

## **Comparison of Gelatin and Collagen Scaffolds for Fibroblast Cell Culture**

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### **Abstract**

Gelatin and collagen were used to produce the scaffold for fibroblast cell culture. The properties of scaffolds obtained from type A and type B gelatin were compared to scaffold obtained from collagen, which is widely used in skin substitute. Porous scaffolds were prepared by freeze drying and dehydrothermal (DHT) crosslinking method. DHT treatment time was performed at 24 and 48 h and the degree of crosslinking was determined by 2,4,6-trinitrobenzene sulphonic acid (TNBS). The morphology of scaffolds was investigated by scanning electron microscopy (SEM). The compressive modulus and swelling ratio of the scaffolds were reported. To confirm the applicability of the scaffolds as a skin substitute, in vitro cell adhesion and cell proliferation tests were employed in this study. The gelatin scaffolds showed comparable properties, especially cell proliferation, to those of collagen scaffolds but the rapid degradation rate of gelatin was the limiting factor of using gelatin in wound healing. However, gelatin scaffolds could be modified to reduce the degradation rate and used substitute collagen scaffold to reduce the cost of materials for scaffold fabrication.

**Keywords:** Gelatin, Collagen, Scaffold, Skin tissue engineering

### **Introduction**

Traditionally, severe burns which cause full thickness wounds must be treated by using cadaver skin, animal skin or autograft. Obviously, these treatments bring to the issues of immune rejection and availability of skin source. Skin substitutes, constructed from biocompatible polymers, have been developed for full thickness wound healing due to their advantages in reducing scar formation and accelerating the wound healing without any infection.<sup>(3)</sup> Skin substitute requires many characteristics in order to heal the wound perfectly. Firstly, it should mimic the natural dermis as much as possible. Secondly, it should have biocompatibility. Thirdly, it should have a high surface area for cell attachment. Fourthly, it should have good mechanical integrity which is suitable for treatment handling. Finally, it is necessary for the skin substitute to be produced

from biodegradable materials. The degradation rate of scaffold or skin substitute is required to match the rate of tissue formation.<sup>(2)</sup> Therefore, biopolymers play an important role in tissue engineering as scaffolds for cells because of their suitable characteristics. In an effort to find suitable biomaterial candidates for fabricating scaffolds, gelatin was chosen in this study because it is a derivative of collagen that is the major constituent of skin, bones and connective tissue. Gelatin does not exhibit antigenicity, and practically, it is one of the most convenient proteins to use because it is much cheaper than collagen. The chemical, physical and biological properties of gelatin scaffolds, including crosslinking degree, morphology, swelling ratio, compressive modulus, degradation rate, cell attachment, and cell proliferation were studied to compare with those of collagen scaffold.

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## Materials and Methods

### Materials

Type A gelatin (lab grade, 116g bloom, pH 4.5, pI 9, Ajax Finechem, Australia) and type B gelatin (pharmaceutical grade, 152g bloom, pH 5.64, pI 4.9, Geltech Co., LTD., Thailand) were used. Collagen solution (pH 3.1) was purchased from Nitta Gelatin Inc. (Tokyo, Japan). All other chemicals used in this work are of analytical grade.

### Preparation of the scaffolds

Type A and type B gelatin were swollen in deionized water at room temperature and then dissolved at 37°C under agitation to obtain 0.6wt% (w/w) solutions. The solutions were then degassed centrifugally. After measuring pH of the solutions, 1 ml of the solution was poured into each well of polystyrene 24-well plates and frozen at -50°C overnight prior lyophilized at -50°C for 24 h (PowerDry LL3000, Heto, USA). The resulting freeze dried gelatin scaffolds were crosslinked by dehydrothermal (DHT) treatment at 140°C<sup>(6)</sup> for 24 and 48 h in a vacuum oven (VD23, Binder, Germany). The collagen scaffolds, obtained from collagen solution (6.06 mg collagen in 1 g collagen solution in HCl), were prepared by lyophilization and DHT crosslinking techniques as previously described for the case of gelatin scaffolds. Table 1 listed six experiments of gelatin and collagen scaffold preparations as well as the pHs of their solutions.

**Table 1.** Experiments of gelatin and collagen scaffolds and pH of the solutions.

Solution concentration	Scaffold type	DHT treatment time (h)		pH
		24	48	
0.6wt%	Type A gelatin	0.6A-24	0.6A-48	5.15
	Type B gelatin	0.6B-24	0.6B-48	5.80
	Collagen	0.6C-24	0.6C-48	3.22

### Determination of crosslinking degree

The determination of crosslinking degree was carried out by modifying the method of.<sup>(1)</sup> Briefly, about 5 mg of the scaffolds was weighed into a test tube where 1 ml of 0.5% TNBS solution

and 1 ml of 4% sodium hydrogen carbonate (NaHCO<sub>3</sub>, pH8.5) were added. It was then heated in a water bath maintained at 40°C for 2 h. The uncrosslinked primary amino groups of gelatin and collagen in the scaffolds would react with TNBS and form a soluble complex. This solution was further treated with 2 ml of 6 N HCl at 60°C for 1.5 h. The absorbance of the solutions was determined at 415 nm after suitable dilution spectrophotometrically. The crosslinking degree was then calculated by the following equation:

$$\text{Crosslinking degree (\%)} = \left( \frac{1 - \text{Absorbance of crosslinked scaffold}}{\text{Absorbance of uncrosslinked scaffold}} \right) \times 100$$

The values were expressed as the mean±standard deviation (n=2).

### Morphological observation

The morphology of scaffolds was investigated by scanning electron microscopy (JSM-5400, JEOL Ltd., Tokyo, Japan). In order to observe the scaffolds inner structure from cross-sections plane, the scaffolds were cut with razor blades. The cut scaffolds were placed on a Copper mount and coated with gold prior to SEM observation.

### Mechanical testing

A universal testing machine (No. 5567, Instron, USA) was used to determine the slope from 5 to 30% strain of the stress-strain curves of the scaffolds (dimension: d = 14.5 mm, h = 5 mm) at a constant compression rate of 0.5 mm/min. The compressive modulus was determined and reported as the mean±standard deviation (n=5).

### PBS swelling property

The water sorption capacities of the scaffolds were determined by swelling them in phosphate buffered saline (PBS) at 37°C, pH 7.4. Known weights of the scaffolds were placed in the PBS solution for 5 h. The wet weights of the scaffolds were determined by first blotting the scaffolds on a lint-free paper (Kimwipe) to remove excess water, and then weighed immediately. The swelling ratio of the scaffold, W<sub>sw</sub>, was calculated from the equation:

## Comparison of Gelatin and Collagen Scaffolds for Fibroblast Cell Culture

$$W_{sw} = \frac{(W_t - W_o)}{W_o}$$

$W_t$  represented the weight of the wet scaffolds, and  $W_o$  was the initial weight of the scaffolds. The values were expressed as the mean±standard deviation (n=3).

### ***In vitro* biodegradation**

The biodegradation of scaffolds was investigated using 1 ml phosphate buffered solution (PBS, pH 7.4) at 37°C containing 1.6 µg/ml lysozyme.<sup>(5)</sup> The concentration of lysozyme chosen corresponded to the concentration in human serum. The lysozyme solution was refreshed daily to ensure continuous enzyme activity. Every other day, samples were taken from the medium, rinsed with deionized water, freeze dried and weighed. The experiment was done triplicates for each different scaffolds. The extent of *in vitro* degradation was expressed as a percentage of the remaining weight of the dried scaffold after lysozyme treatment.

$$\text{Remaining weight (\%)} = \left( \frac{W_d}{W_o} \right) \times 100$$

$W_o$  denoted the initial weight of the scaffolds, while  $W_d$  was the remaining weight of the scaffolds at time t. The values were expressed as the mean±standard deviation (n=3).

### ***In vitro* cell adhesion and proliferation tests**

For cell attachment, L-929 cells (60,000 cells per scaffold) were seeded onto the scaffolds placed in 48-well tissue culture plates in 10%DMEM containing serum. At 5th h after the culture, scaffolds were rinsed with PBS and 350 µl/well of MTT (0.5 mg/ml) was added and incubated at 37°C in 5% CO<sub>2</sub> incubator (Series II 3110, Thermo Forma, USA) for 30 min to establish cell viability. DMSO was used to elute the ice crystals of MTT and the absorbance of the solution was measured at 570 nm using a spectrophotometer (Thermo Spectronic, Genesys 10UV scanning). The treatment of the scaffolds without cells was used as the control.

For cell proliferation, L-929 cells (30,000 cells per scaffold) were seeded onto the scaffolds

instead. The medium was changed every other day. At each time interval, 24<sup>th</sup> and 48<sup>th</sup> h, scaffolds were rinsed with PBS and the MTT treatments were performed as mentioned previously. The extent of *in vitro* cell viability was expressed as a percentage of cell adhesion and proliferation.

$$\% \text{ Cell adhesion} = \% \text{ Cell proliferation} \left( \frac{= \text{Number of cells by MTT}}{\text{Number of seeded cells}} \right) \times 100$$

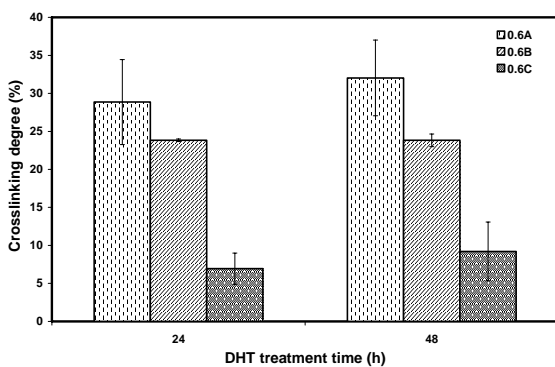
All experiments were run in duplicate. All data were expressed as mean±standard deviation (n = 3).

## **Results and discussion**

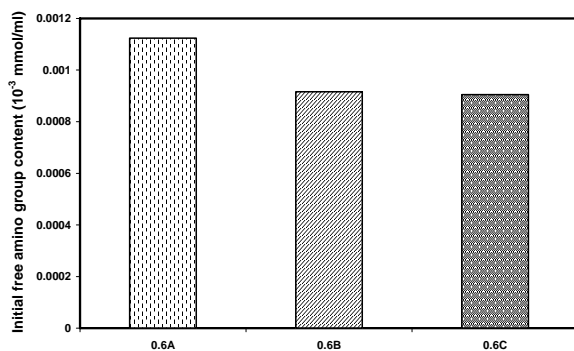
### **Crosslinking degree**

The results from TNBS showed that the extent of crosslinking of gelatin scaffolds was a function of gelatin type and DHT treatment time, as presented in Figure 1. With the increasing DHT treatment time from 24 to 48 h, the crosslinking degree increased from about 28% to 32% for type A gelatin scaffolds and from about 24% to 25% for type B gelatin scaffolds. The difference of crosslinking degree between scaffolds from two gelatin types was the result of the difference in initial free amino group content. Collagen from various connective tissues had different amino group contents.<sup>(4)</sup> Because type A gelatin was derived from porcine collagen while type B gelatin was derived from bovine collagen, amino group contents in both gelatin types were different. The amount of initial free amino group in each gelatin scaffold using β-alanine as a standard<sup>(7)</sup> could be seen obviously in Figure 2. Before the DHT treatment, type B gelatin scaffolds showed less initial free amino group contents than type A gelatin scaffolds. A lesser amount of initial free amino group contents resulted in the less crosslinking in type B gelatin. On the other hand, type A gelatin originally had more free amino group contents so that there were abundant amino groups to link each other, leading to the higher crosslinking degrees. Furthermore, the difference of crosslinking degree may be due to a difference of molecular structure of both gelatin types. The results corresponded with a recent study by Tabata, *et al.*<sup>(6)</sup> reporting that type A gelatin could be crosslinked more than type B gelatin could. They proved that DHT crosslinking could occur only if the amino and carboxyl groups were close to each

other, which meant that the gelatin molecules were closer to each other because the transition temperature from random coil to helix conformation is higher. Therefore, the higher degree of crosslinking of type A gelatin might be due to the tighter packed molecules than that of type B gelatin. For collagen scaffolds, the degree of crosslinking was much lower than that of type B gelatin even though their initial free amino group contents were relatively equal. This could be caused by the structure of collagen. Collagen was composed of three chains, twisted together in a tight triple helix. The tight triple helix of collagen might hinder the crosslinking.



**Figure 1.** Crosslinking degree of gelatin and collagen scaffolds with 24 and 48 h DHT treatment times (n=2).

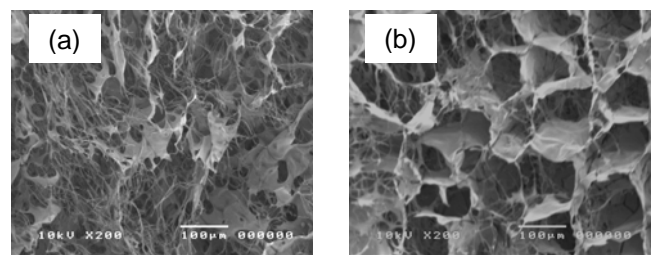


**Figure 2.** Initial free amino group content in gelatin and collagen scaffolds (uncrosslinked scaffolds, n=2).

### Morphology of the scaffolds

In general, porosity, pore size and orientation of porous scaffold were indispensable elements of biological activity of biomaterials having an open-pored structure. The structures of

the scaffolds, as illustrated in Figure 3, seemed to be influenced by type of material. 0.6A scaffolds showed fiber-like structure, as seen in Figure 3(a), while 0.6C scaffolds showed membrane-like structure with interconnected pores, as seen in Figure 3(c). For 0.6B scaffolds, the structure showed some fibers among the continuous wall. The difference in structure of the scaffolds might be due to the nature of material from various sources or the difference in producing process. The dispersion of molecules in the solvent of triple helix and hydrophobic collagen could be different from that of random coil and hydrophilic gelatin.

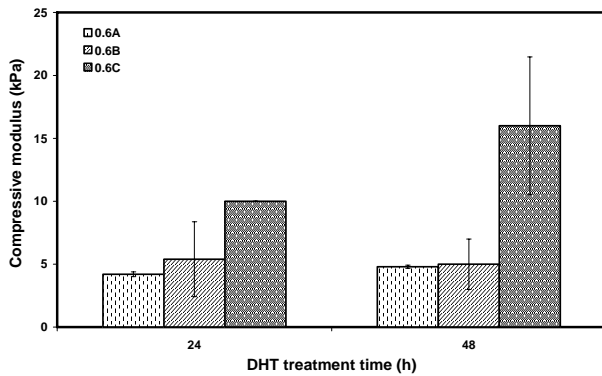


**Figure 3.** SEM micrographs of vertical cross-sections of gelatin scaffolds 48h DHT) : (a) 0.6A and (b) 0.6B.

### Mechanical properties of the scaffolds

One of the most important properties of the scaffolds is mechanical strength. To maintain the scaffold when used as a skin substitute, the scaffold has to be strong enough in order to support extensive vasculatures, the lymphatic system, nerve bundles and other structure in the skin. Therefore, the scaffold should have an appropriate compressive modulus to absorb forces when they are implanted into the wounds. The compressive modulus of gelatin and collagen scaffolds, elucidated in Figure 4, was strongly affected by material type rather than DHT treatment time. The 0.6C scaffolds showed a significant higher compressive modulus than 0.6A and 0.6B scaffolds. This was reflected by the structure of both biomaterials. Random-coil structure of gelatin provided low mechanical property while the triple helix structure of collagen provided the higher mechanical property. In addition, an increase in DHT treatment time could slightly improve the mechanical properties of the scaffolds as longer DHT treatment time allowed more crosslinking within the structure of the scaffolds. As a result, the more crosslinked scaffolds could resist more compressive force.

## Comparison of Gelatin and Collagen Scaffolds for Fibroblast Cell Culture



**Figure 4.** Compressive modulus of gelatin and collagen scaffolds with 24 and 48 h DHT treatment times (n=5).

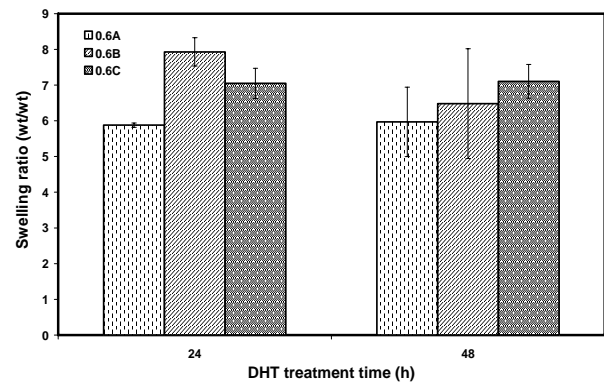
### PBS solution adsorption

The swelling ability of scaffold plays an important role during *in vitro* culture. When the scaffold was capable of swelling, it allowed the pore sizes to increase in diameter thus facilitating the cells not only to just attach but also to migrate inside the scaffolds and grow in a three-dimensional fashion, during *in vitro* culture studies. In this study, the swelling ratios at 5th h were investigated since it was the time for the initially cell attachment. The results showed that the swelling ratios of all scaffolds showed no significant difference, as presented in Figure 5. Gelatin is widely known for its hydrophilicity, which allows gelatin to absorb water up to 10 times of its dry weight. For collagen scaffold, it could be implied from SEM photographs in Figure 3 that morphology of 0.6C scaffolds had much less porosity than that of 0.6A and 0.6B scaffolds. With this advantage of the structure, 0.6C scaffolds could retain PBS solution. Therefore, the dense morphology of 0.6C could compensate the disadvantage of its hydrophobicity. Furthermore, the scaffolds also showed a slight decrease in swelling properties with as increasing DHT treatment time because of the effect of crosslinking.

### *In vitro* biodegradation behavior

All scaffolds were tested with respect to the *in vitro* biodegradation rate by lysozyme. Both 0.6A and 0.6B scaffolds degraded rapidly in lysozyme solution because of its random coil structure and its hydrophilicity. Therefore, the result of gelatin scaffolds was not reported in this

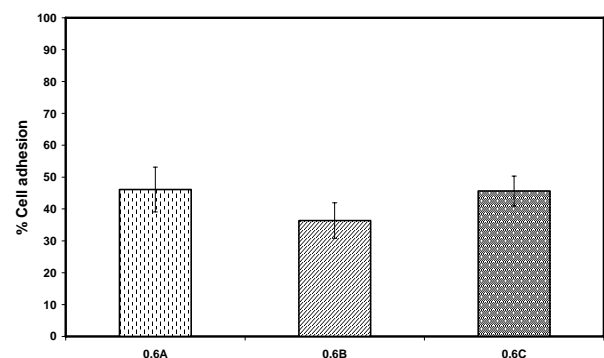
test. The remaining weight of the collagen scaffolds depended on DHT treatment time. The 0.6C-24 completely degraded within a week while the 0.6C-48 h could remain up to three weeks. This could be explained by the structure of collagen and gelatin. The triple helix structure made collagen more difficult to degrade because there was less opportunity for molecules to contact the enzyme; moreover, collagen was hydrophobic so it was difficult to dissolve in enzyme solution.



**Figure 5.** Swelling ratio of gelatin and collagen scaffolds with 24 and 48 h DHT treatment times (n=3).

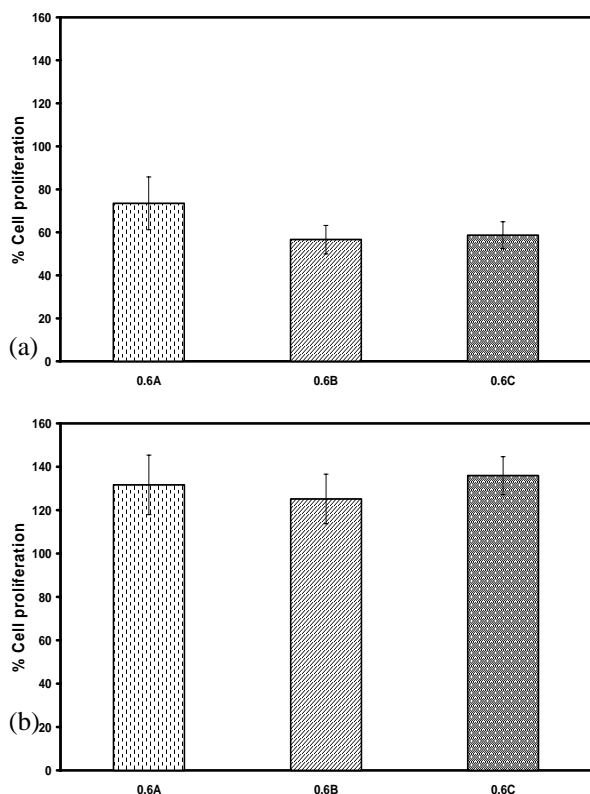
### *In vitro* cell adhesion and cell proliferation

Figure 6 revealed the number of fibroblast cells attached on each scaffolds at 5 h after the culture. It should be pointed out that there was no significant difference in initial cell attachment on 0.6A and 0.6B scaffolds comparing to 0.6C (control) scaffolds ( $p > 0.05$ ).



**Figure 6.** % L929 cell adhesion on different scaffolds at 5 h after culture (Number of seeded cells = 60,000 cells/scaffold, n=3).

Figure 7(a) and (b) showed the number of fibroblast cells proliferated on each scaffolds after 24 and 48 h of the culture, respectively. The results showed that the proliferation rates in all scaffolds were not significantly different. Furthermore, it could be concluded that type A or type B gelatin scaffolds could induce cell proliferation as good as the collagen scaffolds could, at the total solid weight of 0.6 percents.



**Figure 7.** % L929 cell proliferation on different scaffolds in 10%D MEM at each time of the culture (a) 24 h, (b) 48 h (Number of seeded cells = 30,000 cells/scaffold, n = 3).

## Conclusion

The results proved that gelatin scaffolds from both gelatin types possessed comparable properties, especially the cell proliferation, to those of collagen scaffold. The disadvantage of gelatin scaffolds is the rapid degradation in enzyme. Therefore, the gelatin scaffolds should be further modified by blending with other materials or improving the crosslinking technique to slow their degradation rate.

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