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PREPARATION AND CHARACTERIZATION OF BIOACTIVE FUNCTIONALIZED SILK-BASED FILMS FOR WOUND HEALING

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ABSTRACT

In this study, an effort has been made to develop a ready-to-use bioactive wound dressing material using natural silk fibroin biomaterial functionalized with drugs and PRP.The flexible silk films were prepared by incorporating 3% dextrose (w/w) in the silk films which acted as a plasticizer. The dextrose modified silk films showed higher water holding capacity than the normal silk films. After evaluating the results of the comparative study done in between the silk films and the dextrose modified silk films, it was found that the flexibility as well as the hydrophilicity of silk fibroin films increased upon the addition of dextrose. Additionally, two more types of dextrose modified films were prepared, the PRP coated dextrose modified films and the drug coated dextrose modified films. The films were microscopically examined for detecting cytotoxicity and the results showed that the films were not toxic. An in-vivo wound healing test on mice models indicated that the PRP coated and the drug coated films resulted in a good wound recovery effect, similar to a commercial wound dressing material. The increased hydrophilicity of these films along with the combined results of drugs and PRP have balanced the moist environment at the wound site, which improved the wound healing compared with the other types of films. This systematic approach to wound healing biomaterial wound dressings may demonstrate a useful strategy to select formulations for further study towards new treatment options for chronic wounds in the future.

Keyword: Hydrophilicity, Functionalize, Pathological,Proliferation

1.INTRODUCTION

A wound, whether a minor scrape or a major injury, needs to be properly taken care of. Health care professionals from all around the world face an increasing number of patients suffering from different wounds that are difficult to treat and heal on a day-to-day basis(1). This has led to a huge demand for smart dressing materials to combat delayed healing and scarring problems.

Wound dressings such as gauzes, lints, plasters, bandages, cotton wool, hydrogels and semi-permeable films etc. assist in protecting the wounds from external contamination and promote wound healing. Bioactive wound dressing is a new and a promising approach for wound treatment. These wound dressings are synthesized from biological materials such as silk, chitosan, collagen, alginate, hyaluronic acid, and elastin. All these biomaterials have unique properties that promote the process of wound healing (2).

Among the different types of the biological materials available for producing bioactive wound dressings, silk fibroin (SF) obtained from *Bombyx mori* silkworm has gained significant importance as a bioactive wound dressing. Silk materials have shown to promote wound healing since the 1990s. In 1990, Tsubouchi developed a silk fibroin-based wound

dressing that could accelerate healing and could be peeled off without damaging the newly formed skin (2).

Silk is a natural polymer present in the glands of arthropods such as spiders, silkworms, mites etc with an excellent tissue regeneration property because of specific amino acid sequence (GAGAGS) which leads to the faster healing of wounds. It has also got NMF – Natural Moisturizing Factor which is very similar to what the body produces in normal human skin. These properties make silk protein one of the most suitable biomaterial for wound healing(3).It has a genetically tailorable composition which makes it easy to mold it into various shapes such as films, membranes, hydrogels etc (4). Silk is derived from a non-mammalian source having a similar composition to mammalian dermal tissue and therefore behaves similar in its biological properties. This makes silk fibroin free from any sort of microbial contaminations detrimental to human health, which may be present in other proteins such as collagen sourced from bovine tissues.

Several studies done on silk fibroin have shown the efficacy of silk when used as dressing materials for the treatment of wounds used either in pure forms or in amalgamation with herbs, monosacharides, drugs or growthfactors (5)(6)(7)(8). During the wound healing process, the silk dressings protect the injury and contribute to the faster healing of the wounds to the recovery of epidermal tissues.

Similarly, in the recent times, a growing number of studies have reported the beneficial effects of platelet-rich plasma (PRP) in wound healing(9). PRP has shown to contribute to the healing of wounds by shortening the wound healing duration by delivering abundant growth factors at the site where it is used. Because of such abilities, it is expected to become one of the most common substances in accelerating wound repair in patients with chronic wounds in the near future. However, little is known about the synergistic effects of PRP and silk in wound healing.

Thus, to gain an insight in the effects of silk films as a dressing material in wound healing, when used alone and in combination with drugs and PRP separately, this research was initiated. The films were prepared and studied in terms of its functions such as hydrophilicity, porosity, protein adsorbing capacity, drug retaining capacity etc. Similarly, the impact of functionalizing those films by PRP and drugs separately was also examined in terms of healing of wounds in the animal models.

2.MATERIALS AND METHOD

The silk cocoons were purchased from Nepal Silk farming, Khopasi. Distilled water was used throughout thestudy.

2.1. Fibroin extraction from silk cocoons

Figure 1 shows the depiction of the overall process of fibroin extraction from silk. For the fibroin extraction from silk cocoons, first 2L ultrapure water was heated until it started to boil. 45g of Na_2CO_3 was added into the boiling water. The silk cocoons were cut into dime size. The cocoon pieces were added into the water once the water started to boil. The cocoons were occasionally stirred with the help of a spatula to promote a good dispersion. When the cocoons started to appear in a cottony form, it was removed and cooled. It was then soaked in 1000L of cold distilled water .The excess water was squeezed which gave off glue like residues called sericin. This process was repeated thrice. After the third wash, silk was removed and squeezed well. Finally, the silk appeared in a thread like form which was spread well in the Aluminum foil and left to dry overnight. This process ensured the removal of sericin and extraction the silk fibroin from silk cocoons.

The recipe for degumming of silk is shown in Table1.

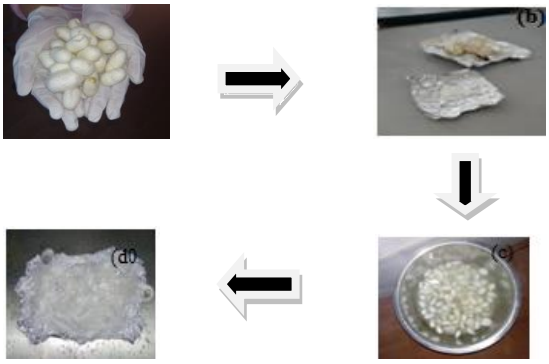


Figure 1: Extraction of fibroin from silk cocoons((a): Silk cocoons (b): Cut cocoons and chemicals(c): Boiling cocoons and chemicals in distilled water (d): Dry fibers)

Table 1: Recipe for degumming of silk cocoons

S.n	Particulars	Proportion
1	Distilled water	2 L
2	Cocoons	55g
3	Na_2CO_3	45g
4	Temperature	80°C

2.2. Dissolution of silk fibroin

For the dissolution of silk fibroin, 10g of degummed silk fibroin fibers was dissolved in a mixture containing3.32g of $CaCl_2$ and 70ml of formic acid by continuous stirring in a magnetic stirrer. The temperature was maintained at 60°C.

2.3. Preparation of Normal Silk films and Dextrose Modified Silk films

To prepare Normal Silk Films (NSF), 15 ml of the silk fibroin solutions was casted in Polystyrene Petri dishes (9 cm diameter). As for the Dextrose Modified Silk Films (DMSF), 3g of dextrose was incorporated in the silk fibrion solution (mixture of 10% fibrion, 3.33% $CaCl_2$ and70% formic acid).

The dishes containing the silk solution were placed in a laminar hood to allow fast evaporation of formic acid within 24h. Then the dishes were left untouched for a week to let the solutions cast into films. After the Silk Fibroin (SF) films dried, they were pricked out with the help of needles and the resulting films were directly immersed in water for 2h to remove the $CaCl_2$ and residual formicacid.

Table 2 shows the recipe for the dissolution of silkfiber.
Table 2: Recipe for the dissolution of silk fiber

S.n.	Particulars	NSF Proportion	DMSF Proportion
1	$CaCl_2$	3.32g	3.32g
2	Formic acid	70ml	70ml
3	Cocoons	10g	10g
4	Dextrose	-	3g
5	Temperature	60°C	60°C

2.4. Purification of silk films

The resulting NSF and DMSF were directly immersed in water at room temperature to remove $CaCl_2$ and residual formic acid. The films were washed each hour with distilled water until the pH came to neutral. The neutrality was tested with the help of pH meter where calibration was followed by phosphate buffer tablet. The silk films were then stored in moist environment untiluse**.**

2.5. Bacterial Contamination test

2.5.1. Preparation of Agar solution:

The Agar nutrient solution was prepared by dissolving 28g of agar nutrient in 1L distilled water and was sterilized in autoclave at 121°C temperature and 15lb/inch^2 pressure.

2.5.2. Agar Test

Then the agar solution was allowed to cool in laminar flow until it reached 50-60°C which was then poured into Petri dishes. The small sizes of silk films were placed around the Petri dishes with the help of forceps. One of the Petri dish only with agar nutrientwas considered as controlled test. Then all the Petri dishes was placed in incubator and incubated for 24 hours at 37°C temperature.

2.6. Characterization of the films by FTIR

Secondary structure of the silk blended scaffolds was analyzed by Fourier transform infrared spectroscopy (FTIR).

2.7. Functional testing of the films

The developed silk films was evaluated for six additional functions through the functional tests such as water uptake test, weight loss test, contact angle determination test, porosity test, bovine serum albumin absorption test and the drug retain test.

2.7.1. Hydrophilicity tests

2.7.1.1. Water *absroption* test

For this, both the NSF and DMSF were accessed after immersion in an ISS solution for time period ranging from 1-42 hours at 37°C in an incubator. The percentage of water absorption was calculated in different time intervals using the following equation:

Water absorption (%) = (Wf−Wi) Wi ×100 %

Where,

Wi= initial weight of the dry films

Wf=wet weight of the films after taken out from the PBS solution

The experiment was repeated thrice and the percentage of water uptake was calculated.

2.7.1.2. Contact angle Measurement

Hydrophilicity of the films was also determined with the help of contact angle goniometry. The water droplet angle formed between liquid and solid surface of NSF and DMSF was analyzed by sessile drop method where the total volume of water droplet was kept at 2µl. And the contact angle of each sample was calculated as the average of four readings obtained from NSF and DMSF samples.

2.7.1.3. Weight loss Test

The dried silk film was cut into circles of 3 cm diameter and was immersed in 10ml of PBS at room temperature for the period of 1 to7 days. Every day, two readings at an interval of 12 hours were taken. At the 7^{th} day, all the samples were dried at 65°C in an oven for 24hrs and the remaining masses were measured. Experiments were performed in 5 replicates and the weight loss % of the developed samples was calculated as:

Weight loss(%) = [(Wdi – Wdf)/Wdi] X 100 %

Where,

Wdi= initial weight of dry films

Wdf=final dry weight of films after removed from the oven

2.7.2. *Porosity* Test

The porous nature of the film was studied by the liquid displacement method using hexane as the displacement liquid. Films samples with a diameter 3cm were immersed in a known volume (V1) of hexane in a graduated cylinder for 5 min. The porosity of the scaffolds (ε) was obtained by

ε=V1-V3/V2-V3

The total volume of hexane-impregnated films along with hexane was recorded as (V2). The residual hexane volume in the cylinder after removal of hexane-impregnated films was recorded as (V3).

2.7.3. Determination of drug retain ability of developed film

To determine drug retaining capacity of the films, drug coated films were immersed in 10ml, Phosphate Buffered Saline (PBS) at 37°C. The buffer was removed for every 24 hours from the system and assessment of drug content was confirmed by Ultraviolet visible spectroscopy. The drug release was studied for 15 days at time interval of 4 days.

2.7.4. Bovine serum *albumin* (BSA) adsorption Test

The protein adsorption capacity of NSF and DSMF were carried out by using BSA as the protein. The varied concentration of BSA was added to phosphate buffer saline solution, and different silk film samples were added into mixture of BSA and PBS solution. These samples were incubated at 37°C for a day and the amount of protein absorbance was determined by using UV spectroscope at 595 nm.

2.8. Preparation of drug coated films

The Dextrose modified silk films were soaked in a mixture containing 0.15g Placentrex and 10ml PBS and left for 48 hours stored in 4°C in order to prepare the drug-coated films.

2.9. Preparation of PRP coated films

Platelet rich plasma was purchased from Nobel hospital, Sinamangal upon the donation of 300ml of blood by the donors. The activated PRP was stored at 22 ºC in an incubator in a VDRL shaker to prevent the clot formation in its packet until use. It was instructed that the efficacy of PRP decreases in three days so it was changed at every 3 days.

To prepare the PRP coated films the films (1.5 cm radius) were soaked in 10 ml PRP for 10 minutes.

2.10. Cytotoxicity Test

Besides investigating the functions of the films, it was very important to check the biocompatibility of the films. An initial approach to assay the biocompatibility of new materials is to test its cytotoxicity in vitro.

The hela cells were cultured in the in the laboratory. The silk samples were sterilized and cut to 0.1cm^2.The films were then transferred to 96-well plates and the cells were seeded at a density of 1.0x10, 000 and maintained at 37°C and 5%CO_2 for 48h. After the incubation period, the MTT was added to each sample. After 4h, the MTT was removed and the samples were washed. The UV absorbance of each samples were measured based on the absorbance of MTT-formazon by the Eliza plate reader.

2.11. In vivo Test

Before starting the in vivo test, ethical clearance was received from Nepal Health Research Council to initiate the in vivo test in the research.

Thirty rats of same species, age, gender and weight were used for the study.

- Rat species :Albino rats(white)
- Age :2 and half months
- Weight :150 gm(avg)
- Gender : Male

2.11.1. *Wound Induction*

Local anesthesia was supplied to all the rats during the wound induction and the wounds were induced by a pathologist on the dorsal surface of each rat penetrating dermis. The size of the wound was kept 1x1cm.

2.11.2. Treatment of the animal models in vivo

The wounded animal models were treated with the - prepared films as grouped below in Table 3.

Table 3: Grouping of the animal models receiving various treatments

S.n.	Coding	Treatment for the wounded animal models
1	Group-1	Tegaderm Tape (-ve control)
2	Group-2	Normal silk film (+ve control)
3	Group-3	Dextrose modified silk films (DMSF)
4	Group-4	Drug Coated silk films
5	Group-5	PRP coated silk films

2.11.3.Wound measurement

Evaluation of wound closure was done by the measurement of wound size with the help of a Vernier Caliper. The size of the wounds was measured from the horizontal and vertical direction in 2nd day, 4th day, 6th day and 8th day.

2.11.4.Biopsy sample preparation

For a biopsy sample, small tissue from the wound area was removed from each group of animal model on the 3rd day, 6th day and 9th day.

2.11.5.Preparation of histological slides

The fixed biopsy samples were left in a Pathology Lab for the preparation of histological slides.

2.12. Statistical analysis

The results at the end were analyzed statistically using two-way analysis of variance (ANOVA) based on the wound size and its reduction.

3. RESULTS AND DISCUSSION

3.1. Fibroin extraction from silk cocoons

The degumming of silk fibers was carried out in order to ensure the removal of toxic sericin protein. It was observed that there occurred a slight decrease in weight after the degumming process. 5g of cocoons after degumming resulted to 3g of fibers.

3.2. Preparation of silk films

Both NSF and DMSF were prepared. The films were highly flexible and appeared to be transparent. The concentration of Dextrose was maintained 3% throughout the project because in a concentration more than that the films became brittle and powdery. The developed films were yellowish in color as shown in the figure 2.

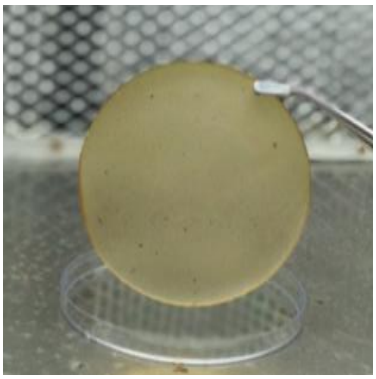


Figure 2: Silk Films

3.3. FTIR-Characterization of films

In order to confirm the structural change, the developed films were investigated by FTIR spectra on a FTIR spectrometer, IR-Prestige-21 model.

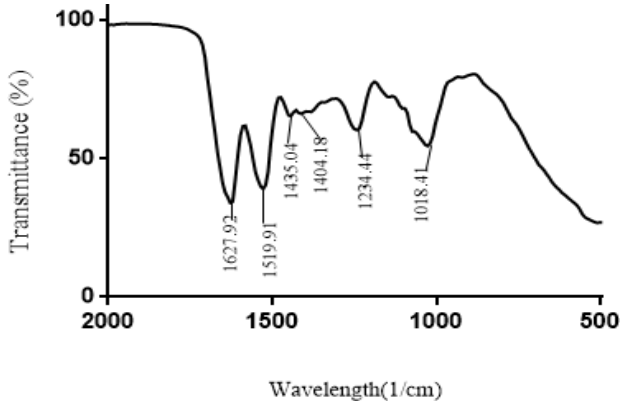


Figure 3: FTIR characterization of NSF

The structure and blending nature of NSF and DMSF were investigated through FTIR spectra as shown in figure 3 and 4. Normally, silk fibrion consist of characteristics variation in IR spectra that represent three amide groups (Amide I, Amide II, Amide III) which have been commonly used for the analysis of secondary structure of silk films. The region within 1700-1600 cm^{-1} is assigned to amide I (C=O stretching), 1600-1500 cm^{-1} to amide II (secondary N\H blending) and 1200-1300 cm^{-1} to amide III (C-N and N-H functionalities)(10). From the figure, the peaks at 1620-1635cm^{-1} represent amide I, 1512-1543cm^{-1} is for amide II and 1226-1257 cm^{-1} represents amide III. As a result the presence of protein in the silk film was confirmed.

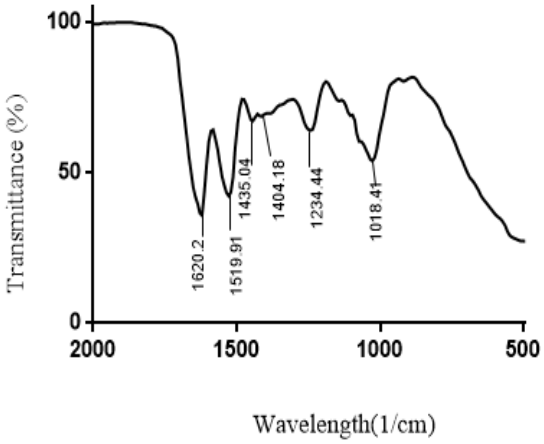


Figure 4: FTIR characterization of DMSF

In order to see the structural change in the dextrose modified silk film the FTIR of DMSF was carried out. The figure shows FTIR spectra of silk film loaded with 3g of dextrose. Here, the characteristics band specially

amide I had been shifted from the range 1620-1635cm^{-1} to 1612-1643 cm^{-1} where as other region were unaffected. It means the content of dextrose within the silk film has very less effect on the secondary structure of silk fibrion in crystalline region.

3.1. Functional Testing of films

The films prior to the cytotoxicity and in-vivo testing were subjected to 6 additional functional tests. The results of which are given below:

3.1.1. Hydrophilicity tests

The ability to up take fluids from the wound surrounding plays an important role in tissue engineering as well as in wound healing process(6). The absorption properties of wound dressing plays a crucial role in controlling the accumulation of wound exudates in a wound dressing thereby preventing the accumulation of bacteria.

3.1.1.1. Water Absorption Test

The material to be used as wound dressing should be efficient to absorb fluid for preventing the accumulation of exudates that can cause infection by the bacteria.

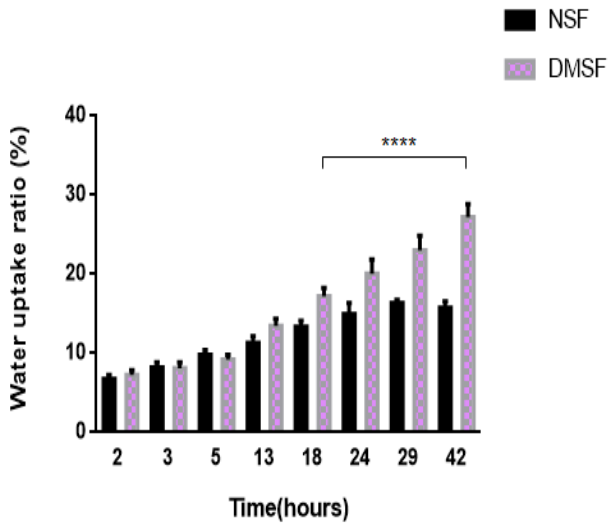


Figure 5: Water absorption (%) of NSF and DMSF

(Statistical differences was calculated using two-way Anova and Sidak's Multiple Comparision Test, Mean ±SD; n=5)

The water absorption ratio of both NSF and DMSF kept increasing gradually and in the 24th hour it increased significantly ($p<0.001$) in the case of the DMSF as shown in figure 5. In case of NSF the ratio was increasing in the same linear fashion and from the 29th hour it started to show a slight decrease.

Thus, the water absorption percentage of NSF was 15% and that of the DMSF was 27%.

The results of the water uptake ability of different NSF and DMSF showed that the films possessed a good hydration capability by their ability to absorb saline. This increase in absorption is due to highly hygroscopic property of dextrose molecules. In a study carried out by Srivastava et al. (6), it was found that the water absorption percentage of DMSF increased significantly upon the increase of the content of Dextrose. The NSF showed 15% of absorption while the films with 15% dextrose incorporated showed 63%.It can concluded that the higher the dextrose content, higher will be absorption.

3.1.1.2. *Contact Angle Measurement*

The results of the contact angles measurement were a basis for indicating the wettability of the films It was anticipated that the NSF and DMSF would differ in terms of wettability. When the contact angles were being measured, it was found that the water droplet on NSF was stable and the water droplet on the DMSF started to spread making it difficult to measure the contact angle.

The NSF showed water contact angle of 59.02 ±0.7°. DMSF showed the water contact angle of 45.15 ±0.3°. A significant reduction ($p< 0.001$) in the contact angles of NSF and DMSF was observed as shown in figure 6. This result showed that the surface of the NSF was successfully modified with the incorporation of dextrose and the wettability of NSF was significantly enhanced. This in accordance to various studies previously done on wettability of silk films and surface modified silk films (11).

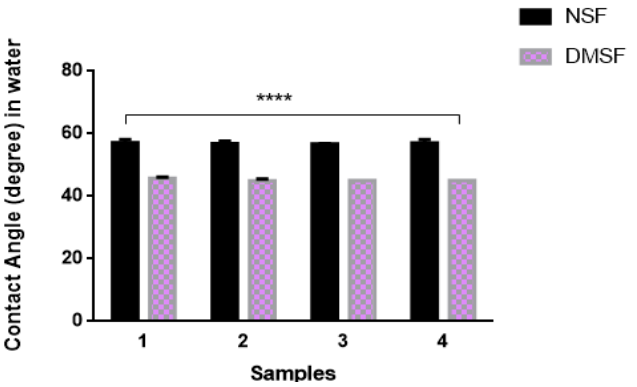


Figure 6: Water contact angle of NSF and DMSF

(Statistical differences calculated using two-way Anova and Tukey's Multiple Comparision Test, Mean ±SD; n=4)

3.3.1. Weight loss Test

In case of weight loss test, the weight of the films changed in an uneven pattern as shown in figure 7.

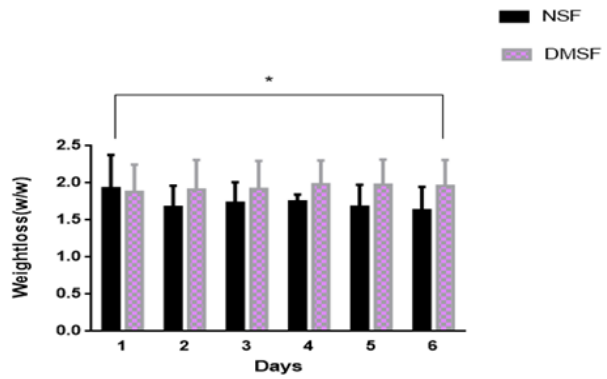


Figure 7: Weight loss in %(W/W) of NSF and DMSF

(Statistical differences calculated using two-way Anova and Tukey's Multiple Comparison Test, Mean ±SD; n=5)

A decrease ($p<0.05$) in weight of the NSF was observed at the very beginning followed by a slight increase and remained stable thereafter as shown in figure 7. While in case of DMSF an increase ($p<0.05$) in the weight was observed followed by stability in its weight from there on.

At the end of the experiment, NSF attributed to 1.7% weight loss while DMSF attributed to 1.9% weight loss from its original weight.

In this study, the weight loss by both of the silk films at different time interval was same. However, the total weight loss by dextrose modified silk film was slightly greater than that of normal silk film. Various factors are responsible for the weightloss such as the degumming time(12), soluble nature of Dextrose molecules.

3.3.2. Porosity Test

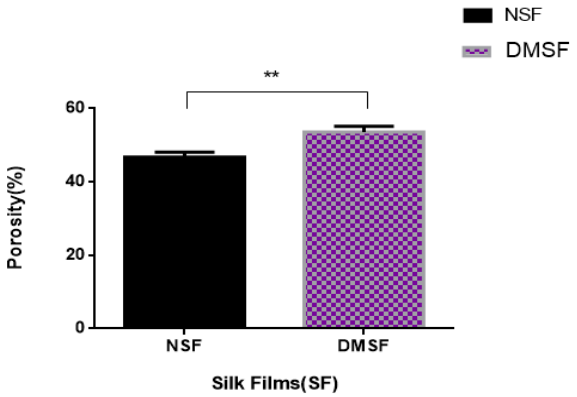


Figure 8: Porosity (%) of Silk films

(Statistical differences calculated using two-way Anova and Tukey's Multiple Comparision Test, Mean ±SD; n=5)

The percent porosity of NSF was found to be 46.8± 1.3% and that of DMSF was found to be 53.60 ± 1.5 %. The hydrophilic property of DMSF constituted to the increase in the porosity ($p<0.01$) of DMSF as shown in figure 8.

In regards to a study carried out by Rucksanti et al in Chiang Mai University (13), it was found that the modified porous films are appropriate for biomedical applications. The films that can absorb more are more porous. Hence, the results of the porosity test of the NSF and DMSF showed that with a slight increase, dextrose has a better porosity than NSF. This is due the better water absorption capacity of DMSF.

3.3.3. Drug Retain Test

The results of the drug-retain test is shown in figure 9. The UV-absorbance was different ($P<0.01$) of the NSF and DMSF in Day1.The absorbance decreased in a linear fashion until the 15th day in which they were equal. The drug retain ability by developed silk films loaded with Placentrex drug was analyzed by UV

spectrometer at 324nm. The porous nature of DMSF constituted to the low drug retaining ability of the DMSF. The retention capability also depended on the amount of drug coated(5).

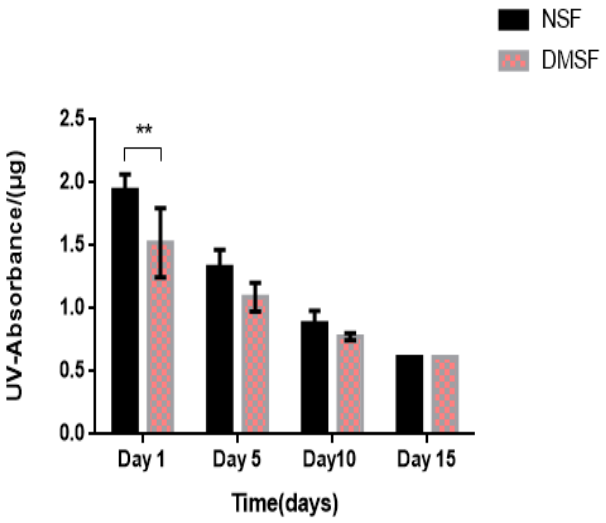


Figure 9: Drug retain ability of NSF and DMSF

(Statistical differences were calculated using two-way Anova and Sidak's Multiple Comparision Test, Mean ±SD; n=5)

3.3.4 Bovine Serum Albumin(BSA) Adsorption Test

The plasma protein adsorption of different silk films was evaluated by BSA adsorption technique. The graphical representation of the UV-absorbance of NSF and DMSF is shown in figure 10. The graph was plotted against the different concentrations of Bovine Serum Album. The amount of protein adsorbed in NSF was found to be lesser ($P<0.05$) than DMSF.

The result of different concentration of BSA adsorbed by NSF and DMSF within 24hrs showed that the amount of protein absorbed in DMSF was comparatively more than that of NSF. This is because of the hydrophilic nature and flexibility of DMSF. The presence of dextrose in silk film is intercalated between the polymer chains of silk fibroin.

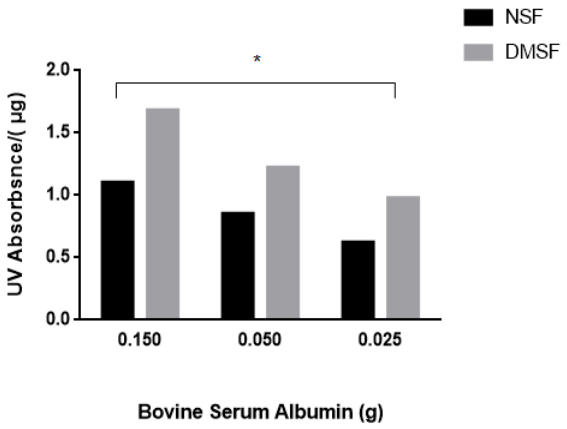


Figure 10: BSA adsorption in NSF and DMSF

(Statistical differences were calculated using two-way Anova and Tukey's Multiple Comparision Test, Mean ±SD; n=3)

As a result, the interchain space increases with increase in flexibility of silk film. The interaction of DMSF with the water molecules forms a hydrated chain. Thus formed hydrated chain can affect the composition of blood and the flexibility of DMSF. This somehow blocks the microsurface protein, resulting in the adhesion of plasma protein(14). The higher bovine serum albumin adsorption on the films surface will lead to lower levels of fibrinogen adsorption thus limiting the formation of thrombus and embolism on surface(14).In light of good blood compatibility the films appealed to be a promising biocompatible material.

From the comparison of the functions between NSF and DMSF it was evident that dextrose modified films had enhanced properties in terms of hydrophilicity, porosity, protein adsorption capacity which would result into improved healing when used as a dressing material. Therefore, two more types of films (Drug coated SF and PRP coated SF) were prepared by incorporating dextrose in it.

3.4. Cytotoxicity Test

Hela cell growth was assayed by 5groups when subjected to MTT. The picture showing the adherence of hela cells forming the formazon crystals is showing in figure 12. It was found that the hela cells adhered

and spread on silk fibrion films. The highest percentage of cells viability was seen in drug-coated silk films which was 93.55%.All the other silk films had a cell viability % ranging from 84%-89%. The lowest cell viability was shown by the control group which was 78%. The result highlights the potential of using silk fibrion films in –vivo.

When the films were studied for cell viability, it was found that there was higher cells adhesion and proliferation onto DMSF film as compared to SF films. This can be explained on the basis of combined effect of increased hydrophilicity and wettability. The increased hydrophilicity and wettability of DMSF films due to dextrose contributes higher adhesion and proliferation of cells as compared to SF film. In addition, various researchers have studied that nanophase roughness is responsible for higher adhesion and proliferation of cells(10) .The surface roughness of DMSF films further helped in anchoring and proliferation of cells. In a study carried out by Nogueria et.al, (15)high affinity of endothelial cells to silk fibroin films was explored

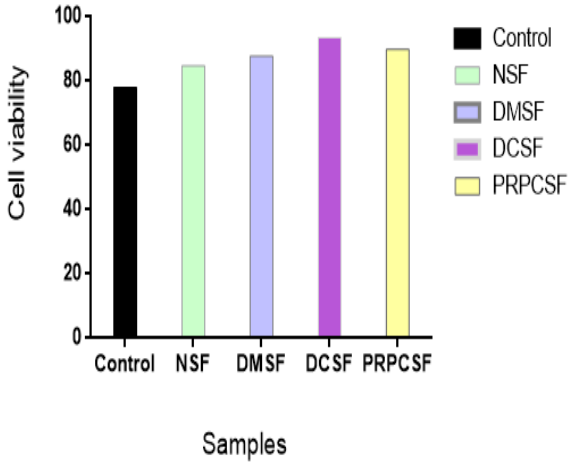


Figure 11: Cell viability of all 5 groups (Control, NSF, DMSF, Drug-coated SF, PRP-coated SF) assayed by cytotoxicity test (mean; n=3)

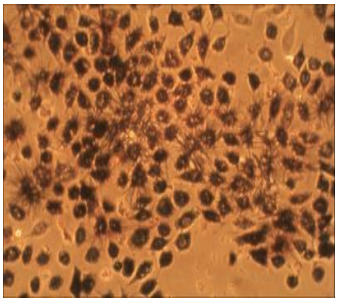


Figure 12: The production of crystals of formazon by Hela cells when subjected to MTT

3.5. In vivo Testing

Wound analysis

When the films were tested in the animal models, the results of the assessment of the histological slide and wound measurement were all in the favor of silk films.

Visual observance of the wound showed a gradual decrease in the wound size. The initial elliptic 1x1cm wound contracted forming an irregular shape having a decreased area. The different silk films and the Tegaderm dressing were applied on the excisional wounds. The length of wound was calculated with the help of Vernier Caliper on days 2nd, 4th, 6th, 8th and 10th and the extent of wound healing was calculated by comparing wound size at each time with the original wound size on day 0 as shown in the figure 17 below.

In terms of the wound area, a significant difference ($p<0.0001$) was observed in between the DMSF grouped animal models and PRPCSF animal model. Similarly, difference ($p<0.01$) in the wound area was observed between NSF-PRPCSF and DCSF-PRPCSF.Thus, a maximum reduction in the wound area was shown by PRP coated silk films from day 6 onward. Similarly, the drug-coated films and the DMSF showed comparatively similar effects in terms of wound-closure. In this study, the wound size had slightly increased up to 4th day with the formation of scab and after 4th day the wound had started to close.

The healing rate in terms of days of wound treated with the functionalized silk films was fast as compared to negative control In addition to DMSF placentrex drug enhanced the wound healing rate. Here, the PRP and the Placentrex played their parts.PRP speeded up the healing process by increasing the concentration of

growth factors in the wounded area. When used with silk, not just the growth factors from PRP were accumulated on the wound but the amino acids (GAGAGS) present on silk which boosted the healing process.

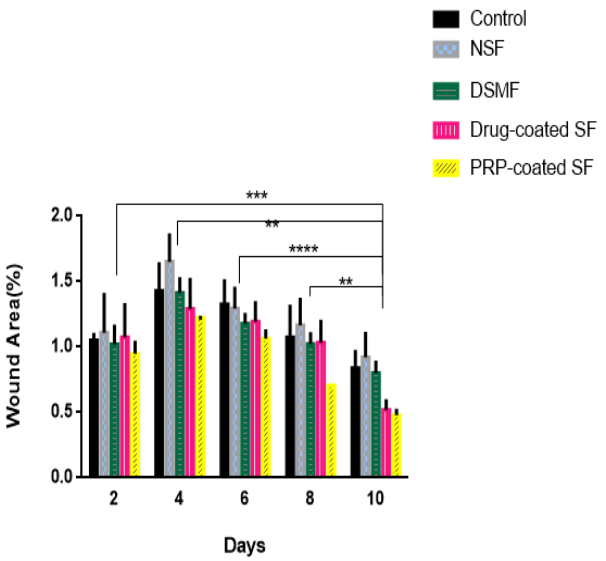


Figure 13: Wound area evaluation in different animal models

(Statistical differences were calculated using two-way Anova and Tukey's Multiple Comparision Test, Mean ±SD; n=5)

Similarly, Placentrex is a drug mainly used in healing of chronic-non healing wounds. When infused in silk, a protein polymer which is itself used as a healing material, their combined effects lead to a faster healing. Thus, the wound closure with the drug and PRP coated on DMSF was significantly greater than that of the wound closure with DMSF and NSF.

Table 4 shows the pictures of wounds of the animal models receiving different treatments at certain intervals.

Table 4: In vivo testing in animal models receiving 5 different treatments

(Tegaderm Tape, NSF, DMSF, Drug-coated Silk films, PRP coated Silk films) (figure on new page below)

3.7 Histological Slides Evaluation

The comparison of the wound healing rate with the application of normal and functionalized silk films over the Tegaderm dressing were analyzed through the histological slides at days 3rd, 6th and 9th.The histological slides of the specimens

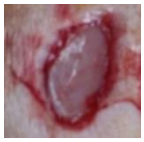
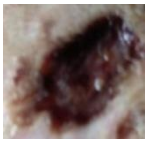
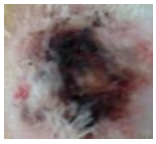
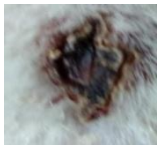
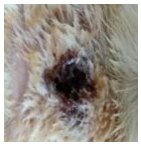
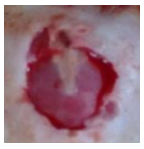
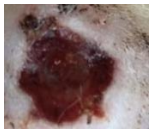
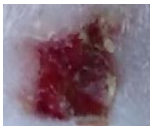
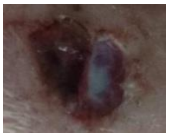
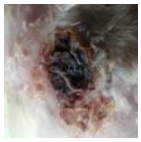
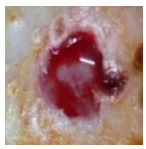
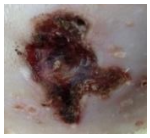
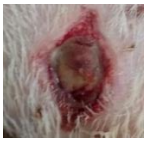
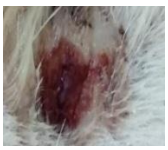
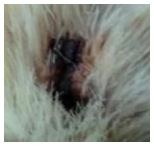
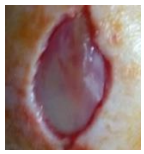
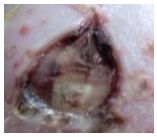
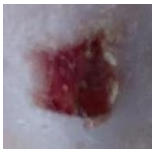
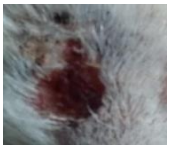
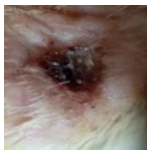
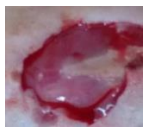
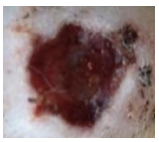
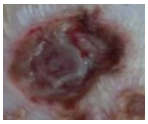
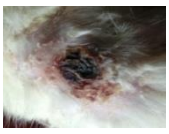
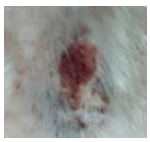
obtained from Biopsy samples showed the presence of various cells such as platelets, eosinophils, basophils, neutrophils, macrophages and lymphocytes in the phase of inflammation.

The healing was indicated by the presence of fibro collagen, formation of blood vessels and muscles, sweat and sebaceous glands along with the formation of hair follicle.

The comparison of the wound healing rate with the application of normal and functionalized silk films over the Tegaderm dressing were analyzed through the histological slides at days 3rd, 6th and 9th.The analysis showed reduced inflammation in the wound treated with different developed silk films and functionalized silk films. Whereas in the animal models treated with Tegaderm dressing, the inflammation was seen to be present up to 9th day. Also, the presence of ulcer was also observed in the histological slides of the negative control group. This may be as result of bacterial infection that the animal models had been prone to or different environmental factors such as the humidity and temperature. Compared to other groups, the DMSF coated with PRP had initially shown higher inflammation, which was found in the form of bundles in the histological slides. The infiltration of platelets along with neutrophils, macrophages and lymphocytes were present at the 3rd day of specimens of PRP-coated groups. As the time extended, the inflammation rate gradually decreased with the formation of fibro collagen, sweat duct, sebaceous duct and the proliferation of skin. The presence inflammation was confirmed by the presence of neutrophils and lymphocytes. Additionally, the PRP coated films had shown minimum scar formation at the time of wound closure. Similarly, the inflammation decreased with increase in growth of capillaries, formation of fibroblast and increment of collagen in the drug loaded silk film. The formation of sweat gland, sebaceous gland was comparatively lower in the Drug-coated films. The NSF and the DMSF also showed the presence and proliferation of cells such as neutrophils, monocytes and macrophages and the formation of muscle, collagen, but it was comparatively lower.

Table 4: In vivo testing in animal models receiving 5 different treatments

(Tegaderm Tape, NSF,DMSF,Drug-coated Silk films,PRP coated Silk films)

Days / Treatment	0	2	6	8	10
Tegaderm Tape					
NSF					
DMSF					
Drug-coated DMSF					
PRP-Coated DMSF					

4. CONCLUSION

Thus, the overall result indicates that the films act as a preventing barrier for wound infection and serves as a fine healing material. Hence the developed silk films may be used as a supporting material for wound dressing to avoid infections suggesting a promising potential for these silk-based biomaterials in wound healing. In conclusion, the result of this thesis demonstrates that silk as biomaterial, when used alone and in combination with monosachrides, drugs and PRP has the necessary prerequisites to become a benchmarked polymer for medical wound healing applications.

5.RECOMMENDATION

This research showed a promising result in-vivo. Some really interesting performances were obtained in the animal models and the healing rate in terms of days was fast. Hence, it is recommended to extent the research and try on a human setting as well.

This work has demonstrated that silk as a biomaterial is non-toxic, anti-bacterial, and anti-inflammatory. But, to find the exact mechanism behind such actions further studies needs to be done. So, further, a more detailed research in this aspect is recommended.

Silk is an emerging biomaterial in the biomedical engineering field playing humongous roles in areas such as wound healing, tissue engineering, and artificial skin grafting for years etc. It carries an immense potential to be used in nanotechnology as well as in drug-delivery systems where biocompatibility, mechanical stability, biodegradability plays a pivotal role.

The agro climatic condition of Nepal has a potential for Sericulture development, especially in yielding bivoltine cocoons and raw silk of international quality. Therefore, it is recommended study more about silk fibroin especially when utilized as films, foams or fibers as it may offer a 'new' alternative biomaterial for use as matrices in tissue engineering where mechanically robust, long-term degradable materials are needed.

6. ACKNOWLEDGEMENT

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