International Journal of PharmTech Research
CODEN (USA): IJPRIF ISSN : 0974-4304
Vol.5, No.4, pp 1774-1785, Oct-Dec 2013

Manuka Honey Loaded Chitosan Hydrogel Films for Wound Dressing Applications

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Abstract: A hydrogel film composed of chitosan and honey was developed using two different solvents namely acetic acid and lactic acid. Based on the Minimum Inhibition Concentration (MIC) value of Manuka honey (*Leptospermum scoparium*), two different concentrations of honey were experimented. The hydrogel film formation was effective in case of Chitosan - 1% Lactic acid - 6%Honey (CLH6), which also showed significant results in water vapour transmission and water absorption. This may be because of the hygroscopic nature of honey which helps in absorbing more water. The film also satisfies the strength and elongation requirements of a wound dressing with higher zone of inhibition against *Staphylococcus aureus* and *Escherichia coli*, the most wound infecting bacteria. CLH6 film was also soft, flexible and exhibits its potential to be used as a wound dressing.
Keywords: Hydrogel film, *Leptospermum scoparium*, Chitosan, Lactic acid, Wound dressing.

Introduction

Several types of wound dressings have been commercialized in recent years and they possess certain important limitations like incorporation of antimicrobial agents, the antimicrobial agents have cytotoxic effects leading to delayed wound healing and several dressings adhere to the surface of the wound and damage the newly formed epithelium. Nowadays bacterial resistance to the antimicrobial agents poses a very serious threat to public health[1,2,3,4]. Therefore, alternative antimicrobial strategies are urgently needed, and thus this situation has led to a re-evaluation of the therapeutic use of ancient remedies, such as plants and plant-based products, including honey[5,6,7]. At present, even though large variety of honeys are marketed, *Leptospermum scoparium* (*L.scoparium*) honey, which is light and heat stable, has been reported to have an inhibitory effect on around 60 bacteria species including aerobes and anaerobes, gram-positives and gram-negatives[8-16].

Cellulose, chitin, chitosan and gelatin are widely used natural polymers in regenerative medicine, implantable materials, controlled release carriers or scaffolds for tissue engineering[17]. Chitosan, one of the most abundant polysaccharides found in skeletal materials of crustaceans cuticles of insects, and cell walls of various fungi, is prepared by N-deacetylation of chitin[18,19]. Chitosan is biodegradable[20], biocompatible[21,22], non toxic and exhibits bioadhesive characteristics[23,24].

Chitosan is used in wound dressings because of its decreased wound healing times and improved tissue organization[25]. It is soluble in dilute organic acids[26], allowing the hydroxyl and amino groups to be utilized in chemical reactions. The amino groups in chitosan become protonated, which results in a positively charged polysaccharide that can attract and promote cell adhesion. Hence chitosan has shown to facilitate wound healing, reduce serum cholesterol levels and stimulate the immune system[27,28]. Since chitosan degrades before melting, it is necessary to dissolve it in an appropriate solvent before casting into films. It is preferable to dissolve chitosan in acetic acid/lactic acid due to its non-toxicity and ease of removal[29]. The properties of chitosan film depends on the solvent used, morphology, which is effected by molecular weight, degree of N-acetylation, solvent evaporation, and free amine regenerating mechanism[30]. It exhibits antimicrobial activity against bacteria[31,32], Fungi[33],and has rapid blood clotting property[34]. It has reported that chitosan is used in wound healing[35-38]. It would be more effective in hydrogel form for chitosan to protect and contract the wound in a suitably moist healing environment[39].

Materials And Methods

Materials

Chitosan was purchased from Sigma Aldrich Chemicals, Ltd (Bangalore, India). Acetic Acid was purchased from HI-PURE chem industries, (Chennai, India) and Manuka honey was obtained from MediHoney, Canada, Derma Sciences Inc. Viscose nonwoven was obtained from Birla Viscose, Birla Cellulose.

Chitosan and Chitosan-Honey film preparation Method

The films were prepared by modifying the method described by Bai-Shuan Liu et al. using casting technique. Briefly, a chitosan aqueous solution of 2% (w/v) was prepared in distilled water that contained two different solvents 1% (w/v) acetic acid and 1%(w/v) lactic acid. The solutions were prepared by mixing overnight. Then 5ml of 5% sodium bicarbonate was added dropwise. The resulting solution was filtered, left to stand until the air bubbles disappeared, casting onto the petri dish and dried in an oven at 40º C for 24 hours.

Chitosan-Honey films were prepared by adding 6% & 10% honey to the above solution before pouring the solution onto the glass plate. The prepared films (Figure 1& Table 1) were stored in an airtight glass container at 25±1 ºC and relative humidity 60-65% until further use.

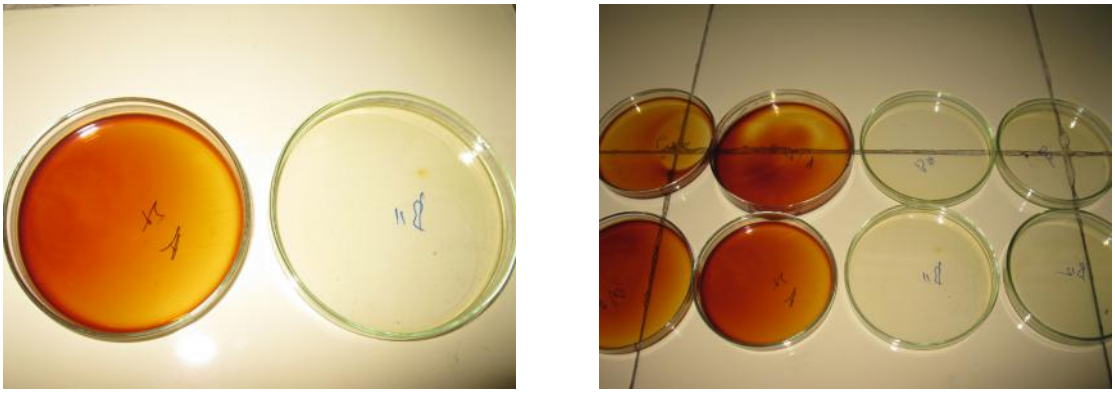

Figure 1 a) Prepared samples of Blank Chitosan and b) Chitosan- Honey Film

Table 1 Different sample proportions selected for experiment

S.No.	Sample	Sample Proportion
1	CA	Chitosan- 1% Acetic Acid
2	CL	Chitosan- 1% Lactic Acid
3	CAH6	Chitosan- 1% Acetic Acid- 6% Honey
4	CLH6	Chitosan- 1% Lactic Acid- 6% Honey
5	CAH10	Chitosan- 1% Acetic Acid- 10% Honey
6	CLH10	Chitosan- 1% Lactic Acid- 10% Honey

Characterization of chitosan – honey films

Thickness

A micrometer was used to measure the thickness of the film with least count of 0.001 mm prior to all the tests. Film strips in specific dimensions and free from air or bubble or physical imperfection were held between two clamps and the thickness of the film sample was measured using a micrometer at five locations (center and four corners), and the mean thickness was calculated. Samples with air bubbles, nicks or tears and having mean thickness variation of greater than 5% were excluded from the analysis.

Weight of the film

To determine weight uniformity of the each film, five specimens of size 2.0 cm x 2.0 cm of all films were weighed on electronic balance and mean weight was calculated.

Folding endurance

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

Degradation properties

All the dried samples were weighed (Wo) before the experiment. Then the samples were immersed in solvents for different time periods using the area/volume ratio = 0.1 cm^{-1} , following each immersion the sample were carefully removed from the medium and weighed after drying at 40°C and constant final weight (W_f) being verified. The degradation index (Di) was calculated based on the mass loss using the equation.

% Degradation = [(Wo-Wf)/Wf]x100

Water Vapour Transmission (ASTM E 96-95)

To measure the water vapour penetration, dessicant method was used. The films were cut and placed on top open bottles containing 5 gms. of silica gel and held in place with a screw lid (test area: 4.9 cm^2). The bottles were conditioned in a desicator containing silica gel for 12 hours. The bottles were then placed in a desicator containing NaCl at 30° C (75% relative humidity). The equilibrium vapour penetration was determined by weighing the bottles at 6, 12 and 24 hours, respectively.

The water vapour Transmission (WVT) was calculated as follows:

$$\text{WVT in g./hr.m}^2 = G/t.A$$

Where, G- Change in weight of Silica gel(gms)

t- Time during which G occurred

A-Test Area (m^2)

Swelling ratio

The water uptake was assessed gravimetrically. The weights of the completely dried films were determined with an analytical balance. Strips of chitosan and chitosan-honey films (2x 2 cm^2) were immersed in deionized water at 37° C in an incubator for 12 and 24 hours. The resultant swollen film was gently blotted with filter paper to remove excess surface water and weighed again. The water uptake of the film is the increase in weight, expressed as a percentage.

The water uptake of different samples was calculated using the following method:

Water uptake (%) = 100 x $(W_2-W_1)/W_1$

Where,

W_1 is the weight of completely dried sample

W_2 is the weight of swelled sample at 37° C for 12 hrs, 24 hrs.

Mechanical Properties

Tensile Strength and % Elongation (ASTM D 882-12)

Tensile strength was evaluated using an Instron Universal Testing instrument (Model 4206, Instron Ltd., Japan) with a 2 kg load cell. Film of the required dimension without any air bubbles or physical imperfections was held between two clamps positioned at a distance of 3 cm. During the measurement, the top clamp was pulled at a rate of 100 mm/minutes and the force and elongation were measured upon breaking the films. The results from film sample that broke down between the clamps were used. Measurements were run in triplicate for each film. Tensile strength and percent elongation were calculated by applying the following equations:

Tensile strength = Force at break (N)/ Initial cross sectional area of the sample(mm^2)

Elongation % = Increase in length at breaking point mm/ Initial length mm x100 %

Bioevaluation Properties

Antimicrobial property -- Agar diffusion test (SN 195920: 1992)

Plate Count Agar (PCA) plate was prepared and 100 µl of the selected dilutions of respective bacterial cultures were spread plated in duplicate. The wound care product with the diameter of 2cm ± 0.1cm was taken for the analysis. Both the sides of samples were pre sterilized under ultra violet radiation for 15 minutes. Sterile bacteriostasis agar was dispensed in sterile petridishes. Broth cultures (24 hours) of the test organisms were used as inoculum. Using sterile cotton swab, the test organisms (Escherichia coli, Pseudomonas aeruginosa & Staphylococcus aureus, Bacillus Subtilis & Fungi- Candida) were swabbed over the surface of the agar plate. Pre sterilized samples were placed over the swabbed agar surface by using sterile spatula and forceps. After placing the samples, all the plates were incubated at 37ºC for 18 to 24 hours. After incubation the plates were examined for the zone of bacterial inhibition around the fabric sample. The size of the clear zone was used to evaluate the inhibitory effect of the fabric.

Microbial Penetration

The films ability to prevent microbial penetration was tested by placing the films on open 10-mL vials containing 5 mL of nutrient broth and held in place with a screw lid. The negative control was a vial closed with a tightly packed cotton ball, while the positive control was an open vial. The tested vials were placed in an open environment for 1 week. The cloudiness of the nutrient broth in any vial was recorded as microbial contamination.

Scanning Electron Microscope (SEM) Study

The morphology and surface topography of the film was examined by Scanning Electron Microscope. Spherical samples (5 mm to 2 mm) were mounted on the SEM sample stab using a double-sided sticking tape and then coated. The coated samples were observed under the SEM and photomicrographs of suitable magnifications were obtained.

Statistical analysis

Five readings were taken in all measurements and expressed as means ± standard deviations. Single factor analysis of variance (ANOVA) was employed to evaluate the statistical significance of the results. Statistical significance was associated with a probability $P<0.05$.

Results And Discussions

Physical Properties

Table 2 Physical properties of samples

Samples	Thickness (mm) (Mean± SD)	Weight (gms.) (Mean± SD)	Folding Endurance (Mean± SD)
CA	0.228±0.024	0.142±0.035	161.4±8.6
CL	0.222±0.029	0.174±0.054	155.2±10.9
CAH6	0.230±0.027	0.260±0.071	204.4±25.0
CLH6	0.254±0.031	0.226±0.065	244.0±38.9
CAH10	0.260±0.032	0.252±0.064	179.4±35.1
CLH10	0.300±0.037	0.238±0.110	190.6±32.9

Table 2. shows the thickness of prepared films, ranged between 0.222-0.300 mm. To obtain the same thickness, same glass plate and equal volume of the prepared solutions were used and hence the deviation in thickness was minimized. Chitosan-honey films shows slightly higher thickness compared to blank chitosan films. The weight of 2cm x 2cm films were measured for all the films, which ranged between 0.142 gms. - 0.260 gms. Chitosan-Lactic Acid- 6% Honey showed maximum folding endurance of 244 compared to other films.

Degradation properties

Degradation % was calculated for chitosan and chitosan-honey films as shown in Figure 2. The films were soaked in solvent for 1hour and observed continuously. The mass loss was very rapid in the initial 30 mins and then minimum reduction was observed. It is observed that the degradation % increases with increase in honey content and slight increase is found in lactic acid films compared to acetic acid films. This shows that the degree of crosslinking reduced when the honey content is increased and the solution medium used for the immersion test influences the stability of the film.

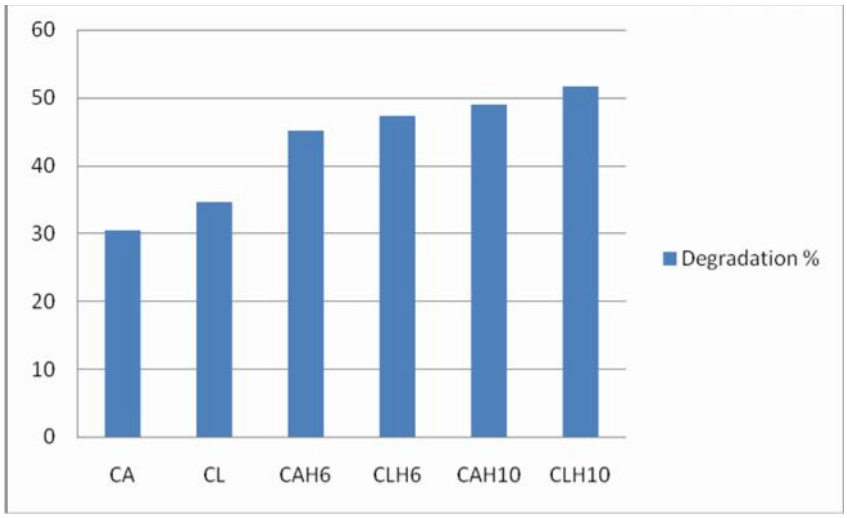

Figure 2 Degradation % of film samples

Transmission Properties

Water vapour Transmission

Permeability of moisture through the film for wound dressing is important to keep the wound moist and comfortable to help in the healing process. It was reported that the film must be permeable to the extent that a moisture exudates under dressing was maintained, preventing excess fluid absorption and evaporation leading to the desiccation of the wound bed[40].

Figure 3. represents the water vapor transmission(ASTM E 96-95) results of different formulation after 6,12 and 24 hours respectively. The vapor transmission was measured under steady-state conditions. Hence, the contribution of the moisture absorbed by the film can be considered negligible. The water vapour transmission for chitosan honey films ranges between 7000-11000 g /m^2 /day, which is comparable to the Opsite® wound dressing.

Thickness and water vapour transmission vary in inverse proportion, which was confirmed by the literature[41,42]. It can be seen that the rate of vapour penetration increases at a faster rate between 6-12 than 12-24 hrs. This trend was significant in transmission of almost all formulations. These results supported by Kim et al, who also mentioned water vapor penetration was significantly affected by degree of deacetylation of chitosan, solvent pH, and type of acid, which interacted significantly with each other.

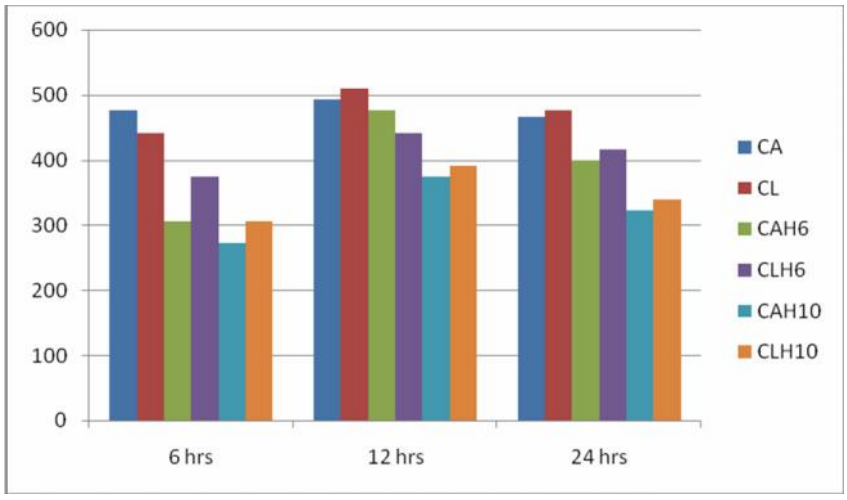

Figure 3 Water Vapour Transmission (g/m^2/h.) of the samples

Absorption properties

Swelling ratio

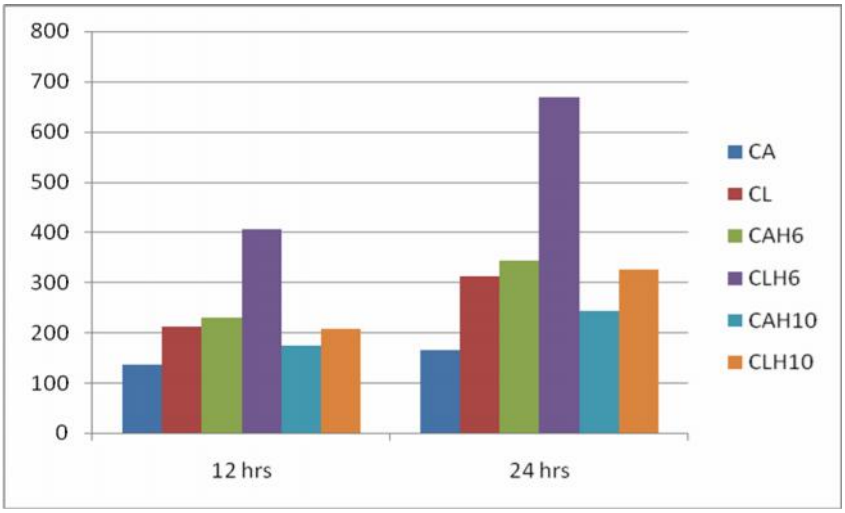

Figure 4. Swelling ratio of the samples

The result shows the average equilibrium water uptake of Chitosan films with different solvents and honey concentrations. The water absorption capacity of the films ranged from 135-668%. Water absorption capacity increased with the addition of honey but there is a significant decrease when the honey concentration reaches 10% with Acetic acid as solvent. These results are expected since the hydrophilic nature of the chitosan reduced on addition of acetic acid, as it reduces the amorphous region of the film.

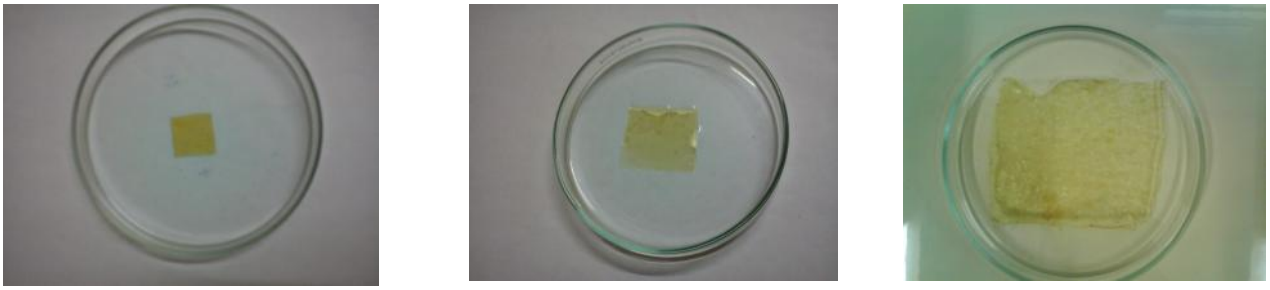

Figure 5 Appearance of the swollen film

Mechanical Properties

Tensile test and elongation %

The studies on tensile testing is an indication of tensile strength and elasticity of the prepared films. The films suitable for wound dressing should preferably strong but flexible. It was noted that the films with acetic acid shows significantly more strength but low elongation compared to the films with Lactic acid. That can be explained by the fact that lactic acid had one hydroxyl group instead of hydrogen in the structure compared with acetic acid, which induced electrolyte instability in the solutions. The intermolecular arrangement of chitosan in an aqueous solution is influenced by the peculiarity of acid solutions such as ionic strength and the degree of dissociation, molecular weight of chitosan dissolved in acetic acid was larger than that dissolved in the other acid solutions.

In acetic acid solution, chitosan forms dimers indicating that the intermolecular interaction is relatively strong, which suggests that the chitosan films prepared with acetic acid had tighter structure than those prepared with other acid solutions[43]. Hence it was expected that lactic acid acts as both solvent and plasticizer, chitosan-lactic acid films are more elastic. In the similar manner, the increase in honey content in the film reduces the strength and increases elongation at break[44].

Table 3 Tensile strength (N/mm^2) and Elongation % at break of the samples

Samples	Tensile Strength (N/mm^2)	Elongation at break %
CA	69.12	16.80
CL	43.16	34.17
CAH6	52.42	18.37
CLH6	38.00	28.99
CAH10	46.78	20.55
CLH10	37.23	26.92

Generally, it is known that there is an inverse relationship between tensile strength and elongation of biopolymer film. Though elongation values of chitosan films were much lower than those of commercial high density polyethylene (HDPE) or low density polyethylene (LDPE) films, their mechanical properties with higher TS and reasonable E values suggest a high potential for film use in medical and other applications[45].

Bioevaluation properties

Antimicrobial test- Agar Diffusion Test

The zone of inhibition with various films against S.aureus and E.coli was different in agar disc diffusion technique as shown in Table 4. Blank chitosan films shows minimum bacterial inhibition compared to chitosan honey film, which can be explained by the inherent antimicrobial property of chitosan. As the concentration of honey was increasing, the mean diameter of zone of inhibition was increased with chitosan honey films. The bacterial inhibition is higher in all the cases of *S.aureus* than *E.coli*. Hence it is clear that both Chitosan and Honey contributes to antibacterial property in prepared films and the addition of honey increases the zone of inhibition.

Table 4 Zone of Inhibition of the samples against *S.aureus* and *E.coli*

Samples	Zone of Inhibition(mm)	
	S.aureus	*E.coli*
CA	23	22
CL	22	-
CAH6	27	20
CLH6	29	24
CAH10	30	22
CLH10	31	25

As per the literature, honey is hygroscopic, which means that it can draw moisture out of the environment and dehydrate bacteria, and its high sugar content and low level pH can also prevent the microbes from growth[46].

Microbial Penetration Test

Figure 6 represents the microbial penetration test method. In the microbial penetration tests, the positive control tubes were tested to ensure that the nutrient broth was suitable for bacterial growth, while the negative control tubes were tested because it represents a condition free from intrinsic bacterial contamination. The results showed that the microbial contamination was not observed in all the formulation of chitosan and chitosan honey films covered tubes and the negative control tubes. Only the positive control tubes had bacterial contamination.

Figure 6. Microbial penetration test

This indicates that the developed composite films have good potential for use as wound dressing because of their ability to bind the negatively charged bacteria to the positively charged amino groups of the chitosan polymer by reducing the primary wound contamination because of their ability to protect the wound from secondary bacterial infection.

Morphological Studies by Scanning Electron Microscopy

Chitosan-lactic acid-6% honey film surface morphology was examined using Scanning Electron Microscopy. The dried film samples were mounted on a metal stub with double-sided adhesive tape .The morphological structures of the films were studied and the images were taken at accelerating voltage of 3 kV. The surface smoothness of the sample was at 1900X.

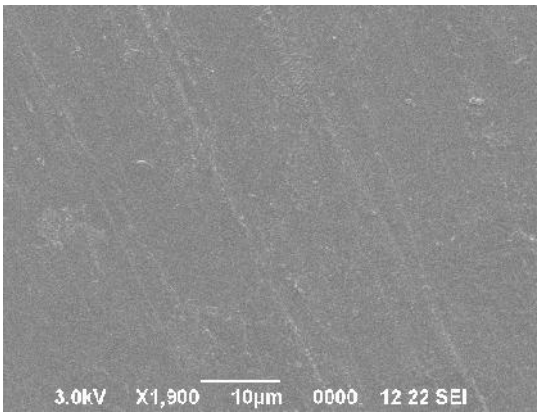

Figure 7 Scanning Electron Microscope image of the sample

Statistical Analysis

To identify the effect of variation in cross linking agent and addition of different concentrations of honey ANOVA were performed between the samples for all the characterization studies in specific reference to wound dressing. Even though the test results shows variation numerically between the sample, the statistically there is no significant difference between the chitosan honey samples ($p>0.05$). There was a significance difference between blank chitosan and chitosan-honey samples in almost all characterization studies ($p<0.05$).

Conclusion

From this study, it is concluded that the film produced from Chitosan-Lactic acid with 6% Honey satisfies all the requirements of wound dressings like thickness, weight, folding endurance and degradation. The film has water absorption of 668%, water vapour transmission of 9984g/m^2/day, tensile strength 38N/mm^2, elongation 28.99% and antimicrobial activity against *S.aureus* and *E.coli*, which also arrests microbes transmitting from outside environment to wound bed. Further investigations in chitosan-honey hydrogels will more precisely delineate the mechanisms in wound healing.

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