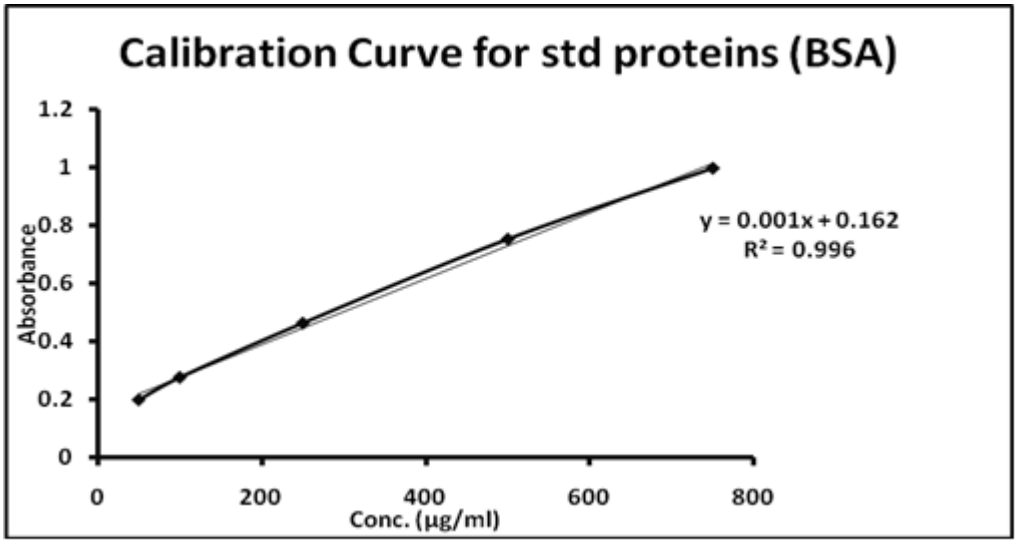
2.4.1. Total Protein Content

Total protein contents (TPC) has to be determined by using biuret method.

Table 2.1: Procedure for biuret testing

Pipette out into Test tube	Test (T)	Std. (S)	Blank (B)
NSF	0.05 ml	-	-
Protein Std.- 1	-	0.05 ml	-
Biuret Reagent- 3	2.5 ml	2.5 ml	2.5 ml

The standard proteins (BSA) of varying concentrations of 200, 400, 600, 800 µg/ml were prepared. All required reagents as mentioned in the Table 8.1 were added to the NSF and standard proteins and kept in water bath at 37^{0}C for 10 mins. The optical density of test (T) and Std (S) was measured at 555 nm by UVspectrophotometer (Jasco V 530, Jasco, Japan). A calibration curve of Absorbance Vs Concentration was plotted for the standard proteins and the concentration of test proteins (NSF) was determined by using the equation obtained from the calibration curve.



The Absorbance of 6% Silk Fibroin solution containing silver nanoparticles was found to be 0.5486. Thus the content of silk fibroin was found to be 3.86g/100ml.

2.4.2 Density of NSF

Density of the NSF was determined by using a precise specific gravity bottle. Hence the density was calculated by using formula:

$\rho = \frac{W_2 - W}{W_1 - W}$

Where; W_2= Weight of Specific gravity bottle+ Weight of the NSF

W_1= Weight of Specific gravity bottle+ water

W= Weight of Specific gravity bottle

Density of the 6%SF solution containing silver nanoparticles was determined as 1.01.

2.4.3 pH

pH of the SF before three days dialysis and after dialysis was observed by using digital pH-meter.

During the preparation of silver nanoparticles the pH of the silk fibroin solution was adjusted to 7.5-8.5. But reduction in the pH (7-7.5) was observed after 24 hrs. Thus the liberation of hydrogen ions during in situ reduction might have shown such behavior.

2.4.4. UV-visible spectroscopy

UV-visible spectroscopy is the most common method used to confirm the formation of silver nano particles, since nano-silver exhibit surface Plasmon resonance (SPR) at about 400 nm and this was the basis for our further study. All colloidal solutions along with SF dialysate prepared for the study were scanned between 200- 800 nm.

UV visible spectra was recorded for silk fibroin solution containing 10^{-4} M $AgNO_3$ (Curve 1 in Figure 2.5) and 10^{-3} M $AgNO_3$ (curve 2 in Figure 2.5). Both the solutions showed characteristic absorption band at about 400-420 nm due to Surface Plasmon Resonance phenomena of Silver nanoparticles indicating formation of Silver nanoparticles. The positions and shapes of the peaks are related to the dispersions, sizes, and shapes of the colloidal particles.

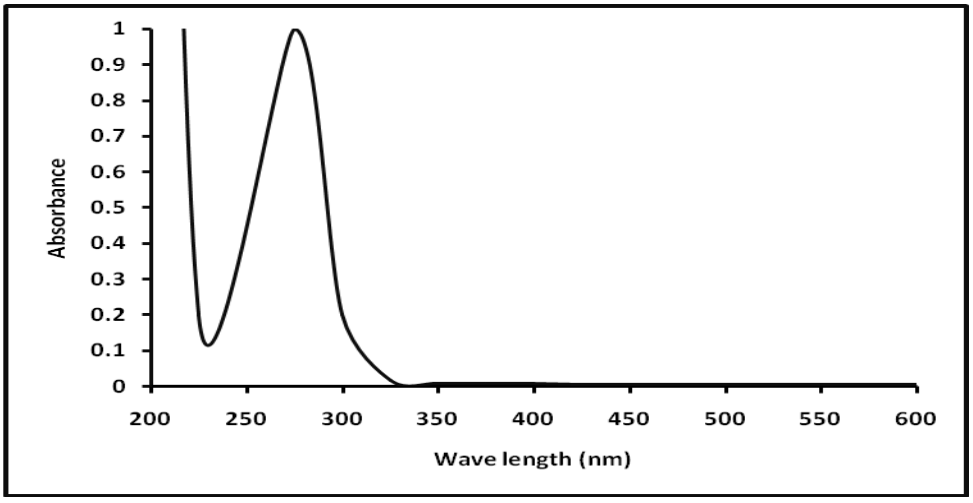


Figure 2.4: UV visible spectra for silk Fibroin solution

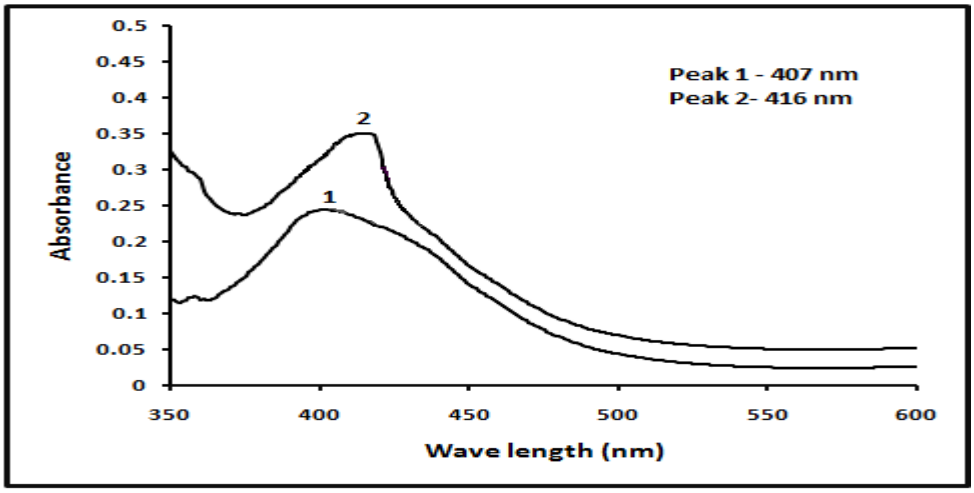


Figure 2.5 UV visible spectra for Nanosilver loaded silk fibroin solution

1. Silk fibroin containing 10^{-4}M $AgNO_3$ 2. Silk fibroin containing 10^{-4}M $AgNO_3$.

2.4.5. X-Ray Powder Diffractometry (XRPD)

The powder obtained after the spray drying of SF and colloidal NSF was used to record the X-ray diffraction patterns. Philips PW 1729 X-ray diffractometer (PW 1729, Philips, The Netherlands). Samples were irradiated with monochromatized CuKα radiation (1.542 A°) over 0° to 70° diffraction angle 2θ range. The absence of sharp peaks in XRD pattern of silk fibroin powder indicated its amorphous nature. On the other hand Nanosilver loaded silk fibroin powder showed peaks at 2θ =38.18°, 44.32° and 64.50° (Figure 2.4). These peaks are due to {111}, {200} and {220} crystal planes of silver. Presence of crystal planes confirms the face central cubic crystalline structure of silver and also ensures that silver metal is in zero valent state.

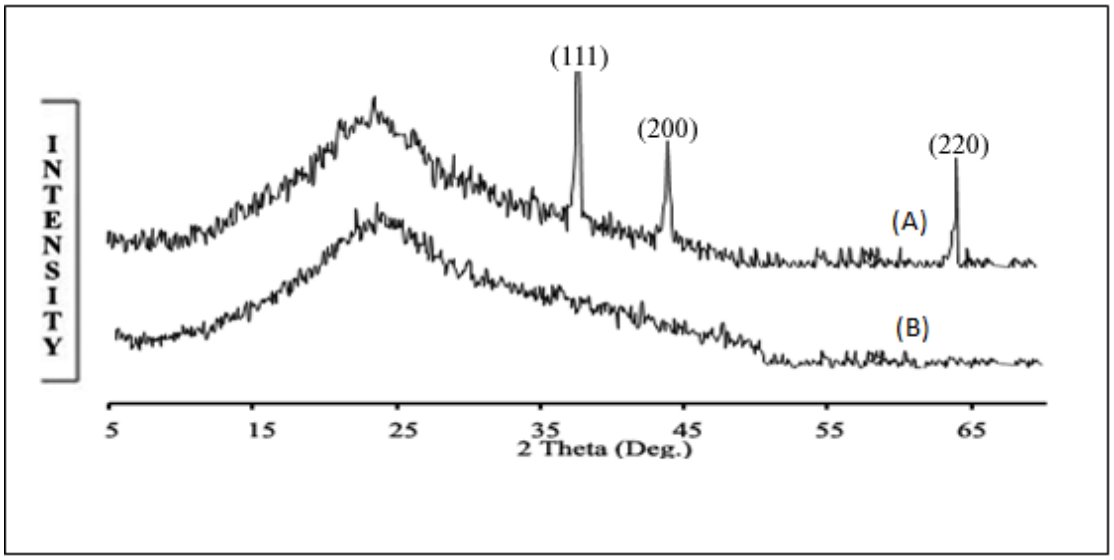


Figure 2.6 XRD graph of (A) Spray dried Nanosilver loaded silk fibroin powder and (B) Spray dried Silk fibroin powder.

2.4.6. Fourier Transform Infrared (FTIR) Spectroscopy

Infrared spectra of spray dried powders of SF and NSF was determined by using JASCO FT/IR 4100. Small amounts of samples were triturated along with activated Potassium Bromide (KBr) and the samples were scanned from 600 to 4000 cm^{-1}.

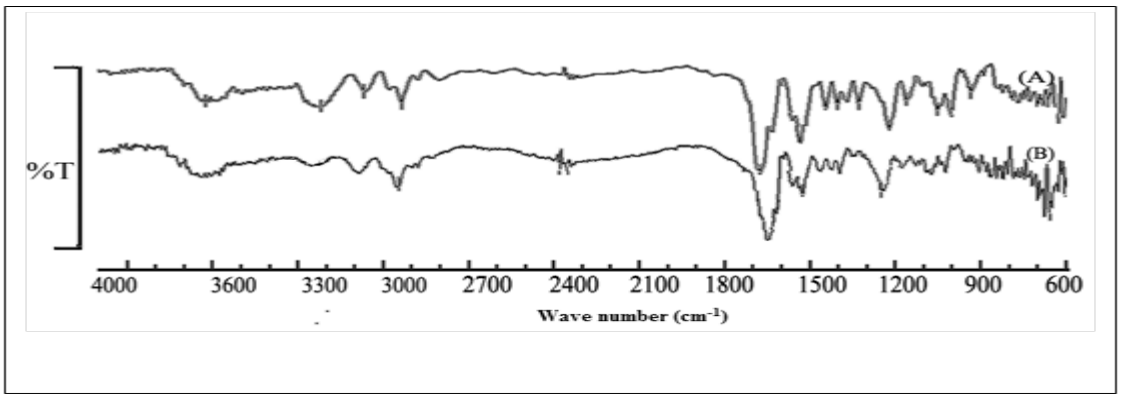
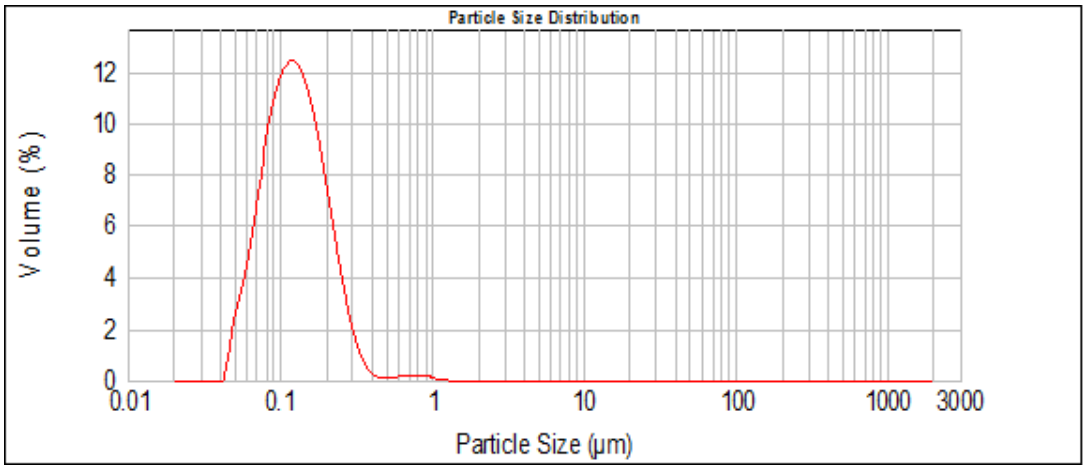


Figure 2.7 FT-IR spectra of (A) Spray dried Silk fibroin powder and (B) Spray dried Nanosilver loaded silk fibroin powder.

2.4.7. Particle Size Analysis

Particle size analysis was done using the technique of Laser light diffraction. Malvern Mastersizer 2000 SM which works under the principle of Laser light diffraction was used to analyse the particle size of freshly prepared samples of NSF. The particle size analysis was performed on NSF. The particle size of silver nanoparticles was found to be 126nm (Figure). The Silver nanoparticles with such a small size will be suitable for biological activity.



2.4.8. TEM

TEM measurements of the colloidal solution of NSF were performed on a Philips model CM200 instrument operated at an accelerating voltage of 200 kV. Samples for transmission electron microscopy TEM analysis were prepared by placing drops of the NSF on carbon-coated TEM copper grids. The mixtures were allowed to dry under the IR lamp. TEM analysis revealed the spherical shape of the Silver nanoparticles with size in the range of 30-50 nm (Figure 2.8). It has been reported that particles less than 200 nm size are most suitable for biological applications. Moreover, Silver nanoparticles were found to be embedded in the proteinous structure of silk fibroin indicating their involvement with the silk fibroin proteins.

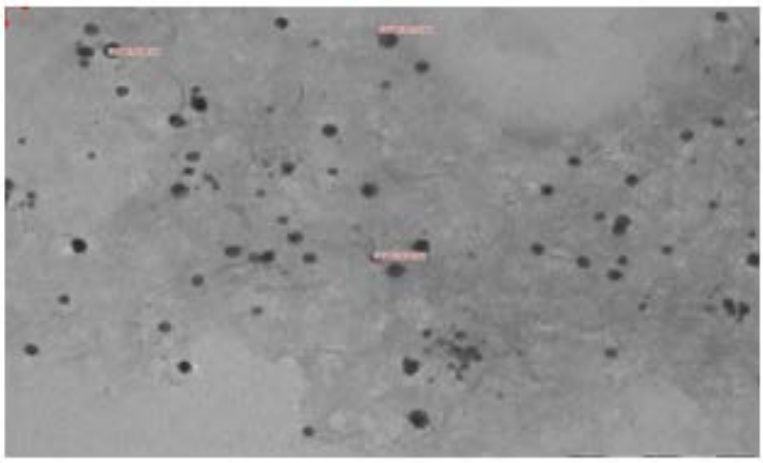


Figure 2.8 TEM of NSF

2.4.9. Determination of Silver content

The silver content present in colloidal solution of NSF was determined by using Atomic Absorption Spectroscopy (AAS) (Varian spectrAA 220 spectromete). The silver content of the NSF solution was determined and it was found to be 28.5 ppm

2.4.10. Antimicrobial Studies

Determination of antimicrobial activity of NSF, SF and Silver nano solution against clinically isolated strains of *Staphylococcus aureus*:*Staphylococcus aureus* obtained from NCL Pune was used to carry out the antimicrobial studies. The enriched samples were smeared on nutrient agar medium plates. The wells were made on the nutrient agar medium plates. The solutions of different concentrations of silver nano particles present in NSF, silver nano solution and SF were added to the well. The plates were then incubated at 37°C for two days. The zone of inhibition was calculated with the help of Vernier calliper. The silver nano sol^{n} (SNs) used for the anti microbial study was prepared by chemical reduction of $AgNO_3$ solution 10^{-3}M using 1%w/v Hydrazine Hydride solution as the reducing and concentration of silver was determined.

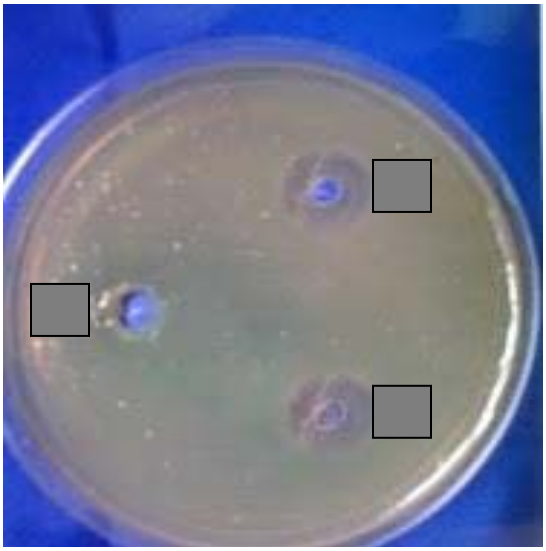


Figure 2.9 showing Anti microbial effect of (A) NSF (B) SNs and (C) SF.

The antibacterial response of NSF and SNs could be attributed to the increased permeability of cell wall by the Silver nanoparticles. The results thus confirmed that the antibacterial activity of the NSF was solely due to the Silver nanoparticles.

Table 2.3 Showing Anti microbial effect of (A) NSF, (B) SNs and (C) SF against *S. aureus.*

Formulation	MIC of Silver nanoparticles against *S. aureus.*	Zone of Inhibition
NSF (A)	6 ppm	11mm
SNs (B)	6 ppm	11mm
SF (C)	-	0

2.5. Formulation of Nanosilver loaded Silk Fibroin Gel (NSF gel)

The Carbopol 934 was added to the NSF containing 6% w/v silk fibroin along with 10^{-3} M $AgNO_3$, Silk Fibroin (6 %w/v), Silver nano solution and distilled water and the pH was adjusted to 6.8-7.5 by addition of triethanolamine (TEA) and was allowed to stand for an overnight to obtain Nanosilver loaded silk fibroin (NSF), Silk Fibroin (SF), silver nano soln (SNs), and plain Carbopol gels respectively.

2.6. Characterization of NSF gel

2.6.1. Rheological Tests

The Rheological characterizations of Silk fibroin (SF), Nanosilver loaded silk fibroin(NSF), silver nanoparticles (SNs) and Carbopol gels were performed by using Viscotech Rheometer (Rheological instrument) using cone-plate geometry with the diameter of the cone being 25 mm and angle of cone is 10, operating in the oscillation mode. The gap was maintained at 0.5 mm. all the Rheological measurements were performed at 25^0C. The results of creep recovery test of silk fibroin SF, nanosilver loaded silk fibroin NSF, silver nanoparticles SNs and plain carbopol gels are shown in Figure 9.14. The percent creep-recovery for SF, NSF, SNs and plain carbopol gels was found to be 82%, 82.60%, 57.40%, and 57.04%, respectively Table (2.4). This clearly

indicates the ability of SF and NSF gels to regain its original structure after application of stress was relatively higher than SNs and plain carbopol gels. There was no significant difference in the percent creep recovery of SF and NSF gel indicating the preservation of elastic character of silk fibroin proteins even after in situ reduction of $AgNO_3$ to silver nanoparticles by silk fibroin proteins. Moreover absence of silk fibroin in SNs and plain carbopol gels showed lower values of percent creep recovery. Hence creep recovery studies shows the NSF had more elastic Nature than viscous one.

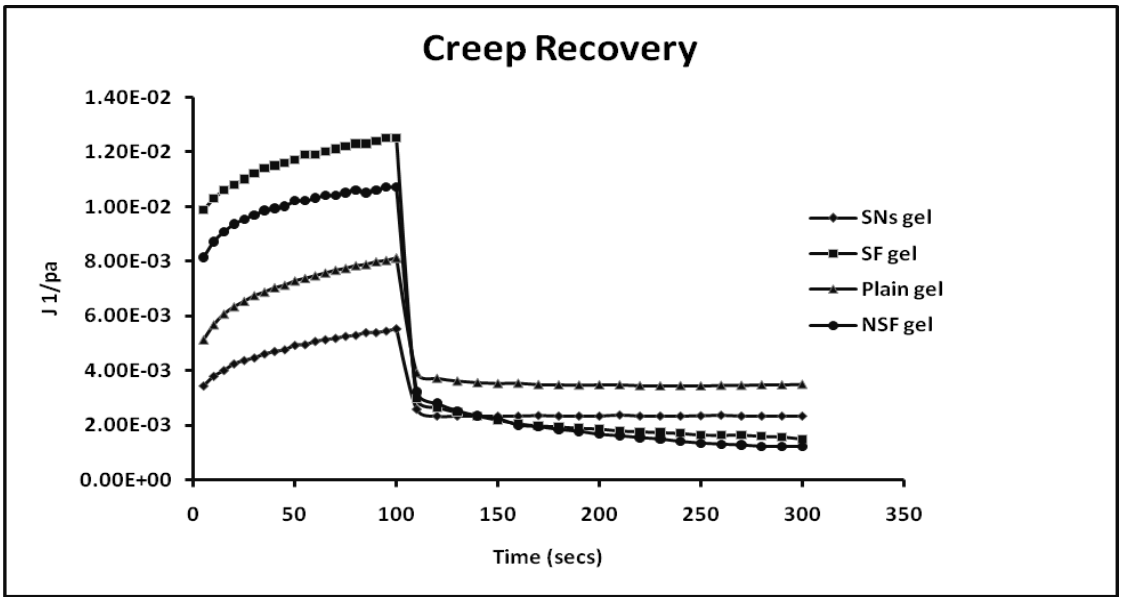


Figure 2.14 Creep recovery test for SF, NSF, SNs and plain carbopol gels.

Table2.14. Percent creep recovery of the prepared gels

Formulation	%Creep recovery
Silk Fibrion gel (SFgel)	82%
NSF gel (NSF gel)	82.59%
Silver nano gel (SNs gel)	57.50%
Plain Carbopol gel	57.02%

2.6.2. Spreadability of gel

The spreadability of the gel was determined using the following technique. 0.5gm of gel was placed within a circle of 1 cm diameter premarked on a glass plate over which a second glass plate was placed. A weight of 500 gm was allowed to rest on the upper glass plate. The increase in the diameter due to spreading of the gel was noted.

The spreading diameter for NSF gel was found to be 4.2 ± 0.22 cm.

2.7. Wound Healing Study in rats

2.7.1 Animal Selection:

Male albino rats of wistar strain weighing 180-250 gm were used for the present study. Animals were kept in laboratory for 3-4 days with free access to food and water.

2.7.2. Groups

The screened animals were divided into six groups each containing 5 animals. The formulations used for a group includes.

1) Optimized Silk fibroin gel (SF gel).
2) Optimized Nanosilver loaded silk fibroin gel (NSF gel).
3) Silver nano gel (SNs gel).
4) Carbopol gel.
5) Positive Control (Soframycin).
6) Negative Control.

2.7.3. Excision Wound Model in rats

Under light ether anesthesia, the animal was secured to operation table in its natural position. An impression was made on the dorsal thoracic central region 5 mm far away from the ears, by using around seal of 2 cm diameter. The skin of the impressed area was excised to the full thickness to obtain a wound area of 250 mm^2. Haemostasis was achieved by blotting the wound with cotton swab soaked in normal saline solution. The prepared formulations were applied on wound by weighing the same quantity of formulations i.e. 1 gm at every day till wound was fully healed. The animals were then placed back into their individual cages and following parameters were noted:-

a. Biophysical parameters

The observation of percentage wound closure has to be made on 3^{rd}, 6^{th}, 9^{th} and 12^{th} days and subsequently on every day till complete wound closure occurred and scar size was noted. Wound area was measured by retracting the wound on a millimeter scale. The degree of wound healing was calculated as percentage closure in wound area from original wound area using the formula:

Percentage Closure = {1- (A – D)/ (A- O)} × 100

Where, A-O = Wound area on day zero and

A-D = Wound area on corresponding days

The mean and S.E. values were calculated. The number of days for complete epithelization was noted. The scar shape and area were traced and measured.The biophysical parameters of the wounds were studied after 15 days. Figure 9.16 show the graph of percent wound closure on 15^{th} day of the study. Wound treated with the nanosilver loaded silk fibroin (NSF) gel showed the highest percent wound closure (97.44%) when it was compared to the other formulations such as SF gel (75.05%), SNs gel (76.27%) and plain carbopol gel (49.45%) (Figure 2.15).The percent (%) wound closure for the positive control (soframycin) and negative control (Untreated) was found to be 79.48% and 47.79% respectively. Thus the percent (%) wound closure of SF gel and SNs gel had no significant difference even though they had different mode of action on wound healing process.

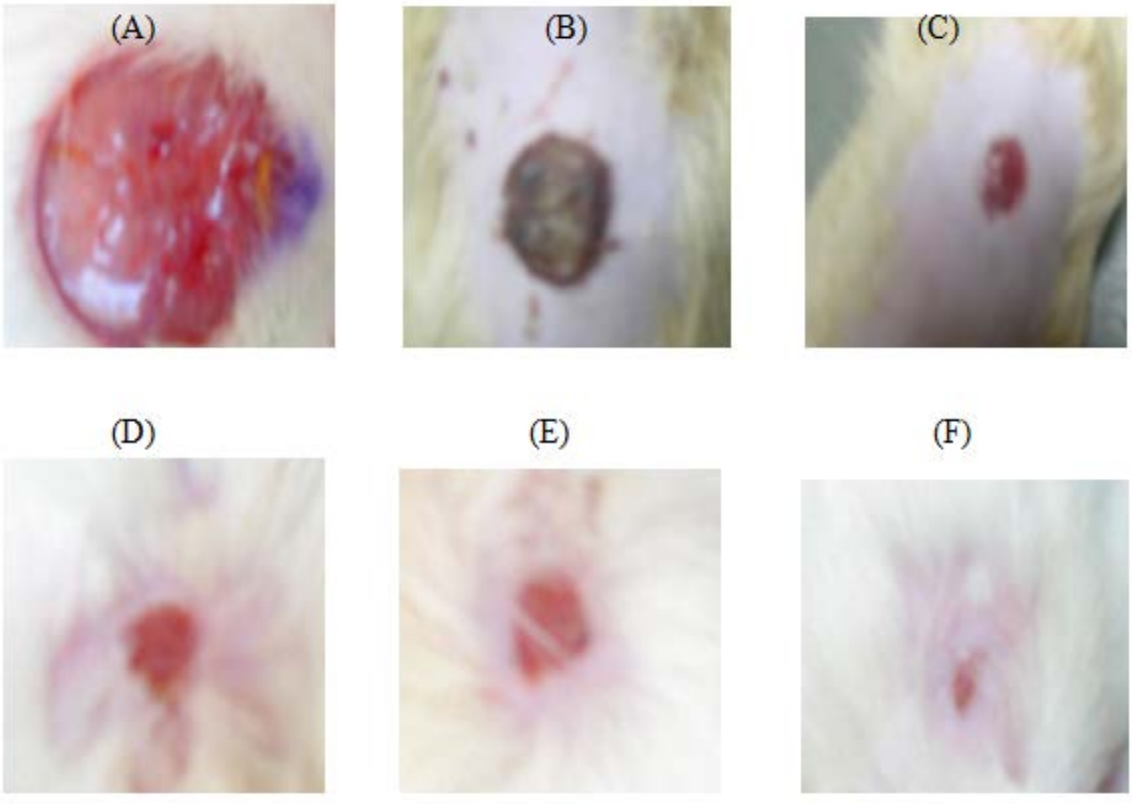


Figure 2.15: Excision wound healing studies on rats showing (A) Wound on day 0 and wound closure on 15^{th} day for (B) Negative control, (C) Positive control, (D) SNs gel, (E) SF gel (F) NSF gel

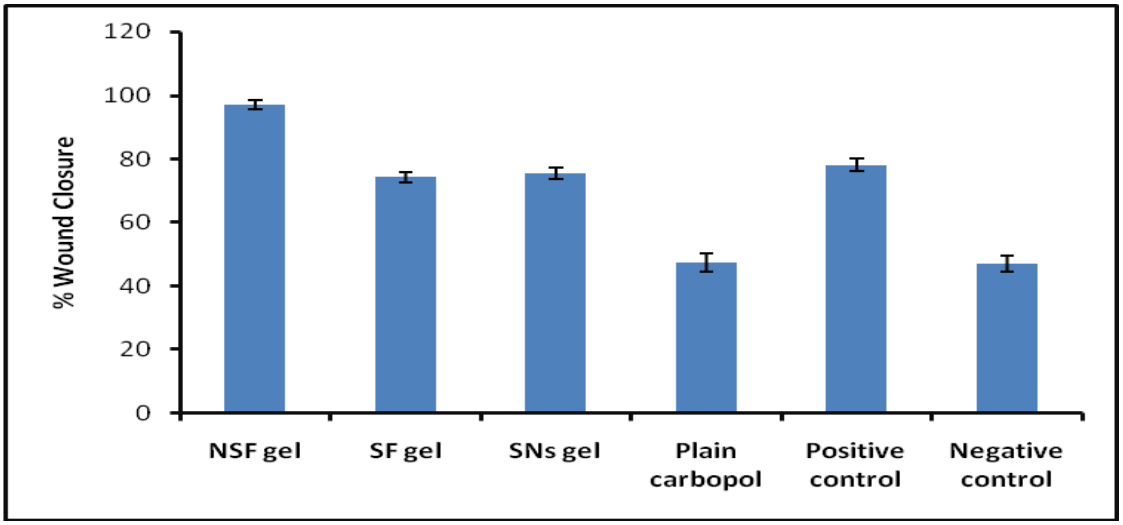


Figure 2.16 Percent wound reduction of different animal groups on Day 15

In the present study the NSF gel promoted the wound healing accurately, which is indicated by the highest percent wound closure compared with marketed preparation of Soframycin cream. Thus the wound healing activity of NSF gel was stronger than the marketed formulation as indicated by the excision wound model.

2.6.2. Histological parameter

Portion of the healed wound (on 15th day) was cut and subjected to histopathological studies Histological examination of hematoxylin and eosin (H & E) stained sections showed a large number of cells, such as fibroblasts ,macrophages, and neutrophils in (1) the NSF group when compared to (2) SF,(3) plain Carbopol, (4) SNs, (5) positive control and (6) negative control groups (Figure 2.17).

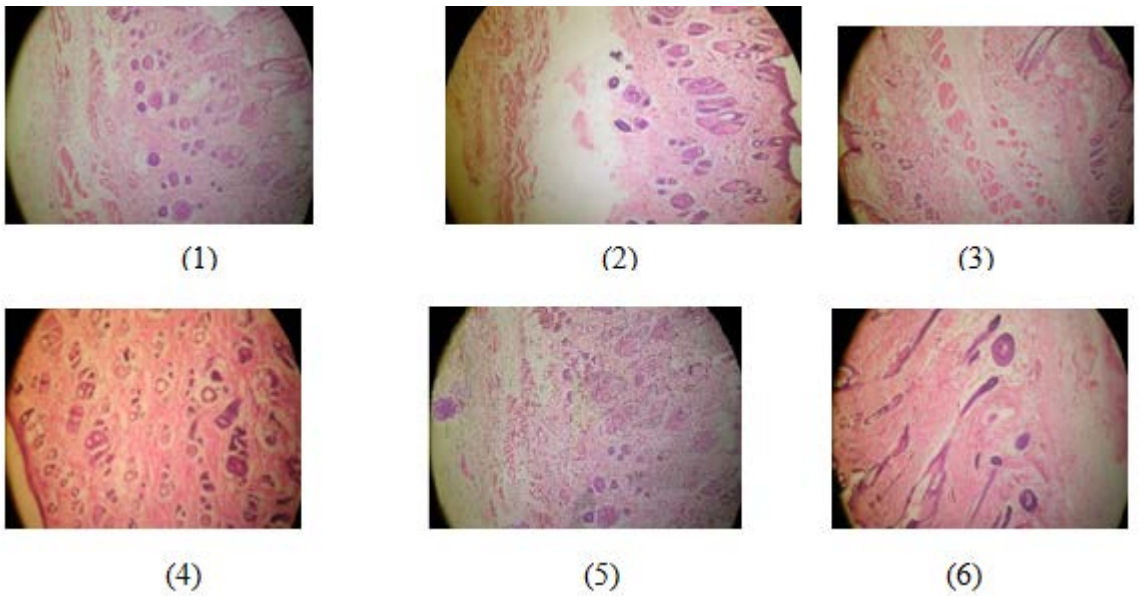


Figure 9.17. Histological analysis of H&E stained section of the granulation tissue of, (1) SF group, (2) Plain carbopol, (3) SNs group, (4) NSF group ,(5) Positive control group and (6) Negative control on 15th day of wound healing at 100 x magnifications.

It has been reported that the fibroblast play an important role in the epidermal cell multiplication. The silk fibroin has been reported to increase the fibroblasts in the wounded tissue. Silk fibroin present in NSF gel treated group indicated speedy recovery of the wound when compared to other groups. The inflammatory cells played an important role in initiating the wound healing process but completely healed wound is devoid of inflammatory cells indicating complete recovery of the wound tissues. The granulation tissue treated with NSF gel exhibited a fewer number of inflammatory cells which were abundant in other groups confirming

complete recovery of the wounds at 15th day. And the Silver nanoparticles (SNs) present in NSF gel reduced microbial contamination of the wounds in turn accelerating the wound healing process. The SF gel treated group did not show complete wound closure on 15th day may be due to microbial contamination of the wounds. Similarly SNs gel treated group failed to show complete wound closure on 15th day since it was devoid of cell growth promoting agent.

NSF gel played a dual role in wound healing process i.e. silver nanoparticles act as antimicrobial agent and silk fibroin acted as epidermal cell growth promoter leading to faster percent wound closure.

Conclusion:

Nanosilver loaded silk fibroin gel was prepared successfully by *in situ* reduction of Ag+ ions to zero valent silver atoms (silver nanopartilcles) at room temperature using silk fibroin as the reduction agent. The prepared gel had more pronounced wound healing activity than marketed formulation which is believed to be due to the dual role played by NSF gel wherein silk fibroin acted as epidermal cell growth promoter and silver nanoparticles acted as antimicrobial agent leading to faster percent wound closure.

This investigation also proposes the need for such combinations of a cell growth promoting agent with anti–microbial agents in the treatment of wounds.

REFERENCES:

1. Wikipedia; http://en.wikipedia.org/wiki/Wound. Accessed on 15 Jan 2015
2. Morton J.J.P., Malone M.H. 1972. Evaluation of vulnary activity by an open wound procedure. Journal of Arch. Int. Pharmacodyn.Ther. 199, 117-126
3. Shimura K., Kikuchi A., Katagata Y., Ohtomo K. Hyodo A., 1976. Studies on Silk Fibroin of *B. mori*. I. Fractionation of Fibroin Prepared from the Posterior Silk Gland. Journal of Biochem. 80-84, 693-702.
4. Pak T.S., Takhtaganova L.B., Kristallovich E.L. 2007. Enterosorbent based on natural silk fibroin and its mechanochemical modification with deoxypeganin hydrochloride. Pharmaceutical Chemistry Journal. 41, 20-24.
5. Quitain T.A., Daimon H., Fujie K., Katoh, S., Moriyoshi T. 2006. Microwave-Assisted Hydrothermal Degradation of Silk Protein to Amino Acids. Ind. Eng. Chem. Res. Journal. 45, 4471–4474.
6. Tsubouchi Kozo. 1999. Development of skin protectants from silk. Brain Techno News, 72, 13-15.
7. Tsubouchi, K., Yamada, H., Takasu, Y. 2007. Material for activating epidermal cell multiplication. United States Patent 6440740, 27, Aug
8. Sugihara A., Sugiura K., Morita H., Ninagawa T., Tobe R., Izumiya M., Horio T.,Abraham N. G., Ikehara S., Tubouchi K 2000. Promotive Effect of Silk Film on Epidermal Recovery from Full- Thickness Skin Wounds. Society for Experimental Biology and Medicine. 225, 58-64.
9. Patakfalvi R., Papp S., 2007. The kinetics of homogeneous nucleation of silver nanoparticles stabilized by polymers. J. Nanoparticle Res. 9,353–364.
10. Shimura K., Ohtomo K., Kikuchi A., Katagata Y., Hyodo A., 1976. Studies on Silk Fibroin of Bombyx mori. Journal of Biochem. 80, 693-702.
11. Sondi I., Salopek-Sondi B., 2008. Silver nanoparticles as antimicrobial agent: a case study of E.coli as a model for Gram- negative bacteria. Journal Colloid Interf Sci. 277, 177–182.
12. Reddy M.R. 2009. Innovative and Multidirectional Applications of Natural Fibre, Silk - A Review. Academic Journal of Entomology. 2,71-75.
13. Chun D., Yong seol S., Cho D. T., Tak R., 1977. Drug Resistance and R Plasmids in Salmonella typhi Isolated in Korea. J. Antimicrob. Agents Chem. 11, 209-213.
14. Dibrov P., Dzioba J., Gosink K., Hase C., 2002. Chemiosmotic Mechanism of Antimicrobial Activity of Ag+ in Vibrio cholera. J. Antimicrob. Agents Chemo. 46, 2668-2670.
15. Hyde H.J., Wipper C. 1962. Molecular weight of silk fibroin. J. Polymer Sci. 50, 1083-1088.
16. Christensen K.L., Pedersen G., Kristensen H.G., 2008. Preparation of redispersible dry emulsions by spray drying. International J. Pharm. 202,187-194.
17. Chun D., Yong seol, S., Cho D. T., Tak R., 1977. Drug Resistance and R Plasmids in Salmonella typhi Isolated in Korea. J. Antimicrob. Agents Chemo. 11, 209-213.
18. Minoura N., Aiba I.S., Gotoh Y., Tsukada M., Imai Y., 1999. Attachment and growth of cultured fibroblast cells on silk protein matrixes. Journal of Biomedical Material Research. 29, 1215-1222.
19. Quitain T.A., Daimon H., Fujie K., Katoh S., Moriyoshi T. 2006. Microwave-Assisted Hydrothermal Degradation of Silk Protein to Amino Acids. Ind. Eng. Chem. Res. 45, 4471–4474.
20. Sondi I., Salopek-Sondi B., 2006. Silver nanoparticles as antimicrobial agent: a case study on E.coli as model for the Gram- negative bacteria. Journ. Colloid Interf Sci. 279, 177–182

21. Wang H., Qiao X., Chen J., Wang X., Ding S., 2008. Mechanisms of Pollyvinylpyrrolidine in the preparation of silver nanoparticles. Mat. Chem. Phys. 94,449–453.

22. Torres L.G., Iturbe R., Snowdenb M.J., Chowdhry B.Z., Leharne S.A. 2007. Preparation of o/w emulsions stabilized by solid particles and their characterization by oscillatory rheology. Colloid Surfac. A 302, 439–448.

23. Shao Z.Z., VollratF.2002.Materials: surprising strength of silkworm silk. Nature. 418, 741-1741

24. Shahverdi A., Fakhimi A., Minaian S., Shahverdi H., 2007. Synthesis and effect of silver nanoparticles on the antibacterial activity of different antibiotics against Staphylococcus aureus and E. coli. Nanomed. 3, 168-174.

25. Russell A.D., Hugo W.B., 2004. Antimicrobial activity and action of silver Progressive Med. Chem. 31, 351–370.