MODULATION OF THERMAL PROPERTIES OF BOVINE COLLAGEN SCAFFOLD USING VARIATION OF FABRICATION TEMPLATE

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ABSTRACT: It has been a major challenge in the creation of synthetic or natural materials to favour the regeneration of the damaged tissues and organs. The fabrication methods and techniques of the scaffold directly influence with physical, chemical, and biological function of the cells when applied in *in vivo* and *in vitro*. Design of scaffolds for tissue regeneration requires an intimate knowledge of precise relationships between structure and biological function. These scaffolds can have a broad range of morphological properties, such as interconnecting (open pores), non-connecting (closed pores), or a combination of both. The physicochemical structure of collagen scaffold influences the repairing and tissue building to take place without contraction and scar formation. The goal of the current study is to study the relation of volume and area of exposure to freeze-drying process using different sizes of aluminium tray to achieve different thermophysical properties of collagen scaffolds. Freeze-drying of collagen scaffold is useful because the production of a glassy amorphous material with a T_g above room temperature (>32 ^oC) makes the scaffold stable and can be stored on the shelf. From this endeavor, we hypothesized that dimension of fabrication template and volume are able to influence thermal properties of collagen scaffold's glass transition temperature (T_g).

KEY WORDS: *Collagen, Scaffold, DSC, T_g analysis, and Tissue engineering.*

1. INTRODUCTION

Engineering of highly precise and well-defined microarchitecture onto a biomaterial is often complex and tedious, and the scaffolds with these features are susceptible to surface and material disintegration during physical manipulation. Other attempts to biofunctionalized existing materials with topological features face problems with controlled degradation and alteration to its topography and biophysical features [1]. At present, there exists an unmet need to enable the translation of microarchitectural principles into actual clinical applications. In wound healing, the physicochemical structure of collagen scaffold influences the repairing and tissue building to take place without contraction and scar formation [2]. There are substantial bodies of research that detailed studies and characterizations of every relevant structural feature implicated in the processing method [3]. Often these methods such as the use of harsh chemicals and electrical field can be damaging to collagen structure as the fabrication of collagen scaffold require the temperature to be around 4 to 6 °C to prevent the denaturation of native collagen structure

[4]. The process requires highly specialized and costly apparatus which make it difficult to scale up.

Freeze-drying is an effective and widely used method in the fabrication of scaffolds. It is a transformation process of free water available into ice and the removal of the ice crystals through sublimation process. The fabrication of collagen scaffold into specific structures by manipulating freezing conditions such as volume, container size and addition of additives to influence the geometrical and physical properties of the scaffold is not new [5]. Technically, water is a diamagnetic material that is susceptible to manipulation thermodynamically [6]. In this project, lyophilization or freeze-drying process was used to fabricate collagen scaffold. Three different dimensions of pan were used to observe whether it will affect the collagen scaffold structure and properties.

2. MATERIALS AND METHODS

2.1. Extraction and purification of collagen from bovine

The experiment began with the extraction process by skinning the bovine legs to separate tendon using a scalpel. The tendon was cleaned and kept in the freezer. After the tendon was frozen, the tendons were immersed in 0.5 M acetic acid (CHCOOH) (Sigma-Aldrich, UK) for a week and then the mixture was blended in a blender. The mixture was poured into a 50 mL centrifuge tube then, a 5 g of sodium chloride (NaCl) (Sigma-Aldrich, UK) was added into the mixture by using spatula. The tube was shaken until the mixture becomes homogeneous and was stored in refrigerator for 2 days at 4 °C to let the solution separate. Then, dialysis process was carried out on the collagen using dialysis membrane with molecular cut off ranging from 20 to 30 kDa and sodium phosphate dibasic (Na_2HPO_4) (Sigma-Aldrich, UK) solution with pH 7.2 was used as buffer for 7 days. Dialysis buffer was changed twice a day. Dialysis was done to further purify and separate collagen from other unwanted proteins.

2.2. Freeze-drying of collagen sheet in different dimension of fabrication template

After extraction of collagen process, the collagen suspension was put in a -20 °C freezer for one night. The collagen suspension was freeze-dried using freeze-drier with -80 °C temperature and <100 mmTorr pressure for vacuum condition. The collagen was freeze-dried for 4 days to reach -80 °C temperature as the freezing rate for the freeze-drier is -20 °C per day. After 4 days, the freeze-dried collagen suspension will be in powder form. The powdered form of collagen was dissolved in 33 mL of 0.05 M of acetic acid with 5 mg/mL collagen concentration and was chilled overnight. It was then poured into 15×10×3.5 cm^3 stainless steel aluminium trays and was placed in -20 °C freezer overnight. After that, the aluminium tray was placed into pre-cooled freeze-dryer. The temperature of the freeze-dryer must be maintained at -20 °C at all times for 60 minutes as the freezing rate will influence the pore structure of the scaffold. The aluminium tray was placed in freeze-dryer for the next day 4 days. The protocol was then repeated with 8.5×8.5×4 cm^3 stainless steel aluminium tray and plastic petri dish with dimension of 1.5 cm height and 10 cm diameter.

2.3. Crosslinking of collagen scaffold using dehydrothermal treatment and glutaraldeyde

Cross-linking process was to introduce covalent cross-link between polypeptide chains of the collagen fibre without denaturing the collagen into gelatin. After freeze-drying, the collagen was in sheet form. The collagen sheet was carefully detached from the aluminium tray. For dehydrothermal treatment (DHT) process, the collagen sheet was put in aluminium

foil wrapper and was put into oven of temperature 105 °C together with 50% of carbon dioxide, CO_2 overnight. After overnight in the oven, the collagen sheet was taken out and cut into 1×1 cm^2 small pieces and placed into 12 well plates filled with control (0.05 M acetic acid) and 0.1 M of glutaraldehyde (GA) (Sigma-Aldrich, UK). The collagen sheet was left to be treated with glutaraldehyde overnight. After overnight of glutaraldehyde crosslinking, the collagen sheet was washed with distilled water 3 times before storing the sheet in 0.05 M of acetic acid until further use. The sheet was dried overnight in fume hood to remove any remaining acetic acid. Then the scaffold was analyzed by using differential scanning calorimeter (DSC).

2.4. Differential scanning calorimetry (DSC)

The T_g of collagen scaffolds were measured using DSC (Perkin Elmer DSC Pyris-1, Massachusetts, U.S.A). The scaffold was cut into 1x1 cm^2 then measured over and placed in an aluminium pan in the cell of the DSC unit. An empty pan was used as the inert control. The crucibles were then closed by pressing aluminium cap, which was pierced by a needle on the top for degassing. Nitrogen was used as the purge gas. DSC was performed from -60 and 60 °C at a heating rate of 1°C per minute. The computer software (Perkin Elmer) for the apparatus plotted and analyzed the thermal analysis curves and the values of endothermic heat were normalized to sample weight and presented in units of ($mJ.mg^{-1}$). The amount of energy released was subjected to the amount of free water inside the hydrogel.

3. RESULTS AND DISCUSSION

3.1. The effects of freeze-drying, dehydrothermal treatment (DHT) and glutaraldehyde (GA) crosslinking on the fabrication of materials

The aim of freeze-drying, DHT and crosslinking with glutaraldehyde was to produce a stable material with controlled degradation rate. The physicochemical effect of different sizes of aluminium trays and petri dishes during freeze-drying, DHT and glutaraldehyde crosslinking are able to influence the rate of degradation and equilibrium water content of the scaffold. From Table 1, collagen sheet made from 8.5x8.5x4 cm^3 and 30.4x1.5 cm^3 containers were more stable to degradation after 18 hours in comparison to collagen sheet made from larger container 10.5x8.5x3. These collagen sheets also appeared strong. Treatment with DHT and GA also greatly improved the rate degradation although there were no apparent differences in equilibrium water content (EWC) of in all samples.

Table 1: Observations of collagen scaffold after freeze-drying

Scaffold [cm^3]	Time [hour]				EWC [100%]
	0	1	3	6	
15×10×3.5	Solid	Start to break	90 % degraded	Dissolved	-
15×10×3.5 (with 0.1M glutaraldehyde)	Solid	Solid	Start to break	60 % degraded	-
8.5×8.5×4	Solid	Solid	Solid	10 % degraded	105 ± 7
8.5×8.5×4 (with 0.1M glutaraldehyde)	Solid	Solid	Solid	Solid	103 ±10
31.4×1.5	Solid	Solid	Solid	Solid	105 ± 10
31.4×1.5 (with 0.1M glutaraldehyde)	Solid	Solid	Solid	Solid	110 ± 8

The characterizations of crosslinked and non-crosslinked collagen scaffolds were conducted using Differential Scanning Calorimetry (DSC). In this work, we measured the changes T_g and enthalpy of collagen scaffold with varying area of exposure to freeze-drying and the use of GA as crosslinker. DSC measurements are effective measurements to study the strength and synergistic interaction of collagen polymers [7]. From Fig. 1, the first endothermic peaks observed in control and 0.1 M GA were between 38.12 °C and 107.06 °C; which corresponded to dehydration process of surface water. Then the second endothermic phenomenon observed in the 1.0% GA was in the temperature range between 132.96 °C and 179.71 °C which can be related to the loss of hydrogen bonds, indicating formation of bonds and linkages [8]. The information of interval temperature, onset and enthalpy for Fig. 1 is detailed in Table 2. From this data, it showed that collagen sheet which treated with GA had higher enthalpy of -123.47 compared to the control at -5.72 and -115.97 resulting from the formation of two peaks. Both collagen scaffolds were structurally weak and form cracks in the middle of aluminium tray due to the large size area exposed to freeze-drying, where ice nucleation was more concentrated in the centripetal direction (Table 3). Crosslinked collagen scaffold also exhibited sharp peak of glass transition event with relaxation, indicating the formation of stable bonds by GA crosslinked in the collagen.

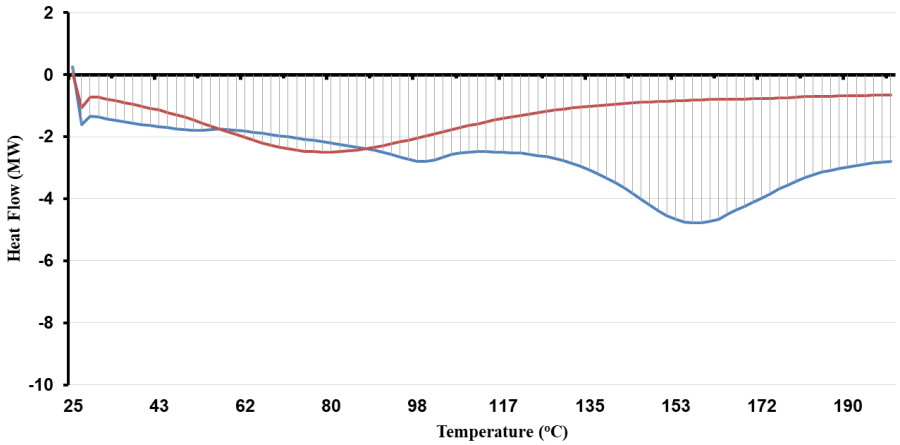
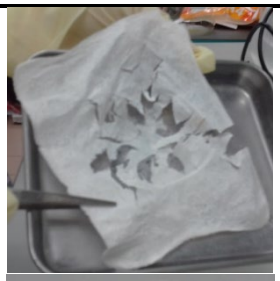
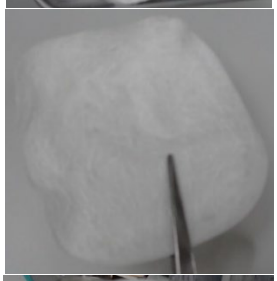
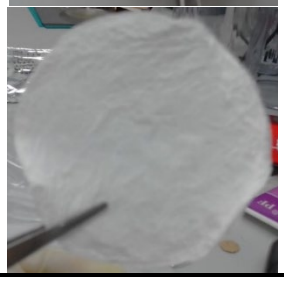


Fig. 1. DSC thermogram of collagen scaffold fabricated in a 15x10x3.5 aluminium tray without glutaraldehyde (blue) and with 0.1 M glutaraldehyde (red) heated from room temperature to 200 °C at 10 °C per minute.

Table 2: DSC Analysis for collagen scaffold fabricated in 15x10x3.5 cm^3 aluminium tray

15×10×3.5 [cm^3]	**Peak 1**		**Peak 2**		**T_g [°C]**
Control	Integral (mJ)	-20.21	Integral (mJ)	-409.38	42
	ΔH ($J.g^{-1}$)	-5.72	ΔH ($J.g^{-1}$)	-115.97	
	Onset (°C)	90.03	Onset (°C)	132.96	
	Peak(°C)	99.24	Peak (°C)	157.3	
	Endset (°C)	107.06	Endset (°C)	179.71	
+0.1M Glutaraldehyde	Integral (mJ)	-533.31			52
	ΔH	-123.74			
	Onset (°C)	38.12			
	Peak (°C)	78.26			
	Endset(°C)	126.76			

Table 3: Observations of collagen scaffold after freeze-drying and DHT using different size and dimension of aluminium trays and petri dish

Size and dimension of tray [cm^3]	Collagen scaffold after freeze-drying	Collagen scaffold after DHT	Observation
15×10c×3.5			The scaffold covered the surface, with breaking in the middle. The sheet was thin and easily break.
8.5×8.5×4			The scaffold covered all the surface area of the pan and was thick and strong. The thickness of the scaffold was evenly distributed throughout and easily removed from the surface.
31.4×1.5			The scaffold covered the surface area and appeared thick and strong. The thickness was evenly distributed and did not stick to the surface.

From Fig. 2, the first endothermic peak observed in control and 1.0% GA were 99.61°C and 153°C, respectively. Higher peak temperature in crosslinked collagen scaffold was corresponding to dehydration process of surface water in the temperature range between 149 °C and 172 °C. This could explain the formation of bonds between collagenous polymers [7]. In comparison to collagen scaffolds made in larger aluminium tray, the T_g of these scaffolds were higher. Degradation studies also showed that these scaffolds were stronger and stable, and last longer in degradation studies. Addition of GA crosslinker in the scaffold also delayed degradation up to 6 hours after immersion in distilled water. Table 4 shows the thermodegradation information of both scaffolds made in 8.5x8.5x3 cm^3 aluminium tray. From this data, it was obvious that the thermal degradation of controlled scaffold without the crosslinker was broader with higher enthalpy at -331.11 $J.g^{-1}$ while narrow and sharp peak of enthalpy was formed in the crosslinked scaffold with -75.72 $J.g^{-1}$. The sharp peak of crosslinked sample revealed glass transition with enthalpy relaxation where after the initial climb, the signal will drop again before leveling out which was common in crosslinked polymeric sample.

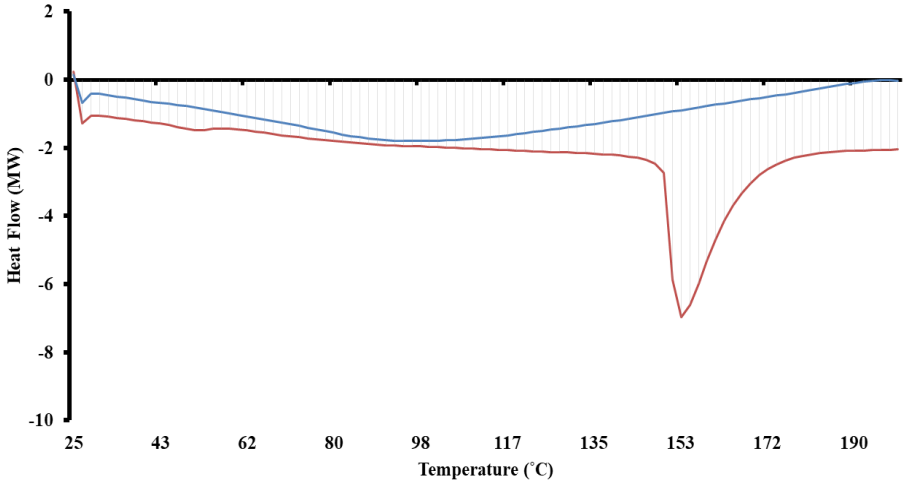


Fig. 2. DSC thermogram of collagen scaffold fabricated in a 8.5x8.5x3 cm^3 aluminium tray without glutaraldehyde (blue) and with 0.1M glutaraldehyde (red) heated from room temperature to 200°C at 10°C per minute.

Table 4: DSC Analysis for collagen scaffold fabricated in 8.5x8.5x4 cm^3 aluminium tray

8.5×8.5×4 [cm^3]	**Peak 1**		**Peak 2**	**T_g [°C]**
Control	Integral (mJ)	-850.95		60
	ΔH ($J.g^{-1}$)	-331.11		
	Onset (°C)	30.87		
	Peak (°C)	99.61		
	Endset (°C)	192.22		
+0.1M Glutaraldehyde	Integral (mJ)	-333.94		145
	ΔH ($J.g^{-1}$)	-75.72		
	Onset (°C)	149.8		
	Peak (°C)	153.05		
	Endset (°C)	172.62		

From Fig. 3, the first endothermic peak observed in control and 0.1M GA were 99.71°C and 122°C respectively. The T_g for these control and crosslinked scaffolds were 52°C and 90°C. Broader peak was observed in control sample, with onset at 90.92 to 107.00 °C and 124.31 to 160 °C (Table 5). From all results in Fig. 2-4, they showed that T_g will generally be low in control samples without GA (non-crosslinked) when the chains were highly flexible and loose. All results showed that, crosslinked scaffolds exhibited higher T_g in comparison to control samples, indicating the samples were stronger due to formation of bonds in the polymer, requiring higher temperature for transition as the chains were rigid and becoming in orderly fashion [9]. Although both non-crosslinked and crosslinked samples did not show any significant and apparent differences in EWC, the thermal properties of collagen scaffold were significantly altered using larger aluminium tray, which exposed more area for freeze-drying process.

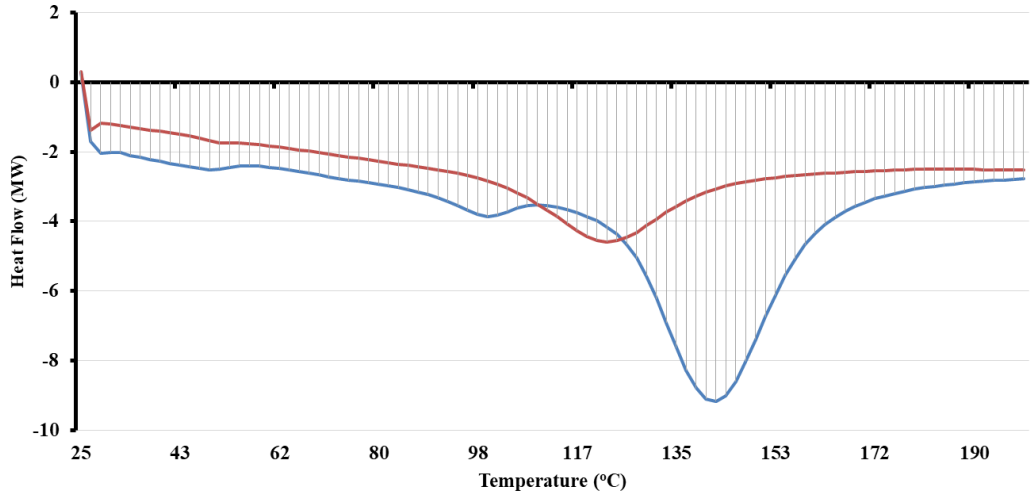


Fig. 3. DSC thermogram of collagen scaffold fabricated in a 31.4x1.5cm^3 without glutaraldehyde (blue) and with 0.1M glutaraldehyde (red) heated from room temperature to 200 °C at 10 °C per minute.

Table 5: DSC Analysis for collagen scaffold fabricated in 31.4x1.5 cm^3 petri dish

31.4×1.5 [cm^3]	Peak 1		Peak 2		T_g [°C]
Control	Integral (mJ)	-25.58	Integral (mJ)	-817.89	52
	ΔH (J.g^{-1})	-4.77	ΔH (J.g^{-1})	-152.59	
	Onset (°C)	90.92	Onset (°C)	124.31	
	Peak (°C)	99.71	Peak (°C)	141.64	
	Endset (°C)	107.00	Endset (°C)	160.86	
+0.1M Glutaraldehyde	Integral	-309.15			90
	ΔH (J.g^{-1})	-71.56			
	Onset (°C)	101.67			
	Peak (°C)	122			
	Endset	142.68			

4. CONCLUSION REMARKS

From the fabrication process of collagen scaffold, it can be concluded that the aluminium tray with a dimension of 8.5×8.5×4 cm^3 with DHT and 0.1M GA crosslinking greatly improved collagen scaffold integrity and degradation rate. The physical properties of the collagen scaffolds fabricated using the above-mentioned condition were thick, strong and the collagen concentration was evenly distributed throughout the scaffold. Our hypothesis on the relationship of size and exposure of collagen to freeze-drying to modulation of scaffold polymer properties was confirmed with the revelation that the smaller the aluminium tray, heat was distributed evenly and formed stronger scaffold. Addition of GA as crosslinker made the scaffold stronger due to bond and linkage formations in the collagen structure.

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