HETEROPOLYSACCHARIDE OF KGM-XANTHAN HYDROGELS FOR THE REMOVAL OF BACTERIA IN A WOUND MODEL

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ABSTRACT: This project focused on the development of polysaccharides hydrogels from Konjac Glucomannan (KGM) and Xanthan (XG) gum in removing the bacterial load on wound. This new therapy design is due to the traditional wound care methods using antibiotic and normal hydrogel cannot adequately treat biofilm infected wounds to healing process. For the treatment of bacterial infected wound, bacterial removal need to be done physically and manually by debridement, which is very painful and require the use of general anesthesia. Therefore, to reduce the formation of slough and necrotic tissue by the formation of bacterial biofilm, there is a need of specific hydrogels that will able to remove bacterial from the wounded site so it will prevent further infection and enhance the healing process. This study focused on the design and optimization of polysaccharide based hydrogels for the wound treatment. Polysaccharide based hydrogels from KGM and XG were developed at different ratio to optimize the physical and chemical properties of the hydrogel that relate to the effectiveness of bacterial removal. Film casting technique was used in the development of the hydrogel film. KGM and XG were selected because of their natural polymers properties which are non – toxic, biocompatible and safe to be used. The hydrogels were tested *in vitro* for bacterial removal efficacy, using *E.coli* species colonies cultured on a gel inside a petri dish to represent the condition of bacteria infested wound bed. Hydrogel with 50:50 % composition blend of KGM-XG was found to be the most effective in the removal of *E.coli* colonies with $13x10^3$ CFU/mL at 95% water content. Morphological studies of hydrogels showed flat interfacial morphologies, except the sample with 100% of XG without KGM. The results present in this study suggested that the biological activities and adhesion of the hydrogels were not controlled only by the chemical structure but also the composition of compound and the amount of water present in the hydrogels.

KEY WORDS: *Hydrogels, glucomannan, xanthan, bacterial removal, wound model.*

1. INTRODUCTION

Bacterial biofilms prominently *Pseudomonas aeruginosa* are being recognized as a major detrimental to chronic wound healing especially in diabetic foot ulcers (DFU) [1]. The gram negative bacteria, *P. aeruginosa* can cause clinical problems and severe tissue damage in diabetic foot ulcers resulting from high-resistance to antimicrobial agents [1]. In recent years, hydrogels have gained attention for its benefits in wound dressing. The appearance of bacterial resistance to antibiotic and antimicrobial agents bring to the discovery of new therapeutic design of new material that able to remove bacteria physically from the wounded site.

Generally, hydrogels are water-swollen, three-dimensional cross-linked polymer network that can respond to the fluctuations of the environmental stimuli [2]. Hydrogels contain 90% of water helps to regulate fluid exchange from wound surface. The high-water content of hydrogels will create a moist healing environment that promote a granulation, epithelialization and autolytic debridement. Because of diabetic wound is complex and unpredictable, the therapy design should be accordingly to the conditions of the wound, where either biodegradable or non-biodegradable hydrogel can be used for the purpose [3]. In this study, the experiments will be focused on the design and optimization conditions to the effectiveness of polysaccharides hydrogels to remove bacterial load on agar, which represents infected wound bed.

In particular, physicochemical properties of polymer's surface are important factors such as hydrophobicity, hydrophilicity and roughness in defining the effect on the adhesion of bacterial [4]. Hydrophobicity is a crucial hydrogel's surface property. Microbial cells adhesion to surfaces can be categorized into two which are passive and active [5]. Passive adhesion involves van der Waals forces and hydrophobic interactions forces between surfaces and cells. Van der Waals' interactions is when all molecules attract one another by a group of attractive forces collectively. In simplified definition, these forces include discussion as the attractive forces that are generated by polarizing molecular dipoles [6]. These kinds of interactions can manipulate physicochemical properties of the polymer surface by adding hydrophilic binding groups to the polymer [7].

In vitro, bacteria tend to adhere to hydrophobic surface in greater numbers and form biofilms which will often cause an inflammatory response and infection in human body, while hydrophilic surface is harder for protein to attach since the hydrophilic brush could inhibit protein's adsorption. A previous study has identified that the water content inside the hydrogels will affect the bacterial adhesion. Bacteria will adhere with higher affinity to low-energy, which is hydrophobic surfaces than to high energy, hydrophilic surfaces [8]. Surface topography of biomaterial is another important factor in molecular scale which can affect bacterial to adhere onto surface for initial adhesion [9]. The actual geometry of a surface can show a complex mix of features even the areas considered very smooth. In fact, the surface roughness of biomaterial was demonstrated as major factor of biofilm deposited on the contact lens study [10]. The irregularities behavior of polymeric surfaces will promote bacterial accumulation and adhesion, due to the increased surface area and depression which provide additional and favorable sites for colonization [11]. However, a linear relation of bacterial adhesion with surface roughness is not always verified as the bacterial adhesion is significantly increased for small increase in roughness but have no significant effect for larger increase [12].

2. MATERIALS AND METHODS

2.1. Preparation of Hydrogels

Materials were obtained from the following manufacturers: *A. konjac* Koch (KGM) without any purification (99% GM content) from Health Plus Ltd, U.K and Xanthan gum (XG) powder from House of Ingredients Sdn Bhd, Selayang. The formulation data for five sets of hydrogels with different compositions at different ratios of KGM and XG that were mixed with 100 mL of distilled water can be referred to Table 1. The powder was weighed in plastic weighing boat by using analytical balance and placed into 250 mL of cleaned beaker. 100 mL of distilled water was measured in measuring cylinder and poured into the same beaker then mixed homogenously for 30 minutes using magnetic stirrer. the mixed solution was poured into petri dish. The weight of hydrogels in petri dish before drying

process were recorded. Then, the hydrogels were dried in fume hood cabinet for 4 days to reduce the contamination. Every petri dish was labeled with percent of KGM : XG represented the compositions of konjac and xanthan gum powder in the hydrogels. During the drying process, the cover of the petri dish was opened halfway and the lid was kept pointed down. After 4 days, the weight of dried hydrogels were recorded.

Table 1.Material compositions in hydrogels

No	KGM : XG (%)	KGM : XG (g)
1	100 : 0	1.00 : 0
2	70 : 30	0.70 : 0.30
3	50 : 50	0.50 : 0.50
4	30 : 70	0.30 : 0.70
5	0 : 100	0 : 1.00

2.2 Characterization of the hydrogels

2.2.1. Physical integrity of the hydrogels

Physical appearance of hydrogels were observed before and after drying process. The determination of stability structure was done by immersed the 2x2 cm of dried hydrogels samples into distilled water over 30 minutes. The state of physical changes of hydrogels were recorded after each 10 minutes period.

2.2.2. Analyzation of chemical structure of hydrogels using FTIR

Each dried hydrogel sample was cut into 1x1 cm to be characterized using FTIR. The FTIR spectrophotometer recorded the changes in confirmation of chemical structures of hydrogels that are sensitive to surrounding changes. The FTIR showed the spectra of polysaccharide hydrogels with their characteristics absorption and identified the band positions and the deformation/formation of hydroxyl groups in the hydrogels.

2.2.3. Hydrogels morphology studies using FESEM

Field emission scanning electron microscope (FESEM) was used to characterize the morphologies of hydrogels' surface and cross section. For surface analysis, the hydrogel was cut into 1 cm^2, sputter coated with gold and imaged using FESEM (Jeol JSM-6700F). For cross section analysis, the squared hydrogel was cut vertically at the center, and sputter coated with gold. This was then imaged using the same FESEM model. Magnification of 250-1000x were utilized to observe the morphological differences between the hydrogels.

2.2.4. Hydrogels degradation study using DSC

The dry samples were weighed (approximately 5-15 mg) in aluminium DSC pans. The empty pan used as a reference Analyses were started at 25 ^oC and continued up to 250 ^oC, with a linear increase of 10 ^oC min^{-1}. Data were analyzed using STARe evaluation software (Mettler Toledo). The glass transition temperature (T_g) normally based on the onset temperature or midpoint of the glass transition temperature range. The significant differences in glass transition temperature of sugars are due to the molecular structure of sugars and interactions between sugar molecules.

2.2.5. Determination of water content

The determination was done by immersed the 2 x 2 cm of dried hydrogels samples into distilled water for 10 minutes. The weight before and after the immersion was recorded for calculation. The amount of absorbed water is calculated as swelling ratio and being expressed in the Equation (1).

$$WC : [(W_s - W_d) / W_s] \times 100 \quad (1)$$

where W_s : the weight of swollen hydrogel

W_d : the weight of dried hydrogel

2.3 Bacterial culture

2.3.1 Media preparation

To prepare the broth media, 25 g of LB Broth (Luria – Bertani) powder was dissolved with 1 L distilled water in 1000 mL of Schott bottle. The broth was sterilized by autoclaving at 121°C for 15 minutes. After the sterilization, the media was left cool down and covered with aluminium foil. The media was stored in chiller at 4°C until further used. For preparing agar plates, 37 g of LB agar powder (Luria – Bertani) was dissolved in 1 L distilled water. The agar solution was sterilized by autoclaving at 121°C for 15 minutes. After the sterilization run, the agar solution was left cool down until 55°C – 60°C to ensure that the bottle can be held in bare hand. The solution was ensured not cold down too long, otherwise the agar will start to be solidified. While the agar was cooling, the bench of laminar flow hood was wiped down with 70% ethanol to sterile the working area in order to prevent the contamination. Each petri dish was marked with date and description of media. When the agar bottle was cool enough to be held, the lid of petri dish was lifted halfway only enough to pour the agar solution. After that, the agar solution was quickly poured into the petri dish. The plates were tilted in order to spread the agar and the lid immediately replaced. The agar were allowed to be solidified about 10 – 15 minutes. After they were totally solidified, the agar were stored in the chiller at 4°C with inverted position before used to avoid the dew droplets of condensation drop onto agar.

2.3.2 Preparing overnight culture

A sterilized 50 mL of Erlenmeyer flask was labeled with date, description of media and bacterial species. The mouth of LB bottle was flamed and 25 ml of LB Broth was pipetted into the flask. A well-defined colony about 1 until 4 mm diameter on a streaked plate was located. The inoculating loop was flamed with Bunsen burner until it showed red hot glow. The loop tip was cool down by stabbing several times onto agar plate and after that a visible cell mass from the selected bacterial colony *Escherichia coli* was scrapped up. The colony was transferred into the flask by immersing the loop into the broth and agitated to dislodge the cell mass. The mouth of flask was flamed before and after transferred the colony. The flask was covered with aluminium foil and sealed with parafilm. The culture was incubated overnight for 12 until 18 hours in shaking incubator at 37°C, 170 rpm.

2.3.3 Plating culture for a wound model

After overnight culture, 0.l mL of each diluted bacterial suspension was transferred onto the agar plate using 1000 µl of micropipette. The mouth of flask was flamed before transferred the inoculum. The L-shape spreader was wiped out with 70% ethanol and then flamed to sterilize it. The inoculum was spread evenly onto agar by spreader. The petri dish

was sealed with parafilm and then incubated with inverted position in incubator at 37ºC for 24 hours until a confluent growth of colonies is formed. The *E.coli* species colonies cultured on a gel inside a petri dish was used to represent the condition of bacteria infested on a wound bed.

2.4 Bacterial removal test

The sample of hydrogels (70:30 %, 50:50 % and 30:70 % of KGM: XG) was cut off into 1 x 1 cm. The samples were soaked into distilled water for 2 minutes and then placed on top of confluent bacterial colonies on the agar plates for 30 minutes. After that, the hydrogels were removed and immersed in the 3 mL broth for another 30 minutes. The total viable of bacterial population densities were determined by doing a serial dilution (10^1) and plating onto fresh nutrient agar. All the experiments were run in duplicate to get an average for a better result.

2.5 Validation

2.5.1 Colony forming unit (CFU)

CFU was used in the counting of the viable bacterial or fungal cells. For the validation of this study, the total viable count of bacterial population that attached onto hydrogel's surface was determined by using colony forming unit technique. 1 mL of broth solution with the immersed hydrogel was pipetted into a tube that containing 9 mL of another broth solution. This dilution was mixed well as labeled as 10^1 of serial dilution. The dilution (0.1 mL) was plated onto fresh agar and incubated overnight. The CFU was calculated by using the formula in Equation (2).

$$\text{CFU/ mL} = (\text{number of colonies x dilution factor}) / \text{volume of culture plate} \quad (2)$$

If the plate of the 10^1 dilution produced 90 colonies, then the number of bacterial colonies in 1 mL of original sample will be calculated as in Equation (3):

$$\text{Bacterial / ml} = (90 \times 10^1) / 0.1 \quad (3)$$

Thus, the total number of colony forming units are: 9,000 CFU / ml. This calculation was repeated for every duplicated experiment of other samples to compare the total number of bacterial colonies that attach onto hydrogels' surface.

2.5.2 Optical density (OD)

The equation relation of CFU / ml to OD can provide useful information in comparison to bacterial concentrations that attached onto hydrogels. Bacterial concentrations for the diluted cells were used for determination of the colony forming unit (CFU) by serial dilutions of bacterial culture and determined by UV-Visible spectrophotometer at 600 nm by measuring the absorbance of the bacterial solutions. A graph was plotted to show the relationship between hydrogels' compositions and CFU/mL and OD_{600nm}.

3. RESULTS AND DISCUSSION

3.1 Observation on the hydrogels' physical integrities

The dried sample of hydrogels were transparent, very soft for 100% KGM and brittle for 100% Xanthan, and those with higher concentrations of xanthan appeared slightly yellowish. The physical integrity of hydrogels was observed over 30 minutes and the hydrogels with single composition of KGM and XG degraded faster compared to blend hydrogels (Figure 1). In this case, the hydrogels with 100% of KGM and 100% of XG were also appeared fragile and partially degraded after 10 minutes,and totally dissolved after 30

minutes. Meanwhile, blend KGM-XG hydrogels were appeared with rigid and stable structure over 30 minutes. The observations on the hydrogel's physical integrities are summarized in Table 2

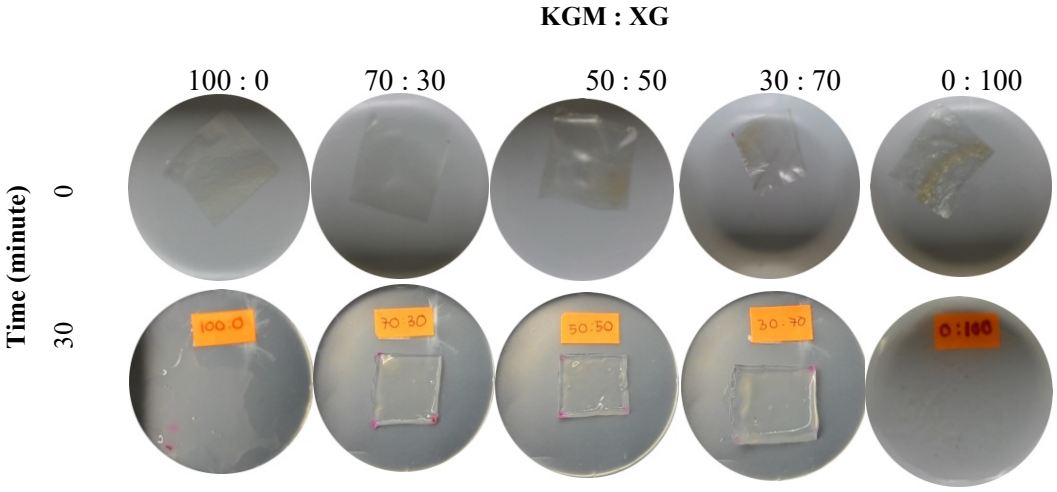

Figure 1. Pictures of hydrogels swelling and breakdown after immersion in water for 30 minutes

Table 2. Qualitative observations of the physical integrities of hydrogels

KGM : XG (%)	Time (minute)			
	0	**10**	**20**	**30**
100 : 0	Solid	Start to break	90 % degraded	Dissolved
70 : 30	Solid	Solid	Solid	Solid
50 : 50	Solid	Solid	Solid	Solid
30 : 70	Solid	Solid	Solid	Solid
0 : 100	Solid	Start to break	70 % degraded	Dissolved

3.2 Observation on the hydrogels' morphologies and surfaces by FESEM

Characteristic morphology of dried hydrogels samples were observed by FESEM. All hydrogels were opaque and possessed no particular texture or porousity on the surface (Figure 2). Each sample of hydrogel with different concentrations of KGM and Xanthan was conducted at magnification 1000x. The surface of (A,F) 100% KGM without XG, (B,G) crosslinked 70% KGM with 30% XG, (C,H) crosslinked 50% KGM with 50% XG, and (D,I) crosslinked 30% KGM with 70% XG were showed flat and even surfaces, but uneven and brittle for (E,J) 100% XG without KGM.

Further analysis of the hydrogel 's cross section (the hydrogels were cut vertically from the centre) showed intricated sponge-like morphologies embedded inside the hydrogel. It can be observed the blend KGM-XG hydrogels had much better polymeric network, where the composition of helical structure XG was evident. The cross section of KGM-XG containing hydrogels appeared to have regular and linear structures, while single composition of KGM and XG containing hydrogels appeared with irregular and brittle structure.

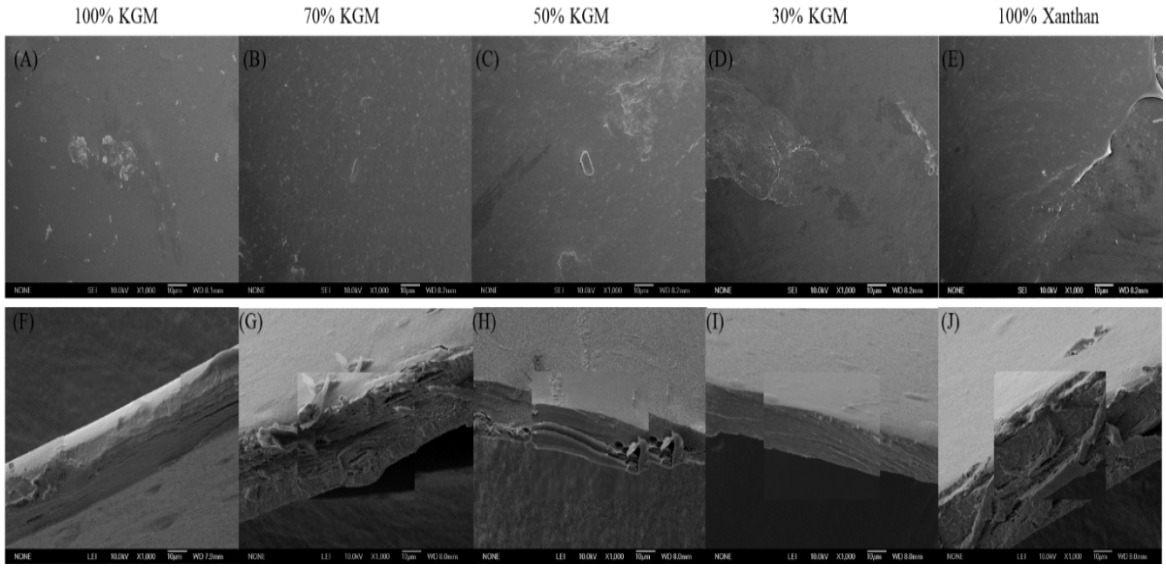

Figure 1. The morphologies of the surface and cross sections of (A, F) 100% KGM without XG, (B,G) crosslinked 70% KGM with 30% XG, (C,H) crosslinked 50% KGM with 50% XG, (D,I) crosslinked 30% KGM with 70% XG and (E, J) 100% XG without KGM observed using FESEM.

3.3 Observations of molecular interactions of KGM-XG using FTIR

The molecular interactions of KGM: XG is examined using Fourier Transform Infra-red (FTIR) as shown in Figure 3. All sample of hydrogels displayed the presence of –OH groups (hydroxyl) which gave broad band between 3650 - 3000 cm^{-1}. Generally, the presence of hydrophilic and hydrophobic moieties in hydrogels verified by FTIR spectroscopy give them a unique ability of to be soluble in both organic and aqueous solvents. Hydrophobicity is a crucial hydrogel's surface property where hydrophobic molecules have to be conducted in living physiological conditions [13]. C–H broad (alkyl) group can be observed at stretching band (2850–3000 cm^{-1}). Also, band from C=O (carbonyl) group that is associated with aldehyde group was identified at 1700 – 1720 cm^{-1}. The band positions of KGM: XG were marked to be higher at 1370 cm^{-1} and 1371 cm^{-1} of KGM-XG blend hydrogels where the assignment shifted from 1369 cm^{-1} in 100% KGM and 1374 cm^{-1} for 100% XG spectra. Carbon–oxygen single bonds displayed stretching bands in the region 1248 -1100 cm^{-1}. The assignments were observed to be lower as increasing of XG concentration in hydrogels where the positions were shifted from 875 cm^{-1} in 100% KGM to 873 cm^{-1} in blend KGM-XG, and 872 cm^{-1} in 100% XG.

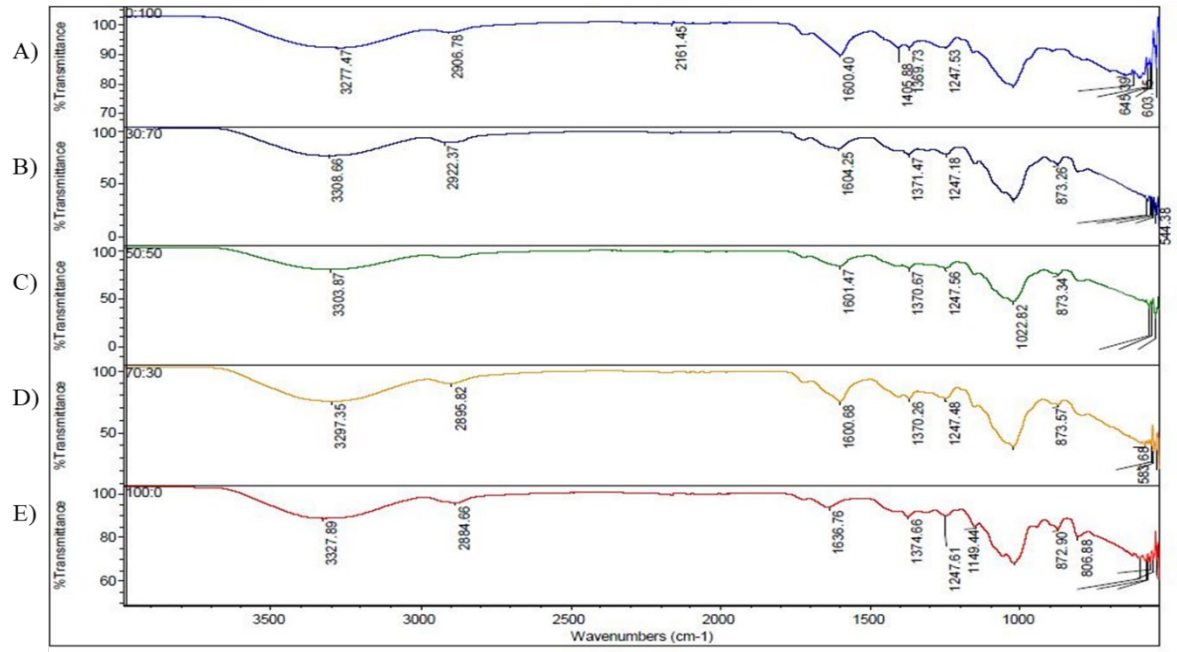

Figure 2. FTIR spectra of (A) 100% KGM without XG, (B) crosslinked 70% KGM with 30% XG, (C) crosslinked 50% KGM with 50% XG, (D) crosslinked 30% KGM with 70% XG and (E) 100% XG without KGM

3.4 Characterization of KGM: XG hydrogels by DSC

Calorimetric analyses for hydrogels was done using DSC and carried out over the full range of studied compositions. The hydrogels in dry condition were subjected to heating process between 25 to 250ºC at a heating rate of 10ºC per minute. The details of the thermograms of dried hydrogels in Figure 4 are summarized in Table 2 From the thermograms, it was clear that the hydrogel's endothermic peak temperature increased with increasing XG concentrations in blend KGM-XG, reflecting the increase of phase separation. The result in Figure 4 suggested that the glass transition temperature (T_g) of hydrogels depends on the concentrations of KGM and the presence of XG. The higher concentration of KGM led to a denser KGM morphology in the hydrogel. Peaks observed for 70:30%, 50:50%, and 30:70 % of blend KGM-XG hydrogels at 103ºC, 85ºC and 99ºC respectively, depicting the morphological changes with the presence of different concentrations in the copolymer. The glass transition (T_g) for KGM containing hydrogels were observed at 60-90ºC. The melting of the amorphous substance of 100% KGM show a sublimation process with a broad radius curvature. As KGM concentration decreases with increasing concentration of XG, the radius curvature at the onset and offset decrease significantly but increasing area. The Tg of KGM which can be observed in the temperature between 60-90ºC, started to deplete after more than 50% of XG addition in the blend. This is due to the different chemical and physical compositions of XG and KGM, with helical structure and linear, respectively.

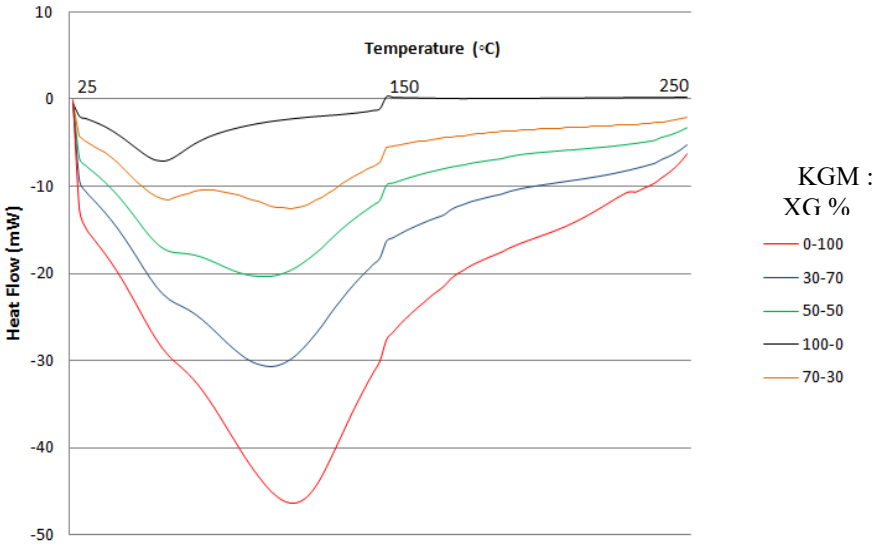

Figure 3. DSC thermograms of 100% KGM, blend KGM-XG, and 100% XG containing dried hydrogels. The dash line in purple refers to the endothermic peak of KGM, and the dash line in orange is for Xanthan

Table 2: Summary of the DSC thermogram and characteristic peak temperature in hydrogel samples

No	KGM : XG (%)	No of peak	Type of peak	Tg (°C)	Peak temperature (°C)
1	100 : 0	1	Endothermic	64.92	90.33
2	70 : 30	2	Endothermic	79.76	103.95
3	50 : 50	2	Endothermic	86.36	85.36
4	30 : 70	2	Endothermic	90.33	99.29
5	0 : 100	1	Endothermic	85.67	110.67

3.5 Attachment of bacterial colonies onto KGM-XG blend hydrogels

Up to this experiment, only KGM-XG blend hydrogels were used to examine the bacterial adhesion onto hydrogel since their physical properties which are remained stable

after being immersed in water. Otherwise, the result might not be correct because both 100% KGM and 100% Xanthan hydrogels started to degrade faster when contacting with water. Since the optical density of a bacterial solution can be easily determined using a spectrophotometer, the equation relating to OD to CFU/ml would provide useful information on total biofilm viable count which is essential for expression of bacterial concentrations. Based on these two relationships, a relation was derived between CFU/mL and bacterial concentrations. In general, the spectrophotometer was set at a wavelength of 600 nm. Total biofilm viable count was calculated as colony forming unit (CFU/mL). The measured data on the relationship between blend KGM:XG hydrogels ratio and CFU/ml and OD of E.coli is shown in Figure 5. The bacterial cells showed almost a linear relationship with a slope of 976.99 CFU/mL/OD indicating that 1.0 OD corresponds to 976.99 CFU/mL. This relation was also represented by a linear regression line with Y= 976.99x + 12.439. From the graph, it can be seen that 50:50 % of blend KGM : XG hydrogel attract greater number of bacterial colonies from petri dish which 13 x 103 CFU/mL, then followed by 70:30% (8.9 x 103 CFU/mL) and 30:70% (4.3 x 103 CFU/mL) of blend KGM:XG hydrogels.

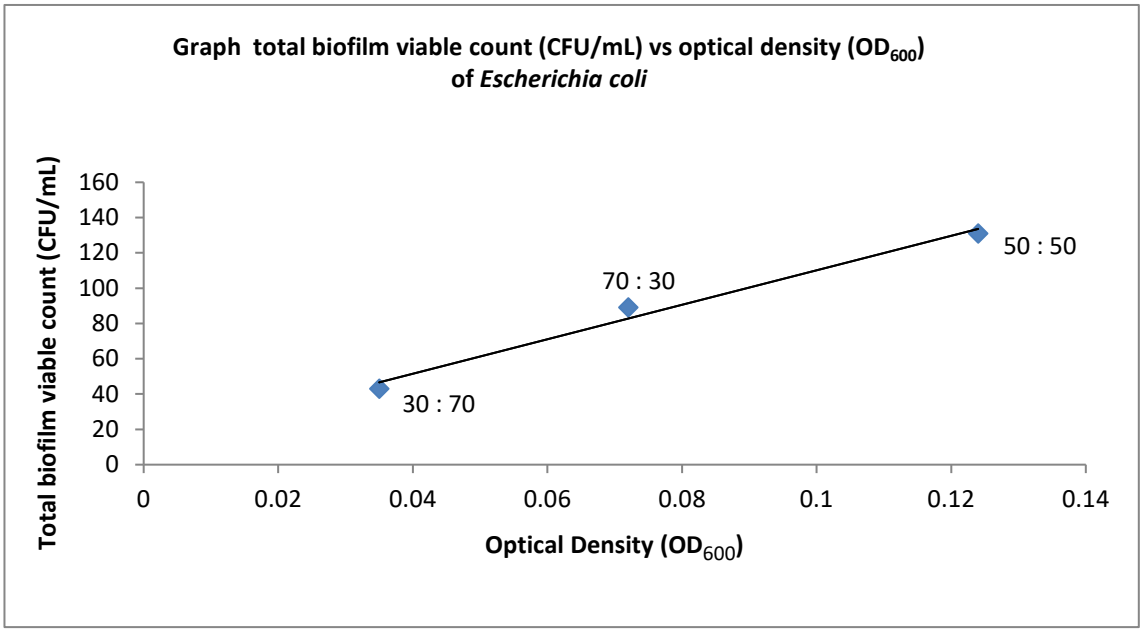

Figure 4. Graph of total biofilm viable count (CFU/ml) adhered to 30:70, 70:30 and 50:30 KGM : XG hydrogels ratio

4. CONCLUSION

In summary this project explores the use of polysaccharide based hydrogels for medical applications. The aim of this project was to demonstrate the potential of KGM and XG in the development of hydrogels for bacterial removal activity during the wound healing process. The choice of polysaccharide as a base of hydrogels was because its properties that consists of bacteria's envelope polysaccharide that will help the bacterial cell recognize the carbohydrate moities. To achieve this, a series of objectives were identified: 1) to produce polysaccharide-based hydrogels, 2) to optimize the ratio of KGM:XG in the production of hydrogels and 3) to understand the effect of hydrophobicity and hydrophilicity in the removal of bacteria from the petri dish. The hydrophilic interaction between hydrogels and

bacterial species plays important rule in adhesion of bacterial colonies to the hydrogels' surface.

Characterizing analysis showed all hydrogels are biodegradable and biocompatible which nontoxic to be used. Initially the attempts were made to produce KGM, XG and KGM-XG hydrogels with crosslinked properties that would benefit wound healing [14]. However, the nature of single composition of KGM and XG hydrogel that breaks easily when contacting with water limits their use in experimental of bacterial removal test, especially when placing the medium onto the agar plate, and can result in the pre-dissolution state. Therefore, the blend KGM-XG hydrogels with greater strength and mechanical properties were conducted by placing onto the confluent bacterial colonies on the agar. These hydrogels have different biological and chemical properties, where the hydrogels which containing lower water content had remarkable properties for bacterial to successfully attach onto the hydrogel. The water content inside the hydrogels presented in this project are equally important for degradation profile and hydrophilicity-hydrophobicity determination.

Increasing the degree of crosslinking to the desired mechanical property of the hydrogel could be achieved. However, too high degree of crosslinking will decrease the % elongation of the hydrogels then creates a more brittle structure. Hence, it would be necessary to have an optimum degree of crosslinking to achieve a relatively strong and yet elastic hydrogel [3]. The interpenetrating phases with different degradation profiles and swelling responses to physiological conditions provided multiple controls over bacterial adhesion onto the hydrogels. A detailed study on the characterizations of the hydrogels would also be advantageous to further understand the factors that influence bacterial - polymer interactions. An adhesive polysaccharide hydrogels will aid the healing process of wound by attaching onto the bacterial colonies and remove them physically from the wound, which that essential in the prevention of further infections. Due to limitation of time for research and facilities, it would be necessary in the future to conduct more repeat experiments for better results. In brief, KGM-XG based hydrogels showed great potential for biomedical applications of the wound treatment especially for diabetic foot ulcer (DFU) where the hydrogels able to reduce the bacterial infections as well as lower the use of antibiotic which speed up the recovery time and reduce the cost in treatment.

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