

Lactic Acid-Chitosan Films' Properties and Their *in vivo* Wound Healing Activity

T. S. Moe, T. A. Khaing

Abstract—Chitosan is a derivative of chitin, a compound usually isolated from the shells of some crustaceans such as crab, lobster and shrimp. It has biocompatible, biodegradable, and antimicrobial properties. To use these properties of chitosan in biomedical fields, chitosan films (1%, 2%, 3% and 4%) were prepared by using 1% lactic acid as solvent. The effects of chitosan films on tensile strength, elongation at break, degree of swelling, thickness, morphology, allergic and irritation reactions and antibacterial property were evaluated. *Staphylococcus aureus* and *Escherichia coli* were used as tested microorganisms. *In vivo* wound healing activities of chitosan films were investigated using mice model. As results, Chitosan films have similar appearance and good swelling properties and 4% chitosan film showed the better swelling activity and the greatest elongation ratio than the other chitosan films. They also showed their good activity of wound healing in mice model. Moreover, the results showed that the films did not produce any unwilling symptoms (allergy or irritation). In conclusion, it is evident that the chitosan film has the potentiality to use as wound healing biofilms in the biomedical fields.

Keywords—Chitosan, wound healing, antibacterial activity.

I. INTRODUCTION

CHITOSAN is derived from chitin by removal of most of the acetyl substituent on the copolymer usually by hydrolysis, to leave a copolymer having generally from 13 to 17 percent of N-acetyl glucosamine monomer units and correspondingly from 87 to 83 percent of glucosamine units in its structure [1].

Today, we know that chitin and chitosan (collectively called as chitinous substances) are found in abundance in nature and are renewable sources and this very fact has attracted much interest in developing new applications from these simple substances. In addition, chitinous polymers are biocompatible, biodegradable, non-toxic materials and this has expanded the possibility for the use of their derivatives in applications in medicine [2].

Chitin and its derivatives have many properties that make them attractive for a wide variety of applications from food, nutrition and cosmetics to biomedicine agriculture and environment. Their antibacterial, anti-fungal and anti-viral properties make them particularly useful for biomedical applications such as wound dressings, surgical sutures and as aids in cataract surgery and periodontal disease treatment [3]. Moreover, Chitosan's ability to be made into gels, films,

membranes, fibres and beads as well as powders, flakes or solutions has led to many commercial and biomedical applications [4].

In this study, we have showed the effectiveness of chitosan films on the wound healing properties. The effects of chitosan films on mechanical properties (tensile strength, elongation at break, degree of swelling, thickness), morphology, allergic and irritation reactions and antibacterial property were evaluated. *In vivo* wound healing assay suggested that the chitosan films were good in healing properties.

II. MATERIALS AND METHODS

A. Materials

Chitosan with degree of deacetylation (DD) of 85-95% was gifted from Department of Biotechnology, Kyaukse and all other chemicals used in this experiment were of analytical grade. *Staphylococcus aureus* and *Escherichia coli* provided from Department of Biotechnology, Mandalay Technological University were utilized in antibacterial test.

B. Preparation of Chitosan Film

Chitosan films were prepared by modifying the method of Sparkes. Briefly, 1%, 2%, 3%, 4% chitosan solution in 1% lactic acid were prepared respectively and stirred for 1-2 hours. 3 g of gelatin was dissolved in 10 ml of distilled water by warming. This warm solution was added to the above chitosan solutions. After thorough mixing, 7 ml of 5% aqueous sodium bicarbonate was added drop wise. The pH of the solution was ~ 6. Then 2 g of glycerol in 10 ml of distilled water were added. The mixture was stirred and allowed to stand for 5-10 minutes. The foam on top was then skimmed off and the solutions were poured into the trays and dried to get the films. To test the antibacterial activity, the aliquots of the chitosan solutions were also kept at 4°C [5].

C. Determination of Degree of Swelling

The films to be tested were cut into 1" x 1" size with 0.25 mm thickness. The film pieces were immersed in distilled water at room temperature for 24 hours. The swollen gel form of the film pieces were pressed with cellulose paper to remove the excess water on the surface of the films and weighed accurately. Then the swollen gel films were dried at 70 °C in an oven until they reached the constant weight and the weights were determined. The equation for the calculation of degree of swelling is shown in below [6].

$$\text{Degree of Swelling (\%)} = (W_a - W_b) / W_b \times 100$$

T. S. Moe and T. A. Khaing are with the Department of Biotechnology, Mandalay Technological University, Myanmar (e-mail: thesumoe@gmail.com, tinayekhai2009@gmail.com).

where

Wa = Weight of chitosan film at swollen state

Wb = Weight of chitosan film at dried state

D. Measuring the Tensile Strength and Elongation

Both ends of the test pieces were firmly clamped in the jaws of a testing machine. One jaw was fixed and the other was movable. The movable jaw moved at a rate of 10 inch/ minute. The resultant data was showed at the recorder. This procedure was repeated for three times for each result. The elongation (%) was calculated by using the following equation [7].

$$\text{Elongation (\%)} = \Delta L \times 100 / L$$

where

ΔL = different length

L = original length

E. Determination of Thickness

The thickness of the chitosan films was measured using slide clipper by reading accurately, changing different area of films thickness and calculated the mean values.

F. Scanning Electron Microscopic (SEM) Analysis

For the characterization of morphology, the scanning electron microscopic analysis of modified chitosan and chitosan films were examined and recorded. The SEM analysis was carried out at the University Research Centre (URC), University of Yangon, Yangon.

G. Antimicrobial Activity Assay

Antibacterial activity test against *Staphylococcus aureus* and *Escherichia coli* was performed by agar well diffusion method. The plates were filled with Muller-Hinton agar medium. Single colony from the stock culture was streaked evenly in 6 directions over the entire surface of agar plate with a sterile wire loop to obtain uniform inoculums. After the plate was inoculated, 6-mm wells were made on the Muller-Hinton agar medium by using a punch. Four different concentrations of chitosan stored at 4°C were introduced into each well. The control solution was prepared by using the previously mentioned chitosan solution preparation method without containing the chitosan. This control solution was used as the negative control. Then the plates were placed in the incubator at 35°C for 18 hours. After 18 hours of incubation, the plates were examined and the diameter of each zone of complete inhibition were measured and recorded in millimeter by ruler [8].

H. In vivo Wound Healing Activity Assay

The following procedure was based on [9]. Mice (*Mus musculus*) of both sexes weighing 40 g were used in this experiment. After shaving on the back, mice were anaesthetized with chloroform. Then superficial round wounds (about 8mm diameter) were prepared on the upper back of each mouse using a sharp pair of surgical scissors and forceps. The wound of each mouse was treated with the chitosan films

and modified chitosan gel. Commercially available product (Bioplacenton) was used as positive control and methylated spirit was used as negative control.

I. Allergic and Irritation Test

The chitosan films were pasted to the normal skin of the arms of 9 human volunteers. The volunteers were carefully observed for the development of untoward symptoms such as irritation, itchiness and scratching.

III. RESULTS AND DISCUSSION

A. Appearance

All the chitosan films made were similar in appearance and the rigid with light transparency appears in yellow colour, and it can withstand the bending or folding without damages.

B. Degree of Swelling

The degree of swelling is intimately related to the material strength of the chitosan films. Thus, in the wound dressing film preparation and applications, the swelling condition was predetermined by the application requirements. Table I shows the degree of swelling (%) of the four different concentrations of the chitosan films in solvent lactic acid after 24 hour. As shown in the Fig. 1, 4% chitosan film absorbs more water than any other chitosan films. It was found that the degree of swelling increased with the increase in chitosan concentration. This is in agreement with the explanation by Mahlous, M., et al, saying that the higher the chitosan concentration, the higher the equilibrium swelling ratio [10].

Sample ID	Degree of Swelling (%)
1% chitosan	1324.379
2% chitosan	1324.819
3% chitosan	1401.713
4% chitosan	1445.710

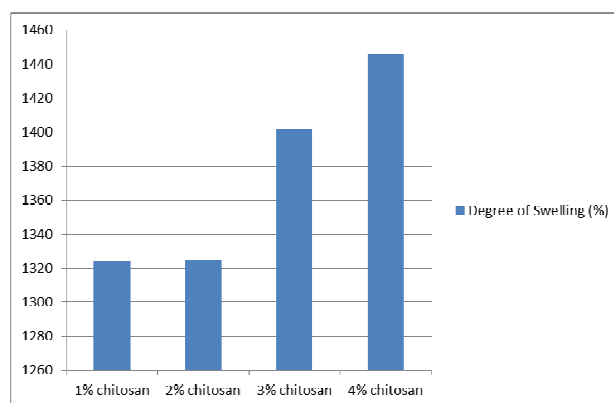


Fig. 1 Degree of swelling (%) of four different concentrations of chitosan films prepared with lactic acid.

C. Tensile Strength

It should be born in mind that evaluation of the mechanical properties of chitosan films is essential for application as

medical devices. In addition the mechanical characteristics of a material should be determined of which it is often necessary to improve them to make the material suitable for the desired application.

The tensile strength of the chitosan films are shown in Table II. The tensile strength of 1%, 2% and 3% chitosan films prepared was not significantly very different. But the maximum value of tensile strength, 5.5960 lbf was found with 4% chitosan films and this 4% film has highest tensile strength if we compare with the other films (Fig. 2).

TABLE II
MECHANICAL PROPERTIES OF CHITOSAN FILMS (TENSILE STRENGTH AND THICKNESS)

Sample ID	Tensile Strength (lbf)	Thickness (mm)
1% chitosan	4.2533	~ 0.25
2% chitosan	4.1980	~ 0.25
3% chitosan	4.1220	~ 0.25
4% chitosan	5.5960	~ 0.25

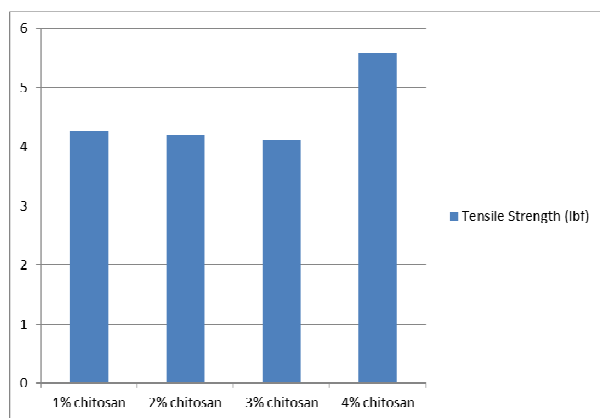


Fig. 2 Tensile strength of four different concentrations of chitosan films prepared with lactic acid.

D. Elongation

The elongation of the chitosan films are tabulated in Table III. The elongation % of 2%, 3% and 4% chitosan films were not greatly different. But the value of 1% chitosan film is significantly lower than that of the others and the elongation of 4 % chitosan has the highest value (Fig. 3).

TABLE III
MECHANICAL PROPERTIES OF CHITOSAN FILMS (ELONGATION AT BREAK (%))

Sample ID	Elongation at Break (%)
1% chitosan	54.875
2% chitosan	84.793
3% chitosan	88.358
4% chitosan	89.543

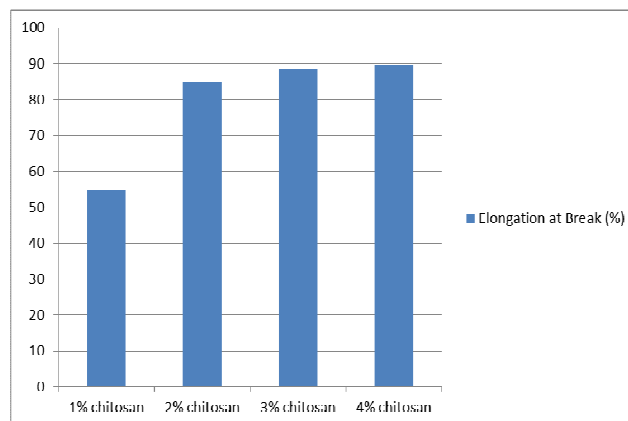


Fig. 3 Elongation at break (%) of four different concentrations of chitosan films prepared with lactic acid.

E. Thickness

To obtain the same thickness, the same tray and the same volume of the prepared chitosan solutions were used. Therefore, all the resultant chitosan films had the same thickness of ~ 0.25 mm. (Table II)

F. Allergy and Irritation

Allergic and irritation test were conducted with 1% to 4% chitosan films. As in the clinical deployment, any types of films in used must be safe, and the human sample test is dispensable. It was observed that the allergic or untoward symptoms did not occur in all volunteers.

G. Antimicrobial Activity

To detect the antimicrobial activity, four different concentrations of the chitosan stored at 4°C were used. The clear zone widths were observed to be varied from 10.75 to 12.42 mm in diameter (Table IV). However, the bacteria grew again after 7 days. No clear zone was observed between the control solution and the bacterial colonies. Therefore, it could be suggested that the chitosan has the antibacterial activity, especially bacteriostatic activity and it can be used as an antibacterial agent to prevent the infection for a certain time (Figs. 4 and 5).

TABLE IV
ANTIBACTERIAL ACTIVITY OF CHITOSAN AGAINST *S. AUREUS* AND *E. COLI*

Sample ID	Clear Zone Width (mm)	
	<i>S. aureus</i>	<i>E. coli</i>
1% chitosan	12.42	11.58
2% chitosan	11.58	11.44
3% chitosan	11.00	11.26
4% chitosan	10.75	11.03
Control solution	-	-

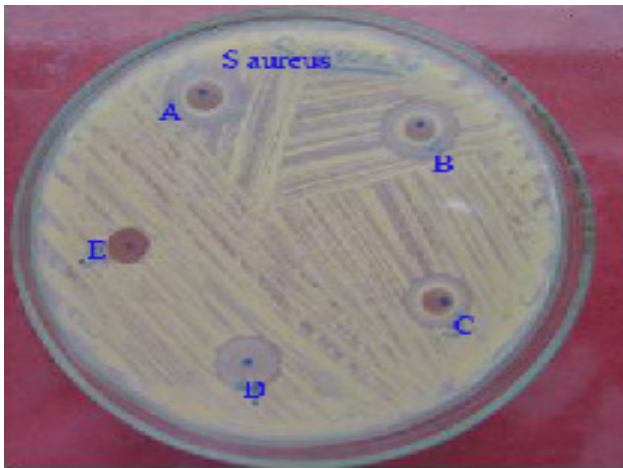


Fig. 4 Culture plate of *S. aureus* showing zones of inhibition caused by chitosan (A - 1% chitosan, B - 2% chitosan, C - 3% chitosan, D - 4% chitosan, E- control solution)

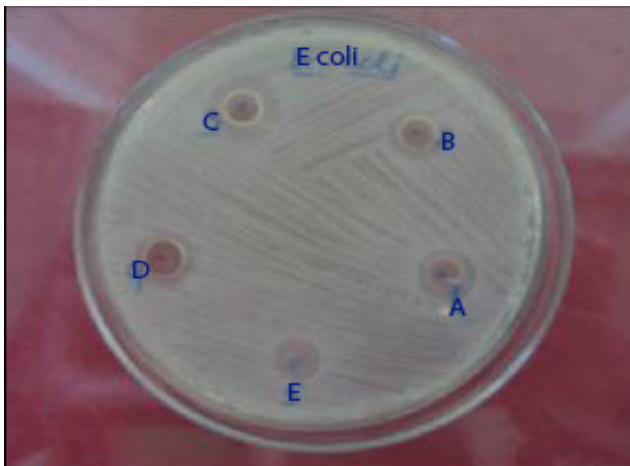


Fig. 5 Culture plate of *E. coli* showing zones of inhibition caused by chitosan (A - 1% chitosan, B - 2% chitosan, C - 3% chitosan, D - 4% chitosan, E- control solution)

H. Wound Healing Activity

The mice that were treated with four different concentrations of chitosan films have better healing result comparing to positive and negative control mice. Although 3% chitosan film has the best wound healing activity, it was observed that the higher the chitosan concentration, the better the healing results appeared for the other films prepared in 1%, 2% and 4% chitosan concentration. Figs. 6 (a), (b), and 7 show the wound of mice treated with chitosan films and their wound healing activity.

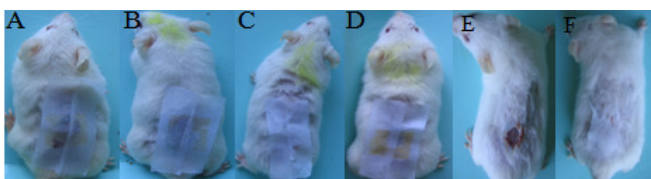


Fig. 6 (a) Representative mice after treatment with chitosan films (day 1)

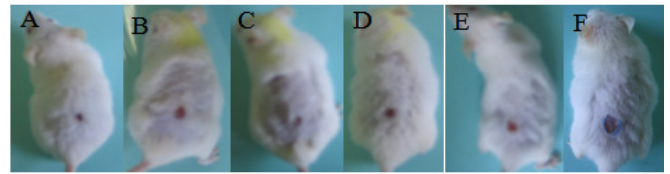


Fig. 6 (b) Representative mice after treatment with chitosan films (day 8) A - 1% Chitosan Film B - 2% Chitosan Film C - 3% Chitosan Film D - 4% Chitosan Film E - Positive control F - Negative control

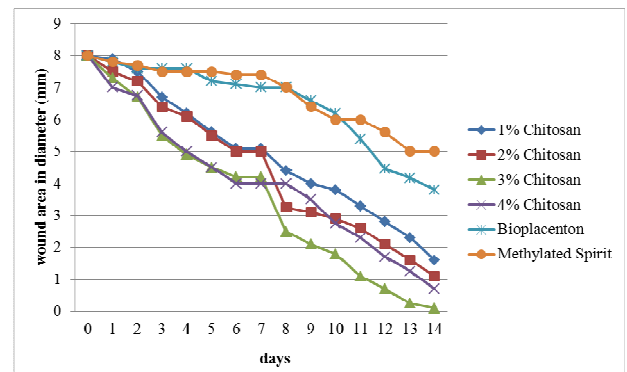


Fig. 7 *In vivo* wound healing activity of chitosan

I. SEM Examination on the Morphological Feature of Chitosan Films

According to the SEM photographs, in the chitosan films prepared with the lactic acid, it could be seen irregularly distributed chitosan on the films. As a result, chitosan in higher concentration of 4% was thoroughly distributed chitosan in the films (Figs. 8 (a)-(d)).

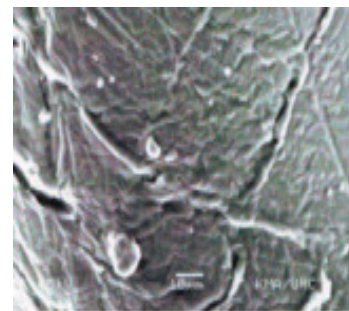


Fig. 8 (a) SEM photograph of 1% chitosan film prepared with lactic acid

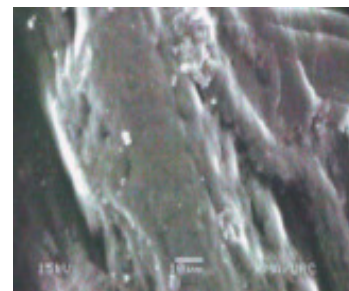


Fig. 8 (b) SEM photograph of 2% chitosan film prepared with lactic acid

ACKNOWLEDGMENT

We are very grateful to Mandalay Technological University (MTU), Ministry of Science and Technology for providing the facilities and support.

REFERENCES

- [1] Hayes, E. R.: N, O- Carboxymethyl Chitosan And Preparative Method, United State Patent No. 4, 619, 95, October 28, (1986).
- [2] Thatte M. R. Synthesis and Antimicrobial Assessment of water Soluble Hydrophobic Chitosan Derivatives Bearing Quaternary Ammonium Functionality, Ph.D Thesis, Agricultural and Mechanical College, Louisiana State University, (2004).
- [3] Chitin, SSS Biotic Pvt, Ltd, (2004)
- [4] Klokkevold, P. R: Effect of Poly-Acetyl- Glucosaminoglycan (Chitosan) On Osteogenesis *in vitro*, Master of Science in Oral Biology, University of California, Los Angeles, (1995).
- [5] Sparkes, B.G. and Murray, D.G.: Chitosan based Wound Dressing Materials. United State Patent No. 4,572,906, February 25, (1986).
- [6] Yoshii, F. et al.: Heat Resistance Polyvinyl Alcohol Hydrogel. Radiat Phys Chem 2: 169,(1995).
- [7] Than Than Aye. Hydrogel From Polyvinyl Alchol. Ph.D. Thesis. Yangon Technological University, Yangon (2002).
- [8] Schlegel, H.G.: General Microbiology. 7th Ed., Cambridge University Press, (1993).
- [9] Ishihara, M., et al.: Photocrosslinkable Chitosan as a Dressing for Wound Occlusion and Accelerator in Healing Process. Biomaterials 23, (2002)833-840.
- [10] Mahlous, M., et al. Radiation Synthesis of Antibacterial PVA/ Chitosan Hydrogel, Advances in Chitin Science-Volume X, Alp Ofset, Ankara, Turkey, (2007) 376-381.

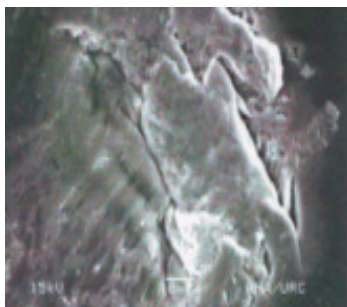


Fig. 8 (c) SEM photograph of 3% chitosan film prepared with lactic acid

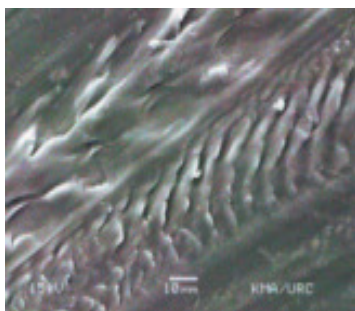


Fig. 8 (d) SEM photograph of 4% chitosan film prepared with lactic acid

IV. CONCLUSION

According to the cited publications, chitosan is famous for its effectiveness in different fields. Here, we had tried to confirm the effect of locally available chitosan on wound healing activity. The chitosan films were prepared by using four different concentrations and constant gelatin concentration. Lactic acid was used as the solvents to prepare the chitosan films. The chitosan films prepared has strong yellow color. The higher the concentration of chitosan, it was observed that the more tensile strength, elongation at break and thoroughly distributed of chitosan in SEM photographs. So it showed more wound healing activity than the others. The physical and mechanical properties of the chitosan films can be managed by changing the concentration of polymers and plasticizer to get the films that has the desired characteristics for the application of different purposes. Moreover, chitosan films exhibit the bacteriostatic activity and wound healing activity when these films are detected with the *in vitro* antimicrobial test and *in vivo* wound healing test using microbes and mice model respectively. It did not show any irritant reactions such as itchiness and skin Rash. Therefore, all the results suggest that the chitosan films could be useful for biomedical applications.