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Effect of Calcium Chloride as a Crosslinking Agent in the Fabrication of Alginate/Beeswax Biofilm

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Abstract: Brown algae are the source of alginate, a form of polysaccharide that is non-toxic, biocompatible, and biodegradable. Calcium chloride, a known crosslinking agent of alginate is assumed to act as complexing carboxylate anions of alginate by its bivalent calcium iron. Beeswax has a lot of medicinal promise when it comes to the treatment of wounds. Alginate biofilm's ability to heal wounds will be enhanced even more due to the special properties of beeswax. The solution casting technique was employed to create the alginate and beeswax biofilm that was used in this investigation. This technique allowed the amount of beeswax to be modified from 1 to 5 grammes. Following that, the calcium chloride was sprayed onto the biofilm at concentrations of 0.5w/v%, 1.0w/v%, and 1.5w/v% to act as a crosslinking agent. Scanning electron microscopy (SEM), Fourier transform infrared spectroscopy (FTIR), contact angle measurement of surface-wetting, and atomic force microscopy (AFM) were used to characterise the alginate/beeswax biofilm surface roughness. The microstructure that is shown by SEM demonstrates that the biofilm changes from having a reasonably smooth surface to having a rough structure when beeswax is added. Alginate and beeswax belong to the same functional group, which was made clear by the FTIR research. The hydrophilic properties of the alginate molecule were precisely targeted by modifications to the molecule. The surface roughness of the alginate/beeswax biofilm increased, according to AFM data, with increasing amount of beeswax.

Keywords: Sodium Alginate, Beeswax, Calcium Chloride, Physiochemical, Wound Healing

1. Introduction

Skin is the most important natural barrier which protects the internal organs and helps in preventing body dehydration. If skin is damaged in any way, it would lose its protective mechanism and allow for the microorganisms to enter, form colonies, infect the wounded site and later delay the healing process.

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In the worst cases, it can cause life threatening complications. Skin wound is a disruption of skin structure and function; includes acute burns of the skin as well as chronic ulcers. Regardless of so many medical advancements, wound healing still remains an inefficiently managed area [1-2]. Basically, there are four relatively distinct phases in wound healing process that include hemostasis, inflammation, proliferation, and remodeling . The ultimate goal of wound healing is to have a speedy recovery with minimal scaring [1, 3].

Currently, a series of wound healing material, ranging from conventional gauze to modern materials, are available. Moisture-retentive materials have been preferred in chronic wound management, owing to their ability to provide a moist environment, which is essential for effective chronic wound healing. Film materials or dressings are known to be one of the most popular choices. Film dressings are simple, thin and semipermeable for effective water vapour and oxygen exchange. When in contact with wound exudates, the film transforms into a gel, creating a moist environment around the wound area [4]. Therefore, natural biopolymers such as alginate, collagen, beeswax and chitosan have been studied because of their importance in formulation of different dressings for healing of burns and other types of wounds. This is due to several favourable characteristics including biocompatibility, biodegradability and some structural similarities with human tissues, as well as their implication in the repair of damaged tissues and consequently skin and tissue regeneration [5-6].

Alginate is known as one of natural based hydrogels from anionic polysaccharide group. It has very good biocompatibility and hydrophilicity characteristics. Particularly, the structure of alginate consists of (1-4)-linked β-D-mannuronic acid (M unit) and α-L-guluronic acid (G unit) monomers, which the later monomer tend to form an 'egg-box' or a gel formation after ionic cross-linked with divalent metal cation such as Ca^{2+}[7]. Alginate dressings are characterized by the formation of a gel due to the exchange between the ions present in the dressing and wound exudate. This gel creates a moist environment that promotes healing and facilities easy removal. This together with its high tissue compatibility, low toxicity and good mucoadhesive properties allow alginates to be used as biomaterials for wound dressings [5, 8].

Beeswax has long been used in pharmaceutical and traditional medicine. It was also discovered that beeswax was a significant substance used in conventional medicine to treat wounds caused by burns and abrasions [9]. Moreover, beeswax has the capability to inhibit antimicrobial activity of certain types of bacteria and also against the *Candida albicans (C. albicans)* [10].

Calcium chloride, a known crosslinking agent of alginate is assumed to act as complexing carboxylate anions of alginate by its bivalent calcium ion. It is known that with increasing concentration of calcium chloride, the amount of Ca^{2+} ion per unit volume of the liquid increases and more Ca^{2+} will bond to the alginate chains. It was found that the number of -COO- groups decreased due to the combination of $Ca^{2+}$and –COOH group of sodium alginate. This condition resulted in weak electrostatic repulsion between –COO- groups.

2. Materials and Methods

2.1 Materials

The materials used in the experiment to fabricate the alginate/beeswax biofilm is listed in Table 1. Table 2 and Table 3 shows the composition of alginate/beeswax biofilm.

Table 1: Listed materials used to fabricated alginate/beeswax biofilm

No. of Items	Name	Chemical Formulae	Manufacturer
1	Alginate	$C_6H_9NaO_6$	SIGMA-ALDRICH
2	Beeswax	$C_{15}H_{31}CO_2C_{30}H_{61}$	SIGMA-ALDRICH
3	Calcium Chloride	$CaCl_2$	SIGMA-ALDRICH
4	Distilled Water	H_2O	-

Table 2: Composition of Sodium Alginate Biofilm

Sodium Alginate (g)	Distilled Water (ml)
1	100

Table 3: Composition of Beeswax

Sodium Alginate Solution (w/v%)	Beeswax (g)
1	0
	1
	2
	3
	4
	5

2.2 Methods

While stirring distilled water using magnetic stirrer, poured 1 gram of alginate into the vortex of distilled water and for 1 hour until this solution fully dissolved. Before adding beeswax into sodium alginate solution, 1 to 5 gram of beeswax was measured by using analytical electronic balance and added into sodium alginate solution. After the sodium alginate mixture poured in petri dish, the mixture was crosslinked with calcium chloride concentration of 0.5w/v%, 1.0w/v% and 1.5w/v%. Utilizing the spraying method, after added beeswax and stirred for 30 minutes the mixture was crosslink directly as shown in Figure 2. The mixture was fully submerged in calcium chloride for 2 minutes in the petri dish. The petri dish is emptied of excess calcium chloride solution. A solid film is then created by allowing the solid-liquid film or “agar-agar” to dry for 2–3 days at room temperature as shown in Figure 1. For the aim of characterising it, the film was produced and then sliced into squares that were around 1 cm 1 cm in size. Table 4 shows the composition of calcium chloride for 1w/v% of sodium alginate.

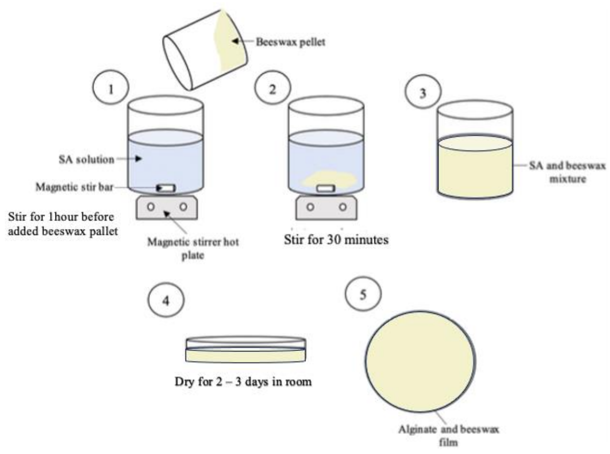


Figure 1: Schematic diagram for preparation of alginate and beeswax biofilm

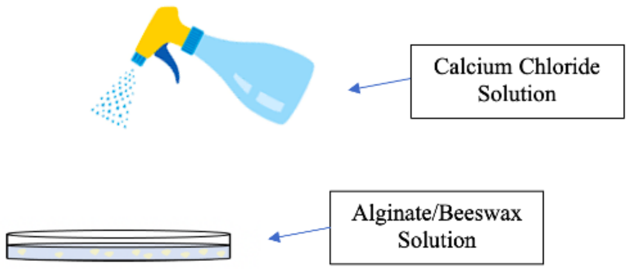


Figure 2: Schematic diagram for Crosslinking of Sodium Alginate and Beeswax film with Calcium Chloride

Table 4: Composition of Calcium Chloride for 1w/v% of Sodium Alginate

Sodium Alginate Solution (w/v%)	Beeswax (g)	Calcium Chloride concentration (w/v%)
1	0	0.5
		1.0
		1.5
	1	0.5
		1.0
		1.5
	2	0.5
		1.0
		1.5
	3	0.5
		1.0
		1.5
	4	0.5
		1.0
		1.5
	5	0.5
		1.0
		1.5

3. Results and Discussion

The findings of tests using Fourier Transform Infrared Spectroscopy (FTIR), Scanning Electron Microscope (SEM), Contact Angle Estimation using Goniometer and Atomic Force Microscopy (AFM) were used to characterise and understand the morphology of biofilms. Figure 3 and Figure 4 shows alginate/beeswax biofilm that fabricated successfully.

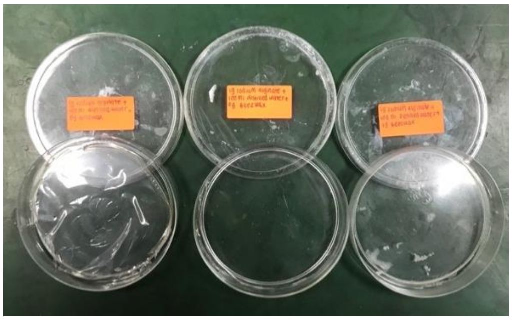


Figure 3: Specimens for 1w/v% of sodium alginate crosslinks with 0.5w/v% concentration of calcium chloride

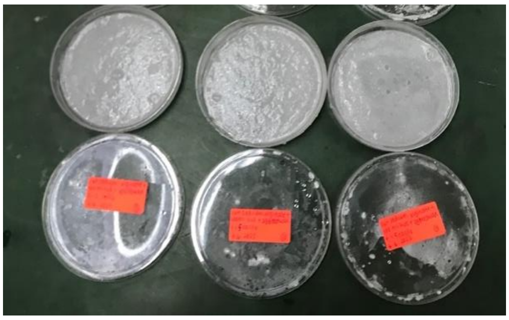
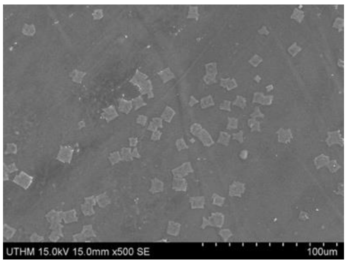
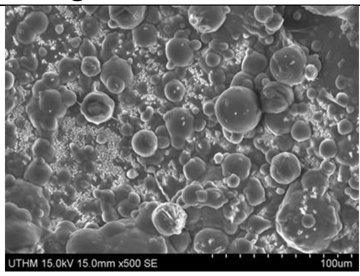
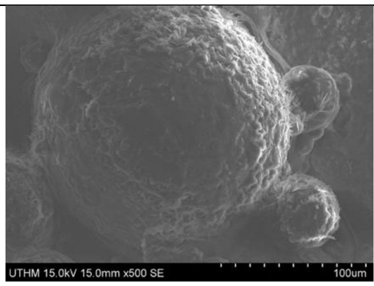


Figure 4: Specimens for 1w/v% of sodium alginate with 5g of beeswax crosslinks with 0.5w/v% concentration of calcium chloride

3.1 SEM analysis

A scanning electron microscope (SEM) was used to check the composition and distribution. To better examine the sample, three magnification levels of 500x, 1000x, and 2000x were used

Table 5: SEM image of alginate/beeswax biofilm crosslinked with 0.5w/v% of Calcium Chloride

Magnifications of 500x		
		
(1a) 1w/v% Sodium Alginate (SA)	**(2a) 1w/v% SA + 3g Beeswax**	**(3a) 1w/v% SA + 5g Beeswax**

Referring to Table 5 of (1a) 1w/v%, the uniform and grey background is sodium alginate and the small particles shape of square are the effects of crosslink alginate/beeswax biofilm with concentration of calcium chloride at 0.5w/v%. Compared to the film with 3g (2a), the film with 5g (3a) comprises bigger particles with a rougher surface. Some of the tiny beeswax particles were seen to stick to the larger ones. Concentration of calcium chloride at 1.5w/v% shows that more existent of the small particles shape of square and concentrated that effected because of high concentration of calcium chloride and smoother rounded and less various size of particles.

Element	Weight%	Atomic%
C K	31.71	45.02
O K	35.22	37.54
Na K	8.14	6.04
Cl K	14.28	6.87
Ca K	10.66	4.53

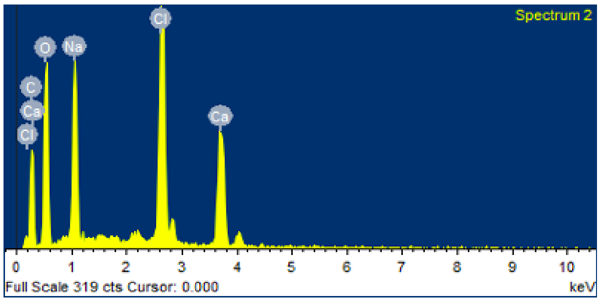


Figure 5: List of element composition of Alginate Biofilm crosslinked with 0.5w/v% of Calcium Chloride

The alginate/beeswax biofilm that had been cross-linked with calcium chloride was examined using energy dispersive X-ray scanning electron microscopy (EDX) to determine its element composition. Sodium alginate, beeswax and calcium chloride are just a few examples of materials that may be quickly and easily analysed for their element composition using energy dispersive X-ray technology. Figure 5 shows the existence of element composition in the alginate/beeswax biofilms crosslinked with calcium chloride at concentration of 0.5w/v% for 0g, 3g and 5g of beeswax.

3.2 FTIR analysis

The configuration and functional group of alginate/beeswax biofilm can be tested with FTIR spectroscopy by determined their wavenumber within the range of 4000-500 cm^{-1}. The wavenumber within the range 4000-1500 cm^{-1} were focused to determine the functional group region or diagnostic region because functional group gives different peaks in different substances. Meanwhile, the wavenumber within the range 1500-500 cm^{-1} called as finger print region were less focused because this region mostly will be compound specific and that is the reason to focus more on functional group region.

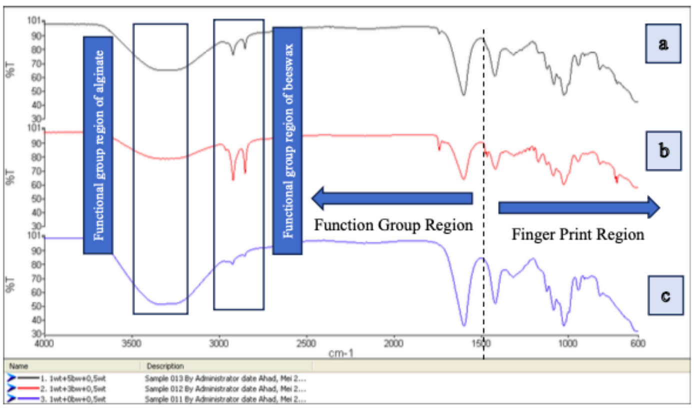


Figure 6: FTIR spectra for (a) 1w/v% of sodium alginate with 5g of beeswax crosslinked with 0.5w/v% of calcium chloride, (b) 1w/v% of sodium alginate with 3g of beeswax crosslinked with 0.5w/v% of calcium chloride, (c) 1w/v% of sodium alginate with 0g of beeswax crosslinked with 0.5w/v% of calcium chloride

Figure 6 shows the strong signals within a wavenumber range of 2919.58 cm^{-1} – 2849.04 cm^{-1} showed that the increase in intensity represent aliphatic CH_2 asymmetric and symmetric stretching that are effects of the interaction of beeswax blended with sodium alginate solution, comparing to sodium alginate biofilm detected within a wavenumber range of 2919.58 cm^{-1} – 2849.04 cm^{-1} that whereby its increased the volume spikes of the hydrophobic component [11]. The detected band peak at 1736.41 cm^{-1} shows that esters and free fatty acids are present in the alginate/beeswax biofilm with 3g of beeswax. The band peak at 1170.57 cm^{-1} results from the vibrations of C=O stretching and C-H bending, which also suggests the existence of esters in the beeswax pellet. With the exception of a slightest shift in wavenumber, this result is nearly identical to the research done on virgin beeswax as shown in Figure 7.

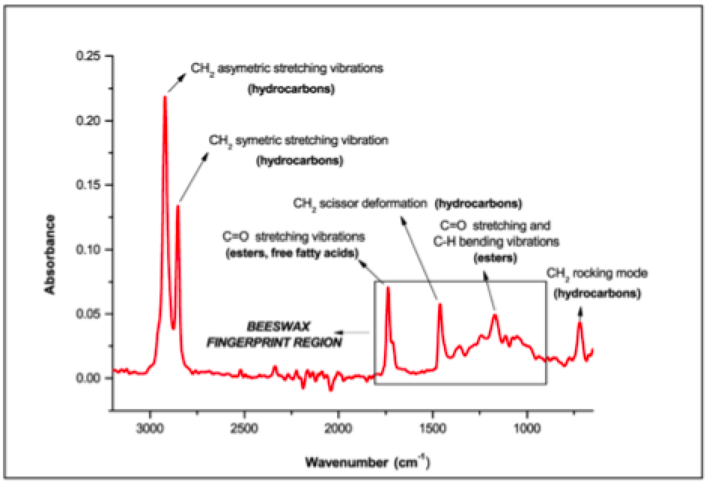


Figure 7: Figure 4.12: FTIR spectrum for virgin beeswax [12]

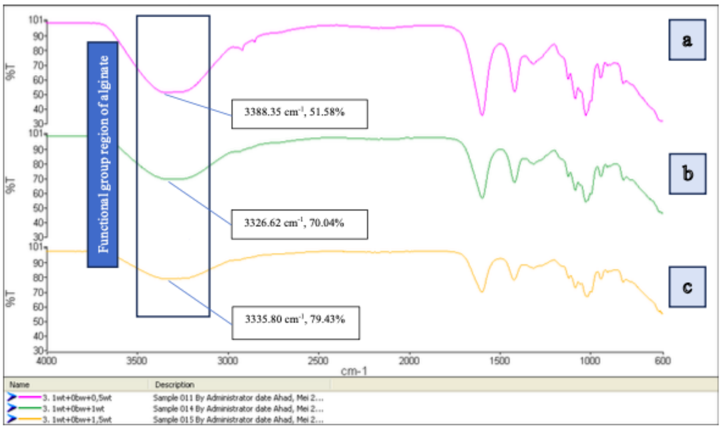


Figure 8: FTIR spectra for (a) 1w/v% of sodium alginate with 3g of beeswax crosslinked with 0.5w/v% of calcium chloride, (b) 1w/v% of sodium alginate with 3g of beeswax crosslinked with 1.0w/v% of calcium chloride, (c) 1w/v% of sodium alginate with 3g of beeswax crosslinked with 1.5w/v% of calcium chloride

Figure 8 shows that the comparison of FTIR spectra for alginate/beeswax biofilm that contain 3g of beeswax with three different concentration of calcium chloride which affect the transmittance percentage which related to the intensities of the biofilm at functional group region for beeswax, (Rb). Figure 8 show that big different percentage of transmittance, this shows that the effect of calcium chloride as a crosslinking agent in the fabrication of alginate/beeswax biofilm that contain 3g of beeswax with different concentration influent the percentage of transmittance which represent as the light passed through the sample.

3.3 AFM analysis

The surface topography of alginate/beeswax biofilm was tested using Atomic Force Microscopy (AFM) to observe the effect of calcium chloride as a crosslinking agent in the fabrication of alginate/beeswax biofilm with different parameters. Figure 9 shows 2D and 3D AFM image of alginate/beeswax biofilm with concentration calcium chloride of 0.5w/v%.

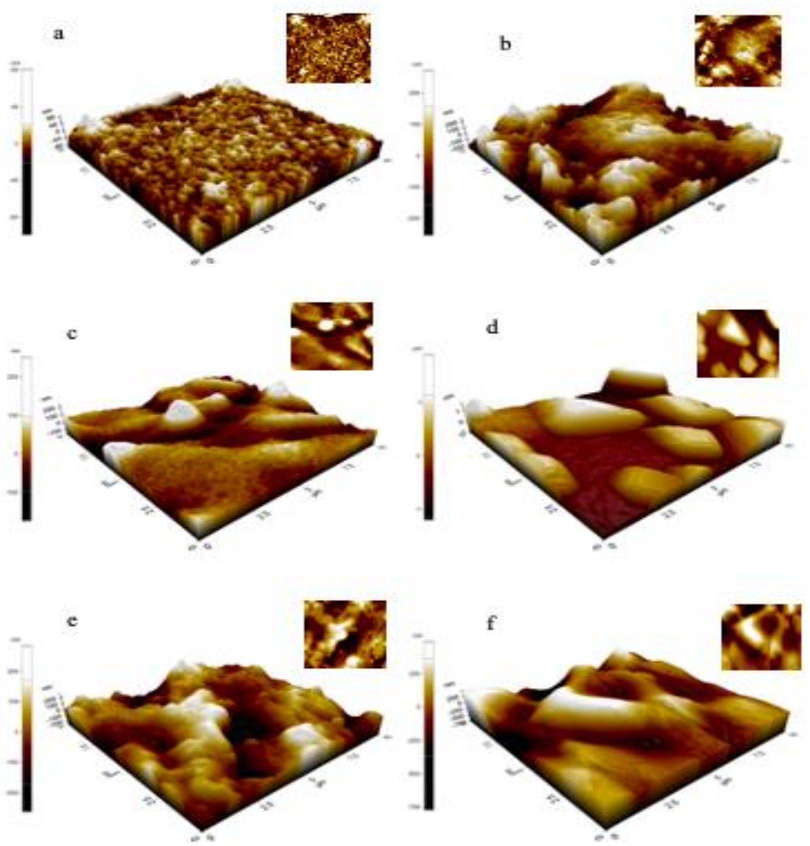


Figure 9: 2D and 3D AFM images of alginate/beeswax biofilm with concentration calcium chloride of 1.0w/v% (a) 1w/v% SA, (b) 1w/v% SA + 1g beeswax, (c) 1w/v% SA + 2 g beeswax, (d) 1w/v% SA + 3 g beeswax, (e) 1w/v% SA + 4 g beeswax and (f) 1w/v% SA + 5 g beeswax

Table 6: Roughness parameter for alginate/beeswax biofilm crosslinked with concentration calcium chloride of 0.5w/v%

Film	Surface Roughness Parameter		
	Rpv (nm)	Rq (nm)	Ra (nm)
1w/v% SA	258.805	23.997	17.410
1w/v% SA + 1g Beeswax	253.185	27.422	19.288
1w/v% SA + 2g Beeswax	852.350	101.191	75.673
1w/v% SA + 3g Beeswax	603.781	88.713	69.243
1w/v% SA + 4g Beeswax	1861.000	272.000	209.000
1w/v% SA + 5g Beeswax	787.698	77.755	56.189

Table 8 shows on roughness parameter for 1w/v% sodium alginate with 0g – 5g of beeswax crosslink with calcium chloride. Compared to previous finding, the effect of calcium chloride as a crosslinking agent in fabrication of alginate/beeswax biofilm plays a large role in increasing the surface roughness

Concentration of calcium chloride at 1.0w/v% shows the increase the mean value of peak to valley value (Rpv), root mean square roughness (Rq) and average roughness (Ra). This proves that concentration of calcium chloride at as a crosslinking agent in fabrication of alginate/beeswax biofilm can increase the surface roughness.

The concentration of calcium chloride at 1.5w/v% reduce the mean value of peak to valley value (Rpv), root mean square roughness (Rq) and average roughness (Ra). This proves that high concentration of calcium chloride as a crosslinking agent in fabrication of alginate/beeswax biofilm can decrease the surface roughness.

3.4 Contact angle analysis

The surface wettability of alginate and beeswax films was evaluated using contact angle measurements. Figure 4.10 and Figure 4.11 shows the contact angle value for alginate /beeswax biofilm with 0g and 5g of beeswax crosslinked with concentration of calcium chloride at 0.5w/v%

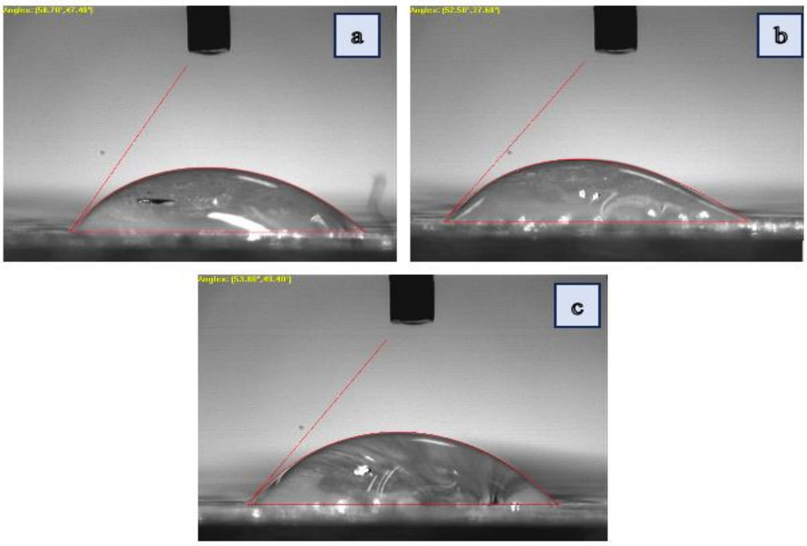


Figure 10: Contact angle of alginate biofilm crosslinked with 0.5w/v% of Calcium Chloride

According to theory, as there is more beeswax in the sample, the contact angle value should increase. The contact angle values consistently decreased as beeswax was introduced, and they were lower than the alginate/beeswax biofilm values with 0g beeswax. The theory may be confirmed by the contact angle values for alginate/beeswax biofilms with 1–5 g of beeswax, which show a steady increase in contact angle values with beeswax content.

Table 7: Contact Angle value for Alginate/Beeswax Biofilm that crosslinked with 0.5w/v% of Calcium Chloride

Sample	Contact Angle (°)			
	1st	2nd	3rd	Average
1w/v% SA	47.40	37.60	49.40	44.80
1w/v% SA + 1g Beeswax	43.40	40.50	39.80	41.23
1w/v% SA + 2g Beeswax	41.30	38.60	29.80	36.57
1w/v% SA + 3g Beeswax	17.40	13.00	19.00	16.47

1w/v% SA + 4g Beeswax	15.00	0	0	15.00
1w/v% SA + 5g Beeswax	20.00	0	0	20.00

The previous findings for contact angle values for 1w/v% of sodium alginate with 0 – 5g of beeswax without crosslink with calcium chloride recorded consistent increase of contact angle when 1w/v% of sodium alginate mixed with beeswax. Alginate/beeswax biofilm with 0 – 4g of beeswax recorded contact angle below 90° which indicates the surface is hydrophilic and has a good surface wettability meanwhile alginate/beeswax biofilm with 5g of beeswax recorded contact angle above 90° which indicates the surface is hydrophobic and has a worst surface wettability. Comparing the lowest concentration of calcium chloride result with the previous findings without crosslinking, contact angle with the lowest concentration of calcium chloride shows the values of contact angle reduced compared to without crosslink and proves that the effects of calcium chloride as a crosslinking agent in the fabrication of alginate/beeswax biofilm reduce the values of contact angle.

The contact angle value for alginate/beeswax biofilm that crosslinked with 1.0w/v% and 1.5w/v% of calcium chloride which is much lower than Table 12 due to higher concentration of calcium chloride. Most of the value in concentration of calcium chloride at 1.5w/v% recorded values of 0° since the high concentration of calcium chloride which is at 1.5w/v% reduce the ability of the biofilm to contain the water because the angle below than 90° that indicates the surface is hydrophilic and has good surface wettability.

Due to the thermal shock when crosslink the alginate/beeswax solution directly after poured into petri dish will effect of rapid thermal changing which a process when a component experiences sudden shifts in thermal stress and strain of significant magnitude whenever its heat change and temperature gradient change. The material may become less ductile as a result of thermal shock, which will reduce the component's normal low cycle fatigue (LCF) life and thermal fatigue life [13]. Thermal shock is more likely to occur in materials with low thermal conductivity and high coefficients of thermal expansion. Due to crack by thermal shock reduce the ability of the biofilm to contain water which indicates the hydrophilic properties and has a good surface wettability.

4. Conclusion

The objectives of this research were achieved, which is fabrication alginate/beeswax biofilm via solution casting method and performing the characterization and testing such as FTIR, SEM, AFM and contact angle measurement to investigate the physiochemical properties of alginate/beeswax biofilm and to investigate the effect of calcium chloride as a crosslinking agent to the fabrication of alginate/beeswax biofilm. Several attempts were carried out in order to find the best way of crosslinking the solution. The process of finding the better way of dissolving the beeswax with sodium alginate solution to get homogenous alginate/beeswax solution.

The characterization was performed and the result for SEM shows that more particles were observed by adding more beeswax into the alginate solution. The result of energy dispersive X-ray scanning electron microscopy (EDX) confirms the existent chemical composition inside alginate/beeswax biofilm. The result of Atomic Force Microscopy revealed the consistent increase in surface roughness in 1w/v% of alginate/beeswax biofilm. According to the FTIR data, the 1w/v% sodium alginate film demonstrated consistent results since vibrational bands from the sample's beeswax and sodium alginate pellets were visible in the FTIR spectrum. Alginate/beeswax biofilm that contain 3g of biofilm with concentration of calcium chloride at 0.5w/v%, 1.0w/v% and 1.5w/v% shows consistent increase percentage of transmittance due to the increase of calcium chloride concentration within the wavenumber range of 2800 - 3000 cm^{-1} compared to alginate/beeswax biofilm that contain 5g of beeswax shows that small different percentage of transmittance at concentration of calcium chloride at 1.0w/v%. Concentration of calcium chloride at 0.5w/v% and 1.5w/v% shows consistent increase percentage of transmittance due increasing of calcium chloride concentration. Lastly, the values for the contact angle for the 1w/v% sodium alginate film with 0–5 g of beeswax consistently show a decrease in value. The contact angle values in all the data are below 90°, indicating that the surface is hydrophilic and has good surface wettability. Most of the contact angle for concentration of calcium chloride at 1.5w/v% unable to detect the reading because alginate/beeswax biofilm contains 0-5 g beeswax with concentration of calcium chloride at 1.5w/v% reduce the ability to contain water. The theory may confirm that beeswax will increase the hydrophobic properties of biofilm, due to thermal shock while crosslinking the alginate/beeswax solution causing the surface of film to crack.

Recommendation

For recommendation, fabricating alginate/beeswax biofilm with 2w/v% of sodium alginate with 0g – 5 g of beeswax at concentration of calcium chloride at 0.5w/v%, 1.0w/v% and 1.5w/v% to compare the result with 1w/v% of sodium alginate with 0g – 5 g of beeswax at concentration of calcium chloride at 0.5w/v%, 1.0w/v% and 1.5w/v%. Next, use spray method to crosslink until the calcium chloride solution filled the whole petri dish and let it soak for 15 - 20 minutes. Furthermore, instead of using heat, dissolve the beeswax into its solvent before adding into sodium alginate solution. Besides, determine the antibacterial properties by doing antibacterial testing. Lastly, alginate/beeswax solution should rest for 1 hour until the temperature reduce to room temperature before crosslink with calcium chloride to prevent from thermal shock.

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