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***In vitro* and *in vivo* evaluation of a polylactic acid-bioactive glass composite for bone fixation devices**

Gwenaëlle Vergnol,^{1,2} Nathalie Ginsac,^{1,3} Pascaline Rivory,^{1,2} Sylvain Meille,^{1,3} Jean-Marc Chenal,^{1,3} Sandra Balvay,^{1,2} Jérôme Chevalier,^{1,3,4} Daniel J. Hartmann^{1,2}

¹Université de Lyon, CNRS, Villeurbanne, France

²Université Claude Bernard Lyon1, MATEIS UMR5510, F-69373 Lyon 8, France

³INSA-Lyon, MATEIS UMR5510, F-69621 Villeurbanne, France

⁴Institut Universitaire de France, 103 bd Saint Michel, F-75005 Paris, France

Abstract: Poly(lactic acid) is nowadays among the most used bioabsorbable materials for medical devices. To promote bone growth on the material surface and increase the degradation rate of the polymer, research is currently focused on organic–inorganic composites by adding a bioactive mineral to the polymer matrix. The purpose of this study was to investigate the ability of a poly(L,D,L-lactide)-Bioglass[®]RV (P(L,D,L)LA-Bioglass[®]VR 45S5) composite to be used as a bone fixation device. *In vitro* cell viability testing of P(L,D,L)LA based composites containing different amounts of Bioglass[®]VR 45S5 particles was investigated. According to the degradation rate of the P(L,D,L)LA matrix and the cytocompatibility experiments, the composite with 30 wt % of Bioglass[®]RV particles seemed to be the best candidate for further investigation. To study its behavior after immersion in simulated physiological conditions, the degradation of the composite was analyzed by measuring its weight loss and mechanical properties and by proceeding with X-ray tomography. We demonstrated that the presence of the bioactive glass significantly accelerated the *in vitro* degradation of the polymer. A preliminary *in vivo* investigation on rabbits shows that the addition of 30 wt % of Bioglass[®]VR in the P(L,D,L)LA matrix seems to trigger bone osseointegration especially during the first month of implantation. This composite has thus strong potential interest for health applications.

Key Words: bioactive glass, polylactic acid, biomaterial, bio-compatibility, degradation

INTRODUCTION

Over the past three decades, bioabsorbable polymer devices have been used and experimented in different fields of orthopedic surgery, including fixation of fractures and bone replacement. They are currently used as pins, plates and screws for orthopedic, oral and craniofacial surgeries.^{1–4} Their bioresorbability limits risk of fibrous capsule formation and chronic inflammation often observed with permanent implants and avoids a second operation to remove the implant. Their stiffness similar to bone also avoids stress-shielding phenomenon. Moreover, radio-transparency of polymers facilitates the medical follow-up of the patient. However, they present some drawbacks like an inadequate degradation rate and an inability to fully integrate with bone. It is the case of poly(L-lactic acid) (PLLA) that was reported to degrade in >4 years in humans.⁵ A strategy to improve their osseointegration ability is to fill the matrix of polymer with bioactive ceramics such as hydroxyapatite (HA)⁶ or tricalcium phosphate (β -TCP).⁷ These bioactive ceramics promote better

bone bonding due to the formation of a calcium phosphate layer at their surface after immersion in body fluids. Various authors have already reported the biological properties of HA-PLLA composites.^{6,8–12} Verheyen et al.¹³ have observed by histological analysis that the contact of bone with PLLA-HA composite increases as compared with unfilled PLLA. Recently, attention has been also focused on composites filled with bioactive glass particles such as Bioglass[®] 45S5.¹⁴

Control of the degradation rate of the polymer is of major importance; this rate has to be adapted to the implantation site. Poly(lactic acid) degradation is a complex process that has been extensively investigated.^{1,2,14–18} A number of parameters can affect the polymer degradation mechanism such as crystallinity, chirality, molecular weight, processing conditions, size and shape of the specimens. The addition of an inorganic phase into the polymer matrix increases the complexity of the polymer degradation mechanism. All studies reported that the presence of bioactive glass increased water absorption and weight loss as compared with neat

polymer. This initial rapid weight loss of the composite was attributed to the dissolution of the bioactive glass.^{19–23} When the bioactive glass reacts with body fluids, a local alkaline environment is created, due to the bioactive glass salting-out. In the case of porous composite scaffold, it has been reported that this alkaline environment neutralizes released lactic acid and slows down the polymer degradation, compared with neat polymer.^{19,20} Different results were observed on dense composites. Rich et al.²¹ observed a faster decrease of molecular weight with the addition of bioactive glass S53P4 (50–60 wt %) into a copolymer of caprolactone and lactide (P(CL/DLLA)). Navarro et al.²² noted a fast decrease of the molecular weight of the P(95 L,5DL)LA-G5 (50 wt %) composite after 2 weeks. All studies seem to show that the addition of bioactive glass particles affects polymer matrix degradation, but the molecular weight decrease profile may depend on the balance between the autocatalytic degradation catalyzed by carboxyl end groups and the buffering effect of the bioactive glass dissolution.

Bioglass® 45S5²⁴ and poly(lactic acid)^{25,26} cytocompatibility has widely been demonstrated. One purpose of the present work was to demonstrate the cytocompatibility of the P(L,DL)LA polymer matrix reinforced with Bioglass® particles by estimating the effect of the Bioglass® on the cell proliferation. Several studies of *in vitro* cytocompatibility have already been carried out on composites made of poly(α -hydroxy)esters filled with bioactive glass particles.^{27–30} Most of these studies were performed on porous scaffolds, or on thin films. To our knowledge no study was reported about the cytocompatibility of dense composites.

In a previous work,³¹ we showed that 45S5 Bioglass® particles in a P(L,DL)LA matrix induced hydroxyapatite precipitation when immersed in simulated body fluid (SBF). The thick layer of HA at the surface of composites containing 30 and 50 wt % of Bioglass® gave optimal conditions for bioactivity. Materials used for bone reparation devices have to be biocompatible, to promote bone growth and to degrade with a time that matches bone growth. These first results unfortunately showed a too fast polymer degradation in the case of the composite containing 50 wt % of Bioglass®, observed by a strong weight loss and a disintegration of the composite after only few days of immersion in SBF.

In line with this previous work, we here propose to focus on the characterization of the behavior of P(L,DL)LA - Bioglass® composites *in vitro* and *in vivo*. The first aim of this work was to study the biocompatibility of composites with different contents of Bioglass® by indirect cytocompatibility testing. To determine the degradation kinetic of the composite, degradation behavior of the selected composite was analyzed *in vitro* in a phosphate buffer saline solution (PBS). Last, *in vivo* biocompatibility and osteoconductive properties of this composite were assessed with intramuscular and intraosseous implantations in rabbits.

MATERIALS AND METHODS

Materials

A poly(L,DL)lactide (P(L,DL)LA) with a 70 L/30DL ratio and an inherent viscosity between 5.7 and 6.5 dL g⁻¹ was supplied

by Boehringer Ingelheim, Germany (Resomer® LR708). 45S5 Bioglass® particles were obtained from high purity SiO₂, Na₂CO₃, CaCO₃, and P₂O₅ powders (Noraker Company). The powders were weighted, mixed and melted in a platinum crucible for 4 h at 1400°C with a de-carbonation step (5 h at 950°C). The melt was then quenched in water, ground by planetary milling in ethanol to a fine powder and sieved to obtain particles with a mean size of 3.5 μ m (particle size distribution of 1–15 μ m). Composite granules with 20, 30, and 50 wt % (i.e., 11, 17, and 30 vol %, respectively) of Bioglass® particles were processed following a patented protocol (Patent WO2008116984 (A2)).³² Briefly, the Bioglass® particles were first put in suspension in acetone (100 g/500 mL), then the polymer was added progressively and mixed to reach the desired bioactive glass/polymer ratio. The suspension was then agitated for few hours to reach a complete dissolution of the polymer in the solvent, and to reach a viscosity of 10¹ Pa s⁻¹. The suspension was then precipitated in water. The precipitate was milled and sieved to obtain granules of a mean diameter of 1 mm. Polymer and composite granules were molded by injection (145°C, 150 bars) into plates or cylinders. For better clarity, the polymer will be named 'P' hereafter and the composites with 20, 30, and 50 wt % of Bioglass® will be named respectively C20, C30, and C50.

In vitro biocompatibility study

The indirect cytotoxicity test was assessed by an extract-based method according to NF EN ISO 10993-5. MG63 osteoblastic cells, originally isolated from a human osteosarcoma (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium containing stabilized L-Glutamine (DMEM, PAA, Pasching, Austria), supplemented with 10% (v/v) fetal bovine serum (FBS, PAA, Pasching, Austria), 1%(v/v) antibiotic antimycotic reagent (PAA, Pasching, Austria). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂.

Cylindrical specimens (length 3 mm, diameter 10 mm) of polymer and composite C20, C30, and C50 were sterilized by gamma irradiation at a minimum dose of 25 kGy (Synergy Health, Marseille, France). Specimens were then immersed in DMEM at a ratio of 0.1 g mL⁻¹ at 37°C for 24 h without stirring according to the standard procedures. The pH of the extract increased with the time to reach around 8.8 at 24 h and over 9 at 72 h whatever the composition of the composite (C20, C30, or C50). The material extraction vehicles were then stored at 4°C to be added on cells after each viability measurement for 1-, 6-, and 10-days testing.

Cells were seeded at a density of 5000 cells per well in 96-well culture dishes (cell culture treated polystyrene). After 24 h of culture, the medium was removed and replaced by the material extraction vehicle, at various concentrations (1, 10, 25, and 50% v/v) in the culture medium and placed at 37°C in a humidified atmosphere containing 5% CO₂. Cells cultured without any extract solution were taken as a positive control. Cell proliferation was evaluated after 1, 6, and 10 days. This *in vitro* test was replicated three times for each material.

The PrestobluTM assay (Invitrogen, Carlsbad, CA) was applied to quantify cell viability and proliferation. This assay uses a nontoxic indicator and is thus nondestructive, compared with the MTT assay. PrestobluTM is a blue non-fluorescent, cell permeable compound (resazurin-based solution), which is reduced by living cells into a fluorescent compound (resorufin). The cells were rinsed twice with DMEM. 150 μ L of PrestoBlueTM reagent, 10% m/v, was added to each well and incubated for 1.5 h at 37°C under 5% CO₂ atmosphere. Fluorescence intensity values were collected at excitation and emission wavelength of 535 and 610 nm, respectively with the Infinite Pro200 fluorimeter (Tecan, Männedorf, Switzerland). The mean values of fluorescence measurements obtained from fluorimetric tests and their corresponding standard deviation (\pm SD) were calculated. The results are expressed as a percentage of the values obtained on a plastic control (cell culture treated polystyrene) tested during the same experiment.

In vitro degradation

The *in vitro* degradation of cylindrical P and C30 samples (length 12 mm, diameter 6 mm) sterilized by gamma irradiation at a minimum dose of 25 kGy was assessed according to the standard ISO 15814. 36 samples were immersed individually in test tubes with 17 mL of phosphate-buffered saline (PBS), pH 7.4, at 37°C for 15 days up to 6 months under static conditions. Every 15 days, three specimens were removed from the solution and analyzed.

Water absorption and weight loss. Specimens were weighted (wet weight), rinsed with deionized water, and dried under vacuum until the weight was stabilized (dry weight). Every 15 days, water absorption and weight loss were measured on three specimens of polymer and composite C30. Water absorption was determined using the following equation:

$$\text{Water absorption(\%)} = \frac{W_{t,\text{wet}} - W_{t,\text{dry}}}{W_{t,\text{dry}}} \times 100 \quad (1)$$

where $W_{t,\text{wet}}$ is the weight of the wet specimen at time t and $W_{t,\text{dry}}$ is the weight of the dried specimen at time t .

The percentage of the weight loss was determined using the following equation:

$$\text{Weight loss(\%)} = \frac{W_0 - W_{t,\text{dry}}}{W_0} \times 100 \quad (2)$$

where W_0 is the initial weight of the specimen.

Microcomputed tomography (CT). Samples were observed with a X-ray tomograph (V|tome|x, Phoenix|X-ray, Wunstorf, Germany) using the following scanning parameters: source voltage: 70 kV, intensity: 240 μ A and voxel resolution: 10 μ m. One specimen of polymer and composite C30 was observed every 15 days.

Compressive test. Every 15 days, compressive tests were performed on an INSTRON 8502 machine on three specimens

of polymer and composite C30, with a cross-head speed of 1 mm min⁻¹ in accordance with NF EN ISO 604:2002.

Molecular weight measurement. Polymer and composite specimens were dissolved in chloroform (10 mg mL⁻¹) and filtered (0.45 μ m) for molecular weight measurements. Average molecular weight measurements were obtained by SEC with a PLgel 5 μ m MIXED-C column (Polymer Laboratories, Shropshire, GB) in chloroform at a flow rate of 0.5 mL min⁻¹ using both a differential refractive index and a multi-angle light scattering detector (miniDAWNTM TREOS, Wyatt Technology, Santa Barbara, CA) with a dn/dc value of 0.2. Measurements were conducted on one polymer and one composite specimen C30 before immersion and after 6 months of immersion in PBS.

In vivo study

The *in vivo* study was carried out using cylindrical implants of polymer and composite C30 obtained by injection molding (length 10 mm, diameter 2 mm for intramuscular implants; length 6 mm, diameter 2 mm for intraosseous implants). Specimens were then sterilized by gamma irradiation at a minimum dose of 25 kGy.

All animal experiments were performed with the approval of the local Veterinary School ethical committee (RTI2B UCBL/ENVL Lyon No. 0936/2009). Fifteen healthy female New Zealand rabbits aged between 12 and 18 months and with a mean weight of 5 kg were used for this study. Trimethoprim 4% (v/w) and Sulfadiazine 20% (v/w) (20 mg kg⁻¹, Borgal®, Intervet, Whitby, Canada) were administered preoperatively and twice a day for 4 days post-operatively. Operated rabbits were firstly anesthetized by an intramuscular injection of ketamine (35 mg kg⁻¹, Imalgène® 1000, Merial, Lyon, France) and xylazine (2 mg kg⁻¹, Rompun® 2%, Bayer HealthCare, Gaillard, France). Anesthesia for the surgical procedure was then maintained with a mixture of isoflurane in oxygen. Back of the animals, as well as their legs, were shaved and scrubbed with an antiseptic fluid (Vetidine savon®, Vetoquinol, Lure, France). A lateral parapatellar arthrotomy was performed under sterile conditions. The patella was dislocated medially and the knee was placed in full flexion. Cylindrical osseous critical-sized defects (length 3 mm, diameter 2 mm) were created in the left lateral femoral condyle and in the right lateral femoral condyle with a drill (diameter 2.0 mm) from the antero-distal part of the femoral condyle to the posterior proximal direction. These defects were then filled, via press fit, with polymer and composite specimens in the left and right lateral femoral condyle, respectively. After cutaneous and sub-cutaneous incisions, polymer and composite specimens were then implanted in the left and right paralumbar regions, respectively. After implantation, the subcutaneous tissue and skin were closed layer by layer with resorbable sutures. The animals received a postoperative pain medication and preventive analgesia as well as preventive antibiotic therapy. Trimethoprim 4% (v/w) and Sulfadiazine 20% (v/w) (20 mg kg⁻¹, Borgal®, Intervet, Whitby, Canada) were administered twice a day for 4 days

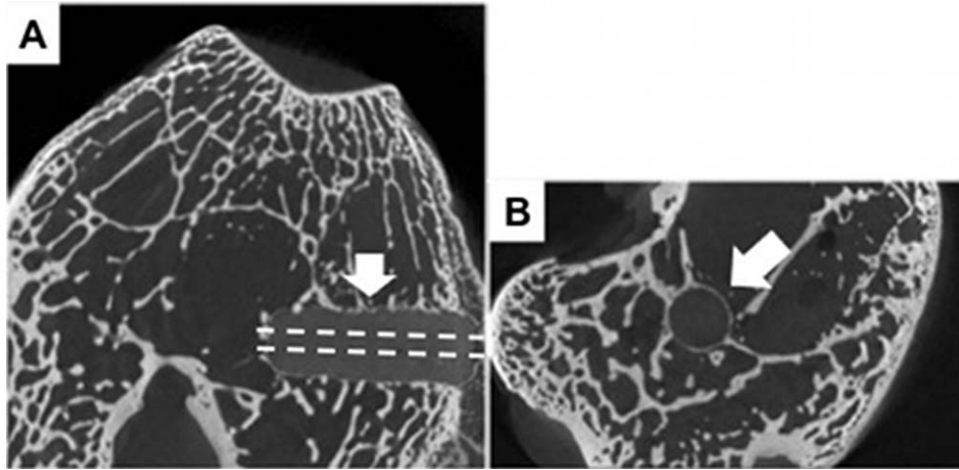


FIGURE 1. Computed tomography imaging of the implants in the lateral femoral condyle at 1 month after implantation. (A) Transversal plane of the condyle with a composite implant C30 in the lateral right condyle. The central longitudinal slice cut for histological analysis is represented by dotted lines. (B) Sagittal plane of a condyle with a polymer implant in the lateral left condyle.

post-operatively. After all surgical procedures, the rabbits were allowed to wake up on a heated carpet. They were free of activity in their cages. The animals were closely monitored for infections and other complications. Rabbits were sacrificed after 1, 3, and 6 months by intracardiac injection of sodium pentobarbital euthanasia solution (Dolethal®, Vetoquinol, Lure, France).

After sacrifice, the intramuscular implants were removed and immersed in 70% ethanol. Femoral condyles were cut on bone diaphysis with a manual saw, got free of any muscles or fibrous tissues, and conserved at 7°C in gauze dipped in a saline solution, before computed tomography examination.

Three groups of animals were distinguished as a function of the time of implantation: 1, 3, and 6 months, with five animals per group. For the 1- and 3-months groups, 4 specimens were implanted per rabbit: 2 intraosseous specimens and 2 intramuscular specimens. For the 6-months group, only 2 intraosseous specimens were implanted per rabbit.

Three-dimensional micro-computed tomography (3D CT).

The 3D CT was performed on bone specimens within 24 h after sacrifice. Measurements were realized on a X-ray tomograph (V|tome|x, Phoenix|X-ray, Wunstorf, Germany) with the following scanning parameters: source voltage: 60 kV, intensity: 260 μ A and voxel resolution: 12 μ m. As soon as CT was realized, specimens were rapidly immersed in 70% ethanol. Bone and soft tissue appeared with different grey levels due to different linear X-ray attenuation coefficients (Figure 1). Bone tissue, having higher attenuation coefficient, appeared in white, whereas soft tissues appeared in dark. Computed tomography was also performed on polymer and composite C30 specimens (length 12 mm, diameter 6 mm) implanted in femoral condyles of dead rabbits, as “control.”

Histological analysis. Intramuscular specimens were collected immediately after sacrifice, dehydrated with a graded ethanol series (70° and 100°), soaked in xylene to remove

the alcohol and embedded in methylmethacrylate resin (MMA). Blocks were then cut with a Shandon Finesse microtome (Thermo Scientific, Waltham, MA), equipped with a tungsten blade, in thin slices of 10 μ m and stained using the Masson–Goldner method. These slices were then examined by light microscopy using a Leica DM2000 microscope coupled with Leica DFC 450 digital camera. Biological reactions in surrounding tissue were examined for each specimen. Several parameters were analyzed (granuloma, eosinophil granulocytes, plasma cells, fibroblast/collagen and angiogenesis) and classified according to the following grading system based on their corresponding intensity (0: absence; 1: limited; 2: moderate; 3: marked; 4: severe).

Bone explants were cut transversally on both sides of the implant after X-ray tomography, using a low speed oscillating Isomet saw (Buehler, Lake Bluff, IL) equipped with a 15 HC diamond blade (length 0.3 mm, diameter 10.2 mm). Then bone specimens were fixed and embedded following the same protocol as the intramuscular specimens. Histological slices were obtained by a microcutting and grinding techniques adapted from Donath.²⁶ One central longitudinal slice per specimen was prepared with a diamond wheel saw (Well 3241, ESCIL, Chassieu, France, diameter 220 μ m, grain size: 40 μ m, length 10 mm). These slices of 150 μ m were then manually sanded between two glass-slabs with silicon carbide powder 180, 360, and 600 (ESCIL, Chassieu, France) until obtaining a thickness of \sim 105–110 μ m. Bone slices were then stained (Paragon staining) for histopathological examination (Biomatech SA, Chasse sur Rhône, France). The analysis was performed using a NIKON Eclipse E600 microscope coupled with a NIKON DN100 digital camera. Bone formation and biological reactions in surrounding tissue were examined for each specimen.

RESULTS

***In vitro* cytocompatibility**

The amount of viable cells as determined by the Presto-Blue™ assay is shown in Figure 2. According to these

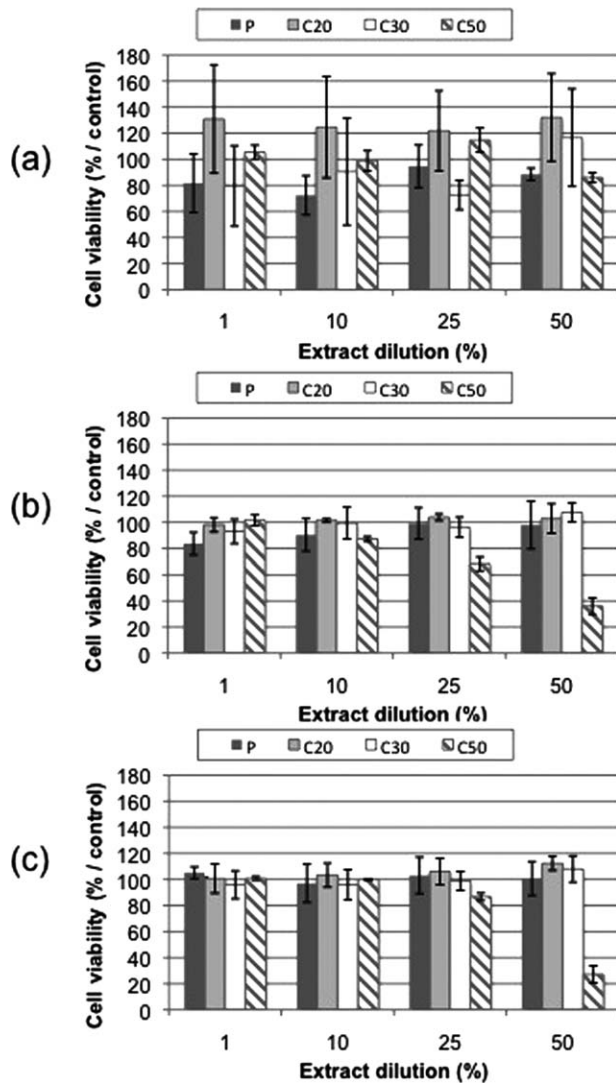


FIGURE 2. Indirect cytotoxicity evaluation of polymer P and composites C20, C30 and C50, after 1 day (a), 6 days (b) and 10 days (c) of cell culture.

results, after 24 h, the amount of cells cultured in presence of the C20, C30, C50 and polymer material extracts didn't show any significative differences whatever the dilution extract. At 1-day post-seeding the amount of cells was surprisingly less important with the polymer extract. Considering that cells have no time to get used to this new environment after 24 h of incubation, the cell viability was determined after 6 and 10 days of incubation with the material extracts. After 6 days of incubation of cells with material extracts, the results showed no cytotoxicity effect of the polymer and composites C20 and C30. Values of cell viability for 50% diluted extract were close to the value corresponding of the control (plastic). For the composite C50, a lower cell viability can be noticed after 6 days of incubation in comparison with the other materials. The cell viability decreased with the increase of the concentration of extract from 102% ± 4% for 1% extract to 36% ± 6% for 50% extract. After 10 days of incubation, these results were

confirmed. Cell proliferation was similar for the polymer and the composite C20 and C30 whatever the extract dilution. However the composite C50 presented a lower cell viability value, especially for 50% diluted extract as compared with the composites C20 and C30.

According to these results and the previous study about the *in vitro* "bioactivity" properties of these composites³¹ (showing that 20 wt % of Bioglass® was not sufficient to trigger bioactivity and that 50 wt % was already questioned because of a rapid degradation in SBF), the composite C30 was chosen for the next part of this work.

In vitro degradation

Figure 3 shows the weight loss as a function of the immersion time for both the polymer and the C30 composite. Polymer weight was rather stable during the 6 months of immersion. By contrast, the composite weight loss increased progressively with time. After 15 days of immersion the composite already lost 15% of its initial weight. We noticed that the weight loss of composite samples tended to slow down with an aging of PBS solution. This weight loss accelerated after each PBS change, at 78 and 162 days of immersion. After 6 months of immersion, the composite lost 35% of its initial weight.

The water absorption into the polymer was lower than 1% all over the immersion period (data not shown). The presence of bioactive glass increased the water absorption. Indeed, the composite absorbed 100% of its weight approximately after 98 days.

After 6 months, polymer specimens appeared not degraded [Figure 4(A)], whereas some cracks were observed at the periphery of the composite specimens already after 15 days [Figure 4(B)].

It can be observed in Figure 5 that the compressive strength of the polymer did not change significantly during the first 3 months of immersion. On the contrary, after 14 days of immersion, the compressive strength of the composite decreased by 60%. After 3 months, compressive strength of the composite was about 5 MPa, whereas it was still about 80 MPa for the polymer. Compressive tests were stopped at this time because the composite specimens were too degraded to be tested.

Table I summarizes the results of the polymer and composite weight average molecular weight (Mw) evolution during the elaboration process chain (granule preparation, injection molding, gamma sterilization) and after immersion in PBS during 6 months. There is a significant decrease of the molecular weight of the polymer and the composite after every step of the process. However, the molecular weight of the composite sample was observed to decrease much faster than polymer one.

The molecular weight of both materials also decreases during immersion in PBS. After 6 months of immersion in PBS, polymer and composite have lost 17 and 49% of their average molecular weight after sterilization, respectively. As during the overall process chain, it appears that the addition of Bioglass® particles accelerates the degradation of P(L,DL)LA during immersion.

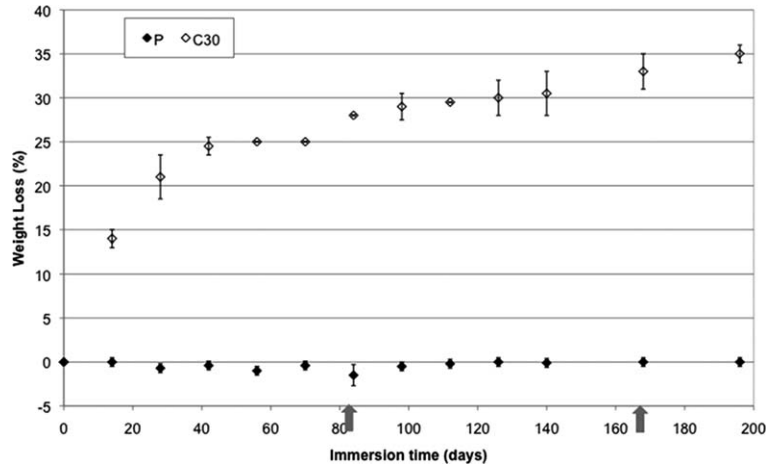


FIGURE 3. Evolution of weight loss of polymer P and PDLLA/Bioglass® composite C30 in function of immersion time in PBS (The arrows indicate solution changes).

In summary, the polymer did not show any sign of degradation after 6 months of immersion. Only its molecular weight is decreasing slowly, but it still lies well above the critical molecular weight of entanglement (around $20,000 \text{ g mol}^{-1}$) correlated to a quick drop of the mechanical properties. On the contrary, the composite C30 exhibited an important decrease of the physical and mechanical properties after immersion in PBS for 6 months: the molecular weight more rapidly reaches values for which the mechanical properties are affected.

***In vivo* biocompatibility**

After euthanasia, bone specimens were observed. Fibrocartilage callus was observed on four bone specimens after 1 month and on one specimen after 3 months. This reaction was a normal physiological reaction occurring at the beginning of the bone fracture healing. No inflammation signs were observed on the joints. It can be noticed that one rabbit from the 1-month group died 15 days after the surgery due to a microbiological infection, and one rabbit from the 3 months group died also after 15 days, for digestive reasons.

Semi-quantitative histopathological analysis of intramuscular implanted specimens is presented in Table II. The results showed a minimal inflammatory reaction, consisting on a granuloma that decreased between 1 and 3 months after the implantation. Connective tissue containing a large number of macrophages associated with lymphocytes surrounded polymer and composite implants. Topographic views of an implant surrounded by striated muscle are presented in Figure 6. Macrophages presented an abundant cytoplasm, eosinophil, with cytoplasmic limits well-defined, regular nucleus, with epithelioid aspect [Figure 6(A)].

At a distance of some polymer implants, a matrix ring with fibroblasts and collagen [Figure 6(A)] colored in green by Masson-Goldner stain was observed. For both polymer and composite, at 1 and 3 months post-implantation, minimal inflammatory reaction remained similar. The implant was well integrated in the muscle with very little fibrous tissue encapsulation.

Tomography observations of intra-osseous implanted specimens did not show visible degradation on polymer and composite implants, up to 6 months of implantation

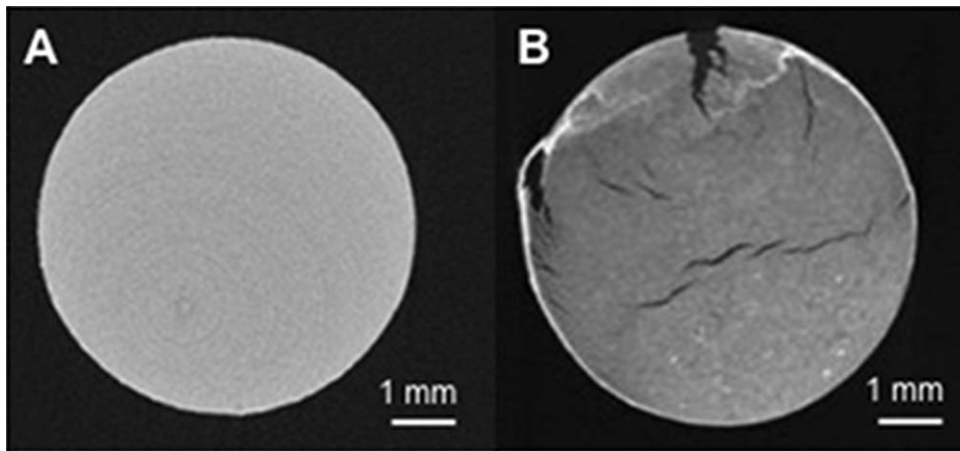


FIGURE 4. 2D slice of a CT reconstructed volume of a polymer P sample after 6 months (A) and a composite C30 sample after 15 days (B) of immersion in PBS.

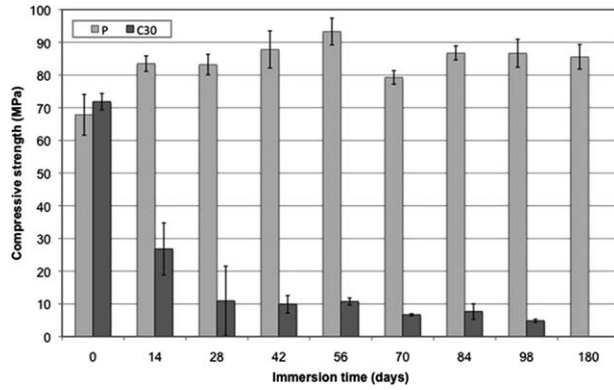


FIGURE 5. Compressive strength of the polymer P (light gray) and the composite C30 (dark gray) in function of immersion time in PBS.

(Figure 7). The images revealed a layer of bone around all the implants as soon as 1 month of implantation. This bone layer suggests the formation of new bone *in vivo*. It could be also argued that this bone layer was the result of bone pushed during the surgery. To assess this hypothesis, scan was also conducted on a “dummy” composite cylinder implanted in femoral condyle of dead rabbit [Figure 7(E)]. This bone layer was not present around the “dummy” implant.

Therefore X-ray tomography clearly demonstrated a white line surrounding the implants as soon as 1 month and clearly observed in the 3D picture (Figure 8).

At 1-month after implantation, histopathological analysis of the intra-osseous implants showed a thin line of extracellular matrix frequently observed at the interface of the implants. Different cellular types were encountered on polymer and composite materials (Table III). Limited macrophages associated with a few multinucleated osteoclasts like cells were observed. Limited lymphocytes were encountered only on the composite. A thin woven bone tissue running along the polymer and composite implants was observed on portion on the implants positioned within the cancellous bone tissue [Figure 9(B)]. No closed contact was detected between bone material and polymer at 1 month after implantation [Figure 9(A)]. In contrast, bone trabeculae were closed to the C30 composite implants [Figure 9(B)].

TABLE I. Changes in Polymer P and Composite C30 Weight Average Molecular Weight After Injection Molding, Sterilization Gamma and Immersion During 6 Months in PBS

Materials		M_w (g mol ⁻¹)	I_p
Polymer	Polymer granules	745,000	1,25
	After injection molding	260,100	1,21
	After sterilization	66,200	1,17
	After 6-months immersion in PBS	54,900	1,17
Composite C30	Composite granules	355,400	1,72
	After injection molding	87,600	1,77
	After sterilization	41,900	1,33
	After 6-months immersion in PBS	21,500	1,14

TABLE II. Semiquantitative Histopathological Analysis of the Polymer P and the Composite C30 Implanted in Dorsal Muscular Tissue After 1 and 3 Months

	Polymer P		Composite C30	
	1 month	3 months	1 month	3 months
Granuloma	3	2	3	2
Plasma cell	1	1	1	1
Eosinophil granulocyte	0	1	1	1
Fibroblast and collagen	1	1	1	1
Angiogenesis	0	0	1	1

Index: (0) Absence; (1) Limited; (2) Moderate; (3) Marked; (4) Severe.

After 3 months of implantation, inflammatory reaction decreased as compared to 1 month in both polymer and composite implants (data not shown). Neo-formed bone, implant osseointegration and bone remodeling around the polymer implants were well defined at this long term of implantation. A limited bone formation was observed around the composite C30 implant.

After 6 months of implantation, the inflammatory reaction characterized by macrophages and lymphocytes infiltration was associated with a few areas of osteolysis. Bone formation and osseointegration were increased for the polymer [Figure 9(C)]. For the composite C30, the inflammation intensity was maintained as compared to 3 months and a moderate grade of bone formation was observed around the implant. In addition, moderated close contact with the bone was visualized [Figure 9(D)]. No evidence of polymer degradation was visible at any time. However, a limited evidence of composite degradation was visible for the composite at 6 months [Figure 9(D)]. Indeed, some cells with a phagocytosis activity showed intracellular fragments of material. However, no significant dissemination was observed in the peripheral zone and the structure of the implants was preserved.

DISCUSSION

Cytocompatibility

Indirect cytocompatibility evaluation showed an absence of cytotoxicity of polymer and composites C20 and C30. The composite C50 seemed to be less cytocompatible, in the conditions of testing chosen in this work. The decrease of cell proliferation with the C50 extract cannot be totally related to an increase of pH since it was superior to 8.8 for all the composites after 24 h of extraction. More likely, this apparent cytotoxicity of C50 extracts for a high ratio of dissolution must also be correlated to the large amount of ion release (mainly silicon ions) from the glass particles to an extent which does compromise cell cultures. To our knowledge no study about indirect cytocompatibility assay of dense composite of PLA/bioactive glass was reported. Most of the authors have reported cell viability assay conducted by directly seeding cells on materials.^{28,30,34} The effect of silicon ion release from the Bioglass particles was already noted by Blaker et al.²⁸ who had to incubate PLA/bioactive glass foams for 24 h in culture medium before cells seeding

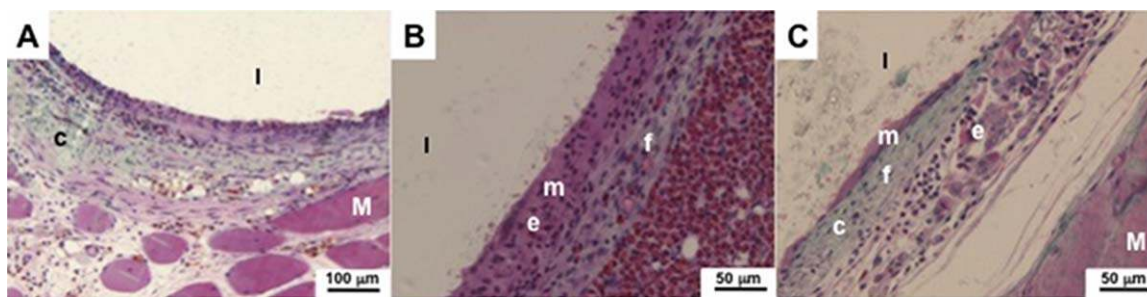


FIGURE 6. Histological observations of sections of the dorsal muscle after Masson-Goldner stain. One month post-implantation (A) Polymer P implant, original magnification $\times 40$; (B) Composite C30 implant, original magnification $\times 20$. Three months post-implantation (C) Composite C30 implant, original magnification $\times 40$. (I = implant; M = muscle; e = epithelioid-giant-cellular reaction; c = collagen; m = macrophage; f = fibroblast).

in order to prevent the sudden release of high ion concentrations from the Bioglass®. Using direct cytocompatibility testing, Lu et al.³⁴ observed that a PLGA composite filled with 10% and 25% of 45S5 Bioglass® supported a greater osteoblast growth and differentiation compared with the composite filled with 50% of Bioglass®. They correlated this result to the presence of a threshold in the Bioglass® content that is optimum for osteoblast growth. They did not correlate this result with the pH values or ion release. Other authors^{28,30} working on highly porous PDLA/Bioglass® scaffolds (cylinders of length 3 to 5 mm and diameter 8 mm) with different filler ratios (5 and 40 wt %), did not observe neither a significant difference in cell growth with an increase of Bioglass® content nor an increase of pH for high amount of Bioglass®. This can be explained in all cases by the immersion (incubation) of the specimens in culture medium before cell seeding. Our dense specimens C50 have a more important Bioglass® concentration leading to a larger ion release in the extract, potentially compromising the cell cultures in the conditions of testing. We would not talk strictly about “cytotoxicity” since this is rather an increase of pH associated to a high concentration of silicon ions released from the Bioglass® than the release of toxic elements which is responsible of the negative results for C50 extracts in a large ratio of the culture medium.

Choice of a preferred composite composition

Despite a better *in vitro* “bioactivity” of the composite C50 described in a previous study,³¹ this material has been dismissed for the next part of this work because of its apparent lower cytocompatibility and also because our previous work showed a strong visual degradation during bioactivity tests, only after few days. In the same way, the composite C20 presents the better cytocompatibility but the least bioactivity.³¹ The composite C30 was thus chosen as a compromise between the bioactivity behavior and the good cell proliferation in the presence of its extract. The degradation behavior of this composite was then compared with the polymer P(L,DL)LA.

In vitro degradation

First of all, we observed that the molecular weight of P(L,DL)LA is degraded at every step of the process, and to a

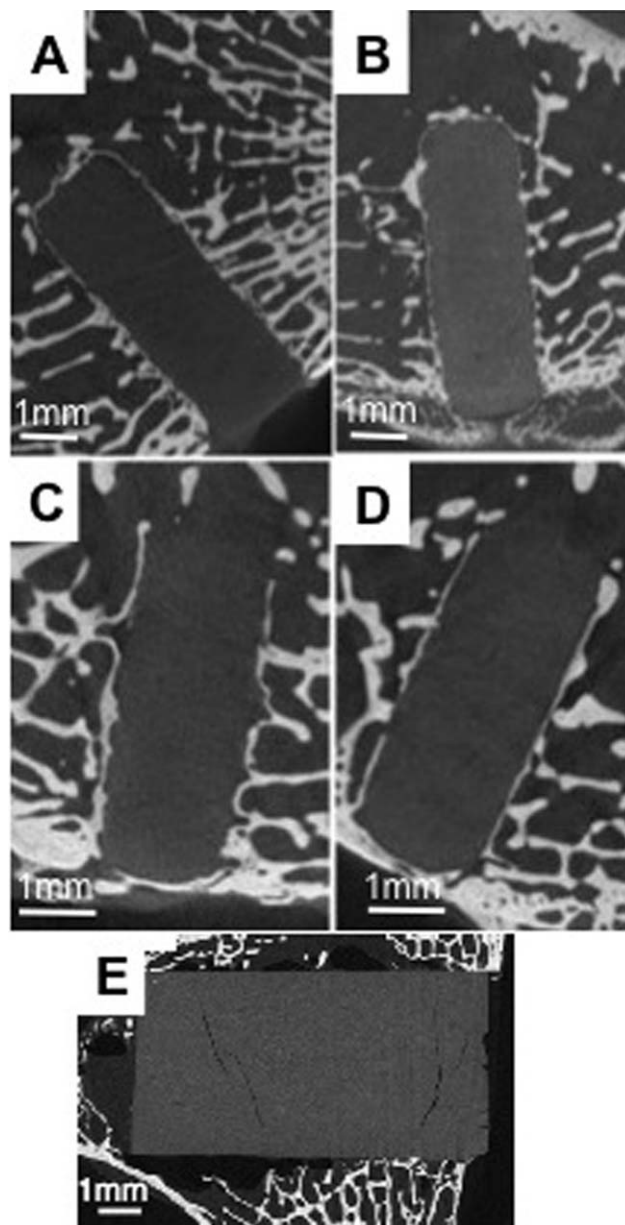


FIGURE 7. Tomography observations of polymer P and composite C30 at 1 month (A and B) and at 6 months after implantation (C and D) and of the composite C30 implanted in a dead rabbit (E).

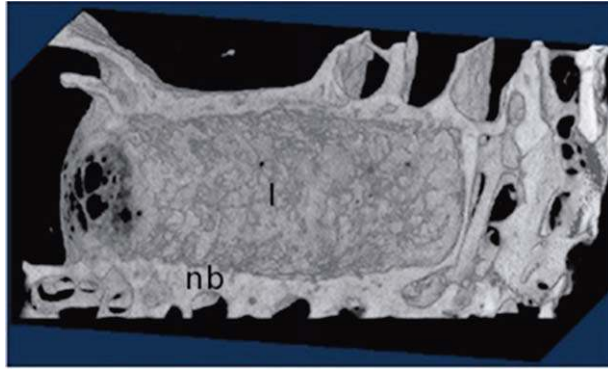


FIGURE 8. The 3D reconstruction of the bone around a composite C30 implant after 6 months of implantation (with ImageJ software). (I = implant; nb = newly formed bone).

larger extent in the composite, that is, in the presence of Bioglass® particles. A first reduction of molecular weight takes place during the composite granules elaboration. Mechanical mixing of the polymer in acetone in presence of Bioglass® particles, during several hours, may generate drastic conditions for the polymer matrix. Ions released by Bioglass® particles (SiO²⁻, SiO⁻) could catalyze the break of the ester linkage, as it happens with hydroxyl anions which catalyze the hydrolysis of polyesters. The second decrease of Mw takes place during the injection step:

- For the neat polymer, from raw granules to injected specimen, several phenomena (thermal degradation, shearing and hydrolysis catalyzed by temperature) induce the Mw decrease of the polymer (around 40%). It is indeed generally accepted that Polylactic acids are thermally degraded from around 150°C.
- For the composite, hydrolysis and shearing (increased by the filler) could explain the even larger decrease of Mw during the injection step.

At the end of the process chain, the Mw of the two materials is different, which already shows that Bioglass®

catalyzes the hydrolysis of the P(L,DL)LA. This is consistent with the results of Blaker et al.³⁵ Gamma sterilization of the polymer also reduced considerably its molecular weight. Claes et al.¹ reported a decrease of 72% of the molecular weight of P(L,DL)LA gamma-sterilized by ⁶⁰Co irradiation with a dose of 28 KGy at 45°C. Tuomo et al.³⁶ noticed a similar decrease of the inherent viscosity from 6.1 to 0.9 dL g⁻¹ of a self-reinforced PLLA-bioactive glass (19 wt %) composite gamma-irradiated with a minimum dose of 25 kGy. Suuronen et al.³⁷ observed a Mw decrease of 77% after gamma sterilization on the SR-PLLA. This observation highlights the influence of the sterilization on the properties of the polymer matrix.

Another purpose of this study was to determine the effect of Bioglass® on the polymer *in vitro* degradation rate. After 6 months in PBS, the polymer did not show any signs of degradation. No weight loss or water absorption was noticed. Maquet et al.²⁰ have observed a PDLLA weight loss of more than 2% after 16 weeks of immersion in PBS. This study realized on a highly porous scaffold is, therefore, not comparable with a dense polymer behavior. The present study showed that Bioglass® addition allowed to accelerate the polymer degradation during immersion in PBS. This hypothesis was confirmed by the high percent of water absorption observed with the composite C30. This acceleration of the degradation with the addition of bioactive glass particles was confirmed by several authors.^{21,22} This phenomenon was correlated to the important absorption of water, promoted by the hydrophilic character of the bioactive glass particles. Fluid penetration in the composite matrix could be related to the presence of the P(L,DL)LA/Bioglass® interfaces and surface cracks that facilitated fluid penetration and leaching of small polymer chains. Indeed, Rich et al.²¹ observed that composites with smaller bioactive glass particles absorbed more water than composites with bigger bioactive glass particles. Smaller bioactive glass particles had a higher area/volume ratio and more interfaces in the composite and therefore a more important surface available to absorb water. Navarro et al.²² found that the presence of calcium phosphate glass promotes morphological

TABLE III. Semi-quantitative Histopathological Analysis of the Polymer P and the Composite C30 Implanted in Femoral Condyles of Rabbits After 1, 3, and 6 Months

		Polymer P			Composite C30		
		1 month	3 months	6 months	1 month	3 months	6 months
Inflammatory reaction	Lymphocytes	0	0	1	1	1	0
	Macrophages	1	1	1	1	1	1
	Osteoclasts	1	0	0	1	0	0
Tissue integrity	Intervening soft tissue	1	1	1	1	1	1
	Osteolysis	1	1	1	1	1	0
Bone neoformation	Bone remodeling	0	2	2	0	0	2
	Newly formed bone	1	2	2	1	1	2
	Osseointegration	0	1	2	1	0	0
	Osteoconduction	1	2	2	2	2	2
	Neovascularization	2	2	2	2	2	2
Implant degradation		0	0	0	0	0	1

Index: (0) Absence; (1) Limited; (2) Moderate; (3) Marked; (4) Severe.

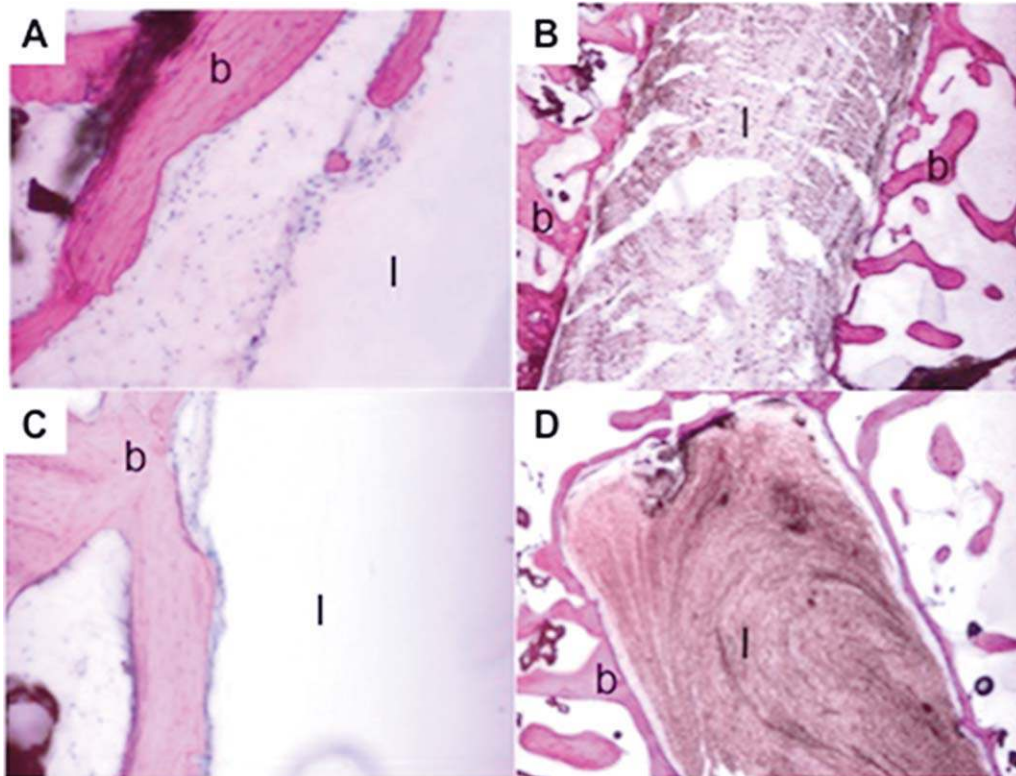


FIGURE 9. Histological observations of a central longitudinal section of the specimen implanted in the lateral condyle. One month after implantation: (A) Polymer P specimen, original magnification $\times 10$; (B) Composite C30 specimen, original magnification $\times 2$. Six months after implantation: (C) Polymer, P original magnification $\times 10$; (D) Composite C30 specimen, original magnification $\times 2$. (l = implant, b = bone).

changes such as the formation of cracks during immersion in a simulated body fluid at 37°C , which induced a faster degradation of the composite.

After 6 months, the molecular weight decrease of the composite was in agreement with other studies.³⁴ However, the loss of $\sim 60\%$ of the compressive strength during the first 15 days was very rapid compared with other studies in the literature.^{23,35} Tuomo et al.³⁵ observed a decrease of $<2\%$ of bending strength after 15 days of aging on a P(L,DL)LA reinforced with 19 wt % of bioactive glass 13-93 having a similar molecular weight ($42,000 \text{ g mol}^{-1}$). This difference can be explained by the different nature of bioactive glass and the difference of process. Their composites were produced by solid-state extrusion and then self-reinforced by die drawing. This process that oriented the polymer chains can be at the origin of the better initial mechanical properties. Similarly Shikinami et al.²³ noted a decrease of $<10\%$ of the initial bending strength on the composite PLLA-HA (30 wt %) after 5 weeks in PBS. This limited decrease in the bending strength compared with our study can be explained by the higher initial molecular weight of their composite ($180,000 \text{ g mol}^{-1}$), a different process (compression molding) and by the nature of polymer especially its crystallinity. Verheyen et al.⁸ observed indeed an similar important decrease of the mechanical properties on a PLLA/HA (80/20 and 70/30) composite with a molecular weight of $125,000 \text{ g mol}^{-1}$. After 3 weeks

of immersion, bending strengths of the polymer and the composite were reduced of 50%.

It is difficult to compare polymer and composite C30 *in vitro* behaviors because their molecular weights were different. However, it appeared that the addition of Bioglass® particles accelerates the polymer degradation by promoting water penetration. Indeed, the presence of particles in the matrix generates filler/matrix interfaces that promote water penetration. This increase in water absorption was correlated with a significant weight loss of the material. These results indicate the possibility to modulate the degradation rate of the polymer by adding Bioglass® to the polymer matrix. This is relevant for bone repair applications knowing that the degradation rate of temporary implants must match the rate of formation of new bone tissue.

***In vivo* study**

In vitro assays have shown an absence of cytotoxicity for both polymer P and composite C30 materials. However, this characterization is not enough to highlight the tissue response to materials. Only an osteoblastic cell line was chosen. In addition, the toxicity of the material could be overestimated during *in vivo* experiments, due to different environments. Indeed, pH regulation of the body, as well as dynamic environment, is not reproduced in *in vitro* experiments. *In vivo* implantation allows the evaluation of materials over long time periods and in different types of tissues.

Intramuscular implantation allows determining the reactivity of the surrounding tissues toward the material. Firstly, our implantation study has revealed a right biocompatibility of both polymer and composite materials. Moreover, the muscle implantation showed that neither the polymer nor the composite C30 had an osteoinductive property. No bone neoformation was observed.

The second objective of this *in vivo* study was to demonstrate significant osteoconductive properties of the composite implant compared with the polymer one. One month after implantation in the femoral bone tissue of rabbits, the composite implants showed an earlier osseointegration in comparison with the neat polymer implants. Both implants exhibited a good tissue tolerance without any inflammatory reaction. After 3 and 6 months of implantation no significant histological difference was observed between polymer P and composite C30 implants with minor inflammatory reaction and a satisfactory biocompatibility.

Several authors have shown that the addition of bioactive ceramic to biodegradable polymer induced bone ingrowth and bone tissue substitution. Furukawa et al.⁹ have shown new/neo bone formation after 2 weeks of implantation on PLLA-HA (30 and 40 wt %) composites whereas limited bone contacts were observed in unfilled PLLA implants. In the same way, Aunoble et al.⁷ have observed by histological analysis a better bone strengthening with the β -TCP/PLLA (60 wt %) composite than with the polymer. At the present, only one study did not show an increase of bone growth with the addition of bioceramic particles. Prokop et al.³⁸ implanted P(L,DL)LA filled with 10% of β -TCP in the femoral condyles of sheep with no significant improvement of bone formation with the composite. The nature of the mineral phase and the lower amount of β -TCP added in the polymer matrix can be an explanation of this phenomena.

No noticeable polymer or composite degradation was observed after 1 month of implantation. As expected some composite C30 degradation was observed after 6 months of implantation, as primarily described in *in vitro* degradation study. However, several authors have already observed significant signs of degradation after 6 months, as Aunoble et al.⁷ on β -TCP/PLLA (60 wt %) composites implanted in the femoral condyle of rabbits. These authors have not observed clear degradation of PLLA, even after 6 months of implantation. The polymer looks like preserved whereas some composite implants had broken up. This increased degradation compared with our study can be explained by the higher amount of the filler and their different nature.

The *in vitro* degradation study has shown that incorporation of Bioglass® particles in the polymer matrix accelerated significantly the composite degradation. This result illustrates the positive effect of adding Bioglass® to the polymer matrix. Indeed, a brief resorption of the composite implants is essential to the bone healing, avoiding inflammation reaction as the result of degraded polymer. Otherwise, the degradation kinetics from *in vitro* to *in vivo* tests cannot be extrapolated. During *in vitro* degradation, the materials were immersed in an aqueous solution and were, therefore,

permanently submitted to a hydrolytic environment. On the contrary, the material was less affected by the hydrolytic reaction from the fluid during implantation because the bone tissue surrounded it.

CONCLUSION

The P(L,DL)LA-Bioglass® 45S5 composite associates advantages of both constituting phases, the ease of processing and the mechanical strength of the polymer matrix and the bone growth ability promoted by the Bioglass®. Addition of Bioglass® clearly accelerates the degradation of the composite *in vitro*. Biocompatibility of the P(L,DL)LA-Bioglass® 45S5 (30 wt %) composite was confirmed both *in vitro* and *in vivo*. Implantation studies have revealed that presence of Bioglass® accelerates bone integration. Kinetics of *in vitro* and *in vivo* degradation assays were not similar, highlighting the limits of the correlation between these two types of tests due to the different environments and the limited relevance of *in vitro* tests to simulate the actual *in vivo* degradation rate. The *in vitro* tests can be considered as a preliminary study before the *in vivo* study in order to determine the degradation of material or not. A complementary *in vivo* study at long time of implantation and with a quantitative histomorphometric analysis would be interesting to define the degradation kinetics of the composite and to precise the new bone formation. All these results suggest that the P(L,DL)LA-Bioglass® composite is a promising material for bone fixation devices applications.

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