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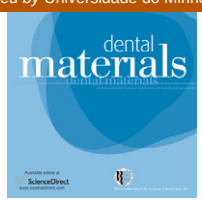

Asymmetric PDLLA membranes containing Bioglass® for guided tissue regeneration: Characterization and *in vitro* biological behavior

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ABSTRACT

Objective. In the treatment of periodontal defects, composite membranes might be applied to protect the injured area and simultaneously stimulate tissue regeneration. This work describes the development and characterization of poly(D,L-lactic acid)/Bioglass® (PDLLA/BG) composite membranes with asymmetric bioactivity. We hypothesized that the presence of BG microparticles could enhance structural and osteoconductivity performance of pure PDLLA membranes.

Methods. The membranes were prepared by an adjusted solvent casting method that promoted a non-uniform distribution of the inorganic component along the membrane thickness. *In vitro* bioactive behavior (precipitation of an apatite layer upon immersion in simulated body fluid, SBF), SEM observation, FTIR, swelling, weight loss and mechanical properties of the developed biomaterials were evaluated. Cell behavior on the membranes was assessed using both human bone marrow stromal cells and human periodontal ligament cells.

Results. Just the BG rich face of the composite membranes induced the precipitation of bone-like apatite in SBF, indicating that this biomaterial exhibit asymmetric osteoconductive properties. SEM images, DNA content and metabolic activity quantification revealed an improved cell adhesion and proliferation on the composite membranes. Composite membranes also stimulated cell differentiation, mineralization, and production of extracellular matrix and calcium nodules, suggesting the positive effect of adding the bioactive microparticles in the PDLLA matrix.

Significance. The results indicate that the proposed asymmetric PDLLA/BG membranes could have potential to be used in guided tissue regeneration therapies or in orthopedic applications, with improved outcomes.

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1. Introduction

Periodontitis is a disease that destroys the tooth-supporting tissues, including the alveolar bone, periodontal ligament (PDL) and cementum. This is the major cause of tooth loss in human adults [1]. The treatment of periodontal defects can be a complex process which may require surgery, but as a rule does not result in surgical intervention. However, periodontal defects, if left empty after open flap debridement, are filled with epithelial and fibroblasts, which are the first cells to reach the defect area, generating a core of fibro-epithelial tissues that does prevent the occurrence of an adequate regeneration process of the periodontal tissues [2]. In this context, Guided Tissue Regeneration (GTR) strategies consist in the application of a membrane that acts as a physical barrier to protect the defect site, preventing the epithelial cells, fibrous and gingival connective tissues to reach the injured area. The creation of segregated space for the invasion of blood vessels and osteoprogenitor cells protects against the growth of non-osteogenic tissues. This procedure favors the regeneration of lost and damaged tissue since it promotes cell repopulation of the periodontal ligament and adjacent alveolar bone. However, acting solely as physical barriers is a limitation on the clinical effect of these membranes: they provide no osteoconductive effects, enabling only minor contributions for new cementum and bone formation, which, by definition, is not true periodontal tissue regeneration [3]. Each side of an implanted membrane is in contact with a distinct biological environment, in which the osseointegration should be ideally promoted just in one of the faces. Nevertheless, this asymmetric bioactive behavior is almost inexistent in currently used GTR membranes and represents a possible challenge toward the development of innovative systems for the regeneration of periodontal tissues. GTR membranes can be obtained from natural or synthetic materials, either bioabsorbable or nonresorbable. Degradability is one of the most important requirements for GTR membranes and intends to avoid second surgical removing procedure. Natural resorbable collagen membranes have been widely used, not just because collagen is concretely one of the components of the alveolar bone and periodontal ligament but also because this material meets almost all the criteria required [4]. Collagen, however, presents some drawbacks such as its cytoxicity and xenogenic origin, poor mechanical strength and fast biodegradation by enzymatic activity [4,5]. In order to avoid these undesirable characteristics, maintaining the desirable ones, synthetic materials have been more frequently used, predominantly those from the poly(α-hydroxyesters) family [6]. The chemical properties of these polymers allow its hydrolytic degradation and the elimination of the resulting products by natural pathways [7]. Moreover their processing is easy compared to other polymers and the variety of existent molecular weights and copolymers permits a wide range of physical, mechanical and degradation rate related adjustments. Poly(D,L-lactic acid), PDLLA, is an amorphous polymer, with interesting mechanical properties and with degradation times in the order of 12–16 months [8]. It exhibits excellent biocompatibility *in vivo*, high mechanical stability and the possibility to be combined with drugs [9–11]. Nevertheless, PDLLA is not osteoconductive. Among different strategies that could be used to improve bioactivity in polymeric systems [12], the combination of osteoconductive inorganic particles has been widely used [7]. Bioglass® is a well known bioactive ceramic and has the ability to enhance the osteoblast activity and attachment between the biomaterial and the surrounding bone tissue, possibiliting the bone growth on the materials surface. Furthermore its dissolution products can control the gene expression in order to control the osteogenesis and consequently the production of growth factors [8], as well as counteracting the acidic degradation of the poly(α-hydroxyesters) providing a pH buffering effect [13,14].

In this work, Bioglass® microparticles were compounded with PDLLA to form a membrane using a solvent casting methodology. The conditions were optimized for the preparation of membranes exhibiting preferentially the BG in one of the sides of the membrane. It is envisioned that, upon implantation, the membrane side richer in BG could be faced to the defect side in which bone ingrowth should be stimulated while the more hydrophobic PDLLA rich side should act mainly as a barrier to avoid the invasion of soft tissue. Some relevant properties of the developed membranes were characterized and their biological performance was assessed, using two distinct cell types: human bone marrow stromal cells (hBMSC) and human periodontal ligament cells (hPDL).

2. Materials and methods

2.1. Materials

Poly(D,L-lactic acid) (PDLLA), (M_n = 31,750 and M_w = 100,000) with an inherent viscosity of 1.87 dL/g was purchased from Purasorb® (PURAC Biochem, The Netherlands) and was used as received. The 45S5 Bioglass®, with the composition: 45 SiO_2, 24.5 CaO, 24.5 Na_2O and 6.0 P_2O_5 in wt%, was supplied by US Biomaterials Corp. (Florida, USA). The particle size of the Bioglass® particles (BG), measured by laser scattering analysis (Coulter LS 100 particle size analyzer, Coulter, USA), was found to be lower than 20 μm. All the other reagents and solvents used were of reagent grade and were used without further purification.

2.2. Preparation of PDLLA and PDLLA/Bioglass® membranes

All PDLLA membranes were prepared based on a solvent casting technique. The PDLLA films were prepared by dissolving 0.50 g of PDLLA in 30 mL of chloroform. After total dissolution, the solution was transferred to a Petri dish with 9 cm of diameter and covered with an aluminum sheet. The Petri dish was settled in a horizontal position to facilitate the formation of a cast film with uniform thickness. The assembly was kept in a hood for 24 h, and chloroform was allowed to evaporate at a very slow rate. Then, the films were vacuum dried for 48 h at 40 °C.

The PDLLA/BG membranes were prepared in the exact same process as the pure PDLLA membranes. The PDLLA/BG dispersions were prepared by dissolving 0.40 g of PDLLA in 30 mL of chloroform. After total dissolution, 0.10 g of Bioglass®

was dispersed in the above solution. During solvent evaporation the particles were deposited by gravity to the bottom side, creating an asymmetric of 80/20 PDLLA/BG membrane along the thickness.

2.3. Bioactivity tests

For the *in vitro* bioactivity tests an acellular simulated body fluid (SBF) (1.0×) with ions concentration nearly equal to human blood plasma was prepared [15]. Sample membranes of $20 \times 15\,mm^2$ were cut from the original processed films for the bioactivity tests. Three replicates for each sample were immersed in 45 mL SBF for 2, 5, 7, 14 and 21 days at 37 °C. After being removed from SBF the membranes were gently rinsed with distilled water and dried at room temperature.

2.4. Physico-chemical characterization

2.4.1. Scanning Electron Microscopy (SEM)

Qualitative information of the morphology of PDLLA and PDLLA/BG membranes surfaces, before and after the immersion in SBF, was obtained using a Scanning Electron Microscope, SEM (Nova NanoSEM 200-FEI Company, USA), at an accelerated voltage of 5 kV. Before being observed by SEM, the membranes were gold coated.

2.4.2. Fourier Transform Infrared Spectroscopy (FTIR)

The chemical structure of each side of the composite membrane were analyzed using FTIR. Spectra were recorded in an IR Prestige 21 FTIR spectrophotometer (Shimadzu, Japan) with the attenuated total reflection accessory (128 scans, resolution $4\,cm^{-1}$) in the spectral range 2000–400 cm^{-1}.

2.5. Mechanical characterization

The tensile properties were determined using an INSTRON 4505 Universal Machine (Instron Int. Ltd., USA) equipped with a 1 kN load cell, with a loading rate of $5\,mm\,min^{-1}$, up to 20% of strain, at room temperature. Samples were analyzed in dry and wet conditions. For the wet condition, samples were immersed for 3 h in PBS before being tested. The values reported represent an average of at least five testing specimens. Tensile stress was taken as the maximum stress in the stress-strain curve. Tensile modulus was estimated from the initial linear section of the stress-strain curve.

2.6. Cell culture studies using hBMSC and hPDL

Two types of cells were used in this study, namely human periodontal ligament cells (hPDL) and human bone marrow stromal cells (hBMSC). Both cells types were collected from two different donors.

hPDL were obtained from human third molar according to the following procedure. After extraction, the teeth were washed three times for 10 min in PBS with 100 units/mL penicillin and streptomycin. PDL tissue was scraped from the middle third of the root with a scalpel blade, to avoid contamination by epithelial or pulpal cells. The freed portions of the periodontal ligament were minced and transferred to a small culture flask, filled with 5 mL alpha minimal essential medium (α-MEM, Gibco) with 10% (v/v) fetal calf serum (FCS, Gibco), 50 mg/mL ascorbic acid (Sigma), 10-8 M dexamethasone (Sigma), 50 mg/mL gentamycin (Gibco) and 10 mM sodium β-glycerophosphate (Sigma). Cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and medium was replaced every 2–3 days. Upon reaching confluence, cells were released with trypsin/EDTA (0.25%, (w/v) crude trypsin and 1 mM EDTA, pH 7.2) and sub-cultured for two passages in standard culture flasks. The cells were then frozen in liquid nitrogen until used for the experiments.

Human BMSC were isolated from bone blocks of human iliac crest biopsies of donors. The biopsies were discarded tissues during standard surgical procedures at Radboud University Nijmegen Medical Center (Nijmegen, The Netherlands). The bone blocks were cut into small pieces and subsequently placed in a 50 mL tube to which 20 mL alpha-minimal essential medium (α-MEM) was added. After that the tube was shaken vigorously and the medium with cells was collected. This procedure was repeated several times. The collected medium with cells was plated in culture flasks (T175, Greiner Bio-one) and expanded in proliferation medium. Cells were characterized and showed stem cells phenotype. Additionally, a multipotential differentiation test was applied, demonstrating their stem cells capacity. Cells were cultured at 37 °C in a humid atmosphere with 5% CO_2 and its passage was performed at 80% confluence using trypsin EDTA (Gibco).

Specific culture medium (O^-) was used for each cell type. The hPDL O^- was composed of α-MEM (Gibco) with 10% fetal bovine serum (FBS, Greiner Bio-one) and 100 units mL^{-1} penicillin/streptomycin (Gibco). The hBMSC O^- was composed of α-MEM (Gibco) with 15% FBS (Greiner Bio-one), 1% L-glutamine, 1% ascorbic acid (Sigma), 100 units mL^{-1} penicillin/streptomycin (Gibco) and 1%, by volume added to each cell culture flask, basic fibroblast growth factor (bFGF). After the first generation, cells were plated at a density of 5000 cells/cm^2 in culture flasks (T175, Greiner Bio-one). The culture medium was changed twice a week. Cells from passage 3 (hBMSC) and 5 (hPDL) were used in the biological experiments.

All Gibco products are from Life Technologies BV, Breda, the Netherlands, all Greiner Bio-one products from Greiner Bio-one BV, Alphen aan de Rijn, the Netherlands, and all Sigma products from Sigma–Aldrich Chemie B.V., Zwijndrecht, the Netherlands.

2.6.1. Cell seeding

Metal rings (15 mm diameter × 3 mm thickness) were glued to the membrane with RTV silicone adhesive (Nusil Technology, Carpinteria, CA), to keep them in the bottom of the culture well, preventing fluctuation. Prior to cell seeding, the samples were sterilized with 70% (v/v) ethanol for 60 min and then washed three times immersed in PBS. The samples were placed in 25-well plates and soaked in cell culture medium overnight. After removing the culture medium, 50 μL of a cell suspension with a 2.0×10^4/sample cell density, was seeded onto the surface of each sample. After incubation for 4 h at 37 °C in a 5% CO_2 atmosphere incubator, osteogenic medium (referred as O^+), specific for each cell type, was added to the seeded samples, according to the type of assay performed. The hPDL O^+ was composed of α-MEM (Gibco) with 10% FBS (Greiner Bio-one), 1% ascorbic acid (Sigma), 1%

b-glycerophosphate (Sigma), 1% dexamethasone (Sigma) and 100 units mL^{-1} penicillin/streptomycin (Gibco). The hBMSC O^+ was composed of a-MEM (Gibco) with 15% FBS (Greiner Bio-one), 1% L-glutamine, 1% ascorbic acid (Sigma), 1% b-glycerophosphate (Sigma), 1% dexamethasone (Sigma) and 100 units mL^{-1} penicillin/streptomycin (Gibco). On the control groups, cells were seeded directly on the well-plates and osteogenic medium was added immediately.

2.7. Cell adhesion, proliferation and metabolic activity

2.7.1. DNA content

After the different experimental time points, medium was removed from the wells and the samples were washed twice with PBS. The analysis was performed on the supernatant of the substrates after day 1, 3, 7, 14 and 28 of culture. Cells were lysed using milliQ with subsequent sonification for 10 min between two cycles of freeze/thaw from −80 °C. The supernatant was stored at −20 °C until further analysis. A PicoGreen dsDNA Quantification Kit (Molecular Probes, Eugene, USA) was used according to manufacturer's instructions. To each 100 μL sample, 100 μL PicoGreen working solution was added. The samples must incubate for 2–5 min at room temperature, in the dark. After incubation, the fluorescence was measured on a fluorescence cuvette reader (microplate fluorescence reader, Bio-Tek, Winooski, USA) with a 485 nm excitation filter and a 530 nm emission filter.

2.7.2. AlamarBlue® staining

Cell metabolic activity was measured using AlamarBlue® staining (Invitrogen) according to the instructions of the manufacturer. A solution was made with AlamarBlue and culture medium in a proportion 1:9 (v/v) and was placed at 37 °C for 5 min. The medium was removed from wells and replaced with the solution. Plates were incubated (37 °C and 5% CO_2) for 4 h. After incubation, 200 μL of each sample solution was transferred to 96-well plates (Greiner Bio-one). Fluorescence was measured using a microplate reader (FL 600; Bio-Tek) at 570 nm. The assay was performed on day 1, 3, 7, 14 and 28 of culture.

2.7.3. Scanning Electron Microscopy (SEM) observation

Adhesion of both cell types (hBMSC and hPDL) on membranes was analyzed by SEM ($n = 2$). After day 3 and day 28 time points, cells were fixed in 2% (v/v) glutaraldehyde in 0.1 M sodium-cacodylate buffered solution, for 5 min. Cells were rinsed in cacodylate buffered solution, dehydrated in a series of ethanol dilutions in water (70%, 80%, 90%, 96% and 100% (v/v)), 1 h in each, and dried in tetramethylsilane (TMS, Merck) to air. Finally, specimens were sputtercoated with a thin layer of gold, and examined in a JEOL 6310 scanning electron microscope.

2.8. Cell differentiation and mineralization

2.8.1. Alkaline Phosphatase activity measurements (ALP)

The same supernatants as used for PicoGreen assay were also used to measure alkaline phosphatase (ALP) activity (Sigma). To each 80 μL of sample, 20 μL of 0.5 M Alkaline Buffer (Sigma) was added. Thereafter 100 μL substrate solution 5 mM paranitrophenylphosphate (PNP, Sigma) was added to each well. After 60 min of incubation at 37 °C, 100 μL stop solution (0.3 M NaOH) was added to each well. Finally, ALP activity was measured at 405 nm using an ELISA microplate reader (Bio-Tek Instruments Inc, USA).

2.8.2. Von Kossa staining

Cells were fixed with 2% glutaraldehyde, stained with fresh 5% silver nitrate ($AgNO_3$), washed with distilled water, developed with 5% sodium carbonate (Na_2CO_3) in 25% formalin, and fixed with 5% sodium thiosulphate ($Na_2S_2O_3$). Stained samples were observed under a Leica MZ12 stereomicroscope and images were captured.

2.8.3. Calcium content

Calcium content was assessed after 21 and 28 days of culture to obtain information about mineralized matrix formation. The samples were rinsed twice with milliQ. 1 mL of acetic acid was added to each sample. The samples were incubated overnight under vigorous constant shaking and the acetic acid with the diluted calcium was frozen and kept at −20 °C, until further investigation. After thawing, the calcium content was determined using the OCPC method. Optic density was read with an ELISA reader (Bio-Tek Instruments Inc, USA) at a wavelength of 570 nm. Bare membranes were also assessed in order to further exactly quantify and distinguish cellular from acellular mineralization on the membranes.

2.9. Statistical analysis

All samples were measured in triplicate. Biological tests were performed twice, excepting Von Kossa. All results are presented as mean ± standard deviation. Statistical analysis of experimental data was performed using an unpaired ordinary ANOVA with standard parametric methods. Calculations were performed in InStat (v. 3.0 GraphPad Software Inc, San Diego, CA). Statistical significance was set to p-value ≤ 0.1 (*), to p-value ≤ 0.01 (**) and to p-value ≤ 0.001 (***).

3. Results

3.1. Membranes characterization

3.1.1. Bioactivity, morphology and microstructure

The surface morphology of PDLLA and PDLLA/BG membranes was analyzed using SEM – see Fig. 1a. A flat, smooth, non-porous surface was observed on the PDLLA membranes with no evidence of surface irregularity (A_1). Both upper and bottom faces of the composite membrane (see scheme in Fig. 1b) were also analyzed. The upper face of the composite PDLLA/BG 80/20 membrane is also smooth (B_1) but the bottom face (C_1) presents some asperities, homogeneously distributed in the surface corresponding to the BG particles that were preferentially deposited in this side of the membrane. The image suggests that the particles are well incorporated in the polymeric matrix.

The bioactive character of the produced composite membranes was tested *in vitro* by immersing the materials in SBF – see evolution of the morphology of the surfaces in Fig. 1a. No Ca–P layer was formed on the surface of pure PDLLA

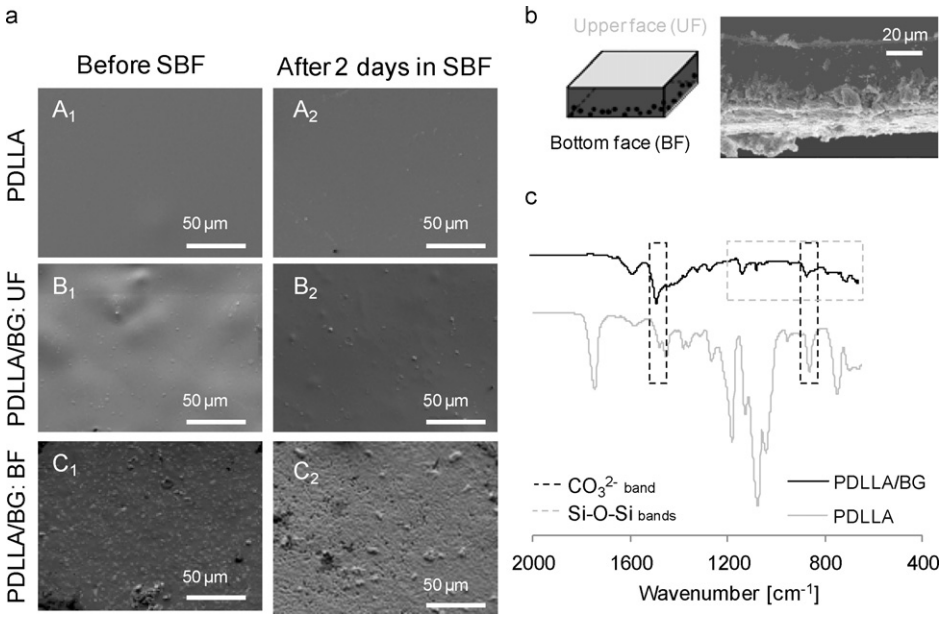

Fig. 1 – *In vitro* bioactivity tests of the developed membranes: (a) SEM micrographs of the surfaces of the PDLLA (A_1) and PDLLA/BG membranes, from upper and bottom face (B_1 and C_1), before and after 2 days of immersion in SBF (A_2, B_2, and C_2). The scale bar represents 50 μm. (b) Schematic design of the PDLLA/BG membrane and respective SEM image, evidencing the distinct faces. (c) FT-IR spectra of both sides of the composite membrane: bottom face rich in BG (dark gray line) and upper face exposing basically PDLLA (light gray line).

membrane (A_2) or on the upper face of the composite membrane (B_2), even after 21 days of immersion in SBF. Only the membranes face enriched with BG presented a bioactive character, where an uniform ceramic layer could be detected after only 2 days of immersion in SBF (C_2).

The morphological analysis was complemented with FTIR – see Fig. 1c. The characteristic FTIR bands of the upper face are located at 750 and 865 cm^{-1} (CH band); 1042, 1090, 1138, and 1186 cm^{-1} (=C—O stretch); 1375 cm^{-1} (CH_2 wag); 1452 cm^{-1} (CH_3 band) and 1751 cm^{-1} (C=O stretch, ester group) that are the characteristic bands of PDLLA [16]. The spectrum of the bottom face of the membrane indicates the presence of BG particles: 867, 1454 cm^{-1} (CO_3^{2-} bands); 1200–700 cm^{-1} (Si—O—Si bands) [17,18].

In order to understand the behavior of the membranes in an aqueous environment, the weight loss and swelling ratio were measured up to 4 months.

In the system studied in this work, no significant swelling was observed in the membranes even when BG is incorporated (data not shown). Within the time period analyzed the weight loss of the PDLLA membrane was not significant: after 120 days of immersion in PBS, the weight loss was about 12%. For the case of the composite membrane we could detect a faster weight loss up to 50 days (data not shown), that could be explained by the partial dissolution of the inorganic component.

3.1.2. *Mechanical properties*

Tensile mechanical tests were performed for the PDLLA and PDLLA/BG membranes. Both dry and wet specimen conditions were assessed; the last condition simulates better the behavior of these materials in the oral cavity environment. Representative stress-strain curves are shown in Fig. 2. Table 1 shows the maximum strain and stress obtained in these experiments as well as the tensile or Young modulus of the membranes. The addition of BG in the PDLLA membranes significantly reduced the maximum strain and a small reduction of the maximum stress could also be detected. Young modulus values were very similar between both types of membranes, in both dry and wet conditions.

3.2. *In vitro hBMSC and hPDL culture*

3.2.1. *Cell adhesion, proliferation and metabolic activity*

DNA content was quantified at different culture time points for both membranes – see Fig. 3a. A clear proliferation of

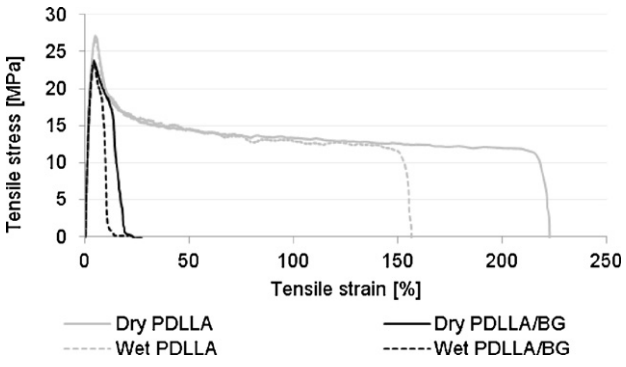

Fig. 2 – Representative stress *versus* strain curves obtained for PDLLA and PDLLA/BG membranes in dry (solid lines) and wet (dashed lines) conditions.

Table 1 – Young modulus, maximum strain and maximum stress of the PDLLA and PDLLA/BG membranes obtained in dry and wet conditions. Values are reported as mean ± SD.

Condition	Young modulus (Mpa)		Maximum strain (%)		Maximum stress (Mpa)	
	Dry	Wet	Dry	Wet	Dry	Wet
PDLLA	713.7 ± 77.9	656.9 ± 38.1	166.2 ± 81.3	141.0 ± 94.1	26.2 ± 1.56	27.4 ± 1.38
PDLLA/BG	743.3 ± 30.3	617.9 ± 38.8	21.9 ± 5.5*	21.6 ± 11.4*	17.7 ± 5.21**	23.1 ± 1.43

* Statistically significant differences in the mechanical properties of the PDLLA/BG membranes when compared to those of the pure PDLLA for $p < 0.1$.

** Statistically significant differences in the mechanical properties of the PDLLA/BG membranes when compared to those of the pure PDLLA for $p < 0.01$.

both cell types onto the PDLLA and PDLLA/BG membranes was observed until 28 days of culture. Significantly higher cell content was found in BG containing membranes seeded with hBMSC after 28 days of culture. Complementarily, cell metabolic activity was measured. As the values between different days for fluorescence intensity of AlamarBlue are not comparable, it was calculated a ratio between the composite and PDLLA membrane samples, in each time point – see Fig. 3b. Therefore, values above 1 means higher metabolic activity for samples with BG than the ones without BG. Average cell growth and their metabolic activity was typically higher in the PDLLA/BG group than in the PDLLA group, for hBMSC. PDLLA and PDLLA/BG membranes cultured with hBMSCs and hPDL were further studied using SEM – see Fig. 3c. After 3 days of culture, hPDL spread perfectly on the PDLLA/BG membranes (B_3). On day 28, there was a very dense extracellular matrix (ECM) deposition for both hBMSC and hPDL on PDLLA/BG membranes (B_{28} and D_{28}), which hampered the distinction of the cells morphology and the examination of surface roughness in the material. Still at this time point, (C_{28}) the adhesion of hBMSC could be detected, either spread in an elongated shape, polarized with lamellipodia (see (*) on inset image of C_{28}) or in some cell agglomerates.

3.2.2. *Cell differentiation and mineralization*

Alkaline phosphatase activity was measured to assess the osteogenic differentiation potential of the cells cultured in the developed membranes. The results obtained from this assay (Fig. 4a) were normalized by the DNA content measured for the same sample. No detectable ALP activity could be seen during the first time points, being consistent with the undifferentiated state of the cells. After day 14, and specially day 28, it can be seen significant ALP activity of the cells in the membranes. At 28 days of culture, higher ALP activity was detected for the composite membranes cultured with hBMSC, compared to the pure PDLLA membranes and even to the control group. In alignment with the ALP results, statistically significantly higher calcium content was found in the PDLLA/BG membranes (Fig. 4b). Von Kossa staining allowed observing the formation of mineralization nodules by hPDL on composite membranes, after 28 days of culture (Fig. 4c). No nodule formation was detected on PDLLA membranes (data not shown). In fact, there was an increasing calcium deposition, over time (21–28th day), statistically significant for the hBMSC on composite membranes.

4. Discussion

In this study, the incorporation of BG in PDLLA membranes modified their physico-chemical and biological properties. The asymmetric distribution of the BG particles along the thickness in the composite membrane permitted to induce a bioactive character in one of the sides of the membrane. The incorporation of BG particles has been reported to play an important role in polymer surface wettability [19]. Its introduction in polymer matrices can modify both surface and bulk properties of the composite, by enhancing both hydrophilicity and water absorption [20]. The faster weight loss in the composite membranes could be related to the slow dissolution of the inorganic component. Such process is consistent with the event of the formation of an apatite layer upon immersion in SBF.

The mechanical properties of the membranes were not significantly compromised with the introduction of BG. Adequate mechanical integrity is known as an important requirement for membranes in guided tissue regeneration. To adapt the desired shape and to support the stresses of the surrounding native tissue at the site of implantation, membranes should be strong but flexible [3]. Previous works reported also a reduction of the strength upon reinforcement in composites of poly(α-hydroxyesters) [21,22]. The behavior observed in the membranes may be due to the increase in pore size by introducing Bioglass® in the pure PDLLA matrix. Moreover, water can affect strongly the molecular wettability and the viscoelastic properties of poly(lactic acid) [23]. However, in the membranes prepared in this work swelling was almost inexistent and no significant plasticization effect of water took place, as reflected in the tensile mechanical properties at 37 °C.

The inorganic component had a positive impact in the adhesion, proliferation, differentiation and mineralization of hBMSC on PDLLA/BG membranes. The enhanced hBMSC proliferation and metabolic activity showed is in accordance with some previous studies [24–26] that reported the promotion of cell proliferation by Si and other released products from bioactive glasses. Sun et al. showed an enhanced metabolic activity of hBMSC on akermite ceramics verified using AlamarBlue staining, which has a composition very similar to BG [27]. Other studies [28–32] reported the enhancement of osteoblast cell proliferation by the influence of BG particles corroborating the results herein obtained for PDLLA/BG membranes, using hBMSC. Higher concentrations of DNA were observed in BMSC cultured when 45S5 Bioglass® was added to a new ceramic

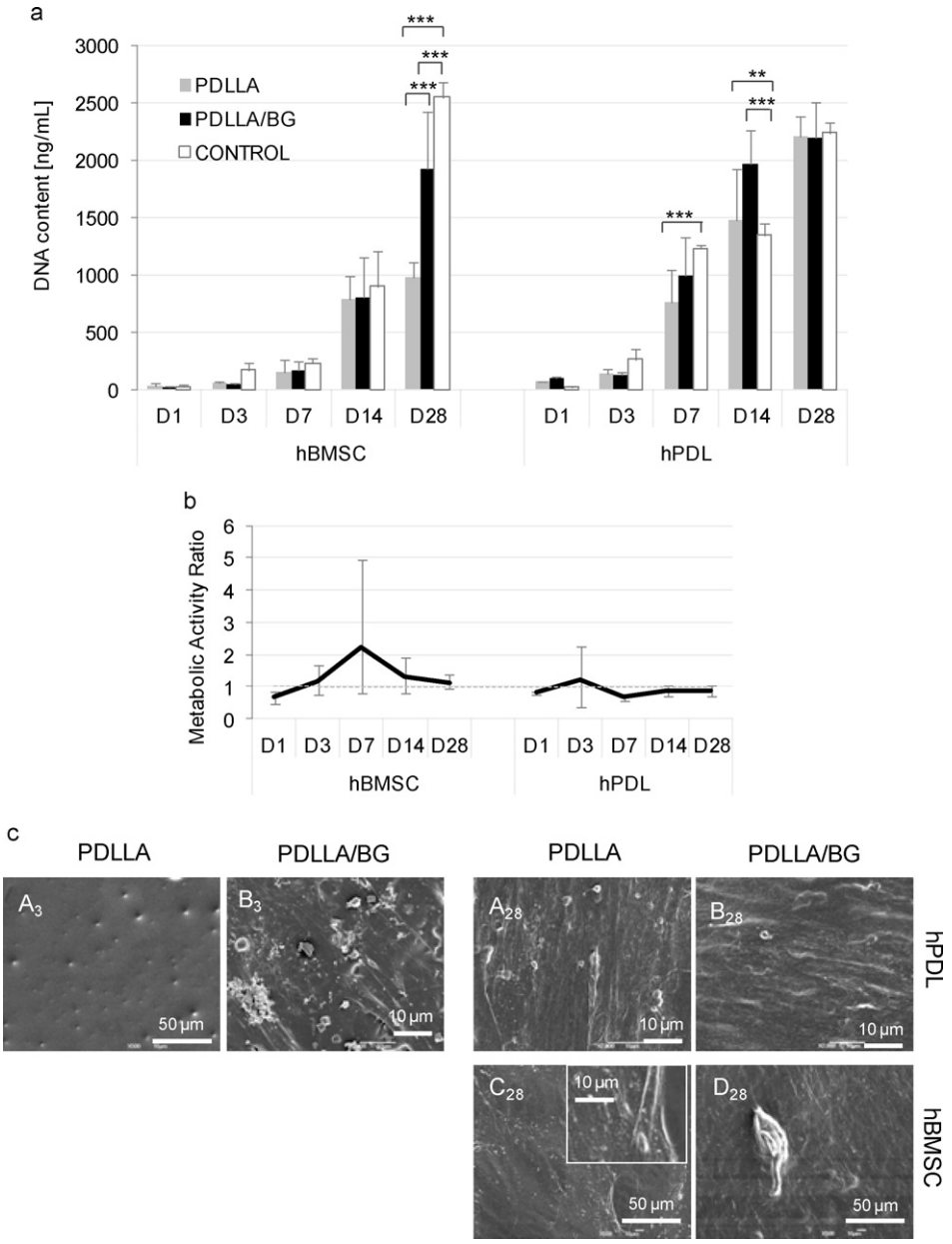

Fig. 3 – Results obtained from DNA and AlamarBlue assays at 1, 3, 7, 14 and 28 days of culture. (a) DNA content, in ng/mL, of hBMSC and hPDL cells seeded onto each membrane type and controls (plastic cell culture in polystyrene). (b) Metabolic activity ratio between the samples with and without BG (solid lines) for both cell types, where the border line (dashed light gray line) is $y = 1$. Values are reported as mean ± SD ($n = 3$). (**) shows significant differences for $p < 0.01$ and (***) for $p < 0.001$. (c) SEM micrographs of PDLLA and PDLLA/BG membranes after cultured with hPDL and hBMSC cells. The subscripts indicate the incubation time.

based on hydroxyapatite compared to the pure material [33]. High contents of bioactive component may lead to a negative effect on cell behavior. For example, cell studies with scaffolds of PDLLA, containing different contents of BG (5 and 40 wt%) showed enhanced proliferation and ALP activity for the 5% ones [34]. Due to the subsequent augmented and prolonged ion release and increase of pH, the materials with the largest BG concentration appeared to fail. Exceptions have been also reported. For example, Helen et al. obtained better results with PDLLA/BG 30 wt% comparing to 0 or even 5 wt% [35]. Thus it alerts to the need of an ideal polymer/BG particles proportion in order to achieve the pretended results. Many studies have examined the ability of BG to enhance not just cell proliferation [36,37] but also the extracellular matrix (ECM) production [38], which is in accordance with the results achieved in this study. Once that osteoblasts mature and start depositing ECM, this production of ECM is a signal of cell differentiation [39]. While some authors [24,25] claim that the addition of BG particles has no effect on ALP activity of rat primary culture osteoblasts and murine osteoblasts, others state that this

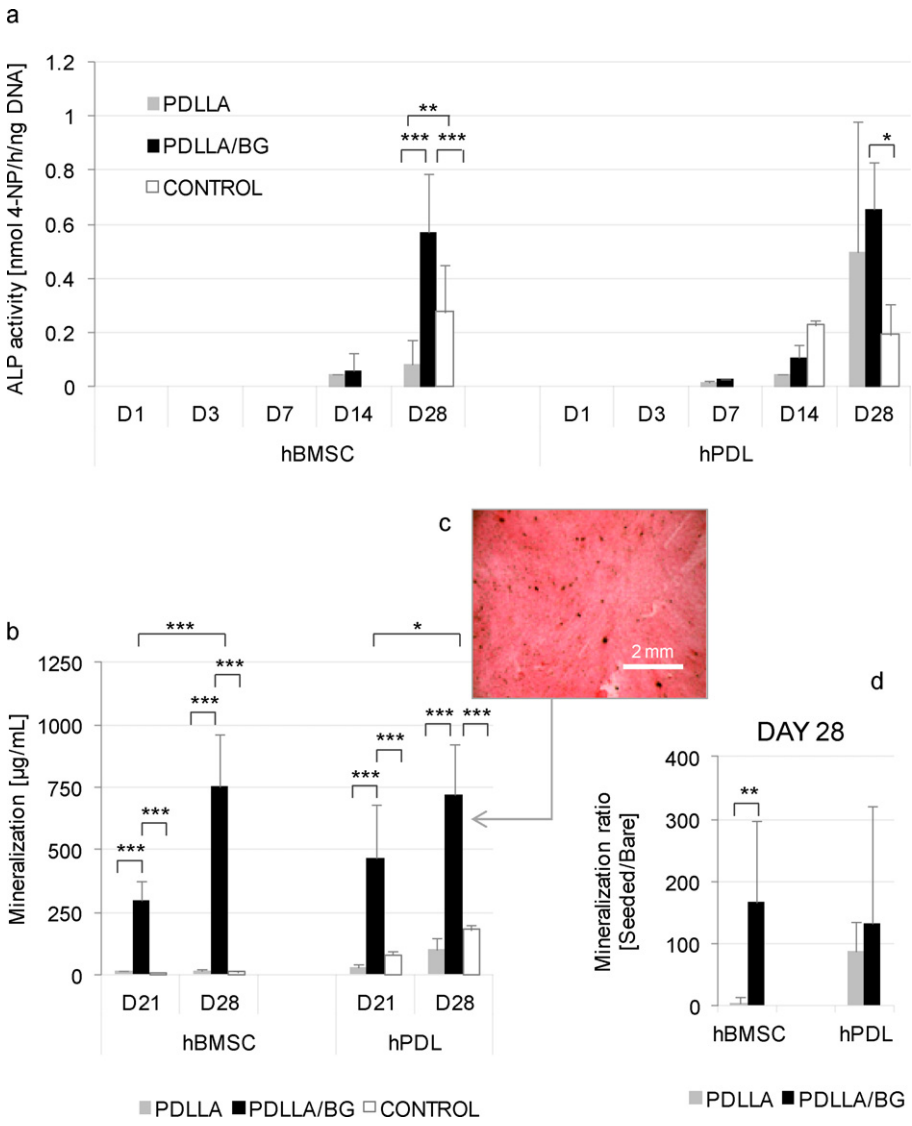

Fig. 4 – (a) Alkaline phosphatase activity, normalized by the DNA content, of hBMSC and hPDL cells on PDLLA and PDLLA/BG membranes and controls (plastic cell culture in polystyrene) at 1, 3, 7, 14 and 28 days of culture. Values are reported as mean ± SD ($n = 3$). (*) shows significant differences for $p < 0.1$, (**) for $p < 0.01$ and (***) for $p < 0.001$ and evaluation of the mineralization of hBMSC and hPDL cells on PDLLA and PDLLA/BG membranes and controls (plastic cell culture in polystyrene), after 21 and 28 days of culture. (b) Calcium content quantification and comparison between 21 and 28 days of incubation. (c) Calcium nodules formation, verified by Von Kossa staining at day 28 of incubation, on PDLLA/BG membrane cultured with hPDL cells. (d) Ratio of mineralization between seeded and bare membranes. Values are reported as mean ± SD ($n = 3$). (*) shows significant differences for $p < 0.1$, (**) for $p < 0.01$ and (***) for $p < 0.001$.

addition has stimulatory effects on the ALP activity of human primary osteoblasts [40], BAF cells [35,41] and hBMSC [27]. This provides a clear indication of a more extended osteogenic differentiation of hBMSC on the PDLLA/BG composite membranes.

Calcium content and Von Kossa results were favorable to the composite membranes, results which are in accordance with other related studies. For example, a stronger and earlier calcium phosphate mineral formation in bioactive composites was observed for rat bone marrow cells [42]; faster nodule formation in porous bioactive glass scaffolds, as compared with control cultures [26] using human primary osteoblasts, among others previous studies [43,44], demonstrating that BG causes an increase of calcium in the medium, which is a modulator of intracellular events. Bone nodules consist of differentiated osteoblasts, extracellular matrix, and associated minerals, and their formation characterizes a late stage of osteoblast differentiation [45], being a good index of osteogenesis *in vitro* [45–47]. Both of the mineralization assays indicated that BG improved mineralization, complementing each other information.

Therefore, our results suggest that the obtained asymmetric bioactive PDLLA/BG 80/20 membrane, with osteoconductive properties in just one of the faces, could have potential

use in the regeneration of distinct tissues, namely periodontal ligament and bone.

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