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Bioinspired gelatin/bioceramic composites loaded with bone morphogenetic protein-2 (BMP-2) promote osteoporotic bone repair

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ABSTRACT

There are currently several commercialized products approved by the Food and Drug Administration and the European Medicines Agency based on the use of recombinant human BMP-2 for the treatment of non-unions long fractures and spinal fusion. However, the adverse effects recorded with the use of BMPs suggest the need for drug delivery carriers that allow reducing the required doses and improve their cost-effectiveness. Herein, we have developed a new osteoconductive scaffold that reduces the required doses of BMP-2 for promoting bone regeneration in an osteoporotic defect model. The composite is, in brief, a gelatin-based 3D scaffold reinforced with either calcium sulfate or hydroxyapatite as an inorganic osteoconductive biomaterial. To this end, the organic/inorganic composite systems showed high hydration capacity and good *in vitro* degradability. The incorporation of 7.5% (m/v) ceramic compounds resulted in scaffolds with stiffer Young modulus (179 and 75 kPa for $CaSO_4$_7 and HA_7, respectively) than bare gelatin hydrogels (48 kPa). Studies with human bone-marrow derived mesenchymal stem cells (hBM-MSCs) revealed that the 3D scaffolds promote cell adhesion and proliferation along with osteogenic differentiation capabilities. Specifically, downregulation of stemness (*Nanog*, *Oct4*) genes and upregulation of osteogenic markers (*ALP*, *Col1a1*, *Fmod*) by two fold were observed over 10 days under basal culture conditions. Promisingly, the sustained *in vitro* release of BMP-2 observed from the porous reinforced scaffolds allowed us to address the critical-sized osteoporotic mice calvarial defects with a relatively low growth

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factor doses (600 ng BMP-2/scaffold) compared to conventional doses at 2–15 micrograms. Overall, this study demonstrates the promising potential of osteoconductive gelatin/calcium bioceramics composites as osteogenic growth factors delivery carriers for bone-regeneration *via* ultra-low growth factor doses.

1. Introduction

Osteoporosis is one of the most frequent bone disorders. It is characterized by the decrease in the bone mass and consequent deterioration of bone microstructure, which results in increased bone fragility and susceptibility to fracture [1]. It is estimated that more than 9 million osteoporotic fractures occur every year worldwide [2]. In adulthood the mass and volume of the bones remain constant because of the coordinated homeostasis of bone formation and resorption by osteoblast and osteoclasts, respectively. Behind the pathophysiology of osteoporosis is the loss of bone turnover regulation, *i.e.* excess of bone resorption against bone formation [3]. The current treatment for osteoporosis consists of systemic administration of antiresorptive drugs (*e.g.* bisphosphonates) that inhibit osteoclasts activity, and anabolic agents (*e.g.* teriparatide) that promote bone formation by stimulating osteoblast activity. Importantly, it has been demonstrated that, during the development of this pathology, bone marrow derived mesenchymal stem cells (BM-MSCs) reduce their ability to differentiate into osteoblasts and increase their differentiation to adipogenic lineage [4]. Therefore, the poor osteogenic capacity due to the disruption of BM-MSCs differentiation balance makes the repair of bone defects after an osteoporotic fracture particularly challenging.

The synergistic integration of therapeutically active growth factors and drug delivery platforms is a promising approach for the treatment of osteoporosis. Among the various growth factors, bone morphogenetic protein-2 (BMP-2), which belongs to the transforming growth factor β (TGF-β) superfamily, is one of the most studied [5]. Importantly, BMP-2 induces the osteogenic differentiation of MSC and osteoprogenitor cells during bone healing and formation [6]. What is more, it has been observed that BMP proteins are key growth factors in order to boost osteoinduction [7,8]. However, unfortunately, the administration of BMP-2 is complex and usually results in a therapeutic effect only *via* supra-physiological concentrations, which in turn can facilitate adverse reactions in the patient. For instance, concentrations of BMP-2 range from micrograms to tens of micrograms in rodents, and up to tens of milligrams in human [9,10]. The development of systems that are intrinsically more osteoconductive with reduced dependence on BMP-2 to promote bone regeneration would address these problems and improve the osteogenic capabilities within the bone tissue engineering field [11]. These carriers would ideally allow not only the controlled release of the growth factor to customize its exposure at the site of injury, but provide the essential cues to promote the adhesion, proliferation and extracellular matrix deposition by host cells, which are already partially differentiated (*e.g.* preosteoblasts). To achieve this goal, bioinspired organic/inorganic composite systems that resemble the natural composition of bone tissue have extensively been proposed [12,13].

Gelatin, as a denatured form of collagen, is one of the main constituents of living bone. Thus, an interesting biomaterial to be used for the organic phase of the proposed composite systems. Its intrinsic ability to create polyion complexes with charged therapeutic agents has made this biodegradable protein particularly fruitful biomaterial for biomedical applications [14]. On the other hand, calcium phosphate and calcium sulfate-based bioactive ceramics can stimulate bone formation and thereby potentially improve the osteoconductivity of the composite.

In the present study, we present a gelatin-based 3D scaffold to support the osteogenic commitment and the release of osteogenic BMP-2 growth factor by incorporating calcium ceramic compounds for osteoporotic bone tissue engineering purposes. For this aim, the gelatin network was enzymatically crosslinked and the therapeutic agent was loaded to the preformed scaffolds. Different ratios of calcium sulfate and hydroxyapatite (HA) were successfully incorporated into the matrices and the swelling, degradation and mechanical properties were evaluated. Furthermore, the analysis of the microstructure and chemical composition were evaluated. The biological performance of the developed scaffolds was assessed by culturing human bone-marrow derived mesenchymal stem cells (hBM-MSCs) on the scaffold surface and cellular adhesion, proliferation, viability and osteogenic differentiation capacity were determined. hBM-MSCs differentiation fate was assessed through the analysis of the expression levels of both stemness (*Nanog*, *Oct4*) and osteoblast-related genes (*Col1a1*, *Runx2*, *Fmod*) by RT-PCR assay. Finally, bone regenerative potential of the developed 3D scaffolds was investigated in osteoporotic mice critical-size calvarial defects.

2. Materials and methods

2.1. Materials

Gelatin from bovine skin Type B (~225 g Bloom), calcium sulfate dihydrate, hydroxyapatite particles, Cell Counting Kit-8 (CCK-8), Triton X-100, collagenease P, bovine serum albumin (BSA), *p*-nitrophenyl phosphate (pNPP) and calf intestinal alkaline phosphatase (CIAP) were purchased from Sigma Aldrich, Spain. Microbial Transglutaminase (100 U/g) was kindly supplied by Ajinomoto Foods Europe, France. Phosphate Buffered saline (PBS), Trypsin, fetal bovine serum (FBS), Penicillin-Streptomycin solution, Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12), Pierce™ BCA Protein Assay Kit, 4,6-diamidino-2-phenyindole dilactate (DAPI), AlexaFluor 488-phalloidin, LIVE/DEAD® kit and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) were obtained from Fisher Scientific, Spain. Calcium Detection Kit was purchased from Abcam and TRIsure™ reagent from Bioline. Recombinant human bone morphogenetic protein-2 (rh-BMP-2) was purchased from GenScript and ELISA kit for rh-BMP-2 detection from Peproptech, UK.

2.2. Fabrication of 3D scaffolds

Enzymatically crosslinked gelatin-based 3D scaffolds were prepared by the freeze-drying technique. Initially, 1 g of gelatin from bovine skin was dissolved in 3 mL distilled water under constant stirring at 40 °C for 1 h. Then, different concentrations (3.75, 7.5 and 15% (*w/v*)) of calcium sulfate salt or HA particles were mixed with gelatin in order to obtain different reinforced scaffolds with low, medium or high grade, respectively. These reinforced gelatin dispersions were mixed with the microbial transglutaminase enzyme solution at a 1.5:1 (*v*/v) ratio to reach the final scaffolds composition of 20% (w/v) gelatin and 20 U/g gelatin enzymatic activity. A scaffold without addition of reinforcement materials was also prepared for comparison. Afterward, the hydrogels were casted into polystyrene molds and punched out to obtain desirable sized cylindrical 3D scaffolds. These structures were immersed in ethanol 70% (v/v) for 15 min and thereafter two washings with PBS were performed, in order to discard any remaining ethanol. Subsequently, the samples were first frozen at −80 °C, and freeze-dried after.

2.3. Swelling ratio and degradation profile

The swelling behavior of the gelatin-based scaffolds was determined in PBS at 37 °C under constant shaking (300 rpm). The initial dry weight of each sample was measured and, then, scaffolds were immersed in PBS at 37 °C. The wet weight of the samples at different time intervals (1, 2,

5, 10, 15 and 30 min, 1, 2, 7, and 24 h and 6 days) were measured after removing the excess water from the surface. The swelling ratio was calculated following Eq. (1), where Ws is the weight of the wet sample and the Wo corresponds to the initial dry weight of the same sample.

$$\text{Swelling Ratio} = (Ws - Wo)/Wo \quad (1)$$

To study the stability of the samples under physiological conditions, both hydrolytic and enzymatic degradation tests were performed. For the hydrolytic degradation test, the samples were first immersed in PBS until swelling equilibrium was reached (2 h) and weighed then. This value was considered as initial weight and the samples were immersed in PBS and maintained at 37 °C during 9 days. At each time point, samples were taken out and the weight of each sample was recorded. The degradation rate was determined by the weight of the remaining matrices following Eq. (2), where Wi is the initial weight of the sample and the Wt corresponds to the weight of the sample at different time intervals.

$$\text{Weight of scaffolds } (\%) = (Wt/Wi)*100 \quad (2)$$

For the enzymatic degradation test, collagenase P solution at 0.02% (*w/v*) concentration was used to immerse the samples. In these case, when the samples were completely degraded the total protein and calcium content of the supernatants were determined by commercially available kits, following the instructions of the manufacturer.

2.4. Physicochemical characterization

2.4.1. Compressive mechanical properties

The compressive mechanical behavior of the developed scaffolds was analyzed by monotonic uniaxial unconfined compression, following a protocol described elsewhere [15]. A universal mechanical testing (Instron 5548) equipped with 50 N load cell was used. Prior to the test, the cylindrical samples were immersed in phosphate buffer saline (PBS) for 24 h and the size of the scaffolds was measured prior to the compression (1 mm height and 10 mm diameter).

Tests were performed at a rate of 1 mm/min, at room temperature. The Young modulus was determined from the slope of the engineered stress-strain curves in the 10–20% strain linear region. Same experimental settings were applied to measure at least six different samples per each composition.

2.4.2. Scanning electron microscopy (SEM) and energy dispersive X-ray spectroscopy (EDAX) analysis

In order to determine the porosity of the hydrogels SEM analysis were performed. SEM images were acquired with SEM Quanta FEG 250 Analytical ESEM instrument operating at an accelerating voltage 2 kV in high vacuum mode after gold sputtering the freeze dried hydrogels with 5 nm. ImageJ software was used to measure the pore size of the collected SEM images and average pore size was calculated from at least 40 measurements from each sample. EDAX analysis was performed with Oxford Instruments 80 mm^2 X-Max silicon drift detector connected to the SEM instrument after gold sputtering with 5 nm.

2.4.3. Fourier transform infrared spectroscopy (FTIR) analysis

The chemical composition of the lyophilized scaffolds was assessed by means of FTIR spectroscopy. FTIR spectra of the freeze-dried hydrogels were recorded on attenuated total reflection (ATR) mode using a PerkinElmer Spectrum 100 FTIR spectrophotometer with an ATR accessory after background subtraction. Spectra were recorded over the range of 4000–500 cm^{-1} with 16 scans at a resolution of 4 cm^{-1}. The collected spectra were baseline-corrected and normalized using PerkinElmer Spectrum software and the average of four spectra were used for the analysis.

2.4.4. X-ray diffraction (XRD) analysis

The XRD patterns of the freeze dried hydrogels were collected with a Huber G670 powder diffractometer in the 2θ range of 3 to 100° in steps of 0.005° using CuKα1 radiation (λ = 1.54056 Å) for 10 min. The data were collected in transmission mode from a rotating flat plate sample inclined 45° relative the primary beam.

2.5. In vitro release of bone morphogenetic protein–2 (BMP-2)

The *in vitro* release of BMP-2 growth factor from the developed formulations was evaluated with 4 mm diameter scaffolds and Protein Lobind Eppendorf tubes. First, previously prepared and lyophilized scaffolds were loaded with 600 ng of BMP-2 as described in our previous paper [16] and they were incubated overnight for the protein adsorption. Afterward, a washing step with 1 mL of PBS was performed to remove the unbound protein fraction before the sampling phase was initiated. The *in vitro* release assay was performed at 37 °C under mild orbital agitation and at each time point, all the volume of PBS in tubes was collected and replaced with fresh one. After collecting the last sample, the scaffolds were degraded with collagenase P solution in order to determine the amount of growth factor remaining in the scaffolds. All the samples were kept frozen until the determination of the BMP-2 concentration by commercial ELISA kit following the manufacturer's guideline.

2.6. Biocompatibility study

The preliminary cellular compatibility of the scaffolds was assessed as described by Echave MC et al. [16], following the guideline ISO 10993 (Biological evaluation of medical devices guideline: cytotoxicity on extracts and cytotoxicity by direct contact). Both the cytotoxicity produced by cell-scaffold direct interaction and the indirect toxicity of the scaffolds were determined by the evaluation of the cells metabolic activity trough the CCK-8 assay. The metabolic activity of cells without contact with scaffolds or the extracts was considered as 100% viability.

2.7. In vitro cell studies: human bone-marrow derived mesenchymal stem cells (hBM-MSCs) seeding on the developed systems

2.7.1. hBM-MSC expansion and seeding

hBM-MSC were expanded in complete basal medium consisting of DMEM/F12 culture medium, supplemented with 10% (*v*/v) FBS and 1% (v/v) penicillin/streptomycin solution. Cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2 and the split of the cells was done when reached confluence. hBM-MSC were used at passage 3–5 for all the cellular studies. Before the seeding of the cells on the top of the 3D systems, all the scaffolds were exposed to UV light for 15 min and hydrated with the basal culture medium. The hydrated scaffolds were placed in wells of the same size in ultra-low attachment microplates (Corning™ Costar™ Ultra-Low Attachment Microplates) and 10^5 hBM-MSC/scaffold in 30 μL of medium were seeded onto the surface of the scaffolds. These scaffolds were kept in the CO_2 incubator for 1.5 h and then 1.5 mL of complete basal medium was added to each well. The culture medium was changed every 2–3 days during the experiments.

2.7.2. Live/dead viability assay

Cell viability at the enzymatically crosslinked gelatin-based substrates was evaluated using Live/Dead viability assay (Life Technologies) according to the manufacturer's protocol. After 2 and 10 days of culture, three samples of each group (GEL, $CaSO_4$_7 and HA_7) were incubated in calcein-AM/ethidium solution for 30 min and fluorescence micrographs were taken using inverted fluorescence microscope (Nikon TMS). Flow cytometry (MACSQuant Analyzer, Miltenyi Biotec) was used to quantify the viability of the cells seeded on the surface of the scaffolds at day 10. First, cells were detached from the scaffolds with trypsin-EDTA and dyed after with Live/Dead kit, following the guidelines.

2.7.3. Cell adhesion, proliferation and metabolic activity determination

Immunofluorescence staining was performed to study the adhesion and proliferation of hBM-MSCs cells on the scaffolds. The cell-seeded scaffolds were washed with PBS and fixed with 3.7% (*v*/v) formaldehyde for 10 min after 7 days of culture. The cells were permeabilized with 0.1% (v/v) Triton X-100 for 5 min and the blocking of the samples was performed with 1% (*w/v*) BSA for 30 min. The F-actin filaments were stained with 165 nM AlexaFluor 488-labelled phalloidin for 30 min at room temperature protected from light. After washing the samples with PBS three times, the nuclei were stained with 300 nM DAPI solution. The samples were observed under inverted fluorescence microscope (Nikon TMS).

The proliferation of hBM-MSCs on the 3D scaffolds was evaluated by means of metabolic activity determination following CCK-8 assay. The cell-seeded samples were rinsed with PBS after 2, 7 and 10 days of incubation and 350 μL of fresh medium containing 35 μL of CCK-8 kit reagent was added to each scaffold. The samples were incubated for 4 h at 37 °C and the optical density of the generated formazan was measured by Tecan Infinite M2000 microplate reader.

2.7.4. Alkaline phosphatase (ALP) activity and staining

The secretory form of ALP from the cells was evaluated spectrophotometrically during 3 weeks. The activity was evaluated by determining the hydrolysis of *p*-nitrophenyl phosphate by ALP at pH 9.3. A standard curve with calf intestinal ALP was prepared and 100 μL of pNPP at 0.2% (*w/v*) was added to each sample. The reaction was stopped with 50 μL of NaOH 3 M and absorbance measured at 405 nm. Moreover, for the evaluation of the intracellular ALP activity of hBM-MSC seeded onto the scaffolds at the end of the assay, BCIP/NBT solution was used. After washing the scaffolds three times with PBS, the samples were covered with the solution and incubated protected from light at room temperature for 2 h. Then, the excess of the dye was discarded washing the scaffolds three times with PBS. The stained scaffolds were observed under bright field and imaged with a digital camera. Image J software was used for the analysis of the images and the area covered by the black-violet stained cells was determined by applying a threshold. The entire area of the scaffold was considered as 100%.

2.7.5. RNA isolation and real-time quantitative reverse transcriptase-polymerase chain reaction (q-RT-PCR)

Total RNA was isolated from primary hBM-MSC using TRIsure reagent (Bioline, London, UK). 1 μg of total RNA was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was performed using the SensiFAST Probe Hi-ROX Mix (Bioline) in an ABI Prism 7900 HT Fast Real-Time thermocycler. The genes were screened using Taqman 5′-nuclease probe method (Applied Biosystems) and all transcripts were normalized to the housekeeping gene TATA binding protein (Tbp). Fold-change expression was calculated using the $2^{-\Delta\Delta Ct}$.

2.8. In vivo evaluation of bone regeneration

2.8.1. Osteoporotic mice calvaria defect model

The animal experiments were carried out in conformity with the European Directive (2010/63/UE) on Care and Use of Animals in Experimental Procedures. In addition, the animal protocols were previously approved by the Ethics Committee for Animal Care of the University of La Laguna. The surgeries were carried out under isoflurane anesthesia. The analgesia consisted in buprenorphine (0.01 mg/kg) by subcutaneous route before the surgeries and paracetamol (200 mg/kg) in the drinking water, for 3 days post-surgery. Furthermore, after recovery from the surgeries, animals were allowed free movement, food and water uptake. Experimental osteoporosis was induced to 20 female FVB mice, approximately 16 weeks old, by bilateral ovariectomy, *via* dorsal approach. Immediately after the surgery the mice received 3 mg/kg body weight of dexamethasone-21-isonicotinate (Deyanil retard, Fatro Ibérica, Spain) administered subcutaneously once a week for up to 16 weeks. Then, the animals underwent a new surgery to create the bone defect and simultaneously the scaffolds were implanted. Briefly, the calvaria bone was exposed and a 4 mm circular area was delimited with a biopsy punch. Then, a 4 mm circular trans-osseous defect was made with a trephine bur [17]. The scaffold was inserted in the defect and the skin was stapled. At 8 weeks post-implantation, animals were sacrificed by CO_2 inhalation and the defect area was extracted.

2.8.2. Histology, histomorphometry and immunohistochemistry analysis

The extracted samples were prepared for histological analysis as previously described [18]. Briefly, samples were fixed in paraformaldehyde 4% solution first, decalcified in Histofix® Decalcifier (Panreac, Barcelona, Spain) and dehydrated in a graded series of ethanol after, before they were embedded in Paraplast®. Longitudinal microtome (Shandon Finesse 325) sections with 5 μm thickness were prepared throughout the defect site. The sections were stained with hematoxylin-erythrosin for new bone formation visualization. Bone mineralization was assessed with VOF trichrome staining. Red and brown staining indicates advanced mineralization, whereas less mineralized, newly formed bone stains in blue [19].

Sections were analyzed by light microscopy (LEICA DM 4000B) and computer based image analysis software (Leica Q-win V3 Pro-image Analysis System, Barcelona, Spain) was used to evaluate all sections. A region of interest (ROI) within the defect (12.5 mm^2) was defined for quantitative evaluation of new bone formation. New bone formation was expressed as a percentage of repair with respect to the original defect area within the ROI. From the total bone repair, the areas of mature bone (MB) and immature bone (IB) were determined in the VOF staining sections, and the MB/IB ratio for each experimental group was calculated.

For immunohistochemical analysis, sections were deparaffined and rehydrated in Tris-buffered saline (TBS) (pH 7.4, 0.01 M Trizma base, 0.04 M Tris hydrochloride, 0.15 M NaCl), which was used for all further incubations and rinse steps. Sections were incubated in citrate buffer (pH 6) at 90 °C for antigen retrieval, followed by incubation in 0.3% hydrogen peroxide in TBS buffer for 20 min to inactivate endogenous peroxidase activity. After a rinse step, sections were blocked with 2% FBS in TBS–0.2% Triton X-100 (blocking buffer). The indirect immunohistochemical procedure was carried out by incubating the sections with osteocalcin (OCN) antiserum (1/100) (Millipore, Barcelona, Spain) in blocking buffer overnight at 4 °C. Sections were rinsed three times, then incubated with biotin-SP-conjugated donkey anti-rabbit F(ab0) fragment (1/500) (Millipore, Barcelona, Spain) in blocking buffer for 1 h followed by incubation in peroxidase-conjugated streptavidin (1/500) (Millipore, Barcelona, Spain) for 1 h. Peroxidase activity was revealed in Tris–HCl buffer (0.05 M, pH 7.6) containing 0.005% of 3.3′ diaminobencidine (Sigma, Poole, UK) and 0.01% hydrogen peroxide. Reaction specificity was confirmed by replacing the specific antiserum with normal serum or by pre-adsorption of the specific antiserum with the corresponding antigen. OCN staining was evaluated using computer-based image analysis software (ImageJ, NIH, Bethesda, MD). OCN staining was measured by applying a fixed threshold to select for positive staining within the ROI. Positive pixel areas were divided by the total surface size (mm^2) of the ROI. Values were normalized to those measured from blank scaffolds and are reported as relative staining intensities.

Neovascularization was quantified by determining blood vessel density and vessel surface area within the ROI. For this purpose, sections were immunolabeled with an anti-CD34 monoclonal antibody (1/50) (DAKO, Barcelona, Spain) in blocking buffer overnight at 4 °C. Sections were rinsed three times, then incubated with biotin-SP-conjugated donkey anti-rabbit F(ab0) fragment (1/500) (Millipore, Barcelona, Spain) in blocking buffer for 1 h followed by incubation in peroxidase-conjugated streptavidin (1/500) (Millipore, Barcelona, Spain) for 1 h. Peroxidase activity was revealed in Tris–HCl buffer (0.05 M, pH 7.6)

containing 0.005% of 3.3′ diaminobencidine (Sigma, Poole, UK) and 0.01% hydrogen peroxide. Reaction specificity was confirmed by replacing the specific antiserum with normal serum. Blood vessel density was expressed in absolute values and vessel surface area in mm^2 based on the quantitative evaluation of the ROI.

2.8.3. Micro-CT studies

MicroCT studies were acquired with a Quantum FX imaging system (Perkin Elmer. 940 Winter St. Waltham, Massachusetts. EEUU). This piece of equipment is specifically designed for small lab animals. Acquisition parameters were: Field Of View 20 mm, acquisition time 4.5 min, X-ray energy 50 kV and 200 μA. Image reconstruction was based on

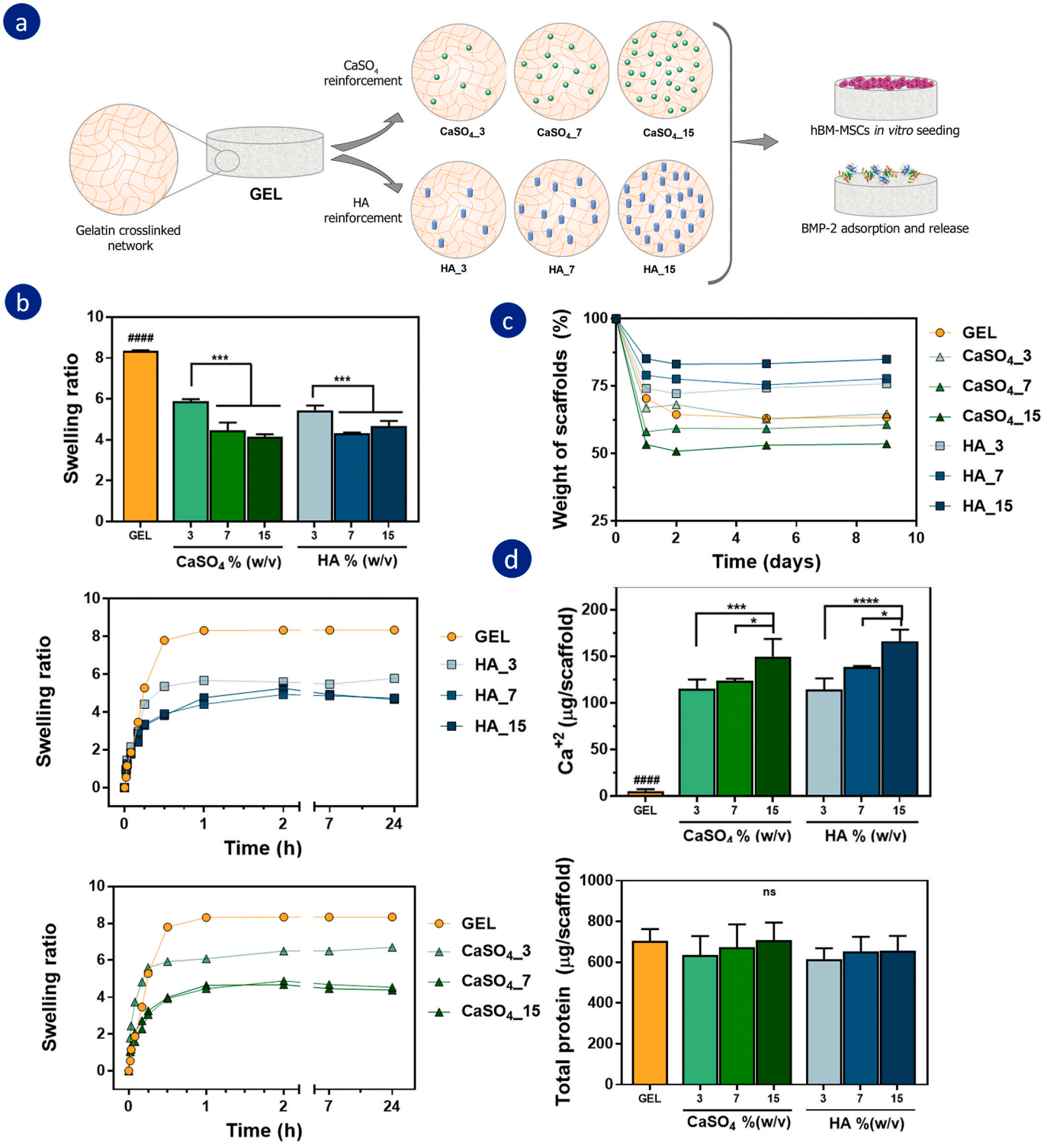

Fig. 1. Development and characterization of reinforced gelatin-based scaffolds. (a) Schematic illustration of the design strategy of gradually reinforced gelatin scaffolds with either $CaSO_4$ or HA and the *in vitro* biological evaluation with hBM-MSCs seeding and BMP-2 growth factor delivery. (b) Determination of swelling properties of the systems. *In vitro* hydrolytic (c) and enzymatic (d, e) degradation of the 3D scaffolds. Statistical significance: ns = no significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$; $^{\#\#\#}p < 0.001$ and $^{\#\#\#\#}p < 0.0001$ compared GEL to all the reinforced scaffolds.

Feldkamp's method. These parameters define a 512 × 512 × 512 voxels image with 0,04 mm pixel size and 0,000064 mm^3 voxel size.

Images were analyzed by Preclinical Imaging Platform staff at Vall d'Hebron Researching Institute using AMIDE software (Copyright (c) 2000–2012 Andreas Loening). Briefly, a fixed size Region of Interest (ROI) was made in the image, centered and adjusted to the bone defect created. Then the volume of the total tissue was measured adjusting the minimum threshold of the selection to the soft tissue. No maximum threshold value was applied in order to include all the voxels of the bone tissue.

In a posterior step, a secondary ROI was created adjusting the threshold to the bone density and including all the voxels with a radiodensity value over this threshold. The threshold values were fixed in both threshold selections due to a previous microCT system calibration. ROI volumes and bone volume/total volume ratio were calculated. Finally, a histogram of each sample was made. For this purpose we created a macro in Slicer software (http://www.slicer.org). Using the ROI that include soft and bone tissues voxels distributions were represented according to the density of each voxel that compounds the image.

2.9. Statistical analysis

The statistical analysis of the data was completed using GraphPad PRISM (7.0) software. The normal distribution of the data was checked by the Shapiro-Wilk test. For normally distributed data, Student's *t*-test or one-way ANOVA were applied for differences between two groups or multiple comparisons, respectively. Tukey *post-hoc* test was applied for multiple comparisons. For non-normally distributed data, Mann-Whitney nonparametric analysis or Kruskal-Wallis test with Dunn's multiple comparisons tests were applied. In all cases, p values <0.05 were considered as significant, represented by symbols described in the graphs. Data are presented as mean ± standard deviation.

3. Results and discussion

The development of organic-inorganic composite 3D scaffolds was driven through the freeze-drying technique. The organic part consisted of enzymatically crosslinked gelatin network, whereas two types of bioceramics were evaluated for the inorganic phase. Thus, the gradual incorporation of HA and calcium sulfate into the polymeric hydrogel resulted in three levels of reinforcement and seven different types of formulations. Some of the composite scaffolds could be seen in Supplementary Fig. S1. First, we characterized the main properties of all prototypes and then preselect only the best candidates for further *in vitro* and *in vivo* assessment (Fig. 1a).

3.1. Swelling capacity and degradation profile

Regarding the water uptake process by dried scaffolds, all the formulations showed great ability to swell. Although the incorporation of fillers does not affect the kinetics of such a process (all the samples were swollen after 1 h), the swelling ratio decreased proportionally with the level of reinforcement (Fig. 1b). These results are in accordance with those presented recently by others in which the swelling ratio of gelatin-HA cryogels decreased with the increase of HA concentration [20–22].

Biodegradability is considered one of the most desirable features that a substitute designed for bone tissue regeneration must show [22,23]. In fact, a temporal structure that supports the neo-tissue growth but its degradation is coordinated with the regeneration process is still challenging for biomaterials designers. The analysis of the scaffold stability under hydrolytic conditions revealed a biphasic degradation profile for all the samples (Fig. 1c). Because the calcium sulfate salt is more water-soluble than HA, the scaffolds reinforced with apatite ceramic showed lower hydrolytic degradation rate [24]. Although the study of the mechanism involved in this degradation is not within the scope of this work, we hypothesize that physical bulk erosion through the diffusion and dissolution of oligomers could be the responsible of the mass loss occurred during the first step [25]. Interestingly, the scaffolds remained stable for several days because the bonds that form the gelatin network were not hydrolytically cleaved. However, when the 3D matrices were immersed in the enzymatic solution that mimics better the *in vivo* environment, complete degradation of the samples was observed. Even so, the *in vitro* recapitulation of the surrounding conditions after the implantation of the scaffold is extremely challenging because of the presence of numerous families of proteases that control the extracellular matrix remodeling [26].

As expected, the calcium content accumulated because of the degradation of the scaffolds was proportional to the amount of inorganic bioceramic incorporated and the total amount of the proteins was the same for all the samples (Fig. 1d). Interestingly, this release of calcium ions as a consequence of the scaffold degradation could potentially promote a chemotactic paracrine effect to induce the recruitment of endogenous osteoprogenitor cells and to lead to their osteogenic differentiation. In fact, Aquino-Martinez R et al. have recently demonstrated similar bone regeneration with cell-free calcium containing scaffolds and scaffold seeded with MSCs in critical-sized calvarial defect model, suggesting the possibility to design cell-free and growth factor-free strategies for bone tissue engineering approaches [27].

3.2. Mechanical characterization

Uniaxial unconfined monotonic compression tests were performed to evaluate the Young modulus of the developed scaffolds (Fig. 2a). The incorporation of inorganic components into gelatin-based organic network resulted in a significant increase in Young modulus from ~48 kPa to >75 kPa, when compared to plain gelatin hydrogels ($p < 0.001$). Interestingly, $CaSO_4$_15 scaffolds showed a 2-fold increase in Young modulus when compared to $CaSO_4$_3 scaffolds (209 ± 30 kPa *versus* 108 ± 9 kPa). Conversely, although the incorporation of HA resulted in a reinforcing effect compared to GEL scaffold, no statistically significant differences were observed between the different HA containing scaffolds. Some studies have demonstrated that hydrogels with a Young modulus similar to our HA-reinforced scaffolds (~60 kPa) induce optimal MSC osteogenesis both *in vitro* and *in vivo* [28]. However, the higher values of calcium sulfate-reinforced scaffolds may also be advantageous, particularly during the surgical insertion. In fact, although the bulk mechanical requirements for osteogenic 3D scaffolds designed to serve as resorbable temporary structures differ from those weight-bearing dense permanent implants, swollen 3D porous scaffolds must present enough stiffness to ensure proper surgical handling and stable graft fixation. In this regard, Zhang B. et al. have recently achieved exceptional results regarding the stable fixation rate, with multifunctional amphiphilic copolymer-HA composite grafts presenting compressive modulus of 126–181 kPa after scaffolds hydration process [29].

Therefore, considering the preliminary examined physical and chemical properties, we limited the further characterization, biological commitment evaluation and *in vivo* bone regeneration performance assessment to scaffolds reinforced with 7.5% of calcium mineral elements and GEL scaffold as the control group.

3.3. Microstructure and elemental composition characterization

The microstructure analysis of the 3D scaffolds was performed by SEM. As shown in the representative SEM images depicted in Fig. 3a, the scaffolds were porous. The pore size of the scaffolds is an important feature which influences the cell-cell interaction, cell migration, proliferation and differentiation processes during the regeneration of the tissue. In the case of these scaffolds, the incorporation of ceramic compounds increased the size of pores, especially with HA inclusion. According to some previous studies, 3D scaffolds intended for bone tissue engineering with a pore size of around 100 μm may favor the migration

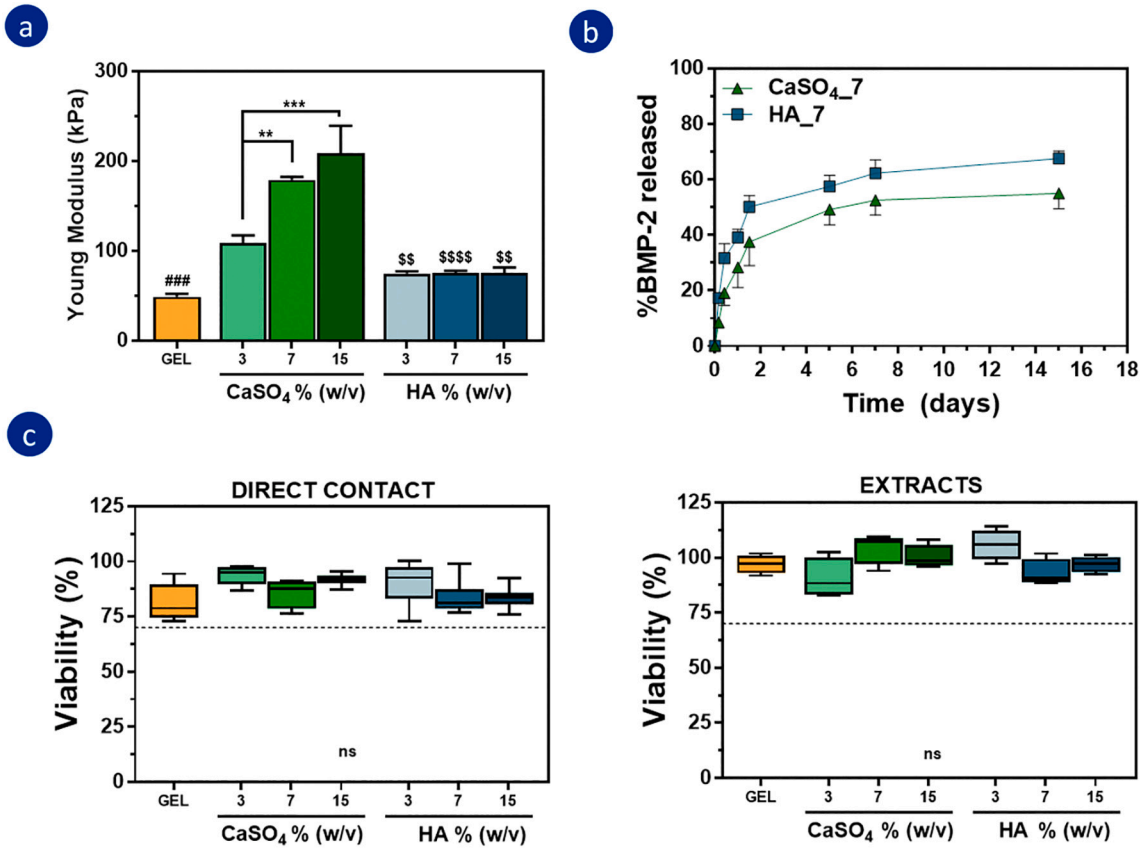

Fig. 2. Further characterization studies of 3D organic-inorganic scaffolds. (a) Compressive mechanical properties of the developed material systems. (b) Biocompatibility properties assessed by extracts and direct contact cell-toxicity assays. (c) *In vitro* cumulative release of BMP-2 from the selected scaffolds. Statistical significance: ns = no significant, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$; $^{\#\#\#}p < 0.001$ and $^{\#\#\#\#}p < 0.0001$ compared GEL to all the reinforced scaffolds.

and proliferation of osteoblast, thus promoting the bone formation [30]. Moreover, an efficient oxygen diffusion and waste and nutrient exchange between infiltrated cells the and the surrounding environment is expected with this range of pore size [31].

To properly examine the effective incorporation of osteoconductive minerals into the 3D gelatin structure, the evaluation of chemical structure was performed by means of EDAX mapping, FTIR and XRD analysis. Regarding EDAX measurements (Fig. 3b), uniform distribution of calcium was noticed in both composite scaffolds. As expected, sulfur and phosphorous elements were observed in $CaSO_4$_7 and HA_7 composite scaffolds, respectively. The presence of reinforcing minerals was confirmed *via* the FTIR spectra. Infrared spectra of raw materials and the scaffolds are shown in Fig. 3c. The characteristic bands of gelatin in 1023 (C-O-C stretching), 1342 (Amide III) and 3289 (Amine peak) cm^{-1} were observed in all the scaffolds. In the case of $CaSO_4$_7 scaffold, the amide I peak of gelatin shifted from 1634 to 1622 cm^{-1}. In addition, characteristic bands of the sulfate groups (SO_4^{-2}) were observed in this composite scaffold: 1112 (ν_4 antisymmetric stretch vibration) and 596, 665 cm^{-1} (ν_4 antisymmetric bending vibration). On the other hand, regarding the spectra of scaffold reinforced with HA, the characteristic Amide II peak of gelatin shifted from 1535 to 1543 cm^{-1}. Moreover, the absorption bands at 601, 558 cm^{-1} (ν_4 bending vibration of phosphate groups) and 1029 cm^{-1} (ν_3 streching vibration of phosphate groups) confirmed the presence of HA within the composite scaffold. The functional incorporation of both reinforcing calcium minerals was further confirmed with XRD analysis (Fig. 3d), since HA associated peaks (002), (211), (300) [32,33] and calcium sulfate corresponding peaks (110), (310), (220) and (−114) [34,35] were recorded in respective composites. Interestingly, the crystalline structure of HA and calcium sulfate remained the same after the composite formation.

3.4. *In vitro* release of BMP-2

The release profile of bioactive BMP-2 factor from the organic/inorganic composite scaffolds (Fig. 1b) demonstrated that the developed biomaterials facilitate the release of the osteogenic growth factor over a period. The first burst release of the protein from the scaffolds was within the first 2 days and just 35–50% of the loaded BMP-2 was delivered in that period. Following that phase, the release of the growth factor was maintained from both gelatin scaffolds reinforced either with calcium sulfate or HA particles and around the 50 and 60% of the loaded dose was delivered until the end of the assay, respectively.

The spatiotemporal release of therapeutic factors at the injured site is a major challenge associated to the growth factors based therapies for tissue regeneration. Due to the short half-life of these biological factors, the use of supraphysiological doses to achieve the desired therapeutic effect is inevitably accompanied by several adverse effects that limit the consolidation and expansion of these therapies in the usual clinical practice [36]. For instance, ectopic bone formation, renal complications and cytotoxic effects have been broadly observed with the use of INFUSE® bone graft clinically approved for lumbar spinal fusion promotion [9]. Even though more advanced and complex systems with growth factors encapsulated in micro and nanoparticles as well as gene-therapy approaches [37,38] are being investigated to improve the spatiotemporal control of the therapeutic agents, it is expected that the bioactive constructs developed through the simple soak loading procedure may improve the strict regulatory concerns regarding the clinical translation [11]. Therefore, we hypothesize that enhancing the

osteogenic drug delivery carriers. Unlike those observed in the repair response from histological analysis, the OCN expression was significantly higher in HA_BMP-2 group compared to $CaSO_4$ scaffolds loaded with osteoinductive growth factor. On the other hand, vascularization analysis at the defect site revealed the presence of higher density and vascular surface area in the experimental groups treated with BMP-2 in relation to the blank groups and the control group (Fig. 7). Uniform distribution of the neovascularization was shown in all the experimental groups.

In order to macroscopically confirm the results of the histological and histomorphometric analysis of the effect of BMP-2 on the regeneration process, a micro-CT analysis was performed in the groups treated with this growth factor. The analysis revealed in both groups repair percentages referred to as BV/TV (bone volume/total defect volume), identical, in the $CaSO_4$ BMP-2 group (53.9%), and somewhat higher in the HA BMP-2 group (66.2%), with respect to those obtained in the histomorphometric analysis. The micro-CT images and videos reveal, in both groups, large areas of radiopaque material compatible with areas of bone repair at the margins of the lesion (Fig. S2, Supporting information). In the HA BMP-2 group, discrete areas of radiopaque material are also observed inside the defect that could correspond to areas of bone repair, however the percentages obtained in this group differ slightly upwards from those obtained by histomorphometry, which could be explained based on the presence of HA in the scaffold, which is detected as radiopaque material not distinguishable from the surrounding bone tissue, and can be counted as repaired area when in fact they are not.

4. Conclusions

In this study, we have successfully engineered bioinspired organic/inorganic composite 3D scaffolds, integrating calcium sulfate and HA bioceramics into the enzymatically crosslinked gelatin network. The reinforced systems showed higher pore size, increased compressive mechanical properties and good biocompatibility profile. In addition, hBM-MSCs exhibited efficient adhesion and proliferative capacities on the organic/inorganic composite scaffolds and osteogenic differentiation patterns were recorded in systems prepared with both bioceramics. The optimization of the experimental conditions and the potential proteomic analysis could elucidate the *in vivo* biological impact produced by the 3D scaffolds after their implantation on the host osteoprogenitor cells. These reinforced scaffolds were capable of promoting *in vitro* sustained release of BMP-2 after loading the therapeutic factor though soak-loading procedure, without the need of complex delivery systems. Interestingly, the osteoconductive gelatin/bioceramics composite functionalized with BMP-2 promoted bone regeneration in osteoporotic mice calvarial defect. Overall, the developed gelatin-based composites may be useful as scaffolds for bone regeneration in osteoporotic defects, avoiding supraphysiological doses and reducing consequently the possible adverse effects.

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Associated content

Micro-CT images and quantification; animal model histomorphometric analisis (PDF).

Animations showing alternately the regenerated area in dorsal and ventral view (MPG).

CRediT authorship contribution statement

Echave MC: Conceptualization, Investigation, Formal analysis, Writing - Original Draft, Visualization.
Erezuma I: Writing - Original Draft, Writing - Review & Editing.
Golafshan N: Investigation.
Castilho M: Review & Editing.
Kadumudi FB: Investigation.
Pimenta-Lopes C: Investigation.
Ventura F: Investigation.
Pujol A: Formal analysis.
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Camara J: Investigation.
Hernáez-Moya R: Investigation.
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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.

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