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Fabrication of Gelatin Scaffolds using Thermally Induced Phase Separation Technique

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ABSTRACT

Gelatin is considered as a partially degraded product of collagen and it is a biodegradable polymer which can be used to produce scaffolds for tissue engineering. Three-dimensional, porous gelatin scaffolds were fabricated by thermally induced phase separation and freeze-drying method. Their porous structure and pore size were characterized by scanning electron microscopy. Scaffolds with different pore sizes were obtained by adjusting the concentration of the gelatin solution. Scaffolds with 3.75% (w/v) gelatin and 5% (w/v) gelatin produced pores ranging from 100 to 450µm. The average pore size increased with an increase in gelatin concentration. The properties of the scaffolds in terms of water uptake were studied. The results showed that when the concentration of the gelatin solution was changed from 3.75% to 5%, the water absorption of the fabricated scaffolds decreased by 104%. The increment in the concentration of gelatin induced a reduction in water uptake in the scaffolds produced.

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1. INTRODUCTION

Tissue engineering [TE] is an interdisciplinary field that uses scaffolds to support cell seeding and biochemical factors to regenerate biological function of a tissue or organ. The biochemical factors involve are such as growth factors and proteins that stimulate proliferation and differentiation cell. Tissue engineering helps to regenerate and repair the damaged tissue by substituting the engineered tissue with an aim to restore the biological functions followed by integrating with the host tissue. Therefore, a significant attention is given to three-dimensional polymer scaffolds for tissue engineering and drug delivery applications [1]. Scaffolds functions as an artificial extracellular matrix to provide mechanical support to the cells and mimic the natural environment of the tissues for regeneration [2].

The ideal scaffold ought to have the following features: (i) three-dimensional structure with high porosity and interconnected pore for nutrients and metabolic waste to be transported to the cells and enabling cell growth (ii) biocompatible (iii) bioresorbable (iv) controlled rate of degradation to match the growth of cell or tissue, (v) surface is chemically favorable for cell attachment, differentiation, and proliferation and (vi) provide suitable mechanical properties [2]. Due to the development of different fabrication process, it is possible to fabricate highly porous scaffolds available in different sizes and shapes with porosities up to 90%. Degradable and biocompatible monomers can be used to modulate the scaffold properties. In in- vitro study, the scaffolds can exhibit a continuous degradation with different degradation rates depending on their material composition and solvent involves. Furthermore, the culture of osteoblast cells on the scaffolds were performed and revealed their biocompatibility. Cell growth on inside and surface of the scaffold, extracellular matrix formation, and starting mineralization were detected by histological and microscopical analyses. Therefore the developed materials should be well-suited candidates for the design of tailor-made matrices in bone tissue engineering [3].

Biodegradable and biocompatible polymers are the most popularly used biomaterials for the fabrication of

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scaffolds due to many attractive mechanical and chemcial properties. Synthetic and natural biodegradable materials such as Poly (lactide) (PLA), Poly (L-lactic acid) (PLLA), Poly (D,L-lactide) (PDLLA), Poly (lactide-co-glycolide) (PLGA), Poly (lactide-co-caprolactone) (PLCL), Poly (D,L-lactic acid-co-glycolide acid) (PDLG), Poly (ε-caprolactone) (PCL), Polyurethane (PU), Poly (ether ester urethane) (PEEUU), collagen, chitosan and gelatin have been used in the fabrication of scaffolds [4].

Gelatin is considered as a partially degraded product of collagen [2]. Collagen has been known to have antigenicity due to its animal origin whereas gelatin has relatively low antigenicity compared to its precursor but it can still retain some of the information signals which may promote cell adhesion, proliferation, and differentiation, such as the Arg–Gly Asp (RGD) sequence of collagen [5,6]. Gelatin has been mixed with chitosan scaffolds to promote cell adhesion, migration, proliferation, and differentiation [7,8]. Gelatin is made of collagen by basic or acidic hydrolysis and its chemical composition is very similar to that of collagen. Therefore, gelatin mimics the chemical composition of natural collagen. Moreover, gelatin can be denatured and the denaturing hydrolysis process eliminates the potential pathogens. Gelatin has been used in tissue engineering [TE] as a natural biopolymer [9].

Different methods are used to fabricate scaffolds, and it is classified as designed manufacturing techniques and non-designed manufacturing techniques. Designed manufacturing techniques are rapid prototyping (RP) technologies that have been applied to the tissue engineering field, and due to their various advantages have attracted much attention in recent years. Non-designed manufacturing techniques include solvent casting/particulate leaching, phase separation, melt molding, gas foaming/high pressure process, electrospinning, emulsion freezing/freeze-drying, and combinations of these techniques [10]. Thermally induced phase separation (TIPS) has been applied in the fabrication of microporous membranes or microcellular foams in scaffolds for tissue engineering, as a drug carrier for controlled release and also medical and chemical industry. Adjusting TIPS parameters, such as types of polymers, polymer concentration, solvent or non solvent ratio and thermal quenching could be obtained materials with distinctive morphologies based on their applications [11]. In tissue engineering, thermally induced phase separation (TIPS) has been explored to produce scaffolds with well interconnected porous structure [12]. This technique is based on changing thermal energy to activate the de-mixing of a homogeneous polymer solution into a multi-phase system domain by a quench route [1]. During the phase separation, the homogenous solution separates in a solvent-rich phase and polymer-rich phase either by liquid–liquid phase separation or solid–liquid de-mixing or mechanism [10]. the solvent is extracted from the scaffolds, different morphologies and characteristics of the materials can be obtained such as closed or open-pore structure, spheres, powders and bead-like morphology based on the system and phase separation conditions. Scaffolds with open pore and well-interconnected morphology are the most applied scaffolds in tissue engineering [12]. TIPS has many advantages such as process high reproducibility, simplicity, high porosity, low tendency to form defects, and the ability to form interesting microstructures with narrow pore size distribution. Moreover, the ability to control the polymer polymorphism using solvents and process parameters is also a unique feature. The key procedures for the TIPS method are as follows (1) allow the polymer of interest dissolved in high temperature, (2) cast the dope solution into the desired shape, (3) induce phase separation and precipitation of the polymer (4) cool the cast solution in a controlled manner, (5) extract the diluent, often via solvent extraction to yield a membrane.

Freeze-drying technique is a simple method which can be used to produce highly porous scaffolds. By using this technique the porosity level can be achieved above 90% and pore size also can be controlled. In this process an appropriate solvent should be used to make the polymer solution, creating an emulsion by homogenizing a mixture of the polymer solution, and a water phase, rapidly cooling the emulsion to maintain the liquid state structure and removing the solvent and water phase by freeze-drying. Therefore, it is possible to produce hardly porous and tough scaffolds using this technique [13]. The porous structure of polymeric scaffolds can be controlled by changing the processing or formulation parameters such as the polymer, polymer solution concentration, solvent freezing temperature and water phase percentage [10]. Therefore, it is crucial to be carefully selecting the parameters for creating an emulsion from two immiscible phases (the polymer solution and the water phase) to obtain scaffolds with desired porous structure and properties.

This work aims to develop a low-cost scaffold, the fabrication of gelatin scaffold by thermal induced phase separation method. The different concentrations of gelatin were used to study the effect of porosity and water uptake of the scaffold depending on its concentration. The objective of this work was focused on the study of the influence of gelatin concentration and on the effect of thermal induced phase separation on the pore size range and average pore size. The effect of gelatin with two different concentrations and their water uptake was also studied. It can be mentioned that although the scaffolds using other polymers using thermally induced phase separation were reported previoulsy but in this paper we reported the fabrication of a new scaffold using a natural polymer, gelatin, which may have better characteristics as tissue engineered scaffolds.

2. MATERIALS AND METHODS

2. 1. Materials The materials used in this study were gelatin polymer and acetic acid. Gelatin polymer was purchased from Sigma-Aldrich. Acetic acid was analytical grade.

2. 2. Fabrication of Scaffolds with Gelatin To prepare gelatin solution, 1.0 g of gelatin polymer was weighed. Then, 20 ml of 2% (v/v) of acetic acid solvent was carefully measured and then was added into a Schott bottle. 1.0 g of gelatin polymer was added into the Schott bottle to prepare into a polymer solution of 5% (w/v). The function of 2% (w/v) acetic acid was used to dissolve the gelatin. Subsequently, the solution was prepared using a hotplate magnetic stirrer with a fixed speed until the polymers were completely dissolved. Due to this condition, another solution was prepared by decreasing the amount of gelatin polymer. For the second trial, 0.75 g gelatin polymer was measured and then dissolved into 20 ml of 2% acetic acid which makes into a gelatin solution of 3.75% (w/v). After about 30 minutes, it was found that both solutions were completely dissolved and used to fabricate the scaffolds. Then, the solution was transferred to two glass vials. The polymer solution in the vials was transferred into a freezer at a temperature of -18°C for solidification. The solidified mixture was kept in the freezer for overnight and then transferred into a freeze-dryer vessel (LABCONCO-Freeze Dry System, Kansas City, MO, USA). The solutions were freeze-dried for 48 hours to sublime the solvent and producing porous scaffolds. The scaffolds specimens were then being taken out from the glass vials by breaking up the glass vials. The scaffolds were then cut into two small slices to test for water uptake, morphology, and pore structure studies.

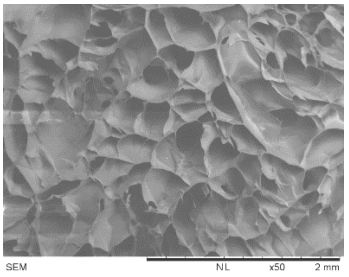
2. 2. Scanning Electron Microscope (SEM) and Energy Dispersive X-Ray (EDX) Analysis The porous structure and pore morphology of the fabricated scaffolds were examined using a Scanning Electron Microscope (SEM, Table Top TM3000). By using SEM, the pores sizes were also determined. In order to study the compositions of elements present in the gelatin fabricated scaffolds, Energy Dispersive X-ray (EDX) spectroscopy analysis was used.

2. 3. Water Uptake The purpose of this water uptake experiment was to measure the water absorption of the gelatin scaffolds. In order to calculate water uptake, each of the samples was pre-weighed using an electronic balance. Each of the samples was then being immersed in distilled water for 2 minutes. After that, the sample was blotted dry on a filter paper in order to remove excess water. Then, the samples were weighed again. The water uptake for each sample was calculated by subtracting the sample weight before and after soaking in distilled water and dividing by its initial weight.

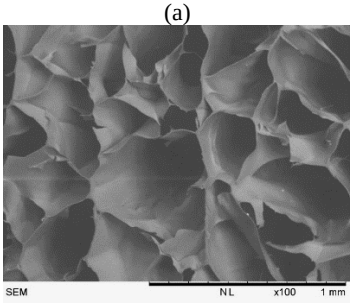
3. RESULTS AND DISCUSSION

In this study, we found that the concentration of the gelatin affects the morphology of the scaffolds. Figures 1a and 1b show SEM of 3.75% gelatin scaffold at a magnification of 50 and x100, respectively. The distribution of pore sizes are shown in Figures 2a and 4a for both at different concentrations of gelatin scaffolds, respectively.

The effect of the gelatin concentrations on the morphology of the gelatin scaffolds was investigated using 3.75 and 5% gelatin. SEM observation of the gelatin scaffolds prepared by thermally induced phase separation method showed a continuous structure of irregular interconnected pores (Figures 1 and 3). The pore sizes for both scaffolds concentration ranged from 100 - 450μm.The obtained result was comparable to a study reported by Liu and Ma [9], where pore sizes of gelatin nanofibers diameter range were from 50-500μm which were more likely the same to the natural collagen fibers. The porosity of the scaffolds could be adjusted by changing the gelatin concentration. The gelatin composition at 3.75% showed minimum pore size of 110 μm, maximum pore size of 406μm and an average pore size was 244μm. The highest pore size in 3.75% gelatin scaffold was within the range of 150 to 200 μm (Figure 2). For the scaffold with 5% gelatin, it was characterised by larger pore size compared to 3.75% gelatin concentration where the minimum pore size was 149 μm, maximum pore size was 420μm and had the average pore size of 249μm (Figure 4). The highest frequency of pore sizes was within the range of 200-250μm.

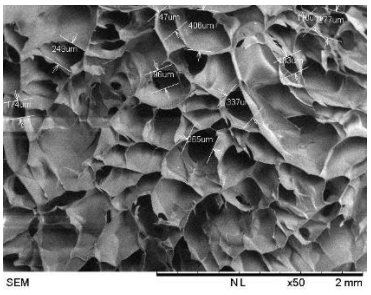


(a)



(b)

Figure 1. SEM micrograph of fabricated gelatin scaffolds (a) 3.75% gelatin scaffold at x50 magnification (b) 3.75% gelatin scaffold at x100 magnification



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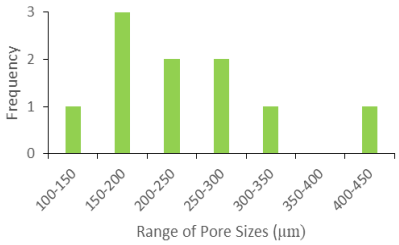
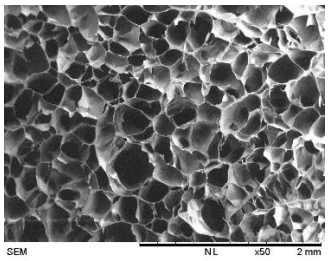


(b)

Figure 2. (a) Pore sizes using SEM micrograph on 3.75% of fabricated gelatin scaffolds (b) Pore size distribution of 3.75% gelatin scaffold



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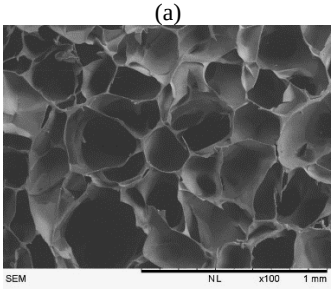
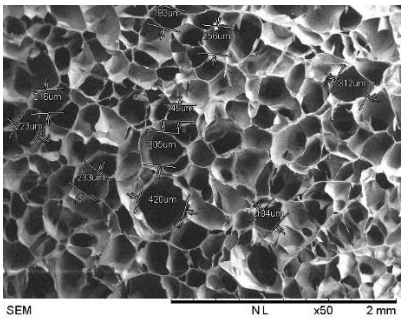


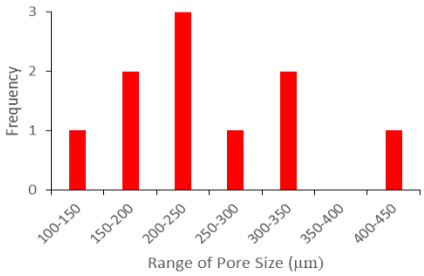
(b)

Figure 3. SEM micrograph of fabricated gelatin scaffolds (a) 5% gelatin scaffold at x50 magnification (b) 5 % gelatin scaffold at x100 magnification

Therefore, from this result, it shows that the average pore size increases with an increase in gelatin concentration. A previous study on gelatin nanofibers reported by Liu and Ma [9], found that average diameter of gelatin decrease with an increase of gelatin concentration [9, 14]. In this investigation, the solvent solidification front proceeded mainly from the bottom to the top of the solution and from the side walls to the centre within a few hours.



(a)



(b)

Figure 4. (a) Pore sizes using SEM micrograph on 5% of fabricated gelatin scaffolds (b) Pore size distribution of 5% gelatin scaffold

As a result, a continuous polymer-rich phase was formed which was in fact the aggregation of excluded polymer from every single solvent crystal. After the sublimation of the solvent and water phase, scaffolds with pores of the similar geometry and water phase crystals was formed. When the temperature of the polymer solution was low enough, both liquid-liquid phase separation and polymer crystallization can occur [9,14]. The kinetic phenomena is important in this situation. The system phase morphology can also be affected by coarsening of liquid phases which can occur during the later stage of phase separation. Coarsening phenomena can be observed in systems which exhibit a phase transition when the temperature is decreased below a critical temperature. In the diffusive process of solutions, droplets that are well separated and have well-defined interfaces can coarsen due to the fact that smaller droplets have higher solubility and hence preferentially dissolve while larger droplets grow [4,9,10].

In order to study the composition of gelatin, EDX analysis was conducted for determining the elemental composition for 3.75 and 5% gelatin scaffold. The results are shown in Figure 5. Both gelatin scaffolds contained organic elements of carbon and oxygen.

The atomic weight % of both elements varied with respect to the concentrations.

In this work, we used the thermally induced phase separation (TIPS) technique to prepare 3D gelatin matrix. The TIPS technique involves several steps, including and dissolving a polymer in a proper solvent, phase separation, solvent exchange and freeze-drying.

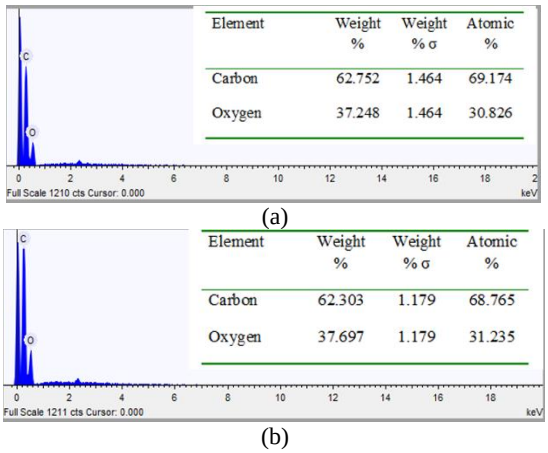


Figure 5. The EDX analysis spectrum and elemental analysis of (a) 3.75% gelatin scaffold (b) 5% gelatin scaffold

Among them, the selection of proper solvent is one of the most important steps in scaffold structure formation. By choosing the acetic acid/water solvents, architecture of gelatin was created. By varying the concentration of gelatin, it gives different pore sizes to the scaffold as mentioned above. So these results indicated that controlling the interactions between gelatin and solvent molecules were critical to create the scaffolds with different pore sizes and their interconnectivity. Moreover, the results may change due to temperature differences during the process [13, 15]. The temperature and duration may affect the crystal formation and sublimation process, producing pores in the scaffolds. Water uptake test for the fabricated scaffolds was carried out for both 3.75% gelatin scaffold and 5% gelatin scaffold. These scaffolds were weighed before immersed in distilled water for 2 minutes. Then, the weight of scaffolds after immersion was measured. Table 1 shows the weight of the samples, and their corresponding water uptake (%).

As a scaffold for tissue engineering, the water absorption by the scaffold not only affects its morphology and structure but also affects the ingrowth of the cells.

It is important for the absorption of body fluid and for a transfer of cell nutrients and metabolites through the materials. Table 1 shows the water uptake of gelatin scaffolds prepared from two different gelatin concentrations.

TABLE 1. Weight and water uptake of the samples

Sample (gelatin) (%)	Weight (g)		Water uptake (%)
	Before	After	
3.75	0.0454	0.4062	794.70
5	0.0397	0.3141	691.18

The results indicated that the scaffolds from gelatin with higher concentration (5%) had lower water uptake than scaffolds with low gelatin concentration (3.75%) with a difference of 104%. Gelatin is a hydrophilic polymer. According to a study, water uptake ability in gelatin can be enhanced when gelatin was added into chitosan [16]. Results show the amount of water retention was higher with an increase in gelatin ratio. That is when gelatin mixes with chitosan, the hydrophilicity of the scaffolds was raised, which leads to an increase in scaffold porosity. As a result, the water uptake ability increased.

A study showed that different concentration of gelatin had influence on porosity and water uptake where an increase in the concentration of gelatin caused decrease in the porosity of the scaffolds and water uptake [4]. Therefore it was concluded that Gelatin scaffolds made from low gelatin concentration had high porosity and large pore size, which offered more space for water uptake; therefore, water absorption became higher [16].

A study by Banerjee et al, (2009) showed that addition of hydrophobic poly (lactide-co-glycolide) (PLGA) microspheres into gelatin scaffolds reduced significant water uptake [17]. The use of PLGA microspheres inside the hydrophilic polymers would change the size and distribution of ice crystals formed in the scaffolds. Furthermore, microspheres may cause changes in the mechanical properties of the scaffold and this will reduce the hydrophilicity of gelatin scaffolds. In the experiment, it was found that low concentrations of gelatin caused smaller average pore size and had higher water uptake compared to the high concentration of gelatin scaffold which resulted in bigger average pore size and had lower water uptake.

4. CONCLUSIONS

Gelatin scaffolds with different pore size structure by thermal induced phase separation technique were successfully fabricated. Different concentrations of gelatin which were 3.75 and 5% caused different average pore size, where the average pore size was larger in 5% gelatin (244 µm) scaffold compared to 3.75% gelatin scaffold (249 µm). Moreover, the water uptake results indicated that the scaffold with a high concentration of gelatin had decreased water uptake than scaffold with low gelatin concentration.

5. ACKNOWLEDGEMENT

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چکیده

ژلاتین به عنوان یک محصول نیمه تجزیه شده کلاژن در نظر گرفته می شود ویک بسپار قابل تجزیه زیستی است که می تواند برای ساخت داربست های بافتی در مهندسی بافت مورد استفاده قرار گیرد. در این بررسی داربست های بافتی سه بعدی متخلخل توسط فرآیند جداسازی فاز ناشی از حرارت و خشک کردن انجمادی ساخته شد. ساختار متخلخل داربست های زیستی و ابعاد حفره های آن بوسیله میکروسکوپ الکترونی روبشی مورد بررسی قرار گرفت. در داربست های بافتی بوسیله تغییر غلظت ژلاتین حفره های با ابعاد متفاوتی بدست آمد. در داربست های با درصدهای وزنی–حجمی ژلاتین 3.75 و 5، حفره هایی به ابعاد 100 تا 450 میکرومتر پدید آمد. متوسط ابعاد حفره ها با افزایش درصد ژلاتین افزایش یافت. همچنین خواص داربست ها در زمینه جذب آب مورد بررسی قرار گرفت. نتایج نشان داد که با افزایش غلظت ژلاتین در محلول از 3.75 به 5 درصد، جذب آب داربست تولید شده به میزان 104 درصد کاهش یافت. افزایش درصد ژلاتین باعث کاهش جذب آب گردید.

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