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Microspheres leaching for scaffold porosity control

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Scaffold morphology plays a key role in the development of tissue engineering constructs. The control of pore size, shape and interconnection is needed to achieve adequate nutrient transport and cell ingrowth. Several techniques are available for scaffold manufacturing, but none allows easy control of morphology and is, at the same time, applicable to a wide variety of materials.

To investigate the possibility of processing a wide range polymers by solvent casting/particulate leaching with accurate control of scaffold morphology, three different porogens (gelatin microspheres, paraffin microspheres and sodium chloride crystals) were used to fabricate scaffolds from commonly employed biodegradable polymers. The outcome of processing was evaluated in terms of scaffold morphology and structure/properties relationships.

Highly porous scaffolds were obtained with all porogens and well defined spherical pores resulted from microspheres leaching. Furthermore, scaffolds with spherical pores showed better mechanical performance and lower flow resistance. Cytocompatibility tests performed showed no evidence of processing residuals released from the scaffolds.

Solvent casting/microspheres leaching, particularly gelatin microspheres leaching, can be used to process a large number of polymers and enables to tailor scaffold pore size, shape and interconnection, thus providing a powerful tool for material selection and optimization of scaffold morphology.

1. Introduction

When an organ loses its functionality, current clinical strategies often require the harvest of scarcely available tissue from a donor or from the patient. With the tissue engineering approach, laboratory-grown cells can be cultured on three-dimensional scaffolds, to create an engineered construct that can be implanted to replace or support the functions of defective tissues.

Depending on the application, the scaffold should meet several different requirements, but a well interconnected pore network is needed for most tissue engineering applications, to achieve better nutrient transport and easier cell migration. Pore size, interconnection and even pore shape can affect tissue ingrowth [1]. Therefore, accurate control of pore morphology and interconnection is a crucial step in the design of tissue engineering scaffolds.

Several processing techniques are available for the fabrication of polymeric scaffolds, such as solvent casting/particulate leaching, gas foaming, thermally induced phase separation, fiber bonding, 3D printing or fused deposition modeling [2–6]. All this techniques differ in terms of obtainable morphologies, range of materials that can be processed and number of parameters to control, but none allows an easy control of pore morphology and is, at the same time, applicable to a wide variety of materials. On the other hand, to evaluate the effect of scaffold morphology on cell migration and tissue organization, as well as for the selection of the optimal scaffold material, a very versatile technique that enables easy control of pore shape, size and interconnection, is highly desirable.

Solvent casting/salt leaching is, in fact, a very simple processing method that can be used, in principle, to fabricate scaffolds from any thermoplastic polymer. However, leaching of salt crystals, typically sodium chloride, often results in poor interconnection and irregular pore shape. To overcome this drawbacks, different modifications of this technique have been proposed, as leaching of microspheres [7] and the partial fusion of particles before casting [7, 8], respectively to overcome poor pore shape and to improve interconnection.

To investigate the possibility of obtaining scaffolds with controlled morphologies from different biodegradable materials by particle leaching, three different porogens (gelatin microspheres, paraffin microspheres and sodium chloride crystals) were here used to fabricate scaffolds from commonly employed medical grade biodegradable polymers. The outcome of processing

was then evaluated in terms of scaffold morphology and structure/properties relationships.

2. Materials and methods

2.1. Materials

Poly-DL-lactide (PLA, Resomer® R 207) and Poly-L-lactide-co-trimethylene carbonate in a 68:32 ratio (PLATMC, Resomer® LT 706) were purchased from Boheringer Ingelheim, polycaprolactone (PCL) from Sigma Aldrich (440744). All solvents were reagent grade and used without further purifications. Paraffin for histological inclusions (Bio-Plast®) and alimentary sodium chloride, gelatin and soybean oil were used.

2.2. Microspheres preparation

Paraffin microspheres were obtained as previously described by Ma and Choi [7], with only minor modifications. Briefly, paraffin was melt at 65 °C and poured in a glass becker containing water and 1% w/v polyvinylpyrrolidone (Fluka), kept at 70 °C on a magnetic stirrer. The emulsion was vigorously stirred and paraffin microspheres were solidified by rapid cooling, obtained by adding ice cold water. The microspheres were then washed with deionized water, dried at room temperature and kept in a dessiccator until use.

Gelatin microspheres were prepared by adapting a technique previously reported for the preparation of microspheres for drug delivery [9]. Gelatin was dissolved in water at a very high concentration and the solution was added to a becker containing soybean oil, heated to 60 °C under stirring. After a few minutes, the emulsion was cooled down to 15 °C in an ice bath, and gelatin droplets were dehydrated by adding cold acetone. Microspheres were then removed from soybean oil, washed several times with acetone and dried.

2.3. Scaffold preparation

Paraffin and gelatin microspheres and NaCl crystal were sieved to obtain different ranges of particle dimensions and leveled in a glass mold to a 4 mm thickness.

To achieve paraffin adhesion, the mold containing the microspheres was placed in oven at 40 ÷ 50 °C for up to one hour, until satisfactory adhesion of microspheres was observed.

A similar procedure was used for gelatin microspheres and NaCl crystals fusion, but the molds were kept in the oven for up to 96 hours with 95% relative humidity and T = 50 °C.

PLA was dissolved in acetone (3% w/v) and cast into the molds containing salt, paraffin or gelatin. PLA-TMC and PCL were dissolved in chloroform and cast on gelatin microspheres only.

After vacuum drying, 10 mm diameter cylinders were punched out from the sheets before dissolving the porogen by washing the scaffolds several times with hexane (paraffin) or deionized water (gelatin and sodium chloride). All samples were dried in air and kept in a dessicator until characterization.

2.4. Characterization

After drying, the scaffolds were weighed with a high precision balance and linear dimensions were measured with a caliper in accordance with ISO 1923 [10]. Pore to material volume ratio (*total porosity*) was then calculated as:

$$\text{Total porosity} = \frac{V_g - W_{sc}/\rho_{\text{pol}}}{V_g} \times 100 \qquad (1)$$

where V_g is the scaffold geometric volume, W_{sc} is the scaffold weight and ρ_{pol} the polymer density.

The volume percentage of open cells *(open porosity)*, that accounts only for penetrable volume of the scaffolds (i.e. for interconnected pores), was evaluated by gas expansion porosimetry. The instrument employed was purpose-built in accordance with ISO 4590 [11, 12] and consists of a glass-tubing manometer filled with water and with one side open to atmosphere. The other end of the manometer is connected via an expansion bulb to a sealed chamber containing the specimens to be tested. In accordance with the Boyle-Mariotte law, if the gas in the chamber is expanded at constant temperature, a reduction in the chamber volume (i.e. the presence of the scaffolds) results in a proportionate variation of the pressure drop. The pressure variation corresponding to a controlled expansion with or without test specimens in the chamber can be read on the manometer and the impenetrable volume (V_i) of scaffolds can be measured if the instrument is calibrated with samples of known volume. The percentage of open cells can be then calculated as:

$$\%\text{open cells} = \frac{V_g - V_i}{V_g} \times 100 \qquad (2)$$

where V_g is the geometric volume of the scaffolds.

To obtain further information about pores interconnection, flow resistance was evaluated by sealing the scaffolds between two rubber rings at the bottom of a measuring tube ending with a shut-off valve and filled with 50 cm of water. To keep the water level as even as possible during flow, the tube was communicating with a large diameter reservoir. Before each test, samples were preconditioned in water for 24 h. Flow resistance of scaffolds was then evaluated as the time needed for 10 ml of water to flow through the scaffold when opening the shut-off valve.

Pore morphology was analyzed after sputter coating the samples with gold, with a Scanning Electron Microscope (SEM, Stereoscan Cambridge 360) using an accelerating voltage of 20 KV.

Creep compression tests were performed at 37 °C in a dynamic-mechanical analyzer (DMA 2980, TA Instruments, equipped with compression clamps) for two different load levels (1 N and 5 N). After creep test, flow resistance of scaffolds was measured again as previously described.

Molecular weight analyses were conducted before and after processing by Gel Permeation Chromatography (GPC, Waters) using dimethylformamide +0.05% w/v lithium bromide as solvent. The chromatography

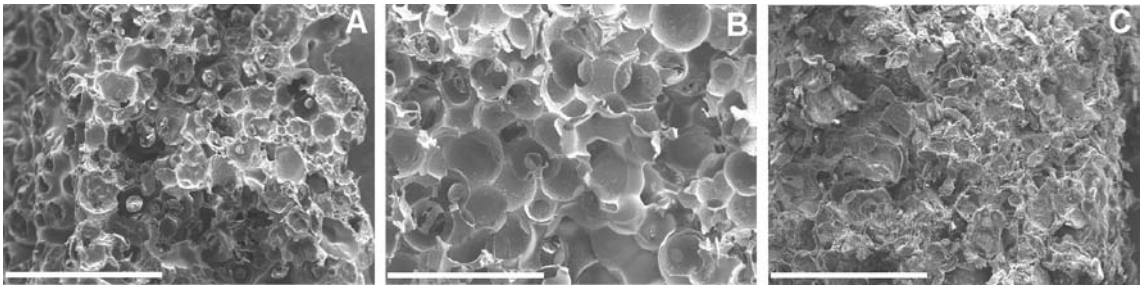

Figure 1 SEM images of PLA gelatin—(A) paraffin—(B), and NaCl—(C) leached scaffolds (200–400 μm particles, scale bar = 1mm).

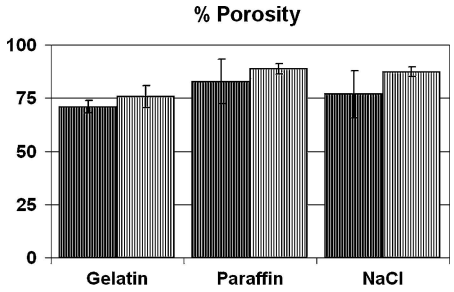

Figure 2 Open (dark bars) and total (light bars) porosity of PLA scaffolds (200–400 μm particles).

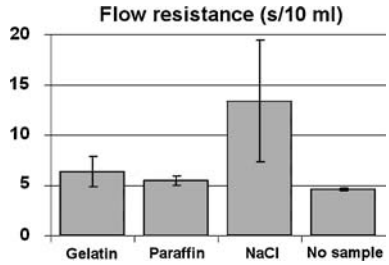

Figure 4 Flow resistance of gelatin-, paraffin- and NaCl–leached scaffolds.

system used was equipped with Styragel columns (HR3 to HR5) and a differential refractometer and was calibrated with PMMA standards, (Polymers Laboratories).

2.5. Cytocompatibility

To detect the presence of possible processing residuals adversely affecting biocompatibility, indirect cytotoxicity tests were performed. Polylactide scaffolds prepared with gelatin, paraffin or NaCl were disinfected with ethanol, dried in a laminar flow hood and incubated respectively for 1, 3, and 7 days at 37 °C and 5% CO_2 in 1 ml of culture medium (EMEM with 2 mM glutamine, 10% fetal bovine serum and 1% penicillin streptomycin). Osteosarcoma MG63 cells (ECACC 865051601) were then seeded at a density of 1.8×10^5 cells/well in a 24 wells plate containing either the scaffolds extracts or medium as control. After 12 and 24 h from seeding, Alamar Blue assay was performed to assess cell viability.

3. Results and discussion

Highly porous PLA scaffold were obtained for all porogens (Fig. 1), with open and total porosity always higher than 70% (Fig. 2). Well defined and interconnected spherical pores, detected by SEM analysis of PLA scaffolds, resulted from the use of both types of microspheres (SEM images are shown in Fig. 3). Conversely, an irregular geometry of pores was obtained for NaCl-leached scaffolds.

Controlling the adhesion of particles was easier when using paraffin, whereas, after a certain extent, increasing gelatin microspheres adhesion resulted more difficult. As a consequence, porosity was found to be slightly lower for gelatin-leached scaffolds with respect to paraffin-leached ones. Good correspondence between open and total porosity was found for microspheres-leached scaffolds, where the porosity seems to be almost completely open. A more appreciable deviation between open and total porosity was observed for NaCl scaffolds (Fig. 2) to indicate the presence of a larger number of totally closed pores.

This result was confirmed by flow resistance analysis. Despite the overall high porosity, flow resistance was significantly higher for NaCl-leached scaffolds, compared to microspheres-leached ones (Fig. 4). Standard deviation of flow resistance was also considerably higher for NaCl-leached scaffolds, to indicate a more irregular structure with non uniform distribution of interconnections between pores. Presumably, both

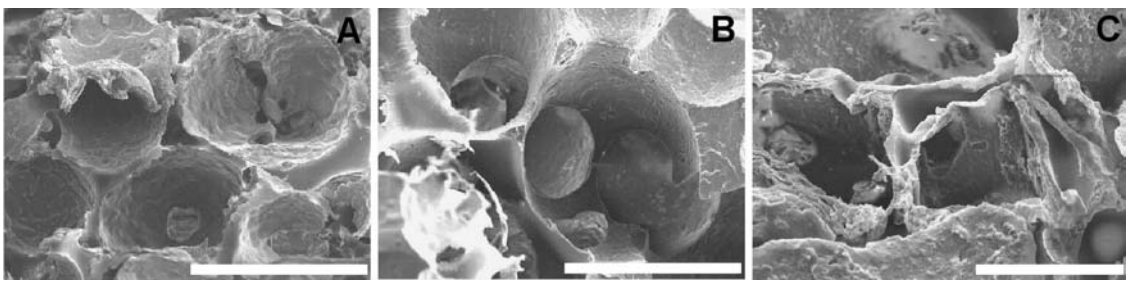

Figure 3 SEM images of PLA gelatin—(A) paraffin—(B), and NaCl—(C) leached scaffolds (200–400 μm particles, scale bar = 300 μm).

TABLE I Weight (M_w) and number (M_n) average molecular weight of the processed polymers before and after scaffold preparation with paraffin (for PLA only) and gelatin (PLA, PLATMC and PCL) microspheres

	PLA-paraffin		PLA-gelatin		PLATMC		PCL	
	M_w	M_n	M_w	M_n	M_w	M_n	M_w	M_n
Before processing	299,000	141,000	299,000	141,000	227,000	124,500	201,500	119,500
After processing	284,000	137,000	290,000	174,000	233,500	125,000	126,000	71,500

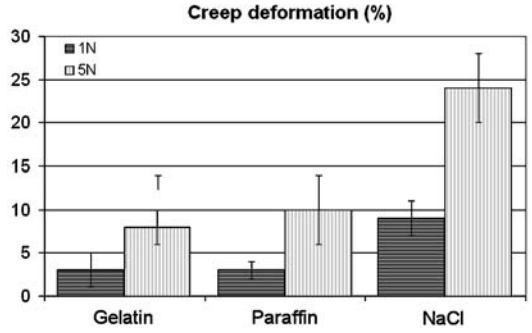

Figure 5 Creep deformation under 1 N or 5 N compression load of gelatin-, paraffin- and NaCl-leached scaffolds.

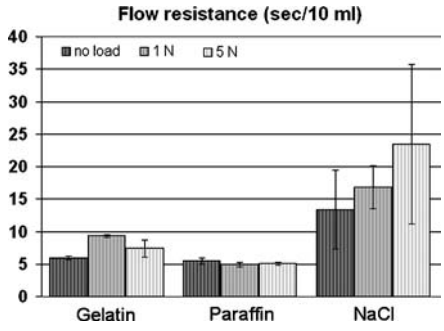

Figure 6 Flow resistance for gelatin-, paraffin- and NaCl-leached scaffolds before and after creep test at 1 and 5 N loads.

the higher percentage of closed cells and the irregular morphology account for the higher flow resistance observed for this type of scaffolds. Microspheres-leached scaffolds, on the contrary, possess very low flow resistance, considering that the time needed for water to flow through the shut-off valve, without any specimen, was approximately 4.50 seconds.

Creep deformation for both loads chosen (1 and 5 N) was also significantly higher for NaCl-leached scaffolds (Fig. 5) with respect to the microspheres-leached ones. By comparing gelatin- and paraffin-leached scaffolds, it can be noticed that deformation after 1 hour is very similar, despite the significant difference in density ($0,156 \pm 0,05$ g/cm^3 for paraffin-leached and $0,3346 \pm 0,06$ g/cm^3 for gelatin-leached scaffolds).

After mechanical testing, flow resistance was found to be unaltered for paraffin-leached scaffolds (Fig. 6), while a more appreciable increase in the time needed for water to flow through was observed for the other two types of scaffolds. According to these results, after loading the open pore structure is well conserved in paraffin-leached scaffolds, while a partial occlusion of pores occurs for gelatin- and NaCl-leached scaffolds and is more substantial for the latter.

From the cytocompatibility tests performed, none of the PLA scaffolds prepared with the three porogens evidenced cytotoxic effects of the extracts in the culture medium In fact, cell viability was not altered in the presence of medium incubated with the scaffolds up to seven days, as Alamar Blue absorbance was always found to be comparable or even higher than control absorbance at the tested time points (Fig. 7).

By using gelatin microspheres as porogen and chloroform as solvent, it was possible to prepare well interconnected porous scaffolds also from PLATMC and PCL (Fig. 8), while no suitable solvent/non solvent combination was found to process these materials with paraffin microspheres.

After processing, no significant variations in the molecular weights of PLA and PLATMC were observed (Table I), whereas a more appreciable reduction was found for PCL before and after the scaffolds preparation, to indicate a higher sensitivity of this materials to the processing technique used.

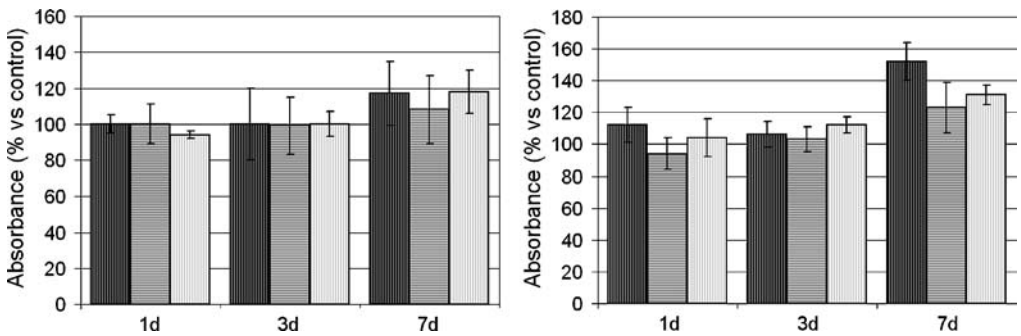

Figure 7 Alamar blue absorbance 12 (left) and 24 (right) after seeding MG63 cells with media incubated for 1,3, and 7 days with gelatin-(darker), paraffin-and NaCl-(lighter) leached PLA scaffolds.

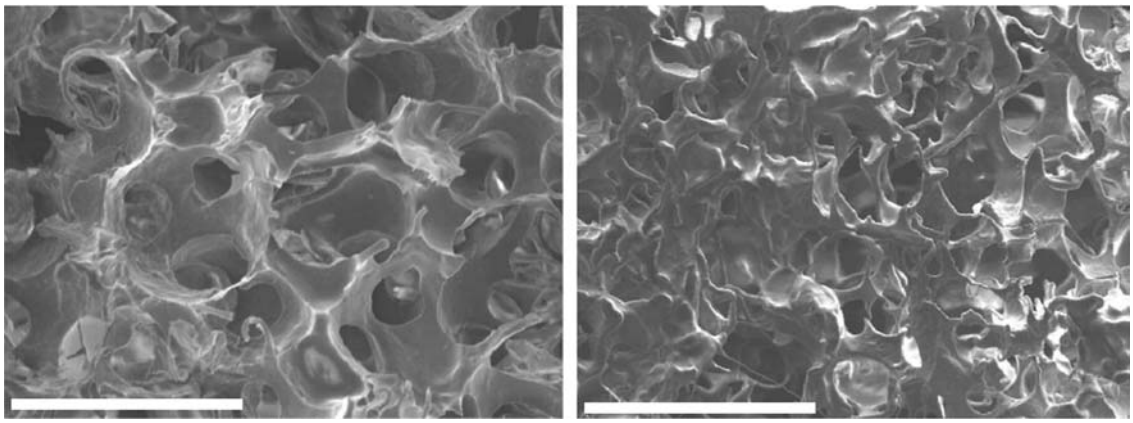

Figure 8 PLATMC (left) and PCL scaffolds prepared with gelatin microspheres (scale bar = 100 μm).

4. Conclusions

Micropsheres leaching can be effectively used to prepare scaffolds from a large variety of biodegradable materials, enabling an easy control of both porosity and pore interconnection. Thanks to their low flow resistance, scaffolds with spherical pores could reasonably be expected to improve fluid exchange and nutrient supply to cells. The regular geometry obtained by microspheres leaching seems to improve scaffolds mechanical performance, and even after loading low flow resistance is better maintained.

Although the control of porosity is somehow easier when using paraffin microspheres, water soluble/chloroform insoluble gelatin greatly helps to broaden the range of thermoplastic polymers this technique can be applied to. Gelatin microspheres leaching allows to effectively compare different materials and morphologies all other conditions being the same, offering a powerful tool for material selection and morphology optimization in the design of scaffolds for tissue engineering.

References

1. S. YANG, K. F. LEONG, Z. DU and C. K. CHUA, *Tissue Eng.* **7** (2001) 679.
2. A. MIKOS, A. THORSEN, L. CZERWANKA, Y. BAO, D. WINSLOW, J. VACANTI and R. LANGER, *Polymer* **35** (1994) 1068.
3. T. FREYMAN, I. YANNAS and L. GIBSON, *Progress in Materials Science* **46** (2001) 273.
4. A.G. MIKOS, Y. BAO, L.G. CIMA, D.E. INGBER, J. VACANTI and R. LANGER, *J. Biomed. Res.* **27** (1993) 183.
5. S. YANG, K.F. LEONG, Z. DU and C.K. CHUA, *Tissue Eng.* **8** (2002) 1.
6. C. LHOMMEAU, H. LEVENE, S. ABRAMSON and J. KHON, *ibid.* **4** (1998) 468.
7. P. MA and J.W. CHOI, *ibid.* **7** (2001) 23.
8. W. L. MURPHY, R. G. DENNIS, J. L. KILENY and D. J. MOONEY, *ibid.* **8** (2002) 43–52.
9. E. ESPOSITO, R. CORTESI and C. NASTRUZZI, *Biomaterials* **17**(1996) 2009.
10. EN IS0 1923 : 1995, "Cellular plastics and rubbers—Determination of linear dimensions".
11. A.BRUGOLA, "Espansi poliuretanici come matrici di compositi biomimetici per la ricrescita del tessuto osseo" Master thesis, Politecnico di Milano, 1999.
12. EN ISO 4590:2003, "Rigid cellular plastics—Determination of the volume percentage of open cells and of closed cells".

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