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Physiochemical Properties of Chitosan Biofilm for Wound Healing Applications

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Abstract: Chitosan is a fiber derivative from chitin, a substance found in the hard outer skeletons such as crabs, crawfish, shrimp, and squid. Chitosan, a common cationic polymer is organically sustainable, biodegradable, biocompatible, non-antigenic, non-toxic, and biofunctional. It may speed up the healing progression by improving the elements of inflammatory cells, macrophages, and fibroblasts. For this research project, glutaraldehyde is incorporated together to form a film for wound healing application. Solution casting method was used to fabricate the films of 1wt% dried in the oven, 2wt%, and 3wt% chitosan while 1wt% dried at room temperature was fabricated using the pipette technique. After that, the characterization and testing that involved were Scanning Electron Microscope (SEM), Fourier Transform Infrared Spectroscopy (FTIR), Atomic Force Microscope (AFM), and contact angle measurement. SEM images showed no porosity is found, rough and uneven surfaces for films of 1wt%, 2wt%, and 3wt%. FTIR spectra revealed that all films showed spectra that chitosan exists in the film. AFM results revealed that the surface roughness of chitosan films decreases with an increasing amount of chitosan and film of 1wt% CS 40ml (O) recorded the highest surface roughness value. Contact angle measurements revealed that as more chitosan was introduced into the homogeneous solution, the contact angle value decrease. It can be concluded that chitosan films can be applied as both hydrophilic and hydrophobic wound dressing materials.

Keywords: Chitosan, Wound Healing, Glutaraldehyde, Films, Polymer, SEM, FTIR, AFM, Contact Angle

1. Introduction

Wound healing refers to the biological process that happens during the regeneration of wounded connective and epithelial tissues. Wound healing is a common and efficient way for the body to mend itself after tissue damage. After an injury, the skin and the tissues under it go through a complicated wound healing progression. Wet-to-dry dressings are commonly used to treat wounds, and the purpose of a wound dressing is to generate and maintain an optimally moist environment. Haemostasis, inflammation, proliferation, and remodeling are all successive steps of wound healing that need highly

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coordinated, conserved, and spatiotemporally controlled activities [1]. Wound infections are expected to affect 2.4–4.5 million people in the United States healthcare system each year, costing over $20 billion [2]. According to the Malaysian Ministry of Health (MOH), 15% of the approximately 3 million diabetic Malaysians would develop lower extreme pustules [3]. This number is constantly growing, with an alarming 7 million Malaysian individuals expected to be impacted by wound diabetes, both diagnosed and undiagnosed by 2025 [4]. Consider a patient who has a history of chronic lower-limb wounds that are difficult to heal. In that case, their quality of life may be affected, their mortality and morbidity rates may increase, and their medical expenditures may increase [5].

Chitosan is a sugar found in the hard external skeleton of crustaceans, including crabs, lobster and prawns. It is used for medication. Chitosan is extracted from the shell of prawn, lobsters and crabs. It's a fibrous substance that might block the absorption of dietetic fat and lipid. Chitosan is a copolymer composed of β-(1 → 4)-linked 2-acetamido-2-deoxy-D-glucopyranose and 2-amino-2-deoxy-D glucopyranose units. Chitin, the major component of exoskeletons of crustaceans, mollusks, and insects, is usually generated through alkaline deacetylation. Sponges, nanoparticles, scaffolds, gels, dressings, and films have all been suggested as ways to speed wound healing using chitosan and its derivatives. Chitosan may adjust its physical and biological features further to meet the requirements of wound healing applications. Chitosan increases cell proliferation proliferates inflammatory cells in granular nerves, and affects macrophage functionality, all of which speeds up the healing process [6].

During in vivo testing, chitosan reduced healing time and scar formation to a minimum. The chitosan derivatives have many treasured properties that make them particularly interesting materials. Chitosan is a unique cationic polymer that is biodegradable, biocompatible, non-antigenic, and non-toxic. It has been shown to quicken wound healing by fixing the elements of inflammable cells, macrophages, and fibroblasts [7]. Chitosan is insoluble in water, soluble fluid arrangements, and basic natural solvents, but it dissolves speedily in watery inorganic and natural acidic media. Chitosan has the chance to accomplish hemostasis and speed up normal tissue repair.

The main objective of this study is to fabricate chitosan film via the solution casting method and pipette technique. Next, to investigate the physiochemical properties of chitosan film. Biomaterials generated from natural sources have been applied as a critical source of therapy for various wounds, and there has been increasing interest in their potential utility in wound healing. One example of a frequently used material in medical and biological applications is biomaterials.

This study also focuses on developing wound-healing materials made from chitosan, a natural biopolymer. Chitosan can stimulate fibrogenesis or fibrous tissue growth, which is necessary for wound healing and repair. These biopolymers are also biocompatible, generate a moist environment, are non-toxic, and have good film-forming capabilities. Chitosan may also boost cytokine levels in wounds by activating macrophage activity.

2. Materials and Methods

The method or process involved in this research was discussed, including the materials involved, sample preparation method and characterization method. Chitosan is used to make biofilm. Chitosan was diluted in acetic acid until homogeneous before been cross-linked with glutaraldehyde. For this research, the characterization methods involved were Scanning Electron Microscope (SEM), Fourier Transform Infrared Spectroscopy (FTIR), Atomic Force Microscope (AFM) and Contact Angle Estimation (Goniometer).

2.1 Material Preparation

Chitosan ($C_{56}H_{103}N_9O_{39}$), acetic acid (CH_3COOH), and glutaraldehyde ($C_5H_8O_2$) are the materials used to prepare the sample. Table 1 shows the list of materials used in this study.

Table 1: List of material in this experiment

No.	Material	Molecular Formula	Manufacturer
1.	Chitosan		
		$C_{56}H_{103}N_9O_{39}$	Sigma-Aldrich
2.	Acetic acid		
		CH_3COOH	Bendosen
3.	Glutaraldehyde		
		$C_5H_8O_2$	Sigma-Aldrich

2.2 Preparation of Casting Thin Film

100ml acetic acid was used to dilute 1wt%, 2wt% and 3wt% chitosan respectively. Then, the mixture was stirred using magnetic stirrer in hot plate for about 1.5 hours until homogeneous solution is formed. This homogeneous solution is distributed 40 ml and 30 ml for 1wt% while for 2wt% and 3wt% were distributed 30 ml and 20 ml respectively. Notice that the time taken consumed for 1wt%, 2wt% and 3wt% solutions completely dissolved in acetic acid might different from one another. For 1wt% chitosan that were drying in room temperature, pipetting method was used to crosslinking glutaraldehyde. Meanwhile casting method was used to crosslinking 1wt% chitosan that were drying in oven. The same process was used to crosslinking 2wt% and 3wt% chitosan with glutaraldehyde because the solution is very viscous. The manufacturing of chitosan was shown in Figure 1. Table 2 – 5 shows the composition of preparing chitosan at different weight percentage.

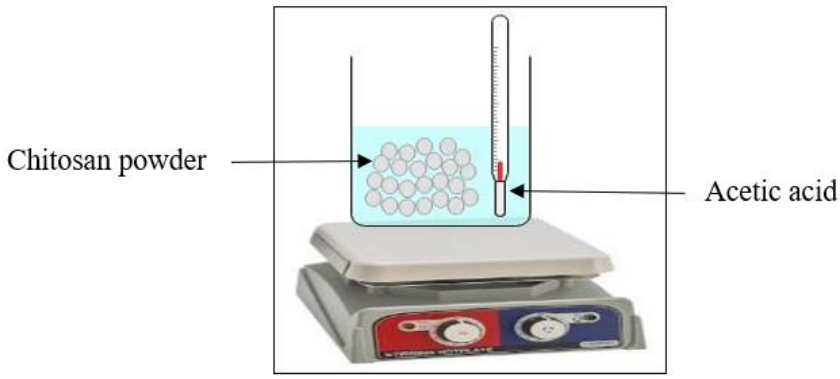


Figure 1: Preparation of chitosan thin film

Table 2: Composition of 1wt% chitosan at room temperature

Composition of chitosan at 1% (room temperature)		
Solution (ml)	40	30
Chitosan (g)	1	1
Acetic acid (ml)	100	100
Glutaraldehyde (ml)	0.75	0.75

Table 3: Composition of 1wt% chitosan at oven

Composition of chitosan at 1% (oven)		
Solution (ml)	40	30
Chitosan (g)	1	1
Acetic acid (ml)	100	100
Glutaraldehyde (ml)	150	150

Table 4: Composition of 2wt% chitosan at room temperature

Composition of chitosan at 2% (room temperature)		
Solution (ml)	30	20
Chitosan (g)	2	2
Acetic acid (ml)	100	100
Glutaraldehyde (ml)	150	150

Table 5: Composition of 3wt% chitosan at room temperature

Composition of chitosan at 3% (room temperature)		
Solution (ml)	30	20
Chitosan (g)	3	3
Acetic acid (ml)	100	100
Glutaraldehyde (ml)	150	150

2.3 Weighing and Mixing Process

Raw material such as chitosan are weighted using the precision electronic balance. The purpose of this process is to obtain a specific amount of composition used. Meanwhile, the magnetic stirrer is used to create a homogenous solution between chitosan and acetic acid.

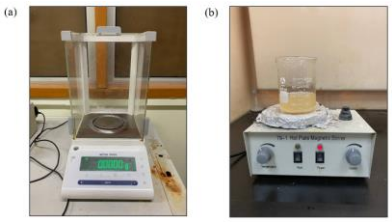


Figure 2: Weighing and mixing process (a) Precision electronic balance and (b) magnetic stirrer

2.4 Pipetting Method

A set of the pipette is used for crosslinking glutaraldehyde with 1wt% chitosan that were drying in room temperature. Homogeneous solution was divided into 2 petri dishes which is 40ml and 30ml. 1 drop of pipette is equal to 0.15ml. Each sample was dripped 5 drops of glutaraldehyde representing 0.75ml. The purpose of using this process is to obtain smooth and thin film when the glutaraldehyde is pumped by applying pipette technique.

Figure 3: Pipetting apparatus to crosslink glutaraldehyde and 1wt% chitosan

2.5 Casting Method

To form biofilm, 150 ml glutaraldehyde is poured into a container. Each sample of 1wt% dried in oven, 2wt% and 3wt% chitosan solution was dipped into the container for few seconds. This process helps the glutaraldehyde to harden forming biofilm structure. 1wt% dried in oven that has been dipped with glutaraldehyde need to be left for 30 minutes to make sure that the sample reach harden state. Next, the excess glutaraldehyde is poured out from petri dishes. Then, put the sample into the oven with a temperature of 50°C for 24 hours. But for 2wt% and 3wt% that has been dipped with glutaraldehyde was left for 24 hours then dry for 7 days in room temperature or until a film is formed. The dried sample was proceeded for characterization.

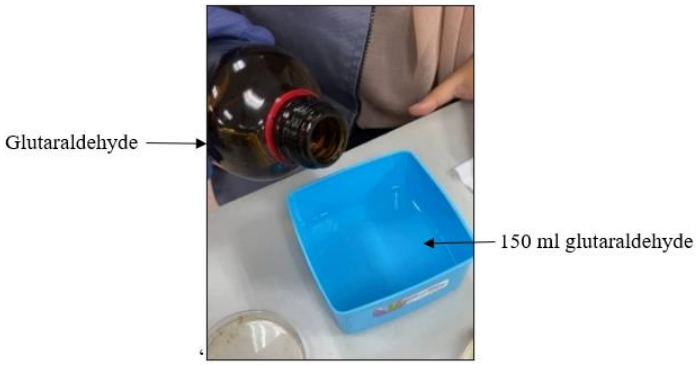


Figure 4: Solution casting method to create chitosan biofilm

2.6 Characterization of Sample

In this study, the characterization and testing methods that involved were Fourier Transform Infrared Spectroscopy (FTIR), Contact Angle Estimation (Goniometer), Scanning Electron Microscope (SEM) and Atomic Force Microscope (AFM).

2.6.1 Fourier Transform Infrared Spectroscopy (FTIR)

Chitosan biofilm was analyzed using FTIR (PerkinElmer Spectrum 100 FTIR Spectrometer, PerkinElmer Inc., The United States of America (U.S.A) to determine their chemical composition. The wavenumber range was from 4000 to 600 cm^{-1}. The resolution used was 4 cm^{-1}. First, the flat shoes were cleaned using ethanol before being fixed onto the pressure knob. Then, chitosan films were put on the metal mounting plate. It was ensured that the films cover the crystal glass. Next, the lever was gently pulled until a 'tap' sound was heard. Force was then applied gently onto the films by turning the pressure knob until slightly tight. The machine then started scanning the film and spectrum appeared on the monitor screen. The amount of each element in the sample will be translated in the form of peaks, with high peaks indicating a high amount and low peaks indicating small amount. FTIR is a favorable method to analyze a sample because it is non-destructive, have a precise measurement, good sensitivity and excellent scanning speed [8].

2.6.2 Contact Angle Estimation (Goniometer)

Contact angle for chitosan films were measured to determine their surface wettability. A Ramé-hart Model 200 F1 230V was used to measure contact angle of a water droplet on substrate in accordance with ASTM D7490. The films were cut into squares measuring 1 cm × 1 cm before being taped onto a glass slide. Then, the glass slide with the film was fixed onto the stage of the machine. Water droplet was then dropped onto the surface of the film. A camera captures the image and the contact angle value was measured.

2.6.3 Scanning Electron Microscope (SEM)

For this research, SEM (Hitachi VP-SEM SU1510, Hitachi Ltd., Japan) was used to visualize the surface of chitosan film. The magnification used was 100x, 500x and 1000x magnification for all sample. First, the surface of the stub that was used to hold the sample was cleaned to remove any debris. Next, carbon adhesive tape was cut into small squares and taped onto the stub. Then, chitosan films were fixed onto the carbon taped and coated with a thin layer of gold to create a conductive surface. The stub was then fixed onto the stage before being assembled into the machine The machine was then closed and switched on for surface morphology analysis.

2.6.3 Atomic Force Microscope (AFM)

Chitosan film was analyzed under AFM (Park XE-100 AFM, Park Systems Corp, Korea) to visualize the surface topography. For this characterization, chitosan film was prepared into small squares before taped into a glass slide. The glass slide was then fixed onto the sample disk inside the machine. The surface topography of the film was then analyzed.

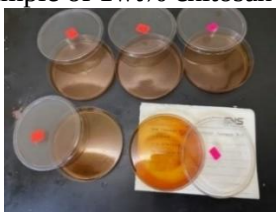
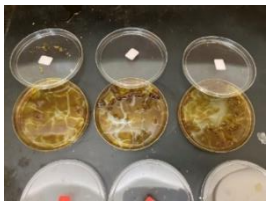
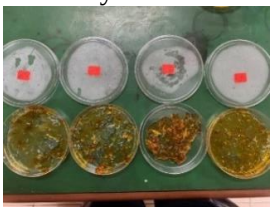
3. Results and Discussion

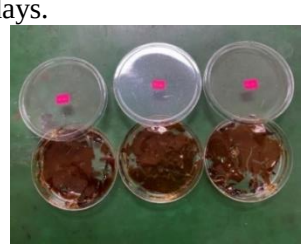
The results and discussion section presents data and analysis of the study. Detailed discussion has been made based on the results obtained from the following characterization which is Scanning Electron Microscope (SEM), Fourier Transform Infrared Spectroscopy (FTIR), Atomic Force Microscope (AFM) and Contact Angle Estimation (Goniometer).

3.1 Fabrication of chitosan film

Table 6 shows the sample of 1wt%, 2wt% and 3wt% chitosan biofilm that were fabricated throughout the semester. The purpose of fabricating the sample is to determine the structure and surface.

Table 6: Fabrication of the sample

Sample	Observation	Rate
a) Sample of 1wt% chitosan 	Sample is too thin and difficult to remove from petri dish. Wet the sample with distilled water but does not work.	Success 50%
b) Sample of 2wt% chitosan that had been immersed in glutaraldehyde for 24 hours. 	Film structure was formed but ruptured in glutaraldehyde solution.	Success 30%
c) Sample of 2wt% chitosan using pipette technique to crosslinked glutaraldehyde. 	The color has changed from yellowish to brown at room temperature. Texture of film is like a jelly that is easily crushed.	Fail
d) Sample of 2wt% chitosan that had been immersed in glutaraldehyde for 15 minutes. 	Texture of jelly is formed but a little bit dry and uneven surface. It's quite thick.	Fail

e)	Sample of 2wt% chitosan that were drying for 4 days in room temperature. 	The texture is like dry jelly but a little hard and easily damaged. Very dry and broke into many pieces.	Fail
f)	Sample of 1wt% chitosan that were drying in room temperature for 7 days. 	Thin film was formed but broke into pieces.	Success 80%
g)	Sample of 1wt% chitosan that were drying in oven for 24 hours at 50℃. 	Sample is thick and shrink because of sudden dry in an oven.	Success 60%
h)	Sample of 2wt% chitosan that were drying in room temperature for 7 days. 	Thin film was formed for 30ml and 20ml. But 30ml is very fragile and darker in color. 20ml is the best film so far.	Success 85%
i)	Sample of 3wt% chitosan that were drying in room temperature for 7 days. 	Thin film was formed for 30ml and 20ml. But 30ml is a little bit hard and very fragile. 20ml is fragile and roll on.	Success 65%

3.2 Morphology Analysis

A focus electron beam is scanned across a surface by Scanning Electron Microscope to produce an image. Allocating the morphology of chitosan is the main objective of this analysis. 1wt% was dried using an oven and room temperature. While 2wt% and 3wt% were dried using room temperature.

Figure 5 shows the microstructure result of chitosan film at magnification 1000x. Film containing 1wt%, 2wt% and 3wt% of chitosan. The chitosan film was cross-linked with glutaraldehyde. The surface of film is flat and no porosity is found on the film. Surface were homogeneous, although some particles could be observed on the surface of cross-linked films. As the film reached its maximum saturation point, acetic acid did not mix well with chitosan powder, causing small cavities to develop on the film surface.

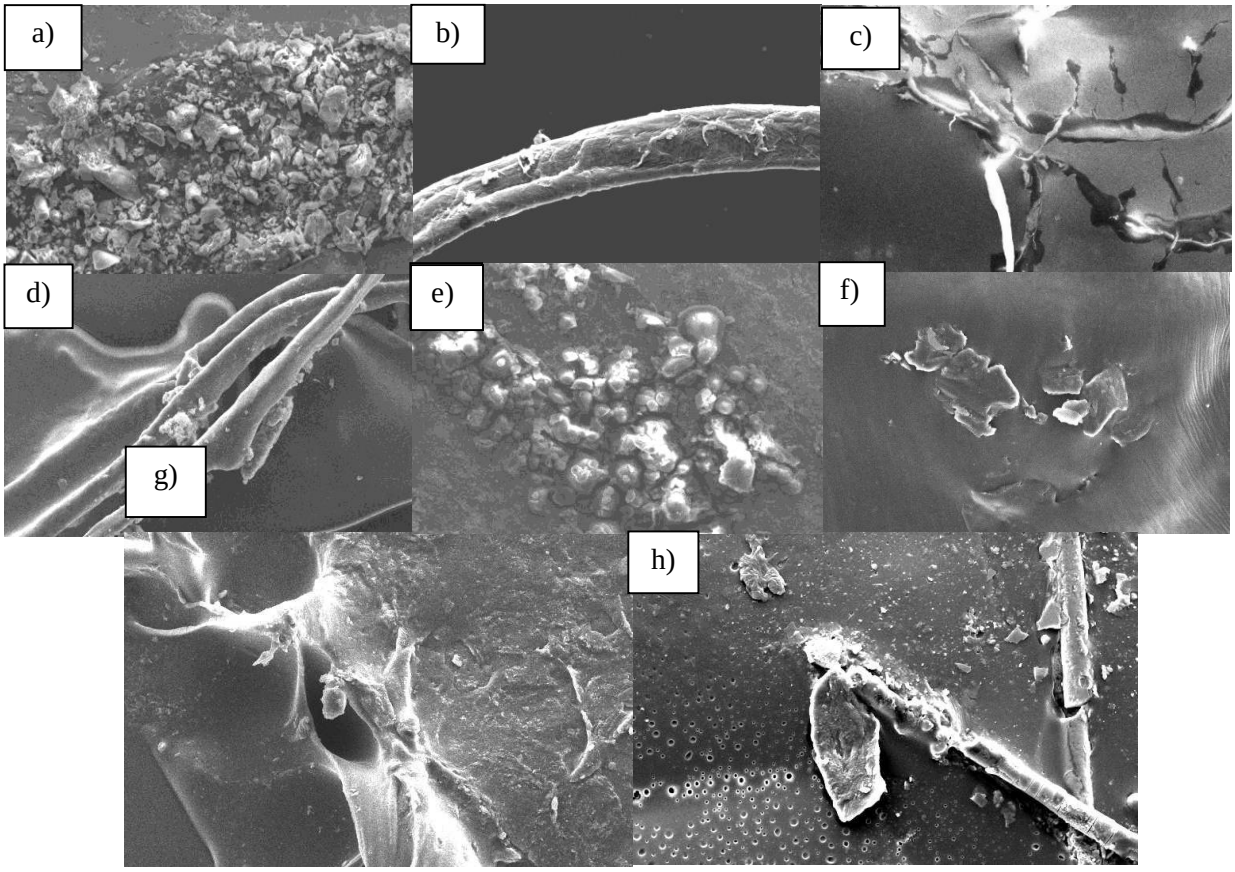


Figure 5: SEM images that dried in room temperature and oven at magnification 1000x of (a) 1wt% CS 40ml-R, (b) 1wt% CS 30ml-R, (c) 2wt% CS 30ml, (d) 2wt% CS 20ml, (e) 3wt% CS 30ml, (f) 3wt% CS 20ml, (g) 1wt% CS 40ml-O and (h) 1wt% CS 30ml-O

3.3 Fourier Transform Infrared Spectroscopy (FTIR) Analysis

Figure 6 shows the FTIR spectra for films of 1wt% that was dried in room temperature and oven. For films of 1wt% chitosan, broad bands were detected at 3360 cm^{-1} and 3376 cm^{-1} respectively. The broad band were contributed by OH stretching vibration band. At 3001-3010 cm^{-1}, small and medium bands were observed for both films, indicating the presence of C-H stretching vibrations band. The peak of 2899 cm^{-1} and 2897 cm^{-1} have been chosen as CH stretching vibrations band. The C=O stretching vibrations band are showing values 1650 cm^{-1} and 1719 cm^{-1}.

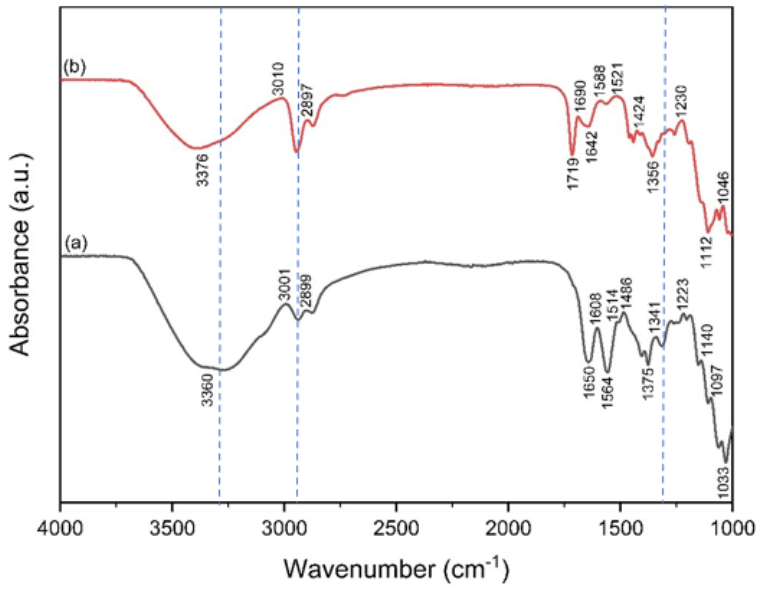


Figure 6: FTIR spectra for (a) 1wt% CS 40ml room temperature, (b) 1wt% CS 40ml oven

Figure 7 shows the FTIR spectra for 1wt% CS 30ml that was dried in room temperature and oven. Peaks observed at 3251 cm^{-1} and 3403 cm^{-1} belong to OH stretching vibrations specifically contributed by intermolecular H bonds respectively. The CH stretching vibrations can be seen at 3000 cm^{-1} and 3010 cm^{-1} showing the group of C-H. Band observed at 2941 cm^{-1} and 2946 cm^{-1} have been chosen as CH stretching vibrations which was C-CH_3.

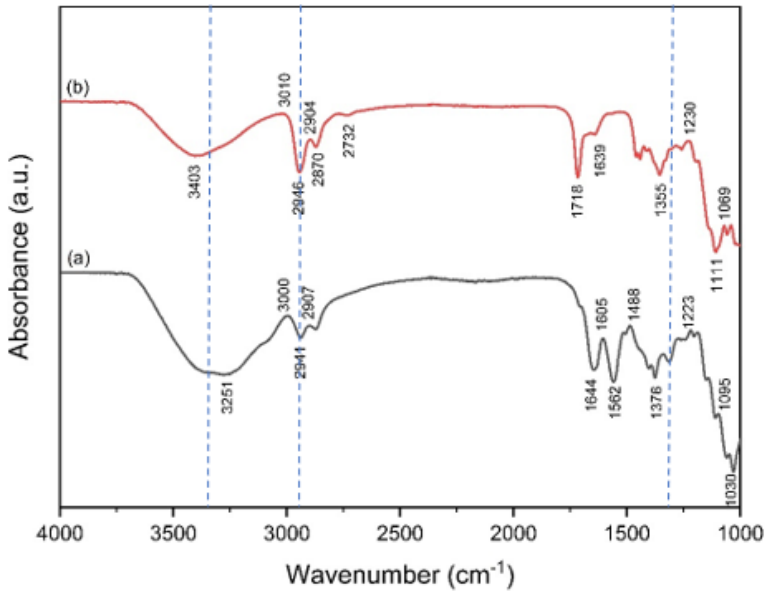


Figure 7: FTIR spectra for (a) 1wt% CS 30ml room temperature, (b) 1wt% CS 30ml oven

Figure 8 shows the FTIR spectra for films of 2wt% and 3wt%. Both films are 30ml volumes. The broad peaks at 3256 cm^{-1} and 3344 cm^{-1} are due to the stretching vibrations of OH group. The sharp IR bands at 3009 cm^{-1} and 3000 cm^{-1} could be assigned to stretching vibrations of C-H. The peaks at 2937 cm^{-1} and 2935 cm^{-1} have a strong CH_2 stretch. IR band at 2894 cm^{-1} and 2895 cm^{-1} is due to CH stretching vibrations. Bands observed at 1694 cm^{-1} and 1697 cm^{-1} were contributed by C=O stretching vibrations which was conjugated. Absorption peaks at 1643 cm^{-1} and 1646 cm^{-1} is due to nonconjugated C=C stretching vibrations.

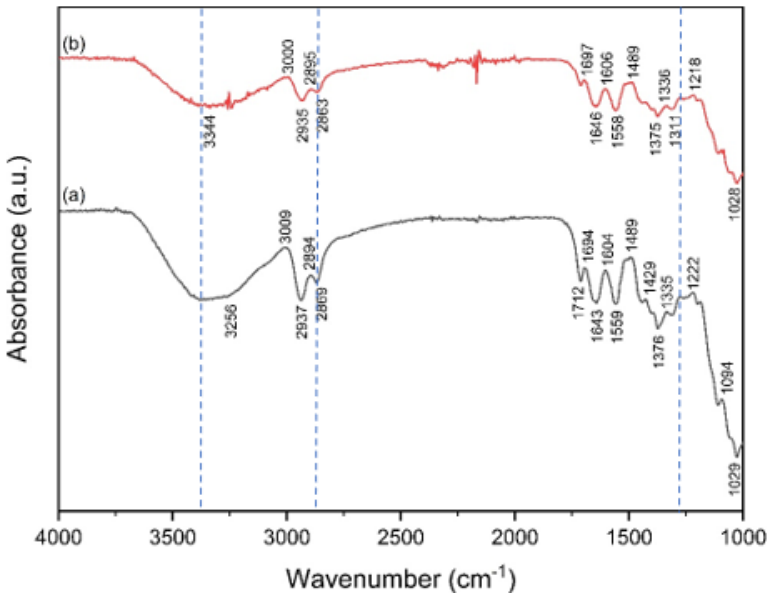


Figure 8: FTIR spectra for (a) 2wt% CS 30ml, (b) 3wt% CS 30ml

Figure 9 shows FTIR spectra films of 2wt% and 3wt%. Both films are 20ml volumes. Peaks observed at 3399 cm^{-1} and 3370 cm^{-1} belong to OH stretching vibrations specifically contributed by intermolecular H bonds respectively. The observed band at 3002 cm^{-1} in both films indicate the presence of C-H stretching vibrations. Small peaks were observed at 2936 cm^{-1} and 2933 cm^{-1} been assigned to CH stretching vibrations, specifically contributed by C-CH_3. The peaks at 2899 cm^{-1} and 2893 cm^{-1} are considered due to the stretching vibrations band of CH group.

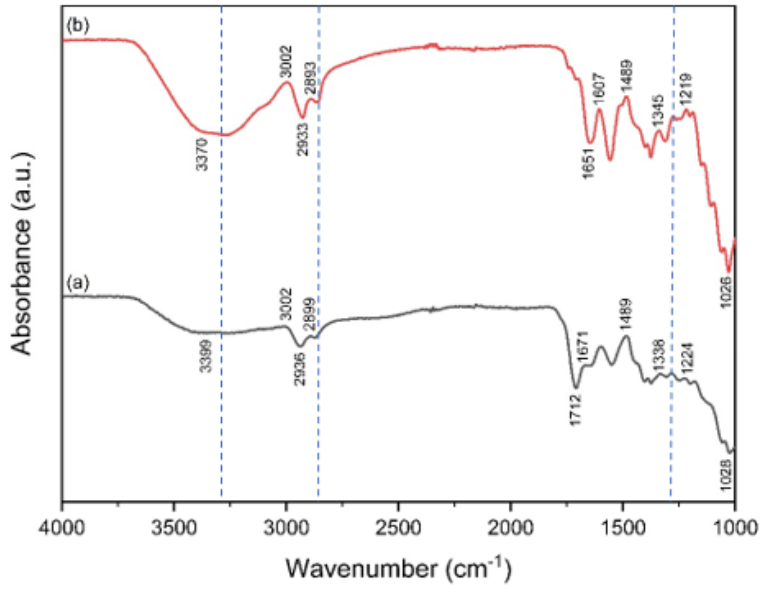


Figure 9: FTIR spectra for (a) 2wt% CS 20ml, (b) 3wt% CS 20ml

Figure 10 shows FTIR spectra films of 1wt%, 2wt% and 3wt%. All films are 30ml volumes. Based on interpretation, the spectra for films of 1wt% CS 30ml room temperature and oven showed the highest intensity, followed by film of 2wt% CS 30ml with moderate intensity and finally, films of 3wt% CS 30ml with the lowest intensity. Film of 1wt% CS 30ml oven recorded the highest intensity for the observed band at 3005 cm^{-1}, which might indicate strong OH stretching vibration.

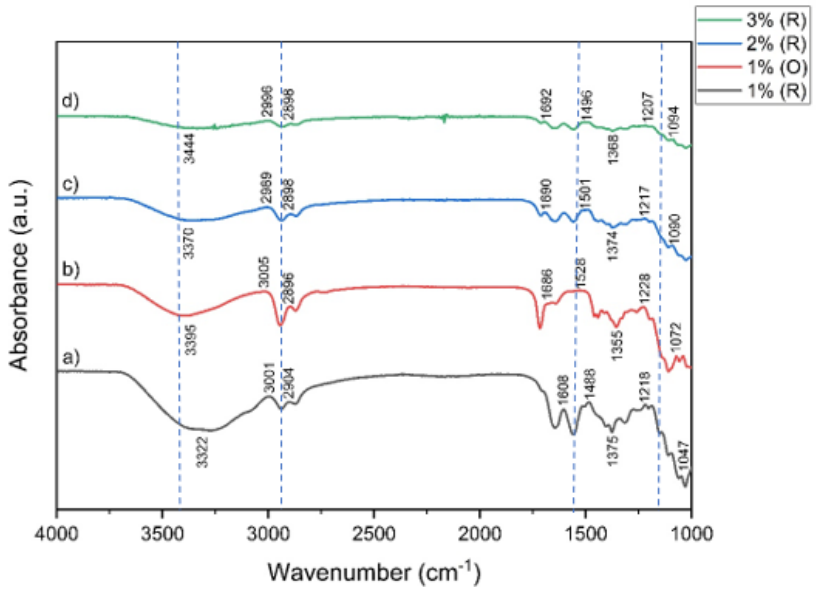
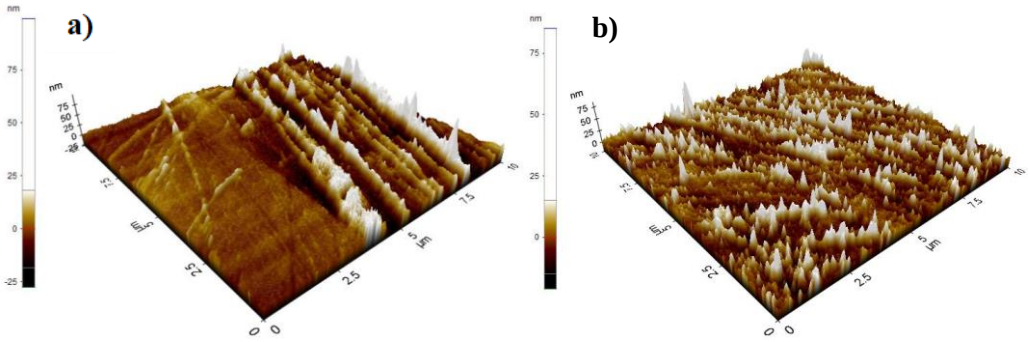


Figure 10: FTIR spectra for (a) 1wt% CS 30ml room temperature, (b) 1wt% CS 30ml oven, (c) 2wt% CS 30ml and (d) 3wt% CS 30ml

3.4 Atomic Force Microscope (AFM) Analysis

The 3D images for chitosan films are presented in Figure 11. The surface roughness of all film was assessed through mean value of peak to valley values (Rpv), root mean square roughness (Rq) and average roughness (Ra), as depicted in Table 7. Overall, film of 3wt% CS 20ml (R) demonstrated the lowest surface roughness value. Film of 3wt% CS 20ml (R) illustrates a smaller aggregate structure, relative to its surface roughness value which is the lowest among all samples. Film of 1wt% CS 40ml (O) showed the highest surface roughness value among all samples which indicate a more prominent and bigger aggregate structure. Film of 3wt% CS 30ml (R) showed second lowest value for surface roughness. Film of 1wt% CS 30ml (O) showed the second highest value for surface roughness. The heights of the aggregate structure are lower than 1wt% CS 40ml (O). Surface roughness of an oven indicate higher value due to cavities. Overall, the surface roughness value for films 1wt%, 2wt% and 3wt% showed consistent results with some films recorded a slightly higher value than other films.



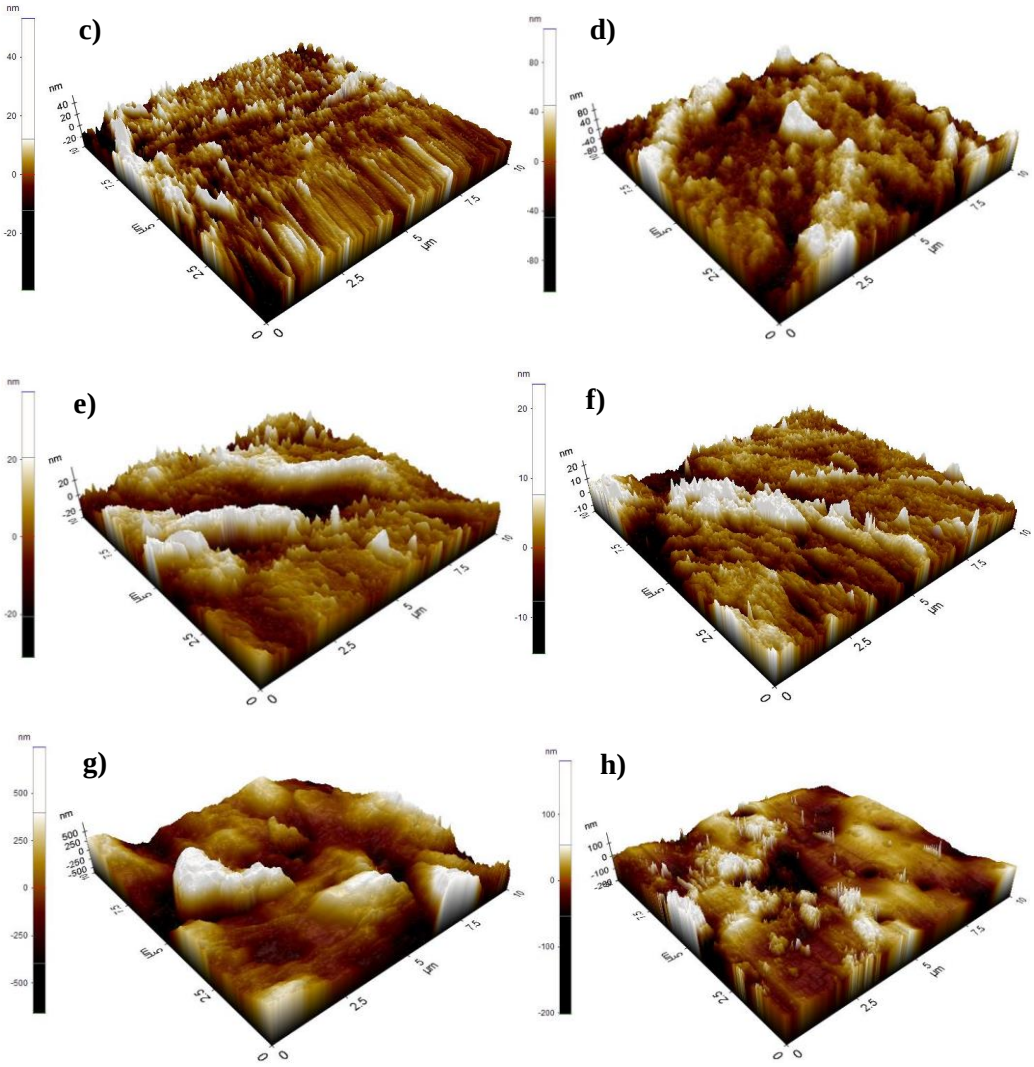


Figure 11: 3D AFM images of film (a) 1wt% CS 40ml room temperature, (b) 1wt% CS 30ml room temperature, (c) 2wt% CS 30ml, (d) 2wt% CS 20ml, (e) 3wt% CS 30ml, (f) 3wt% CS 20ml, (g) 1wt% CS 40ml oven and (h) 1wt% CS 30ml oven

Table 7: Roughness parameters for films of 1wt%, 2wt% and 3wt%

Films	Roughness Parameters		
	Rpv (nm)	Rq (nm)	Ra (nm)
1wt% CS 40ml (R)	23.533	4.698	3.737
1wt% CS 30ml (R)	19.467	4.319	3.475
2wt% CS 30ml (R)	14.767	3.276	2.623
2wt% CS 20ml (R)	29.630	5.922	4.687
3wt% CS 30ml (R)	14.519	3.172	2.550
3wt% CS 20ml (R)	12.326	2.355	1.907
1wt% CS 40ml (O)	92.814	20.455	16.184
1wt% CS 30ml (O)	46.695	9.310	7.135

3.5 Contact Angle Estimation (Goniometer)

Contact angle measurement was carried out to assess the surface wettability of chitosan films. Table 8 shows the contact angle value for films of 1wt%, 2wt% and 3wt% chitosan. Theoretically, the contact angle value started to decrease with increasing amount of chitosan. Film of 3wt% CS 30ml (R) demonstrated the lowest contact angle value 56.17º. The contact angle value for 1wt% CS 40ml (R) and 1wt% CS 30ml (R) increased before decreasing for film 2wt% CS 30ml (R). Fortunately, contact angle value recorded an increment for films 2wt% CS 20ml (R) and 3wt% CS 20ml (R). Film of 1wt% CS 40ml (R) demonstrated the highest contact angle value more than 90º, suggesting that the film might transform into hydrophobic surface and have the worst surface wettability. Chitosan film starts from 1wt% CS 30ml (R) until 1wt% CS 30ml (O) recorded contact angle values below 90º, suggesting that the films have hydrophilic surface and good surface wettability.

Table 8: Contact angle values for films of 1wt%, 2wt% and 3wt%

Films	Contact angle (º)			
	1st reading	2nd reading	3rd reading	Average
1wt% CS 40ml (R)	96.40	94.40	91.70	94.17 ± 1.93
1wt% CS 30ml (R)	93.40	87.50	87.40	89.43 ± 2.81
2wt% CS 30ml (R)	61.20	57.90	60.30	59.80 ± 1.39
2wt% CS 20ml (R)	72.70	76.80	72.30	73.93 ± 2.03
3wt% CS 30ml (R)	49.90	58.20	60.40	56.17 ± 4.52
3wt% CS 20ml (R)	71.40	66.00	83.00	73.47 ± 7.09
1wt% CS 40ml (O)	73.60	68.10	67.80	69.83 ± 2.67
1wt% CS 30ml (O)	65.50	68.40	74.60	69.50 ± 3.80

4. Conclusion

Solution casting method was used to fabricate the films of 1wt% dried in oven, 2wt% and 3wt% chitosan while 1wt% dried in room temperature was fabricate using pipette technique. Characterization and testing such as FTIR, SEM, AFM and contact angle measurement was performed on all films. The SEM images for films of 1wt%, 2wt% and 3wt% chitosan presented flat and uneven surfaces. No porosity is found. As more chitosan was added into the solution, more particles were observed under SEM, correlating with the SEM images that were presented by the films. Based on the FTIR results, films of 1wt%, 2wt% and 3wt% showed consistent results since the vibration bands from chitosan, acetic acid and glutaraldehyde were recorded in the FTIR spectra for the samples. AFM results revealed a consistent increase with some films recorded a slightly higher value than other films in surface roughness for films of 1wt%, 2wt% and 3wt%. This concludes that drying method effect surface roughness of films due to cavities. For contact angle values, films start from 1wt% CS 30ml (R) until 1wt% CS 30ml (O) demonstrated contact angle values below 90°, implying that the surface is hydrophilic and have good surface wettability. Films of 1wt% CS 40ml (R) revealed contact angle values more than 90°, indicating that the surface is hydrophobic and have poor surface wettability. Based on these findings, it can be concluded that chitosan film can be applied as both hydrophilic and hydrophobic wound dressing material.

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