Research Article

Preparation and Characterization of Biomaterial from Collagen, Chitosan and *Hibiscus rosa-sinensis* Nanoparticles

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ABSTRACT

Leather industry accounts for wastes like chrome shavings that contains large amount of type I collagen which can be extracted by alkaline hydrolysis method. The resulting collagen (Co) can be used in wound healing application as it mimics properties of extracellular matrix of the skin and induce wound healing. Chitosan (Ch) extracted from shrimp shells, a linear polysaccharide used in the treatment of wounds and burns due to its haemostatic effect accelerates the formation of fibroblasts and increases early phase reactions related to healing. Collagen along with chitosan, a natural polymer producing muco-adhesive fibers and an attracting alternative for sustained drug release systems will be made into a wound dressing material. Silver nanoparticles (AgNp), synthesized from *Hibiscus rosa-sinensis* on the other hand are used in making a wound dressing material with collagen and chitosan. The prepared wound dressing materials, (Co-Ch-AgNp) was subjected to analysis such as moisture content, porosity measurement, water absorption, and mechanical properties. The functional groups were analyzed by FTIR. The surface morphology was studies by SEM.

Keywords: Chitosan, Collagen, Silver nanoparticles, wound dressing materials.

INTRODUCTION

Numerous varieties of materials have been used in tissue engineering and bone engineering for the scaffold formation. Biodegradable polymers are preferred over synthetic polymers as they have flexibility because the composition and structure can be engineered to specific needs and their design capability to degrade as new tissues develop, thereby not leaving anything foreign to the body. This scaffold material upon which cells can attach, proliferate and differentiate into functionally and structurally appropriate tissue for specific body location into which it is placed should be capable of recreating the *in vivo* microenvironment and that is mainly provided by the extracellular matrix. The scaffolds thus used should be able to incorporate the appropriate biophysical, biomechanical and biochemical cues that facilitate cell proliferation, differentiation, maintenance and function and act as templates for tissue regeneration, to guide the growth of new tissue[1]. Skin repair becomes an important field of tissue engineering in the case of extended third-degree burns. Several wound dressings made of natural polymers are introduced as biomaterials especially in this case[2]. Natural polymers are involved in the repair of damaged tissues and in skin regeneration by inducing and stimulating the wound healing process[3].

These are typically composed of a polymeric network that can contain up to 99 percent or higher water content. The use of three-dimensional polymeric scaffolds for cell targeting is a common strategy for tissue engineering. Recent studies about biocompatible and biodegradable natural/synthetic polymers led to a substantial development of novel types of wound dressings and to great applications in the biomedical area, particularly for regenerative medicine[4].

Altogether, the wound healing process comprises of five stages, which involve complex biochemical and cellular processes. They are hemostasis, inflammation, migration, proliferation and maturation phases[5]. When the proper materials are used, it influences the rate at which the wound is healed[6]. It was studied that the biocomposite film incorporated with neem extract has shown an increase in biostability with addition of neem extract into collagen sheets and also influence the nitric oxide scavenging capacity to the biocomposite films[7]. Inflammation is characterized by erythema, heat, edema, pain and disturbances in function. When any wound occurs, the blood vessels in the bed of the wound will contract and there will be clot formation. During hemostasis, the blood vessels under the wound will dilate and allow antibodies, white blood cells, growth factors, enzymes and nutrients to reach the wounded area[8]. During the proliferative phase, the wound is rebuilt by the process called as "angiogenesis" where the new tissue formed comprises of collagen and extracellular matrix wherein the blood vessels network develop. Finally the outermost layer of the skin, epithelial cells will resurface the wound and this process is known as epithelialization[9]. Wound dressing material prepared using collagen isolated from chrome shavings impregnated with neem extract possess both physical and biological properties required for an ideal dressing material.

It was revealed that neem extract incorporation has imparted a nitric oxide scavenging capacity to collagen biocomposite. Thus, it can be considered as a potential wound dressing material for diabetic wound healing[10].

Collagen matrix acts as support matrix for release of extracts in a controlled manner and also helps in cell adhesion and proliferation. The phenolic compounds like tannin and flavonoid content present in teak leaf have anti-microbial and anti-oxidant properties have contributed to the increase in the rate of wound healing. The fabricated sheet with combined properties of collagen and teak leaf extract reduces the epithelization period[11].

Hibiscus rosa-sinensis, commonly known as shoe-flower is a medicinal plant which has anti-inflammatory and antioxidant property[12]. The extraction of silver nanoparticles from the *Hibiscus rosa-sinensis* will influence the modulation of fibrogenic cytokines and to promote wound healing by accelerating re-epithelization and differentiation of fibroblasts[13]. The biomaterial prepared with natural polymers incorporated with silver nanoparticles as a wound dressing will promote suitable moist environment and it is nontoxic, biodegradable and biocompatible to enhance the wound healing activity[14]. Among the several materials used, silver nanoparticles and natural biodegradable polymers such as collagen and chitosan are considered for preparing biomaterials for the application of wound healing.

Collagen forms more than 30% of the human body and type I collagen is the most abundant in connective tissue and skin[15] Collagen along with hydroxyapatite makes one of the two major components of bone. It makes up 89 % of the organic matrix and 32 % of the volumetric composition of bone. Collagen types I, II and III are the most common collagen types in the humans. In a study, the *in vitro* testing of collagen/hyaluronan/beta glucan to be non-toxic and biocompatible with cultured cells was conducted[16]. It positively influenced the production of extra cellular matrix[17]. Collagen capped nanocomposite were found to be effective under different NaCl concentrations. Chitosan is the most excessive natural polysaccharide after cellulose found on earth and is composed of β (1-4) linked 2- acetamido- 2- deoxy- β- D-glucose. Chitosan is extracted from shrimp shells by alkaline and acid treatment. Its hemostatic effect is used in treating wounds and burns[18]. Chitosan speeds up the formation of fibroblasts and increases the wound healing phases[19]. Silver nanoparticles are considered as a potential antimicrobial agent and have been used in silver-based dressings and silver-coated medical devices. Silver-loaded chitosan nanoparticles also have been used as wound dressing material in tissue engineering applications[20,21]. They have been widely used in treatment for infections in burns, open wounds, and chronic ulcers. They increase the rate of wound closure through the promotion of proliferation and migration of keratinocytes. They differentiate fibroblasts into myofibroblasts by promoting wound contraction[22]. Silver nanoparticles with improved tensile properties lead to better fibril alignments in repaired skin, with a close resemblance to normal skin[23]. The unique property of the collagen, chitosan and silver nanoparticles makes biocomposite sheet one of the ideal materials for the production of 'moist healing' wound dressings. Biodegradable and biocompatible property of biocomposite sheet has numerous pharmaceutical and biomedical applications such as drug delivery system and cells encapsulation[24].

In this study, a composite was prepared containing collagen extracted from the tannery waste, chitosan from prawn shell, and nanoparticles synthesized from *Hibiscus rosa-sinensis* using poly ethylene glycol as a crosslinker. The prepared biocomposite sheet was characterized for its physicochemical properties like ash content**,** moisture content, porosity measurement, water absorption studies, tensile strength and elongation at break and finally FTIR analysis.

MATERIALS AND METHODS

Collagen extraction from chrome containing leather waste (CCLW)

The chrome containing leather waste sample was collected from Central Leather Research Institute, Adyar, Chennai. 50 g of sample was soaked in 5% NaOH and followed by the treatment with 10-15 mL of concentrated sulphuric acid to dechrome the leather and then bleached with 10 mL of hydrogen peroxide until the final collagen obtained was colorless and stored at 4°C[25].

Extraction of chitosan from shrimp shells

The shrimp shells were collected from Chintadripet market and were subjected to the following treatment.

Demineralization

Shrimp shells were demineralized using 1% HCl with four times its quantity. The samples were allowed to soak for 24 h to remove the minerals[26].

The demineralized shrimp shell samples were then treated for one hour with 50 mL of a 2% NaOH solution to decompose the albumen into water soluble amino acids. The remaining chitin was washed with de-ionized water, which was then drained off. The chitin was further converted into chitosan by the process of deacetylation[27].

Deacetylation

The deacetylation process was carried out by adding 50% NaOH and then boiling at 100°C for 2 h on a hot plate. The samples were then placed under the hood and cooled for 30 min at room temperature.

Then the samples are washed continuously with the 50% NaOH and filtered in order to retain the solid matter, which was chitosan. The samples were then left uncovered and oven dried at 110°C for 6 h[28].

Determination of Degree of Deacetylation for Chitosan

Degree of Deacetylation determined using the equation proposed by[29] as given as,

$$DD\ (\%) = 100 - [(A_{1655}/A_{3450}) \times 100/1.33]$$

where, A_{1655} – Amide-1 measure of N-acetyl group. A_{3450} – Hydroxyl bond 1.33 – Value of ratio (A_{1655}/A_{3450}) for fully acetylated chitosan.

Ash content

1 g of sample was taken in a pre-weighed crucible with lid and placed in the muffle furnace and was maintained at 575 ±10°C for 6 h. After cooling, the crucible was removed from the furnace and was cooled to room temperature. This was repeated until constant weight was obtained[30]. The percentage ash was calculated with the following formula,

Ash (%) = Wt. of ash (g)/Wt of sample (g)*100

Moisture content

The samples were dried to constant weight in oven at 105 °C and moisture content was calculated with the formula,

Moisture (%) = $(W_1–W_2/ W_1) * 100$

Where W_1 = weight (g) of the sample before drying, W_2 = weight (g) of the sample after drying.

Silver nanoparticles synthesis

Hibiscus rosa-sinensis (shoe flower) was collected, washed with distilled water and dried under sunlight. The petals were cut into small pieces and boiled under the flame with distilled water. It was filtered and treated with 1mM silver nitrate solution in the ratio 1:10. Then the beaker was kept under the sunlight until the color changes from light red to brown. The absorbance was taken between 300-600 nm. The solution was purified by centrifugation at 10,000 rpm followed by re-dispersion of the pellet with acetone. The solution was dried and nanoparticles obtained were stored at 40°C for further analysis.

Biocomposite sheet formation

Co-Ch-AgNP'S sheets were formed in various compositions cross-linking with PEG and stored at room temperature for further analysis.

Water Absorption Studies

The water absorption capacity of biocomposite sheets prepared was determined by swelling small pieces of each biocomposite of known weight in distilled water at room temperature. The weight of biocomposite sheets was noted after blotting it with filter paper to remove excess and was recorded for every 1 h, 2 h, 3 h and after 24 h. Percentage water absorption of the biocomposite sheets at a given time was calculated from the formula,

Water Absorption = (Ws - Wo)/ Ws*100

Where Ws - the weight of the biocomposite (moist) at given time, Wo - the initial weight of the biocomposite[31].

Porosity Measurement

Biocomposite sheet porosity was determined through liquid displacement method using ethanol as the displacement liquid because of its easy penetration through the pores of the biocomposites and which will not induce shrinking or swelling as a non-solvent of the polymers.

A known weight (W) of the biocomposite was immersed in a graduated cylinder containing a known volume (V_1) of ethanol. The biocomposites sheets were kept in ethanol for 5 min.

The process was repeated until the air bubble stops. The total volume of ethanol and the ethanol impregnated biocomposites was recorded as V_2.

The difference in the volume was calculated by (V_2-V_1). The biocomposite impregnated with ethanol was removed from the cylinder, and V3 is the residual ethanol volume[32]. Thus, total volume of the biocomposite was calculated by

$V = (V_2-V_1) + (V_1-V_3)$

Porosity of the biocomposite was obtained with,

$\epsilon = (V_1-V_3)/ (V_2-V_3)$

FTIR Spectroscopy

Fourier transform infrared spectroscopy was carried out to determine the functional groups in the prepared biocomposites. The spectra were measured in the frequency range of 4000-500 cm^{-1} using Nicolet 360 Fourier Transform Infrared (FTIR) spectroscopy.

SEM Analysis

The prepared biocomposite films were mounted on metal grids with double sided adhesive tape, coated with gold to ~ $500x10^{-8}$ cm thickness using SC7640 sputter coater (Quorum Technologies, Newhaven, UK0 under high vacuum, 0.1Torr, 1-2kV and 50mA at 25°C ± 1°C. The surface morphology of coated sample was examined by scanning electron microscopy (SEM:JEOL JSM-52—TOKYO, JAPAN) at 20kV.

RESULTS AND DISCUSSION

The collagen extracted from the chrome containing leather waste produced 14 % yield with large quantity of the sample being wasted during alkali and acid treatments. Chitosan was extracted by demineralization process and the yield obtained was 28.6 %. Degree of deacetylation decreases the yield and pH of the Chitosan from 7.8 -7.0. The extracted collagen and chitosan were stored at 4°C for further use.

Table 1: Physicochemical properties of collagen and chitosan

Properties	Collagen	Chitosan
Ash content	38.57±0.31	3.03±0.20
Moisture content	85.9±.42	2.17±0.75
Yield (%)	14 ±0.11	28.6 ±.023
DDA %		64.48

Degree of Deacetylation was determined by the equation[33]

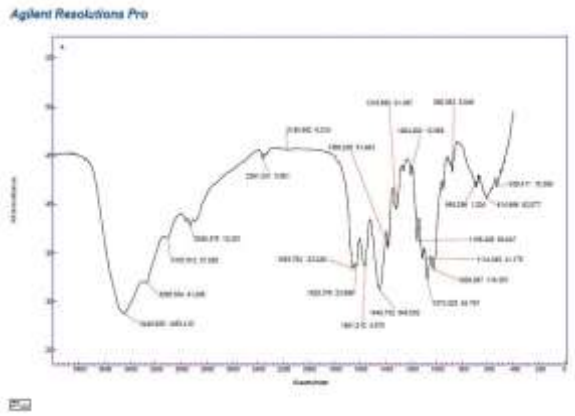


Figure 1: FTIR for calculating Degree of Deacetylation of chitosan

Chitin and chitosan have significant affinities for metal cations. In particular, chitosan has high capacity for collecting heavy metals cations, and it is one of the best adsorbents occurring in nature[34]. It adsorbs a wide variety of metal cations such as copper, mercury, cadmium, iron, nickel, zinc, lead and silver. The metal adsorption ability is influenced markedly by the degree of deacetylation. The samples prepared by deacetylation in homogenous solutions, however showed maxima at around 50% deacetylation, and these maxima correspond to the deacetylation degree of the water soluble chitin[35]. The degree of deacetylation is calculated by the following formula:

DD (%) = 100 – [(1629.375/ 3448.629) x 100/ 1.33] = 64.48 %

Where, 1.33 is the value of ratio A1655/A3450 for fully N-acetylated chitosan. By calculating with the above formula, the degree of deacetylation was found to be 0.64 for the extracted chitosan (Fig.1).

Silver nanoparticles synthesis

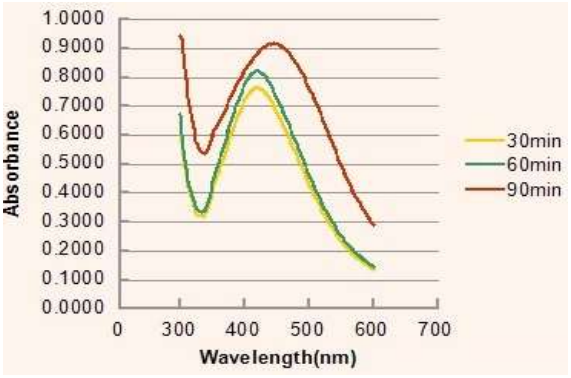


Figure 2: UV absorbance for silver nanoparticles

The absorption spectra in the UV visible region were recorded at the room temperature. The optical density was measured between 300 – 600 nm. The synthesized silver nanoparticles from *Hibiscus rosa-sinensis* exhibiting an adsorption peak around 432 nm (Fig.2). Usually the biologically synthesized nanoparticles will be in the range of 400-450 nm[13].

Table 2: Antibacterial Properties of Collagen, chitosan and silver nano particles

Collagen (w/v %)	Chitosan (w/v %)	AgNP'S (mg)	Antibacterial test
5	20	1	No zone formed
5	20	2	No zone formed
5	20	3	No zone formed
5	20	4	Zone formation (1.5mm)
5	20	5	Zone formation (15mm)

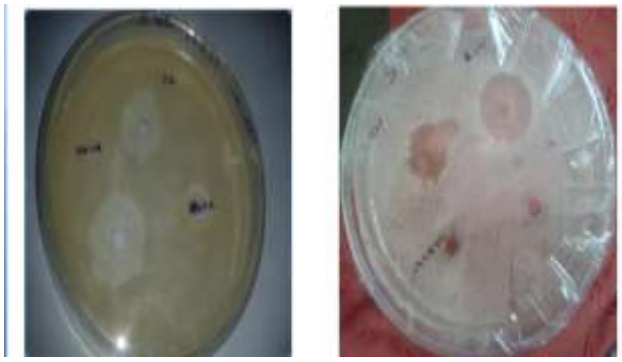


Figure 3: Zone of inhibition of Chitosan, Collagen and AgNPS in petri plates with cultures of *E.Coli* and *Staphylococus aereus*

Biofouling is a major problem faced today by many microbiologists and scientist. Various antifouling agents for example like chlorine or antibiotics etc. were being used until recently, but chemicals showed harmful side-effects to human beings and the microbes slowly developed resistivity to antibiotics. The silver nanoparticles demonstrate excellent antibacterial activity alone or within the polymer matrix. From the table 2, it was observed that the antibacterial effect of biocomposite film increases with increasing concentration of silver nanoparticles. The petri dishes with discs indicates that the silver nanoparticles are equally effective to both *E.Coli* and *Staphylococus aereus* (Fig.3).

Porosity study of biocomposite sheets

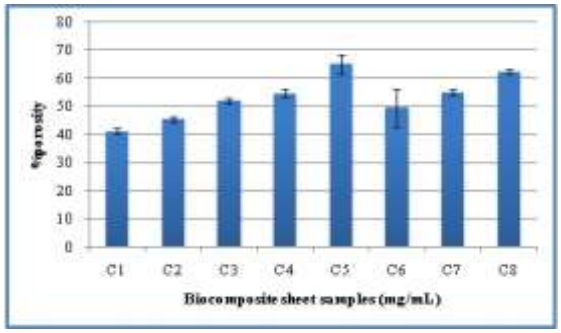


Figure 4: Porosity studies for biocomposite sheet ($p<0.05$)

Pore size, surface area and porosity of bio composites sheets play an essential role for cell attachment and migration in wound healing[35]. Porosity of the material is evaluated using liquid displacement method and a porosity of minimum of 40 % to 65% was found for all the materials (Figure 4).

Water absorption studies

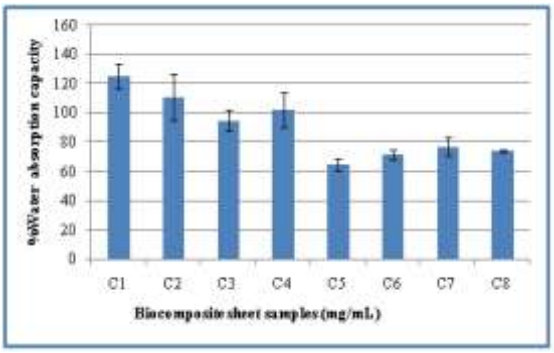


Figure 5: Water absorption studies for biocomposite sheet ($p<0.05$)

The prepared biocomposite sheets should possess good water absorption capacity and it should also retain its shape intact for wound healing. The bio composites prepared was evaluated for water absorption capacities (Figure 5) and the results shows more than 100 % water absorption for all the bio composites which makes its suitable to be applied for wounds and all the bio composites retained its shape till the end of testing[36].

Tensile Strength

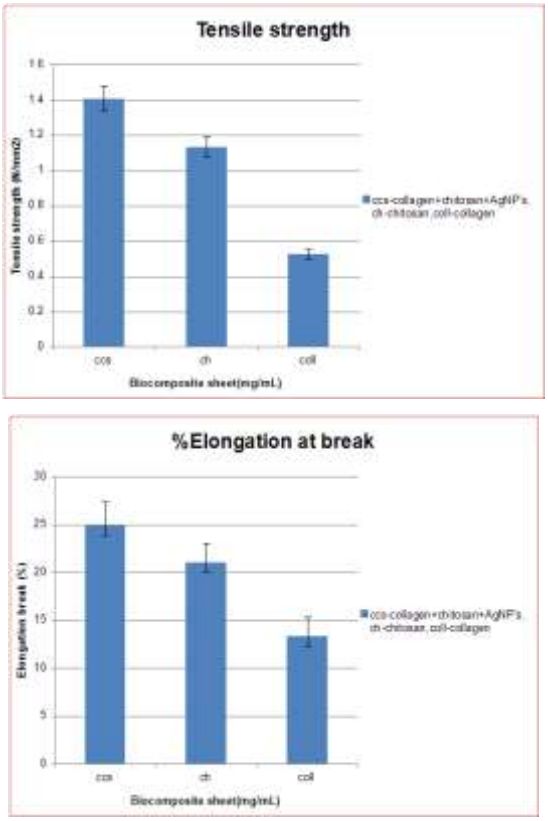


Figure 6: Tensile strength and elongation at break of bio composites with 0.5 mL of PEG as cross-linker. ($p<0.05$)

From the graph, the mechanical properties of biocomposite sheet the combination sheet collagen, chitosan and silver nanoparticles (1:4:1) was found to have good mechanical properties compared with collagen and chitosan alone sheet. Tensile strength is an important parameter for a wound dressing material to ensure easy handling. Percentage elongation is also found to be moderate for Collagen-Chitosan-Silver nano particles combination, which is suitable for the wound dressing material[37]. From the Fig.6, it can be concluded that the combination of chitosan, collagen and silver nanoparticles for fabricating the biocomposites posses the required mechanical strength for wound dressings[38].

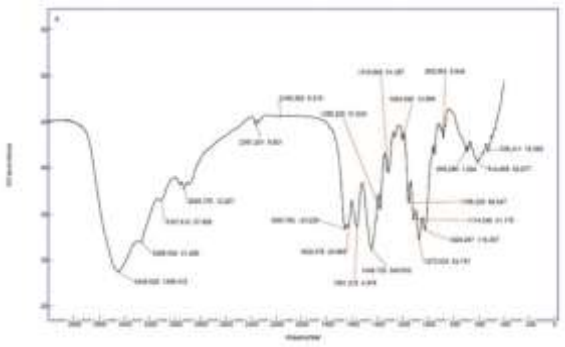


Figure 7: FTIR spectra for extracted chitosan

The characteristics peaks of extracted chitosan was observed from the Fig.7 and the functional properties are explained in the below table. The following absorption bands, 2361.37 2361.34 cm^{-1} representing C-N asymmetric band stretching, 1629.37 cm^{-1} representing amide I band and C-O stretch of acetyl group showing 1073.73 cm^{-1}, amide II showing 1561.21. Then, 2926.37 and 2361.24 cm^{-1} represents the symmetric CH_3 and asymmetric CH_2 stretching and 1073.52 showing skeletal vibration involving bridge C-O stretch.

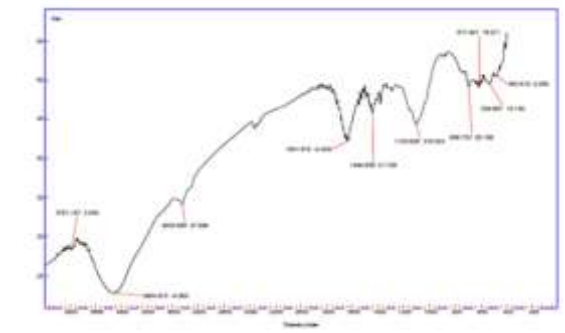
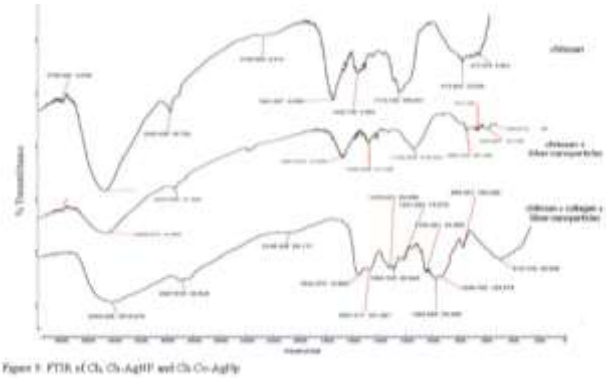


Figure 8: FTIR spectra for silver nanoparticles

Fig.8 displays FTIR spectra of silver nanoparticles. It was used to identify the potential biomolecules present in the flower extract which is responsible for reducing, stabilizing and capping the bio reduced silver nanoparticles[33]. FT-IR analysis revealed that the carbonyl groups from the aminoacids residues and from the proteins extracts of the flower had a strong ability to bind metal indicating that the proteins could possibly form the layer covering the metal nanoparticles and prevent agglomeration and thereby stabilize the medium. The

FTIR spectrum results of the synthesized AgNPs, crude *H. sinensis* petal extract and silver nitrate salt. The synthesized AgNPs showed strong bands at 3791.14, 3454.67, 2025.5, 1641.61, 1444.03, 1103.62 and 617 cm^{-1} which corresponds to O–H stretching, H–C–H asymmetric stretching, C≡N stretching, C−C=C symmetric stretch, N=O bend and C−O stretch, respectively. The carboxyl and amide groups indicate the presence of secondary amines which is a signature marker of proteins confirming the biofabrication of the nanoparticles by the action of the protein or phytochemicals[36].



It was revealed from the Fig.9, that chitosan has 3 characteristic amide peaks and the presence of amide peaks at 1643.27cm^{-1}, 1567.3cm^{-1}, and 1321.05 cm^{-1} in FTIR results confirms the presence of chitosan in the prepared sheet[34]. Collagen has 3 characteristic amide peaks and its presence can be used to confirm the presence of collagen in the prepared sheets. Amide peaks at 1649cm-1, 1555cm-1, and 1242 cm-1 were obtained in the FTIR results of composites made of collagen. There is a slight shift in amide peaks of collagen, chitosan and silver nanoparticles biosheet, which was not present in the extraction of chitosan and silver nanoparticles, which confirms showed the successful incorporation of collagen extract in the combination sheet.

SEM Analysis

The Fig.6, 7 and 8 shows the SEM pictures of Collagen and chitosan (Co-Ch), Silver nanoparticles and Collagen-Chitosan and silver nanoparticles (Co-Ch-AgNP).The collagen and chitosan pictures shows the crystalline and fibrous structure, the nanostructure of AGNP are clearly visible. The Fig.7 shows the composites of Co-Ch- AgNP and the silver nanoparticles are evenly distributed in the composite film.

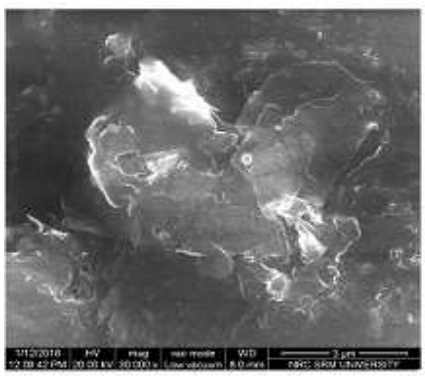


Figure 10. SEM image of Co-Ch

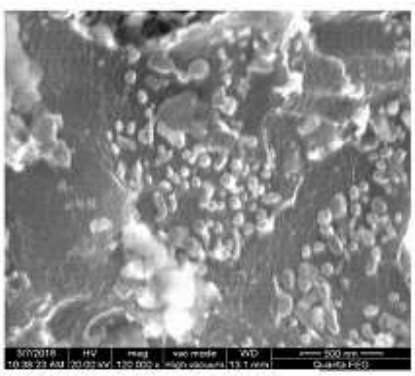


Figure 11. SEM image of AgNP

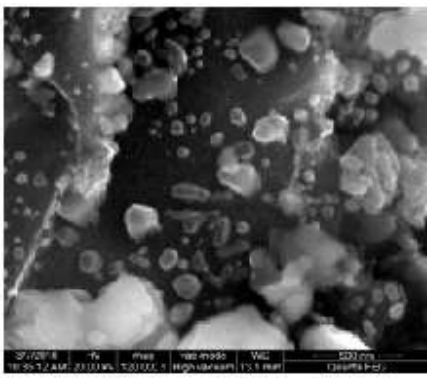


Figure 12. SEM image of Co-Ch-AgNP

A composite film prepared from the mixture of collagen, chitosan and silver nanoparticles showed superior characteristics comparing with chitosan and collagen films. A film prepared from collagen was known for the fast degradation and mechanical strength are the limit further uses especially as a wound dressing material. Chitosan gave strength and flexibility to the film. The silver nanoparticles incorporated increases the antibacterial property. The green synthesized AgNPs from the petal extract of *Hibiscus rosa-senisis* demonstrated to possess an effective antimicrobial effect against the four pathogenic microbes which were with accordance to the results[38]. The Co/Ch//AgNp is more flexible than pure collagen and chitosan bio composite films and are also homogeneous and no macroscopic imperfections seen on these films.

CONCLUSION

Leather industry wastes are one the most dangerous threat to the environment. Collagen isolated from chrome shavings and chitosan extracted from shrimp shells waste helps to reduce the environment pollution. Silver nanoparticles isolated from *Hibiscus rosa-sinensis* were impregnated with collagen and chitosan. The bicomposite sheets were formed under different stoichiometric ratio to determine the mechanical strength and other physicochemical properties.

From the characterization studies it is revealed that the prepared wound dressing biocomposite sheets shows more than 100% water absorption capacity and retained its shape for wound healing. Porosity is evaluated using liquid displacement method and porosity was found to be above 60 % for the biocomposite sheets.

The presence of good mechanical and antibacterial property makes the biocomposite sheet can be further used for *in vitro* studies on wound healing.

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REFERENCES

1. Dutta RC, Dutta AK, Cell-interactive 3D-scaffold; advances and applications. Biotechnology advances, 27(4), 2009, 334-339.
2. Boucard N, Viton C, Agay D, Mari E, Roger T, Chancerelle Y, Domard A, The use of physical hydrogels of chitosan for skin regeneration following third-degree burns. Biomaterials, 28(24), 2007, 3478-3488.
3. Huang S, Fu X. Naturally derived materials-based cell and drug delivery systems in skin regeneration. Journal of Controlled Release, 142(2), 2010, 149-59.
4. Chandran S, Amritha TS , Rajalekshmi G, Pandima Devi M, Potential wound healing materials from the natural polymers-A review. Int J Pharm Bio Sci, 6(3)(B), 2015, 1365–1389.
5. Dai T, Tanaka M, Huang YY, Hamblin MR, Chitosan preparations for wounds and burns: antimicrobial and wound-healing effects. Expert review of anti-infective therapy, 9(7), 2011, 857-879.
6. Rathinamoorthy R, Sasikala L, Polysaccharide fibers in wound management. International Journal of Pharmacy and Pharmaceutical Sciences, 3(3), 2011, 38-44.
7. Chandran, Amritha TS, Rajelakshmy G, Devi M, A preliminary *in vitro* study on the bovine collagen film incorporated with *Azadirachta indica* plant extract as a potential wound dressing material, International Journal of PharmTech Research, 8(6), 2015, 248-257.
8. Shankar M, Ramesh B, Kumar D, Babu M, Wound healing and its importance- a review. Der pharmacologiasinica, 1(1), 2014, 24-30.
9. Diegelmann R.F., Evans M.C. Wound healing: an overview of acute, fibrotic and delayed healing. Front Bioscience, 9(1), 2004, 283-289.
10. Chandran S, Amritha TS, Rajalekshmy G, Pandima Devi M, Collagen – *Azadirachta indica* (Neem) Leaves Extract Hybrid Film as a Novel Wound Dressing: *In vitro* Studies. Int. J. Pharm. Sci. Rev. Res., 32(2), 2015, 193-199.
11. Amritha, Pandima Devi M, *In vitro* studies on the wound dressing prepared using collagen and Teak leaves (*Tectona randis*). Journal of Pharmacy Research, 10(2), 2016, 90-98.
12. Divya MJ, Sowmia C, Dhanya KP, Joona K, Screening of antioxidant, anticancer activity and phytochemicals in methanolic extract of *Hibiscus rosa-sinensis* leaf extract, Research Journal of Pharmaceutical, Biological and Chemical Sciences, 4(2), 2013, 1308-1316.
13. Shabana S, Muzammil MS, Parsana S, Silver nano scaffold formation by flowers of *Hibiscus rosa-sinensis*. International Journal of Herbal Medicine, 1(2), 2013, 169-174.
14. Thomas, V, Yallapu M, Sreedhar B, Bajpai SK, Fabrication, characterization of chitosan/nano-silver film and its potential antibacterial application, Journal of Biomaterials Science. Polymer Edition, 20(14), 2009, 2129-2144.
15. Avery NC, Bailey AJ. Restraining cross-links responsible for the mechanical properties of collagen fibers: natural and artificial. Collagen, Springer US, 2008, 81-110.
16. Danisovic L, Novakova ZV, Bohac M, Bakos D, Vojtassak J. *In vitro* testing of modified collagen/hyaluronan/beta-glucan scaffold. OnLine Journal of Biological Sciences, 13(2), 2013, 40-45.
17. Ma L, Gao C, Mao Z, Zhou J, Shen J, Hu X, Han C. Collagen/chitosan porous scaffolds with improved biostability for skin tissue engineering. Biomaterials, 24(26), 2003, 4833-4841.
18. Khor E, Lim LY, Implantable applications of chitin and chitosan. Biomaterials, 24(13), 2013, 2339-2349.
19. Paul W, Sharma CP, Chitosan and alginate wound dressings: A short review. Trends in Biomaterials & Artificial Organs, 18(1), 2004, 18-23.
20. Mishra M, Kumar H, Tripathi K, Diabetic delayed wound healing and the role of silver nanoparticles. Dig J Nanomater Bios, 3(2), 2008, 49-54.
21. Azuma K, Izumi R, Osaki T, Ifuku S, Morimoto M, Saimoto H, Okamoto Y, Chitin, Chitosan, and Its Derivatives for Wound Healing: Old and New Materials. Journal of functional biomaterials, 6(1), 2015, 104-142.
22. Liu X, Lee PY, Ho CM, Lui VC, Chen Y, Che CM, Wong KK, Silver nanoparticles mediate differential responses in keratinocytes and fibroblasts during skin wound healing. ChemMedChem., 5(3), 2010, 468-475.
23. Kwan KH, Liu X, To MK, Yeung KW, Ho CM, Wong KK, Modulation of collagen alignment by silver nanoparticles results in better mechanical properties in wound healing. Nanomedicine: Nanotechnology, Biology and Medicine, 7(4), 2011, 497-504.
24. Kato Y, Onishi H, Machida Y, Application of chitin and chitosan derivatives in the pharmaceutical field. Current Pharmaceutical Biotechnology, 4(5), 2003, 303–309.
25. Deepachitra R, Ramnath V, Sastry, TP, Graphene oxide incorporated collagen-fibrin biofilm as a wound dressing material. Royal Society of Chemistry Advances, 4(107), 2014, 62717-62727.
26. Trung TS, Han WW, Qui NT, Ng CH, Stevens WF, Functional characteristics of shrimp chitosan and its membranes as affected by the degree of deacetylation. Bioresource Technology, 97(4), 2006, 659-63.
27. Huang M, Khor E, Lim LY, Uptake and cytotoxicity of chitosan molecules and nanoparticles: effects of molecular weight and degree of deacetylation. Pharmaceutical Research, 21(2), 2004, 344-353.
28. Muzzarelli RAA, Rochetti R, Determination of the degree of deacetylation of chitosan by first derivative ultraviolet spectrophotometry, Journal of Carbohydrates and Polymers, 5(2), 1985, 461-72.
29. Domszy JG, Roberts GAF, Evaluation of infrared spectroscopic techniques for analyzing chitosan. Die MakromolekulareChemi, 186(8), 1985, 1671-1677.
30. Shilratan W, Gopal S, Milind N, Jignesh D, Rakesh K, Satish R, Pooja D, Physicochemical and functional characterization of chitosan prepared from shrimp shells and investigation of its antibacterial, antioxidant and tetanus toxoid

entrapment efficiency. International Journal of Pharmaceutical Science, 26(2), 2014, 215-225.

31. Rao KP, Joseph KT, Naydamma Y, Grafting of vinyl monomers on to modified collagen by Ceric ion-studies on grafting site, Leather Sci, 16, 1969, 401-408.
32. Zhang R, Ma PX, Poly(α-hydroxyl acids)/hydroxyapatite porous composites for bone tissue engineering: Preparation and morphology, J. Biomed. Mater. Res, 44, 1999, 446-455.
33. Shabana S, Muzammil MS, Parsana S, Silver nano scaffold formation by flowers of hibiscus rosasinensis". International journal of herbal medicine, 1(2), 169-174.
34. Paul S, Jayan A, Sasikumar C S, Cherian SM, Extraction and purification of chitosan from chitin isolated from sea prawn (fenneropenaeus indicus). Asian Journal of Pharmaceutical and Clinical Research, 7(4), 2014, 201-204.
35. Kurita K, Sannan T, Iwakura Y., J Applied Chem Polymer Science, 23, 1979, 511.
36. Muzzarelli Raa, Natural chelating polymers: Oxford: Pergamon Press, 1973.
37. Yang SF, Leong KF, Du ZH, Chua CK, The design of scaffolds for use in tissue engineering. Part I. Traditional factors. Tissue Engg, 6, 2001, 679-689.
38. Gole, A., Dash, C., Ramakrishnan, V., 'Pepsin gold colloid conjugates: preparation, characterization, and enzymatic activity', Langmuir, 17, (5), 2001, 1674–1679.
39. Sadhasivam, S., Shanmugam, P., Yun, K.S.: 'Biosynthesis of silver nanoparticles by Streptomyces hygroscopicus and antimicrobial activity against medically important pathogenic microorganisms', Colloid Surf. B, 81(1), 2010, 358–362.

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