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Development and characterisation of biodegradable film from natural polymers

P Ganesan[a] & P Kanmani

Department of Textile Technology, PSG College of Technology, Coimbatore 641 004, India

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This study is focused on the preparation of silk fibroin based bio degradable films with the combination of various concentrations of chitosan (1:1 and 2:1), which is used as a drug releasing model. Scanning electronic microscope has been used to observe the morphology of prepared films and the chemical compounds are studied by Fourier transform infrared. Required properties for successful wound dressing such as water uptake %, thickness, folding endurance and water absorption capacity are examined. Also, the mechanical strength (tensile strength and elasticity), liquid culture test, % degradation, folding endurance and water uptakes of the films are analysed gravimetrically. Antimicrobial activity against *Escherichia coli* is evaluated quantitatively using Agar diffusion test (AATCC 100) and liquid culture test (BS EN ISO-14119, 2003). The obtained films are found homogenous without phase separation. The traces of both silk fibroin and chitosan are found evenly distributed in the film. Blend proportion of 2:1 shows rougher surface, and better results in term of antibacterial property are obtained against Gram negative bacteria *Escherichia coli* .

Keywords: Antimicrobial activity, Bio degradable film, Bio protein polymer, Chitosan, Fibroin, Silk, Wound healing

1 Introduction

Silk is a protein fibre and naturally produced by silkworm. Each silk fibre consists of two insoluble fibroin fibres which are coated by sericin glue-like protein[1-5]. Sericin can be dissolved in polar solution such as hot water and acid or base solutions. It comprises highly hydrophilic and bulky amino acids[3-8]. Fibroin is also insoluble and does not dissolve readily with many solvents, except in salt solution including lithium bromide (LiBr) and calcium chloride ($CaCl_2$). Silk fibroin is used for various applications, such as tissue engineering and drug delivery system. Silk fibroin shows excellent properties including high strength, biodegradability, biocompatibility and minimum inflammatory[3,6,8,9]. Silk fibroin shows characteristics of vibration bands in their FTIR spectra, including 1630–1650 cm^{-1} for amide I (C=O stretching), 1540–1520 cm^{-1} for amide II (secondary N\H bending) and 1270– 1230 cm^{-1} for amide III (C–N and N–H functionalities)[39]. Chitosan and silk fibroin blends with various proportions are used to develop biodegradable film for the purpose of quick wound healing[10,11]. Chitosan is a natural and copolymer of N- acetyl-D- glucosamine (Glc-NAc) and D-glucosamine (GlcN) that is produced by alkaline deacetylation of chitin[10-14]. Chitosan is a weak base and the pKa value of the D-glucosamine residue is approximately 6.2-7.0 (refs 11,15-17). Chitosan is insoluble in neutral and alkaline *p*H values, but is soluble in acidic media. Chitosan shows biodegradable, biocompatibile, absorption, non-toxic, antimicrobial properties and flexibility[13,18-20]. The reaction of chitosan is more versatile than cellulose due to the presence of NH_2 groups[19,21-24]. Chitosan is available in various forms, Silk has the properties of high strength and biocompatibility and it does not cause thrombosis when introduced into the body[25-29]. The aim of this study is to produce wound healing films using silk fibroin solution and chitosan powder.

2 Materials and Methods

Bombyx mori silk cocoon was obtained in raw form, from the farmers of Coimbatore district. Chitosan and dialysis was purchased from Hi Media Learning Bio Science Company, Mumbai. Sodium carbonate, lithium bromide, acetic acid, dialysis bag and polystyrene plate were obtained from M/s Merck. Deionized water was used in all experiments.

2.1 Preparation of Silk Fibroin Solution

Silk cocoons, cut into small pieces were boiled for 60 min separately. The mixture was treated separately with 300mL of 0.5% w/v aqueous solution of sodium carbonate at 90-100° C for 60 min. Silk fibres were washed with hot water till neutral *p*H, and dried

[a] Corresponding author.
E-mail: ganeshg007@gmail.com

overnight. The extracted silk was dissolved in 9.3M lithium bromide solutions at 60 °C and incubate for 4 h. This solution was dialyzed at 4°C for 3 days in distilled water using a dialysis bag for removing the salts. The solution was then subjected to centrifugation at 9000 rpm for 20 min at 4 °C. This procedure was carried out twice, finally filter the silk fibroin in the form of aqueous solution[3-5].

2.2 Preparation of Chitosan Solution

Chitosan solution of 1% (w/v) was prepared by mixing 1g of chitosan powder and 100mL of 2% acetic acid solution. The acetic acid acts as a plasticizer during film formation. The contents were then stirred using magnetic stirrer at room temperature until completely dissolved.

2.3 Preparation of Film

The silk fibroin / chitosan film was prepared by mixing 10mL of 1% (w/v) of chitosan and silk fibroin solutions homogeneously. The solution as poured on the polystyrene plates before drying in an oven at 40 °C for 3 days to obtain blended films. The films were carefully removed from the plate and stored in desiccators for future use.

2.4 Physical Characterisation of Film

Thickness (ASTM D-882)

The thickness of the fibroin / chitosan films was measured using micrometer with least count of 0.001mm prior to all the tests. The film thickness was measured using a micrometer at five locations (center and four corners) by reading accurately, and the mean thickness was calculated.

Weight of Film (ASTM D-882)

To determine weight uniformity of the each film, five specimens of the size 2.0 cm of all films were weighed on hi accuracy micro balance (Cole - palmer) and mean weight was calculated.

Swelling Ratio

The fibroin / chitosan film was cut in 1×1 cm size and dried in vacuum at room temperature for a week. After initial weighting, film was kept in a beaker with 50mL of distilled water at 37° C for 72h. Then the film was taken out after 72h carefully, and then excess amount of water was removed from the surface of the films with filter paper. Swelling ratio was calculated using the following equation:

$$\text{Swelling ratio \%} = [(W_S - W_D)/W_D] \times 100$$

where W_S is the weight of swollen films in g; and W_D, the weight of the dry films in g.

Folding Endurance

It is determined to find out the flexibility of film which is needed to handle the film easily and for comfortable and secured application of film on the wound. It is determined by repeatedly folding one film at same place till it breaks or folded up to 300 times manually. The number of times, a film could be folded at the same place without breaking gives the value of folding endurance.

2.5 Degradation Properties

The samples were weighed in dried condition (W_o) initially. Then they were immersed in a solvent for 1h using the area / volume ratio = 0.1 cm^{-1}. The samples were then removed from the medium and dried at 40°C. Final weight (W_f) was noted after drying the samples[11]. The degradation index (D_i) was calculated based on the mass loss using the following equation:

$$\text{\% Degradation } (D_i) = W_o - W_f / W_f$$

where W_o is the initial weight of film; and W_f the final weight of film.

2.6 Tensile Strength and Elongation

Tensile strength and elongation at break % of the films were measured (ASTM D 638-03) by a Universal Testing Machine with a 10N load cell. Film with a dimension of 4 cm × 2 cm was made using the clamp distance as 1 cm and the top clamp was pulled at the rate of 50 mm / min after fixing the samples in between two clamps. Then the sample was pulled apart, and the force and elongation were measured upon breaking the samples.

2.7 Surface Characterization of Film

SEM study

The surface morphology of the silk fibroin and chitosan sample was studied by scanning electron microscope (JEOL, JSM-6390LV, Japan) with an accelerating voltage of 20 kV after gold coating.

FTIR Study

The functional groups of silk fibroin, chitosan and their interaction were studied using Fourier-transform infrared (FTIR) spectra (Perkin–Elmer India Pvt. Ltd). The IR spectra in the absorbance mode were obtained in the spectral regions of 400 - 4000 cm^{-1}. Each spectrum of the samples was acquired by accumulation of 32 scans with a resolution of 4 cm^{-1}.

2.7 Bio Evaluation of Film

Liquid culture test (optical density measurements) (BS EN ISO – 14119, 2003) was used for bio evaluation of film. In this liquid culture test, each film was cut into squares (1cm^2). Two samples were immersed in 20mL nutrient broth in a 25mL universal bottle. The medium was inoculated separately with 200l of *E. coli* / *S. aureus* in its late exponential phase, then transferred to an orbital shaker and finally rotated at 37° C and 200 rpm. The culture samples were periodically measured for the microbial growth profiles during the incubation time (0, 2, 4, 8, 12, 24 h). The optical density (O.D. 600) was measured at 600nm using a spectrophotometer.

2.8 Anti-microbial Test

Agar diffusion method (AATCC 100 quantitative method) was used and 50mL of nutrient broth (13g for 1000mL) was prepared and autoclaved. Then *Escherichia coli* was inoculated in the media and incubated for overnight.

In Agar preparation method, 1g of Agar powder and 13g of nutrient broth were mixed and autoclaved at 100 ^oC. Three petri dish and cotton swabs were also autoclaved. The Agar media was poured into the petri dish and left for solidification. The *Escherichia coli* (Gram negative) was swabbed over the agar media. The positive and negative control samples were also tested and incubated for 24 h (refs 35-38).

3 Results and Discussion

3.1 Physical Properties of Film

The physical properties of the films prepared in different ratio are shown in Table 1. The thickness of the film with 2:1 blend proportion (chitosan and silk fibroin) is 0.12 mm and the weight of the film is 0.294 g. This ratio shows higher thickness and weight due to the high amount of chitosan blended with fibroin. The swelling ratio of chitosan and silk fibroin (2:1 ratio) increases slightly due to high humidity absorption character of chitosan content. The 2:1 blend ratio shows the higher number of folding endurance due to the concentration of chitosan. The increase in degradation properties of silk fibroin and chitosan blend films is observed when the chitosan content is increased. This is because the chitosan has its own inherent behaviour to be soluble in water or other moisture related characteristics.

3.2 Mechanical Properties of Film

The tensile strength and elongation at break % are summarized in Table 1. It is found that the tensile strength is higher for 2:1 blends than for 1:1 blends due to increase in chitosan content. The elongation % also increases in 2:1 blend ratios, as the concentration of chitosan solution and acetic acid give the flexibility of the material.

3.3 Film Appearance and Surface Morphology

The appearance of the silk fibroin and chitosan blend films is found uniform yellowish, and strong enough to withstand deformation (Fig. 1). This observation is conducted to examine the surface and internal micro architectures of porous chitosan and silk fibroin film. It is clearly observed from SEM that the film is porous and the pores are most suitable for the uptake of exudates, which is essential for wound healing process. Figure 2 shows SEM micrographs of the prepared silk fibroin and chitosan film.

3.4 FTIR Analysis

The FTIR spectra of pure silk fibroin show the absorption peaks at 3375.78 and 3360.00 cm^{-1}, thus conforming the presence of carboxylic group (O-H) in

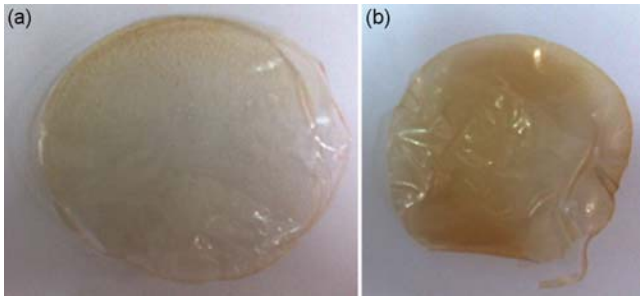

Fig. 1 — Chitosan and silk fibroin blended film (a) 1:1 and (b) 2:1 ratio

Table 1 — Physical and mechanical properties of silk fibroin chitosan film

Chitosan & silk fibroin ratio	Physical properties					Mechanical properties	
	Average thickness mm	Average weight of Film g	Folding endurance No of times	% Degradation	Average swelling ratio %	Average tensile strength MPa	Elongation at break %
1 : 1	0.07	0.1262	170	62.5	40	52.4	6.4
2 : 1	0.12	0.294	215	78	49	58.3	14.4

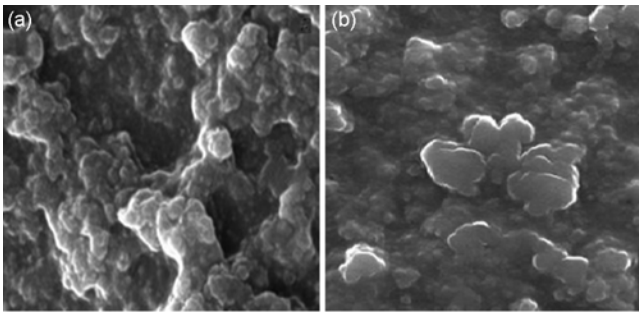

Fig. 2 — SEM images of chitosan and fibroin blend film (a) 1:1 and (b) 2:1 (magnification × 100)

the main structure of silk fibroin. The peaks at 829.39 cm^{-1} (C-H bend Para) and 729.09 cm^{-1} (C-H bend Ortho) show the presence of aromatics in the structure. These observation indicates the trace of silk fibroin [29-30,39].

The FTIR spectra of silk fibroin and chitosan films (1:1) show peaks at 3379.29 and 1199.72 cm^{-1}, indicating the presence of alcoholic group (O-H) stretch attached with the side chain while the absorption peak at 1311.59 cm^{-1} shows the presence of C-N stretch (aryl amines), may be due to the presence of primary and secondary amines in the chitosan and fibroin structures. The absorption peak at 1357.89, 1153.43, 1118.71 and 1083.99 cm^{-1} conform the presence of amine groups. The same absorption peaks can be seen in both 1:1 and 2:1 blend ratios. Based on the results, it has been concluded that both the samples have the traces of silk fibroin and chitosan [29-32,39].

The FTIR spectra of silk fibroin and chitosan blend films (2:1) show the absorption peaks at 3375.43 and 829.39 cm^{-1}, indicating the presence of alcoholic group in main chain of the structure. A peak at 729.09 cm^{-1} conforms the presence of alcoholic group. The peaks at 3360, 1627.92, 1357.89 and 1311.59 cm^{-1} conform the presence of aryl amine. This conforms that there are traces of fibroin [33, 34,39].

3.5 Bio Evaluation of Chitosan and Silk Fibroin Film

Figure 3 shows the antimicrobial activity of blended chitosan / fibroin film against *Staphylococcus aureus* and *Escherichia coli*. The antimicrobial activities of blended chitosan and fibroin film are found more effective against Gram-positive bacteria than against Gram-negative bacteria. In fact, one of the reasons for the antimicrobial property of chitosan, is that the positively charge amino group interacts with negatively charged microbial cell membranes.

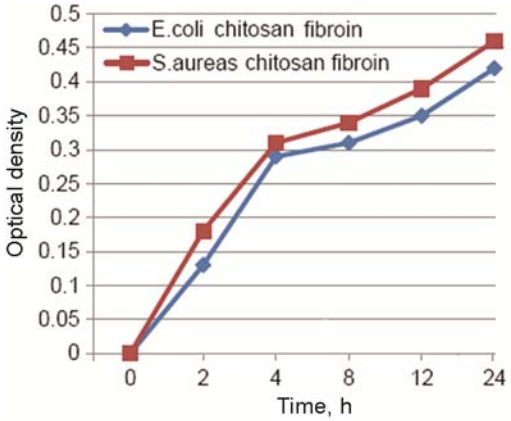

Fig. 3 — Liquid culture tests for chitosan and silk fibroin film

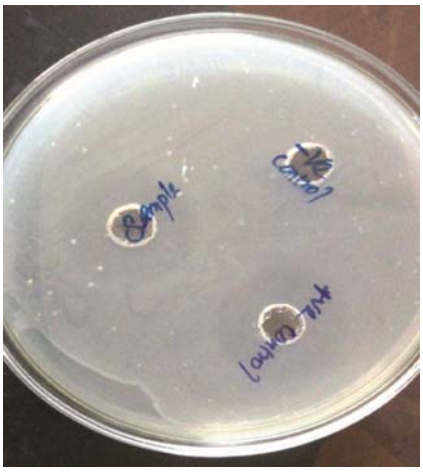

Fig. 4 — Antimicrobial activity of film (zone of inhibition in mm)

3.6 Antimicrobial Activity of Film

Figure 4 shows the antimicrobial activity (zone of inhibition) of the prepared film. The findings show antimicrobial activity against Gram negative bacteria (*Escherichia coli*). The prepared sample shows 26 mm zone of inhibition around the sample. The positive control shows 24 mm zone of inhibition and negative control shows no zone formation [36-38].

4 Conclusion

The FTIR results show the amine (N-H) and hydroxyl (O-H) groups well dispersed in the films. The absorption peaks conform the traces of both silk fibroin and chitosan in the film. The silk fibroin and chitosan blended film could be prepared by simple evaporation technique. The obtained blend films are found homogeneous without phase separation. SEM shows that the silk fibroin and chitosan are bound evenly distributed on the surface of film. The SEM micrographs also show rougher surface for 2:1 blend

proportion than for 1:1 blend ratio. The physical and mechanical properties are studied as per the standards. From the liquid culture tests and antimicrobial activity evaluation, better results are obtained against the Gram negative bacteria, such as *Escherichia coli*.

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