ABSTRACT

A facile procedure for fabrication of a poly(urethane urea)-based porous scaffold with proper porosity, pore size and mechanical strength for bone tissue engineering is reported in this work. For this purpose, sodium chloride (inorganic porogen) and polyethylene glycol (polymeric porogen) in particulate form with pre-defined mesh sizes were mixed with the polymer and subjected to compression moulding process under optimum condition. Leaching out the impregnated porogen particles by soaking in water (as a safe solvent) led to the final scaffold with desired morphology. The porogen ratios and contents were verified in relation to pore morphology and mechanical properties of the scaffolds. Porosity and pore size of the scaffolds were independently controlled by the ratio and the particle size of the added porogen. An increase in pore interconnectivity was observed as the sodium chloride/polyethylene glycol ratio was increased. Scaffolds with a total porogen content of 80-85 (wt%) displayed acceptable mechanical properties for bone tissue engineering applications. Our results revealed that a highly porous three-dimensional scaffold (>85 (v%)) with a well interconnected porous structure could be achieved by this combinatory process. The scaffold with a NaCl/polyethylene glycol ratio of 60/25 exhibited a suitable morphology for osteoblast cells attachment and growth.

INTRODUCTION

Scaffold is a key component in tissue engineering for bone regeneration. It is a porous structure that plays the role of a synthetic extracellular matrix, and permits adhesion, proliferation and differentiation of the cells. Due to its direct effects on cell adhesion and growth, its porosity and pore size are the determining factors for any 3D scaffold used in bone tissue engineering. In addition, scaffolds for bone regeneration should meet some other criteria to serve this function, including mechanical properties similar to those of the bone repairing site, and biodegradability at a rate adequate to restore new tissue remodelling. Therefore, it can be concluded that to take advantage of a successful tissue engineering approach for bone healing, fabrication of a scaffold with a desired morphology is just as important as a proper material to be chosen for its fabrication.
In recent years, several methods have been developed for the preparation of porous polymeric scaffolds including: electrospinning [1-3], freeze drying [4-7], liquid-liquid [8-11] and liquid-solid phase inversion [12-14], thermally induced phase separation [15,16], gas foaming [17,18], solvent casting/particulate leaching [19-22], and a combination of these methods [23-26]. More recently, rapid prototyping techniques involving the processing of polymer melts and powders, such as precise extrusion [27], three-dimensional printing techniques [28-31] and fused deposition modeling [32-34] have also received considerable attention.

Each of these methods has its own advantages and drawbacks. For example, despite enhanced mechanical properties of the scaffolds produced by electrospinning, formation of a scaffold having appropriate pore size for bone tissue engineering applications is very difficult to achieve by this method [1]. Freezing at a relatively high temperature induces a low nucleation rate and thus a low crystal growth rate which leads to a small number of large sized solvent crystals. As a result, the average pore size obtained by freeze-drying increases with increasing freezing temperature, although it may be complicated to obtain scaffolds with proper pore size by this method.

In methods based on phase separation, the reproducibility of the structure is an issue due to strict requirements in controlling the critical parameters such as polymer concentration, quenching temperature and solvents [4]. Complex free-form features can be readily produced by computer-aided design (CAD) models. However, these techniques are time consuming and require sophisticated equipments. The maximum porosities obtained are limited to 80%.

In methods based on particulate leaching, porosity and pore size can be independently adjusted by simply controlling the former by the amount of leachable particles and the latter by the size of them. However, the resulting scaffold shows limited interconnectivity which would leave an adverse impression on cell seeding and ingrowth [15]. To improve the pore morphology, this method has been combined with a number of different methods including freeze drying [6], gas foaming [17], solvent casting [21], coagulation and compression moulding [24] and polymer blending [35-37]. However, from a manufacturing viewpoint, consumption of a large amount of organic solvents (which are typically hazardous to environment and living species), sophisticated facilities, as well as a rather limited control over the shape and the geometry of the fabricated scaffolds are main challenges confronted by material scientists engaged in tissue regeneration.

Therefore, preparation of porous structures from polymer melt as a convenient route attracts much attention, as it allows rapid, reproducible and economically feasible fabrication of scaffolds with complicated shapes and geometries [37]. Inspired by the common method of porous structure fabrication from bioceramics [38] sintering of compacted particles was developed to address the manufacturing issues with biodegradable polymers [24]. As a general methodology, a mixture (as solids) of a biodegradable polymer and a water-soluble inorganic salt (usually sodium chloride) both meshed to the desired sizes are compacted at temperatures ranging between the \( T_g \) and \( T_m \) of the polymer [39].

Very recently, we have developed a new simple and versatile method for preparation of biodegradable poly(urethane urea)s (PUUs) with tailor-made physical and chemical properties as well as mechanical properties [40]. In addition, preliminary study of fibroblast cell interaction with these polymers showed a good level of biocompatibility.

In this work, we have scheduled on a technique for the preparation of porous 3D scaffolds of highly homogeneous and porous structure with well-interconnected pores network from these newly developed PUUs. To the best of our knowledge no work has been reported in the literature using a combination of inorganic and organic porogens in compression moulding and particulate leaching method. Just a similar technique was already reported for porous structure fabrication from PCL [41], PDLLA and a poly(ether ester) block copolymer [24] where the only porogen employed was NaCl.

Aiming to improve the pore morphology of the final scaffolds, a leachable polymer (a second porogen) was also employed in our study. Here, we show how this second. Here we show how this second porogen can produce scaffolds with more homogeneous morphology in comparison with the structures produced using NaCl particles as the sole porogen.
porogen. This study has been accomplished by examining the scaffolds ability to support osteoblast cells attachment and their ingrowth.

EXPERIMENTAL

Materials and Methods
Polycaprolactone diol (PCL) with $M_n = 4155$ Da from Introx Chemicals (UK) was dried at 80°C under vacuum for 24 h. Polyethylene glycol (PEG) with $M_n = 3937$ Da from Aldrich (USA) was partially dried under vacuum at 80°C for 24 h and was subjected to azeotropic distillation with toluene prior to use. Hexamethylene diisocyanate (HDI) from Merck (Germany) was purified via vacuum distillation. Benzoic acid from Merck (Germany) was freed from any absorbed moisture by keeping in a vacuum oven at 60°C for 5 h. Dimethyl sulphoxide (DMSO) from Merck (Germany) was distilled over CaH₂ under reduced pressure and stored on a 0.4-nm molecular sieve. PEG with $M_n = 35000$ Da and NaCl as porogens were purchased from Aldrich (USA) and Bahman Co. (Iran), respectively and used as received. Glutaraldehyde was obtained from Sigma-Aldrich (USA) and used as received.

Poly(urethane urea) Synthesis
PUU was synthesized using the method reported elsewhere [40]. Briefly, a mixture of PCL and PEG, with a molar ratio of 4/1 was used as the polyols for the preparation of PUU. The diols were reacted with HDI in a reactor at 85°C till the NCO content reached the theoretical value as determined by titration against dibutyl amine. For the preparation of a prepolymer (ITPP), the overall ratio of NCO/OH was kept at 2/1. Dimethyl sulphoxide (DMSO) was added to the reaction vessel to dissolve the prepared ITPP. Addition of an equimolar quantity of benzoic acid with respect to ITPP, dissolved in excess amount of DMSO, led in total transformation of ITPP intermediate to PUU.

Scaffolds Preparation
Freeze Drying
Samples of poly(urethane urea) (PUU) were dissolved in DMSO to obtain solutions of 4% and 8% (w/v) concentrations. For scaffold preparation, a beaker (9 cm in diameter) was filled to 1 cm height with the polymer solution. The beaker was placed into the freeze dryer and cooled down to -10°C in 10 h. Then, the solvent was removed under vacuum at 10°C for 3 days (solution melting point of about 20°C). The porous cylinder was easily removed from the container.

Phase Inversion
Dimethyl sulphoxide (DMSO) and water were used as solvent and non-solvent, respectively [42]. The concentration of the polymer solution was 8% (w/v). The polymer was dissolved in DMSO at 100°C upon stirring. Then, non-solvent was added dropwise to the polymer solution upon vigorous stirring until a cloud point was reached. At this time, the solution was poured into a crystallizer (70 mm in diameter and 40 mm height) and dried to solidify in an air circulated oven for 7 days.

Modified Combinatory Particulate Leaching
A combination of compression moulding, heating, polymer etching and particle leaching was used in this method, with water being the only solvent used. Polyethylene glycol (PEG) with $M_n = 35000$ Da with the density of 1.127 g/cm³ and NaCl with the density of 2.165 g/cm³ were used as the leachable particles in this double porogens particulate leaching study. PEG was used as the porogen of choice in the present study on the basis of its solubility in water and outstanding biocompatibility [43]. The procedure was repeated to produce discs containing salt and PEG at different ratios. Poly(urethane urea) (density 1.19 g/cm³) and PEG pellets were separately milled using a Fritsch GmbH laboratory mortar grinder (Pulverisette2, Germany) under liquid nitrogen atmosphere. Milled polymer particles (both PUU and PEG) and NaCl crystals were sieved to yield particles of <250μm size.

At first, mixtures of the salt, PEG and PUU particles at different ratios were prepared by mechanical mixing. The mixtures were compression moulded in a stainless steel mould (at 1500 psi) to obtain disc-shaped composites of 10 mm diameter × 3 mm height. The compression moulded composites were then heated at 80°C for 15 min and finally the salt and
PEG particles were leached out by water. The solvent was replaced every 5 h for 3 days until the removal of NaCl and PEG was completed.

**Polymer Characterization**

For determination of molecular weight an Agilent 1100 modular gel permeation chromatography system (US) with a RI (refractometer index) detector (US) was used. Density of the synthesized polymer was measured with a six column density measuring apparatus (Davenport, UK) based on gradient density.

Differential scanning calorimetry was performed on a Netzsch DSC 200 F3 (Germany) to determine the melting point of the products. The runs was performed with a scanning rate of 10°C/min over a temperature range of -100°C to 130°C under nitrogen atmosphere.

**Scaffolds’ Morphological Studies**

The porous structure of the scaffolds was studied by scanning electron microscopy technique (SEM). The scaffolds were fractured in liquid nitrogen and after coating with gold (10 mA, 30 s) they were examined by a Vega Tscan scanning electron microscope (Czech Republic) operating at an accelerating voltage of 10 KV. Complete removal of NaCl particles was confirmed by SEM/energy dispersive X-ray analysis (EDXA) (Czech Republic) at the scaffolds' fractured surfaces.

**Mechanical Properties of Scaffolds**

The mechanical properties of the scaffolds were measured by a MTS testing machine (5500R, US) armed with a 200 N load cell in compression mode. The tests were performed on the disk-shaped samples (10 mm diameter × 5 mm thick) applying a crosshead speed of 0.5 mm/min.

**Porosity Measurements**

Two different methods were followed for evaluation of scaffolds porosity. In the gravimetric method the
following equation was applied:

\[
\text{Porosity} = (1 - \nu_s) \times 100 \quad (1)
\]

\[
\nu_s = \frac{\rho_{app}}{\rho_{bulk}} \quad (2)
\]

In this equation, \( \nu_s \) is the solid volume fraction and \( \rho_{app} \) and \( \rho_{bulk} \) are densities of polymer in porous and non-porous forms, respectively. The volume of each disc-shaped scaffold sample was calculated and then the ratio of weight to volume was considered as its apparent density. Moreover, a Thermo Finnigan Pascal porosimeter (Italy) was used to determine porosity of the scaffold samples.

**Osteoblasts Harvesting and Culture on PUU Scaffolds**

The human osteogenic sarcoma cell line G-292 clone A141B1 (Pasteur Institute of Iran) was used to investigate the cell seeding on PUU scaffold samples. The cells were maintained in Dulbecco's Modified Eagles Medium (DMEM; GIBCO, Scotland) supplemented with 100 iu/mL penicillin, 100 \( \mu \)g/mL streptomycin and 10% fetal bovine serum (FBS). Then, these cells were cultured at 37°C, humidity 85% and atmosphere of 5% CO\(_2\) within an incubator. After 3 to 4 days of culturing (confluent monolayer formation), the cells were harvested by trypsinization with 0.25% trypsin.

Cell suspension with a concentration of \( 2 \times 10^4 \) cells/mL was prepared for the cell seeding assessment. Scaffold samples were sterilized using UV (20 min of UV sterilization was applied on each side) and then they were placed in a 12-well culture plate where one well was left with no sample as control under the completely sterilized conditions. After placing the samples, exactly 2 mL of the cell suspension was applied on each sample as well as the control well in the culture plate. Plates were then incubated for 72 h.

After the incubation period, seeded cells on the scaffold were assessed under a Nikon TE-100 inverted microscope (Nikon Instruments Europe, UK) for cellular attachment and morphological determinations. After completion of the incubation, the scaffolds were washed twice with PBS and fixed with 2.5% glutaraldehyde in PBS buffer. Cells in fixative solution was kept at 4°C for 24 h. Scaffold samples were dehydrated in an descending series of different concentrations of ethanol starting from 96, 90, 80, 70 and 50% followed by drying in the critical point dryer machine (CPD). The samples were coated with gold for conducting SEM observations.

**RESULTS AND DISCUSSION**

**Poly(urethane urea) Synthesis**

The segmented PUU was prepared through the polycondensation reaction of an in situ generated AB-type macromonomer consisting of terminal isocyanate and amine groups [40]. In order to render the prepared PUU to be biodegradable under biological condition of human body with appropriate rate of biodegradation suitable for bone tissue engineering, a mixture of PCL and PEG polyols was chosen as soft segment of the polymer.

The synthetic route for the preparation of PUU is outlined in Scheme II. The reaction of PCL/PEG polyols with excess amount of HDI led to ITPP. The reaction duration was controlled by determination of NCO content of prepolymer and comparing experimental data with ultimate theoretical value that could be reached at 1/2 molar ratio of (PCL+PEG)/HDI. Then, chain extension reaction and formation of PUU was performed by adding benzoic acid and excess amount of DMSO to the same reaction pot. The reaction proceeds efficiently with evaluation of carbon dioxide and subsequently a viscous solution obtain. Molecular weight, density and the melting point of the polymer were 40000 Da, 1.153 g/cm\(^3\) and 65°C, respectively.

**Scaffolds Preparation and Optimization**

Two different scaffolds were obtained by adjusting the PUU concentration before freeze-drying. The morphology and porous structure of the scaffolds were studied with scanning electron microscopy and related micrographs are shown in Figures 1a and 1b. The measured quantities of the pore size and the porosity of these scaffolds are collected in Table 1.

Figure 1a shows SEM micrograph of the freeze-dried scaffold from an 8% PUU solution
The pore structure appears discontinuous in some areas as is displayed in Figure 1a, which is in close agreement with the observations by other researchers [5]. A skin layer has been formed at the surface of the scaffold and a compression modulus of 2.4 kPa was recorded for this sample. The average pore size of this sample was about 28±10 μm. The diameter of the cells dictates the minimum pore size which varies from one type of cell to another. In this case, an optimum range of pore size of 40-100 μm for osteoid ingrowth and 100-350 μm for regeneration of bones is emphasized [43]. However, the pore size of our freeze-dried scaffold sample was smaller than what is adequate for successful osteoblast cells in-growth support.

The pore size increased to some extent and reached about 45±25 μm when the polymer concentration was decreased to 5% which is still 0.2% above the critical concentration of the polymeric solution. An obvious reduction in the ratio of closed/open cells was obtained as the polymer concentration was decreased in the freeze-dried solution (Figures 1a and 1b). Again, similar to the scaffold sample produced from 8% polymer solution, a skin layer was formed on the scaffold surface. Although a slight improvement was observed in the pore size of this scaffold sample prepared from polymer solution concentration of higher than 8%, the mechanical properties decreased considerably and the compression modulus dropped to 170 Pa which is far below the expected threshold for bone tissue applications. Similar changes were observed in the mechanical properties in relation to variable concentrations as reported by other research works [44].

Our efforts were not successful for enlarging the pore size to about 100 μm by reducing the concentration of the polymer solution in drying method. Meanwhile, as indicated in Table 1, this method led to fabrication of scaffold samples with unsatisfactory mechanical properties for application in bone tissue replacement. Although preparation of scaffolds having porosity up to 98% by freeze-drying has been reported for some polymeric systems [4] our results in this study showed that this technique is not applicable to PUU material.

We then switched to phase inversion technique to prepare 3-D porous poly(urethane urea)-based scaffold. In this method, structures with almost uniform cross-sections (Figure 1c) and cellular pores were obtained. The compression modulus of this scaffold sample was registered at about 1.8 MPa (Table 2) which stood remarkably higher than those reported for polyurethanes by Goglovsky et al. [43]. The remarkable increase in mechanical properties, although still well below the acceptable limits for most of the bone regeneration applications, was...
predictable as a direct result of urea linkages present in PUU structure.

It is possible to enhance the porosity of scaffolds prepared by this method through various amounts of a porogen with adequate particle size. However, this strategy may lead to the scaffolds of more feeble mechanical properties. The difficulty in complete removal of a large quantity of DMSO as solvent from Table 1. Porosity, pore size and mechanical strength of the scaffold samples prepared by freeze-drying and phase inversion methods.

<table>
<thead>
<tr>
<th>Scaffold samples</th>
<th>Preparation method</th>
<th>Polymer concentration (w/v%)</th>
<th>Porosity* (%)</th>
<th>Average pore diameter (µm)</th>
<th>Compression modulus (Pa)</th>
<th>Compression strength (Pa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>Freeze drying</td>
<td>8</td>
<td>84.7</td>
<td>28±10</td>
<td>2400±50</td>
<td>405±10</td>
</tr>
<tr>
<td>F2</td>
<td>Freeze drying</td>
<td>5</td>
<td>90.5</td>
<td>45±25</td>
<td>700±15</td>
<td>233±7</td>
</tr>
<tr>
<td>PS1</td>
<td>Phase inversion</td>
<td>8</td>
<td>78.1</td>
<td>84±65</td>
<td>7100±100</td>
<td>1920±22</td>
</tr>
</tbody>
</table>

* Measured with a Thermo Finnigan Pascal porosimeter.
the prepared scaffolds was another drawback of this method.

Searching for an alternative methodology with the capability to produce scaffolds of improved mechanical properties and controlled pore size geometry, next, we tried salt leaching technique. A method based on a combination of compression moulding, heating and particulate leaching, and utilization of NaCl and PEG particles as porogens which were applied in this work.

A mixture of NaCl and PUU particles which contained 70% salt was prepared by mechanical mixing. The mixture was compression moulded and the composite was then heated at 80°C for 15 min and finally the salt was leached out in water. The salt concentration of 70% was chosen on the basis that at smaller salt content, complete removal of salt particles through salt leaching would not be possible due to lack of pores interconnections [24]. SEM micrograph of a representative scaffold sample prepared by this technique is shown in Figure 2a. Although prepared in a very simple and quick way by this approach, the disc lacked the necessary integrity in its structure and large cracks were observed in the final disc which possessed a laminate-like structure (inset, Figure 2a).

Efforts to improve the structure of the scaffold samples were failed by lowering the salt content to about 55%, increasing the temperature up to 90°C and increasing the heating time to 20 min. Though, successful preparation of poly(ethylene oxide) scaffolds by sintering was already reported that showed acceptable mechanical properties and pore morphology [24]. Considering the appropriate thermal behaviour of the polymer [40] we decided to use PEG (\(M_n = 35000\) Da) as porogen. Discs with PEG/PUU ratio of 70/30 were prepared in a similar way and heat treated at 80°C. Leaching out the PEG particles from these discs resulted in well integrated composites. However, the scaffold samples prepared from these discs lack the appropriate pore morphology and configuration as it is evident in SEM micrograph (Figure 2b).

To address the structural integrity and pore morphology homogeneity, we examined a combination of PEG (which melts upon heating) and salt particles in a double porogen particulate leaching methodology. An overall result of the salt/PEG/PUU ratios employed as well as the pore diameter range, the average pore size and the porosity of the scaffolds prepared by this modified method are tabulated in Table 2. In this study, the same particle size was kept unchanged for all components of different scaffolds except the SNP4, for which the PUU particles were sieved to yield particles of 80-250 μm.

From the data in Table 2, a remarkable increase in porosity is concluded (from 77% to about 86%) when percentage of total porogen particles increased from 80 to 85%. The morphological differences due to different salt/PEG ratios in scaffold samples of a total porogen concentration of 80 and 85% are presented in Figures 2c-2f, respectively. A close observation of the data in Table 2 and Figures 2c-2f reveals an improved pore morphology and interconnectivity which are obtained when PEG particles are added as a second porogen. This is possibly due to the effect of the

**Table 2. Different formulations of the scaffold samples prepared by modified method.**

<table>
<thead>
<tr>
<th>Scaffolds samples</th>
<th>PUU particle size (μm)</th>
<th>Porogen size (μm)</th>
<th>Porogen content (wt%)</th>
<th>Average pore diameter (μm)</th>
<th>Porosity* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NaCl</td>
<td>PEG</td>
<td>NaCl</td>
<td>PEG</td>
<td></td>
</tr>
<tr>
<td>SN1</td>
<td>0-250</td>
<td>0-250</td>
<td>-</td>
<td>70</td>
<td>176±30</td>
</tr>
<tr>
<td>SP1</td>
<td>0-250</td>
<td>-</td>
<td>0-250</td>
<td>30</td>
<td>164±50</td>
</tr>
<tr>
<td>SNP1</td>
<td>0-250</td>
<td>0-250</td>
<td>0-250</td>
<td>40</td>
<td>155±110</td>
</tr>
<tr>
<td>SNP2</td>
<td>0-250</td>
<td>0-250</td>
<td>0-250</td>
<td>45</td>
<td>141±100</td>
</tr>
<tr>
<td>SNP3</td>
<td>0-250</td>
<td>0-250</td>
<td>0-250</td>
<td>60</td>
<td>153±70</td>
</tr>
<tr>
<td>SNP4</td>
<td>80-250</td>
<td>0-250</td>
<td>0-250</td>
<td>25</td>
<td></td>
</tr>
</tbody>
</table>

* Measured with a Thermo Finnigan Pascal porosimeter.
Figure 2. SEM Micrographs of porous polymeric scaffolds prepared by compression moulding, heating and particulate leaching: (a) SN1, particle size <250 μm (60% NaCl), (inset: PUU disc appearance), (b) SP1, porosity: 61.3, particle size <250 μm (80% PEG), (c) SNP1, porosity: 77.1%; particle size <250 μm (30% NaCl, 50% PEG), (d) SNP2, porosity: 81.4%; particle size <250 μm (40% NaCl, 40% PEG), (e) SNP3, porosity: 86.9%; particle size <250 (for the polymer and NaCl) and 80-250 μm (for PEG), (50% NaCl, 35% PEG), and (f) SNP4, porosity: 87.6%; particle size 80-250 μm (65% NaCl, 20% PEG).
co-porogen (PEG) as a mediator, to improve the adhesion between the adjacent particles.

The process of scaffold preparation by polymer/double porogens mechanical mixing, heat treatment, and solvent leaching overrides the disadvantages of the previous methods. Interestingly, porosity, mechanical properties and average particle size, all increased simultaneously as the overall porogen load was increased from 80% to 85% and particles < 80 μm were cut from the PUU meshed sample. A comparison between the mechanical properties of SNP2 and SNP4 indicates that the mechanical properties increased to a large extent while the particle size was changed from < 250 μm to 80-250 μm, most possibly because the cells' wall thickness increased accordingly. Polymeric scaffolds of high porosity and pore interconnectivity are obtained while the porosity and the pore size can be independently controlled by porogen particle size and the total content, respectively.

Since PEG was used in the PUU backbone and also as a porogen in this study, the detection of residual amounts of PEG (used as etching polymer) in the scaffolds was extremely difficult by spectroscopic techniques. However, PEG has been considered as a biocompatible material and a trace amount of this polymer remaining in the resulting scaffolds is not an issue. A typical energy-dispersive X-ray analysis of the scaffolds demonstrates no elemental Cl or Na in the scaffold samples (Figure 3) which confirms that after leaching, the salt was almost entirely removed.

The mechanical properties of the scaffolds retaining the best morphologies were measured in compression mode. Figure 4 illustrates the corresponding stress-strain curves of the samples SNP2-4. The results showed that the SNP2 scaffold (80% total porogen content) had the highest modulus. By increasing the amount of the porogen up to 85% with the same particle size (SNP3 scaffold) the modulus decreased considerably. However, the scaffold prepared with the same percentage of porogen with particle sizes between 80 and 250 μm (SNP4 scaffold) the modulus again increased to about 3.2 MPa. The high modulus of the sample SNP4 in comparison with that of SNP3 can be possibly due to a higher salt to PEG ratio used in the former. Homogeneous pore morphology can reportedly lead to enhanced mechanical properties, i.e., compression modulus [45]. As the same porogen size range was used throughout our
Figure 5. SEM Micrographs of (a) G292 cells spread on the control (culture plate PS) and (b) scaffold SNP4 which were taken after 72 h post-seeding.

shows that scaffolds with compression strength between 0.7 MPa [36] up to about 7 MPa [47] have been used for different bone tissue applications.

Cell Culture Assay
As the chemical structure of the scaffolds was the same for all samples in this study and the SNP4 holds the best mechanical and morphological properties, this sample was chosen as the best scaffold for cell experiment. The interaction of G292 cells with SNP4 scaffold was studied and compared with a control sample. Three days after cell seeding, SEM micrographs of samples were recorded to study the cells spreading and attachment on the SNP4 scaffold (Figure 5). Typically the scaffold surface was covered with cells which appeared as spindle shape with needle-like projection. More interestingly, the cells freely penetrated into the pores of the scaffold which is a good sign of proper morphology of the prepared scaffold. Similar behaviour has been already reported for chondrocytes cells on polyurethane scaffolds [48,49].

CONCLUSION
In this work new PUU based scaffolds with interconnected pores and controlled porosity with porosities between 77% and 87% were fabricated using a mixture of PEG and salt particles in a double porogen particulate leaching methodology. A combinatory technique for preparation of porous polymeric scaffolds has been developed that involves mechanical mixing, compression moulding, heat treatment and salt and PEG leaching. Preparation of highly porous scaffolds with an interconnected network of homogeneously dispersed pores and well-defined pore morphology becomes possible using this newly developed methodology by maintaining an appropriate porogens ratio. Due to the semi-crystalline nature of the polymer, no collapsed pore was observed in the scaffolds. According to our results, scaffold SNP4 holds promise as a good candidate with desirable mechanical and biological properties for bone tissue engineering applications.

This technique is simple and avoids any hazardous organic solvents. Tailoring of structures was
possible by controlling the porogens ratio. The scaffolds exhibited mechanical properties comparable to those required in bone regeneration application. A preliminary cell culture experiment confirmed the ability of selected scaffold, with interconnected and homogeneously dispersed pores, to support and ingrowth of osteoblast cells.

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