Freeze-dried Carboxymethyl Chitosan/starch Foam for Use as Hemostatic Wound Dressing

Nalintorn Jungprasertchai
Chula: Chulalongkorn University
Piyachat Chuysinuan
Chulabhorn Research Institute
Pongpol Ekabutr
Chulalongkorn University
Pimolpun Niamlang
Rajamangala University of Technology Rattanakosin Faculty of Engineering
Pitt Suphaphol (pitt.s@chula.ac.th)
Chulalongkorn University

Research Article

Keywords: Carboxymethyl chitosan, starch, foam, hemostatic, wound dressing

Posted Date: March 10th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-297098/v1

Version of Record: A version of this preprint was published at Journal of Polymers and the Environment on August 19th, 2021. See the published version at https://doi.org/10.1007/s10924-021-02260-w.

Freeze-dried carboxymethyl chitosan/starch foam for use as hemostatic wound dressing

Nalintorn Jungprasertchai[1], Piyachat Chuysinuan[2] Pongpol Ekabutr[1], Pimolpun Niamlang[3], and Pitt Supaphol[1*]

[1] *The Petroleum and Petrochemical College, Chulalongkorn University, Bangkok 10330, Thailand*

[2] *Laboratory of Organic Synthesis, Chulabhorn Research Institute, Bangkok,Thailand*

[3] *Department of Materials Engineering, Faculty of Engineering, Rajamangala University of Technology Rattanakosin, 96 Mu 3 Phutthamonthon Sai 5 Road, Salaya, Phutthamonthon, Nakorn Pathom, 73170, Thailand*

*Corresponding authors. E-mail addresses: pitt.s@chula.ac.th (P. Supaphol), Tel: +66827965329, Fax: +6625538943

Abstract

Prolonged bleeding is a general complication that occurs after tooth extraction or oral surgery. Thus, patients must apply pressure and use absorbable wound dressings to stop bleeding and prevent blood loss. This method can stop bleeding and create blood clotting. However, some people have bleeding disorders or cannot stop bleeding with applied pressure after oral surgery. Therefore, hemostatic foam dressing has been an interesting material that can be used to stop bleeding and not damage blood clotting in the wound site. In this study, foam was prepared using a blend of starch with carboxymethyl chitosan and glyoxal as a crosslinking agent. The foam was formed by freeze-drying. Carboxymethyl chitosan/starch foam can absorb and hold water rapidly as analyzed by blood clotting assay and maximum swelling. Thus, carboxymethyl chitosan/starch foam (CM/starch foam) in a 1:4 ratio with 1% glyoxal can also absorb blood very well with suitable properties. It is non-cytotoxic to human dermal fibroblast cells by MTT assay and has good mechanical properties in a wet environment.

Keywords: Carboxymethyl chitosan; starch; foam; hemostatic; wound dressing

1. Introduction

Prolonged bleeding is a common problem. It is usually caused by either trauma or surgery of the soft tissue. Platelet activation during the primary hemostatic releases a number of important cytokines, which start the healing process via chemotactic signals to inflammatory and resident cells. If a wound continues to bleed, healing is delayed because of the disturbed formation of blood clots. Thus, a wound is typically filled with wound filler after receiving damage, such as gauze or foam to allow pressure to be passed on and distributed evenly over the wound bed. Then, the wound is sealed with an adhesive film dressing following the formation of granulation tissue to accelerate wound healing. Therefore, wound filler is very important for absorption and to stop bleeding in a wound. The wound filler should inhibit bleeding, be easily removed without trauma to the wound, nontoxic, anti-inflammatory, and antibacterial. It should also have the ability to absorb excess exudates and be flexible. In commercial usage, gauze is usually used because of its ease of application to large and irregular wounds. Previous results found that wound contraction was more pronounced for foam than for gauze. This cause observed that foam could easily promote wound healing [1, 2]. Recently, foam wound dressings have been utilized for the treatment of wound dressing because they can quickly absorb water from blood to concentrate on native elements of coagulation at the site of bleeding, are non-occlusive and gas-permeable owing to their open cell structure, and absorb excess exudates [3]. Thus, foam is the best material in this work to heal a wound. Further, foam is a candidate for other applications. The typical types of foam used are polyurethane foam or silicone foam. They commonly use a synthetic polymer with a hemostatic agent, which increases hemostatic efficiency. However, polyurethane foam and silicone foam have many disadvantages as well, such as some solvents used being toxic and the price of hemostatic agent being expensive. For this reason, this study tried to avoid using chemical solvents by water and decreased the cost of materials by using natural materials that are easily found and wildly used in medical application. Hence, materials used in this work are obtained from natural products, which are corn starch and carboxymethyl chitosan (CM) [4].

CM is a material sourced from chitosan and obtained from nature, such as shrimp. The properties of CM include water-soluble, non-cytotoxic, biocompatible, and biodegradable. It has various uses in biomedical applications, especially for wound healing and for hemostatic materials. To improve the mechanical properties of CM and decrease the cost of hemostatic

foam, corn starch was used in this properties [5]. Corn starch is a material for biocompatible hemostasis, biocompatible adhesion prevention, tissue healing promotion, absorbable surgical wound sealing and tissue bonding. Moreover, the starch rapidly absorbs fluid from the body without complications [6]. The advantages of corn starch are its high viscosity and high strength. The drawbacks of corn starch include its inflexible properties and the potential to damage the wound site. Accordingly, we will add plasticizer to improve the flexibility of the foam. It may help to improve the mechanical properties of the foam. Glycerol is a common plasticizer and used in food products because it is nontoxic, low-cost and water-soluble as well as having biocompatibility [7]. To blend corn starch with CM, a crosslinking agent is very significant because corn starch and CM can be dissolved in water and be degraded by tissue fluid such as the lymph in wounds. We have to crosslink corn starch and CM by using a cross-linking agent to protect degradation. Glyoxal is generally used as a crosslinking agent because it is non-toxic in low concentrations, is low cost, easy to use and has high potential to crosslink by aldehyde group. Therefore, we have to vary the amount ratio of glyoxal to protect toxicity [8, 9].

For foam formation, there are many methods used to prepare the foam by using starch as a primary role material, such as baking and extrusion. According to sensitivity with high temperature, using a freeze-drying method is one of the best choices for foam formation. As such, this work focuses on a freeze-drying method because it is a common and easy method for making sponge or foam. It is used for materials that are heat-sensitive, such as proteins. Freeze-drying can effectively remove solvents. Moreover, the product of a freeze-drying method is light weight.

The objective of this study was to evaluate the safety of CM and corn starch with a glyoxal crosslinking agent in various ratios as well as to study the morphology, water absorbance, mechanical properties, and blood clotting index of foam for used as a low-cost, disposal, hemostatic material.

2. Materials and methods

2.1 Materials

The starch used to fabricate the foam was of practical grade and obtained from Sigma-Aldrich, USA. CM was purchased from Xi′an Lukee Biotech Co., Ltd., China. Glycerol and

glyoxal 40% aqueous solution were purchased from Sigma-Aldrich, USA. Ethanol 96% in AR grade and Acetone in AR grade were purchased from RCI Labscan, Thailand.

2.2 Fabrication of CM/starch foam

Hemostatic foam dressing was prepared by freeze-drying method. Briefly, corn starch powder was dissolved in water to obtain 5% w/v solution under constant stirring to form a homogeneous solution. This reaction solution was kept stirring at 100 °C for 1 hour. CM solution was prepared at a concentration of 1 % w/v in distilled water with mechanical stirring at 500 rpm and heating at 90 °C until a homogeneous solution. All solutions were cooled to room temperature and air bubbles in the solutions were removed by settling at room temperature. All solutions were mixed together by varying the ratios between CM and corn starch at 1: 1, 1:3, 1:4, and 0:1 by weight. Then, 1% v/v of glycerol was added to the mixing solution in all formulas with mechanical stirring at 500 rpm until a homogeneous solution was formed. Glyoxal with the concentrations of 1 % and 3 % w/v was used as crosslinking agent into each formula, respectively. The ratios between CM and corn starch at 1: 1, 1:3, 1:4, and 0:1 by weight with 1% w/v glyoxal crooslinking agent was coded as CM1:ST1-G1, CM1:ST3-G1, CM1:ST4-G1, and Pure Starch-G1, respectively. The compositions of different formulations of CM/starch at 1: 1, 1:3, 1:4, and 0:1 by weight with 3% w/v glyoxal crooslinking agent was coded as CM1:ST1-G3, CM1:ST3-G3, CM1:ST4-G3, and Pure Starch-G3, respectively. Subsequently, the solution was stirred with a mechanical stirrer at room temperature. The homogeneous solution was left at room temperature for 3 hours to continue the crosslinking reaction. Finally, the solution was frozen at -20 °C for 24 hours and then freeze-dried for 48 hours to obtain CM/starch foam.

2.3 Compression test

The compression test for CM/starch foam.were performed and the samples with cylinder-shaped in a diameter of 15 mm and a height of 10 mm were used (A.B. Castro-Cesena et al., 2016) [10]. The samples were rehydrated in phosphate buffer solution (PBS) at room temperature for 30 min. The mechanical properties of samples were determined by a universal testing machine (Lloyd) in compression mode. The constant speed of compression deformation

was set to 1.25 mm/min with a 500 N load cell at room temperature. The measurements were repeated for triplicate and the average means ± standard deviation values were reported.

2.4 Gel Fraction

Gel fraction of hydrogels was evaluated by measuring their insoluble parts after extraction from the distilled water [11]. The CM/starch foam were cut into cylinder shape with a diameter of 15 mm and a height of 10 mm. The dried samples were weighed (W_i) and immersed in distilled water for 24 h, then the samples were dried in a vacuum oven at 70 °C to constant weight (W_e). Each of samples were carried out in triplicate and the gel fraction was calculated using equation. (1):

$$\text{Gel fraction (\%)} = \left(\frac{W_e}{W_i} \right) \text{x } 100 \qquad (1)$$

Where W_i and W_e are the weight of dried foam and weight of the dry samples after removing soluble part.

2.5 Surface Morphology

The surface morphology of samples was analyzed by scanning electron microscopy (SEM) to observe the homogeneous blending of the foam. For that purpose, the foam was vacuum-coated with gold and analyzed with a Hitachi/S-4800 scanning electron microscope.

2.6 *In Vitro* Blood Clotting

The vitro blood clotting followed S.Y. Ong *et al.* (2006) [12]. All samples were cut and placed into flat-bottom bottles. The bottles were pre-warmed at 37 °C for 5 minutes. Afterwards, 0.3 ml of mixing blood and ACD (anticoagulant citrate dextrose) ratio 9:1 was slowly dropped on each sample, followed by 0.03 ml of 0.2 M $CaCl_2$. The bottles and samples were kept at 37 °C for 10 minutes. Subsequently, 10 ml of DI water was carefully added into each bottle without disturbing the blood clot. 5 mL of solution was sampling and diluted 5 times with distilled water. All sampling was kept at 37 °C for 60 minutes. The blood clotting test was determined by UV-Vis spectrophotometer at 542 nm. The blood clotting index (BCI) was calculated by equation (2):

$$\text{BCI Index} = \frac{100 \text{ x (absorbance of blood sampling solution)}}{\text{(absorbance of ACD blood in DI water)}} \qquad (2)$$

149 **2.7 *In vitro* cytotoxicity assay**

150 **2.7.1 Cell culture**

151 Human dermal fibroblast cells were purchased from GIBCO, Grand Island, NY
152 and culture in a 24 well culture plate with Dulbecco′s Modified Eagle′s Medium (DMEM) with
153 10% fetal bovine serum (FBS) and 1% antibiotic/antimycotic solution. The culture plate was
154 incubated at 37 °C under humidified atmosphere comprising 5% CO_2 air.

155 **2.7.2 Indirect cytotoxicity of CM/starch foam**

156 The indirect cytotoxicity studies of CM/starch foam was demonstrated using MTT
157 assay adapted from ISO 10993-5 standard test. The samples with 1.5 cm in a diameter circular
158 shape were sterilized by 75 % ethanol for 30 min and washed with phosphate buffer solution
159 (PBS) three times and then placed in the culture medium for 24 h in humidified, 5% CO_2 at 37
160 °C to produce extraction media. The human dermal fibroblasts were seeded into 24-well plate
161 with a density of 4×10^4 cells/well and incubated for 24 h. After this, the extraction medium
162 replaced with the DMEM medium and incubated further another 24 h. Cell viability was
163 determined using MTT assay. In brief, a 500 µL MTT solution was added to each well and
164 incubated for 2 h At 37 °C. The MTT solution was replaced by 900 µL of DMSO and 125
165 µL/well glycine buffers (pH 10) to dissolve purple formazan crystals. The optical density (OD)
166 value in each well was quantified at 540 nm by using a microplate reader and the cell viability
167 (%) was calculated related to nontreated control cells using the following equation (3):

168 $$\text{Cell viability (\%)} = \frac{OD\ test}{OD\ control} \times 100 \quad (3)$$

169 **2.8 Swelling behaviors**

170 Water absorption of CM/starch foam was investigated by immersing the sample
171 in phosphate buffer solution (PBS) at pH 7.4. The foams were cut into cylinder shape with a
172 15 mm diameter and thickness 10 mm, then the initial weight of dried samples was measured
173 as the Wi (g). Each foam samples was immersed in15 mL of PBS solution. The swollen samples
174 were removed at each time points (5, 15, 60, 180 min) and excess water was removed on the
175 surface by filter paper. The weights of the swollen samples were recorded as the Ws. The
176 swelling ratio as a function of immersion time were calculated from the following equation (4):

177 $$\text{swelling ratio (\%)} = \left(\frac{W_s - W_i}{W_i} \right) \text{x } 100 \quad (4)$$

178 Where W_s is the weight of foam at equilibrium swelling and W_i is the initial dried
179 sample weight.

180 **2.9 The Porosity**

181 The porosity of the foams was examined via liquid displacement method following
182 the protocol described by Ong *et al.* [12] In brief, the foams were immersed in a known volume
183 (V_1) of absolute ethanol for 15 min. The total volume of absolute ethanol and the foam was
184 recorded (V_2). Thereafter, the ethanol-impregnated foam was removed and the residual volume
185 of ethanol was recorded (V_3). % porosity was calculated by equation. (5):

186

187 $$\% \text{ Porosity} = \frac{(V_1 - V_3)\,x\,100}{V_2 - V_3} \qquad (5)$$

188 **2.10 Statistical Analysis**

189 All data was in the form of mean ± standard deviation (SD). Statistical analysis
190 was achieved by SPSS 12 using a one-way analysis of variance (ANOVA) followed by Scheffe's
191 post hoc multiple comparisons test and $p < 0.05$ was considered significant.

192 **3. Results and Discussion**

193 **3.1 Preparation of CM/starch foam**

194 The CM/starch foam (Figure 1) was prepared in a different ratio between
195 carboxymethyl chitosan and starch with different concentrations of glyoxal. The color of the
196 physical appearance of foams for each formula shows that it has a yellowish color in specimens.
197

198

199 **Figure 1.** Appearance of CM/starch foam in different ratios and concentration of glyoxal after
200 retaining PBS solution

3.2 Gel Fraction

The gel fraction was the method used to calculate the efficiency of crosslinking by using dissolved material into the water and keeping it for several days. In many experiments, results are shown when you increase the amount of crosslinking agent. The structure will increase in mechanical properties and swelling properties as well as the stability of the substance. In this experiment (Figure 2), gel fraction was detected at one day because the application of this work uses materials in the short term. The ratio between CM and starch with 1:1 shows no difference when adding increased glyoxal, likely because the reaction between glyoxal and the free-amino group of CM has high reactivity more than for reactions of CM with the hydroxyl of starch [13].

The effect of crosslinking increases with higher concentrations of the free-amino group. In contrast, ratios of starch increase cause the percentage of crosslink to increase because some of the glyoxal can react with the hydroxyl group of starch. By the way, the ratio between CM and starch 1:4 shows that the content of starch is higher than CM. Thus, the amount of hydroxyl group is more than the amino group. The gel fraction of 1 % glyoxal is not different from another ratio when comparing between concentrations of glyoxal. In this case, the gel fraction decreases because the efficiency of glyoxal with starch is good at high temperatures or in low pH. However, this experiment used heat treatment only. The results were shown to be similar to those by Uslu et al. [8]. When the percentage of glyoxal increases with a starch solution, efficiency decreasing. Moreover, glycerol and glyoxal are water soluble. Thus, they can dissolve in water and some parts of substances are destroyed, which causes loss of weight in the sample. From the statistical results, it can be shown that there is no significant difference except in CM1: ST4, which shows a significant difference. Overall, the results show the stability of foams with a high average gel fraction (Over than 80 %).

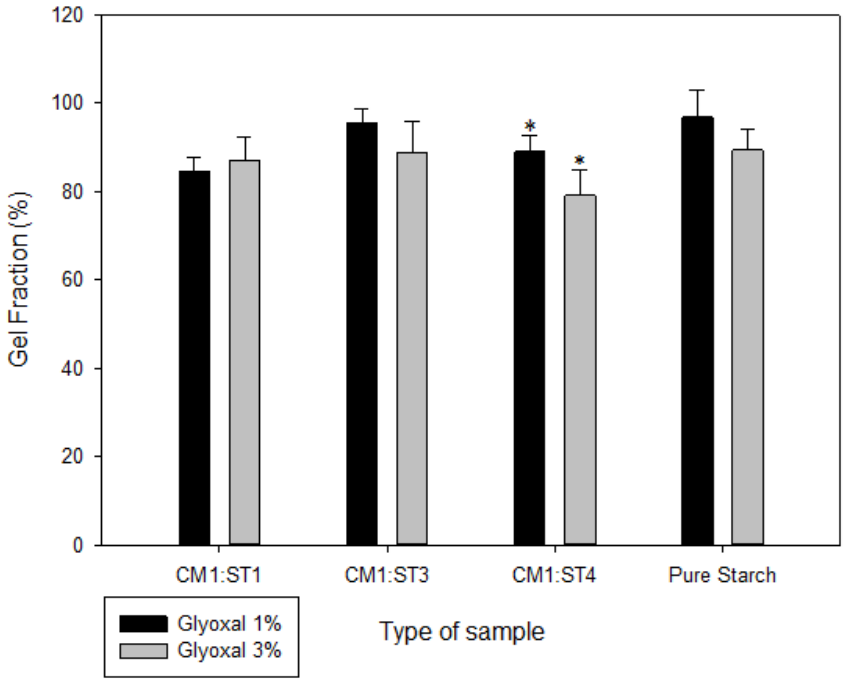


Figure 2. Gel fractions (%) of foam * indicates the significantly different at the 0.05 level ($p<0.05$)

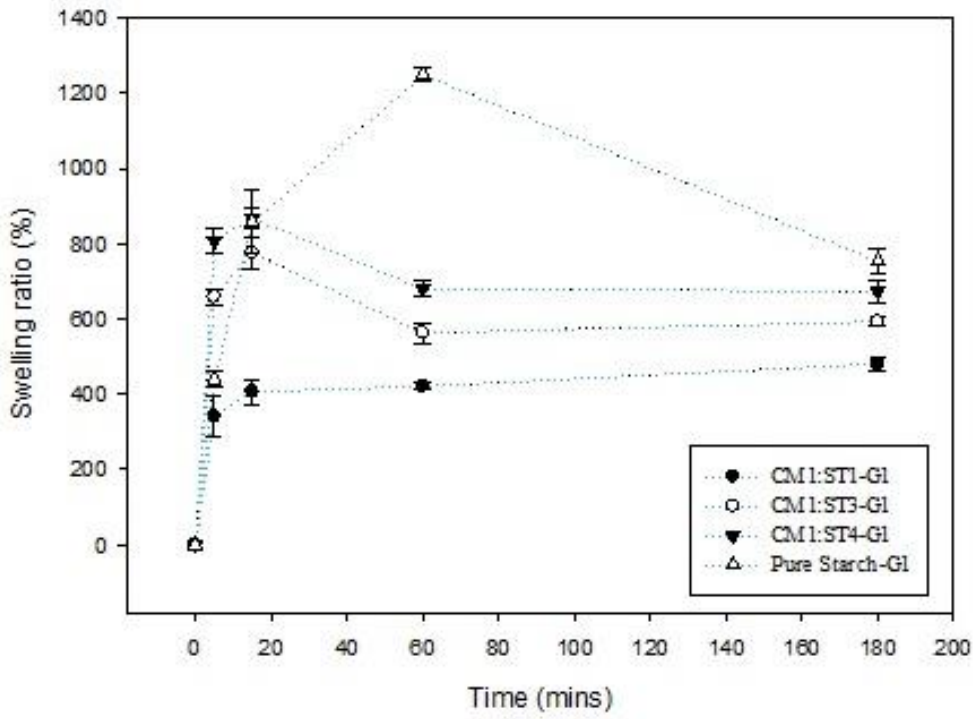
3.3 Swelling behaviors

The swelling ratio of foam was measured in a PBS buffer (pH 7.4) in order to evaluate the capacity of foam to hold a wet environment, which is an important factor when applied onto an open wound surface. The foam was weighed and then incubated in a phosphate buffered solution (PBS, pH 7.4) [14].

The results showed the ability of the foam with water to significantly influence keeping the blood plasma and solution from the body. Swelling properties of CM/starch foam are represented (Figure 3). From the results of maximum swelling, the result of using glyoxal 1 % (w/v) indicated that the mixture between CM increased to maximum in the first 15 minutes and then slowly dropped with increasing time. This is because starch and CM are in higher amounts in the hydrophilic group (Figure 3a). Thus, foam can absorb maximum solution in the first 15 minutes, but will decrease effectiveness over time. The swelling could be decrease slowly because some parts of the starch, such as glycerol content and some parts of uncrosslinking, are dissolved in the water. Uslu et al. showed the result of maximum swelling with increasing time. When increasing the concentration of glyoxal, maximum swelling dropped because the ability to crosslink was low.

The relationship between maximum swelling (%) and gel fraction (%) was in the same direction. For pure starch in different percentages of glyoxal, the gel fraction showed no different result. In terms of maximum swelling, it shows the same trend. The result of 3 % glyoxal shows maximum swelling at 1 hour, which then decreases slightly (Figure 3b). The result shows that increasing the percentage of crosslinking decreases water absorbance. The previous result explained that glyoxal is commonly used in the paper industry. The trend of % glyoxal in various ratios was in the same direction. From the results, the ability of 1 % has maximum swelling at 15 minutes and 3 % maximum swelling at 1 hour. This result can be applied to the time used to absorb the bleeding of blood because bleeding should be stopped as soon as possible to prevent excessive blood loss [8, 15].

(a)



(b)

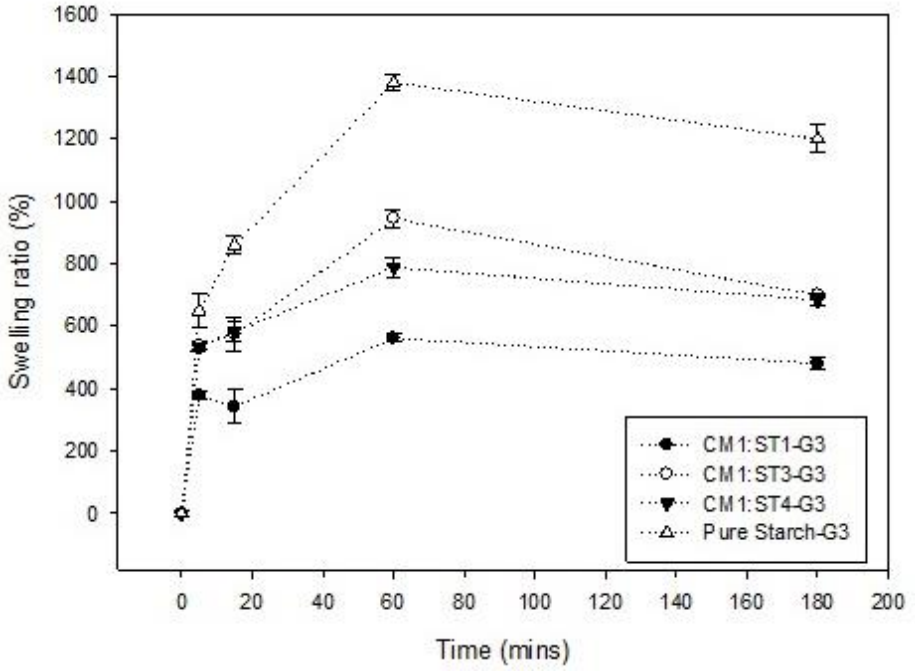


Figure 3. Swelling ratio (%) of foam with increasing of times

3.4 Structure Characterization

The morphology of foam with different ratios between CM and starch with different concentrations of glyoxal (1 % and 3 % by weight) were analyzed by SEM (Figure 4). SEM micrographs of the cross-sections of the foams showed that morphology changed with 3 % concentration of glyoxal. These SEM micrographs reveal the presence of an open macro porous framework. It is well confirmed that the composites of CM and starch foams usually present a porous structure. Although SEM does not provide quantitative information about porosity, it can be seen that foams with 3 % glyoxal have smaller pores than 1 % glyoxal. The concentration of glyoxal directly affects the porous size of foam. (4A) and (4B) showed the cross section of starch with glyoxal in different concentration only. The picture shows that increasing the concentration of crosslinking, decreases the quantity of pores. When combining CM with starch in ratios of 1:1, 1:3, 1:4 by weight with 1 % glyoxal (1A, 2A and 3A), it exhibits the rough surface because a low concentration of glyoxal may be make some parts of CM interact together and infiltrate into starch to react with it. 3 % glyoxal and CM and starch in various ratios (1B, 2B, 3B) show smooth surfaces because the amount of glyoxal is sufficient

to react between CM and starch. Therefore, increasing the amount of glyoxal makes foam have smaller pores and a smooth surface.

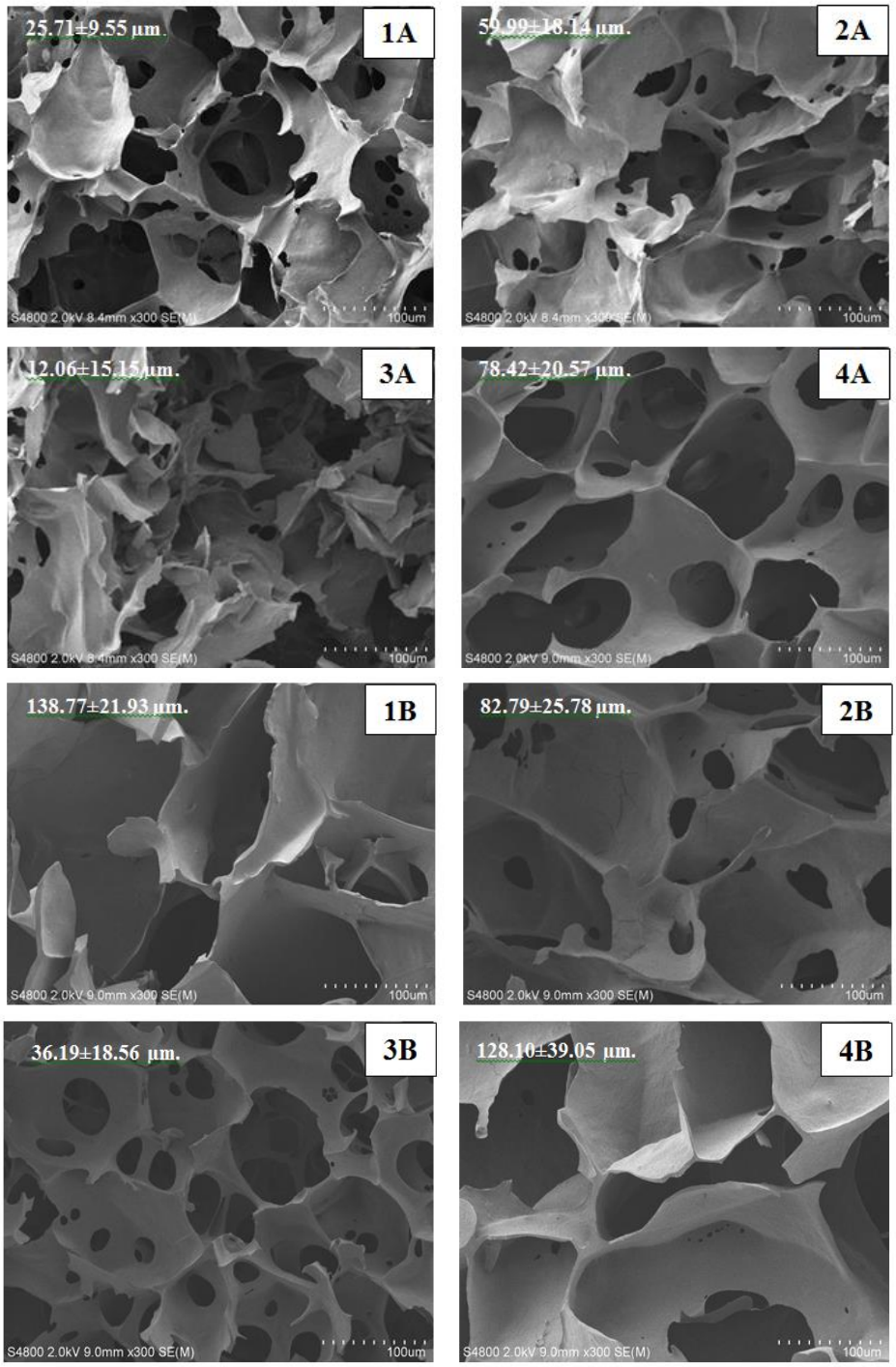


Figure 4. Representative SEM micrographs showing the cross section of CM: Starch foam. (1A) 1:1, (2A) 1:3, (3A) 1:4, (4A) Pure Starch with 1 % glyoxal. And (1B) 1:1, (2B) 1:3, (3B) 1:4, (4B) Pure starch with 3 % glyoxal

3.5 Porosity (%)

The most important thing for a material is porosity because it can indicate the ability of pores to absorb the liquid solution from a wound [11]. Porosity is the measure for percentage of pores in each sample. The original porosity of foam is measured through the ethanol replacement method [16]. The result shows that the porosity of 1 % glyoxal is more than 90 percent (Figure 5). 1 % glyoxal shows a loose structure. Thus, crosslinking was added into the substance with increased concentration. The structure of foam was dense because polymer chains have connected between each other. The morphology of the samples shows that when increasing the crosslinking percentage causes the structure to collapse and lose porosity because the polymer chains are packed closely. However, CM1:ST4-G3 shows a significant difference because the effectiveness of crosslinking in gel fraction is lower. Therefore, the structure is loose.

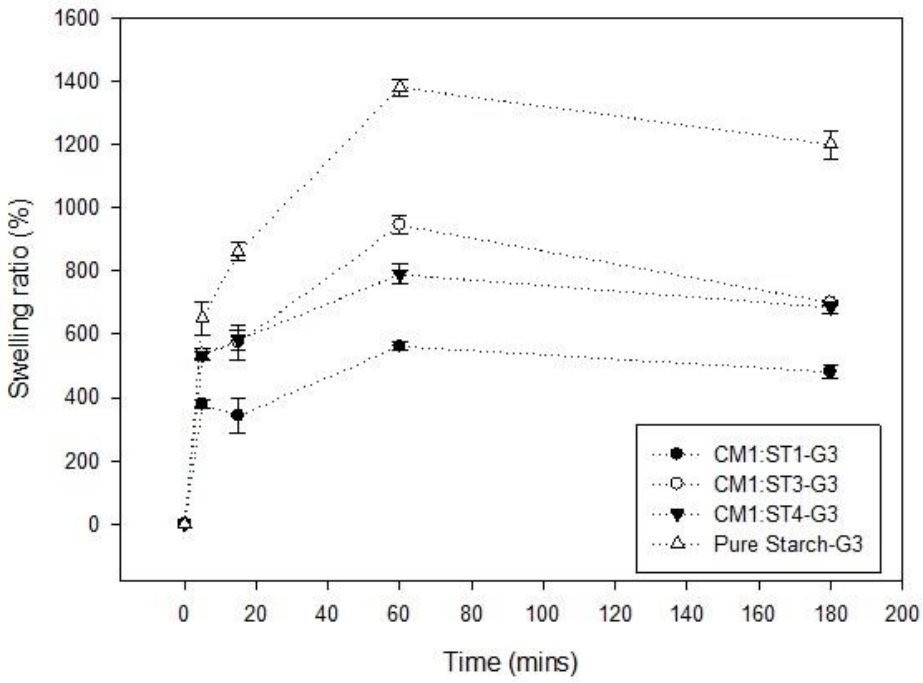


Figure 5. Porosity of CM: starch foam in various ratios.

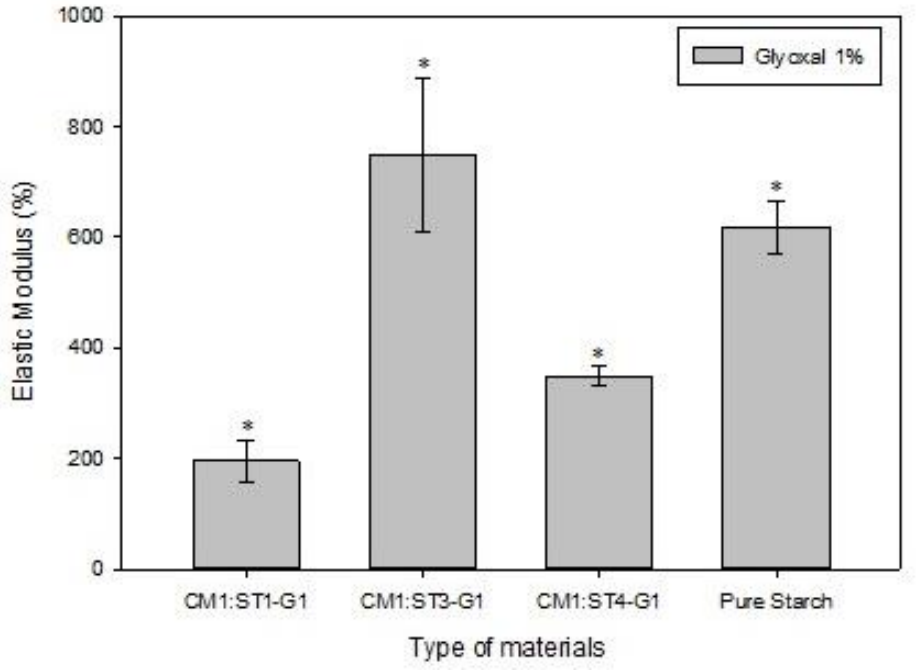
* indicates the significantly different at the 0.05 level ($p<0.05$). Error bars show one standard deviation of the mean

3.6 Mechanical Properties

Compression tests were performed on all fabricated foam samples. Foams were loaded at a strain rate of 1 x 10^{-3} s^{-1} to 75 % strain using a LOYD machine with a 500 N load cell. Elastic modulus was calculated from the slope of the stress-strain curve in the linear region over a 2 % strain range within all data between 0 and 10 % strain [10].

For application use with a wound, material was used that should have low elastic modulus. This means that low elastic modulus is comfortable to the wound and does not damage the wound. Figure 6 represents the elastic modulus of CMC/starch foam. The behavior of foam shows low modulus because the foam is a soft material. Similar to all bio-composites, CM and starch exhibited elastic modulus curves characteristic of elastic foams. However, differences such as crosslinking concentration and the addition of CM or concentration of starch led to structural characteristics that directly affected the mechanical response of the foams. For 1 % glyoxal (Figure 6 a), the ratio between CM: ST was 1:3 and had the highest elastic modulus of 748.82 ± 16.64. The ratio between CM: ST was 1:1, which had the lowest elastic modulus of 195.38 ± 36.59. The pure starch shows low elastic modulus because crosslinking has lower efficiency with a hydroxyl group. This method controlled the temperature and crosslinking time. Thus, crosslinking for hydroxyl was slower than for the free-amino group [8]. The suitable material with 1 % glyoxal is CM1:ST1 because of its lower elastic modulus.

For 3 % glyoxal (Figure 6 b) the pure starch has the highest elastic modulus was 11641.73 ± 998.99. The ratio between CM: ST was 1:3, which has the lowest elastic modulus of 168.76 ± 28.60. Pure starch shows the highest elastic modulus because the percentage of glyoxal is too high to react with the hydroxyl group of starch. In the physical properties of pure starch, the hardest material is shown. The lowest is CM1:ST3 because of the previous reason from gel fraction. 3 % glyoxal has low mechanical defect in the specimen and trapping of the free amino group in the large particle in the starch phase. The result between 3 % glyoxal and 1 % glyoxal is different and significant. (a)



(b)

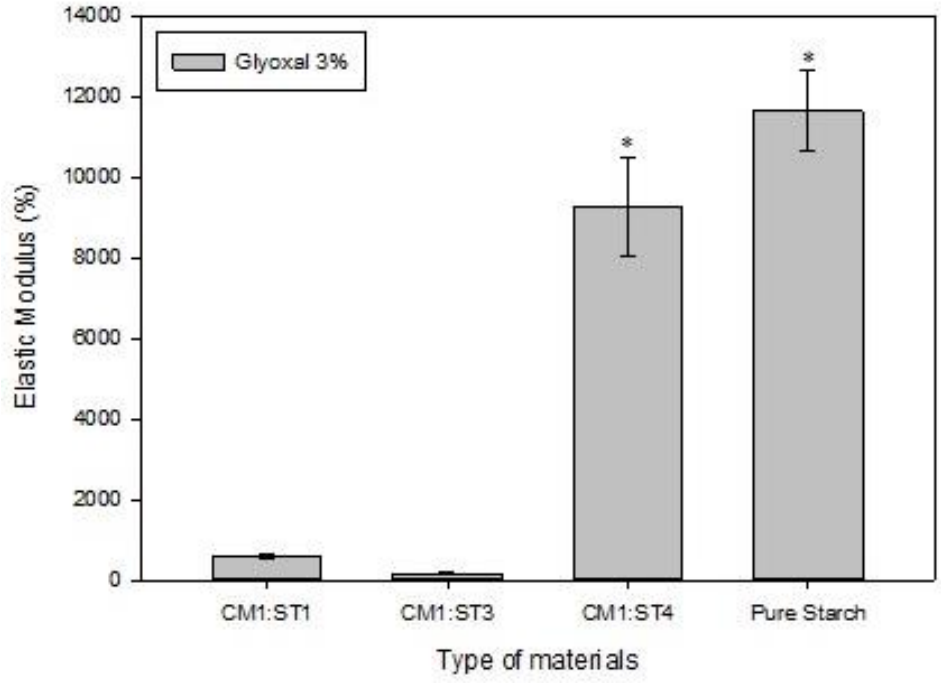


Figure 6. Elastic modulus of CM/starch foam in various ratios with (a) 1 % and (b) 3% w/v of glyoxal

*indicates the significantly different at the 0.05 level ($p < 0.05$). Error bars show one standard deviation of the mean.

3.7 Cytotoxicity

Cytotoxicity is a method used to determine cell availability and cytotoxic effects by using a colorimetric assay that indirectly measures the metabolic activity of living cells based on the reduction of MTT reagent [17]. Cytotoxicity was examined by the viability of cells. The results are shown in Figure 7. For standard testing, non-toxic material can be used with humans because it must have a percentage of cell viability greater than 80 %. The low percentage of cell viability is toxic material. In this research, we used human fibroblast cells to be representative for cytotoxic testing. We can estimate the amount of released glyoxal from chitosan-starch foam dressing. Glyoxal has the same structure as glutaraldehyde, but it has a short chain. Thus, we needed to test cytotoxicity to confirm that glyoxal can be used in a hemostatic wound dressing. A cultural-grade polystyrene well plate is generally used as positive control in the MTT assay. Moreover, a polystyrene plate can promote a higher cell growth rate because of the distribution of nutrients, which is different in a polystyrene plate. In the results, the ratio of CM and starch with glyoxal in various percentages show cell availability greater than 100 %. We illustrate that other factors such as the microstructure of the foam directly influences the viability of the cells seeded on the foams. In this case, higher absorbance is interpreted as the higher number of cells attached to the scaffolds. Other reports show that the incorporation of starch to foam increased cell viability. As dental fillers, however, these materials should prevent volume reduction after tooth extraction. An ideal graft material placed in extraction sockets should also promote healing to allow initiation of osteogenesis [10].

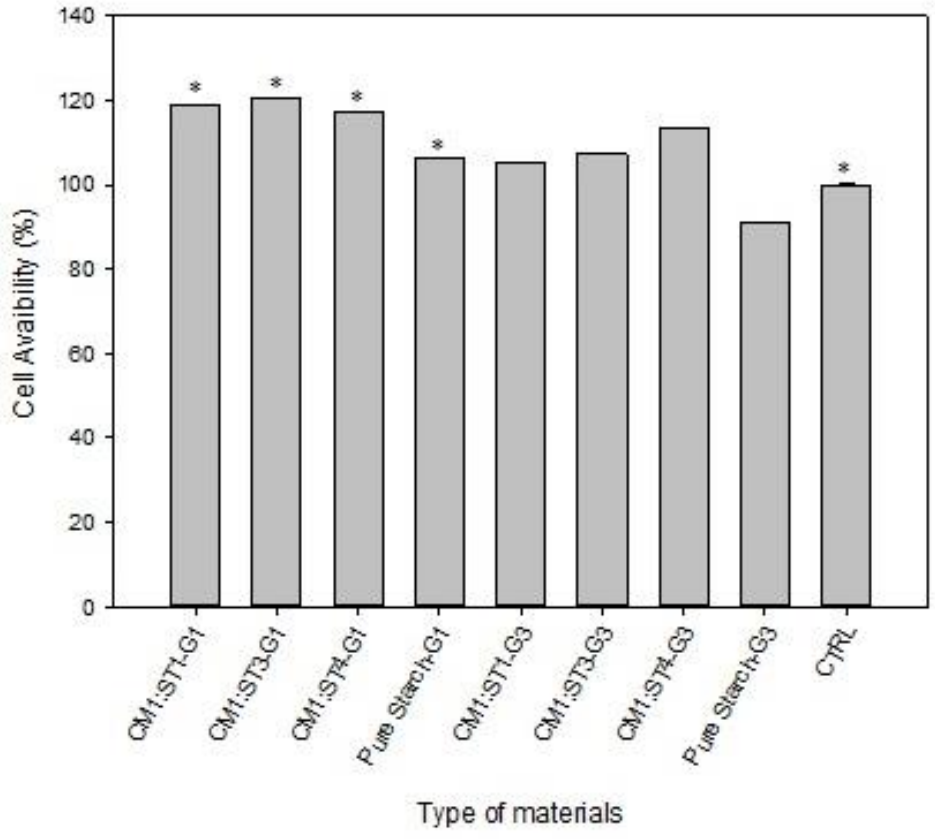


Figure 7. Viability of human dermal fibroblast cells on CM/starch foam (n = 3/group). Error bars show one standard deviation of the mean.

* indicates the significantly different at the 0.05 level ($p<0.05$)

3.8 Blood Clotting Index

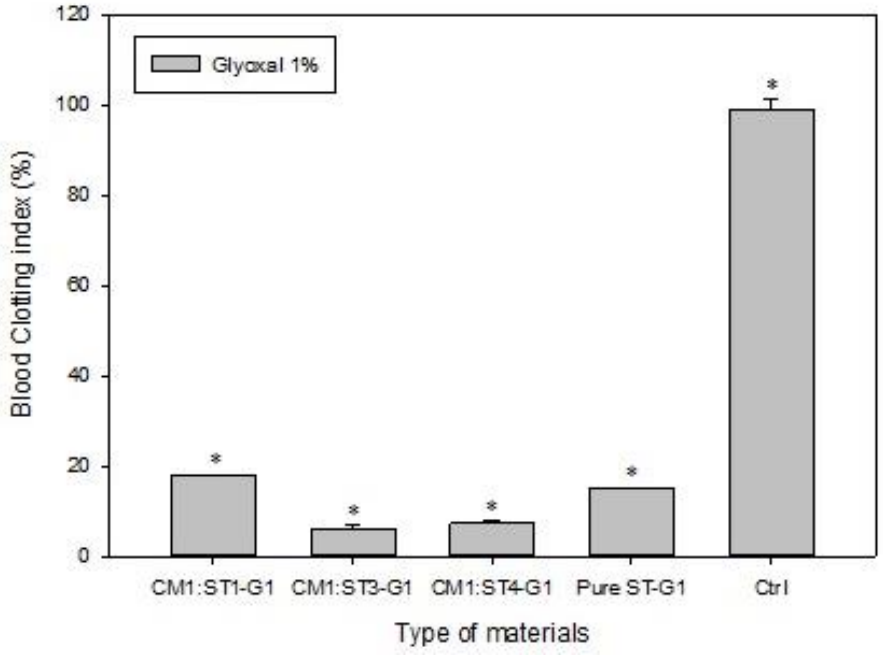
Uncontrolled hemorrhaging is the main cause of death. Thus, absorbable hemostatic agents have been developed to prevent prolonged bleeding [18]. An ideal hemostatic agent should be lightweight, easy to store, and able to be rapidly applied to a hemorrhaging wound. It should be conformable to the wound, allowing the hemostatic agent to reach areas of injury that may be difficult to access with direct pressure. The dressing should cause minimal local tissue reestablishment, be easily removable from the wound, and not carry particles which can spread systemically. The dressing must not be washed away by rapid bleeding from high-flow blood vessels [19]. Many experiments have demonstrated that CM can be applied for hemostasis and the acceleration of wound healing. However, more studies are necessary to determine whether CM has significant effects on the coagulation and fibrinolytic functions of an organism after absorption [20]. Another study shows that CM attracted and activated the

404 platelets most effectively, probably because of its carboxyl functional group being similar to
405 that of collagen fiber in the vascular endothelium [5].

406 The results of various ratios for CM/starch foam with 1 % glyoxal on blood clotting
407 index (BCI) (Figure 8 a) show that the ratio CM:ST;1:3 had the lowest BCI. It can be described
408 that CM can trap the blood clotting very well and release some of the red blood cells that were
409 not trapped in the porous. In our experiment, increasing the starch ratios into the mixtures
410 showed a decrease in blood clotting index. In previous experiments [10], the granules of starch
411 act as molecular sieves that locally dehydrate blood by absorbing its fluids and molecular
412 components, concentrating platelets and other serum proteins on the surface of the scaffolds.
413 This activates the clotting process. However, the blood-clotting response of the foams was
414 improved when starch was doubled from 3-4, i.e. foam 1:3 and 1:4 clotted more blood than
415 foam with composition 1:1 ($P < 0.05$) after 5 min of contact with blood ($P < 0.05$).

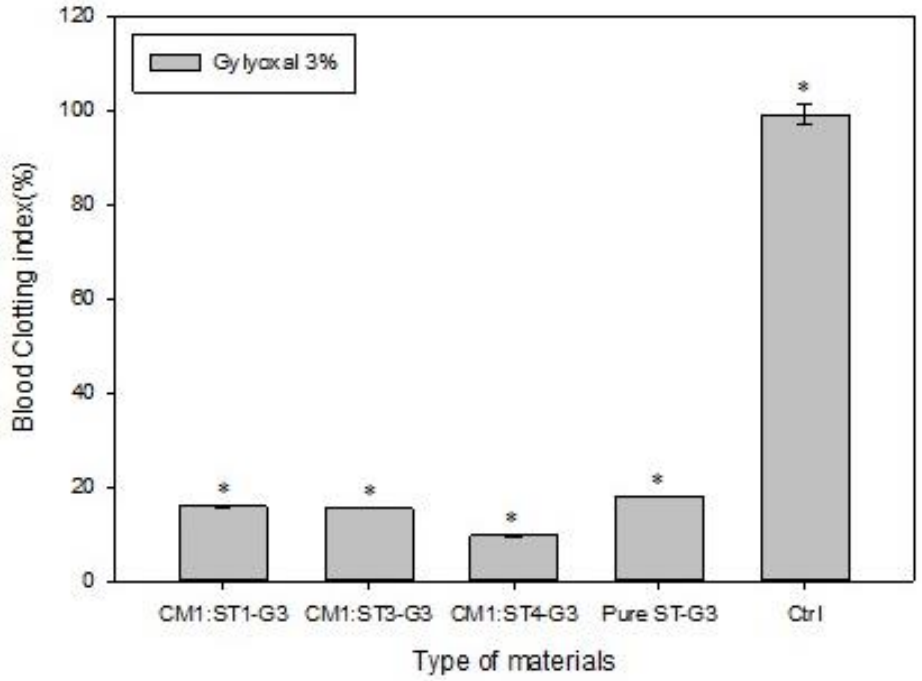
416 The results of various ratios of CM/starch foam with 3 % glyoxal on blood clotting
417 index (BCI) (Figure 8 b) show that the ratio CM:ST; 1:4 is the best for use with 3 % glyoxal.
418 We can describe that using a higher concentration of glyoxal makes the structure of foam
419 collapse inside of it. Thus, it is clear that BCI is higher than using 1 % concentration. The
420 ability to absorb foam depends upon the absorbed blood platelets, porosity, efficiency of
421 crosslinking, and attractiveness of the functional group and blood platelets.
422
423
424
425
426
427
428
429
430
431
432
433
434
435 (a)

436



437

438 (b)



439

440 **Figure 8.** The effect of various ratios of CM/starch foam with (a) 1 % and (b) 3 % glyoxal on

441 blood clotting index (BCI)

442 * indicates the significantly different at the 0.05 level ($p<0.05$) Error bars show one standard

443 deviation of the mean.

444 **4. Conclusions**

Various ratios between carboxymethyl chitosan/starch were blended with glyoxal in concentrations of 1 % and 3 % (w/v). Subsequently, the mixture solution was freeze-dried for 24 hours into foam. The results showed a significant amount of carboxymethyl chitosan and starch with blood clotting properties. The ratios between carboxymethyl chitosan/starch were significant in the role of swelling, porosity and blood clotting properties. The porosity of the materials shows the highest percentage in CM1:ST3-G1. The mechanical properties are the highest at pure starch-G3. Therefore, increasing the concentration with carboxymethyl chitosan will decrease the mechanical properties in low elastic modulus. Crosslinking is significant with mechanical properties. From experimentation with gel fraction and maximum swelling, it was shown that this foam is suitable for use in the short term because gel fraction is high in initial time (24 hours) and maximum swelling of 1 % and 3 % glyoxal is 15 minutes and 1 hour, respectively. The morphology and porosity of carboxymethyl chitosan/starch foam is dependent on the crosslinking concentration. Blood clotting index showed the efficiency of carboxymethyl chitosan to stop bleeding. Carboxymethyl chitosan/starch foam successfully enhances swelling, blood clotting, cytotoxicity activity and good mechanical properties for use as a hemostatic dressing. Costs can be reduced by using starch material that is low-cost, easy to find and can stop bleeding rapidly.

Acknowledgements

The authors acknowledge financial support received from the Research Pyramid, Rachadaphiseksomphot Endowment Fund (GCURP_58_02_63_01) of Chulalongkorn University. This work was supported in part by (1) the Petroleum and Petrochemical College, Chulalongkorn University, (2) the 90 th Anniversary of Chulalongkorn University Fund (Rachadaphiseksomphot Endowment Fund) (3) the Center of Excellence for Petroleum, Petrochemicals, and Advanced Materials (CE-PPAM) (4) The Royal Government of Thailand Scholarship 2562.

References

1. M. Malmsjo, R. Ingemansson, Effects of green foam, black foam and gauze on contraction, blood flow and pressure delivery to the wound bed in negative pressure wound therapy. J Plast Reconstr Aesthet Surg. 64 (2011) 289-296.
2. H. Larjava, Oral wound healing: cell biology and clinical management: John Wiley & Sons; 2012.
3. S-h. Hsu, K-C. Hung, C-W. Chen, Biodegradable polymer scaffolds. J. Mater. Chem. B 4 (2016) 7493-7505.
4. X. Liu, Y. Niu, KC. Chen, S. Chen, Rapid hemostatic and mild polyurethane-urea foam wound dressing for promoting wound healing. Mater Sci Eng C Mater Biol Appl. 71 (2017) 289-297.
5. W. Janvikul, P. Uppanan, B. Thavornyutikarn, J. Krewraing, R. Prateepasen, In vitro comparative hemostatic studies of chitin, chitosan, and their derivatives. J. Appl. Polym. Sci.102 (2006) 445-51.
6. X. Ji, C. Xing , X. Shi, J. Chen, Modified starch material of biocompatible hemostasis. Google Patents; 2008.
7. HW. Tan, AR. Abdul Aziz, MK. Aroua, Glycerol production and its applications as a raw material: A review. Renewable and Sustainable Energy Reviews. 27 (2013) 118-127.
8. M-K. Uslu, S. Polat, Effects of glyoxal cross-linking on baked starch foam. Carbohydr Polym.87 (2012) 1994-1999.
9. Q. Yang, F. Dou, B. Liang, Q. Shen, Studies of cross-linking reaction on chitosan fiber with glyoxal. Carbohydr Polym.59 (2005) 205-210.
10. AB. Castro-Cesena, TA. Camacho-Villegas, PH. Lugo-Fabres, EE. Novitskaya, J. McKittrick, A. Licea-Navarro, Effect of starch on the mechanical and in vitro properties of collagen-hydroxyapatite sponges for applications in dentistry. Carbohydr Polym. 148 (2016) 78-85.
11. Z. Ajji, I Othman, JM. Rosiak, Production of hydrogel wound dressings using gamma radiation. Nuclear Instruments and Methods in Physics Research Section B: Beam Interactions with Materials and Atoms. 229 (2005) 375-380.

503 12. S-Y. Ong, J. Wu, SM. Moochhala, M-H. Tan, J. Lu, Development of a chitosan-based
504 wound dressing with improved hemostatic and antimicrobial properties. Biomaterials.
505 29 (2008) 4323-4332.
506 13. CM. Vaz, LA. de Graaf, RL. Reis, AM. Cunha, In vitro degradation behaviour of
507 biodegradable soy plastics: effects of crosslinking with glyoxal and thermal
508 treatment.Polym Degrad Stab.81 (2003) 65-74.
509 14. SY. Lee, T. Kamarul, carboxymethyl chitosan enhanced scaffold porosity and
510 biocompatibility under e-beam irradiation at 50 kGy. Int J Biol Macromol. 64 (2014)
511 115-22.
512 15. S. Iannace, L. Sorrentino, E. Di Maio, Biodegradable biomedical foam scaffolds. (2014)
513 163-187.
514 16. SAA. Ghavimi, MH. Ebrahimzadeh, MA. Shokrgozar, M. Solati-Hashjin , NAA.
515 Osman, Effect of starch content on the biodegradation of polycaprolactone/ starch
516 composite for fabricating in situ pore-forming scaffolds. Polym Test. 43 (2015) 94-
517 102.
518 17. Y. Zhang, Z. Chang, W. Luo, S. Gu, W. Li, An J. Effect of starch particles on foam
519 stability and dilational viscoelasticity of aqueous-foam. Chin. J. Chem. Eng. 23 (2015)
520 276-280.
521 18. X. Huang, Y. Sun, J. Nie, W. Lu, L. Yang, Z. Zhang, Using absorbable chitosan
522 hemostatic sponges as a promising surgical dressing. Int. J. Biol. Macromol.75 (2015)
523 322-329.
524 19. M. mani, V. Ebenezer, R. Balakrishnan, Impact of Hemostatic Agents in Oral Surgery.
525 BJR Suppl. 7 (2014) 215-219.
526 20. D. Fu, B. Han, W. Dong, Z. Yang, Y. Lv, W. Liu, Effects of carboxymethyl chitosan
527 on the blood system of rats. Biochem. Biophys. Res. Commun. 408 (2011) 110-114.
528

Figures

Figure 1

Appearance of CM/starch foam in different ratios and concentration of glyoxal after retaining PBS solution

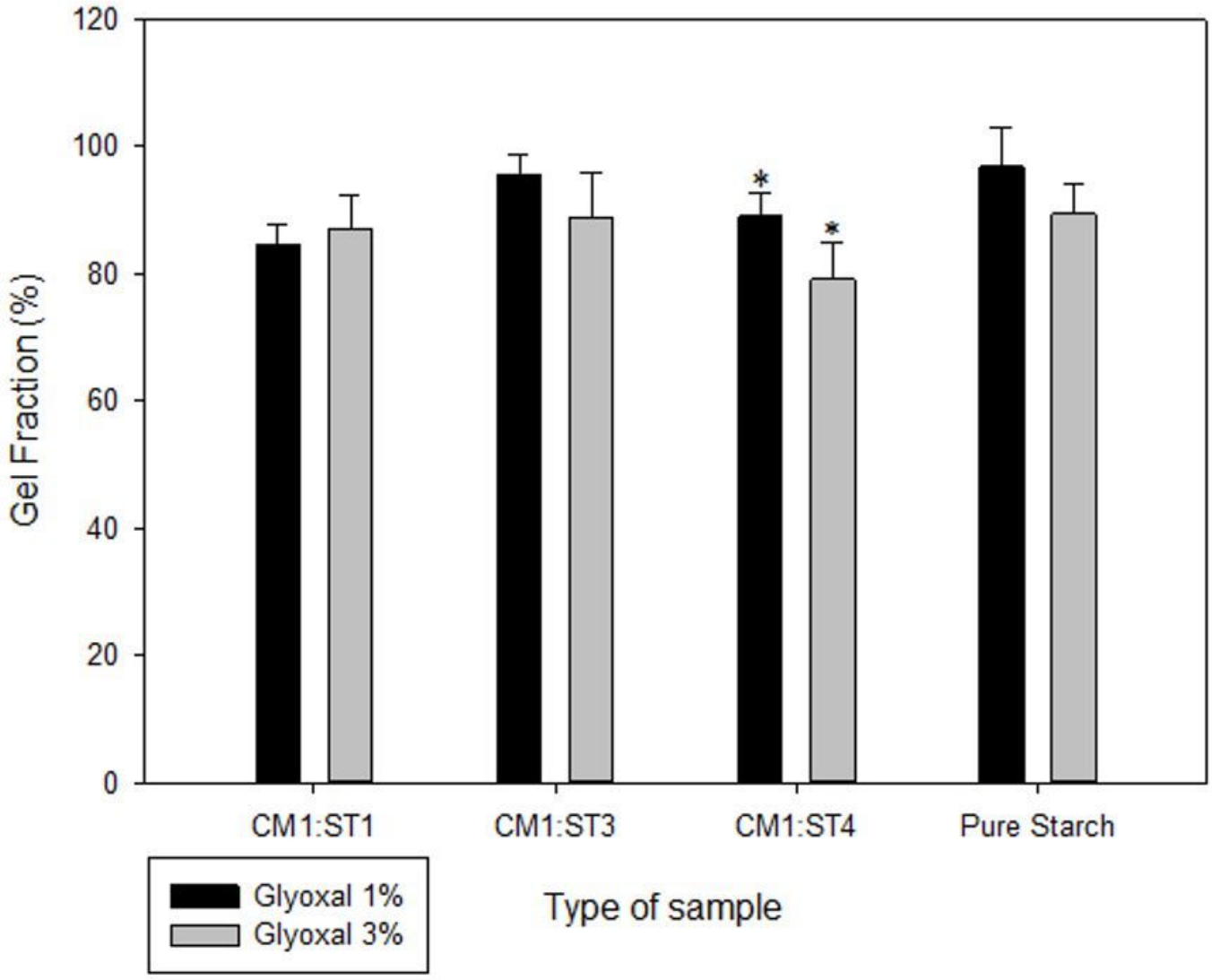
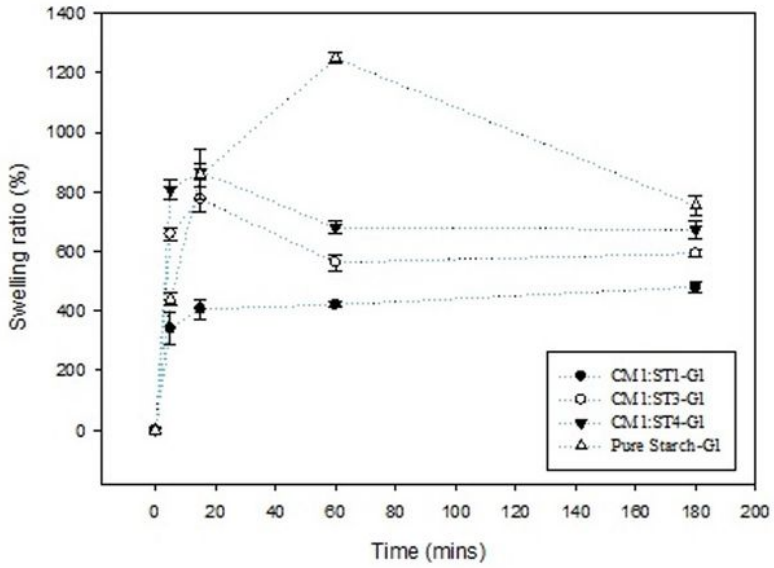


Figure 2

Gel fractions (%) of foam * indicates the significantly different at the 0.05 level ($p<0.05$)

(a)



(b)

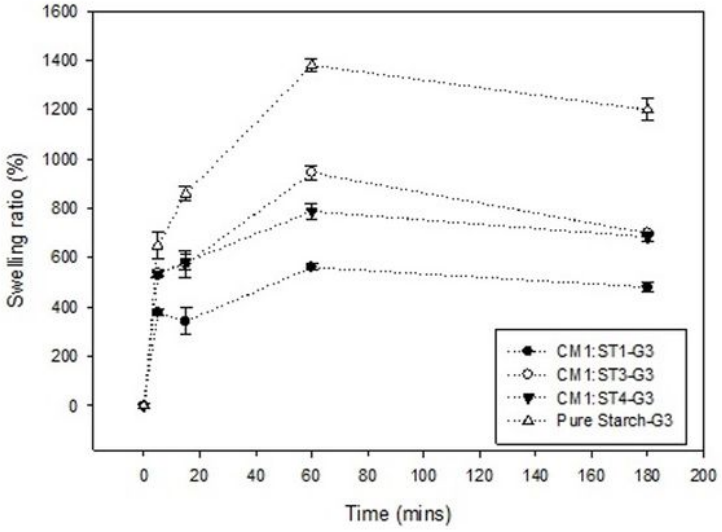


Figure 3

Swelling ratio (%) of foam with increasing of times

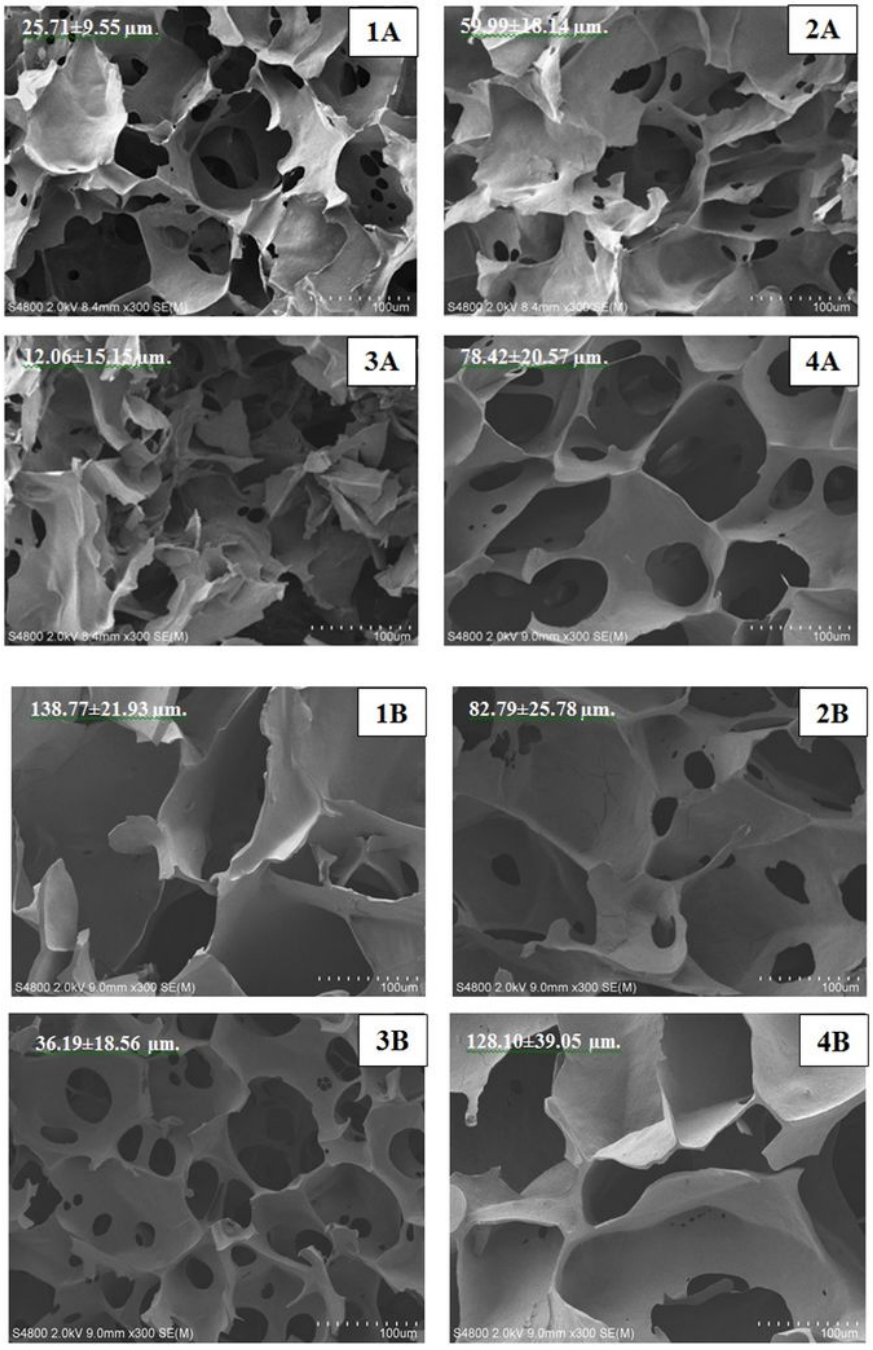


Figure 4

Representative SEM micrographs showing the cross section of CM: Starch foam. (1A) 1:1, (2A) 1:3, (3A) 1:4, (4A) Pure Starch with 1 % glyoxal. And (1B) 1:1, (2B) 1:3, (3B) 1:4, (4B) Pure starch with 3 % glyoxal

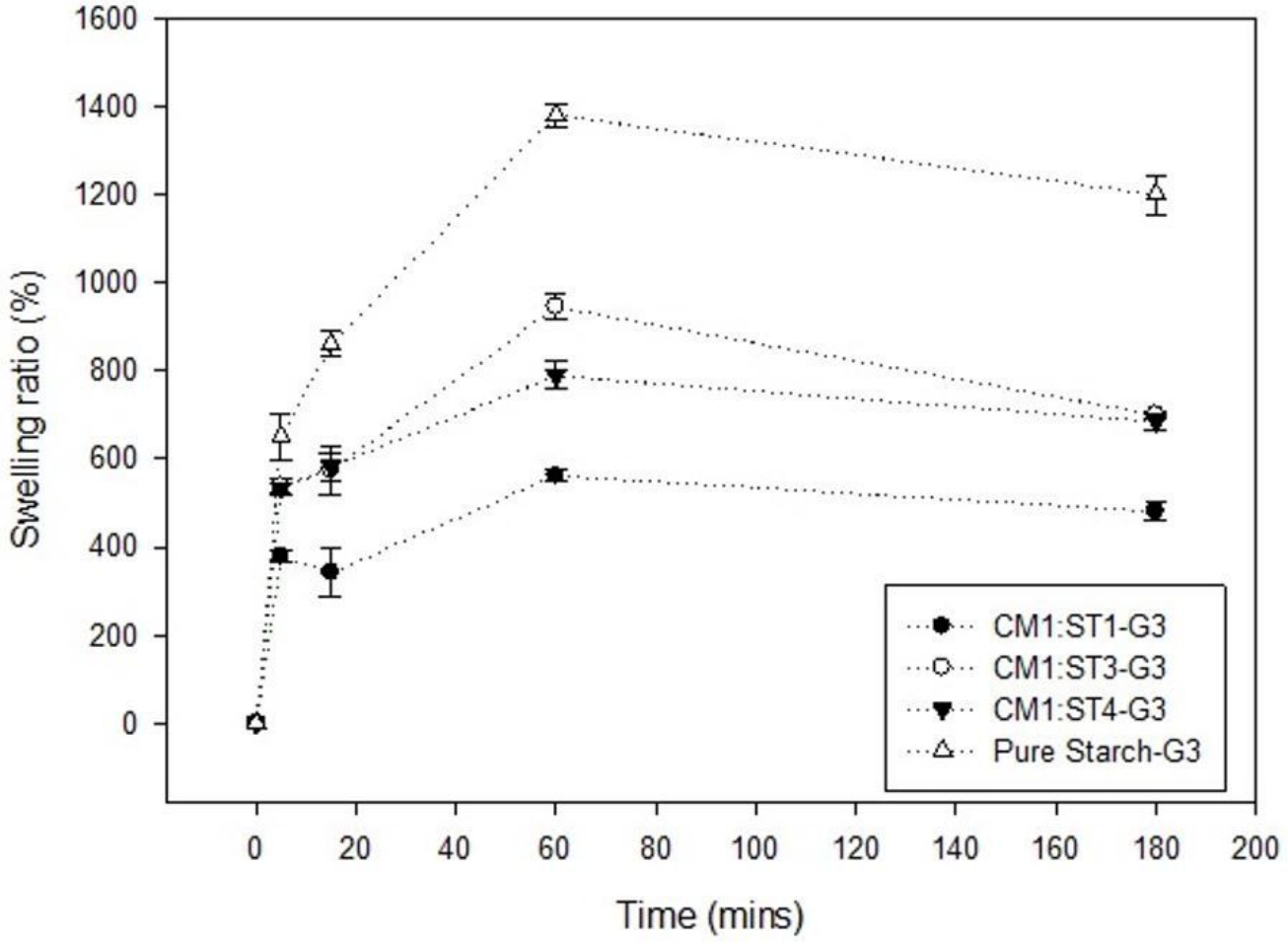
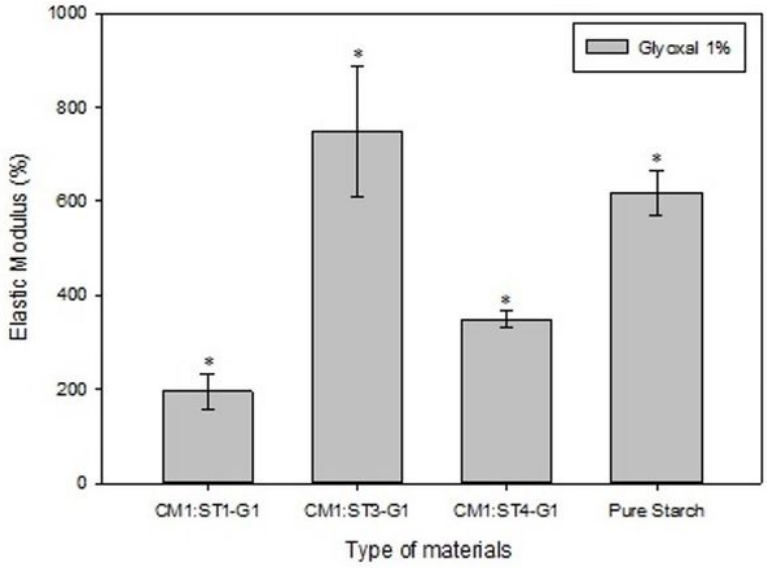


Figure 5

Porosity of CM: starch foam in various ratios. * indicates the significantly different at the 0.05 level ($p<0.05$). Error bars show one standard deviation of the mean

A



(b)

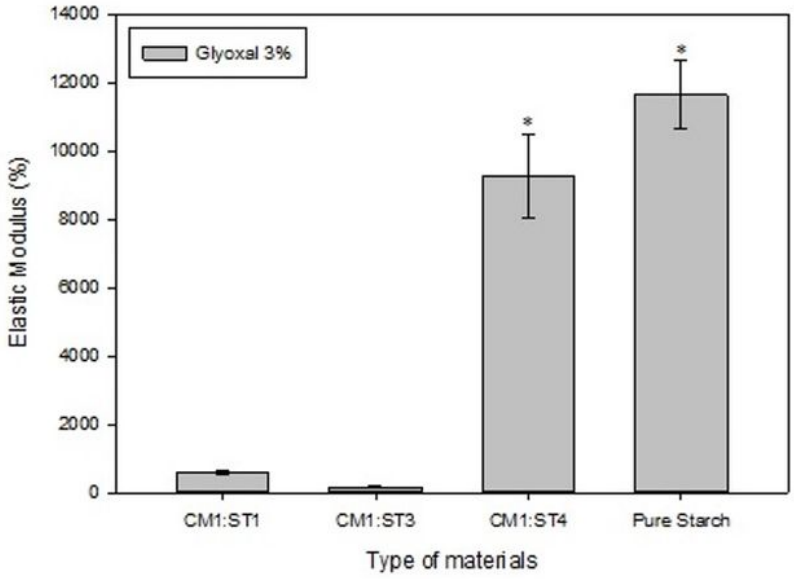


Figure 6

Elastic modulus of CM/starch foam in various ratios with (a) 1 % and (b) 3% w/v of glyoxal *indicates the significantly different at the 0.05 level ($p < 0.05$). Error bars show one standard deviation of the mean.

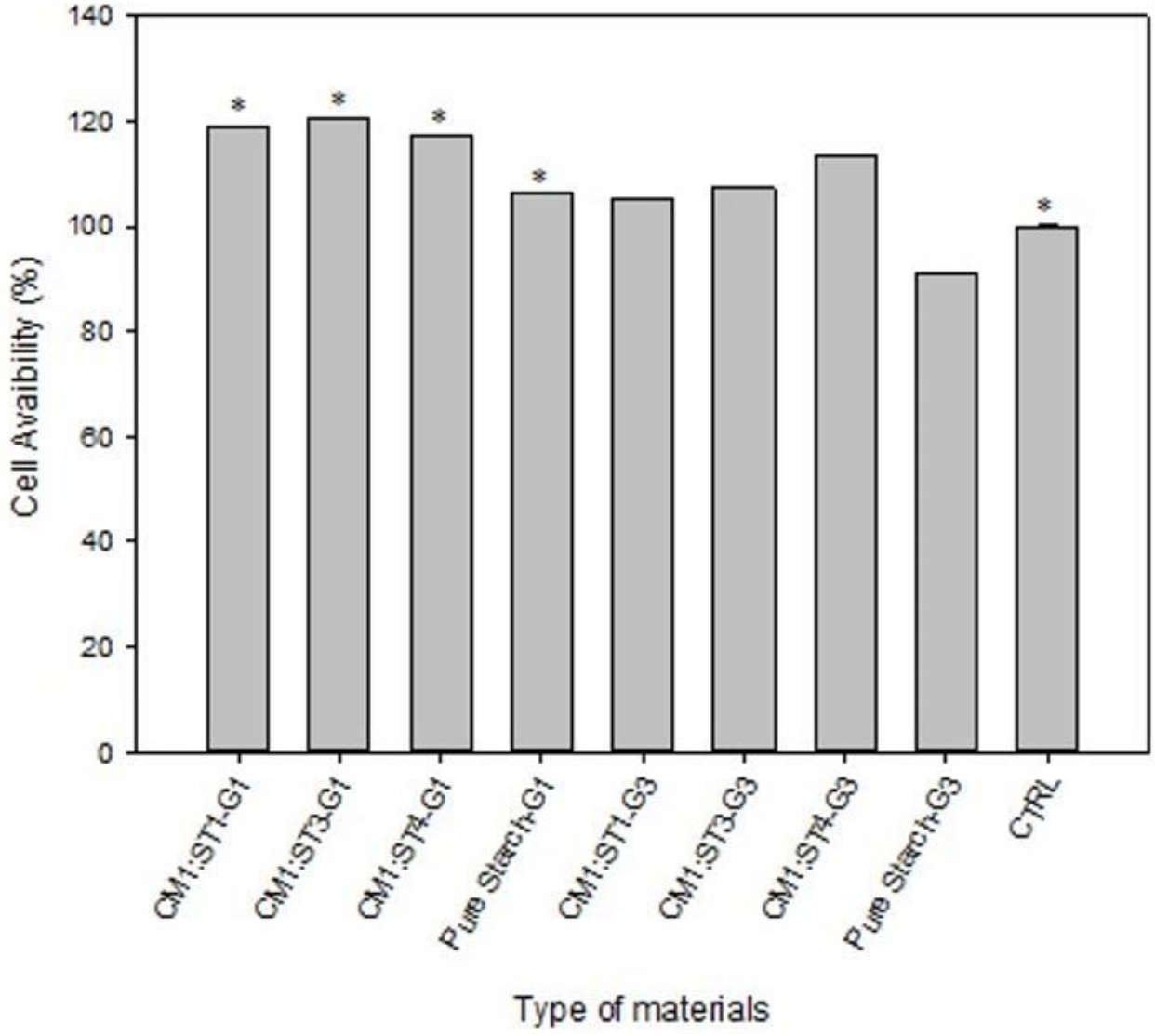
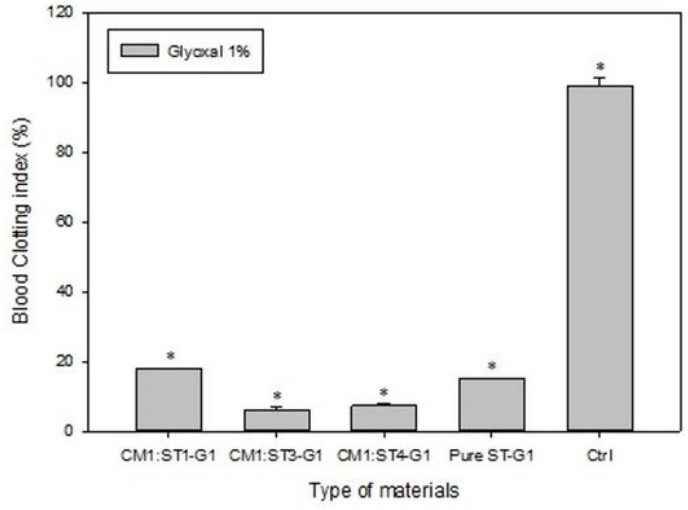


Figure 7

Viability of human dermal fibroblast cells on CM/starch foam (n = 3/group). Error bars show one standard deviation of the mean. * indicates the significantly different at the 0.05 level ($p<0.05$)

(a)



(b)

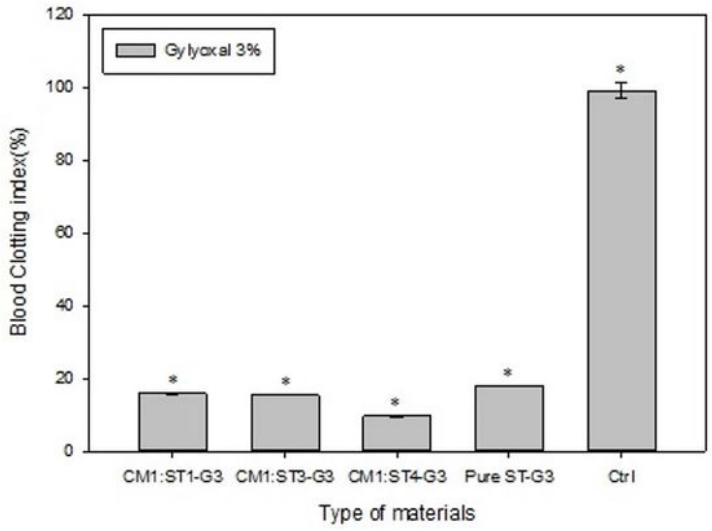


Figure 8

The effect of various ratios of CM/starch foam with (a) 1 % and (b) 3 % glyoxal on blood clotting index (BCI) * indicates the significantly different at the 0.05 level ($p<0.05$) Error bars show one standard deviation of the mean.