Full length article

3D printing of hierarchical porous biomimetic hydroxyapatite scaffolds: Adding concavities to the convex filaments

Joanna Konka\textsuperscript{a,b}, Judit Buxadera-Palomero\textsuperscript{a,b}, Montserrat Espanol\textsuperscript{a,b}, Maria-Pau Ginebra\textsuperscript{a,b,c,\ast}

\textsuperscript{a} Biomaterials, Biomechanics and Tissue Engineering Group, Department of Materials Science and Engineering, Universitat Politècnica de Catalunya (UPC), Av. Eduard Maristany 16, 08019 Barcelona, Spain

\textsuperscript{b} Barcelona Research Center in Multiscale Science and Engineering, Universitat Politècnica de Catalunya (UPC), Av. Eduard Maristany 16, 08019 Barcelona, Spain

\textsuperscript{c} Institute for Bioengineering of Catalonia (IBEC), The Barcelona Institute of Science and Technology, Baldori Reixac 10-12, 08028 Barcelona, Spain

A R T I C L E  I N F O

Article history:
Received 21 February 2021
Revised 22 July 2021
Accepted 29 July 2021
Available online 4 August 2021

Keywords:
3D printing
Hydroxyapatite
Gelatin
Biomimetic
Concavity, Bone Regeneration
Porous filament

A B S T R A C T

Porosity plays a key role on the osteogenic performance of bone scaffolds. Direct Ink Writing (DIW) allows the design of customized synthetic bone grafts with patient-specific architecture and controlled macro-porosity. Being an extrusion-based technique, the scaffolds obtained are formed by arrays of cylindrical filaments, and therefore have convex surfaces. This may represent a serious limitation, as the role of surface curvature and more specifically the stimulating role of concave surfaces in osteoinduction and bone growth has been recently highlighted. Hence the need to design strategies that allow the introduction of concave pores in DIW scaffolds. In the current study, we propose to add gelatin microspheres as a sacrificial material in a self-setting calcium phosphate ink. Neither the phase transformation responsible for the hardening of the scaffold nor the formation of characteristic network of needle-like hydroxyapatite crystals was affected by the addition of gelatin microspheres. The partial dissolution of the gelatin resulted in the creation of spherical pores throughout the filaments and exposed on the surface, increasing filament porosity from 0.2 % to 67.9 %. Moreover, the presence of retained gelatin proved to have a significant effect on the mechanical properties, reducing the strength but simultaneously giving the scaffolds an elastic behavior, despite the high content of ceramic as a continuous phase. Notwithstanding the inherent difficulty of in vitro cultures with this highly reactive material an enhancement of MG-63 cell proliferation, as well as better spreading of hMSCs was recorded on the developed scaffolds.

Statement of significance

Recent studies have stressed the role that concave surfaces play in tissue regeneration and, more specifically, in osteoinduction and osteogenesis. Direct ink writing enables the production of patient-specific bone grafts with controlled architecture. However, besides many advantages, it has the serious limitation that the surfaces obtained are convex. In this article, for the first time we develop a strategy to introduce concave pores in the printed filaments of biomimetic hydroxyapatite by incorporation and partial dissolution of gelatin microspheres. The retention of part of the gelatin results in a more elastic behavior compared to the brittleness of hydroxyapatite scaffolds, while the needle-shaped nanostructure of biomimetic hydroxyapatite is maintained and gelatin-coated concave pores on the surface of the filaments enhance cell spreading.

© 2021 The Authors. Published by Elsevier Ltd on behalf of Acta Materialia Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/)

1. Introduction

The recent breakthroughs in the additive manufacturing technologies have opened up new possibilities in the field of bone grafting, enabling the fabrication of synthetic bone grafts with
patient-specific architectures [1,2]. However, as important as the external shape of the implant is, the internal pore architecture of the scaffold plays an essential role as well [3,4]. There is a general agreement that there is a need of interconnected pores ranging from 50 to 500 μm to promote cell migration and vascularization [5,6]. In this respect, direct ink writing (DIW), which consists in extruding a paste through a nozzle following a pre-designed pattern, allows an accurate control of pore dimensions, and therefore seems to be an appropriate technique to design scaffolds meeting this requirement. However, not only pore dimensions are relevant. Other geometrical parameters are known to have direct implications in the biological performance of the scaffolds. In this respect, we recently demonstrated that calcium deficient hydroxyapatite (CDHA) foams, with an open network of concave pores induced a significantly higher amount of bone in a canine model, both ectopically and orthotopically, than the same CDHA obtained by DIW, which resulted in arrays of cylindrical rods with convex surfaces [7,8]. In the latter, bone formation initiation occurred in the concavities of the turning of filaments or in the crossings of the filaments. Thus, although robocasting allows controlling the macroporosity and pore shape of printed scaffolds, the fact that microextrusion results in convex surfaces is a limitation as far as bone regeneration and osteoinduction are concerned. Hence, there is a growing need for further improvements in scaffold manufacturing processes by 3D-printing that would take into account the biological effects mentioned above. With this in mind, the aim of this work was to develop a method to introduce concave surfaces into calcium phosphate scaffolds obtained by DIW.

The possibility of incorporating additional porosity into the filaments obtained by DIW in ceramics for a variety of application was previously tackled by refining the DIW method or combining it with other processing techniques. Feng et al. [9] developed a method to obtain multi parallel channel structures by modifying the nozzle used in DIW. Although this strategy allowed obtaining continuous aligned concave channels inside the filaments, the pores were not exposed on the surface of the filaments and lacked interconnectivity. A different approach was proposed by Moon et al. [10], who used camphene dendrites as sacrificial material in alumina suspensions for generation of interconnected macropores after eliminating them via freeze-drying. This resulted in anisotropic macropores with little to no control of their geometry. Minas et al. [11] combined DIW with emulsion/foam templating. They developed ultrastable alumina emulsions and foams, suitable for DIW, through the modification of alumina powders with short amphiphiles. In the latter two cases a post-printing thermal treatment was required to sinter the ceramic particles.

In the current study, we propose to use a self-setting ink based on calcium phosphates, which does not require a high-temperature sintering process. Different formulations have been proposed, based on α-tricalcium phosphate (α-TCP) and polymeric binders [12–17] that harden at low-temperature by a hydrolysis reaction resulting in a calcium deficient hydroxyapatite (CDHA). They have several advantages, in the sense that not only do they result in a composition and structure very close to the mineral phase of bone [12,13,18], but also that the mild processing opens the door to developing hybrid structures, incorporating organic components like extracellular matrix proteins, other biomolecules or even cells [12,14,15,17]. We propose to take advantage of this feature to incorporate gelatin microspheres as a sacrificial material in the DIW printing paste. The starting hypothesis is two-fold. On the one hand, that the release of the gelatin may result in the formation of concave pores with controlled size and distribution in the convex filaments, and on the other hand, that the presence of the retained gelatin may result in an enhancement of the mechanical and biological properties of the CDHA scaffolds. This strategy has the benefit that can be easily scaled up by increasing the size of gelatin microspheres and filament diameter. Thus, theoretical pore size ranges which are reported for optimum bone ingrowth to be between 50 to 500 μm [5,6], might be achieved achieving the processing parameters during microspheres synthesis and the printing conditions.

2. Materials and methods

2.1. Preparation and characterization of gelatin microspheres

Gelatin microspheres were synthesized by modifying previously mentioned methods [19–21]. In this work, 40 mL of either 10 (w/v) % or 15 (w/v) % aqueous solutions of gelatin type-B (Rousselot® 250 LB8, France) at 50 °C were added dropwise to 400 mL olive oil previously heated up to 50 °C under continuous stirring at 1000 rpm to obtain gelatin microspheres of small-size (S-μs) or large-size (L-μs) respectively. The two MS sizes were chosen to explore their effect on the final properties of the scaffold. After 10 minutes, the solution was put in an ice bath for 30 min under constant stirring to allow for the gelation of the microspheres. Thereafter, 400 mL of cold acetone (4 °C) were added to the mixture and stirred for another hour. Additional 200 mL of acetone at 4 °C were added to the solution and stirred for 5 minutes to facilitate subsequent filtration. Upon filtration with the use of a table bench filter paper the microspheres were rinsed with acetone, air dried and sieved. The S-μs were sieved with a 40 μm sieve (Fritra, Spain) and L-μs using combined sieves of 40 μm and 100 μm. The microspheres were used as-prepared and without any cross-linking step to allow their gradual dissolution.

The size distribution of the microspheres was analyzed by laser diffraction (Mastersizer 3000, Malvern Instruments, UK). The dispersing media used was 96 % ethanol and the measurements were taken under constant mechanical stirring with previous sonication. The particle refraction and absorption indices were set to 1.54 and 0.01, respectively. The swelling behavior of the microspheres was determined by optical microscopy comparing the size of the microspheres soaked in water (swelled) with microspheres soaked in ethanol (non-swelled).

2.2. Self-setting ink preparation

The solid phase of the self-setting ink was composed of gelatin microspheres (5-μs or/and L-μs) and α-tricalcium phosphate (α-TCP, Ca₃(PO₄)₂), the preparation of which was described previously [22]. Shortly, to obtain α-TCP a 2:1 molar mixture of CaHPO₄ (Sigma Aldrich, St. Louis, USA) and CaCO₃ (Sigma Aldrich, St. Louis, USA) was sintered at 1400 °C for 15 hours and thereon rapidly quenched to room temperature. Milling was carried out with an agate ball mill (Pulverisette 6, Fritschi GmbH) to obtain a fine α-TCP powder, with median particle size of 4.02 μm. The milling sequence was as follows: the powder was first milled with 10 balls (d = 30 mm) for 40 min at 450 rpm and then for 60 min at 500 rpm, followed by the third milling for 60 min at 500 rpm with 100 balls (d = 10 mm). The resulting powder was mixed with 1.5 mm diameter Zirconia balls (Tosoh, Japan) and sieved with a 40 μm sieve (Filtrà, Spain). The liquid phase was composed of a 30 (w/v) % poloxamer 407 solution (Kolliphor® P 407, BASF pharmaceuti-
cals, Germany), to which an amount of distilled water was added, depending on the amount of gelatin microspheres contained in the powder, with a weight ratio of 3:2 (distilled water: gelatin microspheres), in order to compensate for the thickening caused by the absorption of water by the gelatin. Five different ink formulations were prepared, as summarized in Table 1 together with the corre-
spanding abbreviations further used in this study.

Firstly, the α-TCP and gelatin microspheres were mixed at 1000 rpm for 10 s in a dual asymmetric centrifugal mixer (DAC 150,
Speedmixer, USA). Afterwards, the liquid phase was added and mixed at 2050 rpm for 10 s and 3500 rpm for another 10 s. This protocol was repeated twice.

2.3. Computer-aided design and DIW process

A cylindrical model of 6 mm diameter x 12 mm height was designed by using computer-aided design (CAD) software (Solidworks, Dassault Systems, France) and exported to an .stl file. The .stl model was imported to Simplify3D (www.simplify3d.com) to program the printing parameters and generate gcode for numerical control of the 3D printer. The orthogonal pattern, corresponding to 90° rotation between each layer, was used. For each ink composition the printing nozzle had 410 µm diameter (Nordson EFD, USA). The layer height was set to 320 µm, the infill density to 50 %, which corresponded to a distance between filaments of 410 µm (XY plane) and an overlap of 20 % (Z axis). The deposition speed was 12.5 mm s⁻¹.

Here the printing process will only be mentioned briefly as it follows the procedure established before [13]. Firstly, the cartridge (3cc Optimum® Syringe Barrels, Nordson EFD, USA) was filled with the self-setting ink and was further coupled with a tapered dispensing tip (Gauge 22 SmoothFlow Tapered Tips, Nordson EFD, USA). Thereon the assembly was installed in the 3D printer (Paste caster, Fundació CIM, Spain) and triplicate batches of 16 scaffolds for each condition were robocasted. The fabricated scaffolds were stored 24 h in a water vapor saturated atmosphere at 37 °C to ensure cohesion of the samples, and subsequently immersed in distilled H₂O at 37 °C for 6 days to allow for the transformation of α-TCP to CDHA. The hardened scaffolds were stored at room temperature in a desiccator to prevent humidification of the samples.

2.4. Scaffolds characterization

2.4.1. Phase composition, microstructure characterization and gelatin microspheres content

The phase composition was assessed by X-ray diffraction (XRD) using a D8 Advance diffractometer (Bruker, USA). Scanning was performed in the Bragg–Brentano geometry using Cu Kα radiation with the following conditions: 2θ scan range of 20–50°, with a scan step of 0.02°, and a counting time of 2 s per step at 40 kV and 40 mA. The crystallite size of the 3D scaffolds was calculated by using the Scherrer’s equation as follows (Eq. 1):

$$X_{\text{cr}} = \frac{\beta_{1/2}}{\cos \theta}$$  \hspace{1cm} (1)

Where $X_{\text{cr}}$ is the crystallite size (nm), λ is the wavelength of monochromatic X-ray beam (nm) (0.15406 nm for Cu Kα radiation), $\beta_{1/2}$ is the full width at half maximum for the diffraction peak under consideration (rad), θ is the diffraction angle (in degrees), and K is a constant varying with crystal habit and here set to 0.9.

The morphology of the pores in the filaments and the microstructure of the specimens were characterized by scanning electron microscopy (Phenom XL Desktop SEM and JEOL JSM-7001F) with an acceleration voltage of 15 kV (BSD) and 2kV (SED). Previously, the surface of the samples was coated with a carbon evaporated layer using EMITECH K950X.

The specific surface area (SSA) was determined by Nitrogen sorption using the Brunauer-Emmett-Teller (BET) method (ASAP 2020, Micromeritics, USA). Prior to the measurements, the samples were outgassed at a holding temperature of 100 °C for 2 h in vacuum conditions at 10 mmHg.

Thermogravimetric analysis (TGA) was performed to assess the amount of gelatin remaining in the printed specimens after the consolidation process (n=3 for each condition). The analyses were performed under the heating rate of 15 °C min⁻¹ from 25 to 800 °C in an oxygen-saturated atmosphere. DTG (derivative thermogravimetry) curves were calculated to determine the inflection points of the peaks corresponding to the starting and ending point of gelatin decomposition. The amount of gelatin contained in the scaffolds was determined by comparing the curves of each sample with those of the control scaffold between 200 and 700 °C. The amount of poloxamer 407 in the control was obtained by subtracting from the weight loss in the control the corresponding weight loss for a poloxamer 407-free CDHA counterpart, consisting of α-TCP mixed with water with the same L/P ratio and subjected to the same treatment. To estimate the amount of gelatin released, the values obtained were compared to the nominal amounts of gelatin, poloxamer 407, and CDHA in the samples, calculated on the basis of the composition (Table 1).

Confocal Raman microscopy (inVia™ Qontor® confocal Raman microscope, Renishaw Inc.) was used to determine the gelatin content distribution in the cross-sections and surfaces of the scaffolds. Raman mappings were performed with resolution of 20 µm x 20 µm (cross-sections) and 20 µm x 10 µm (surfaces). Mappings of the whole cross-section of individual filaments were made, and an area of 160 µm x 200 µm on the filament surface was mapped as well. The Raman spectra were obtained using x50L (long working distance) objective, 785 nm laser source with 1200 l/min (vis) grating, 150 mW laser power, 0.2 s exposure time, and 25 accumulations. The spectra were treated and analyzed with WiRE 4.4 software. Contour maps were obtained by calculating the ratio between the intensity of the proline domain in gelatin (at ~820 cm⁻¹) [23] to phosphate mode 1 (~962 cm⁻¹) [24] domain in hydroxyapatite. Hence, the increase in the ratio was equal to increase in relative gelatin content for the collected spectra.

Porosity, pore architecture and pore size distribution

The apparent density ($\rho_{\text{app}}$) of the scaffolds was calculated as the average from triplicate results of the mass of the scaffold over its volume, which was obtained measuring the diameter and height of the cylinders with a caliper. The skeletal density ($\rho_{\text{ske}}$) was determined by helium pycnometry (AccuPyc 1330, Micromeritics, USA). The total porosity ($P_{\text{tot}}$) was determined according to Eq. (2) [13,25]:

$$P_{\text{tot}}(\%) = \left(1 - \frac{\rho_{\text{app}}}{\rho_{\text{ske}}}\right) \cdot 100$$  \hspace{1cm} (2)

To visualize the scaffolds’ architecture and quantify their porosity, X-ray computed microtomography (µ-CT) analyses were per-
formed with the SkyScan 1272 microtomograph (Bruker, USA) equipped with a 100 kV/20W maximum power X-ray generator and 11MP X-ray detector. The tomography measurements were carried out with an acceleration voltage and current of 90 kV and 111 μA respectively. The generated beam was filtered with a 0.5 mm thick aluminum and 0.038 mm copper filter. Projection images were collected every 0.2 ° over 360 ° with an exposure time of 8800 ms. The isotropic voxel size (voxel resolution) of the obtained volume was 1 μm. Thereafter, the collected scans were used for reconstructions of samples volume by adjustment of alignment, beam hardening correction, ring artifacts filtering, and image smoothing. The reconstruction procedure was carried out with NRecon reconstruction software (Micro Photonics Inc., USA). Further analysis of collected volume slices was conducted with Python 2.7 and 3.7 (http://www.python.org). Scipy and skimage libraries were used for binary segmentation and calculation of pores volume. Classification between concave pores in the filaments and orthogonal DIW-origin macropores was estimated by using iteration operations over the pore volume array with pore size cut-off between the two regions. Volume rendering of the binary μ-CT slices was executed with 3D slicer (www.slicer.org) software [26] and visualized with Autodesk Meshmixer (www.meshmixer.com).

The open porosity and pore entrance size distribution were characterized by mercury intrusion porosimetry (MIP, AutoPore IV, Micromeritics, USA). The filament porosity was calculated based on the MIP measurements, from the ratio between the volume of mercury intruded in pores smaller than 100 μm and the volume of the filaments, determined by μ-CT.

2.4.2. Mechanical characterization

Uniaxial compressive tests were carried out in a universal testing machine (BIONIX, MTS Systems Corporation, USA) with 2.5 kN load cell. The load was applied in the Z direction, i.e. perpendicular to the printing plane (XY), at a rate of 1 mm min⁻¹. Control samples -- free of gelatin, and scaffolds with different amounts of gelatin microspheres were tested. All measurements were performed in wet state to mimic the in vivo situation. For each condition 12 scaffolds were tested.

Moreover, cyclic loading was performed on the S30 specimens. The measurements were carried in a hybrid rheometer (Discovery RH-2, TA Instruments, USA): 6 samples in wet state were subjected to 10 compression cycles, between the point of unloading (F = 0 N) and a 25 % strain, applied perpendicular to the printing plane (XY) at a rate of 3 mm min⁻¹.

2.4.3. In vitro response of human mesenchymal stem cells (hMSCs) and human osteosarcoma MG-63 cells

Human mesenchymal stem cells were expanded in Advanced Dulbecco’s Modified Eagle’s Medium (advDMEM) and human osteosarcoma MG-63 cells were expanded in Dulbecco’s Modified Eagle’s Medium (DMEM). Both cell culture media were supplemented with 10% foetal bovine serum (FBS), 2 mM L-glutamine, 50 U ml⁻¹ penicillin, 50 mg ml⁻¹ streptomycin, (all from Invitrogen, USA). Two types of robocast scaffolds were tested, namely, the free-gelatin control and the S30 series, using cylindrical specimens 2 mm in diameter and 6 mm in height. The following steps were followed for both hMSCs and MG-63 cells. Firstly, the scaffolds and the cover slips used as control for the immunostaining were sterilized in 70 % ethanol for 30 min, rinsed thrice with PBS, placed in 24-well plate and further preconditioned overnight in cell culture media. Afterwards, the scaffolds were placed in low-attachment well plates. Cells, hMSCs at a passage 6 and MG-63 (MG-63 ATCC CRL-1427) were seeded at respectively 5×10⁴ and 4×10⁵ cells per sample, and were allowed to attach during 4 h at 37 °C and 5 % CO₂. Then, 6 mL of medium were added to each sample. As a control, both hMSCs and MG-63 were seeded at a density of 1×10⁴ cells per well on the glass cover slips. The cells were cultured for 1, 3 and 7 days, with the medium being renewed every day. At each time point, the scaffolds were transferred to a new 48-well plate, washed thrice with PBS and immersed in PrestoBlue™ (Thermo Scientific, USA) during 1h according to manufacturer’s instructions to determine the cell number. A calibration curve with increasing number of cells was prepared. PrestoBlue™ was measured in the fluorescence mode at 560 nm for the excitation and 590 nm for emission with Synergy HTX multi-mode microplate reader (BioTek). The experiment was performed in triplicate.

Cell morphology was characterized by scanning electron microscopy (Phenom XL Desktop SEM) with an acceleration voltage of 15 kV (BSD). The scaffolds were placed in a new 24-well plate, three rinses with PBS were carried out and the cells were fixed in 2.5 % glutaraldehyde solution in PBS. Thereon, the samples were incubated for 1h at 4°C and subsequently washed thrice in PBS and dehydrated in 50, 70, 90, 96 and 100 % ethanol series. Subsequently, they were dried overnight at 37 °C and then the surface was coated with carbon sputtering using EMMTECH K950X.

Cell morphology and adhesion on TCPs, control, and S30 was studied by staining of cytoskeleton and nuclei [27]. Briefly, the cells were permeabilized with 0.05 % (w/v) Triton X-100 (Sigma Aldrich, USA) in PBS for 20 min and subsequently blocked with 1 % bovine serum albumin (BSA, Sigma Aldrich, USA) (w/v) in PBS for 30 min. Thereon, actin fibers were stained by 1h incubation in TRITC-conjugated phalloidin (1:300 in permeabilizing buffer), whereas incubation for 2 min in 4’, 6-diamidino-2-phenylindole (DAPI) (1:1000 in 20 mM glycine in PBS) was used for staining the cell nuclei. In between the steps, the samples were rinsed thrice for 5 min with PBS-glycine (Sigma Aldrich, USA). The cells on the scaffolds and the cover slips were observed with a confocal scanning laser microscope (Zeiss LSM 800, Carl Zeiss, Germany) using a 10x objective. The collected images were processed with Fiji/Image-J package and analyzed to quantify cell spreading and aspect ratio (n=15). To this end, cells with well-defined actin boundaries were marked manually with a freehand selection tool and added to ROI (region of interest) manager. The area was calculated using ImageJ measure Area tool and the aspect ratio as the end-to-end distance of each cell and its perpendicular axis at the longest points.

2.4.4. Statistics

Unless otherwise stated, the experiments were performed in independent triplicates, and the results are shown as mean ± standard deviation. The statistical analysis was performed using either t-student test or ANOVA with post-hoc Fisher’s LSD test with the significance level set at p-value < 0.05.

3. Results

3.1. Characterization of the gelatin microspheres

The particle size distribution of the gelatin microspheres in dry state is displayed in Figs. 1A and 1B. Both small and large gelatin microspheres showed normal distributions, with positive skewness. The median size of the particles, as indicated by D₅₀, were 34.8 ± 1.4 μm and 74.1 ± 2.5 μm for S-μs and L-μs respectively. The swelling behavior of the gelatin microspheres was observed under the optical microscope by immersing the microspheres either in ethanol or in water (Fig. 1C). Swelling was more pronounced for L-μs, whose diameter increased around thrice, whereas for the S-μs two-fold. Fig. 1C revealed also that L-μs were more homogenous in size, with lower tendency to form agglomerations.
3.2. Scaffold characterization: phase composition and microstructure

A specific characteristic of α-TCP based ceramic inks is that instead of a high temperature sintering process they harden through a hydrolysis reaction. In this study the samples were immersed in water at 37 °C to allow for this consolidation process. The X-ray diffraction patterns of the scaffolds revealed a single crystalline phase present, CDHA, which confirms the complete hydrolysis of the α-TCP powder (Supplementary Fig. S1). The reduction in crystallite size calculated by the Scherrer’s formula on the (002) diffraction peak indicated a decrease in crystallinity with the increase in the amount of gelatin microspheres in the scaffolds (Table 2).

During the immersion period, it is expected that simultaneous to the hydrolysis of α-TCP the dissolution and progressive release of the gelatin will take place. The quantity of gelatin remaining in the scaffolds after consolidation was measured by TGA (Fig. 2). In Fig. 2A, the TGA plot along with the DTG curve of pure gelatin microspheres is displayed, showing three main thermal degradation
events. The first event, from around 60 to 150 °C is associated with the loss of water [28]. The second one, from 280 to 380 °C, corresponds to the thermal decomposition of gelatin molecules to endothermic hydrolysis and oxidation reactions, and the third event, between 480 and 620 °C, is related to the combustion of the residual organic matter [28–30].

To assess the contribution of CDHA to the thermal events, the TGA curve of a CDHA sample obtained by the hydrolysis of an α-TCP paste without poloxamer was measured, showing a gradual weight loss due to the evaporation of surface-adsorbed water, below 200 °C, and further loss of the lattice water at higher temperatures [31,32] followed by the phase transformation of CDHA to β-TCP at 780 °C (Fig. 2A).

Fig. 2B shows the TGA plots of the control and the gelatin microspheres-containing scaffolds. In the case of the control scaffold, consisting of CDHA-poloxamer 407, three main thermal decomposition steps were observed. Two of them were consistent with those detected in pure CDHA, namely the one below 200 °C, attributed to the decomposition of surface-adsorbed water and that at 780 °C related to the transformation to β-TCP. However, between 200 and 500 °C another decomposition event was detected, which was superimposed to the loss of lattice water in CDHA, which can be associated to the degradation of poloxamer 407, as previously described [13].

The TGA and DTG curves for the scaffolds containing gelatin microspheres showed four thermal decomposition steps. The first one, below 200 °C was associated to the loss of adsorbed water, which increased with the amount of gelatin microspheres in the scaffolds. The second one encompassed both the decomposition of gelatin and poloxamer 407 and was most intense for the S30 scaffolds, followed by S15L15, S20 and S10. The third one corresponded to the degradation of the remaining gelatin chains and followed the same trend [28]. The last step corresponded to the phase transition of CDHA to β-TCP, having similar intensity for all the specimens and the control.

The comparison between the DTA curves obtained for the different scaffolds and the control allowed calculating the percentages of gelatin, poloxamer and CDHA contained in scaffolds after 6 days of immersion in water at 37 °C, as displayed in Fig. 3C. Moreover, these values were compared with the nominal composition of the scaffolds, to calculate the percentage of gelatin dissolution during scaffold reaction (Fig. 3D). For this calculation it was assumed that the amount of CDHA in the samples analyzed by TGA was equal to the nominal value (Supplementary Table S1). The highest rate of dissolution of gelatin microspheres was observed for S15L15 with a 56.1 % loss of gelatin after consolidation which was followed by S20 (46.5 %), S30 (38.8 %), and finally S10 (23.1 %). The poloxamer 407 binder amount for gelatin-free control was 1.9 % which constituted to 83.5 % loss as compared to the amount in the original ink.

The SEM images showing the morphology of the filaments, containing the pores generated by the partial dissolution of the gelatin microspheres after 6 days of immersion in water are presented in Fig. 3. The filament surfaces are shown in Fig. 3A and 3A’ and the filament cross-sections in Fig. 3B and 3B’. Most pores in the scaffolds containing gelatin microspheres are covered with a layer of gelatin. Moreover, a tendency not only towards increasing pore quantity, but also to increasing pore size was observed with increasing amounts of S-μS in the scaffolds. That is, S30 showed larger pores both on the surface and cross-section of the filaments than S20 and S10. The concave pores in S30 were more homogeneous in size and were also homogeneously distributed through

**Fig. 2.** (A) Thermogravimetry (TG) and derivative thermogravimetry (DTG) of pure gelatin microspheres and polymer-free CDHA, (B) TG and DTG of the CDHA control scaffold and scaffolds with different quantities of gelatin microspheres, (C) Calculated nominal composition (hashed bars) and composition after consolidation as calculated from TGA (no pattern bars) of scaffolds. Bars represent mean ± SD of triplicate TGA measurements for each condition. (D) Amount of the gelatin released (%) from the scaffolds after consolidation. Table S1 in the supplementary information shows the precise concentration values given in (C) and (D).
the filaments. Similarly to S30, S15L15 filaments had a significant quantity of pores. However, they were heterogeneous in size due to the incorporation of two different sizes of gelatin microspheres (S-μs and L-μs). In case of the free-gelatin control only a few isolated small pores were seen in the filaments cross-section but none on the surface. These observations were substantiated by the reconstruction of the 3D μ-CT models of the samples, as depicted in Fig. 3C, the cross-section μ-CT representative images (Supplementary Fig. S2) and the reconstruction videos (Supplementary Movies S1-S5). The porosity of the printed filaments clearly increased with growing amounts of gelatin microspheres, which gave rise to homogeneously distributed pores for S10, S20 and S30, and exhibited a more heterogeneous size distribution for S15L15.

The microstructure in the regions between the pores was similar for all the scaffold formulations, which exhibited the microstructure typical of self-setting α-TCP based inks, consisting of an entangled network of needle-like crystals [13,33], regardless of whether the ink contained gelatin microspheres or not (Fig. 3A' and 3B', and Supplementary Fig. S3). Only in areas close to the pores generated by the dissolution of gelatin microspheres the remnants of the polymeric matter in between the CDHA crystals were observed (Supplementary Fig. S3).

Raman microscopy mapping revealed an increase in the gelatin phase over the whole scanned area of the filaments with the increase in the amount of gelatin microspheres in the scaffolds (Fig. 4). Within the margins of the resolution used, the technique allowed to observe a gradient in the gelatin concentration, caused by the diffusion of gelatin from the microspheres into the ceramic matrix, more marked in the S30 specimens. This was also visible to some degree for S15L15, followed by S20, and S10. As expected, no gelatin was detected for the control. There was a good correlation with the optical microscopy images, the areas with major gelatin content measured by Raman corresponding to the pore concavities observable in the optical microscopy images.
The SSA of the CDHA scaffolds was very similar for all the conditions, as shown in Table 3, which is in good agreement with the microstructural observations (Fig. 3 and S3).

3.3. Porosity characterization

The characterization of the porosity of the scaffolds at different levels, total and filament porosity, is summarized in Fig. 5A and 5B. As expected, the total porosity, calculated on the basis of apparent and skeletal densities of the samples was the highest for S15L15 and S30, followed by S20, S10 and the control.

The μ-CT total porosity as the sum of robocast and filament porosity is represented in Fig. 5A. The 3D robocast architecture of the scaffolds was the same for the samples containing gelatin microspheres, whereas its value was slightly smaller for the free-gelatin control. As expected, the largest filament porosity was for the S30 specimen (33.60 %), followed by S15L15 (24.95 %), S20 (11.56 %), S10 (8.49 %), and the control (0.13 %). It has to be taken into account, however, that the μ-CT settings were adjusted to reach a resolution of 1 μm, and therefore pores smaller than that size are not accounted for by this technique. Moreover, the filament porosity relative to the total volume of the filaments as obtained by μ-CT is depicted in Fig. 5B. This allowed to discern the percentage of the filament volume occupied by the pores, ranging from 67.86 % for the S30 sample to 17.25 % for S10. As expected, the pore count in the filaments for the control was negligible.

The pore entrance size distribution, corresponding to the interconnections between the pores [31] within the filaments of the scaffolds determined by MIP is depicted in Fig. 5C. For the control scaffold a monomodal distribution in the 0–0.1 μm region was observed. The addition of gelatin microspheres into the CDHA scaffolds resulted in a broader and split peak that shifted towards larger sizes (< 1 μm) with the increase in the amount of gelatin microspheres in the structure. Moreover, for the samples containing microspheres an additional broad peak in the region between 5 to 100 μm appeared, which increased with the content of micro-

---

**Table 3**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>S10</th>
<th>S20</th>
<th>S30</th>
<th>S15L15</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSA (m²/g)</td>
<td>17.48 ± 0.64</td>
<td>17.15 ± 2.27</td>
<td>16.81 ± 3.14</td>
<td>17.55 ± 1.70</td>
<td>16.61 ± 2.57</td>
</tr>
</tbody>
</table>

---

Fig. 4. Confocal Raman microscopy mappings and corresponding optical microscopy images of mapped areas (whole area or marked with blue rectangles), showing gelatin content in the cross-sections and surfaces of filaments of control, S10, S20, S30, and S15L15.
spheres: S30–S15L15≈ S20–S10. In this pore size range the curve for the control was flat, indicating that the source of this open porosity for the rest of specimens was solely due to the incorporation of gelatin microspheres. The quantification of the open filament porosity, which includes the interconnected pores left upon partial/total gelatin dissolution and the intrinsic porosity arising from the entanglement of the needle-like CDHA crystals, is illustrated in Fig. 5B. The interconnected filament porosity was the highest for S20 (67 %) closely followed by S30 (64 %) and S15L15 (57 %). Interestingly, although the total open filament porosity was similar between S20 and S30/S15L15, the distribution of entrance pore sizes was different (Fig. 5C). In case of S30/S15L15 the contribution of 1–100 μm entrance pore sizes was higher and 0–1 μm smaller than for S20.

3.4. Mechanical characterization

Representative stress vs. strain plots for the compression tests for the control, S10, S20, S30, and S15L15 scaffolds are displayed in Fig. 6A. The mechanical deformation behavior was observed to change from a brittle behavior (control and S10), to a more ductile (S20), and finally an elastic behavior (S30 and S15L15) with increasing amounts of the gelatin microspheres in the CDHA scaffolds. In Fig. 6B the compressive strength, calculated as the maximum force divided by the nominal section of the scaffold, as well as the elastic moduli of the different specimens are shown. The control samples showed both the highest compressive strength and elastic modulus at an average of 2.49 ± 0.91 MPa and 162.37 ± 54.91 MPa respectively. The elastic modulus dropped with the addition of gelatin microspheres in the inks, up to 99 % and 98 % decrease for S30 and S15L15 respectively. The S15L15 (3.87 ± 4.14 MPa) exhibited high variability between replicates, resulting in higher standard deviation and not always purely elastic behavior, whereas S30 (1.64 ± 0.82 MPa) showed elastic behavior for all replicates. The change of elastic modulus for the other conditions was not as high, but the decrease was still substantial at 83 % (27.36 ± 6.16 MPa) for S20 and 67 % (53.90 ± 15.12 MPa) for S10. The compressive strength just like the elastic modulus dropped with the increasing concentration of gelatin microspheres in the samples up to 92 % for S30 and S15L15, 67 % for S20, and 38 % for S10. Fig. 6C shows the integrity of the different samples after the compressive testing. It can be observed that with the increase of the amount of gelatin microspheres in the CDHA structure their brittle breakage was avoided, especially for S30 and S15L15.

The elastic behavior of S30 specimens was analyzed under cyclic compressive loading. In Fig. 6D and 6E the compressive loading over 10 cycles in axial direction is shown in strain and time domain, respectively. The scaffolds showed elastic behavior over all the cycles and were capable of undergoing repeated loading without permanent deformation. A series of photos depicting one cycle is shown in Fig. 6F and Supplementary Movie S6.

To better understand the mechanical behavior of different scaffolds the samples subjected to the compressive testing were analyzed by SEM (Fig. 7) which revealed the presence of numerous cracks in the ceramic phase of the gelatin-containing scaffolds. Moreover, the presence of gelatin inside those cracks was also observed for all the gelatin containing scaffolds. It is speculated that the polymeric matter in the regions of damage maintained the integrity by attaching the broken pieces together preventing the collapse of the structures and causing their hydrogel-like behavior. This was especially noticeable for S30 and S15L15 where a significant number of cracks in the cement structure was visible, nevertheless, the scaffolds remained whole on the contrary to control which broke into pieces (Fig. 6C). This effect was already observed to certain extent for S10, and was more pronounced with the increase in the amount of gelatin microspheres in the scaffolds.
Fig. 6. (A) Stress vs. strain representative plots for control, S10, S20, S30, and S15L15 scaffolds. (B) Calculated ultimate compressive strengths and elastic modulus for the different specimens. The series labeled with the same letter or number are not statistically different (p > 0.05) when comparing respectively the compressive strength and elastic modulus between the different specimens. (C) Images illustrating the integrity of samples after the compression testing: from the left control, S10, S20, S30, and S15L15. (D) Cyclic compressive loading of S30 in axial direction: in strain domain and (E) time domain. (F) Series of images showing one full cycle of compressive loading shown in (D) and (E).

3.5. In vitro response of human mesenchymal stem cells (hMSCs) and human osteosarcoma MG-63 cells

The in vitro response of MG-63 and hMSCs cultured both on the control and S30 scaffolds is summarized in Fig. 8. The cell number ratios at different time points relative to day 1, for both the control and S30, as obtained by PrestoBlue™, are shown in Fig. 8A and 8B. In general, a different behavior was found for MG-63, which proliferated over time and hMSCs, which number decreased with time. Regarding MG-63, similar results were observed at day 2 for
S30 and the control. At day 7 a significantly higher value was obtained for S30, which can be associated to the increased surface area caused by the presence of concavities. In the case of hMSCs, a significantly higher value at day 3 was observed for S30, whereas at day 7 no significant difference was observed between the conditions.

The immunofluorescence staining (nuclei and cytoskeleton) of MG-63 and hMSCs on the control and S30 samples at day 1, 2 or 3, and 7 of cell culture is shown in Fig. 8C. The MG-63 cell area was found to be significantly higher for the control than for S30 at day 1 (Fig. 8D). In case of cell aspect ratio, no significant differences were observed at either time point. Due to the high number of cells at day 7 for both control and S30, it was impossible to discern the features of individual cells and hence cell area and cell spreading could not be calculated at the mentioned time point. These results are in contrast to the clear differences observed between S30 and control seeded with hMSCs in terms of cell morphology and spreading. The formation of actin stress fibers was visible at each time point for S30, whereas in the case of the control only at day 1. The latter was followed by cell disorganization, a clear decrease in cell spreading (Fig. 8D), and mostly round-like morphology at day 3 and 7. On the contrary, a well-defined cytoskeletal organization distributed homogeneously on the surface of the filaments was observed for S30 at days 1, 3, and 7 with similar elongated and spread morphology (Fig. 8D) and significantly higher cell areas for day 3 and 7 when compared to control.

The cell morphology was further analyzed by SEM, as illustrated in Fig. 5 of the supplementary information. For both cell lines, good adherence of cells at day 1 of culture was observed for both the control and S30, but whereas in the first case they showed a flattened morphology, in the latter they adopted a well-spread stretched one. The differences persisted for days 3 and 7, substantiating the results obtained by immunofluorescence. In case of hMSCs seeded on the control, the morphology started to change from day 3 where the cells seemed to be stressed and showed rounded shapes. At day 7 a significant increase of round-shaped cells was observed. At each time point for S30, the hMSCs and MG-63 cells showed spindle-like shapes stretching themselves either from one side of the pore to the other or were also observed attached to the pore wall.

4. Discussion

The aim of this work was to develop a strategy to improve the control of pore architecture in 3D-printed synthetic bone grafts, with a view to introducing concavities in the structure. This approach is based on numerous studies that have highlighted the role of surface curvature on cell behavior and tissue growth [34,35], a general phenomenon but for which there is particularly clear evidence in the case of calcium phosphate substrates and bone growth, both in vitro [36,37] and in vivo [7,8]. The underlying mechanism is complex. Surface concavity, which is primarily a geometric parameter, may translate into mechanical and chemical stimuli for the cells. On the one hand, the curvature of the surface has a direct impact on the tensile state of the cells, which has been shown to affect cell and tissue growth [34,36]. On the other hand, the presence of concavities results in confined volumes, capable of retaining ionic and molecular species dissolved in physiological fluids, creating specific microenvironments with osteogenic and osteoinductive cues [38–42].

In this study, the incorporation of concave pores in 3D-printed structures was accomplished by adding degradable gelatin microspheres in the self-setting printing inks. This resulted in filaments with well-distributed macropores which features were easily controlled by the size and amount of microspheres incorporated (Fig. 1, Table 1). No significant breakage of the filaments was observed neither during robocasting nor after setting, suggesting that the swelling of the gelatin microspheres once mixed with the α-TCP paste was mitigated when entrapped in the highly viscous α-TCP paste, compared to what was observed in water (Fig. 1C). The XRD analysis showed that the hardening of the scaffolds, associated with the hydrolysis of α-TCP to CDHA, was not hindered by the inclusion of gelatin microspheres in the self-setting inks (Supplementary Fig. S1). However, the addition of gelatin affected the crystal growth of CDHA crystals, resulting in smaller crystal-like sizes (Table 2) for gelatin-containing scaffolds, especially S30 and S15L15, as compared to control. This mild reduction in crystal-like size would be expected to benefit the biological performance of the scaffold as it would help improving the scaffold reactivity without necessarily compromising mechanical integrity.

Both the TGA results (Fig. 2) and SEM images (Fig. 3) demonstrated partial dissolution of gelatin during the 6 days of immor-
sion in water required for the hydrolysis of the α-TCP to CDHA leading to the hardening of the scaffolds. According to the TGA results, between 23 and 56% of the initial gelatin was released from the scaffolds, depending on the amount of microspheres added in the ink. Some of the retained gelatin was clearly visible covering the inner walls of the pores as indicated by SEM (Fig. 3). Moreover, since the dissolution of the gelatin microspheres took place simultaneously to the process of hydrolysis of α-TCP, some gelatin was also retained between the entangled precipitated CDHA crystals as shown in Figs. 3 and Supplementary Fig. S3. Raman microscopy substantiated the presence of a nearly continuous polymeric phase with the increase of gelatin microspheres in the scaffolds – specifically for S30 (Fig. 4).

The scaffolds presented a complex multiscale porosity, ranging from the nanosized pores resulting from the CDHA nanocrystal network (Fig. 3A’ and 3B’, and Supplementary Fig. S3) to the
macro pores generated during the 3D-printing process, through to the intermediate pores generated by partial dissolution of the gelatin microspheres. The control of porosity at different scales is an added value compared to other strategies present in the market, such as the combination of HA granules with a polymeric component, which although allows injectability, lacks macro porosity. Moreover, another distinctive advantage of the approach proposed in the present article is that, unlike what happens when a polymeric phase is combined with non-reactive ceramic particles [43,44], the CDHA nanocrystals precipitate in presence of the gelatin released from the microspheres. As a result, the polymeric component does not encapsulate the calcium phosphate crystals, as clearly shown in Figs. 3 and Supplementary Fig. S3 and indicated by the high specific surface area of all ink formulations, and therefore their bioactive potential remains intact. It is worth reminding that in previous studies such CDHA needle-like nanostructure was seen to improve the scaffold’s osteoinductive capacity [7,45]. This was explained by the superior ionic reactivity of the high specific surface area needle-like crystals, as well as the entrapment/adsorption of relevant proteins/growth factors on the nanorough surfaces. In addition, this unique nanostructure was also shown to generate a favorable osteoimmune environment, triggering osteoblast differentiation and osteogenesis in vitro [46].

In this respect, the reduction of crystallite size brought about by the inclusion of gelatin (Table 2) is expected to increase the reactivity of the scaffolds and improve their performance in vivo.

As expected, the porosity of the 3D-printed scaffolds increased with the amount of gelatin microspheres added (Fig. 5). A homogeneous distribution of pores of similar sizes was obtained for S30, whereas for S15L15 the porosity was more heterogeneous. As observed by SEM (Fig. 3A and 3B) the pore sizes were in good agreement with the dry-state microsphere size distributions shown in Fig. 1A, for S10, S20 and S30 being around 30 μm and smaller, whereas for S15L15 having a bimodal distribution with diameters of around 80 μm (1-μs) and 30 μm (5-μs). These pore sizes are smaller than those reported to be optimum for vascularization and bone ingrowth (50-400 μm) [5,47–49]. However, it has to be considered that in the 3D-printed scaffolds the internal porosity of the filaments is superimposed to the porosity obtained by the 3D-printing process, which falls within the optimal range. The role of the pores in the filaments is to provide concave surfaces for cell adhesion and soluble species retention rather than facilitating vascularization and tissue ingrowth, which is already guaranteed by the 3D-printed porosity.

The total porosity obtained by μ-CT was smaller than that obtained by the gravimetric method for the control, S10 and S20 samples, which is reasonable taking into account that the resolution of μ-CT only allows for the detection of pores larger than 1 micron, overlooking smaller pores which are indeed present in the material. The same happened when comparing the internal filament porosity determined by μ-CT or by MIP. MIP is able to detect pores as small as 6 nm, and in fact, as shown in Fig. 5, all specimens exhibit submicrometric porosity due to intranonic nanopore interconnections arising from the entanglement of CDHA needle-like crystal structure. For this reason, one would expect to find significantly larger values of filament porosity by MIP than by μ-CT, which is actually the case for the control, S10 and S20 specimens. The fact that the values obtained by μ-CT for the S15L15 and S30 samples were very similar or even slightly higher respectively than those obtained by the gravimetric method and by MIP is an indication that the gelatin retained scaffolds was not visible by μ-CT, leading to an overestimation of the filament porosity values and hence the total porosity by this technique, this being especially relevant for high gelatin contents, as S30 and S15L15. Overall, the values of total porosity and filament porosity obtained by gravimetric methods and MIP respectively should be considered more accurate, as these techniques are able to detect pores across the entire size range, without overlooking the volume occupied by the remaining gelatin.

On the other hand, the MIP analysis provided relevant information regarding size distribution of the filament porosity. It is important to stress out that the results provided by MIP do not correspond to pore dimensions, but to the size of pore interconnections. Therefore, the reported values are compatible with the larger pore sizes observed in SEM images (Fig. 3). Direct comparison of MIP curves (Fig. 5C) showed a shift of the pore size towards higher values with increasing number of microspheres, especially visible in the submicrometric range, which can be associated to a larger distance between precipitated crystals, and can be attributed to the increasing amount of water added as the proportion of gelatin microspheres in the ink increased. Moreover, also an increase of pores entrance sizes in the range 10 – 100 μm was observed with the addition of the microspheres, contrasting with close to no porosity in the given range for the control, which can be associated to the pores resulting from the degradation of the gelatin microspheres.

Furthermore, the addition of gelatin microspheres in the self-setting inks triggered significant changes in the mechanical properties of the 3D-CDHA scaffolds (Fig. 6A to 6C). As expected, the mechanical strength of the gelatin-containing specimens dropped significantly when compared with the free-gelatin control, on one hand due to their higher porosity, and on the other to the presence of gelatin as a second phase in the structure. However, an advantageous feature of the incorporation of the gelatin microspheres in the 3D-printed CDHA was the change from the usual brittle ceramic behavior of 3D-CDHA scaffolds [13] to a more ductile for S10 and S20 to hydrogel-like elastic behavior [50] for S30 and S15L15 (Fig. 6A and Supplementary Movie S6). This elastic behavior is especially remarkable considering the high amount of ceramic phase in the structure, from 78 % for S30 to 91 % for S10, and can be attributed to the presence of retained gelatin not only inside the pores, but also as a continuous phase between the precipitated CDHA crystals as confirmed by Raman spectroscopy (Fig. 4), which under compression behaved like an elastomeric damper absorbing most of the applied mechanical energy and avoiding the scaffolds’ disintegration, as shown in Fig. 7. This behavior was however not reported when gelatin in the form of an aqueous solution was used as a liquid phase of CPC [12,31,32], where its addition was shown to increase the mechanical strength of the hardened cements, but they remained essentially brittle. In this respect, it has to be kept in mind that in the present study the mechanical tests were performed in wet state and therefore it cannot be compared to previous studies which mostly report measurements for dry samples. Cyclic compressive loading of S30 specimens (Fig. 6D to 6F) revealed that the samples can sustain repeated compressive stresses without permanent deformation (except for the first cycle) and with the ability to fully recover their shape. The change in stiffness with the first loading cycle is explained by the cracking of the CDHA. However, no further changes were observed in the subsequent cycles indicating that the continuous gelatin phase (Fig. 4) with embedded CDHA was responsible of holding the structure. The continuity of the gelatin phase is expected to play a major role in the strain distribution, preventing local strain accumulation in the gelatin-coated concavities. The high deformation capacity of the scaffold is an advantage because it reduces its fragility, facilitating manipulation during surgery and fitting into complex bone defects without fracturing. However, it can also represent a problem because it is known that high deformations can hinder tissue mineralization and even promote fibrosis [51]. Therefore, the material should not be used in load-bearing applications, but should be restricted to confined defects, where the deformation of the scaffold is minimal. The compressive strength of the scaffolds dropped from 2.5 to 0.2 MPa with the increment of gelatin microspheres.
in their composition, as a consequence also of the high porosity. This is in the lower range of the human trabecular bone, which has been shown to vary between 2.4 and 6.8 MPa, depending on the anatomic site [52], and approaches, for the highest gelatin contents, the behavior of hydrogel scaffolds, for which compressive strength values in the range of 0.02 to 0.16 MPa have been reported [50,53,54]. In spite of the significant reduction of strength, which limits its application to non-load bearing sites, the elastic behavior of the gelatin-containing scaffolds is expected to facilitate their manipulation and allow fitting them to irregular defect sites without the risk of breaking them apart, overcoming the brittle behavior of hydroxyapatite, which has always been a disadvantage for surgeons when they need to accommodate and fit the samples in the fracture site [43,55]. For instance, a relevant field of application would be bone reconstruction in maxillofacial surgery [56,57] in combination with metal ware that provides mechanical stabilization, like internal fixation devices, external fixation, intramedullary pinning or k-wires.

One of the consequences of the high bioactivity of CDHA is the difficulty to perform in vitro studies, as it has been consistently reported, due to the drastic ionic exchanges with the cell culture medium, which cause cell stress and apoptosis, very markedly in hMSCs and to a lower extent to other cells, like osteosarcoma cell lines [58,59]. Such drastic ionic exchanges no longer occur in vivo thanks to continuous fluid renewal [7,8], but rather the specific microstructure and natural reactivity of CDHA is expected to help entrapping relevant proteins for osteogenesis and osteoinductivity, particularly during reprecipitation events, encouraging tissue regeneration [8,33]. In the case of in vitro static cell culture, the ionic exchange can be mitigated by longer pre-incubation times or by increasing the volume ratio between the cell culture medium and the scaffolds [59]. In this study, due to the presence of non-crosslinked gelatin microspheres, the first option - longer pre-incubation times- was discarded to prevent further gelatin dissolution and degradation of the scaffolds. Instead, higher cell culture medium to scaffold volume ratio was used. Although the presence of gelatin within the pores might be expected to have a mitigating effect on ion exchange, this is countered by the fact that the high SSA CDHA crystals are mostly exposed, as shown by SEM images (Fig. 3), contributing to a high reactivity, this effect being amplified on S30 by the smaller crystallite size measured as compared to the control [60]. On the other hand, a positive influence of gelatin on cell adhesion and proliferation in hydroxypatite materials has been reported before [12,61,62] which has been attributed to the presence of the RGDF sequence in gelatin which promotes cell adhesion through integrin mediated mechanisms. Based on these results, we envisaged a positive effect of the presence of retained gelatin on in vitro cell adhesion on the scaffolds, where concave pores coated with gelatin were exposed on the surface of the strands. Although the independent effect of gelatin and the presence of concavities could not be discerned from the study, different behaviours were observed depending on the cell type, MG-63 or hMSC (Fig. 8). Whereas MG-63 were able to proliferate on the scaffolds (Fig. 8A), hMSCs cell proliferation was hindered, in agreement with previous results [59], which can be ascribed to higher sensitivity of these cells to the ionic fluctuations in the culture media (Fig. 8B). Nevertheless, a clear increase in cell spreading was seen for hMSCs in confocal microscopy for S30 when compared to control, which, on the other hand, was not detected for MG-63 cells (Fig. 8C). Despite the results from in vitro cell culture testing are only preliminary, this work proves that the combination of a bioactive substrate (CDHA) with surfaces exposing concavities and the presence of gelatin encourage well-defined cytoskeletal organization and favor cell adhesion and spreading of hMSCs and proliferation of MG-63. Further tuning regarding the size of the concavities would still be required to encourage cellular colonization and enhance cell spreading along the pore walls.

Taking all the above into account, the combination of 3D printing inks with sacrificial materials, such as gelatin microspheres, paves the way to new strategies for tailoring the architecture, texture and mechanical properties of synthetic bone grafts. This can be obtained by incorporation into the calcium phosphate mixture of other degradable phases with different shapes and compositions. Whereas the reduction of the brittleness of the materials is a positive outcome, the mechanical strength should be further enhanced to extend the clinical applicability of the developed materials. Finally, the benefit of having macroporous strands in the 3D printed scaffolds remains to be tested in an in vivo scenario.

5. Conclusions

In this study, a versatile strategy was presented to introduce concave porosities in the convex filaments of 3D-printed calcium phosphate scaffolds, by adding gelatin microspheres as a sacrificial material in self-setting hydroxyapatite inks. This method introduces an additional level of control of the scaffold porosity to that inherent in direct ink writing, and could be used with other porogens, provided they can be dissolved at low temperature and are biocompatible. The presence of concave pores on the surface of the filaments is expected to provide niches with cell-friendly environment for their effective adhesion and proliferation, which remains to be validated in vivo. Moreover, the retention of the typical microstructure of biomimetic hydroxyapatite, with needle-shaped crystals and high specific surface area is expected to contribute also to the osteoinductive and osteogenic potential of the 3D-printed CDHA scaffolds. Finally, the more elastic behavior associated to the gelatin phase is an additional advantage that facilitates their use in surgical practice.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors acknowledge the Spanish Government for financial support through PID2019-103892RB-I00 project. They also thank the Generalitat del Catalonia for funding through projects 2017SGR-1165 and BASE3D 001-P-001646 (co-funded by the European Regional Development Fund), as well as the ICREA Academia award of MPG, the FI-AGAUR scholarship of JK and the Serra Hunter Program of ME.

Supplementary materials


References

J. Konka, J. Buxadera-Palomero, M. Espaillat et al.  
Acta Biomaterialia 134 (2021) 744–759


