Mesoporous bioactive glass/ε-polycaprolactone scaffolds promote bone regeneration in osteoporotic sheep

Supporting information

1. Physico chemical characterization

FTIR spectra (Figure S1.a) show absorption bands at 2800-2900 cm^{-1} and 1700 cm^{-1} corresponding to C-H stretching vibration mode and C=O groups, respectively, of the ε-PCL. The bands at 1080 cm^{-1} and 580 cm^{-1} correspond to the stretching vibration mode of Si-O and the bending mode of O-P-O, respectively, from SiO_2 and P_2O_5 contained in MBG-58S. MBG-PCL and MBG-PCL-zol scaffolds showed the same absorption bands, without distinguishable differences that could be assigned to the presence of the 1% in weight of zoledronic acid. Finally, thermogravimetric analysis indicates that the scaffolds contains 39 % (± 2%) of organic matter, very close to the theoretical 40% of ε-PCL incorporated to the initial MBG-PCL paste (figure S1.b). No significant differences could be observed between MBG-PCL and MBG-PCL-zol scaffolds.

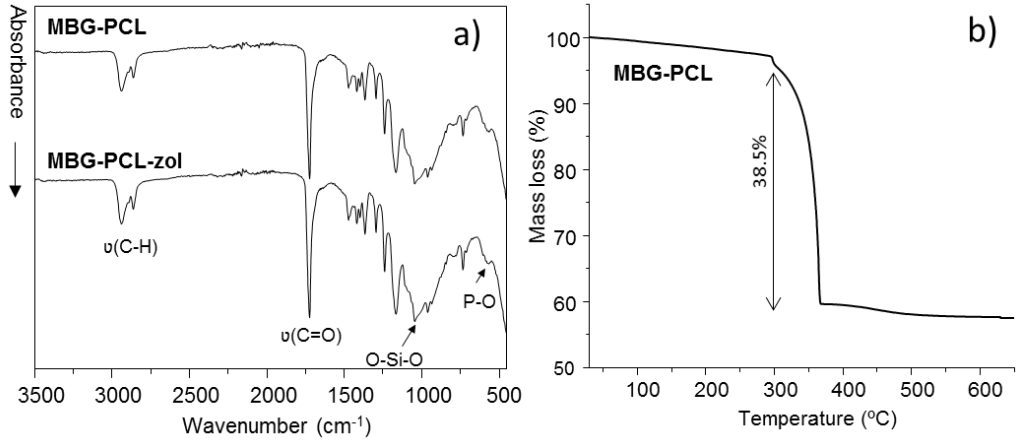

Figure S1. FTIR spectra of MBG-PCL and MBG-PCL-zol (a) and thermogravimetric analysis of MBG-PCL (b).

2. Human Saos-2 osteoblast culture and flow cytometry methods

Human Saos-2 osteoblasts (American Type Culture Collection, ATCC) were seeded in 6 well culture plates (Corning, USA), at a density of 10^5 cells/mL, in 2 mL of Dulbecco´s Modified Eagle´s Medium (DMEM, Sigma Chemical Company, St. Louis, MO, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS, Gibco, BRL), 1 mM L-glutamine (BioWhittaker Europe, Belgium), penicillin (200 μg/mL, BioWhittaker Europe, Belgium), and streptomycin

(200 μg/mL, BioWhittaker Europe, Belgium), under a 5% CO_2 atmosphere and at 37 ºC. Cells were cultured in the presence of MBG-PCL or MBG-PCL-zol scaffolds (1 scaffold per well) for 7 days in these conditions, renewing culture medium after 3 days. Controls in the absence of scaffolds were carried out in parallel. After 7 days, the culture medium was aspirated, the cells were washed with PBS and harvested using 0.25% trypsin-EDTA. Cell suspensions were centrifuged at 310 x g for 10 min and resuspended in fresh medium for the analysis of cell cycle and apoptosis quatification by flow cytometry. Cells were resuspended in PBS (0.5 mL) and incubated with 4.5 mL of ethanol 70% during 4 hours at 4 ºC. Then, cells were centrifuged at 310 x g for 10 min, washed with PBS and resuspended in 0.5 ml of PBS with Tritón X-100 (0.1%), propidium iodide (PI) (20 mg/mL) and RNAse (0.2 mg/mL, Sigma-Aldrich, St. Louis, MO, USA). After incubation at 37 ºC for 30 min, the fluorescence of PI was excited by a 15 mW laser tuning to 488 nm and the emitted fluorescence was measured with a 585/42 band pass filter in a FACScan Becton Dickinson flow cytometer. The cell percentage in each cycle phase: G_0/G_1, S and G_2/M was calculated with the CellQuest Program of Becton Dickinson and the $SubG_1$ fraction (cells with fragmented DNA) was used as indicative of apoptosis. For statistical significance, at least 10,000 cells were analyzed in each sample.

3. Osteoclast differentiation and resorption activity evaluation methods

Murine RAW-264.7 macrophages were seeded on glass coverslips in 6 well culture plates (Corning, USA), at a density of 2 x 10^4 cells/mL, in 2 mL of Minimum Essential Medium Eagle (MEM) Alpha Modification (αMEM, Sigma Chemical Company, St. Louis, MO, USA), supplemented with 5% fetal bovine serum (FBS, Gibco, BRL), 1 mM L-glutamine (BioWhittaker Europe, Belgium), penicillin (200 μg/mL, BioWhittaker Europe, Belgium), and streptomycin (200 μg/mL, BioWhittaker Europe, Belgium). To stimulate osteoclast differentiation, 40 ng/mL of mouse recombinant receptor activator for nuclear factor κ B ligand (TRANCE/RANKL, carrier-free, BioLegend, San Diego) and 25 ng/mL recombinant human macrophage-colony stimulating factor (M-CSF, Milipore, Temecula) were added to the culture medium and cells were cultured for 7 days in these conditions, renewing culture medium after 3 days. MBG-PCL and MBG-PCL-zol scaffolds were immersed in the medium of osteoclast cultures (1 scaffold per well) from either the first day or the sixth day of differentiation until the seventh day. Controls in the absence of scaffolds were carried out in parallel. For confocal microscopy studies, the cells cultured on glass coverslips were washed with PBS, fixed with 3.7% paraformaldehyde in PBS for 10 min, permeabilizated with 0.1% Triton X-100 for 3 min and preincubated with PBS containing 1% BSA for 30 min. Then,

cells were incubated with rhodamine phalloidin (1:40, v/v Molecular Probes) for 20 min to stain F-actin filaments. Samples were then washed with PBS and cell nuclei were stained with 3 μM DAPI (4′-6-diamidino-2′-phenylindole; Molecular Probes) for 5 min. After mounting with Prolong Gold reagent (Thermo Fisher Scientific), cells were examined using a Leica SP2 Confocal Laser Scanning Microscope. Rhodamine fluorescence was excited at 540 nm and measured at 565 nm. DAPI fluorescence was excited at 405 nm and measured at 420–480 nm. In order to evaluate the resorption activity of osteoclasts, RAW-264.7 macrophages were seeded on the surface of nanocrystalline hydroxyapatite (nano-HA) disks and differentiate into osteoclasts for 7 days in the conditions described above, renewing culture medium after 3 days. MBG-PCL and MBG-PCL-zol scaffolds were immersed in the medium of osteoclast cultures (1 scaffold per well) from either the first day or the sixth day of differentiation until the seventh day. Controls in the absence of scaffolds were carried out in parallel. Nano-HA disks were prepared by controlled precipitation of calcium and phosphate salts and subsequently heated at temperatures below the sintering point, as previously described [43]. After 7 days of differentiation, cells were detached using cell scrapers and disks were dehydrated, coated with gold-palladium and examined with a JEOL JSM-6400 scanning electron microscope in order to observe the geometry of resorption cavities produced by osteoclasts on the surface of nano-HA disks.

4. Osteoclasts resorption studies

MBG-PCL and MBG-PCL-zol were immersed in the culture medium from either the first day or the sixth day of osteoclasts differentiation until the seventh day.

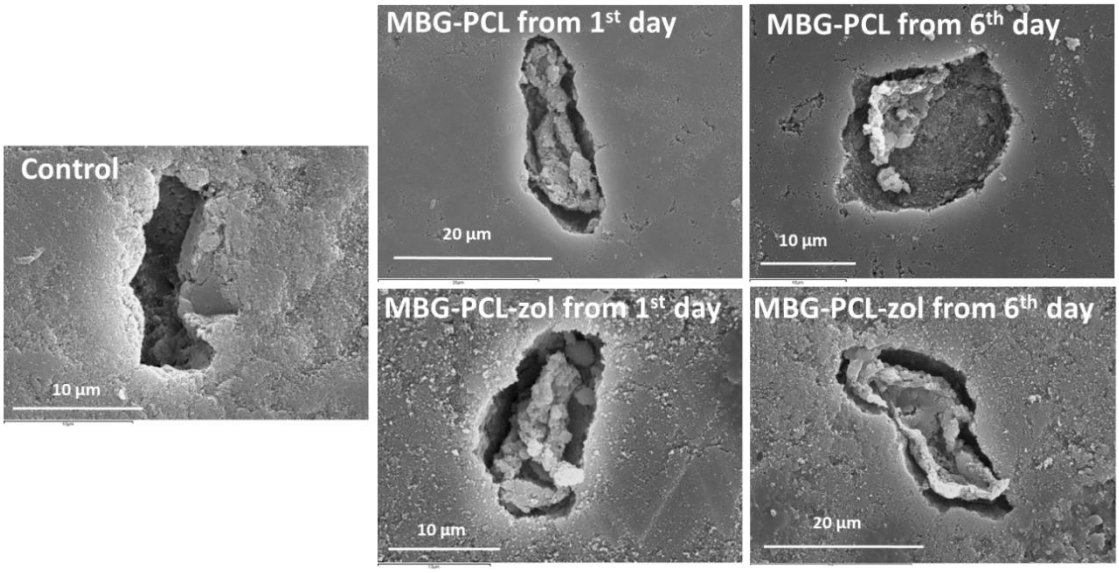

Figure S2. Resorption cavities left by osteoclast-like cells on nanocrystalline hydroxyapatite disks after 7 days of differentiation in the presence of MBG-PCL and MBG-PCL-zol scaffolds The magnification of each image was chosen during the observation of the different samples in order to highlight the resorption carried out by osteoclasts in each condition.

5. Ovariectomy surgical procedure

Intraoperative analgesia was achieved with ketorolac (1mg/kg) and buprenorphine (0.01mg/kg). For postoperative analgesia, meloxicam (0.4mg/kg/24h) was used for three days. The laparoscopic ovariectomy was performed placing one 10 mm and two 5 mm trocars, one at the umbilicus for the telescope and one in each iliac fossa, being the 10 mm trocar located at the right side. The ovaries were removed after sealing the ovarian pedicle with LigaSure™ (Medtronic) and exteriorised by removing the 10 mm trocar.

6. Induction of osteoporotic model

To reproduce similar conditions as osteoporosis in humans, six months before the implantation all sheep underwent, under sterile conditions and general anaesthesia induced by propofol (4 mg/kg) and maintained by isoflurane (1.5%), a laparoscopic bilateral ovariectomy (see supporting information for further details). At the same time, a low-calcium diet (0.5%) and corticosteroids administration (500 mg methylprednisolone via intramuscular injection every 3 weeks) were implemented until the end of the study.

7. Implantation of scaffolds

Cylindrical size defects (10x13mm) were created in each sheep by drilling the cancellous bone of the proximal tibia epiphysis, medial epicondyle of the femur and greater tuberosity of the humerus, under continuous irrigation with cold sterile saline,]. The sample size for each type of scaffold was n = 6. Two defects in each sheep were left empty as control. Once scaffolds were randomly implanted (Figure S3), the muscular and subcutaneous tissue was approximated with absorbable monofilament suture and the skin with absorbable braided suture. Postoperative analgesia was maintained with buprenorphine (0.01mg/kg/8h/3 days) and meloxicam (0.4mg/kg/24h/7 days). Ceftiofur (1mg/kg/24h) was administered for 7 days as prophylactic antibiotherapy. The health condition of all animals was checked daily along

the whole study by an accredited veterinarian. Immediately after the surgical procedure and before the sample removal, a computed tomography (CT) scan was performed (Figure S4).

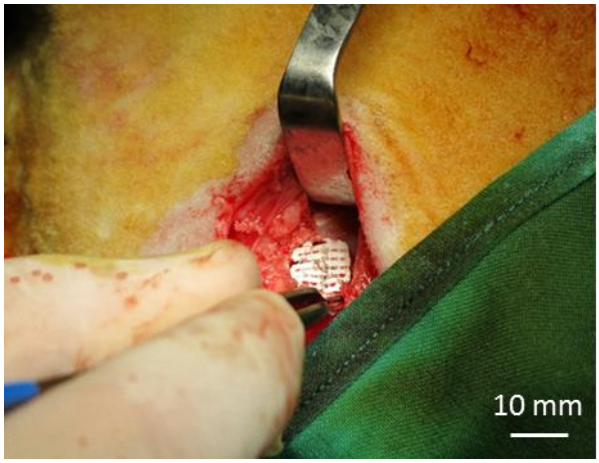

Figure S3. Surgical implantation of MBG-PCL scaffolds

8. Computed tomography (CT) scans

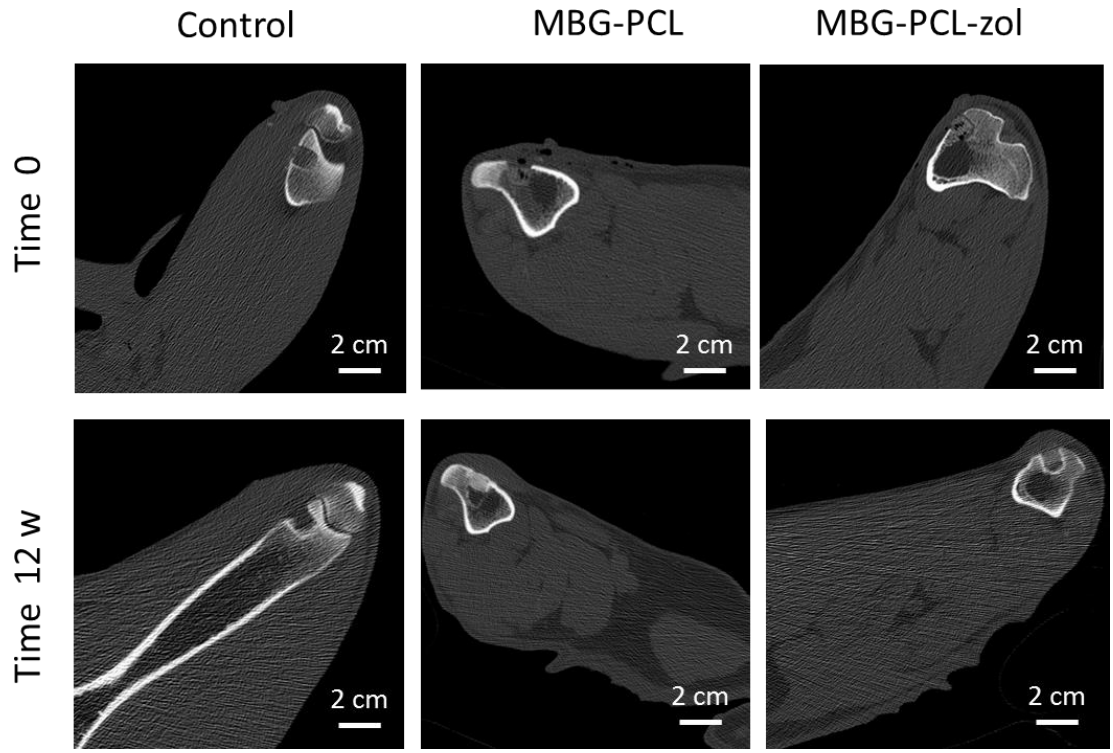

Figure S4. computed tomography (CT) scans performed immediately after the surgical procedure (Time 0) and before the sample removal (12 weeks).

9. Histological overviews

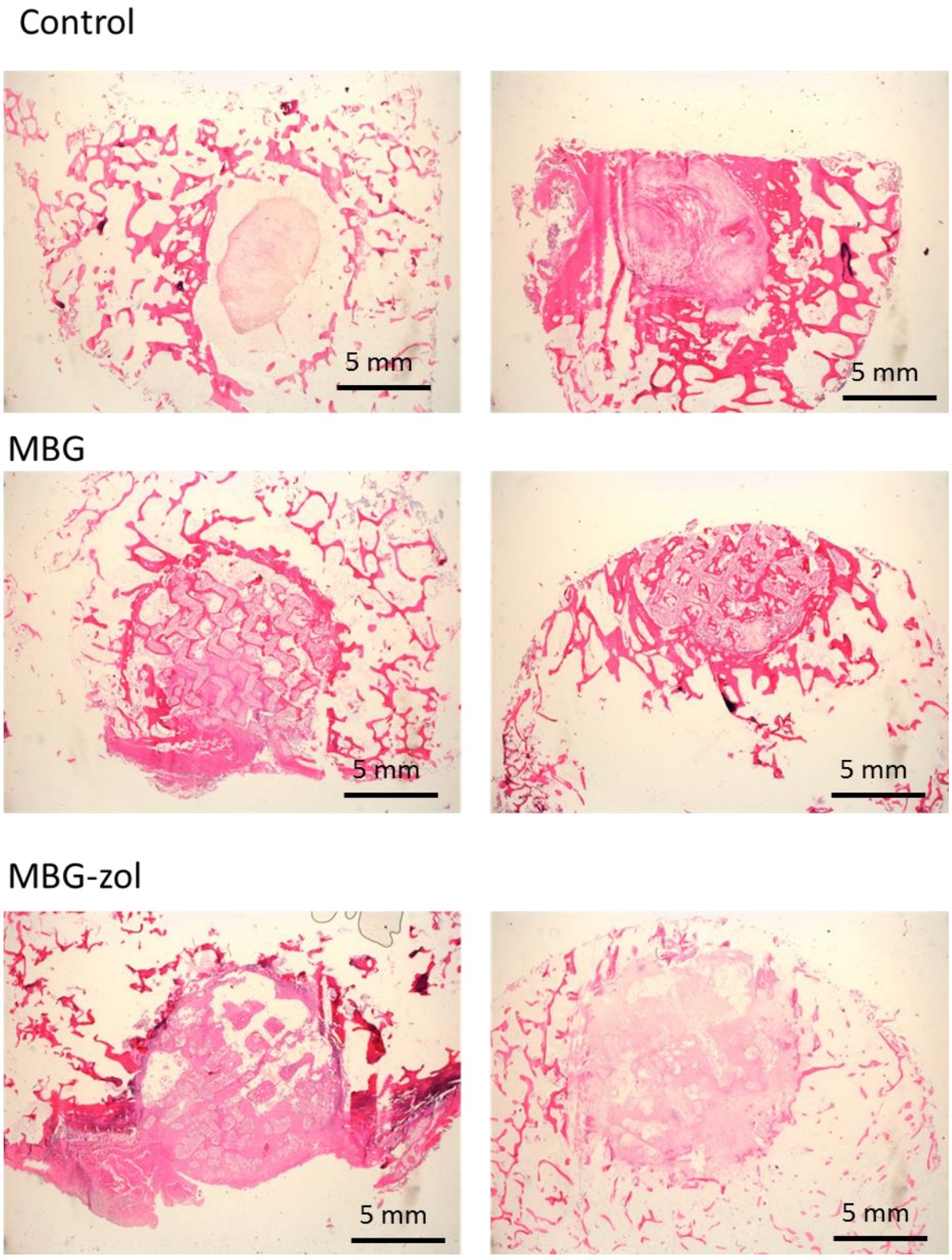

Figure S5. Histological overviews of the samples after 12 weeks of implantation

Table S1. Presence of inflammatory tissue and vascularization after 12 weeks of implantation for each animal.

Sample	Inflammatory tissue (0-4)	Vascularization (0-3)	Osteoblasts presence (0-4)	Osteoclasts presence (0-4)
MBG-PCL	0,1,1,1,1,2	1,2,3,3,3,3	3,3,4,4,4,4	2,2,3,3,3,3
MBG-PCL-zol	2,3,3,3,4,4	1,2,3,3,3,3	1,2,2,2,3,3	1,2,2,3,3,3