EFFECT OF DEHYDROTHERMAL (DHT) TREATMENT ON THE PHYSICOCHEMICAL PROPERTIES OF 3D POROUS CHITOSAN-COLLAGEN-GLYCERINE SCAFFOLD FOR POTENTIAL SKIN REGENERATING TEMPLATE APPLICATIONS

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Abstract. The aims of this study are to develop 3D porous scaffolds from the blending of chitosan, fish scales collagen and glycerine using freeze dry technique and to investigate the effects of dehydrothermal (DHT) treatment on the physicochemical of the scaffolds. The tensile properties of the scaffold were determined using universal testing machine (UTM) while the porosity and degradation rate of the scaffolds were investigated by using common procedures. The crosslinking density was calculated using 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay while the crosslinking reactions were monitored by Fourier transform infrared (FTIR) technique. Scanning electron microscope (SEM) was used to observe the morphology of the 3D porous structure. The results showed that the 3D scaffolds were porous with interconnected pores having pore size between 100 to 200 microns. The ester and amine absorption band were observed in the FTIR spectrum due to crosslink reaction between functional groups of collagen, glycerine and chitosan. The tensile strength of scaffolds increased with increasing DHT temperature for the exposure time of 24 to 48 hours. The highest strength was achieved on DHT treatment at 120^{o}C for 48 hours i.e. 0.56 ± 0.04 MPa. The degradation rate of scaffold decreased after DHT treatment for all temperatures and times. In conclusion, the 3D scaffolds treated with DHT exhibited excellent stability with sufficient mechanical strength and pore size making them suitable for skin regenerating template applications. The roles of glycerine to facilitate the crosslinking between chitosan and collagen were successfully investigated.

Keywords: wound dressing, chitosan, glycerine, fish collagen, 3D porous scaffold

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Introduction

Third degree or full thickness wounds do not heal on their own due to complete damage of the entire layer of skin including epidermis, dermis and underlying subcutaneous fats [1]. If the third degree or full thickness wound or damaged skin is left without medical treatment, it could lead to death. For example, if the loss of skin is 50% in third degree wounds, then the remaining 50 % of healthy skin will be used to cover the 100% wounded area by using split-thickness skin harvests technique. However, the re-epithelialization rate of skin is low by using this technique [2]. Meanwhile, if the third degree wound is bigger than the donor site, then an alternative option is skin graft, where the skin is uniformly perforated and stretched to cover the huge wounded area by using meshing techniques. Unfortunately, the meshing techniques are also associated with several limitations due to the lack of dermis area causing slow epithelialization from the graft margins, thereby resulting in graft contraction, delay in wound healing, scar tissue formation and crocodile-like skin appearance [3]. Therefore, the third degree or full thickness wound or damaged skin is unable to regenerate by using split-thickness skin harvests and meshing techniques. An alternative way to solve the problem is by developing a skin regenerating template for complete healing with minimum scar. Collagen is a well-known material for wound dressing application. However, it has been reported that mammalian sources of collagen are associated with the possibility of zoonosis such as mammalian transmission diseases including bovine spongiform encephalopathy, swine and avian influenza, mouth and foot disease in pigs, bovines and buffalo [4].

To overcome the above problems, several researchers in recent times have focused on the application of fish collagen in skin regenerating template due to their lower production cost, ease of availability of raw materials and lower or no possibility of zoonosis as compared to mammalian source collagen [5]. However, the drawback of fish collagen is their weak mechanical properties and low biostability (fast degradation rate *in vitro* and *in vivo*). The strength and biostability of scaffolds directly influence on cell infiltration, adhesion, proliferation and new tissue development. Thus, the collagen needs to blend with other polymer to enhance their mechanical strength and biostability.

Chitosan is widely used as biomaterial in wound dressing materials due to their good biocompatibility, good mechanical strength, low toxicity and ability to accelerate wound repair [6]. Chitosan is the best choice polymer for the improvement of mechanical strength and biostability of collagen scaffolds. The chitosan/collagen scaffolds have low mechanical strength and 3D porous architecture stability [7]. Glycerin is trihydroxy alcohol, widely used as solvent, humectant and vehicle in various pharmaceutical preparations. Glycerin hydroxyl group was reacted with the fatty acid carboxyl group to form ester bond with one water molecule released per each ester bond formed [8]. Thus, the potential of glycerin to act as crosslink agent between chitosan and collagen was investigated. In this research the 3D porous scaffold of chitosan/collagen was developed using freeze dry method. The potential of glycerin as crosslink agent with the help of dehydrothermal (DHT) treatment was investigated to develop high mechanical strength and biostability for skin regenerating template applications.

Materials and Methods

Chitosan from shrimp shell (medium molecular weight, degree of deacetylation (DDA) above 75%) was purchased from Sigma Aldrich. Collagen type I was extracted from Tilapia scales using enzymatic extraction method. Sodium bicarbonate (purity 99.7%) and glycerin (purity ≥ 99.5%) were purchased from Fisher. Lysozyme was purchased from Thermo Fisher Scientific.

Fabrication of 3D Porous Scaffold. Chitosan powder was dissolved completely in (1% w/v) acetic acid by stirring the mixture for 5 hours to obtain chitosan solution. Hydrolyzed collagen powder from fish scales was mixed in chitosan solution with the ratio of 70:30 (chitosan:collagen) and stirred until it became homogenous. About 20% (w/w) glycerin was added into the solution and stirred for another one hour. The solution mixture was neutralized using sodium bicarbonate solution (5%) until pH 6 was obtained. The bubbles in the solution were removed using a vacuum pump before casting in a plastic mold (11 x 11 x 2 cm) and deep frozen at -20 ºC. The frozen solution was lyophilized in a freeze dryer for 48 hours to produce porous scaffolds. The scaffold was washed three times using phosphate-buffer saline (PBS) and freeze dried for 24 hours.

Dehydrothermal (DHT) treatment of 3D porous scaffold. The effect of DHT treatment was determined on 3D porous scaffold by placing them in the mould and heating in the oven (Memmert, Germany) at temperatures 90 to 120°C) for the periods of 24 hours, 48 hours, 72 hours, 96 hours and 120 hours. The exposed 3D scaffolds (n=3) were then characterized to study the effect of DHT method on the crosslinking density, strength and chemical properties.

Physicochemical Properties Analysis of 3D Porous Scaffold. Surface morphology of 3D porous scaffolds were analyzed using field emission scanning electron microscope (FESEM), model Hitachi SU8020, Japan. The cross section of the sample was cut using sharp razor blade and thereafter coated with platinum using Quorum platinum coater for 300 second. The average pore size of the scaffold was determined from the micrograph by averaging sizes of at least 20 pores using SEM software.

Biostability of the scaffold was analysed by determine degradation rate of porous scaffolds in phosphate-buffered saline (PBS) at 37 ºC from 12 to 120 hours. Initial weight of porous scaffolds were determined and immersed in PBS containing lysozyme enzyme (10000 U/ml) at 37 °C. The biodegradation was calculated using Equation 1.

$$\text{Degradation rate (\%)} = [(W_b - W_a) / W_b] \times 100 \quad (1)$$

where W_b is the weight of scaffold before degradation and W_a is the weight of scaffold after degradation.

Effects of DHT treatment on the chemical reaction between chitosan, collagen and glycerin were monotored using FTIR spectroscopy, model Thermo-Nicolet 6700, USA. The degree of crosslinking was measured using slightly modified TNBS assay as proposed by Bubnis and Ofner [9]. The tensile properties of 3D porous scaffolds were determined by using Instron Universal Testing Machine fitted with 1kN load cell. The cross-head speed kept constant at 1 mm/min and gauge length maintained at 25 mm. The rectangular samples (10 × 40 mm) were cut from 3D porous scaffolds (n=5).

Statistical analysis. The results were expressed in mean ± standard deviation (SD) and statistically significant levels were determined via analysis of variance (ANOVA) with Tukey post hoc test and $p < 0.05$ was considered statistically significant. The SPSS software used was version 28.

Results and Discussion

Microstructure Analysis. Figure 1 shows the microstructures of untreated and DHT treated samples for 120 hours at temperatures of 90°C, 105°C and 120°C. The FESEM results confirmed that DHT treatment does not damage the microstructure of the 3D porous scaffold even when it was exposed for 120 hours at 120°C. The microstructures remained interconnected after heat treatment which indicated the stability of the 3D porous structure at high temperature probably due to the effect of crosslinking of the scaffold. The effect of DHT temperature on the microstructure of 3D porous scaffold has not been reported elsewhere. The presence of chitosan as main composition in the formulation might be another factor that contributed to the stability of the scaffold [6].

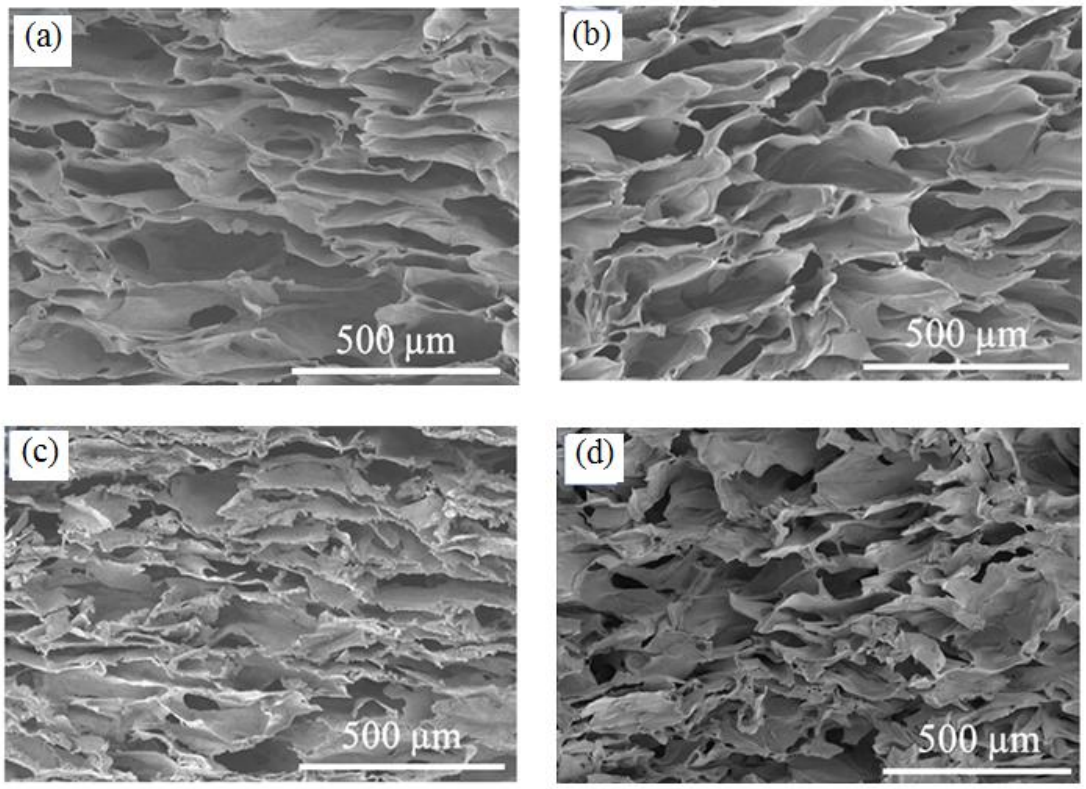

Figure1. FESEM micrographs of cross-sectional images of untreated and DHT treated porous samples at different exposure temperature of (a) untreated, (b) 90°C (c) 105°C and (d) 120°C with exposure time of 120 hours.

From SEM micrographs, the average pore sizes of DHT treated and untreated 3D porous scaffolds were measured and summarised in Table 1. Basically, the average pore sizes of 3D porous scaffolds were within the range of 106 and 162 µm. It was observed that the

pore size decreased as the exposure time and temperature increased. This phenomenon was due to scaffold shrinkage as a result of drying process and also the crosslinking that took place within the polymer matrix. The highest decrease in average pore size was observed for scaffold treated at 120°C. However, all pore sizes of DHT treated scaffolds fell within the range of the required pore size for skin tissue engineering and regeneration applications [10]. The average pore size and microstucture results proved that DHT is a viable method to be used in producing stable 3D porous scaffolds with average pore size in compliance with full requirement of skin tissue engineering and regeneration applications.

Mechanical properties. The effect of DHT treatment on the tensile strength of scaffolds at different temperatures and time of exposure is shown in Figure 2. It was observed that DHT significantly improved the tensile strength of the 3D porous scaffold as compared to untreated samples. Scaffold treated at 90°C exhibited increased tensile strength from 0.17 MPa to 0.41 MPa when heated from 24 to 120 hours. Increase observed in the tensile strength is expected as a result of increase in the crosslink between the polymers in the presence of glycerin in the scaffold matrix with the help of prolonged heating at 90^{o}C.

Table 1. Average pore size of untreated and DHT treated chitosan-fish scales collagen/glycerin 3D porous scaffolds.

Exposure time (hrs)	Pore Size (µm)			
	RT	DHT treated at 90°C	DHT treated at 105°C	DHT treated at 120°C
24	236 ± 134	162 ± 63	140 ± 53	162 ± 60
48	236 ± 134	161 ± 59	120 ± 33	144 ± 68
72	236 ± 134	142 ± 44	114 ± 28	135 ± 43
96	236 ± 134	128 ± 45	112 ± 34	132 ± 62
120	236 ± 134	128 ± 32	106 ± 32	123 ± 57

Similar increment in tensile strength was also observed for scaffold heat treated at 105^{o}C and 120^{o}C but the tensile strength decreased when exposure time increased above 72 hours. Prolonged heating at high temperature would have removed most of the water melecules in the matrix, thus reduction in the flexibility of the matrix and the scaffold became more brittle.

From the tensile test results, it was found that increasing the DHT temperature and exposure time increased the tensile strength up to 48 hours and these results were in agreement with previously reported work from Hough [11]. The highest strength was achieved on DHT treatment at 120^{o}C for 48 hours i.e 0.56 ± 0.04 MPa which was more than twice of the tensile strength of crosslinked collagen/chitosan scaffolds as reported by Liu et al. [12]. The tensile strength results support the argument that the presence of glycerin facilitated the crosslinking process between chitosan and collagen in the scaffolds matrix.

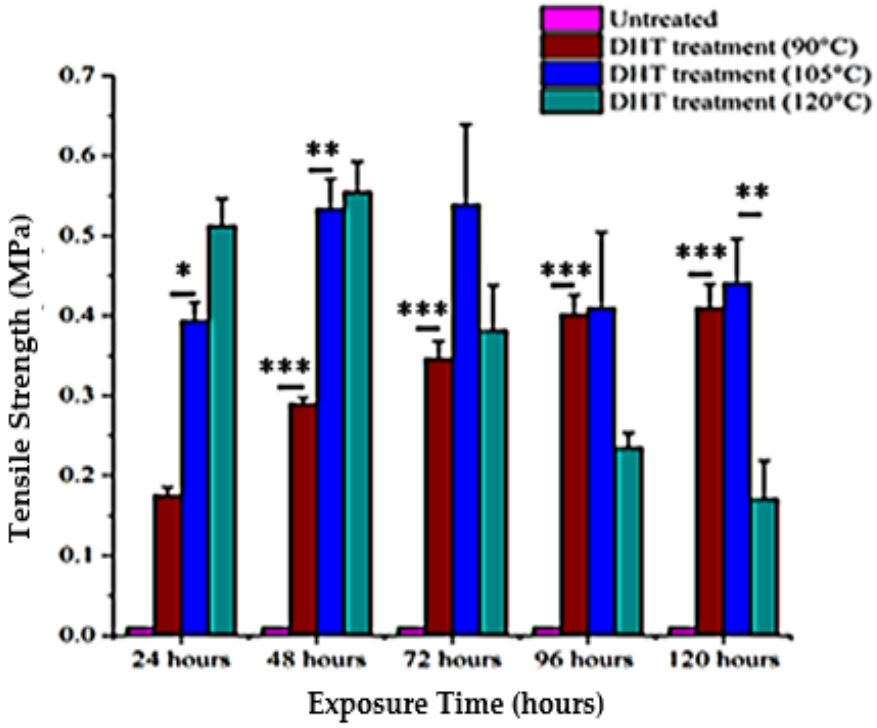

Figure 2. Tensile strengths of untreated and DHT treated chitosan-fish scales collagen/glycerin 3D porous scaffolds (n=3). Asterisks (*) represent statistical significance (* p < 0.05; ** p < 0.01; *** p < 0.001).

Biostability of scaffolds. The enzymatic degradation was carried out for all scaffolds to determine the effect of DHT treatment on the scaffold stability. The degradation rate of untreated and DHT treated 3D porous scaffolds at 90°C, 105ºC and 120ºC on exposure time of 24 to 120 hours are shown in Figure 3. The degradation rate significantly decreased for DHT treated 3D porous scaffolds at 90°C, 105°C and 120°C. It is interesting to note that the degradation rate exhibited little effect (between 45 and 55%) regardless of the heat temperature and exposure time. The results obtained proved that DHT treatment increased the stability of the 3D porous scaffold due to crosslinking within the polymer matrix.

The stability of the scaffolds is required to maintain it integrity during service especially in tissue ingineering applications as previously reported by Liu et al [12]. It can be concluded that DHT treatment succesfully produced chitosan-fish scales collagen/glycerin 3D porous scaffolds which stabilised the 3D structure of the scaffold. The presence of glycerine in the formulation sucessfully facilitated the crosslinking of chitosan and collagen scaffolds and enhanced mechanical strength and biostability of the scaffolds.

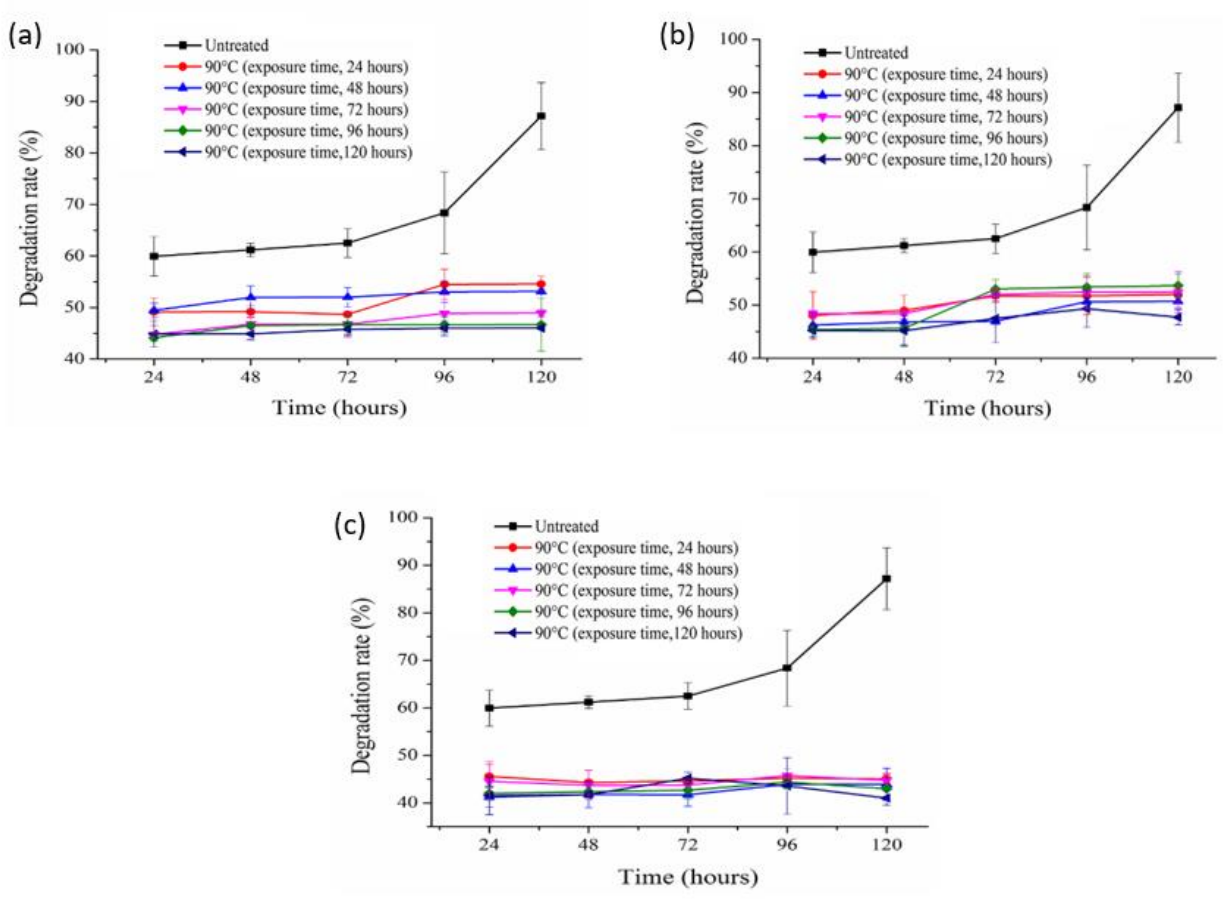

Figure 3. Degradation rate of untreated and DHT treated at (a) 90°C , (b) 105ºC and 120ºC of chitosan-fish scales collagen/glycerin 3D porous scaffolds (n=5).

Crosslinking mechanism and density. The effects of crosslinking on amide and ester functional groups were monitored using FTIR analysis. The amide band was observed at 1550 cm^{-1} whereas ester group band appeared at 1150 cm^{-1} as shown in Figure 4. This peak height at 1550 cm^{-1} was increased with increasing number of amide bonding [13] which corresponds to increasing the crosslink density due to increasing DHT temperature. However, the exposure time did not effect the amide II band crosslinking density as shown in Figure 4. This result shows good agreement with previously reported work by Haugh et al. [11]. The number of ester bonding increased with increasing temperature from 90 to 105 °C and 120 °C however, it decreased with increasing DHT temperature from 105 to 120 °C. The number of ester bonding decreased with increasing exposure time therefore and it was inferred that the ester bond formation was more favorable on DHT treatment at 105 °C at lower exposure time. The ester bond formation of 3D porous scaffolds has not been reported previously on DHT treatment [13].

The proposed crosslinking mechanisms of 3D porous scaffolds are shown in Figure 5. The interactions between chitosan, collagen and glycerin mixture at pH 6.0 were dominated by hydrogen bonding between functional groups [14]. The hydroxyl group from glycerin interacted with hydroxyl groups from collagen and amino groups from chitosan via hydrogen bonding (Figure 5(a) [15]. Glycerin acted as a mediator in between chitosan and collagen (Figure 5(b)). However, upon DHT treatment the hydrogen bonding interactions were transformed into covalent bonding through condensation and esterification reaction. Thus

glycerin facilitated the covalent bonding between chitosan and collagen and provide better mechanical strength and biostability to the 3D scaffolds [16].

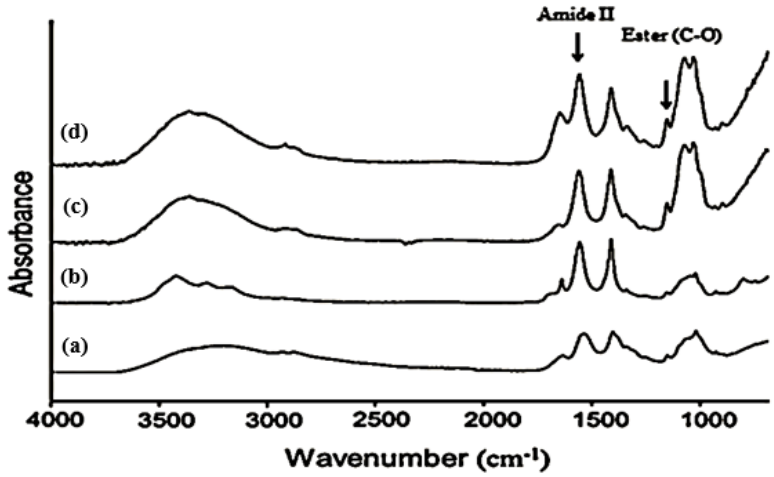

Figure 4. FT-IR spectra of untreated and DHT treated chitosan-fish scales collagen/glycerin 3D porous scaffolds for (a) untreated, (b) DHT treated at 90°C, (c) DHT treated at 105°C, (d) DHT treated at 120°C.

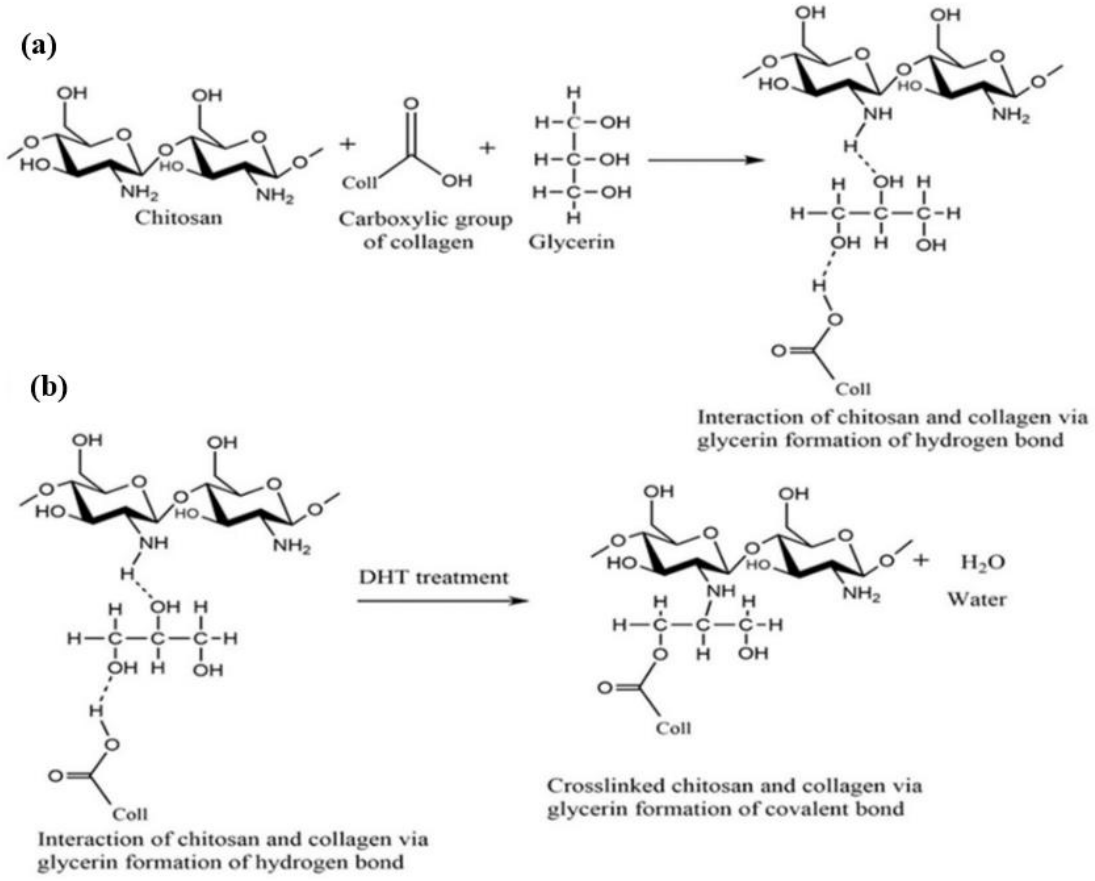

Figure 5. Proposed crosslinking mechanisms of chitosan, collagen and glycerin in 3D porous scaffolds (a) interaction of untreated 3D porous scaffolds via glycerin, (b) crosslinking mechanism of DHT treated 3D porous scaffolds in presence of glycerin.

The degree of crosslinking was determined by reacting the free amino groups in the scaffold with 2,4,6-trinitrobenzenesulfonic acid (TNBS) [9]. The degree of crosslink increased as the exposure time in DHT increased at all DHT temperatures as shown in Figure 6.

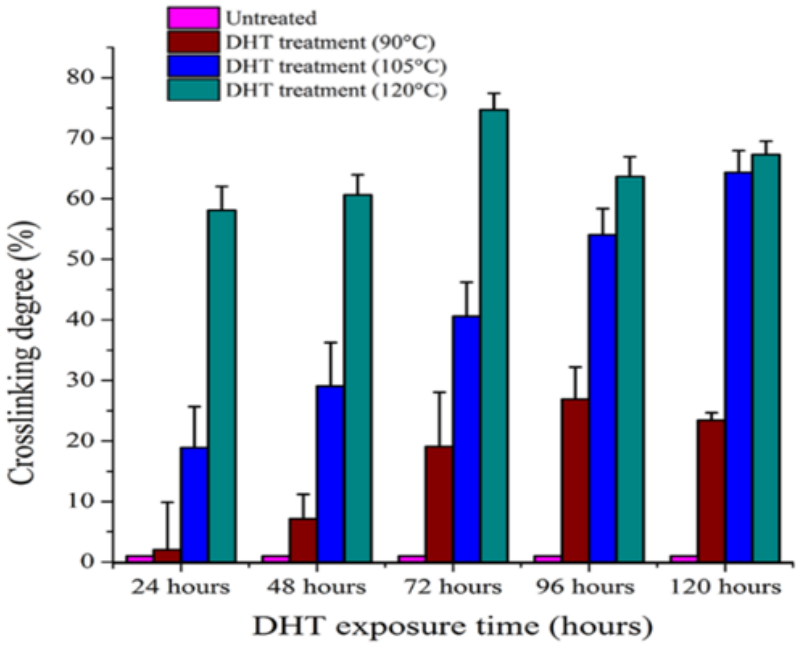

Figure 6. TNBS crosslinking degree of chitosan-fish scales collagen/glycerin 3D porous scaffolds (n=5).

Only scaffold treated at 120ºC showed slight decrease in crosslink density after exposure above 96 hours. The results obtained were supported by the results of degradation rate where as the crosslink increased the degradation rate decreased. This situation occurred probably due to stabilization of the matrix scaffold as a result of formation of network structure through amide and ester bond formation.

Conclusion

The 3D porous chitosan-collagen-glycerol scaffolds were succesfully developed through the blending of chitosan, collagen and glycerin using freze dry method with pore size within aceptable value for skin regenerating template application. A dehydrothermal (DHT) treatment succesfully crooslinked the chiosan and colagen using glycerin as crosslink agent through formation of amide and ester bond between them. The crosslink density was effected by heat treatment as a function of temperature and time. The crosslinking density increased with increasing exposure time at 90°C and 105ºC. It was found that the DHT treatment did not effect the interconnectivity of porous structure however, the average pore size decreased with increasing temperature and exposure time. The tensile strength of scaffolds increased with increasing DHT temperature at 90°C for different exposure times but it decreased for temperature at 105°C and 120°C after exposure time of 72 hours. The stability of scaffolds increased with increasing DHT temperature however, it was noticed that no significant effect

for over exposure for all time. Overall results indicated that the developed 3D porous scaffold met the physical and mechanical properties of skin regenerating template applications.

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Author Contributions

All authors contributed toward data analysis, drafting and critically revising the paper and agree to be accountable for all aspects of the work.

Disclosure of Conflict of Interest

The authors have no disclosures to declare

Compliance with Ethical Standards

The work is compliant with ethical standards

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