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Novel sintering-free scaffolds obtained by additive manufacturing for concurrent bone regeneration and drug delivery: Proof of concept

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ABSTRACT

Advances on the fabrication of sintering-free biphasic calcium phosphate (BCP)/natural polymer composite scaffolds using robocasting as additive manufacturing technique are presented in the present work. Inks with high amounts of BCP powders (45 vol%) containing different HA/β-TCP ratios, in presence of crosslinked polymer, were successfully fine-tuned for extrusion by robocasting. The non-existence of sintering step opened the possibility to obtain drug loaded scaffolds by adding levofloxacin to the extrudable inks. The drug presence induced slightly changes on the rheological behaviour of the inks, more emphasized for the BCP compositions with higher amounts of β-TCP, and consequently, on the microstructure and on the mechanical properties of the final scaffolds. The strong interaction of β-TCP with chitosan difficult the preparation of suitable rheological inks for printing. Drug delivery studies revealed a fast release of levofloxacin with a high burst of drug within the first 30 min. Levofloxacin loaded samples also presented bacteria growth inhibition ability, proving that antibiotic was not degraded during the fabrication process and its bactericidal efficacy was preserved. From the results obtained, the composite scaffolds containing higher amounts of HA (around 80% HA/20% β-TCP) constitute a promising bi-functional synthetic bone substitute for simultaneous local bone regeneration and infection treatments.

1. Introduction

Bone defects caused by several ways, such us, traumatic accidents, tumour resection or osteoporosis, demand major health care challenges to biomedical science and repairing technologies. Also, therapies for bone associated infections often require the removal of infected bone, treating the infection around, and filling the left bone space with a bio-regenerative material [1]. Combined efforts of medical practitioners and material scientists have been undertaken to enable fabrication of bone-regenerative scaffolds incorporating biomolecules with clinically important functionalities as reported in several recent works [2–9]. For instance, addition of antibiotics or chemotherapeutic drugs in the scaffolds could prevent bacterial infection and prevent cancer recurrence, respectively [2–9]. Therefore, as mentioned by Fernandes et al. [10], finding an implanted biomaterial that smartly combines both therapy functions of drug-eluting and regeneration will bring lots of benefits not offered by the conventional medical practice. This challenging approach is likely to become a compulsory target in the near future [10].

A number of biopolymers, such as polycaprolactone (PCL) and poly (lactic-co-glycolic acid, PLGA) have been studied for this purpose [11,12]. However, the exclusive use of polymer scaffolds has shown limited success because of their hydrophobicity, difficulty to control drug release, and low osteoconductivity [2]. Calcium phosphate (CaP) based materials are still the best choice to repair damaged bones [13]. CaP are osteoconductive, might acquire osteoinductive properties

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[14,15], being the most adequate type of material for controlled drug delivery. The trend is therefore finding an optimal combination of biomaterials to obtain tuneable drug-delivery bone scaffolds with proper mechanical strength [1,2,12,16]. Biphasic calcium phosphates (BCP) have been widely investigated for several purposes [17]. As the name suggests, BCP consists of two calcium phosphate phases, commonly a more stable phase (HA) and a more soluble one (β-TCP), assorted in different proportions. This phase's combination offers some advantages over the pure phases, enabling a better control of bioactivity and biodegradation and assuring the stability of the biomaterial while promoting bone ingrowth.

Besides based material, scaffold's architecture on the macro-, micro- and nanoscale is crucial for proper nutrient and waste transport, cellular interactions, mechanical stability, drug delivery and ultimately functional tissue formation [1,11,18]. Mimicking structural and functional properties of bone that could guarantee biochemical affinity with host tissues, as well as, similar response under load exhibited by natural bone is a challenging goal [18–20]. Bone substitutes' architectures presenting interconnecting pores with proper sizes are essential to promote tissue integration and vascularisation. Additive manufacturing (AM) appears as an emergent and promising technique in regenerative medicine, essentially for bone substitute's fabrication, where a precise control of pore shape and size, as well as customized scaffolds to the final size and shape of implant site are possible [19–24]. Robocasting, also called direct write assembling (DWA) was developed for ceramics and uses extrudable aqueous ceramic inks at room temperature that are commonly obtained by mixing ceramic powders in water with few amounts of organic additives [21,23,24]. However, after printing, all green CaPs based scaffolds are commonly submitted to a sintering step at high temperatures (1100–1200 °C) to obtain suitable mechanical properties. This hinders the incorporation of temperature sensitive bioactive molecules in the extrudable ink before sintering. Because of that, bioactive molecules for different therapeutic applications can only be added after sintering by soaking the scaffolds in suspensions with suitable concentrations of the desired biomolecules or drugs [3–5,7,8]. This means that impregnation process limits the amount of biomolecules incorporation to the absorption capacity of the scaffolds, as well as to their surface properties. However, the loading output of the process is very low, since a highly concentrated solution must be prepared in order to incorporate a small amount of drug in the scaffold, and hence the process is neither very well controlled and nor cost-effective. To overcome these difficulties, sintering-free scaffolds fabrication could be the ground-breaking solution, opening the possibility to easily construct bio-inspired and multifunctional ceramic based bone substitutes. F.J. Martínez-Vázquez et al. [16] published the first results related to the sintering-free drug-loaded Si-doped HA composite scaffolds fabricated by robocasting. The biological behaviour and biodegradability of these hybrid (Si-doped HA + gelatin) scaffolds was greater than that of sintered inorganic Si-doped HA scaffolds. But the overall targets are still far from being satisfactorily accomplished. The main drawback was related to the low inorganic contents of inks that strongly limited the filaments strength during printing process and the final mechanical properties of the scaffolds. Optimizing the rheological properties of the inks to obtain appropriate extruded filaments is a key step to fabricate scaffolds by robocasting.

Considering all the constraints stated in literature review and having ahead the future requirements to improve life quality of patients with bone diseases, the main goal of the present work is to develop calcium phosphate based scaffolds, with simultaneous activity on infection and regenerative therapies. This target will be achieved by combining pioneering manufacture specifications, including: (1) preparation of drug-loaded high concentrated calcium phosphate based inks with proper rheological performance, (2) 3D-printing by robocasting technique and (3) sintering stage elimination. The model drug selected for this study was levofloxacin, an antibiotic successfully used in the treatment of bone infection, due to its ability to penetrate into trabecular and cortical bone minimizing the risk of resistance selection [25,26]. The drug delivery behaviour and the antibacterial activity of the resultant 3D scaffolds were also evaluated.

2. Experimental section

2.1. Synthesis and characterization of biphasic calcium phosphate powders

The synthesis of CaP-based powders by aqueous precipitation requires a close control of many parameters such as, reaction pH, ripening time, temperature and stoichiometry of the raw materials [27]. Since the first step of this work was obtaining biphasic calcium phosphate powders (BCP) with different proportions of HA and β-TCP phases, and considering that higher Ca/P ratios and pH values promote the formation of HA phase [28,29], the initial Ca/P ratios and pH values were accordingly varied.

BCP powders with Ca/P ratios of 1.50, 1.59 and 1.65 were obtained via aqueous precipitation, according to a procedure described in a previous work [30]. Briefly, 1.2 M di-ammonium hydrogen phosphate solution [$(NH_4)_2HPO_4$, Panreac, Spain] was added to a calcium nitrate tetrahydrate [$Ca(NO_3)_2.4H_2O$, Panreac, Spain] solution with the concentration required for the planned Ca/P ratio. The reaction occurred at 90 °C for 2 h under constant stirring conditions (1000 rpm) at pH 8, 8.5 and 9, for synthesis of BCP 1.50, 1.59 and 1.65, respectively. These pH values were maintained by adding the required amounts of 8 M ammonium hydroxide solution [NH_4OH, Sigma-Aldrich, Germany]. After the synthesis, the precipitates were separated through vacuum filtration and dried at 100 °C overnight. The prepared powders were calcined at 1100 °C in a Thermolab furnace (Pt30%Rh/Pt6%Rh thermocouple) using a heating rate of 5 °C min^{-1} and 2 h dwelling time at the maximum temperature, followed by cooling to room temperature (RT). The calcined powders were then dry milled for 45 min in a high energetic ball milling up to achieving a mean particle size ~1 μm.

The collection of XRD data for Rietveld refinement studies was performed using High Resolution X-ray Diffractometer (PANalytical X'Pert PRO) with Cu Kα radiation (λ = 1.5406 Å) produced at 45 kV and 40 mA, between 20° and 80° diffraction angles (2θ) with a step size of 0.013° and 200 s time per step. The software TOPAS version 4.2 (Bruker AXS, Karlsruhe, Germany) with the fundamental parameter approach, and the ICDD card numbers of # 04-015-7245 for HA [31] and #04-006-9376 for β-TCP [32] were used for Rietveld refinements.

The particle size and particle size distributions of all powders were measured using a particle size analyser (COULTER LS230, UK) with Fraunhofer optical model. The specific surface area of the powders were measured by the Brunauer–Emmett–Teller (BET) method using a Micromeritics Gemini 2370 V5.00 (Norcross, USA) through the nitrogen gas adsorption after degassing the samples in a Micromeritics Flow Prep 060 (Norcross, USA). Powder morphology was also observed by Scanning Electron Microscopy (SEM, Hitachi S4100, Hitachi High-Technologies Europe, GmbH, Germany).

2.2. Inks preparation and characterization

The first step of ink preparation started with fine tuning the temperature and time dependent gelling/reticulation behaviour of the BCP/chitosan solution system in presence of the crosslinking agent genipin (Challenge Bioproducts, Taiwan) by rheological measurements. A chitosan (low molecular weight, Sigma-Aldrich, Germany) 3 wt% solution was prepared in 0.5 vol% of acetic acid glacial (Pronalab, Portugal) solution at room temperature (RT) overnight under constant stirring. Since cross-linking of chitosan can also occur in presence of only CaP powders [33], the inorganic component in these preliminary studies was fixed at 20 vol% to assess the effects of the other variables. BCP-1.65 powder was selected for these experiments. The test methods employed included temperature, time, stress, and frequency sweeps in oscillatory mode at a constant temperature of 37 °C, except for the