Preparation, Characterization And In Vitro Wound Healing Activity of Collagen-Chitosan Film

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Abstract- *A rise in the incidence of skin burns and chronic wounds present a first health care problem. Hence collagen – chitosan-based wound dressing has been seen as a way to heal wounds without leaving a scar. Collagen is the most ubiquitous protein found in marine organisms. Here, chitosan plays a vital role in improving the stability of the collagen. The Collagen-chitosan films comprised of collagen derived from skins of Catlacatla which were pretreated and lyophilized and confirmed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Thus, in this new approach collagen has been hybridized with the naturally occurring bioactive material chitosan to improve the mechanical properties with enhanced stability, and it can be effectively utilized for the wound dressing in clinical application. Physico-chemical properties and surface morphology of the films were analyzed through SEM and FTIR. Mechanical stability of collagen – chitosan film showed increased tensile strength and elongation at break. The Anti-bacterial and Anti-inflammatory activity of collagen - chitosan films were performed. Also, the invitrobiocompatibility of collagen chitosan films was confirmed by wound scratch assay on fibroblast cells. It was concluded that this present study reveals that the preparation of collagen-chitosan films from a new source of natural collagen could be suitable for the application of wound dressings.*

Keywords- Collagen, Chitosan, Film, Fish waste, Wound dressing

I. INTRODUCTION

Over 50 million tons of fishwaste are generated annually all over the world from fish processing operations (Kristinsson and Rasco, 2000). Hence there is enormous scope for utilization of these fish processing waste for the extraction of value added Bio-functional product like collagen (Välimaa*et al.*, 2019). Collagen is the most abundant protein found in fish waste which amounts about 5-30 % of the total protein (Pati*et al.*, 2010). Collagen maintains the structure and integrity of skin, muscles and bones, which plays a significant role in joint and bone health. Collagen is the most useful biomaterial because of its biocompatibility, biologicalcharacteristics such as skin elasticity, biodegradability and weak antigenicity make collagen the primary resource in a biomedical application (Cho *et al.*, 2014; Lee *et al.*, 2001). Collagen-based wound dressing also finds applications in the treatment of the chronic wound, ulcers and skin burns (Singh *et al*., 2011).

On the other side, biopolymer chitosan possesses properties such as nontoxicity, antimicrobial, biodegradability and ability to form films (Naja i *et al.*, 2014). Furthermore, stimulation of haemostasis and acceleration of tissue regeneration for wound healing are the significant benefits of chitosan (Ninan*et al.*, 2015). Hence blending of biopolymers collagen (cationic) and chitosan (anionic) creates new biomaterial with better properties for the healing of chronic wounds and burns (Indrani*et al.*, 2017).

According to WHO, about 10 lakh people die globally each year from fire accidents. In India, around 7 million people suffer from burn injuries. About 1.4 lakh people die due to burning injuries, and 2.4 lakh people have this disability (Dutta *et al*., 2017). Fires and other accidents related to burn injuries leaves a permanent scar, and physical therapy for those injuries is a time-consuming process for recovery and cure. Hence a new approach is therefore needed for overcoming chronic wounds and burns, this biocompatible collagen-chitosan film based wound dressing helps in cell growth and tissue regeneration of burned skins and recover without side effects since it is a natural collagen. Though there are many collagen-based wound healing treatment available, this is considered to be more economical and ecofriendly since it is utilized from waste sources. Thus the use of collagen – chitosan-based wound dressing is recognized as a prominent way to wound healing.

Thus the present study involves isolation and characterization of collagen from fish waste, preparation of the collagen-chitosan film, and investigates its wound healing property *in vitro f*ibroblast cell lines (L929 cell line).

II. MATERIALS AND METHODS

Collection of fish skin

The skin of the *Catlacatla*(*L.catla*) was obtained from the fish processing factory kasimedufish market, Royapuram, Chennai. Skins were peeled off, and then the samples were washed with the distilled water four times. Cleaned skins were cut into small pieces manually. The sample was frozen at -20 ◦C for further use.

Extraction of collagen

Extraction of collagen from fish skin was carried out following the protocol described by Xu *et al.* (2017).

The first pretreatment of fish skin was done to remove non-collagenous protein. Followed by pretreatment, the skin was washed with distilled water and soaked in 0.5 M acetic acid containing 1% (w/w) pepsin for 48 h to isolate the collagen. The supernatants were collected by centrifugation at 6000 RPM for 15 min. Ammonium sulphate was then added to the supernatant to achieve a final concentration of 0.7 M and dissolved again in 0.5 M aceticacid. The solution obtained was dialyzed against 0.05 M sodium acetate buffer for overnight 12 h. The dialysate was lyophilized by a freeze dryer at -60◦C for three days and stored at 4 ◦C until further use.

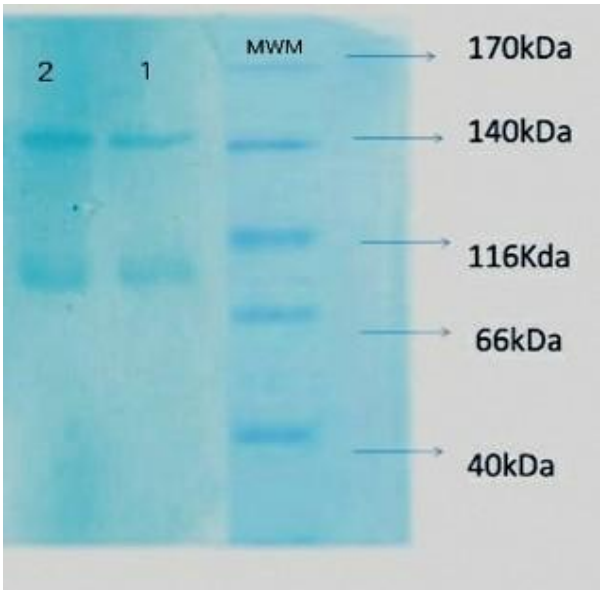

Figure 1: SDS-PAGE pattern of collagen; MKM– Molecular weight market, 1 & 2 – Collagen extracted from fish waste

Figure 2: A typical image of a collagen-chitosan film

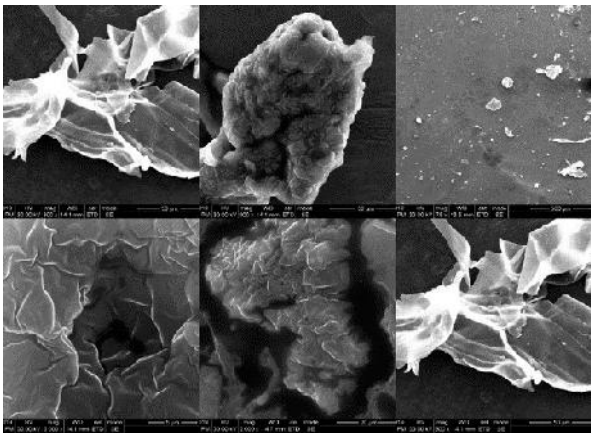

Figure 3: SEM images of collagen-chitosan films

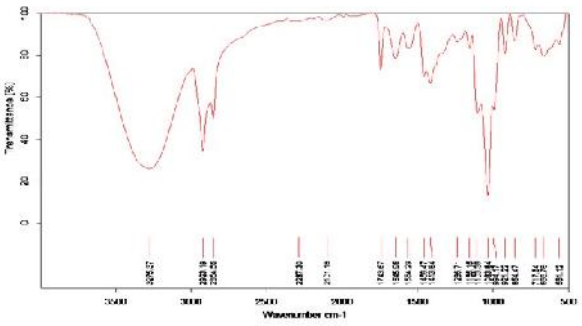

Figure 4: FTIR spectra of collagen - chitosan ilm

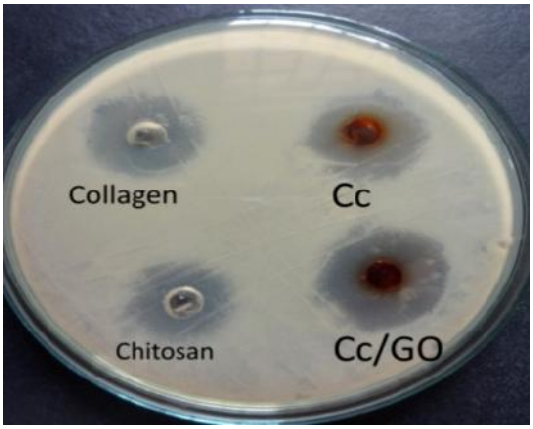

Figure 5: Antibacterial activity of collagen-chitosan ilm

SDS – PAGE analysis

SDS-PAGE did the qualitative estimation of obtained collagen following the protocol described by Sambrook and Russell (2001). The SDS-PAGE was performed using a vertical slab use of a 6.6 % resolving gel and an 8.8 % stacking gel. Then the gel loading buffer was prepared to contain 2% βmercaptoethanol and 0.016 % bromophenol blue, mixed with sample were boiled for 5 min at 100 ◦C. Each boiled samples (10 μl) was injected into the respective well and electrophoresed under a 50mA current. The gel was stained for 45 min in the presence of 0.25 % Coomassie brilliant blue R-250 solution and destained with 3 % methanol until the bands were clear.

Preparation of collagen-chitosan film

The Films were prepared by adding 1 % acetic acid and 3% chitosan (Himedia, India). They were mixed with 25 ml of distilled water. After mixing, gently stir it with a magnetic stirrer for 5 min. Once stirred, 5% of gingelly oil was added for elasticity. Then 5% (w/v) collagen extract were added. After the complete mixing was done, the solution was poured in a plate and allowed to dry in room temperature at vacuum using desiccator (Ismarul*et al.*, 2004).

Morphology of Collagen - Chitosan film

The morphology of the film was examined using Scanning electron microscopy (PhenomPro, CLRI). Samples of the ilms were prepared and fixed under vacuum (approx.16 Pa) for 1 min (current: 15mA in an argon atmosphere). The samples were then observed by SEM operating with an accelerating voltage of 20 kV. Multiple images of various areas of the ilms were recorded for pore size analysis and approximate pore size of the ilms was calculated and reported as a mean ±S.D.

Fourier transform infrared spectroscopy (FTIR)

The FTIR spectra of collagen – chitosan film were obtained from discs in ∼200mg potassium bromide pellet (KBr) under dry conditions. The spectra were recorded using a JASCO FTIR-4700 from 4000cm1to 500cm-1

Anti-bacterial test

Nutrient agar was prepared and poured into the plate. The gram-positive bacteria *staphylococcus aureus* was spread onto the plate. The sample was then added into the well in 20 mm apart from one another. Plates were incubated for 24 h at 36 ± 1◦C, under aerobic conditions. After incubation, confluent bacterial growth was observed. Inhibition of the bacterial growth was measured in mm (Mohanasundaram*et al.*, 2017). **Anti-inflammatory test**.The anti-inflammatory test was done by adding 1 ml of Human blood into the 10ml of saline solution. Then centrifuged for 5 min at 3000 RPM. The supernatant was then discarded. Pellets were collected and suspended using saline solution. Centrifuged again for 5 min. 1 ml of human red blood cell suspension were added to test samples taken in different test tubes at different concentrations (100 – 500 μl). Incubate at 50◦ C for 30 min. Finally, the respective optical density readings were taken at 565 nm using the UV spectrophotometer (Shinde*et al.*, 1999)**.**

$\%\ inhibition = 100 \times ([V_t/V_c] - 1)$
V_t– absorbance of the test sample
V_c– absorbance of control

***In vitro* wound scratch assay**

In vitro wound, a scratch assay was performed on L929 fibroblast cell line. The analysis was done according to the protocol described by Liang *et al.* (2007).

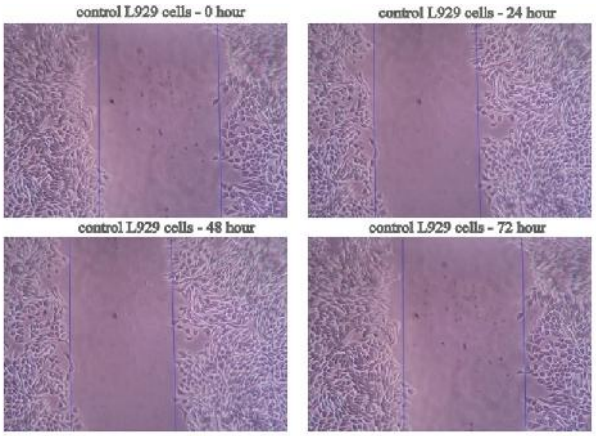

Figure 6: *In vitro* wound scratch assay of control sample on L929 fibroblast cells

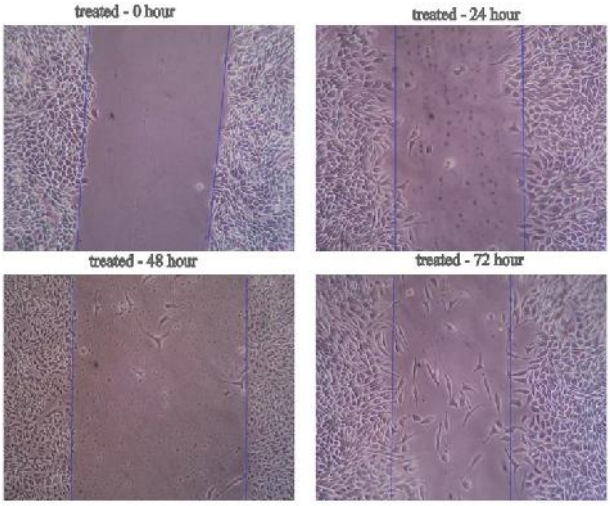

Figure 7: *In vitro* wound scratch assay of collagen – chitosan blend on L929 fibroblast cells

Table 1: Functional groups of collagen - chitosan film

Frequency, cm^{-1}	Functional group
3275	OH Stretch
2923	C-H Stretch in methylene
2854	C-H Stretch in methyl
1743	C=O Stretch
1645	C=C Stretch in Phenyl
1564	Amide III C=C Bending Aromatic
1452 and 1453	Amide I and Amide II
1236	C-O Stretch
1155 and 1103	Aromatic C-H in plane
994	C-H out of a plane

Table 2: Tensile strength parameters of collagen – chitosan film

Biomaterial	Tensile strength parameters	
	Elongation at break (%)	Tensile strength (Mpa)
Collagen	3.05 ± 0.43	5.75 ± 0.33 45.55 ± 5.44
Chitosan	65.43 ± 4.53	
collagen - chitosan film	6.25 ± 0.66	75.43 ± 8.51

Table 3: Inbitiory effect of collngen –chitosan on *S. aureus*

Sample	Zone of inhibition (mm)
Collagen	10mm
Chitosan	9.5mm
Collagen-chitosan	12mm
CC/GO	15mm

Table 4: Anti-inflammatory test of collagen -chi tosanfilm

Sample	concentration(μl) Absorbance at 565 nm	% Inhibition of hemolysis
Control	0.33 ± 0.05	-
(collagen)	0.38 ± 0.02	15
100 μl	0.44 ± 0.03	33
200 μl	49± 0.05	48
300 μl	0.53 ± 0.03	60
400 μl	0. 57 ± 0.03	72
500 μl		

L929 cells were received from NCCS, Pune, India and seeded onto 12 well plates (Tarsons, India) to a final cell density of $1x10^5$ cells/well for wound scratch assay.

Cells were maintained at high glucose Dulbecco's Modiied Essential Medium (DMEM) (Himedia, India) supplemented with 10% of fetal bovine serum (Himedia, India), and 1X of Antibiotic Antimycotic solution (Himedia, India) in humidiied 5% CO_2 atmosphere.

III. RESULTS AND DISCUSSION

Extraction of collagen

The SDS-PAGE results shown in Figure 1 indicates the presence of type 1 collagen with more than one α chain (α1 and α2) and one β chain. The intensity of the α1 was higher that of α2 which was much similar to the bands observed for the type 1 collagen from other fish species and the collagen from *Catlacatla*species was confirmed to be type 1 collagen. Thus the yield of the collagen was obtained to be 9.28 % (Wet weight) which was similar to the Xu *et al.* (2017) results. Similarly, Haratie*t al.* (2020) extracted collagen from tilapia, which showed good thermal and high solubility.

Collagen - chitosan ilms were obtained after vacuum drying Figure 2. Crosslinking of the collagen and chitosan were made out in the ratio 70/30, which give the much better elasticity, stability and fatigue strength. The addition of 1% gingelly oil improved elasticity and smoothness, which shows that increasing the gingelly oil content increases flexibility. Collagen/chitosan in the ratio 70:30 with 1% of gingelly oil

lead to the formation of a ilm with excellent adhesion property.

The collagen and chitosan form the polymer matrices in which chitosan is used for the stability of the protein compounds. The porosity of the collagen chitosan ilms was identified through the SEM morphological study Figure 3. The average pore size of the cc ilms was 64.52 ± 15.43 μm.
This confers a significant measure of intensity over a narrow range of wavelengths at a time. The interaction of collagen and chitosan was analyzed using FTIR spectroscopy. The spectra presented in Figure 4 are characterized by the bands at 1452, 1453 and 1564 cm^{-1} corresponding to the amide I, amide II and amide III of collagen. The bands at the 3275 cm^{-1} are attributed to –OH stretching and 1564c m^{-1} are attributed to the bending amide III aromatic C=C group. The band at 2923 cm^{-1} and 2854 cm^{-1} are attributed to the C-H stretch in methylene and methyl functional group. The band at 1452 cm^{-1} and 1453 cm^{-1} have attributed the asymmetric bending at methyl and methylene. The band at 1645 cm^{-1} gives the C-C stretch in the phenyl. The FTIR spectra of the collagen/chitosan film showed some of the characteristic peaks. The amides I, II and III groups of the collagen-chitosan blend may specify new bond among the linkage contained between the collagen and chitosan (Indrani*et al.*, 2017). The respective functional groups are given in Table 1. **Mechanical stability of collagen – chitosan film**

From Table 2, the tensile strength of the CC film shown as 75.43 ± 8.51 mPa along with the elongation at the break is 6.25 ± 0.66 %. Thus the collagen-chitosan film has a higher thermal intensity strength which can tolerate much temperature than its characteristic temperature. Similarly, mechanical properties of CC film studied by Slimane and Sadok(2018) showed a better tensile strength and elongation at break when chitosan concentration was increased. Hima*et al.* (2010) investigated tensile properties of chitosan – gelatin ilms which showed tensile strength and elongation at break of 5.33 % and 45.42 mPa. Thus results indicated that collagen is mechanically weak compared to chitosan, hence blending of collagen and chitosan enhanced the tensile properties of CC film.

Anti-bacterial test

The anti-bacterial test confirms that the prepared collagen chitosan film exhibit the property of antibacterial activity Figure 5 as an additive of the gingelly oil, which is an enhancer gives the larger scale of the zone over the sample Table 3. This proves that CC/GO has the better enhancement of the antibacterial activity.

Anti-inflammatory test

From Table 4, results show that increasing the concentration also increased the hemolysis. In the present study, 500 μl of collagen – chitosan blend showed the highest hemolytic activity of 72 %, which is low when compared to the control sample. Therefore, this indicates that collagen – chitosan film has an excellent anti-inflammatory profile.

***In vitro* wound scratch assay**

Fibroblast cell proliferation plays a vital role in wound healing as they are involved in collagen production. In vitro wound scratch assay used for testing the ability of growth and stimulation of fibroblast cells (Mimura *et al.*, 2004).

In this present investigation, collagen – chitosan treated fibroblast cells showed growth in 72 h, whereas there was no cell proliferation seen in control (Figure 6 and Figure 7). This indicates that collagen – chitosan film exhibits better results on the wound healing mechanism.

IV. CONCLUSION

Here, the collagen and chitosan used in this study are biocompatible, biodegradable and eco-friendly since its origin from nature itself. Moreover, collagen is the critical component of the human physiological structures, and also the fibroblasts cells proved that having more ability to generate and grow in a three-dimensional fashion. Thus the present study provides evidence that the collagen-chitosan ilms are feasible for the application of the wound dressing with a faster wound reduction process. From the study, it can be concluded that natural collagen extracted from fish skin possess many advantages in the pharmaceutical industry.

V. ACKNOWLEDGEMENT

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Conflict of Interest

All the authors declare no conflict of interest in this study.

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