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Strengthening Scaffold by Using Carbodiimide Crosslinking on Gelatin/Carboxymethylcellulose from Waste Product

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Gelatin and carboxymethylcellose from waste product were selected for wound replacement application. Carbodiimide was used to strengthen the scaffold structure which made from gelatin mixed with carboxymethylcellulose (CMC) using freeze drying method. The mixed scaffold was fabricated in various gelatin/CMC ratios which were 100/0, 90/10, 80/20 and 70/30, respectively. The mechanical characterization of the scaffold was done by using universal testing machine (UTM) to determine compressive modulus. The results showed the highest value compressive modulus which was from gelatin/CMC at ratio of 70/30 with 4.80±1.95 kPa. The compressive modulus from gelatin/CMC at ratio of 80/20 showed the lowest value with 1.48±0.58 kPa. The 3D Finite element analysis could predict stress distribution and shear stress that were consistency with experimental data. It shown deformation behavior of the scaffold which was the similar trend with the experimental result in every case. The strain energy from finite element analysis of 70/30 scaffold showed the highest value. From the results, this condition of scaffold could be used for wound replacement applications.

1. Introduction

The scaffold or skin replacement material which is a biocompatible and biodegradable material, has been widely used and recently available in tissue engineering applications. Its functions have to promote cells and biologically active molecules into functional tissues. Skin loss in patient can cause from various different causes such as diseases, burns and accidents. The main functions of the scaffold are to prevent infection and accelerate the wound healing for skin tissue regeneration. Its properties require suitable conditions for skin to heal the wound (Ma 2004 and Ma 2006). The currently available scaffolds are expensive due to its components and extractions. Therefore, the propose of this research is to produce the cheaper scaffold which has the same functions. The scaffold fabrication has to be designed for supporting and recovery mechanism of skin functions. The scaffold design is depend on tissue engineering applications. The various naturally or synthetically biomaterials can be used to fabricate the scaffolds (Park 2002 and Hollinger 2012 and Wiwatwongwana 2019 and Romero et al. 2018).
The mechanical behaviour of the scaffold is the important functions which can be supported the compressive and tensile strength during implantation and tissue regeneration. Moreover, the appropriate pore size and interconnected pore are need for scaffold structure because it have to provide fibroblast cells to migrate, differentiation and growth in the scaffold. The scaffold should be biocompatible and biodegradable materials. The mechanical properties of scaffold behavior have to be investigated before testing with fibroblast cells. There also have many research studies on finite element models (FEM) of various scaffolds in order to calculate the mechanical behavior of the scaffold and also load transfer from the biomaterial structure to the biological entities. The structure of the scaffold shows a behavior of rubber-like material which can be modeled in the framework of hyperelasticity and its behavior normally has nonlinear stress-strain responses due to the elastomeric behavior (Butcher et al. 2017 and Faghihi et al. 2014 and Kim et al. 2012 and Benitez 2017 and Delong et al. 2008). Gelatin is one of biopolymer that is the most widely used for scaffold fabrication due to positively interacted with cells. There have many research studies approved for *in vitro* biocompatible test for gelatin with fibroblast cells. The results showed that gelatin scaffolds could be able to maintain cells with good

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proliferation of fibroblast cells during culturing (Lee et al. 2003 and Tabata 1998 and Lee et al. 2005). The second biomaterial that is chosen to improve mechanical properties of scaffold is carboxymethylcellulose (CMC). CMC which is a derivative of cellulose obtained from the reaction of cellulose with sodium hydroxide and chloroacetic acid. The example of good mechanical properties of CMC are high viscosity and shear strength which can help for mechanical integrity of the scaffold. The price of CMC is also cheap and easily purchased compared to other polysaccharides. There have many crosslinking methods to strengthen the scaffold structure such as dehydrothermal treatment and chemical treatment such as water-soluble carbodiimide or 1-ethyl-3-(-3-(dimethylaminopropyl)-carbodiimide (EDC) which is a popular reagent for cross-linking scaffolds (Biswal 2004 and Capitani et al. 2000 and Damink 1996). Therefore, this research chose gelatin blended with CMC for scaffold fabrication and crosslink with EDC. The various conditions of gelatin blended with CMC were selected and EDC treatment was used for strengthening the scaffold structure. Finally, the mechanical properties of various scaffold conditions were analyzed by compression test and FEM.

2. Material Fabrication

The conditions of dehydrothermal gelatin/CMC scaffold fabrication was the same as previous research (Wiwatwongwana 2019). Briefly, the scaffold was made from gelatin blended with CMC which could improve for scaffold mechanical structure. Type A gelatin was purchased from BIO BASIC INC, Canada. It was a reagent grade and derived from pork skin with bloom number of 240-270 and pH 4.5-5.5 at 25 ^{o}C. Its viscosity was 3.5-4.5 cps and moisture less than 12.0%. Carboxymethylcellulose sodium salt (CMC) was purchased from Sigma-Aldrich, St. Louis, MO, USA. It was from plant material with medium viscosity with 400-800 cps in a 2% aqueous solution at 25 ^{o}C. The gelatin/CMC solution was prepared by dissolving in deionized water (DI water). The scaffolds were made in four different gelatin/CMC ratios which were 100/0, 90/10, 80/20 and 70/30, respectively. The gelatin/CMC scaffold fabrication was done by preparing gelatin solution by mixing gelatin powder with DI water at 0.8 wt% (w/w) then leaved it at room temperature for 1 hour before stirred it at 50°C for 1 hour. CMC solution was prepared by mixing CMC with DI water at 0.8 wt% (w/w) and then stirred at 70 °C for 30 minutes. The various blending gelatin/CMC ratios were stirred at 50 °C for 15 minutes of each condition. Finally, the solutions were pipetted into 24-well culture plate with volume of 1 ml per well and freezed them for 24 hours at -20 °C. The scaffold was then placed in a Lyophilizer (Freeze-Dry Machine) at -50 °C for 24 hours and put all scaffolds in a humid controlled container to keep all conditions of the scaffolds. All the scaffold conditions were heated at 140 ^{o}C for 48 h or dehydrothermal treatment to strengthen scaffold structure before crosslinking with EDC. The crosslinking methods was done by immersing the scaffold in the 14 mmol/l of EDC solution for 2 hours and then rinse the scaffold with DI water for 3 times by immersing the scaffolds in DI water for 15 mins each time and leave it dry at room temperature for 1 week. The schematic diagram of EDC crosslink gelatin/CMC scaffold fabrication was shown in Figure 1.

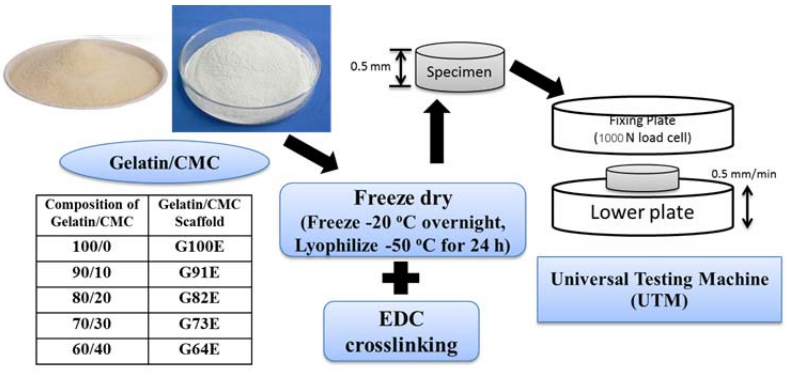

Figure 1: Schematic diagram of EDC crosslink gelatin/CMC scaffold preparation and experimental test.

3. Mechanical Properties Identification

3.1 Geometry and Loading Condition

The examples of EDC crosslink gelatin/CMC scaffolds were shown in Figure 2. Where the porous structure of the scaffold was formed by freeze-drying technique. The compressive testing was performed by using universal testing machine (UTM, Zwick/Roell Z1.0) to collect load-deformation data from experimental test to obtain stress-strain information. The compression rate was 0.5 mm/minute in dry condition at 25 °C (Wiwatwongwana et al. 2012). The EDC crosslink gelatin/CMC scaffolds were divided into 4 mixtures which

were 100/0, 90/10, 80/20 and 70/30, respectively. The compressive modulus was evaluated from initial compressive stress-strain curve to determine the slope from 15% to 25% strain of the scaffolds and expressed as mean ± standard deviation (*n*=5). The significant different of each blending composition was evaluated using a student t-test with 95% confidence interval. The differences were considered to be a statistically significant when $p<0.05$.

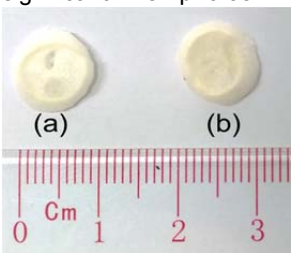

Figure 2: EDC crosslink gelatin/CMC scaffold (a) G100E and (b) G73E.

3.2 Finite Element Modeling

A non-linear elastic material law was used for the model and the finite element code MARC (MSC Software) based on the implicit dynamic approach was used to analyze total strain energy and the Cauchy strain tensor with time response of the scaffolds. The initial mesh of 3D finite element model used for analysis and its boundary conditions were illustrated in Figure 3. The gelatin/CMC scaffold was considered as a three dimensional cylinder deformation bodies with diameter 10 mm and 5 mm of length, while the lower plate and the upper plate were defined as rigid bodies. The four-node tetrahedral solid element type with approximately 80,000 elements was generated on the deformation bodies. In order to achieve a high-accuracy, the fine elements zone of minimum size length 0.01 mm were modeled in the circular edges.

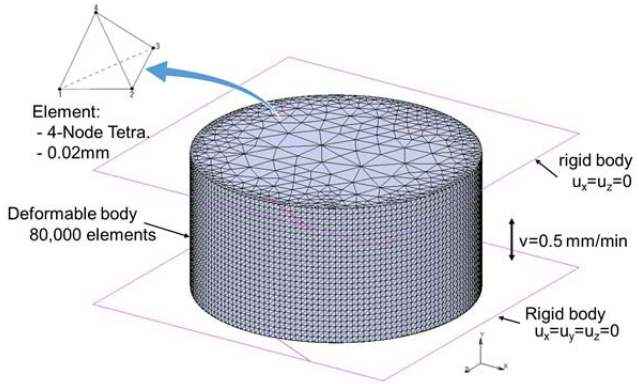

Figure 3: The FEM simulation model of EDC crosslink gelatin/CMC scaffold.

The compressive modulus from experiment were used to analyze the mechanical properties by FEM. The total strain energy time response was obtained from history plot of all increments. The principle strains and stresses as well as the von-Mises stress were calculated for all cylinders. The dilatation stress and octahedral shear strain were calculated since these parameters might be potential mechanical stimuli for tissue differentiation. The material flow of FE models was governed by hereditary integration for FEM Cauchy stress (or true stress) as shown in Eq(1) (Kim et al. 2012).

$$\sigma = \int_0^t 2G(t-\tau)\frac{de}{d\tau}d\tau + I\int_0^t K(t-\tau)\frac{d\Delta}{d\tau}d\tau \qquad (1)$$

Where

σ is Cauchy stress in FEM constitutional equation (kPa)
G is shear modulus in FEM function (kPa)
t is time in FEM constitutional equation (s)
τ is relaxation times (s)
I is unit tensor in FEM constitutional equation
e is strain in FEM constitutional equation
K is bulk modulus in FEM function (kPa)

4. Result

4.1 Compressive Modulus of the Scaffolds

The EDC crosslink gelatin/CMC scaffolds were compressed by UTM with two flat plates to analyze the stress-strain relation of each scaffold condition. Force versus displacement was converted into engineering stress and strain by using of the initial dimensions of each scaffold. The average compressive modulus of all scaffold conditions which represented by circle marker of the scaffolds was plotted as shown in Figure 4. The results showed that gelatin scaffold with 30% CMC (G73E) occurred the highest compressive modulus compared to pure gelatin scaffold (G100E). The compressive modulus of G73E was 4.8 ± 1.95 kPa and the compressive modulus of pure gelatin scaffold was 2.05 ± 1.21 kPa as shown in table 1. However, the compressive modulus of G91E showed slightly higher value compared to pure gelatin scaffold with non-significant different. However, The G82E scaffold showed the lowest compressive modulus (1.48 ± 0.58 kPa) with non-significant different compared to G100E.

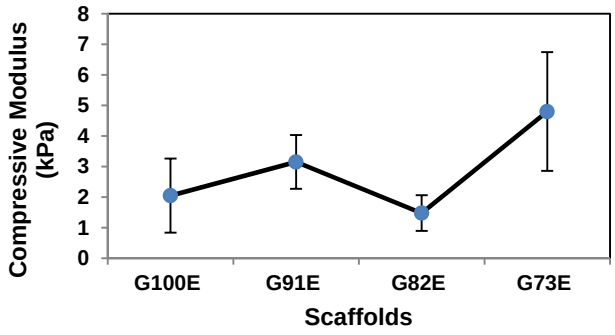

Figure 4: Compressive modulus of EDC crosslink gelatin/CMC scaffold (n=5) (significant different p<0.05 relative to G100E).*

Table 1: Compressive Modulus of EDC crosslink Gelatin/CMC scaffold (n=5)

Scaffold	Average Compressive Modulus (kPa)	SD
G100E	2.05	1.21
G91E	3.15	0.88
G82E	1.48	0.58
G73E	4.8	1.95

4.2 Finite Element Model of the Scaffolds

The mechanical behavior of scaffold should be analyzed due to shear force and compressive force from surrounding tissue during implantation. The scaffold deformation behavior was analyzed by FEM after compressive test analyzing. In order to verify the strengthening scaffold models in terms of compressive modulus, a finite element analysis was made to compare the Cauchy stress with stress in loading direction (S_{22}) shown in Figure 5. The Cauchy stress distribution of difference gelatin/CMC scaffolds was selected at the indentation depth 2.5 mm (50% of thickness). The deformed models of FE results indicated a good consistency with the experimental results (Figure 2). The equivalent Cauchy stress at the centroid of gelatin was analyzed for determine the strength of gelatin scaffold in each condition. The result shown that G73E was the highest of stress which it could summarize that condition of CMC 30 in ratio provided the best in mechanical strength. The G82E scaffold shown the lowest in mechanical strength due to immerse in chemical crosslink agent EDC caused dilute in some part of scaffold structure. The total strain energy-indentation depth from FEM plot of all deformable under uniaxial compression was shown in Figure 6. The scaffold G73E which represented by asterisk purple marker showed the highest of total strain energy compared to other scaffolds at the equivalent time. Whereas, G82E scaffold which represented by rectangular light green marker showed the lowest of total strain energy at the equivalent time. The other scaffolds G73E which represented by orange marker represented the highest in total strain energy-time response. From the total energy plot could confirm that G73E could keep the highest energy in the scaffold which was consistency with the mechanical strength from experimental test.

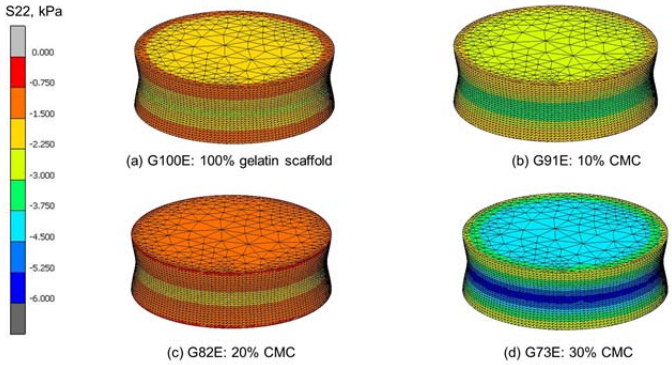

Figure 5: The distribution Cauchy stress of gelatin/CMC scaffolds at the indentation depth 2.5mm (a) G100E, (b) G91E, (c) G82E and (d) G73E.

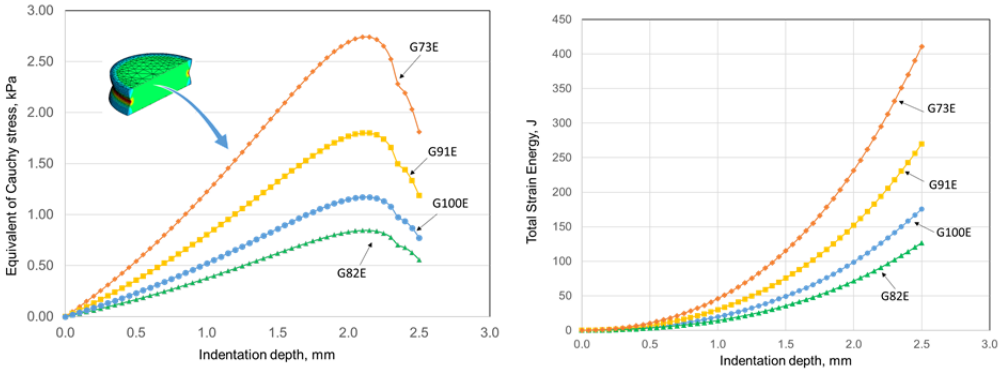

Figure 6: Equivalent Cauchy stress (at centroid) and Total strain energy with respect to indentation depth from finite element analysis under uniaxial compression of G100E, G91E, G82E and G73E scaffolds.

5. Discussion

The mechanical results of this research showed that it could be suggested that CMC and EDC crosslinking could be used as a scaffold strengthening, especially for gelatin scaffold at a condition of gelatin/CMC of 70/30. This condition showed the highest value of compressive modulus was 4.8 ± 1.95 kPa. However, G82E showed the lowest compressive modulus which was 1.48 ± 0.58 kPa. The 3D FEM results which shown deformation was agree with the experimental result in every case. The strength of the scaffold surface was higher than at the scaffold center. The FE models indicated a good consistency with the experimental results. The equivalent Cauchy stress at the centroid of gelatin blended CMC scaffold could be analyzed the strength. The 3D finite model could be used for further investigate and predict the mechanical behavior of the scaffold such as shear stress and shear strain. Total strain energy-time response from FEM plot of all 100 increments of deformed material was illustrated in Figure 6. The scaffold G73E showed the highest value of total strain energy whereas G82T showed the lowest value at the equivalent time. The FEM plot result was in the similar trend of the result from experiment. It could be summarized that scaffold condition of G73E was the strongest structure and supported the most uniaxial compressive load. It might be the best condition for using in further experiment of tissue engineering application. Further research could be focused on the other treatment methods for scaffold strengthening such as glutaraldehyde crosslinking or other scaffold fabrication techniques such as 3D bioprinting technique and particulate leaching which could be compared with freeze drying. The swelling property and biodegradability of the scaffolds should be further analyzed.

6. Conclusion

The scaffolds which made from gelatin blended with CMC and using EDC as the scaffold strengthening were selected in this research. CMC solution was blended with gelatin solution and formed the scaffold at various gelatin/CMC ratio which were 100/0, 90/10, 80/20 and 60/40, respectively. The mechanical characterization of all scaffold conditions was the investigation of compressive modulus by using Universal Testing Machine. The compressive modulus data was used to analyze in finite element model. From the experimental test, all the scaffolds were compressed to 80% deformation. The results showed the maximum compressive modulus of

scaffold which was from gelatin/CMC ratio at 70/30. However gelatin/CMC ratio at 80/20 showed the lowest compressive modulus. It could be implied that increasing of CMC content at appropriate condition could be improved in mechanical property of scaffold structure. The 3D FEM results shown deformation behavior of the scaffold which was the similar trend with the experimental result in every case. The scaffold strength was summarized from 3D finite which the strength at the scaffold surface was higher than at the center. Moreover, the G73E scaffold occurred the highest value of total strain energy from FEM plot at the equivalent time compared to other scaffolds. On the other hands, G82E scaffold revealed the lowest value of total strain energy at the equivalent time. From all the results, it could be summarized that the gelatin/CMC ratio at 70/30 might be useful for tissue engineering applications due to its good for supporting compression. EDC could help for scaffold stiffness. Therefore, the FEM analysis could provide qualitative information regarding to mechanical properties of the scaffold and its deformation behavior. In addition, using of livestock and agricultural waste could be reduced cost for scaffold fabrication and was friendly to environment.

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