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Polycaprolactone / bioactive glass hybrid scaffolds for bone regeneration

*Cédric Bossard,^a Henri Granel,^b Édouard Jallot,^a Christophe Vial,^c Hanna Tiainen,^d Yohann
Wittrant^b and Jonathan Lao^a*

^a. Université Clermont Auvergne, CNRS/IN2P3, Laboratoire de Physique de Clermont, BP
10448, F-63000 Clermont-Ferrand, France. E-mail: cedric.bossard@clermont.in2p3.fr

^b. Université Clermont Auvergne, INRA, UNH, Unité de Nutrition Humaine, CRNH Auvergne,
F-63000 Clermont-Ferrand, France

^c. Université Clermont Auvergne, CNRS, Sigma Clermont, Institut Pascal, F-63000, Clermont-
Ferrand, France

^d. Department of Biomaterials, Institute of Clinical Dentistry, University of Oslo, Geitmyrsveien
69-71, 0455 Oslo, Norway

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Abstract

Bioactive glasses (BG) bond to bone and stimulate bone regeneration, but they are brittle. Inorganic-organic hybrids appear as promising bone substitutes since they associate the bone mineral forming ability of BG with the toughness of polymers. Hybrids comprised of polycaprolactone (PCL) and SiO₂-CaO BG were produced by sol-gel chemistry and processed into porous scaffolds with controlled pore and interconnection sizes. The obtained scaffolds are highly flexible, meaning that PCL effectively introduces toughness. Apatite formation is observed within 24 hours of immersion in simulated body fluid and is not limited to the surface as the entire hybrid progressively changes into bone-like minerals. The degradation rate is suitable for bone regeneration with a 13.2 % weight loss after 8 weeks of immersion. Primary osteoblasts cultured in scaffolds demonstrate that the samples are not cytotoxic and provide good cell adhesion. The *in vivo* study confirms the bioactivity, biocompatibility and suitable degradation rate of the hybrid. A physiological bone made of trabeculae and bone marrow regenerates. The structure and kinetic of bone regeneration was similar to the implanted commercial standard based on bovine bone, demonstrating that this new synthetic PCL-BG hybrid could perform as well as animal-derived bone substitutes.

1. Introduction

BG are a class of bone substitutes first developed by Hench in 1969.¹ They have been the focus of much research as they strongly bond to bone,² they resorb as bone regenerates and their dissolution products stimulate osteoblasts at the genetic level.³ These unique properties have led

to the commercialization of BG, first in the form of monoliths (MEP®, 1985) and later in the form of particulates (PerioGlas®, 1993). However, BG have not been commercialized under a spongy bone-like structure yet. Cancellous bone has a 3-dimensional porous structure that allows cell invasion and fluid flow, thus promoting angiogenesis and osteogenesis.⁴⁻⁵ Although BG scaffolds mimicking bone have been successfully processed, their high porosity combined with the brittle nature of BG prevents them from being viable in cyclic load-bearing applications.⁶ In order to improve the mechanical properties of scaffolds, BG particles can be dispersed in a bioresorbable polymer which introduces toughness to the material. The main drawback of these composites is that the organic and inorganic phases degrade at different rates, thereby causing mechanical instability and release of BG particles *in vivo*.⁷ In BG-polymer hybrids, the interpenetration of the two phases at a molecular level provides more uniform and stable properties.⁷ Another key advantage of hybrid sol-gel materials is the potential for tailoring their degradation rates and their mechanical properties through a controlled covalent linkage between the organic and inorganic chains.⁸

While BG-based hybrid scaffolds have become of increasing interest in the field of biomaterials, their complex synthesis constitutes a chemical challenge that hinders their development. Indeed, the presence of a polymer at an early stage of the sol-gel process forbids thermal treatments, which are commonly employed to incorporate calcium ions into the BG silicate network. Calcium is essential for the bioactivity of BG and it is known to trigger osteoblast proliferation and differentiation.⁹ When processing hybrid materials, calcium alkoxides precursors are preferred to salts, like CaCl₂ and Ca(NO₃)₂, as they allow effective calcium incorporation even at low temperatures.¹⁰ Nevertheless, their high sensitivity to water can lead to premature gelation of the BG sol and make it more challenging to produce a

macroporous structure.¹¹⁻¹² Therefore, although many hybrid scaffolds have been created in the recent years, they either do not contain calcium and possess a limited bioactivity,¹³⁻¹⁵ or they do not incorporate calcium due to the use of salts.¹⁵⁻¹⁷ Only a few BG-based hybrids with well incorporated calcium were obtained, but they were in the form of dense^{12, 18} or fibre¹¹ materials.

50 To avoid the restrictions that arise from the use of calcium, borophosphosilicate glasses SiO₂-B₂O₃-P₂O₅ are a possible alternative as boron is known to accelerate the degradation of BG and induce a faster apatite precipitation on its surface.¹⁹ This means that, even though borophosphosilicate glasses do not contain calcium, they have the potential to be bioactive.²⁰

In a previous work, we developed a protocol to process BG-gelatin hybrid scaffolds with controlled and tuneable porosity and we showed that the calcium was successfully incorporated

55 at room temperature.²¹ Initially, the materials degraded rapidly in simulated body fluid (SBF), which was attributed to the weak interactions (hydrogen bonds and van der Waals interactions) between the organic and inorganic phases. But when a coupling agent was employed, the gelatin chains cross-linked to the silicate network and the degradation was significantly slowed down.

60 Likewise, several authors report the fast degradation of hybrids even with the use of a coupling agent. For example, Poologasundarampillai *et al.* mention a release of 60 % of γ -polyglutamic acid within 2 weeks of soaking in SBF,¹² and Li *et al.* mention a 43 % weight loss of BG-polyethylene glycol monoliths after 3 weeks.¹⁸ An optimal bone substitute should act as a temporary template and degrade at a specific rate (typically more than 6 months)²²; ideally, it

65 should degrade at the same rate as bone grows, so that mechanical properties are maintained. As yet, most of the BG-based hybrids presented in the literature do not meet this requirement.

In the present study, we adapted the aforesaid protocol to process BG-PCL hybrid scaffolds. FDA-approved PCL is a bioresorbable aliphatic polyester that has been suggested for a wide

range of applications such as drug delivery systems, medical devices and tissue engineering.²³ It
70 has a slow degradation rate compared to other polymers (up to 4 years),²⁴ making it more
suitable for long-term degradation applications like bone graft. The apatite-forming ability,
degradation rate, mechanical properties, cytotoxicity and *in vivo* performance of the obtained
BG-PCL hybrid scaffolds were investigated.

75 2. Materials and methods

2.1. Synthesis of a hybrid solution

A BG oligomer solution was obtained using tetraethyl orthosilicate (TEOS) (99 % purity,
Aldrich) and calcium ethoxide (Gelest). TEOS was first hydrolysed in ethanol (absolute,
Aldrich) by adding 2 M HCl (obtained from 37 % fuming, Aldrich) (molar ratio ethanol : H₂O :
80 TEOS : HCl = 3.7 : 2 : 1 : 0.07). The same volume of ethanol was used for the dilution of
calcium ethoxide and the two solutions were mixed together to obtain a sol which was left for
condensation. In parallel, PCL (M_n = 80,000 g.mol⁻¹, Aldrich) was dissolved in an appropriate
solvent and then mixed with the inorganic sol for 2 hours for homogenisation and further
condensation.

85

2.2. Procedure to process scaffold

Scaffolds were obtained using a porogen leaching method. Paraffin microspheres were
fabricated by emulsifying paraffin in hot water as described by Ma *et al.*²⁵ After sieving, the
400-600 μm spheres were stacked in polyethylene moulds and heated at 40°C to partially fuse
90 them. The BG-PCL hybrid solution was then poured onto the stack of microspheres and
centrifuged at 6,000 rpm for a complete infiltration. Gelation and ageing of the materials were

performed at room temperature for 72 h. The materials were immersed in cyclohexane (99.8 % purity, Aldrich) for 24 h to dissolve the paraffin porogen. This operation was renewed twice. Finally, the obtained macroporous hybrid scaffolds were rinsed with absolute ethanol to remove
95 the cyclohexane and dried at room temperature. Pore and interconnection sizes were extracted from Scanning Electron Microscopy (SEM) pictures thanks to the ImageJ software.

2.3. Micro-computed tomography

Micro-computed tomography (microCT) was used to determine the three-dimensional
100 microstructure of the scaffolds. The samples were scanned with SkyScan 1172 microCT imaging system (Bruker microCT, Kontich, Belgium) at 6 μm voxel resolution using source voltage of 40 kV and current of 167 μA without source filter. The samples were rotated 180° around their vertical axis and three absorption images were recorded every 0.4° rotation. The absorption images were then reconstructed with the standard SkyScan reconstruction software (NRecon) to
105 serial coronal-oriented tomograms using 3D cone beam reconstruction algorithm. For the reconstruction, beam hardening was set to 5 % and ring artefact reduction to 10. The image analysis of the reconstructed axial bitmap images was performed using the standard SkyScan software (CTan and CTvox) and included thresholding and despeckling (removing objects smaller than 1000 voxels and not connected to the 3D body). In order to eliminate potential edge
110 effects, a rectangular volume of interest (VOI) with a width and length of 5 mm and a height of 3 mm was selected in the centre of the scaffold.

All images underwent 3D analysis, followed by the quantification of interconnectivity *i.e.* the fraction of pore volume in a scaffold that is accessible from the outside through openings of a certain minimum size.²⁶ A ‘shrink-wrap’ process was performed between two 3D measurements

115 to shrink the outside boundary of the VOI through any openings with a size equal to or larger than a threshold value (0 - 240 μm were used in this study). Interconnectivity was calculated as follows:

$$\text{Interconnectivity} = (V - V_{\text{shrink-wrap}}) / (V - V_m),$$

where V is the total volume of VOI, $V_{\text{shrink-wrap}}$ is the VOI volume measured after shrink-wrap
120 processing, and V_m is the volume of scaffold material.

2.4. Bioactivity evaluation in SBF

Simulated body fluid (SBF) was prepared following Bohner and Lemaitre's recommendations.²⁷ BG-PCL hybrid scaffolds were immersed in SBF at a ratio of 1 mg.mL⁻¹ and
125 put on an orbital shaker with an agitation rate of 250 rpm at 37°C. The scaffolds were glued to the bottom of the flasks to prevent them from floating and placed under vacuum for 5 min to ensure the infiltration of SBF in the pores. After 6 h, 1 day, 3 days and 7 days of interaction, the solutions were filtered with a 0.2 μm paper and the Ca, P and Si concentrations were determined by Inductively Coupled Plasma - Atomic Emission Spectroscopy (ICP-AES). The materials were
130 rinsed with pure ethanol to avoid further mineralization reactions and dried at ambient temperature. SEM imaging was then performed on samples coated with carbon using vacuum sputtering.

Samples were also analysed with PIXE (Particle-Induced X-ray Emission) nuclear microprobe, a technique that is very similar to SEM-EDS or an electron microprobe, but shows increased
135 sensitivity by 100 times. Reacted scaffolds were embedded in Agar resin and cut into slices of about 150 μm thickness with a low speed diamond saw. Quantitative chemical imaging of the obtained cross-sections was carried out at the AIFIRA platform (CENBG, France) using a 3

MeV incident proton beam (beam diameter of 1 μm). An 80 mm² Si (Li) detector (equipped with a 12 μm -thick beryllium window and an aluminium funny filter (a filter with a hole drilled at its centre, placed in front of the detector window) with a tiny hole of 2 mm) orientated at 135° with respect to the incident beam axis was used for X-ray detection. Concentrations in Ca, P and Si were determined using the Gupixwin software after calibration against the NIST 620 (soda-lime glass) standard reference material.

145 2.5. Mechanical testing

Mechanical properties of BG-PCL hybrid scaffolds were assessed using a TA Instruments 2980 Dynamic Mechanical Analyser. Cylindrical scaffolds with a 5 mm height and a 10 mm diameter were compressed at a constant force rate of 1 N.min⁻¹ and their deformation was measured over time. The Young's modulus was determined from the slope of the linear elastic regime using the TA Universal Analysis software. The yield strength was determined at the transition from the elastic regime to the stress plateau.

2.6. Degradation study

The degradation of BG-PCL hybrid scaffolds was investigated in SBF. The procedure to follow the mass of the samples over soaking time was similar to the one used for bioactivity testing. After weighing, scaffolds were glued to the bottom of flasks, immersed in SBF at 1 mg.mL⁻¹ and placed under vacuum for 5 min. Then, they were put on an orbital shaker with an agitation rate of 250 rpm and at a temperature of 37°C for up to 8 weeks. After interaction, the materials were rinsed with absolute ethanol, dried under vacuum and their mass was measured again.

2.7. Surface wettability

The surface wettability of PCL and BG-PCL hybrid was investigated. Scaffolds were crushed and grains of 100 to 150 μm were compressed into disks of approximately 1 mm in thickness. 10 μL of distilled water were dropped on the surface of the disks and pictures were taken with an IDS camera. Contact angle between the water and the disk was determined using the ImageJ software.

2.8. *In vitro* cell adhesion and biocompatibility

2.8.1. Cell culture

Primary osteoblast cells were isolated from 3 days old rats according to Declercq *et al.*²⁸ Briefly, calvaria were dissected and the cells were extracted through different enzymatic digestions (collagenase IA and dispase II) at 37°C. The cells were then cultivated in Minimum Essential Medium Eagle Alpha Modifications (α -MEM) supplemented with 10 % foetal bovine serum and 1 % penicillin/streptomycin at 37°C, 5 % CO_2 until 80 % confluency before being used.

2.8.2. SEM imaging

SEM was performed on primary osteoblasts cultured in scaffolds or on disks. Cells were fixed with a solution of 3 % glutaraldehyde in 0.2 mol.L^{-1} sodium cacodylate buffer (pH 7.4) for 1 h and rinsed in cacodylate buffer three times for 10 min each. Afterwards, the samples were dehydrated using a graded ethanol series (70, 95 and 100 % ethanol, 10 min for each bath). For SEM examination, the specimens of interest were treated in hexamethyldisilazane (HMDS, Delta

Microscopies), dried overnight and coated using a sputter coater equipped with a gold target
185 (Jeol JFC-1300) and observed with a scanning electron microscope (Jeol 6060-LV) at 5 KeV.

2.8.3. Fluorescence microscopy

Fluorescence microscopy was performed on primary osteoblasts cultured 14 days in BG-PCL
hybrid scaffolds under orbital agitation. Scaffolds were washed and incubated in fluorescent dye
190 (CyQUANT™ NF Cell Proliferation Assay Kit, Invitrogen) for 30 min. Pictures were taken with
inverted fluorescence microscope (AxioObserver Z1).

2.8.4. Cell viability

Scaffolds were first incubated in culture medium for 72 h to avoid the initial burst of calcium
195 ions that may damage cells.²⁹ Then, primary osteoblasts were seeded in a 96-well plate at a
density of 3500 cells per well, and cultured for 96 h in α -MEM medium supplemented with 10 %
foetal bovine serum in BG-PCL hybrid scaffolds. The cell viability was determined by a XTT
method using Viability/Proliferation Kit II (Aldrich) in accordance with supplier
recommendations. The optical density was measured through spectrophotometry at 550 nm and
200 compared with the control condition corresponding to the absence of material.

2.9. *In vivo* study

The *in vivo* study was performed at the Centre d'Investigation Clinique–Innovation
Technologique (CIC-IT) Bordeaux. All the procedures for rat handling were based on the
205 principles of Laboratory Animal Care formulated by the National Society for Medical Research
and approved by the Animal Care and Experiment Committee of University of Bordeaux,

France. Experiments were carried out in accredited animal facilities following European recommendations for laboratory animal care. Non-critical size defects in the femoral condyles of 13-week female rats weighing 250 to 300 g were filled with BG-PCL hybrid or commercialized decellularized bovine bone (n = 6 for each material). Bone defects were also left empty as the control condition (sham operation). The animals were anesthetised with isoflurane and a 23 mm³ defect was created by using a round bur with a diameter of 3.1 mm under copious saline irrigation. The bone cavity was washed with saline solution and filled with the material, then the wound was closed with resorbable inner sutures and cutaneous surgical fastener. The sacrifice was carried out after 9 or 15 weeks by CO₂ asphyxiation. The specimens were immediately fixed in 4 % formalin at 4°C for 9 days and then kept in phosphate-buffered saline. Because BG-PCL hybrid is sensitive to temperature, the explants were embedded in the cold-curing resin Technovit® 9100 (Kulzer). The obtained blocks were cut into slices of 7 µm using a RM2255 microtome (Leica) and stained following the Goldner's trichrome method or with von Kossa/MacNeal. Histological sections were eventually observed with a light microscope (Nikon) and analysed using the NIS Elements D software. The different areas (fibrosis, new bone and residual material) were identified and quantified with respect to the surface of the initial bone defect (histomorphometric analysis). Differences between samples were evaluated with a two-way analysis of variance (ANOVA) followed by a Bonferroni's test. Non-coloured sections were also analysed with a PIXE nuclear microprobe (H⁺ 3 MeV).

3. Results and discussion

3.1. Synthesis of a hybrid solution

As mentioned above, calcium alkoxides are required to allow the calcium to enter the silicate
230 network at room temperature. In this work, we opted for calcium ethoxide ($\text{Ca}(\text{OEt})_2$) which has
already proven to be an adequate calcium source for the synthesis of hybrids.²¹ It is worth noting
that $\text{Ca}(\text{OEt})_2$ was chosen over calcium methoxyethoxide used in pioneer studies^{10-11, 30} because
of the safety of the by-products generated (ethanol here, while methanol by-products can be
generated in the above references). This is of special importance in the case of incomplete
235 hydrolysis of the alkoxide since the remaining ligands are likely to be released *in vivo*. As
 $\text{Ca}(\text{OEt})_2$ is extremely reactive towards water, a reduced water-to-TEOS molar ratio of 2 was
used in our synthesis. This effectively delayed the gelation of the BG sol, which happened after
few hours to several days depending on the age of the calcium precursor (hydrolysis reactions
take place with atmospheric humidity in the container). Briefly, TEOS is first hydrolysed in an
240 acidified alcoholic solution to form silanol species. Dilute $\text{Ca}(\text{OEt})_2$ is then added and
condensation reactions occur, resulting in chains of SiO_2 -CaO nanoparticles. The amounts of
precursors were adjusted to reach a BG composition of 75 wt% SiO_2 - 25 wt% CaO known to
induce rapid bioactivity.³¹ Before further condensation leads to gelation, the BG sol is mixed
with the polymer solution so that the organic and inorganic chains entangle to create a hybrid
245 containing up to a 40 wt% BG fraction. Finally, the translucent hybrid solution (Fig. 1) can be
processed to obtain a macroporous structure.

3.2. Procedure to process scaffolds

In the literature, several techniques have been developed to produce scaffolds from a BG sol,
250 such as the polymer foam reticulation³² and the sol-gel foaming process,³³ but they involve a
sintering step and the use of water (respectively) that are not compatible with our hybrid sol – the

presence of polymer forbids thermal treatments whereas water provokes a premature gelation. Here we found a convenient way of generating a 3D-interconnected macroporous structure at ambient temperature and without adding water. A porogen leaching method is employed, which results in a highly porous BG-PCL hybrid scaffold as depicted in Fig. 2a. The pore size is close to the size of the porogen microspheres, which means that it can be readily controlled through the granulometry of the microspheres (ESI 1). Similarly, the interconnection size between pores can be finely tuned by previously heating the stack of microspheres for different times as illustrated in Fig. 2b. Indeed, interconnections originate from the contact point between each porogen spheres and their widening can be achieved through partial fusion of the spheres. Both the pore and interconnection sizes are key parameters to vascularisation and cell migration, and while it is generally admitted that the minimal pore size should be 100 μm ,³⁴ Karageorgiou and Kaplan reported enhanced bone regeneration and formation of capillaries for pores larger than 200-300 μm and interconnections superior to 50 μm .³⁵ To follow these recommendations, the scaffolds in the subsequent sections were produced with paraffin spheres ranging from 400 to 600 μm , which gave pores from 300 to 500 μm (Fig. 2c), and their pre-fusion was carried out to reach interconnections between 150 and 200 μm . According to microCT measurements, the scaffolds have relatively thick struts of $145 \pm 6 \mu\text{m}$ and a total porosity of $68.5 \pm 1.1 \%$ (including barely 0.1 % closed porosity). Interconnectivity analysis (ESI 2) confirms the well-interconnected structure previously observed by SEM and suggests that the mean interconnection size is around 175-190 μm , which fits SEM measurements.

3.3. Bioactivity evaluation

The bioactivity of BG-PCL hybrids was characterized by immersing scaffolds in SBF for various times. The evolution of Ca, P and Si concentrations as a function of soaking time was followed both in the medium (Fig. 3a, ICP-AES) and in the walls of the hybrid scaffolds (Fig. 3b, PIXE). The two techniques gave concomitant results that are typical of BG interaction with SBF.³⁶ At first, Ca and Si ions are released from the samples (Fig. 3b, 6h), resulting in an increase of their respective concentrations in SBF (Fig. 3a, 6h). These dissolution products, known to stimulate osteoblast proliferation and differentiation,^{9, 37-38} trigger the formation of bone mineral by changing the local supersaturation of the medium with regard to apatite precipitation.³⁹ Here the release of Ca ions is followed by a rapid decrease of Ca and P concentrations in the medium (Fig. 3a), which is in accordance with the precipitation of calcium phosphates on the samples (Fig. 3b). Moreover, quantitative analysis on the scaffolds immersed for 7 days in SBF shows a Ca/P atomic ratio of 1.6 in the most reacted areas (data not shown) corresponding to that of biological apatites found in bone.⁴⁰ SEM on this sample further confirms these observations, as Fig. 3c and Fig. 3d reveal the formation of an apatite-like layer on its surface.

The cross-sections of scaffolds were also observed by quantitative chemical mapping using the PIXE nuclear microprobe. After 3 days of soaking in SBF (Fig. 3e-g), phosphorus is found on the surface as well as in the walls of the hybrid scaffolds. This shows that, unlike conventional BG in which apatite only deposits on the outer layer,⁴¹⁻⁴² BG-PCL hybrids have the ability to form apatite throughout the whole volume of the material. This enhanced bioactivity, already observed in BG-gelatin hybrid scaffolds, was previously attributed to the homogeneity of Ca incorporation.²¹

3.4. Mechanical properties

Fig. 4 illustrates the representative compressive stress-strain curve obtained for BG-PCL hybrid scaffolds. Unlike pure BG scaffolds, brittle crushing of the pore walls does not occur and the samples exhibit a ductile behaviour typical of elastomeric or elastic-plastic cellular solids.⁴³ The addition of PCL effectively introduces toughness to the material and the resultant hybrid can potentially be implanted into sites subjected to cyclic loads. However, the samples display low stiffness as evinced by their elastic modulus of 0.49 MPa (\pm 0.03 MPa) and yield strength of 0.036 MPa (\pm 0.003 MPa), which are significantly below those of trabecular bone with the same porosity (95 % porosity).⁴³⁻⁴⁴ Therefore, although BG-PCL hybrid scaffolds may not collapse during handling and during the patient's normal activities thanks to its high flexibility, it is not able to share mechanical load with the host bone. This might not be a major concern since the implant site will progressively recover mechanical properties as new bone grows and bone substitute resorbs. The low stiffness of the scaffolds can be attributed to the polymer used, PCL, which is highly flexible compared to other bioresorbable polymers even at high molecular weight (here, M_n (PCL) = 80000 g.mol⁻¹),⁴⁵⁻⁴⁶ and to the lack of bonding between the organic and inorganic phases.⁴⁷ As reported in the literature, mechanical properties of PCL-based hybrids can be improved by blending PCL with a stiffer polymer such as polyhydroxybutyrate,¹⁵ or through covalent linkage between the PCL chains and the silicate network.⁴⁸

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3.5. Degradation assay

PCL is known to undergo a two-stage degradation process *in vivo*: first, an uptake of water followed by the hydrolytic chain scission of ester bonds causes a decrease in molecular weight, and second, once the molecular weight has reduced to approximately 3000 g.mol⁻¹ the polymer is

320 fully degraded via an intracellular mechanism.²³ Due to the presence of five hydrophobic $-CH_2$
moieties in its repeating units, the de-esterification of PCL is slow and can take longer than 2
years depending on the initial molecular weight of the polymer, whereas intracellular
degradation and subsequent excretion last 4 to 5 months.²⁴ In the present study, the degradation
of BG-PCL hybrid scaffolds was investigated *in vitro* by following their mass in SBF for up to 8
325 weeks (Fig. 5a). The samples degrade slowly with only 13.2 % weight loss after 8 weeks of
soaking in SBF. This degradation rate is more suitable for bone graft applications compared to
many hybrids presented in the literature.^{12, 18} As evidenced by the bioactivity testing (Fig. 3a and
Fig. 3b), the inorganic phase of BG-PCL hybrids mainly dissolves during the first week of
interaction. This implies that the weight losses at 2, 4 and 8 weeks are likely to be caused by the
330 degradation of PCL rather than the dissolution of BG. Hence, it appears that de-esterification
occurs after few weeks in BG-PCL hybrids instead of months or even years in pure PCL. This
substantial difference of kinetics can be explained by the hydrophilicity of BG. Indeed, water
contact angle (θ) measurements (Fig. 5b) on pure PCL ($\theta = 83.4^\circ \pm 3.1^\circ$) and 30 wt% BG / 70
wt% PCL hybrid ($\theta = 56.3^\circ \pm 4.8^\circ$) disks show that the presence of BG increases the surface
335 wettability of the material. Water absorption, which initiates the hydrolytic chain scission of
ester groups, is therefore faster in the hybrid. Moreover, BG is known to induce a local increase
in pH while it dissolves. In addition to an antibacterial effect,⁴⁹ it can be assumed that hydroxide
ions catalyse the cleavage of ester linkages.

340 3.6. *In vitro* cellular behaviour

Cell adhesion on a material surface is a prerequisite to successful cell proliferation and
differentiation.⁵⁰ Primary osteoblasts were cultured on pure PCL (Fig. 6a) and BG-PCL hybrid

(Fig. 6b) disks. Only few cells adhere to the pure PCL substrate and display a spherical morphology indicative of poor adhesion, whereas cells appear well spread on the hybrid substrate and exhibit a favourable spindle shape with elongated filopodia. Cell adhesion on BG-PCL hybrid can be explained by its increased hydrophilicity described previously (Fig. 5b), as more hydrophilic surfaces are highly desirable for osteoblast adhesion⁵¹ and for the regulation of inflammation through the apoptosis of adherent macrophages.⁵²⁻⁵³ Besides, apatite progressively forms throughout the whole material and the obtained bone-like surface may facilitate cell attachment. Indeed, Olmo *et al.* showed that the formation of an apatite layer on 75 wt% SiO₂-25 wt% CaO BG significantly enhances osteoblast attachment.⁵⁴

Primary osteoblasts were also cultured in BG-PCL hybrid scaffolds and the samples were observed by fluorescence microscopy (Fig. 6c) and SEM (ESI 3). The cells adhere on the walls and are well dispersed inside the scaffold, meaning that BG-PCL hybrid scaffolds allow cell colonisation and cell adhesion.

To determine whether the samples are cytotoxic, primary osteoblasts were cultured in BG-PCL hybrid scaffolds and cell viability was examined after 96 h. Results are presented in Fig. 6d and demonstrate that the scaffolds are non-cytotoxic given that no statistical difference is observed with the control conditions.

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3.7. *In vivo* study

The *in vivo* performance of BG-PCL hybrids was evaluated in femoral condyles of 13-week rats. Commercialized bovine bone was also implanted as the reference material. Histology was performed after 9 weeks and 15 weeks of implantation (Fig. 7a-g). Bone tissue was identified thanks to the combination of two staining techniques: collagen appears in blue-green when

365

applying the Goldner's trichrome method (Fig. 7a-c) and calcium is stained in black when using von Kossa/MacNeal (Fig. 7d). The inflammatory response, known to initiate the regenerative healing process,⁵⁵ is minimal at 9 weeks and 15 weeks for both materials (Fig. 7e). This confirms the biocompatibility of BG-PCL hybrids previously described *in vitro*. As the defect size is not
370 critical, bone entirely regenerates in 15 weeks without the need for a filling material (Fig. 7a and Fig. 7f), but doing so an undesirable dense structure is obtained for sham-operated rats (non-physiological bone healing). When bovine bone or BG-PCL hybrid is employed, a physiological bone grows as illustrated by the numerous bone trabeculae after 15 weeks of implantation (Fig. 7b and Fig 7c). Furthermore, vascularised adipose tissue and bone marrow are found abundantly
375 between the trabeculae, which indicates a well-advanced bone reconstruction for both materials. Quantitative analysis of bone reconstruction (Fig. 7f) shows no statistical difference between BG-PCL hybrid and bovine bone. Finally, BG-PCL hybrid appears to be partially degraded after 9 weeks of implantation and even more after 15 weeks: a few of the material is left (Fig. 7c), in contrast with bovine bone that barely degraded (Fig. 7b). Histomorphometry further confirms
380 these observations, as the bone defect contains significantly less residual material after 15 weeks of implantation when using BG-PCL hybrid instead of bovine bone (Fig. 7g).

Histological sections from BG-PCL hybrid samples after 15 weeks of implantation were also analysed with the PIXE nuclear microprobe. Fig. 7h shows that the inorganic compositions of the residual material, trabecular cavity and bone trabeculae are similar and resemble that of bone.
385 Only 5 wt% of Si remains in the hybrid compared to the initial 30 wt% (Fig. 3b), and its low concentration in the trabeculae and trabecular cavity (Fig. 7h) suggests that it was removed from the implant site. In fact, Lai *et al.* proved that in rabbits "the silicon eluted from BG in bone tissue was safely excreted through urine with no adverse physiological effects".⁵⁶ The results

further reveal the apatite-forming ability and thereby bioactivity of BG-PCL hybrids *in vivo*.
390 Indeed, the residual material is greatly mineralised with an atomic Ca/P ratio close to the one
from bone trabeculae (1.8 and 1.7, respectively, Fig. 7i). Bioactivity leads to a strong and stable
interface between the implant and the host bone and considerably reduces the chance of failure.
It is therefore a key advantage of BG-based materials in comparison with other biomaterials like
bovine bone or pure PCL.

395 To conclude this comparative study, the two bone substitutes perform alike in terms of bone
healing and biocompatibility; however, BG-PCL hybrid surpasses bovine bone when it comes to
degradation and bioactivity.

4. Conclusion

400 Despite the difficulties that arise from the synthesis of BG-based hybrid scaffolds (integration
of calcium ions in the silicate network, fabrication of a macroporous structure with a calcium
alkoxide precursor), we were able to process BG-PCL hybrid scaffolds with well incorporated
calcium. The process is relatively simple and allows the control of both the pore and
interconnection sizes. The addition of PCL effectively introduces toughness and the hybrid
405 scaffolds can be implanted into sites subjected to cyclic loads. Even though more work is needed
to increase its stiffness to match that of bone, BG-PCL hybrid appears as a promising bone
substitute from its overall remarkable properties: shortly after implantation, its enhanced apatite-
forming ability observed *in vitro* and *in vivo* may provide a strong interface with host tissues.
Because the material contains BG, it has a hydrophilic surface that favours the initial attachment
410 of cells while the release of osteoinductive Si and Ca ions may stimulate the proliferation and
differentiation of osteoblasts, thus leading to the regeneration of physiological bone comprised of

trabeculae and bone marrow. After few months of implantation, the hybrid still acts as a support for bone growth thanks to its slow degradation *in vivo*. Bone healing (structure and kinetic) is similar when the commercial reference made of bovine bone is employed, with the difference
415 that bovine bone does not degrade.

Conflict of interest

The authors declare no competing financial interest.

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