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Fabrication of Polycaprolactone Scaffold with Gradient Porous Microstructure for Bone Tissue Engineering

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Abstract

Introduction: Selective laser sintering, electrospinning, Layer by Layer Assembly, porogen leaching and additive manufacturing are applied methods in fabrication of gradient scaffolds with limitations such as being expensive or complicated.

Objective: The main purpose of this study was to apply a novel and simple method in fabrication of gradient scaffolds with minimum cost.

Material and Methods: Two types of homogenous and two types of gradient scaffolds were fabricated by combining layer-by-layer assembly and porogen leaching techniques in a new manner. Pore size gradient was created along the radial direction by using paraffin micro particles as porogen and two different size of syringe as mold. The first layer was made in the smaller mold, with a specific size range of porogen and the second layer was fabricated around the inner one using porogens with a different size range from the first layer.

Results: Scanning electron microscope images of scaffolds showed spherical pores and the structure of gradient scaffolds showed the radial gradient with a good adhesion between layers without any detectable interface. The porosity of scaffolds was 77.5 ± 3 % and 61.3 ± 4 % for homogenous and 74 ± 2.8 % and 79.8 ± 2.3 % for gradient scaffolds which are suitable for bone tissue engineering. Mechanical properties of scaffolds were better for lower porosities. The results indicated that gradient porous structure had no considerable effect on mechanical properties. MTT assay and cell morphology tests showed scaffolds biocompatibility.

Conclusion: The applied method is suitable for pore size gradient creation. Gradient scaffolds can be used to investigate the influence of pore size gradient on biologic properties, cells differentiation and cell distribution and bone formation.

Keywords: Polycaprolactone; pore size gradient scaffold; radial gradient; Layer by layer assembly; porogen leaching

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1. Introduction

In recent years, tissue engineering has been applied to restore damaged tissues with the aim of scaffolds, cells and growth factors. The main purpose of scaffold fabrication is providing a desirable environment similar to the extra cellular matrix (ECM) in order to regenerate damaged tissues and organs [1]. Stem cells are able to differentiate into different cell types via some kinds of signals (growth factors), in order to regenerate a specific tissue [2]. Among all materials, polymers can support biochemistry of seeded cells well, so they are widely used to fabricate scaffolds[3]. Polycaprolactone (PCL) is a semi crystalline, hydrophobic and biodegradable polymer with a low degradation rate and melting point of about 60℃. Biocompatibility, processability, non-toxic degradation products and relatively good mechanical properties make this polymer suitable for bone tissue engineering [4].

Structural and zonal differences, mechanical properties and regeneration ability of complicated tissues such as bone and cartilage can be simulated by creation of structural gradient in pore size of scaffolds [5, 6]. In addition, pore size gradient scaffolds can increase cell seeding efficiency, control the zone of seeded cells [7] or be used as a tool of studying the effect of pore size on biological function of scaffolds [8]. Selective laser sintering [9], electrospinning [10], a specific spinning method [11], Layer by Layer Assembly [12], porogen leaching [13], centrifugal method [8] and additive manufacturing [7, 14, 15] are some of applied methods in creation of pore size gradient in a scaffold.

The main purpose of this study was to apply a novel and simple method in fabrication of gradient scaffolds with minimum cost. In this study, PCL pore size gradient scaffolds fabricated with combination of layer-by-layer assembly and porogen leaching techniques. We created pore size gradient along the radial direction by using paraffin micro particles with two different size ranges as porogen and two different size of syringe as mold. The first layer was made in a mold with a smaller diameter, with a specific size range of paraffin particles and the second layer was fabricated around the inner one using paraffin micro particles with a different size range from the first layer. Mechanical performance, morphology, cell attachment and biocompatibility of scaffolds were characterized using compression test, scanning electron microscopy (SEM), MTT (the dye compound of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromidefor) assay (indirect method) and cell morphology test.

2. Materials and Methods

2.1. Scaffolds Fabrication

The material used in fabrication of scaffolds was polycaprolactone (PCL, M_W 80,000; Sigma Aldrich, USA). PCL-acetic acid (Sigma Aldrich, USA) 10 w.% solution was prepared at 40°C. Solid paraffin (Sigma Aldrich, USA) utilized to fabricate porogen particles. In order to fabricate paraffin micro particles, 0.1 w/v.% s aqueous solution of polyvinyl alcohol (PVA, Mw 89,000-98,000; Sigma Aldrich, USA) and 1.5 w/v.% aqueous solution of PVA were prepared. 10 grams of molten paraffin was poured in a beaker containing 12.5 ml of 1.5 w.% aqueous solution of PVA at 60°C. The solution was then stirred for 10 minutes on a heater stirrer. Then was casted into a beaker containing 500 ml of 0.1w/v. % aqueous solution of PVA at 17°C, and stirred at a high speed. By screening these particles through sieves with 30, 40 and 60 mesh sizes, micro-particles were divided in two size ranges of 250 to 420 µm and 420 to 600 µm. Paraffin micro-particles with a specific size range and 10 times of the polymer mass, were poured into a head cut 5 ml syringe. Then polymer solution was casted on paraffin micro particles. Particles were uniformly dispersed in the solution through a mild stirring. After freezing the sample at -20°C for 72 hours, it was freeze-dried (Christ, alpha 1-2 LD) and paraffin particles have been washed by immersing in N-hexane (Sigma Aldrich, USA) for 48 hours while N-hexane was replaced every 8 hours. In order to complete withdrawal of N-hexane, the sample was washed with ethanol 96%.

2.2. Gradient Scaffolds Fabrication

Gradient scaffolds were fabricated in two layers. Two 3 ml and 5 ml syringes were used as molds. The first layer was made in a mold with a smaller diameter, with paraffin micro particles size range of 250-420 µm, with the procedure mentioned above. Then this non-washed layer was placed at the center of the larger mold and fixed with a short needle. In order to fabricate a second layer, paraffin micro particles size range of 420-600 µm were poured around the first layer in the larger mold with 10 times of the polymer mass and were dispersed uniformly after adding the polymer solution. In order to remove the solvent completely, the frozen sample was put in the freeze drier for 72 hours. The scaffold was washed completely with N-hexane and ethanol as mentioned above. The images, type and porogen size range of fabricated homogeneous and gradient Scaffolds are presented in figure 1 and table 1. Two types of homogenous scaffolds and two types of gradient scaffolds named Homog1, Homog2, Grad1 and Grad2 respectively.

In this study, four types of samples fabricated as mentioned in table 1.

2.3. Morphology Studies

After preparation of samples, they were vacuum coated by gold and examined in different magnifications by a scanning electron microscope (AIS2100; Seron Technologies,South Korea).

2.4. Pore Characterization

The pore size of scaffolds was measured by analyzing SEM images using an image analysis program (ImageJ software). In order to measure the porosity of scaffolds (n=3), fluid displacement method was used which is explained in the following [8].

$$VA = V2 - V1 \quad (1)$$

$$VB = V1 - V3 \quad (2)$$

$$\% \text{ porosity} = 100VB/(VA + VB) \quad (3)$$

In this study, N-hexane used as fluid. Scaffold was immersed at a special volume of N-hexane (V_1). Then the volume of N-hexane was measured (V_2). After getting out of scaffold, the final volume of N-hexane (V_3) was measured. V_A and V_B indicate volume of scaffold and volume of N-hexane in pores respectively.

2.5. Mechanical Properties

Mechanical properties of samples (n=3) were studied by compression test. For this aim, cylindrical samples (1 × 1.1 cm) were prepared. Uniaxial compression test with 25 kN load performed using Zwick / Roel Z050, based on ASTM D575 standard. The samples were placed between two jaws of the device and the load was applied at 1mm/min and up to 80% of strain. Elastic modulus and the compressive strength of scaffolds were measured at 80% strain.

Figure 1. a) Inner layer of gradient Scaffold b) gradient Scaffold c) Homogenous scaffolds

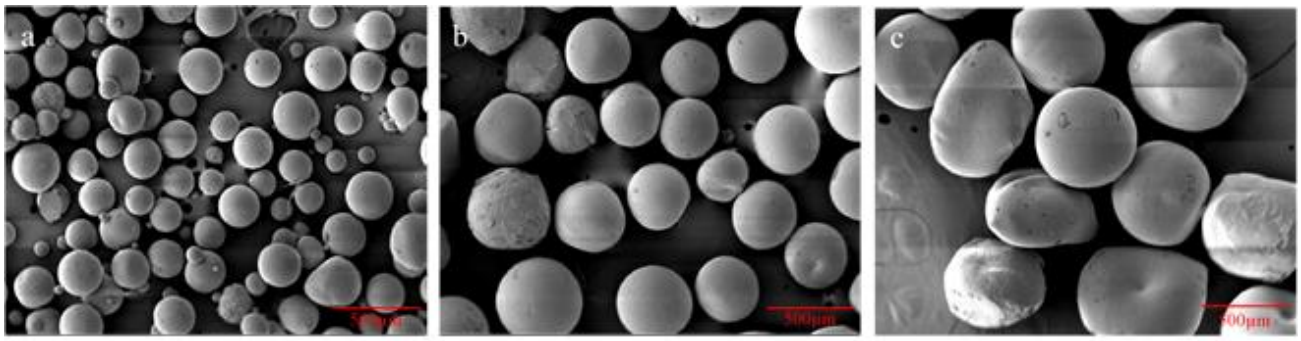

Figure 2. Paraffin micro particles a) 150-250 µm b) 250-420 µm c) 420-600 µm

2.6. In Vitro Cell culture

Before starting in vitro investigation of scaffolds, they were immersed in 1 molar NaOH aqueous solution for 15 minutes to get hydrophilic. Following by sterilization through immersion in ethanol 70% for 2 hours and washing with Phosphate-buffered saline (FBS) and distilled water. In this study, osteosarcoma cells MG-63 (Pasteur Institute, Iran) and cell culture medium with low glucose (Bio Idesabz Co, Iran) were used. The viability of cells was estimated by an indirect MTT assay method. This test was done in an environment similar to the body (at 37 ° C temperature and 7.4 pH). In order to achieve the scaffold extraction, 1ml of medium was added to 5 mg of each sample. The samples were kept in an incubator with 95% humidity and 5% CO_2. Samples were taken out of the cell culture medium after three and seven days. 1×10^4 cells were poured in a 96 wells plate and maintained for 24 hours in a 37°C incubator. The sample extract was added to the 96 wells plate and the cells were exposed to the extract for 24 hours (n=3). After removing the medium, 100 ml of culture medium without color with 10 ml of 12 mM MTT solution (Sigma Aldrich, USA) was poured into the wells. After 4 hours the solution was removed and dimethyl sulfoxide (DMSO, Sigma Aldrich, USA) was added. The concentration of the solution in dimethyl sulfoxide was measured at wavelength of 545 nm using an enzyme-linked immunosorbent assay reader (Stat fax-2100; GMI, Inc., Miami, FL, USA). The viability of cells was obtained as the following:

$$\text{Viability (\%)} = \text{ODS/ODC} \quad (4)$$

Where OD_S is the mean optical density of each sample at the desired time and OD_C is the average optical density of the control group [16].

For cell morphology test, after sterilization of homogenous samples with mentioned procedure, 5×10^4 cells were poured onto the samples in a 24-well plate and the plate was placed at the incubator for 12 hours similar to the MTT test conditions. In order to fix the cells on the scaffold surface, the 4% glutaraldehyde solution was used. After one hour, the glutaraldehyde solution was removed and the sample was rinsed with 40, 50, 60, 70, 80 and 96% ethanol [17]. The surface of the samples was coated by gold and attachment of the cells were analyzed using the scanning electron microscopy.

3. Results

3.1. Paraffin micro particles

Figure 2 images show the morphology of micro particles in three size ranges of 150-250 µm, 250-420 µm and 420-600 µm.

3.2. Scaffold Structure and Morphology

Structure of homogenous and gradient scaffolds are revealed in images of figure 3.

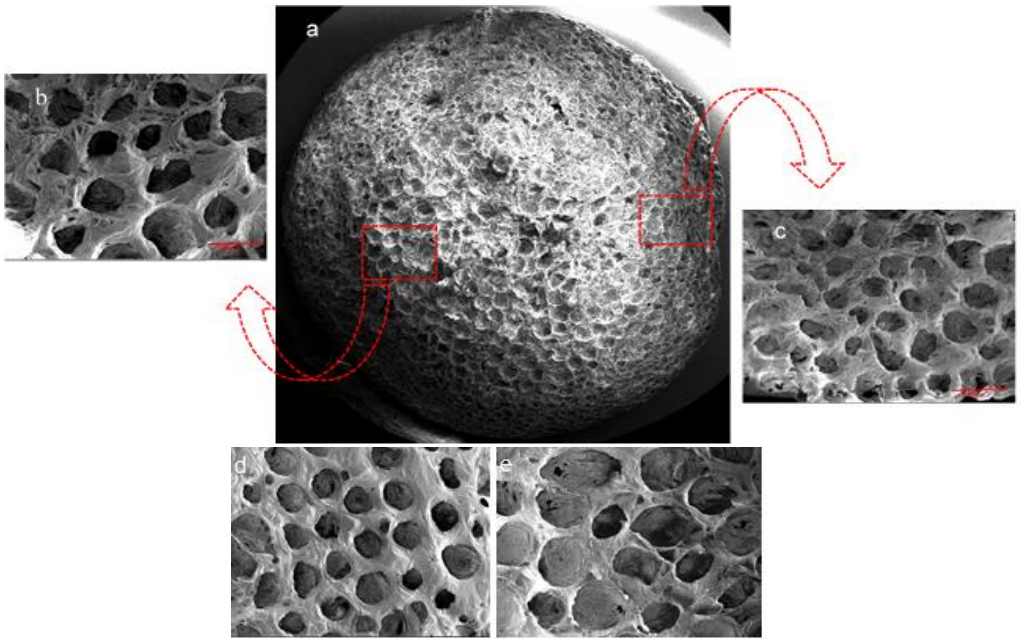

Figure 3. Morphology of a) Grad2 b) inner layer c) outer layer d) Homog 1 and e) Homog 2

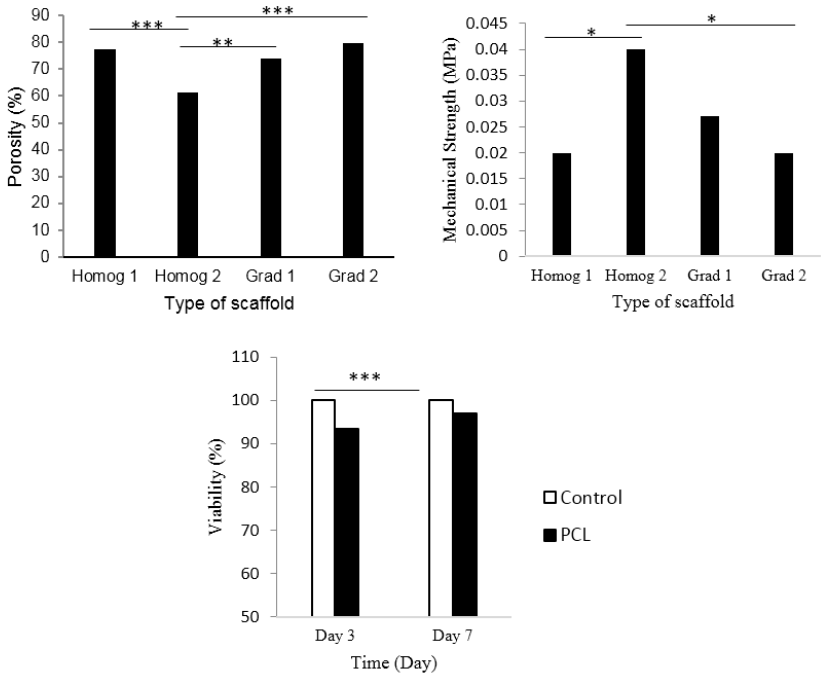

Figure 4 . a) Porosity of scaffolds b) Mechanical strength of scaffolds and c) Viability of cells after 3 and 7 days in contact with scaffold extract

3.3. % Porosity

The porosity of scaffolds measured using fluid displacement method and are shown in figure 4 (a).

3.4. Mechanical Properties

The measured values for compressive strength of samples at 80% strain are given in figure 4 (b).

3.5. In Vitro Studies

Figure 4 (c) shows MTT test results after 3 and 7 days. In addition, SEM images of morphology test are given in figure 5.

4. Discussion and Conclusion

As it is shown in images of figure 2, the micro particles shape changes from spherical to an almost oval form when the particles size increases. Considering the rotational movement (Vortex) and flow of the solution on the heater stirrer, the farther we go from the imaginary axis of the fluid motion, the flow speed decreases and the particle size increases [18]. Therefore, Paraffin particles composed at the lower speeds are larger and non-sphere in comparison to particles made at higher speeds of fluid flow. It is worth mentioning that the shape and size of paraffin particles can be controlled by changing the stirring speed and the concentration of PVA solution [19]. In this study, the composed particles were mainly 150 to 600 µm and the number of particles at size range of 150 to 420 µm were more than the number of particles at the size range of 420 to 600 µm. Microscopic images of the micro particles in figure 2 show microsphere paraffin at three size ranges of 150 to 250 µm, 250 to 420 µm and 420 to 600 µm. In this study, two particle size ranges of 250 to 420 µm and 420 to 600 µm were utilized in fabrication of gradient scaffolds. The average pore size in Homog 1 with paraffin particles size range of 250 to 420 µm was 278.48 ± 11.23 µm and in Homog 2 with paraffin particles size range of 420 to 600 µm was 417.79 ± 14.62. These values are suitable for bone tissue engineering. Considering previous studies, the required minimum pore size for bone growth is 100 to 135 µm and the optimum value is 100 to 400 µm [20, 21]. One of the key parameters in designing of the scaffold is pores wall morphology and their interconnection. These cases are among the important parameters in cell seeding, migration and proliferation, gene expression and the formation of new tissue in three dimensions [22-24]. Scaffold cross section shows pores interconnection and the round and smooth shape of pores indicates the complete elimination of paraffin. As it is obvious in figure 3 few little holes are seen in the scaffold structure, especially in Homog 1; these holes could be created due to incomplete penetration and non-uniform distribution of polymer solution between porogen particles [19].

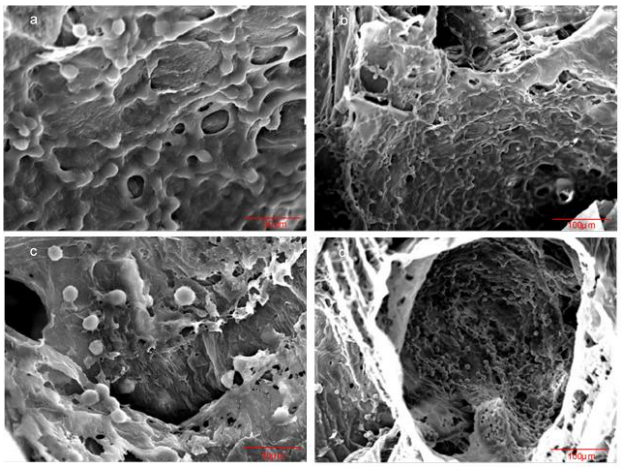

Figure 5 . Cell Morphology on scaffold surface a and b) Homog 1 c and d) Homog 2

Table1 . Scaffold Types and their porogen size range

Scaffold Type	Porogen size range (µm)
Homog 1	250-420
Homog 2	420-600
Grad 1	Inner layer 250-420 Outer layer 420-600
Grad 2	Inner layer 420-600 Outer layer 250-420

The radial gradient is seen in images of figure 3. As mentioned before, the pores in Grad 2 are larger in inner layer than those in outer layer, whereas in Grad 1 the inner layer has smaller pores than the outer layer. The continuity and perfect connection between two layers of gradient scaffolds without detectable interface between layers is clearly seen in figure 3 Therefore, it can be concluded that the utilized material and method is perfectly effective in creation of pore size gradient in scaffolds. Regarding porosity of scaffolds shown in figure 4 (a) the Homog 2 with 61.3 ± 4 % porosity, has the lowest porosity among all four samples. The total porosity of Homog 1 is 77.5 ± 3 %, Grad 1, 74 ± 2.8 % and Grad 2, 79.8 ± 2.3 %. According to previous studies, the required porosity for cell survival and growth is above 70 % (25). So, these values are suitable for bone tissue engineering application.

The porosity differences seen in homogenous scaffolds can be explained as follows. According to a related research, the smaller porogen micro particles can cause non-uniform distribution of polymer solution between them. This may cause hole and bubble creation or can make pore wall thinner and as a result it increases the porosity [19]. According to figure 4 (b), Homog 2 has the Maximum strength and Grad 2 has the lowest strength. Generally, mechanical properties of a scaffold decreases by increasing porosity [19, 26, 27].

As can be observed, Homog 2 with the lowest porosity, has indicated the highest mechanical properties and as expected, Grad 2 with 81% porosity has the lowest mechanical properties.

A figure 4 (b) shows, the difference between mechanical strength of two types of gradient scaffolds is not significant and it can be stated that pore size gradient had no considerable effect on mechanical properties of scaffolds. The little difference between their mechanical strength values can be explained by their different porosities. Moreover, the porosity difference between two homogeneous scaffolds has led to a difference in their mechanical properties. As expected, Homog 2 has better mechanical strength in comparison to Homog 1. As it is seen in figure 4 (c), the viability of cells after 3 and 7 days is over 90% which indicates the complete removal of toxic materials like paraffin and N-hexane. Moreover, the viability of cells after 7 days is higher than 3 days stating viability and proliferation of cells in contact with scaffold extract and biocompatibility of scaffold.

figure 5 shows that cells have penetrated into the pores and have completely covered the inner surface of them. It is also noticed that living cells have started spreading pseudo-podia after 12 hr. These results emphasize scaffold biocompatibility and hydrophilicity of scaffolds after using NaOH.

Pore size gradient, continuity between two layers of gradient scaffolds and lack of detectable interface between them indicated that the method applied in fabrication of gradient scaffolds has been suitable. Fabrication and use of paraffin micro-particles as porogen caused interconnected and spherical pores; however, by using salt or sugar as porogen, pore shape would be limited to crystalline shape of particles and it is not possible to control their interconnection. The results of the pressure test indicate that pore size gradient had no considerable effect on mechanical properties of scaffolds. Due to the low amount of compressive strength of scaffolds, it can be concluded that using PCL as the only material of scaffold is not appropriate. The results of cell toxicity test indicate the biocompatibility of scaffolds for use in bone tissue

engineering and the results of morphology test confirmed the biocompatibility of these scaffolds.

Conflict of Interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest, or non-financial interest in the subject matter or materials discussed in this manuscript.

Ethical Statement

This research involves no human investigations and/or animal studies.

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