Polyurethane (PU) Scaffolds Prepared by Solvent Casting/Particulate Leaching (SCPL) Combined with centrifugation

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Abstract

This article reports an enhanced solvent casting/particulate (salt) leaching (SCPL) method developed for preparing three-dimensional porous polyurethane (PU) scaffolds for cardiac tissue engineering. The solvent for the preparation of the PU scaffolds was a mixture of dimethylformamide (DFM) and tetrahydrofuran (THF). The enhanced method involved the combination of a conventional SCPL method and a step of centrifugation, with the centrifugation being employed to improve the pore uniformity and the pore interconnectivity of scaffolds. Highly porous three-dimensional scaffolds with a well interconnected porous structure could be achieved at the polymer solution concentration of up to 20% by air or vacuum drying to remove the solvent. When the salt particle sizes of 212-295, 295-425, or 425-531 μm and a 15% w/v polymer solution concentration were used, the porosity of the scaffolds was between 83-92% and the compression moduli of the scaffolds were between 13 kPa and 28 kPa. Type I collagen acidic solution was introduced into the pores of a PU scaffold to coat the collagen onto the pore walls throughout the whole PU scaffold. The human aortic endothelial cells (HAECs) cultured in the collagen-coated PU scaffold for 2 weeks were observed by scanning electron microscopy (SEM). It was shown that the enhanced SCPL method and the collagen coating resulted in a spatially uniform distribution of cells throughout the collagen-coated PU scaffold.

Keywords

Polyurethane scaffolds, salt leaching, centrifugation, pore interconnectivity, collagen coating, human aortic endothelial cells

1. Introduction

Heart disease is a major cause of death in the western world. In the past three decades there has been a number of improvements in artificial devices and surgical techniques for cardiovascular diseases. The major drawback of current artificial devices is that they cannot grow and remodel as viable tissues. Tissue engineering offers the possibility of developing a biological substitute material *in vitro* with the inherent mechanical, chemical, biological, and morphological properties required *in vivo* [1].

Scaffolds provide a matrix for guided cell proliferation and are able to control the shape of the regenerated tissue. A high porosity and a high pore interconnectivity of a scaffold are desired to minimize the amount of implanted material and to increase the specific surface area for cell attachment and tissue ingrowth [2-7]. Furthermore, scaffolds for cardiac applications need to be elastomeric and have properties that support cardiac function. In other words, the scaffold must be able to meet the difficult mechanical demands of cardiac tissue without cracking or disintegrating [8]. To achieve the needed elastomeric properties, polyurethanes (PU) as the

typical synthetic polymers are used [3]. PUs have very high flexural endurance compared to most other elastomers, making them prime candidates for cardiac implants [9].

Although several methods have been developed to fabricate macro / micro porous polymer scaffolds, the solvent casting/particulate leaching (SCPL) method is the most commonly used one. This method is characterized by its simple operation and adequate control of the pore size and the porosity by selecting the particle size and the amount of the added salt particles. However, the distribution of the salt particles is often not uniform within the polymer solution. This is because the density of the liquid polymer solution and that of the solid salt are different, and the degree of direct contact between the salt particles is not well controlled [10]. As a result, the interconnectivity of pores in a final scaffold cannot be well controlled. Moreover, the polymer solution and the salt particles are mixed in such a way that the salt particles tend to be wrapped completely by the polymer solution. These wrapped salt particles cannot be easily leached out with water. Thus, most porous materials prepared by the SCPL method are limited to a thickness less than 4 mm [11].

The pore wall surface of a scaffold is also important for tissue engineering because the surface can directly affect cellular response and ultimately the tissue regeneration. Although synthetic materials have been used as tissue engineering scaffolds, they often lack biological recognition. An ideal tissue engineering scaffold should mimic the native extra-cellular matrix (ECM) and positively interact with cells, including enhanced cell adhesion, growth, migration and differentiation [12, 13]. To overcome this drawback of the synthetic polymers, naturally occurring polymers such as collagen have been widely used to modify the synthetic polymers for biological recognition for cardiac tissue engineering [8, 14].

This article reports the preparation of highly interconnected porous PU scaffolds by a simple and effective method, i.e., an enhanced SCPL method using the dimethylformamide (DFM)-tetrahydrofuran (THF) mixed solvent system [15] and involving the use of centrifugation to improve the pore interconnectivity of the scaffolds. The effects of the processing parameters such as the polymer solution concentration, the solvent type, and the solvent evaporation method on the porous structure were examined by scanning electron microscopy (SEM) and the effects of salt particle size on the mechanical properties were investigated. Type I collagen acidic solution was introduced into the pores of the PU scaffolds to coat the collagen onto the pore walls. Finally the HAECs were cultured in the collagen-coated PU scaffolds to study the cellular response of the scaffolds for potential cardiac tissue engineering.

2. Martials and methods

2.1 Preparation of porous PU scaffolds

Porous PU scaffolds were prepared by combining conventional solvent casting/particulate leaching with centrifugation (Fig. 1). The polymer solution was prepared by dissolving polyurethane pellets (Zytar® Z1A1, Biomer Technology Ltd) into dimethylformamide (Sigma) / tetrahydrofuran (Sigma) (volume ratio: 50:50) at room temperature. For comparison, solvent – dioxane was also used. The concentrations of the polymer solutions were 10%, 15% and 20%. The polymer solution was then poured into cylindrical polypropylene containers with desired diameters. Sieved salt particles (212-295 μm, 295-425 μm, and 425-531 μm) from a common table salt (Salpak Pty Ltd, Seven hills, Australia) were added into and mixed with the polymer solution. Subsequently the containers were centrifuged at room temperature at 2500 rpm for 10 min. The optimised (or well-packed) salt amount in a polymer solution was determined by

centrifugation of the salt particle-polymer mixture. Then extra polymer solution was removed with a glass pipette, or more salt particles were added and centrifuged until no pure polymer solution left. The formed salt-polymer mixture then was air-dried, vacuum dried, or freeze dried, followed by salt leaching in distilled water, freezing at -20 ^{o}C and further freeze drying, resulting in a three-dimensional interconnected porous structure. The details of the drying methods were as follows: the centrifuged mixture was air-dried for 7 days or vacuum dried for 3 days. For freeze-drying, the centrifuged mixture was subsequently cooled down (not necessarily frozen) at -80 °C for overnight. After that, the solvent liquid or crystals were removed by freeze drying for a period of 2-3 days in a freeze-dryer (Martin Christ, Freeze Dryer, Alpha 1-2/LD). Collagen-coated PU scaffolds were prepared by dipping the PU scaffolds into a bovine collagen type I acidic solution (type I, pH 3.2, 0.3 tw%, Cellmatrix, Nitta Gelatin, Osaka), and subsequently vacuum-dried. The collagen coating was not cross-linked with glutaraldehyde due to its potential cytotoxicity.

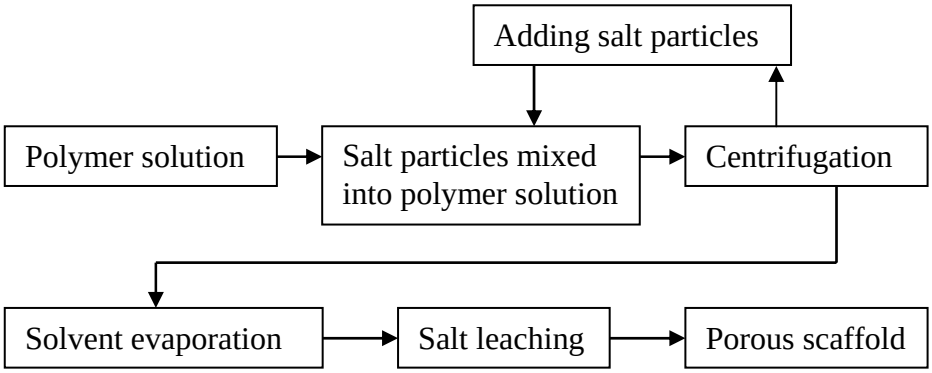

Fig. 1. Steps of the enhanced SCPL method.

2.2 Sample characterization

2.2.1 Porosity of the PU scaffolds

The porosity of a scaffold was determined by measuring the dimensions and the mass of the scaffold and calculated using the following formula:

$$p = \left(1 - \frac{m}{\rho_{polymer} \cdot V}\right) \cdot 100\%$$

where p is the porosity, m is the mass of the scaffold, $\rho_{polymer}$ is the true density of the polymer and V is the volume of the scaffold.

2.2.2 Scanning electron microscopy

The porous structures (e.g. pore size and pore interconnection) of the PU scaffolds and the collagen-coated PU scaffolds were observed by scanning electron microscopy (SEM). For SEM observation, the scaffolds were cut with a razor blade and the scaffold samples were mounted onto aluminium stubs with a carbon tape and coated with a gold film on a sputter coater (BioRad SC500). The porous structures of the scaffolds were then examined using a scanning electron microscope (FEI QUANTA 200) with an acceleration voltage of 15 kV.

2.2.3 Compressive testing of the scaffolds

Compressive tests were performed on cubic shaped specimens of about 4×4×4 mm^3 cut manually. The compressive strain-stress curve of a scaffold was measured at room temperature with a 50N load cell and at a cross-head speed of 0.5 mm/min on a Hounsfield testing machine (Model: H10K/M527). The compressive Young's modulus (kPa) was calculated by taking the

slope at the point of 20% compression (strain = 0.2) from a stress–strain curve. All the given values were means of more than five measurements (± standard deviation).

2.3 In vitro cell culture evaluation

2.3.1 Sample sterilization

The scaffolds with dimensions of 4×4×4 mm^3 were sterilized by soaking them three times in 70% ethanol for 15 min each, then rinsed three times with potassium phosphate buffer solution (PBS) for 15 min before being left to dry overnight in a sterilized laminar hood for cell culture.

2.3.2 Cell seeding and culture

The HAECs (ScienCell Research Laboratories, Carlsbad, CA) at passage 4 were used for this study. The HAECs were first expanded by culturing in an endothelial cell medium (ScienCell Research Laboratories, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (P/S). The medium was replaced after 24 hrs then every 2 days and the cultures were maintained in a humidified incubator at 37 °C and 5% CO_2. After the cells reached 80% confluency, they were harvested using a commonly used procedure. The harvested cells were then re-suspended in the culture medium at a density of 1×10^6 cells/mL. The cell suspension was then dropped into the collagen-coated PU scaffolds (50 µL/scaffold) and cultured in 24-well culture plates. After 1 h of incubation, 2 mL of culture medium was added to each well. Fresh culture medium was added to each well after 12 h to replace the used medium and the cells were cultured under a 5% CO2 atmosphere at 37°C. The cell medium was changed every 2 days during the cell culture up to 2 weeks.

2.3.3 Observation of the cells' penetration and distribution

Collagen-coated PU scaffolds with cultured cells were fixed with a 2.5% glutaraldehyde solution. The scaffolds were then processed by soaking in an osmium tetroxide solution for 1 h, then dehydrated through a series of ethanol solutions with graded concentrations, followed by two changes of 100% amyl acetate for 15 min each. The scaffolds were then dried using a supercritical point dryer (Denton Vacuum critical point dryer) and coated with a gold film before observation under SEM.

3. Results and discussion

3.1 Porous structures of the PU scaffolds made with and without centrifugation

The conventional SCPL method tends to cause closed pores in the porous scaffolds, especially when the amount of salt particles added into the polymer solution is relatively small or when the polymer solution is relatively concentrated or viscous. For a given polymer solution, there should be an optimal salt content that leads to an optimal pore interconnectivity. If a salt content is lower than the optimal content, then the salt particles tend to be wrapped by the polymer solution and isolated pores will be formed. If the salt content is higher than the optimal content, then, there will be no enough polymer solution to fill the gap or space among the packed salt particles, leading to incomplete network of the struts of the porous scaffolds (i.e. some struts will not be formed in the porous scaffolds. The optimal salt amount corresponds to a state of saturation of the polymer solution with the salt particles. For the conventional SCPL method, it is difficult to find out the optimal salt amount. However, centrifugation when combined with the conventional SCPL method can result in the optimal salt amount due to optimal salt particle packing (Fig. 2).

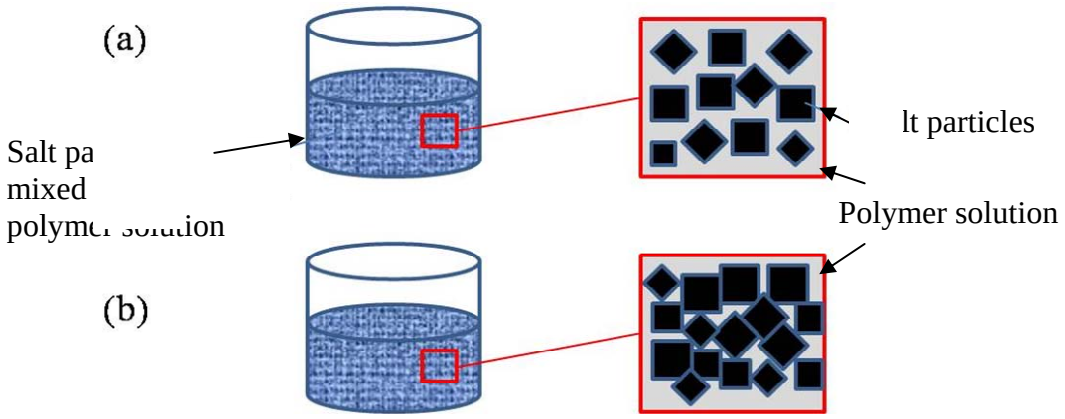

Fig. 2. Geometrical packing of salt particles in a polymer solution for the conventional SCPL (without centrifugation) (a) and the enhanced SCPL (with centrifugation) (b).

In order to achieve the optimal salt content for an optimal pore interconnectivity, a mechanical action of centrifugation was combined with the conventional SCPL method. The centrifugal force enabled the particles to be packed densely or to be in direct contact with each other. After drying in air and salt leaching in water, the desirable porous scaffolds with high pore interconnectivity were obtained, as shown in Fig. 3. It can be seen that the pores of the scaffolds were quite uniform and had a pore size (~ 250 μm) similar to the size of the salt particles. The porosities of the scaffolds were as high as around 92 %; however, with the increase of the polymer concentration, there was a slight decrease of the porosity (about 2 % variation) due to reduced drying shrinkage. Thus, with the use of centrifugation, a high pore interconnectivity, a uniform pore size, and a high porosity even at a high polymer solution concentration could be obtained.

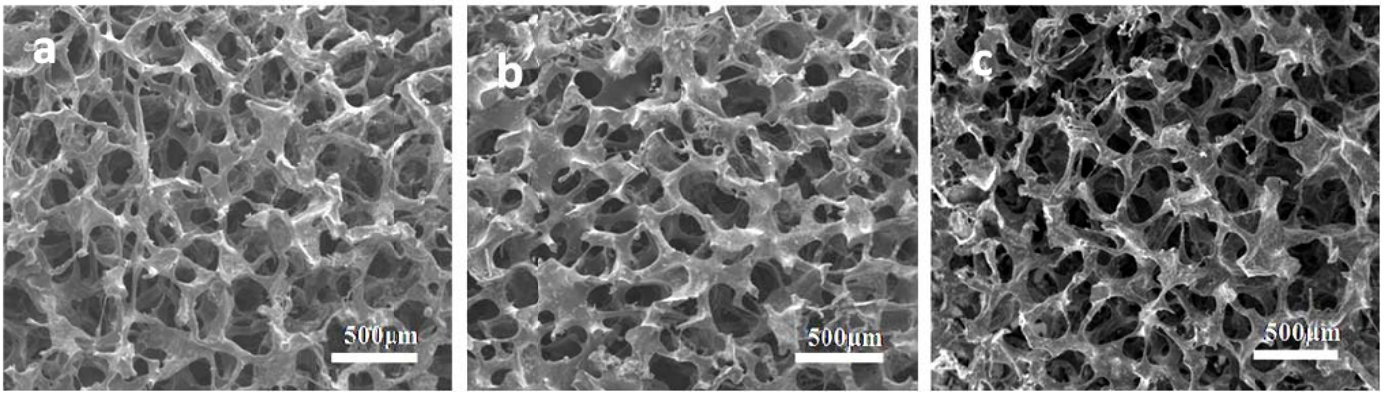

Fig. 3. SEM micrographs of the polyurethane scaffolds prepared by the enhanced SCPL method (i.e. with centrifugation) using different polymer solutions: 10% (w/v) (a), 15 % (w/v) (b), and 20 % (w/v) (c). Other conditions: salt particles of 212-295 μm; DMF-THF mixed solvent; drying in air.

In order to prove the advantage of the effective centrifugation used in the enhanced SCPL method, the disadvantage of the conventional SCPL method should be found out under the condition of the optimal salt content. For this purpose, polymer solutions of 10%, 15%, and 20% (all in w/v) were each mixed with salt particles of a size of 212-295 μm, and the amounts of salt particles added were respectively the same as those as optimised in the enhanced SCPL method. Fig. 4 shows the SEM micrographs of the PU scaffolds prepared by the conventional SCPL method using the optimised salt amounts and using air-drying to remove the DMF-THF solvent mixture. It can be seen that the pore interconnectivity was still high for all the scaffolds even for the conventional SCPL method due to the optimal amounts of salt particles added.

However, the pore sizes were not uniform especially for the highest polymer concentration used. The large pores or voids were thought to be caused by entrapped air bubbles due to higher viscosities of the polymer solutions of higher concentrations.

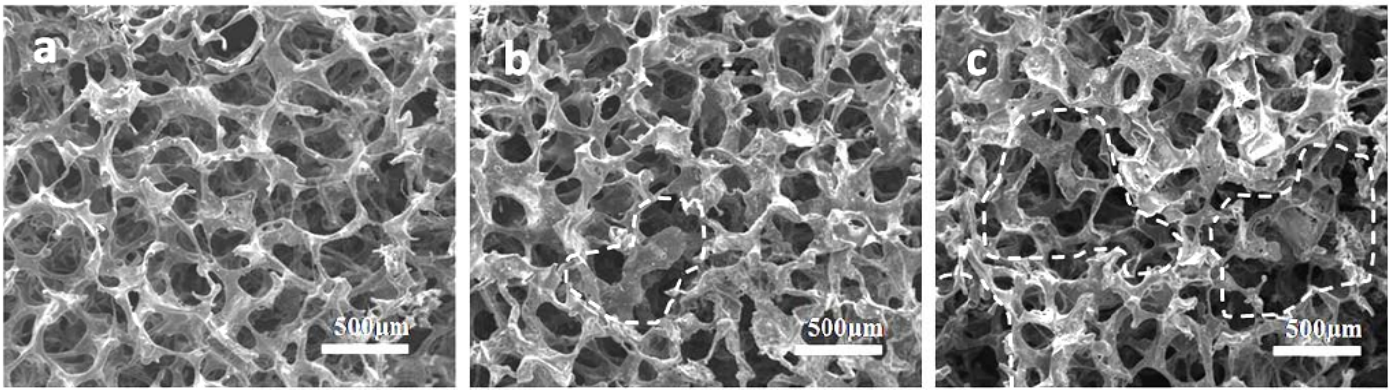

Fig. 4. SEM micrographs of the polyurethane scaffolds obtained by the conventional SCPL method (i.e. without centrifugation) using different polymer solutions: 10% (w/v) (a), 15 % (w/v) (b), and 20 % (w/v) (c). Other conditions: salt particles of 212-295 μm; DMF-THF mixed solvent; drying in air. The dash lines indicate the voids caused by entrapped air bubbles.

Zhang et al. [16] pointed out that if a porogen content in the polymer solution is sufficiently high, the resulting pores must be highly interconnected simply due to close geometric packing. Our study also showed that in order to maximize the number of pores in a scaffold, as many salt particles as possible should be mixed into the polymer solution. However, for the conventional SCPL method, the amount of salt particles added is limited by the viscousity or concentration of the polymer solution [17]; a better pore uniformity might be obtained by decreasing the polymer solution concentration, but this would decrease the compressive modulus of the scaffold. With our enhanced SCPL method, a high and optimal salt amount could be achieved even for a high polymer concentration of 20%. In addition, the thickness of porous polymeric structures prepared by the conventional SCPL method was reported to be < 4 mm [11]. This thickness may limit the wide applications of these scaffolds in engineering complex tissues [18]. However, in our enhanced SCPL method, water for salt leaching could penetrate deeply into the scaffolds because the salt particles were well packed or in close contact, leading to no dense skin layer on the top surface, and thus making a larger scaffold's thickness possible, for example, approximately 8 mm as shown in Fig. 5.

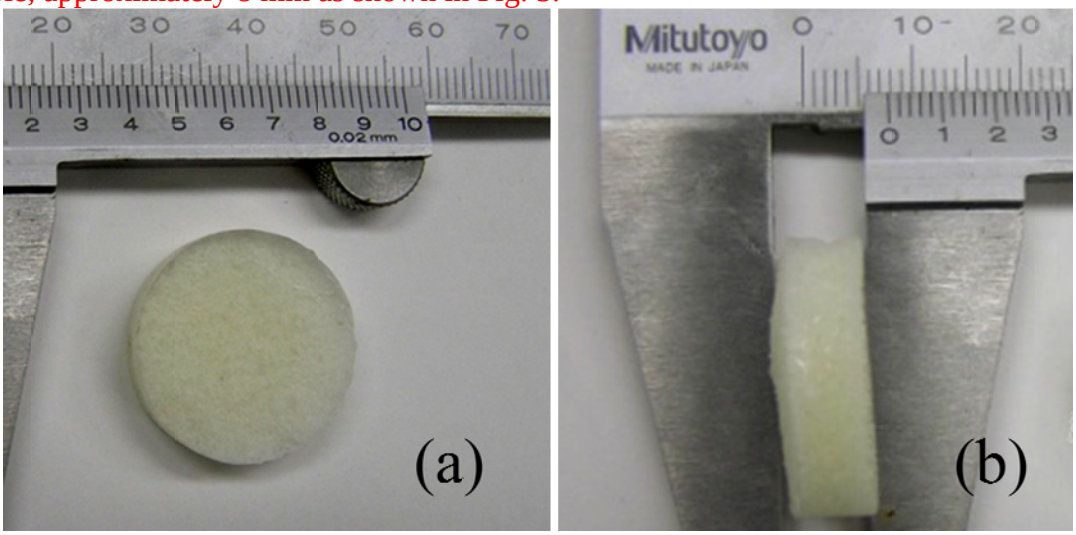

Figure 5. Photographs of a PU porous scaffold prepared by the enhanced SPCL: (a) a top view and (b) a side view. Preparation conditions: 20% PU polymer solution of DMF-THF solvent; salt particles of 212-295 μm; drying in air.

3.2 Effects of solvent evaporation method and solvent type on the pore structures

The pore structures from different solvent evaporation methods were evaluated. The SEM pictures in Fig. 6 show the influence of the solvent evaporation method on the pore structure including the pore interconnectivity. More interconnected pores were found in the scaffolds prepared by air-drying and vacuum drying than those by freeze drying. In the case of freeze drying, the interconnectivity of the large pores was limited due to the small pore windows on the pore walls of the large pores. This may be because the major interconnected pores in the scaffold after salt leaching were solely due to the removal of the adjacent salt particles, which had limited contact areas due to the angular salt shapes; the fast removal of the solvent at a low temperature meant less chance of self-organisation of the struts to change the pore structure. However, for the cases of air-drying and vacuum drying, the polymer solution with salt particles was more thermodynamically active due to drying shrinkage stress and surface tension of the polymer solution, resulting in additional self-organisation or evolution of the pore structure. Recently fused salt or sugar particles were used to improve the pore interconnectivity of polymer scaffolds, but the concentration of the polymer solution studied was limited to 15% due to the higher viscosity of the polymer solution with higher concentrations [4, 10]. The fused salt or sugar was to increase the contact areas. Compared to the method of using the fused salt particles, the present enhanced SCPL method using the DMF-THF mixed solvent could lead to similar interconnected pore structure when using air or vacuum drying.

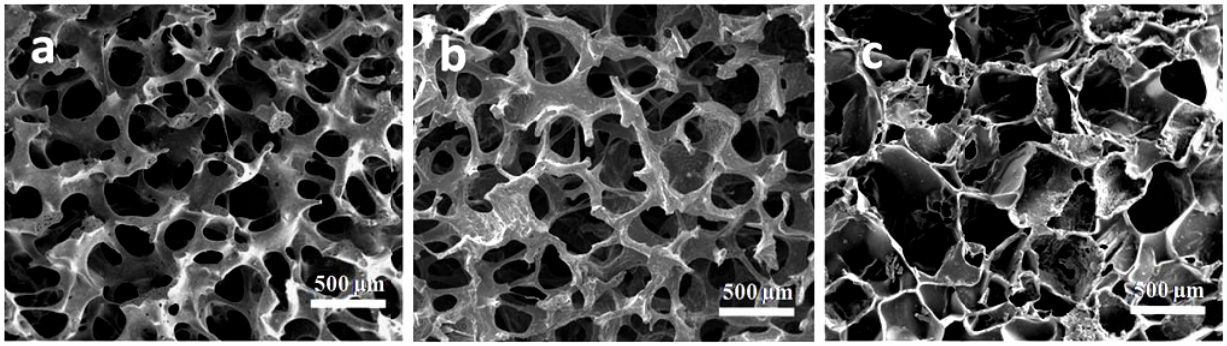

Figure 6. Morphologies of porous polymer scaffolds produced by the enhanced SCPL method with different solvent evaporation methods: (a) air drying, (b) vacuum drying, and (c) freeze-drying. Other conditions: salt particles of 295– 425 µm; and a 15% (w/v) polymer solution of the DMF-THF solvent.

Fig. 7 shows the SEM images of the air-dried pores and the freeze-dried pores from two solvent systems. The scaffolds prepared by dioxane under freeze drying (Fig. 7 (d)) showed micropores on the pore walls of the macropores, which were casued by phase separation and by the sublimation of the solvent crystals (dioxane) during the freez drying, which was the case as dioxane has a freezing point of 11.8 ^{o}C. The micropores in the pore walls for the case of using dioxane and freeze drying were also observed by Heijkants et al. [17]. Freeze drying did not result in micropores in the pore walls of the macropores for the scaffolds using the DMF/THF solvent (Fig. 7 (b)). This was possibly bacause phase separation or solid solvent crystals did not occur, which was due to the low freezing points of DMF (-60.4 ^{o}C) and THF (-108.5 ^{o}C) against the actual temperature in the freeze dryier (> - 60 ^{o}C). Gorna et al. [15] used binary solvent systems for the preparation of scaffolds from polyurethane polymer; the type of a binary solvent system to form the polymer solution strongly affected the scaffolds' pore structure. Gorna et al. showed that the most reproducible porous scaffolds with interconnected pores were from the polymer solution of DMF-THF mixture. However, the authors did not study the effect of the different polymer solution concentrations on the scaffolds' pore structures.

Fig. 7. SEM micrographs showing the morphologies of the PU scaffolds prepared by the enhanced SCPL method using different solvent evaporation methods and different solvents at a salt particle size of 212-295 μm: (a) DMF/THF (50:50) – air drying, (b) DMF/THF (50:50) – freeze drying, (c) dioxane –air drying, (d) dioxane – freeze drying.

3.3 Compressive modulus and porosity of the PU scaffolds

Compressive tests were performed to evaluate the effect of the progen size on the compressive modulus of the PU scaffolds produced by the enhanced SCPL method with a 15% (w/v) polymer solution. Fig. 8 shows the compressive strain-stress curves for different particle sizes. The initial elastic deformation of the struts was followed by subsequent packing and folding of the scaffolds due to the buckling of the struts, typical of porous elastic scaffolds. The curves like those shown in Fig. 8 were used to determine the compressive modulus by taking the slopes. Fig. 9 (a) presents the increase of compressive modulus with the increase of salt particle size. The range of the compression moduli obtained with different salt particle sizes were between about 12 kPa for 212-295 μm and about 27 kPa for 425-531 μm. There was a relationship between the salt size and the porosity; an increase in salt particle size led to decrease in porosity (Fig. 8 (b)). This relationship may be related to the volume of pore space among the packed particles, which had the angular (non-spherical) shapes and a particle size range. A high porosity (>91 vol %) was obtained for the 15% polymer solution concentration and with the 212-295 μm salt size. Thus, an increase in the salt particle size led to a decrease of the porosity, and thus an increase in the compression modulus. It is known that the stiffness of the left ventricle of the heart is around 31 kPa [17], which is similar to the values found for our porous polymer scaffolds, suggesting that these scaffolds could be suitable for growing an endothlial cell layer for cardiac tissue engineering.

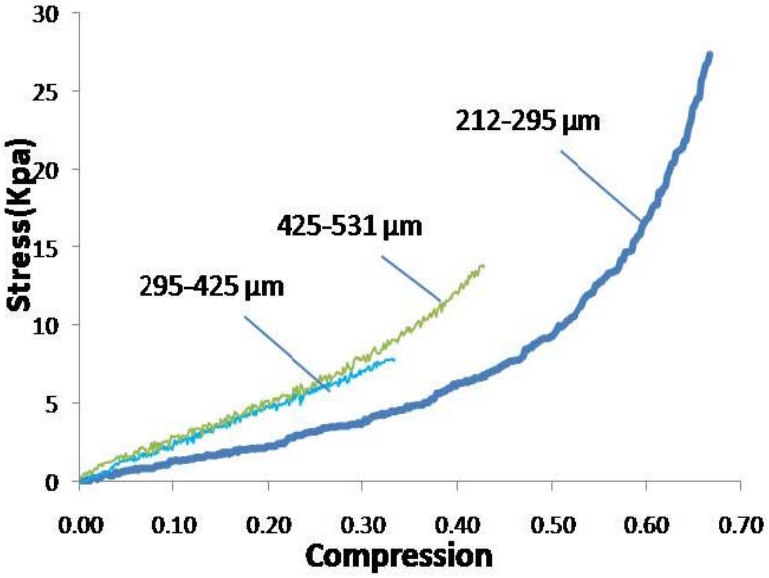

Fig. 8. Influence of the salt size on the compressive strain-stress curve of the PU scaffolds produced by the enhanced SCPL method with a 15% (w/v) polymer solution and drying in air.

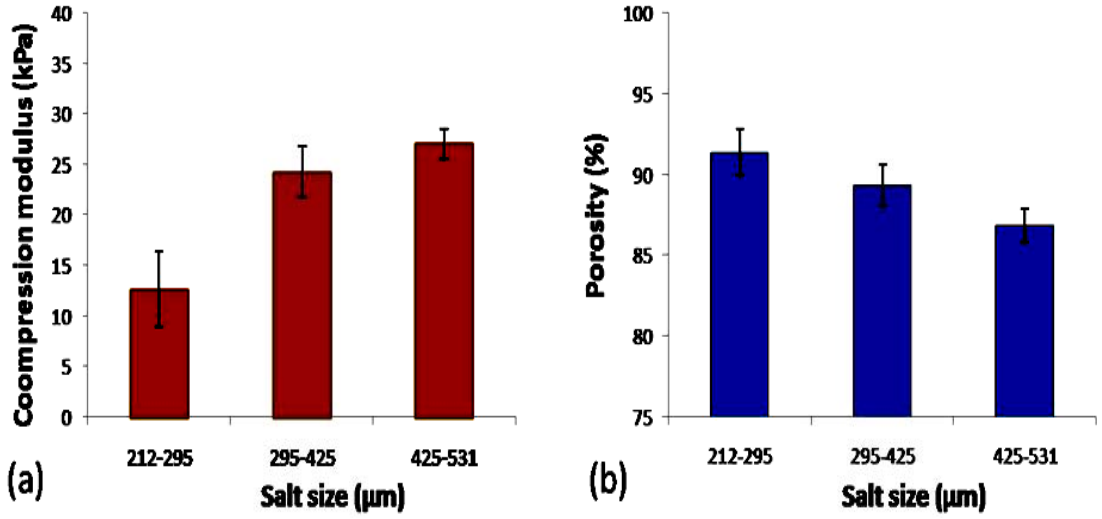

Fig. 9. Influence of the salt size on the compression modulus (a) and the porosity (b) of the PU scaffolds produced by the enhanced SCPL method with a 15% (w/v) polymer solution and drying in air.

3.4 Collagen coating on the PU scaffolds

Much attention has been focused on the synthesis of hybrids of natural and synthetic polymers to combine the advantageous properties of both constituents. Synthetic polymer-derived scaffolds lack cell recognition signals and the surfaces of scaffolds are hydrophobic, which affect the seeding of cells. However naturally derived polymers such as collagen have the advantage of good cell interaction and hydrophilicity, but generally lack adequate mechanical properties and batch-to-batch consistency [19]. To overcome the drawback of the synthetic materials, naturally occurring polymers have been widely used to modify the synthetic materials

[8, 14]. Type I collagen acidic solution was introduced into the pores of the PU scaffolds to coat collagen onto the pore walls throughout the whole polymer scaffolds. Collagen was observed clearly on the surface of the PU scaffold with the collagen coating (Fig. 10), although the porosity was decreased about 5% after the introduction of the collagen coating [20].

Fig. 10. SEM micrographs of a cross-section of the collagen-coated PU scaffold prepared with the enhanced SCPL method and with drying in air (salt particle size: 295-425 μm; using 15% w/v polymer solution of DMF-THF mixed solvent): (a) a general view; (b) a magnified view; (c) and (d) magnified views of the collagen coating on the PU scaffold.

Recently, a new method was developed to nest a collagen micro-sponge into the pores of a polymer scaffold and the collagen micro-sponges were crosslinked [20]. The collagen micro-sponges increased the wettability of the polymer surface, and facilitated cell attachment in the sponges. Although the collagen micro-sponges in the scaffold pores could increase the apparent surface area/volume ratio, the pore size, the porosity and the pore interconnectivity of the scaffolds would be decreased, making it more difficult for the cells to penetrate deeply into the scaffolds. Similarly, diffusion of nutrients and waste products into and out of the scaffold would also be limited [19]. Thus, our collagen coating may be better than collage sponges in the PU scaffolds.

3.5 In vitro cell culture studies

The HAECs were seeded into the collagen-coated scaffolds by adding drops of the cell suspension. The cells cultured for 2 weeks were observed by SEM. The penetration of the cells

into the scaffolds was evaluated using the cross-sections of the scaffolds. The uniform cell distribution and penetration throughout the scaffold was evident (Fig. 11). The HAECs adhered to the collagen-coated PU pore surface. The introduction of the collagen into the pores of the PU scaffold allowed the seeding of cells to interact with the collagen surfaces while avoiding direct interaction with the PU surfaces. The hydrophilicity, as well as the interconnected pore structure, resulted in a spatially uniform cell distribution throughout the collagen-coated polymer scaffold. These results suggest good cell interaction with the collagen-coated PU scaffold.

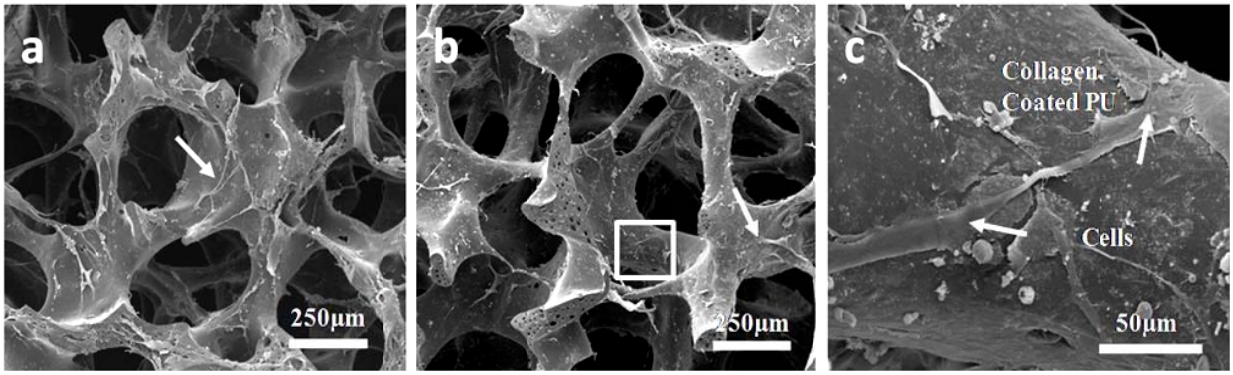

Fig. 11. SEM images of the HAECs on the struts of the collagen-coated PU scaffold prepared with the enhanced SCPL method: (a) top surface; (b) cross-section; (c) enlarged area of (b). Scaffold preparation conditions: salt particles of 295-425 mm; 15% (w/v) polymer solution of the DMF-THF solvent; drying in air.

4. Conclusions

A novel process for preparing three-dimensional polyurethane scaffolds was developed with the aim to enhance the pore uniformity and the pore interconnectivity. The novel process involved the combination of solvent casting/ salt leaching with centrifugation. The effects of different process parameters on the pore structures were studied in order to produce optimal polyurethane scaffolds for cardiovascular engineering. It was shown that more interconnected pores were found in the scaffolds subjected to air-drying and vacuum drying, compared with freeze drying. A decrease in salt particle size led to an increase in porosity. A high porosity (>91 vol %) was obtained using the 15% polymer concentration and a salt particle size of 212-295 μm. The compression moduli obtained with different salt particle sizes ranged from about 12 kPa for 212-295 μm to about 27 kPa for 425-531 μm. The collagen coating on the PU scaffolds resulted in a spatially uniform distribution of cells throughout the collagen-coated PU scaffolds.

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