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Research paper

Biodegradable PCL/fibroin/hydroxyapatite porous scaffolds prepared by supercritical foaming for bone regeneration

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ABSTRACT

Regenerative medicine seeks advanced solutions for bone repair in the form of bioactive synthetic scaffolds by using simple and reproducible processing techniques. In this work, poly-ε-caprolactone (PCL)-based porous scaffolds with improved osteoconductive and osteoinductive properties were processed by supercritical foaming through a careful tuning of components and processing conditions. Composite scaffolds were prepared from various combinations of PCL, silk fibroin and nano-hydroxyapatite (nHA). The green and cost-effective supercritical CO_2 foaming method applied rendered solid scaffolds with 67–70% porosity. The incorporation of fibroin and nHA in the scaffolds increased the compressive modulus, cellular adhesion and calcium deposition. The composite scaffolds were tested *in vivo* in a large-scale calvarial defect model, and bone regeneration was evaluated for up to 14 weeks after implantation. Histomorphometric results showed that all implanted constructs gave rise to the endochondral bone formation and unveiled the synergistic effect of silk fibroin and nHA on the bone repair extent. The information gathered may shed light on the design and processing criteria of bioactive bone scaffolds.

1. Introduction

Synthetic scaffolds are the mainstream alternative to biological grafts to promote bone repair when the natural self-regeneration is compromised (Hutmacher, 2000). Bone tissue engineering pursues the development of scaffolds with accurate porous 3D-structure and biological cues that serve as a platform able to restore native architecture and functions (O'Brien, 2011). Among the technologies available for the preparation of porous scaffolds with an architecture similar to that of the bone, supercritical CO_2 ($scCO_2$) foaming has emerged as an attractive and reproducible green technology (Duarte et al., 2013; Garcia-Gonzalez et al., 2015). In this technique, the apparent glass transition (T_g) and the melting temperature (T_m) of polymers are reduced due to the plasticizing effect of CO_2 under supercritical conditions (temperature and pressure above the critical point of CO_2, *i.e.* 31.1 °C and 72.8 bar). Then, the supersaturation of the CO_2 dissolved in the polymer matrix takes place when the pressure is reduced, leading to the formation of pores from growing nucleation sites (Duarte et al., 2013; Garcia-Gonzalez et al., 2015). Scaffolds composed of either natural or synthetic polymers can be thus obtained by $scCO_2$ foaming operating under mild conditions, avoiding the use of organic solvents and retaining the activity of thermally sensitive compounds such as growth factors (Reverchon and Cardea, 2012; Bhamidipati et al., 2013; Diaz-Gomez et al., 2016a). Supercritical foaming of synthetic polymers such as poly(ε-caprolactone) (PCL) or poly(lactic-co-glycolic acid) (PLGA) has been previously reported to be a successful approach to obtaining porous structures with controlled morphologies that match those of the bone tissue (Diaz-Gomez et al., 2016a,b; Goimil et al., 2017). Even so, the biological and mechanical properties of these structural polymers

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can be improved by their combination with other materials (Bhattacharjee et al., 2015).

Silk fibroin from *Bombyx mori* cocoons is a relevant natural biomaterial that can facilitate tissue regeneration (Wei et al., 2015; Melke et al., 2016). The mechanical strength, biocompatibility, availability and bone-fitting degradation rate make silk an attractive material for bone repair (Keten et al., 2010; Sun et al., 2012; Kundu et al., 2013). The Gly-Ser-Gly-Ala-Gly-Ala amino acid sequences in the primary structure of fibroin facilitate antiparallel β-sheet arrangement, conferring stiffness and mechanical resistance to the structure. The incorporation of silk fibroin into polymeric scaffolds also promotes the attachment, growth and spreading of mesenchymal stem cells (Li et al., 2006; Correia et al., 2012; Park et al., 2016). Silk fibroin degrades slowly with minimum negative impact on the surrounding tissues and has less immunogenic reactions than collagen or PLGA (Rockwood et al., 2011). A variety of polymers have been tested to prepare scaffolds in combination with silk fibroin for the tuning of diverse cellular functions including osteogenic differentiation, metabolism, proliferation and survival (Bhattacharjee et al., 2016). Fibroin is already present in the composition of implantable devices approved by regulatory agencies, such as SERI® (surgical scaffold for support and healing of soft tissues) (Mandal et al., 2012; Clemens et al., 2014).

The combination of organic and inorganic components could be an attractive approach to recapitulate the bone features since it may render bioinspired grafts with optimized performance. Hydroxyapatite (HA) is the main inorganic component of the natural bone matrix and is widely used in hard tissue repair (Ngiam et al., 2009; Wei et al., 2011; Tanodekaew et al., 2013; Chen et al., 2015) because of its good biocompatibility and high osteoconductivity (Chang et al., 2000). HA can also reinforce the mechanical properties of natural and synthetic polymers (Song et al., 2007). Nevertheless, the size of the HA granules plays a relevant role in the erosion rate, so that large HA particles endow the scaffolds with poor mechanical properties and too slow resorbability (Cunniffe et al., 2016). Differently, nanosized HA (nHA) particles are easily degraded by osteoblastic enzymes such as alkaline phosphatase (ALP) (Favi et al., 2016) and can be directly used by osteoblasts to form new tissue (Liu et al., 2009). nHA is also reported to enhance cell adhesion and proliferation as well as to promote calcium deposition, especially when combined with silk fibroin (Liu et al., 2009; Venkatesan and Kim, 2014).

The aim of this study was to develop porous composite scaffolds containing PCL, fibroin and/or nHA using a simple, straightforward and reproducible processing method. $scCO_2$ foaming procedure allowed the scaffold processing in one-step, without using organic solvents and avoiding waste of materials (100% yield). It should be noted that the scaffolds did not include any growth factor or additional active substance in order to elucidate the role that fibroin and nHA in separate and when used together may play in the scaffold performance and the feasibility of development of affordable grafts for bone tissue regeneration. The scaffolds were evaluated *in vitro* regarding the mechanical properties and degradation profile of the scaffolds. Then, the attachment, proliferation and differentiation of MC3T3 pre-osteoblastic cells were evaluated. Finally, the scaffolds were tested *in vivo* for calvarial bone critical defect model in Sprague-Dawley rats, and bone regeneration was evaluated 7 and 14 weeks after implantation. InductOs kit, a commercial collagen-based bone scaffold used for the localized administration of recombinant human Bone Morphogenetic Protein-2 in degenerative disc disease and acute tibia fractures in adults, was used as a reference material (Pekkarinen et al., 2006).

2. Materials and methods

2.1. Materials

PCL (Mw 50 KDa) was from Polysciences (USA); CO_2 (99.9% purity) from Praxair (USA); Alpha Minimum Essential Medium (αMEM) and penicillin/streptomycin from Gibco (Life Technologies, The Netherlands); fetal bovine serum (FBS) and trypsin-EDTA solution (0.25 vol.%) from Sigma-Aldrich (USA); MC3T3 cells from *Mus musculus* (ATCC CRL-2593) from LGC Standards (Spain); and bicinchoninic acid protein quantification assay (BCA) from Thermo Fisher Scientific (USA). Purified water (18.2 MΩ cm at 25 °C) was obtained by reverse osmosis (Milli-Q®, Millipore, USA). All other reagents were analytical grade.

2.2. Fibroin and nHA preparation

For the preparation of silk fibroin, cocoons of silkworms were chopped in 4 or 5 pieces and boiled in 0.02 M Na_2CO_3 for 30 min to remove the glue-like sericin proteins. Then, raw fibroin was rinsed thoroughly with water and dried at room temperature for 3 days. The extracted fibroin was dissolved in 9.3 M LiBr (Acros Organics, Spain) for 3 h at 60 °C to generate a 20% w/v solution that was dialyzed against distilled water for 3 days (Snakeskin Dialysis Tubing 3.5 kDa MWCO, Thermo Scientific, USA) with 8 water exchanges. The resulting 8% w/v fibroin solution was recovered and filtered. Then, silk fibroin was sonicated for 2 min, crosslinked at 37 °C (2–3 days) (Wang et al., 2008) and freeze-dried. Fibroin was finally milled in a mortar to obtain micrometric particles.

nHA was prepared following a method described elsewhere (Ramay and Zhang, 2004). Briefly, a 0.37 M $(NH_4)_2HPO_4$ aqueous solution (400 mL) was slowly poured (1.5 mL/min) into a 0.81 M Ca $(NO_3)_2$ aqueous solution (300 mL, pH 10.4 adjusted with NH_4OH) under vigorous stirring (1200 rpm). Then, the system was stored for 4 days and the white precipitate was recovered by centrifugation at 400 rpm nHA was washed with water until the pH decreased to 7, and 1-butanol was added to prevent nHA from aggregation during the drying process. Finally, the nHA was dried at 80 °C followed by calcination at 500 °C in a furnace for 4 h at a heating rate of 10 °C/min. XRD and FTIR-ATR were performed to confirm the structure of the obtained nHA.

2.3. Scaffolds preparation

PCL, fibroin and nHA at different weight ratios (Table 1) were mixed using a Turbula® (WAB AG Maschinenfabrik, T2C, Switzerland) for 5 min. Weighed amounts of the mixtures (1.8 g)

Table 1
Composition of the mixtures (in weight percentage) that underwent the supercritical processing, and pore structure and mechanical properties of the scaffolds expressed as mean values and, in parenthesis, standard deviation.

Scaffold	PCL	Fibroin	nHA	ε (%)	ε-MIP (%)	ρ_{app} (g/cm^3)	ρ_{skel} (g/cm^3)	ε (MPa)
PCL	100	–	–	68.2 (0.3)	44.9	0.375 (0.003)	1.137 (0.003)	42.79 (2.17)
PCL-Fibroin	85	15	–	67.5 (0.2)	41.0	0.362 (0.002)	1.154 (0.004)	39.42 (2.56)
PCL-nHA	90	–	10	68.2 (0.3)	47.0	0.360 (0.003)	1.178 (0.004)	55.84 (3.27)
PCL-Fibroin-nHA	70	20	10	69.7 (0.2)	49.7	0.369 (0.003)	1.219 (0.004)	50.34 (0.85)