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Gas anti-solvent precipitation assisted salt leaching for generation of micro- and nano-porous wall in bio-polymeric 3D scaffolds

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ABSTRACT

The mass transport through biocompatible and biodegradable polymeric 3D porous scaffolds may be depleted by non-porous impermeable internal walls. As consequence the concentration of metabolites and growth factors within the scaffold may be heterogeneous leading to different cell fate depending on spatial cell location, and in some cases it may compromise cell survival.

In this work, we fabricated polymeric scaffolds with micro- and nano-scale porosity by developing a new technique that couples two conventional scaffold production methods: solvent casting-salt leaching and gas antisolvent precipitation. 10–15 w/w solutions of a hyaluronic benzyl esters (HYAFF11) and poly-(lactic acid) (PLA) were used to fill packed beds of 0.177–0.425 mm NaCl crystals. The polymer precipitation in micro and nano-porous structures between the salt crystals was induced by high-pressure gas, then its flushing extracted the residual solvent. The salt was removed by water-wash. Morphological analysis by scanning electron microscopy showed a uniform porosity (~70%) and a high interconnectivity between porous. The polymeric walls were porous themselves counting for 30% of the total porosity. This wall porosity did not lead to a remarkable change in compressive modulus, deformation, and rupture pressure. Scaffold biocompatibility was tested with murine muscle cell line C2C12 for 4 and 7 days. Viability analysis and histology showed that micro- and nano-porous scaffolds are biocompatible and suitable for 3D cell culture promoting cell adhesion on the polymeric wall and allowing their proliferation in layers. Micro- and nano-scale porosities enhance cell migration and growth in the inner part of the scaffold.

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1. Introduction

The goal of tissue engineering is to augment, replace, or restore complex human tissue function by combining synthetic and living components in appropriate configurations and environmental conditions [1]. Despite significant advances in this field which had lead to engineering simple tissue like skin and cartilage [2], many challenges remain to reach the goal for complex organs. Over the past decade there has been an intense research effort in this area, which has led to the generation of a portfolio of tissue engineering strategies. These include direct injection of isolated and cultured cells or implantation of tissues which have been pre-cultured *in vitro* [3]. In many applications direct injection of a cell suspension has a low efficacy and a scaffold is required to improve and control the environment for cell growth and tissue maturation mimicking the microenvironment around cells [4,5].

Basic requirements for 3D scaffolds are cell/tissue biocompatibility, high porosity, large specific surface, uniform pore distribution, and pore interconnectedness to allow sufficient transport of nutrients and facilitate multicellular processes [6,7]. Such properties affect not only cell survival, signalling, growth, propagation and reorganization, but also play major roles in influencing cell shape modelling and gene expressions that relate to cell growth and the preservation of native phenotypes both *in vivo* and *in vitro* [8].

A large number of biopolymers are available. For instance, both natural polymers, including hyaluronic acid (HyA), collagen, alginate and fibrin, and synthetic polymers as poly(ethylene glycol) (PEG), poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly(lactide-co-glycolide) (PLGA), have been successfully used for production of scaffold for various tissue targets [9–12].

Key requirements for scaffold fabrication methods include control of porosity/pore size, maintenance of mechanical properties, and maintenance of material biocompatibility. Other desirable features include avoiding the use of organic solvents, or if not possible, complete removal of solvent residues after processing. The ability to incorporate bioactive compounds without loss of their biological activity that may occur upon exposure to solvents, high temperature or shear stresses is also relevant [13]. Some methods adopted for scaffold fabrication are fiber bonding, electrospinning, emulsion freeze drying, phase separation, 3D printing, and solvent casting/particulate leaching technique, each one with its advantages and disadvantages [8,14]. Between the main disadvantages we underline the presence

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of residual organic solvent, the limited choice of polymer suitable for application [13] and, especially, the formation of compact polymeric walls which leads to the reduction or inhibition of biomolecules diffusion.

In the *in vivo* micro-environment the cells are subject to many different types of stimuli that regulate their behaviour and their fate. An important part of these stimuli is related to membrane interactions with tethering molecules and to diffusion of soluble factors both humoral and paracrine [15]. Often these paracrine soluble factors have a short lifetime and their release can influence only cells within a limited distance. *In vitro*, the presence of compact polymeric wall obstacles metabolite diffusion enabling high cell proliferation and migration inside 3D scaffolds. Moreover, the inhibition of diffusion of soluble paracrine factors can lead to the formation of non-uniform constructs.

To reduce this phenomenon facilitating biomolecules diffusion though polymeric walls, we aimed to develop a new technique for the production of 3D scaffold with porous walls. For this purpose we coupled the solvent casting/particulate leaching and the Gas Antisolvent (GAS) technique. While the second technique exploits antisolvent properties of pressurized gases to induce polymer precipitation and residual solvent extraction, the first uses an easy to remove particulate to control scaffold porosity. The GAS technique have been applied for producing different polymer morphologies (fiber, scaffolds, micro- and nano-particles) where CO_2 antisolvent properties allowed complete solvent removal [16]. It was shown that temperature, pressure, CO_2 flow rate, composition of polymeric solution determinate the polymer precipitation speed and effect the scaffold micro- and nano-morphology [13]. Exploiting these properties we applied GAS technique to create nano-porous walls and particulate leaching method to control macro-porosity. The GAS process answers to all the cited key requirements for scaffold fabrication methods. The maintenance of mechanical properties was observed and the in vitro biocompatibility was tested seeding murine muscle C2C12 cells. Furthermore the GAS technique has the advantages of using a non-toxic, readily available, environmentally acceptable, non-flammable and inexpensive gas as CO_2 [13,16]. The mild conditions adopted during GAS process allow incorporating protein and other bioactive substance without risk of denaturation [17,18].

2. Materials and method

2.1. Materials for scaffold preparation

Scaffolds were made in HYAFF11, a hyaluronic benzyl esters provided by Fidia Advanced Biopolymers Company (Abano Terme, Italy) and in poly-(lactic acid) (PLA, Resomer L206, Boehringer Ingelheim Pharma, Germany). Dimethyl-sulfoxide (DMSO) and Dichloromethane (DCM) 99.6% (Sigma-Aldrich, Milan, Italy) were used as solvents for HYAFF11 and PLA, respectively. Sodium chloride (NaCl), provided by Sigma-Aldrich (Milan, Italy), was sieved to use crystals with dimension ranging between 0.177 mm and 0.425 mm. CO_2 (99.95%) was supplied by Sapio S.r.l. (Monza, Italy).

2.2. Methods

2.2.1. Solvent casting – salt leaching

Organic solvent was added to 0.5 g of polymer to reach the concentration of HYAFF11 summarized in Table 1 or the concentration of 10/90 w/w PLA/DCM. The solution was stirred for 12 h until polymer was completely solubilized. HYAFF11/DMSO mixture was heated at 40 °C, PLA/DCM solutions were realized at room temperature. Then, the polymer-solvent solutions were distributed on salt beds. The used rates of solution/salt weight are summarized in Table 1. To extract the solvent the samples were kept under vacuum at room temperature for 24 h in the case of HYAFF11/DMSO solution and for 12 h for PLA/DCM solutions. Finally, the samples were leached: the collected samples were washed with distilled water changing water every 12 h for a total of 36 h when the salt was totally solubilized and then dried under vacuum overnight for 12 h.

2.2.2. Gas antisolvent precipitation – salt leaching

Polymeric solutions were prepared as described for solvent casting – salt leaching method. The polymer precipitation and solvent extraction step was then performed through the GAS process using the experimental set-ups reported in Fig. 1. The samples with polymer-solvent solutions distributed on salt beds were loaded in the precipitation chamber (C). The temperature in the precipitation chamber was controlled by the thermostatic bath (TB). Temperature was measured with a Pt 100 Ω resistance located in the upper part of the precipitation chamber. When the desired temperature was reached (see Table 2), CO_2 was added in (C) and the pressure was raised up until 16 MPa using the piston pump (P) (Mod. 2 M–10×70, Officine Meccaniche Gallaratesi, Italy). When 16 MPa were reached, the outflow valve (V3, V4 and V5) was opened and the precipitation chamber was flushed at a constant CO_2 flow rate (8 NL/min) to eliminate the organic solvent. Gas antisolvent flow rate was measured with two flow meters (NGIB212, COMHAS S.r.l., Milan, Italy) and controlled using the fine metering valves V1 (Hoke, model 116G4y) and the lamination valves V4 and V5. The two lamination valves (V4 and V5) (SS-31RS4, Nordival S.r.l., Italy) were dipped in a heated bath to prevent freezing due to CO_2 expansion. CO_2 flushing was stopped when polymer-NaCl beds were completely solvent free (Table 2). Finally, the apparatus was slowly depressurized. The salt leaching step was performed on the collected samples. They were washed with distilled water changing water every 12 h for a total of 36 h when the salt was totally solubilized and then dried under vacuum overnight for 12 h.

2.3. Product analyses

Morphology characterizations were performed by scanning electron microscopy (SEM). The samples were gold sputtered under high vacuum (0.05 mTorr) and their photographs were taken at different magnification from 20× to 1600×.

Table 1
Summary of formulations employed for HYAFF11 scaffold fabrication; each formulation has been identified by a number. The formulations are characterized by different initial HYAFF11 concentrations in DMSO (second column) and by salt percentage (third column). Reported also in the table are the results of mechanical tests and the value of void area due to the salt leaching step estimated from histological images. (n.d., not detectable).

Sample formulation	HYAFF11/DMSO (w/w)	Solution/NaCl (w/w)		Mechanical properties			Void fraction due to salt leaching (%)
				Max deformation (%)	Compressive modulus [Mpa]	Max pressure [Mpa]	
1	10/90	20/80		n.d.	n.d.	n.d.	–
2	10/90	30/70	GAS-SL	17.2 ± 3.3	2.9 ± 0.8	104.8 ± 3.0	–
3	15/85	20/80		n.d.	n.d.	n.d.	–
4	15/85	30/70	SC-SL	9.5 ± 0.8	7.7 ± 0.8	71.1 ± 8.1	67.9 ± 4.4
			GAS-SL	11.1 ± 0.5	6.1 ± 1.2	95.9 ± 8.6	52.2 ± 3.6
5	15/85	50/50		n.d.	n.d.	n.d.	–

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