Rapid Production of Highly Interconnected Porous Scaffolds by Spheroidized Sugar Particles for Tissue Engineering

Doku Mühendisliği İçin Küreselleştirilmiş Şeker Partikülleriyle Yüksek Gözenek Bağlantılarına Sahip Doku İskelelerinin Hızlı Üretimi

Research Article / Araştırma Makalesi

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ABSTRACT

Irregular pore morphology has a significant influence on cell response and the control of pore interconnectivity in the scaffolds constructed for tissue engineering of bone. In this study, we explored a new porogen for particulate leaching technique to fabricate highly interconnected porous scaffold with pores in regular shape. A polymeric blend composed of poly-L-lactide (PLLA) and poly-e-caprolactone (PCL) (with average molecular weights of 220 kDa and 50 kDa, respectively) was used for the construction of the scaffold. Spheroidized sugar particles were produced by using a flame treatment with a Meker burner. This method enabled the formation of homogenous and symmetrical particles with high water solubility. A pigment was blended into this polymeric mixture to investigate the morphology by confocal microscopy. The fabricated scaffolds were thoroughly characterised physically and biologically. Porosity and average pore size values were calculated by m-CT as 83% and 312 mm respectively. Live/dead assay by confocal microscopy demonstrated high cell attachment and cell viability in the scaffolds. This new scaffold fabrication method will be useful for tissue engineering community in the control of scaffold architecture.

Key Words
Porous scaffolds, interconnectivity, spheroidized sugar particles, tissue engineering.

ÖZET

Düzensiz gözenek morfolojisi kemik doku mühendisliği için tasarlanan doku iskelelerinde hücre cevabı ve gözenekler arası bağlantılar üzerinde önemli bir etkiye sahiptir. Bu çalışmada, oldukça yüksek gözenek bağlantıları olan doku iskelelerinin üretiminde partikül ekstraksiyonu yöntemi için yeni bir gözenek oluşturucu üretildi. Doku iskelelerinin oluşturulmasında poli-L-laktik asit (PLLA) ve poli-e-kaprolaktondan (PCL) (ağırlıkça ortalama molekül ağırlıkları sırasıyla 220 kDa ve 50 kDa) oluşan bir polimerik karışım kullanıldı. Bir Meker beki yardımıyla alev ile küreselleştirilmiş şeker partikülleri hazırlandı. Bu yöntemle suda oldukça iyi çözünerek uzaklaştırılabilen homojen ve simetrik partiküllerin oluşturulması mümkün oldu. Oluşturulan doku iskelelerinin gözenek morfolojisinin konfokal mikroskop altında incelenmesi için polimerik karışıma üretim sırasında bir pigment ilave edildi. Üretilen doku iskeleleri fiziksel ve biyolojik olarak karakterize edildi. Gözeneklilik ve ortalama gözenek boyu değerleri mikro-bilgisayarlı tomografi (mikro-CT) ile sırasıyla %83 ve 312 mm olarak belirlendi. Hücre kültürü neticesinde konfokal mikroskop ile canlı/cansız kiti uygulamasının sonuçları, doku iskelelerinde yüksek derecede hücre tutunması ve hücre canlılığına işaret etti. Bu yeni yöntemin doku iskelelerinin yapısal kontrolünde doku mühendisliği çalışan gruplar için oldukça yararlı olacağı düşünülmektedir.

Anahtar Kelimeler
Gözenekli doku iskeleleri, birbirine bağlı gözenekler, küreselleştirilmiş şeker partikülleri, doku mühendisliği.

Article History: Received August 17, 2010; Revised September 24, 2010; Accepted November 17, 2010; Avaliable Online: February 16, 2011.
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INTRODUCTION

Tissue engineering of bone aims to construct viable bone substitutes by using life sciences and engineering approaches and it has been an emerging field in last decades [1]. Main focus has been directed to the use of isolated cells and other growth materials with or without scaffolds. A typical scaffold for tissue engineering of bone should be biocompatible along with the other desired properties such as biodegradability, porous structure and mechanical strength [2]. Polymers which undergo *in vivo* enzymatic or hydrolytic degradation are one of the major classes chosen by the scientist working in this area [3-7]. Poly(lactic acid) and poly-ε-(caprolactone) are FDA approved biodegradable polymers [8,9] and the applications thereof are diverse. They are able to degrade *in vitro* in the presence of water molecules.

Porosity and interconnectivity has been a major concern since nutrient and oxygen intake and metabolite and carbon dioxide removal strictly depend on. Since there has been a wide range of scaffold production methods proposed in the literature [10-17]; particulate leaching is one of the advantageous methods thanks to several benefits such as the control of pore size and percentage porosity. Salt particles are commonly used as porogen; however, the morphology of the resulted pore is quite irregular. This irregularity leads to a heterogeneous pore size distribution in addition to heterogeneous load bearing properties throughout the scaffold. Irregular shaped porogen also resulted in rough contoured edges which adversely affect cell attachment. This study was conducted to produce scaffolds with spherical pores by using water-leachable sugar particles. Production of spheroidized sugar particles, morphological investigation of the obtained scaffolds and *in vitro* biocompatibility results are given.

MATERIALS AND METHODS

Polymers Synthesis

Poly-L-lactide (M_w=220 kDa) and Poly-ε-caprolactone (M_w=50 kDa) were synthesized in nitrogen atmosphere in a glass reactor. Details of the polymerization procedure can be found elsewhere [18]. In order to confirm polymerization, Fourier transform infrared spectroscopy (FTIR, Shimadzu DR 8101, Japan) and nuclear magnetic resonance (NMR, Brucker UltraShield 400, Germany) analyses were conducted. Thermal analyses were performed on the prepared polymers by differential scanning calorimeter (TGA, Shimadzu DSC-50, Japan) at a heating rate of 20°C up to 200°C. We prepare polymeric blends by using these two polymers in a ration of 50/50 w/w. Polymers were dissolved in chloroform, mixed thoroughly and precipitated in methanol. Polymers were characterized by using FTIR, NMR and DSC analyses (Supportive Documents).

Preparation of Spherical Sugar Particles

Granulated sugar (food grade, from any supermarket) was used to produce spherical porogen. Basically, sugar particles were poured into a flame of a Meker burner and undergone periphery melting before dropped into aluminium collector [19]. The schematic illustration of the process is given in Figure 1 [adopted from 19]. The obtained spherical sugar particles were then sieved through 250-355 μm mesh (Reitsch, Germany).

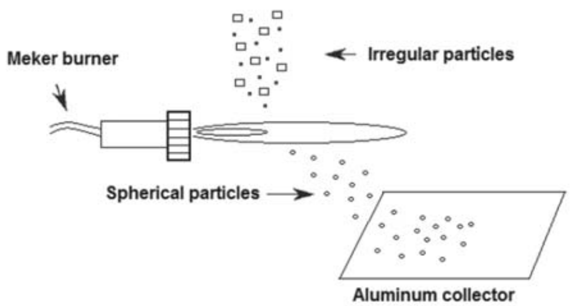

Figure 1. Schematic illustration of the spherical sugar particles production by flame.

Scaffold Fabrication by Using Spheroidized Sugar Particles

Blends from the polymers in the ratio of 50/50 w/w were prepared in chloroform as stated above and mixed with spheroidized sugar particles in the range of 250-355 μm. For confocal microscopic observation of the structure, a pigment dye (Solvent Green 3, Sigma, UK) was used in a ratio of 0.3 % w/w. This porogen/polymer mixture then placed into a cylindrical mould and a syringe filter was placed into one end of the mould while the opposite end used to press the mixture. Chloroform was evaporated gradually then the sugar particles were leached out by using de-ionized water. Following multiple water changes for 3 days, the scaffolds were placed into paper towel and dried in an oven at 30°C for 2 days.

Mechanical Tests

Dried polymeric scaffolds were cut into disks with a diameter of 8 mm and a thickness of 4 mm and sterilized in methanol (70% v/v). In order to investigate the mechanical strength of the scaffolds, a BOSE tester (Electroforce 3200, UK) was used. Four measurements were conducted and an average Young's modulus was calculated.

Morphological Analysis of the Scaffolds

In order to investigate the morphological properties of the resulted monoliths, we used μ-CT (micro-computerized tomography) (Scanco μct40, Switzerland). The X-ray source of the equipment was operated at 40 kVp and 180 μA. Integration time was selected as 200 ms and scans were gathered at a resolution of 6 μm. A reference threshold protocol was used and an average pore size was calculated with a value of 4.4% of the gray scale maxima [20,21].

Cell Response

A human osteoblast-like cell line (MG63, ATCC, UK) was selected to perform *in vitro* cell culture studies. Cell monolayers grown at 37°C in a humidified atmosphere with 5% CO_2 were collected by Tyripsin/EDTA (0.25% Tyripsin/0.02% EDTA, Sigma, UK) and $1X10^6$ cells in a 50 μl of medium were seeded per scaffold and incubated at the same conditions for 2 h before topping up the medium (to a final volume of 5 ml). These constructs were kept in culture for one week in DMEM media supplemented with 10% foetal calf serum, 10% L-Glutamine and 1% Antibiotics. Cell response to the scaffolds in terms of attachment and viability was investigated by live/dead fluorescent dye kit (Fluka, UK) after the 48 hours of culture time. A laser scanning confocal microscope (Olympus FLOUVIEW 300, UK) was used to investigate the fluorescent-labelled constructs. In this protocol, calcein reaction with intracellular esterase resulted in green colour in confocal images and indicated live cells, while red fluorescence resulted from the reaction with ethidium homodimer bound to nuclear acids indicated death cells.

RESULTS

Figure 2 below is the representative optical micrograph of the prepared sugar particles. From the figure, it can be seen that there is a low number of non-spheroidized particles remained following the flame treatment. Nevertheless, Figure 2 clearly proves the efficacy of the flame treatment in the manufacture of spherical sugar particles. It should be noted that these particles were caramelized, at least on their periphery region; therefore required more time for leaching. We reported previously [18] shorter leaching times for the irregular shaped water-leachable sodium chloride particles. Here, the leaching process requires multiple water replenishments throughout 36 hours.

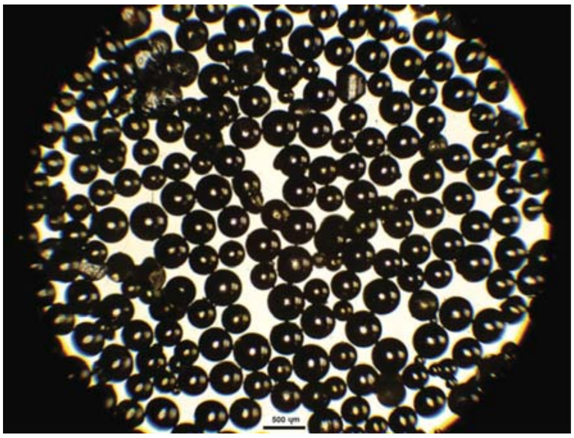

Figure 2. Optical micrograph of the spheroidized sugar particles (sieved, 250-355 μm).

Several properties of the scaffolds prepared in this study are given in Table 1. Average porosity and pore size values were calculated by using μ-CT. As seen from the table, scaffolds have a very high porosity value up to 90% in some regions. Average pore size was found as 312 μm as expectedly. Mechanical tests indicated a Young's modulus value approximately 55 kPa, which is a higher value of those previously reported and achieved by salt leaching techniques [18]. It may due to the

Table 1. Properties of the scaffolds.

Young's Modulus (kPa)	55.0 ± 6.3
Strain (%)	72.0 ± 11.0
Average porosity (%)	83.2 ± 7.8
Average Pore size (μm)	312.0 ± 43.0

phenomenon that, by spherical pore shape, loads can be distributed evenly to the neighbour pores and the overall mechanical strength is increased.

A representative μ-CT image of a scaffold prepared by using spheroidized sugar particles is given in Figure 3 below. Cross section and top view indicates spherical pore formation and homogenous pore distribution. This image created by the device own software and the average porosity and average pore size values were calculated by the same software (given in Table 1).

Human osteoblastic cell line (MG63) grew well on these scaffolds for the period of two weeks in cell culture. Following live/dead kit staining after 48 hours, confocal microscopy images proved that most of the seeded cells were viable. From the Figure 4, it can be said that the cells were proliferated throughout the scaffolds and emitted a high green fluorescent indicating that the cells were happy. Red colour seen from the Figure 4 was due to the pigment blended into polymeric mixture for better visualization of the pore structures by confocal microscopy. The red contours clearly show the spherical pore shape and pore interconnectivity.

DISCUSSION

One of the major design concerns in the development of tissue engineering constructs is the structure of pores and the interconnectivity. Among plethora methods reported for the production of porous polymeric monoliths, particulate leaching technique has been commonly used since it allows better control on porosity, pore size and distribution and interconnectivity. A major porogen used in particulate leaching is sodium chloride; however the resulted pore structure is irregular and the pores have rough surfaces according to the shape

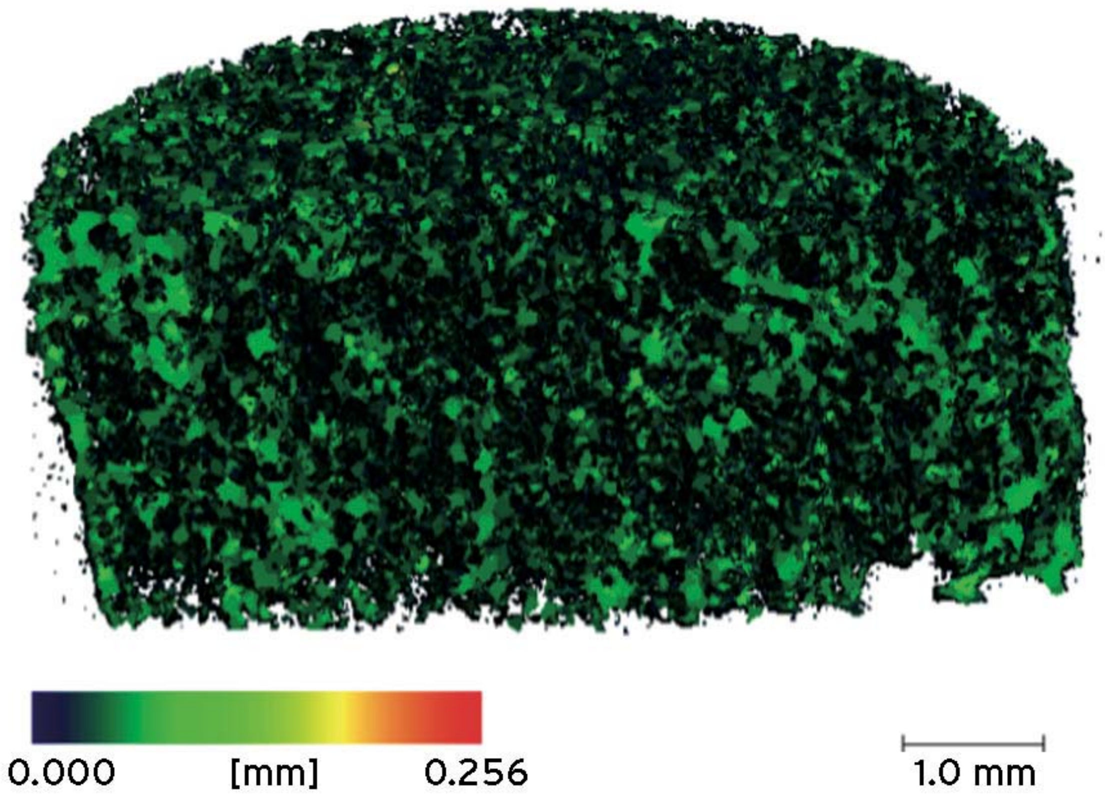

Figure 3. Micro-CT image of the scaffold prepared by spheroidized sugar porogen.

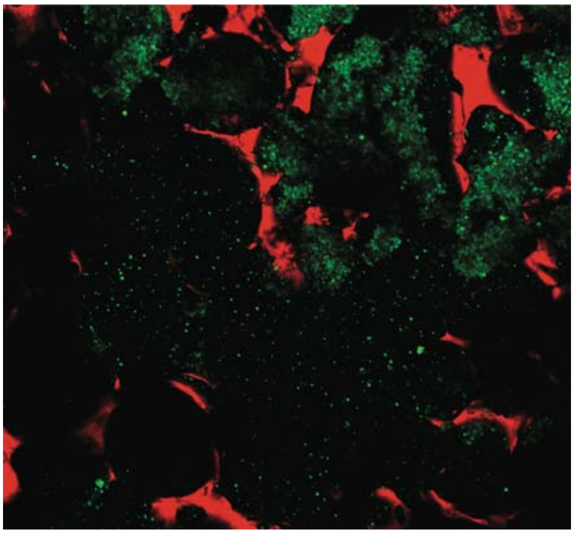

Figure 4. A confocal microscopy image of the scaffold. Interconnectivity is clearly seen from the image. Please note that these scaffolds were incorporated a pigment (Solvent Green 3) for better visualization of the pore morphology.

of the particles. Even though it is an inexpensive and fast technique, an ordered and homogeneous pore structure cannot be achieved. Therefore the use of spherical gelatine porogen was proposed [22] but the total porosity achieved was low. Paraffin microspheres were another attempt but the residual solvents (such as cyclohexane) used to extract paraffin were toxic for the cells. Ma et al proposed fused-paraffin particles to obtain ordered structures [23] and achieved elevated interconnectivity values, but the penetration of polymer solution within these fused-structures was limited. Here, we prepared the polymeric scaffolds with homogeneously distributed pores by utilizing spheroidized sugar particles. This structure resulted in better interconnectivity known as "*sine qua non*" in tissue engineering to allow enhanced cell proliferation, nutrient and metabolite exchange [24]. In addition to basic advantages of sugar to be cheap and readily available, sugar particles can be easily produced in a variety of sizes by a strong flame at around 1900°C. Leaching of porogen is not a problem in this case and all the chemicals are non-toxic. Cell attachment is better on smooth and sleek surfaces and the interconnectivity can be defined or calculated in much more precise manner. Finally, spherical pores can distribute loads homogeneously and the modulus is higher than those prepared by irregular shaped particles.

CONCLUSION

We prepared biodegradable and biocompatible polymeric scaffolds for tissue engineering of bone by using spheroidized sugar particles. Spherical porogen approach is useful in the control of pore interconnectivity and homogenous pore size distributions. Scaffolds with spherical pores yielded higher mechanical strength since the loads can be distributed thanks to pore geometry. This approach is simple and the materials used are all biocompatible and harmless. This technique can be used to produce different tissue engineering constructs such as a cartilage scaffold only by changing the parameters of flame treatment.

ACKNOWLEDGEMENTS

This project was supported by European Union FP7 IEF Marie Curie Actions (PIEF-GA-2009-237762). We kindly acknowledge Mr Yusuf Yesil for technical assistance with the production of sugar particles and Mrs Hu Bin for the confocal microscopy images.

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