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Abstract

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Incorporation of glass-reinforced hydroxyapatite microparticles into poly(lactic acid) electrospun fibre mats for biomedical applications

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Keywords: Biomaterial, Poly (lactic acid), glass reinforced hydroxyapatite, Bonelike[®] bone graft, Electrospun fibres, Bone regeneration.

Abstract:

Tissue engineering is constantly evolving towards novel materials that mimic the properties of the replaced injured tissue or organ. A hybrid electrospun membrane of electroactive poly(L-lactic acid) (PLLA) polymer with glass reinforced hydroxyapatite (Bonelike[®]) microparticles placed among the polymer fibres in a morphology like “islands in the sea” was processed. The incorporation of 60 to 80 wt% Bonelike[®] bone grafts granules with $\leq 150 \mu\text{m}$ into the polymer solution lead to an amorphous polymeric fibre membranes, and a decrease of the average polymer fibre diameter from $550 \pm 150 \text{ nm}$ for neat PLA down to $440 \pm 170 \text{ nm}$ for the hybrid composite. The presence of Bonelike[®] in the polymer mats reduced the activation energy for thermal degradation from 134 kJ.mol^{-1} , obtained for the neat PLLA membranes down to 71

kJ.mol^{-1} , calculated for the hybrid composite membranes. *In vitro* cell culture results suggest that the developed processing method does not induce cytotoxic effects in MG 63 osteoblastic cells, and creates an environment that enhances cell proliferation, when compared to the neat PLLA membrane. The simplicity and scalability of the processing method suggests a large application potential of this novel hybrid polymer-microparticles fibre membranes for bone regenerative medicine.

Introduction

Interaction between cells and scaffolds is one of the most important issues to address during the design of the materials. Cell response depends on different parameters such as surface chemistry, wettability, protein adsorption, surface charge, roughness, and stiffness [1], just to mention the most important ones. Scaffold morphology is one of the key parameters, not only to provide a mechanical support for cell attachment, but it also influences cell morphology, proliferation and differentiation.

Poly(l-lactic acid) (PLLA) is a widely used biomaterial, due to its intrinsic properties, such as low density, easy to process, good biocompatibility and biodegradability, increasing its potential for biomedical engineering applications [2, 3]. Furthermore, PLLA presents interesting electroactive properties, with a piezoelectric coefficient around 10 pC.N^{-1} [4], similar to the one reported for human bone, that can go up to 7 pC.N^{-1} [5], making this material an interesting candidate for bone regeneration strategies. Moreover, PLLA degree of crystallinity can range from a completely amorphous polymer (lower stiffness) to almost 50 % crystallinity (higher stiffness) and this property can be tailored by applying thermal annealing [6]. It is known that mesenchymal stem cells can sense the mechanical elasticity of the physical support, due to their ability of pulling against the matrix [7]. In addition, at the cell-polymer interface, cells can actively modify the implant surfaces, changing their own and even other cells microenvironment stiffness [8]. Further, PLLA with different degree of crystallinity showed to be able to increase the production of aggrecan, characteristic of the extracellular matrix of hyaline cartilage during cell culture of human primary chondrocytes [6].

Different processing techniques can be used to process PLLA polymer into various shapes and geometries, electrospinning being one of the most interesting. This is a

versatile technique to produce membranes that mimic the natural fibrillar structure of extracellular matrix, and cellular adhesion and proliferation are enhanced when compared to the more traditional porous scaffolds [9]. Non-woven electrospun fibrous mats possess high specific surface area and small pore size, and this is pointed as the principal reason for the improved biological response over electrospun materials [10].

Hydroxyapatite (HA) is the major constituent of the bone matrix, and synthetic HA has already demonstrated its excellent biocompatibility with bone and teeth, due to its similar structure to bone mineral phase, bioactivity and osteoconductivity [11, 12]. Nevertheless, it requires high temperatures for scaffold formation, has brittle properties and a low reabsorption rate by the organism (5-15% per year). Therefore, the bone remains brittle and prone to fractures [11]. Moreover, these limitations led to the development of alternative materials based in the combination of HA with bioglass or tricalcium phosphate (α -TCP and β -TCP). Bonelike® is a synthetic bone substitute, consisting of a three-phase material: HA, and TCP, with the α - and β -TCP phases homogeneously dispersed in the HA matrix, resulting in a material with improved mechanical properties and enhanced bioactivity, compared to the commercial HA [11]. Furthermore, the inclusion of ions into Bonelike® composition, such as fluoride and sodium, among others, makes it possible to achieve a chemical composition closer to the mineral phase of bone [13].

Human bone is a hybrid system of HA particles and collagen type I fibres, that assembles in a highly organized porous structure [14]. Electrospinning of HA blended in a polymer matrix, mimics the HA-biopolymer composition and morphology, and improves the mechanical properties of the electrospun scaffolds [15]. Previous works reported the potential of composite electrospun systems, mainly focused in hydroxyapatite (HA) and β -tricalcium phosphate nanoparticles as active fillers for bone regeneration [16, 17]. The use of electrospinning technique allowed to mimic the bone morphology, with polymeric fibres matrix and immobilized ceramic particles. Nevertheless, it was observed that the use of nanoparticles raises issues concerning the immobilization of the particles on fibre surface and consequently exposure of the bioceramics to cells is hindered [18].

In this work, a novel processing methodology was created to obtain self-standing electrospun membranes with a higher weight percentage of glass reinforced modified hydroxyapatite immobilized on the porous spaces between the fibres. The single-step

electrospinning method lead to the creation of a continuous soft polymeric fibrous matrix network, with a random and homogeneous distribution of hard ceramic microparticles exposed to outside the fibres for better interaction with cells, also leading to a material with a micro gradient of mechanical stiffness. Glass reinforced hydroxyapatite (Bonelike[®]) microparticles were synthesized, and carefully dispersed in the PLLA solution and electrospun into a ground collector, to manufacture the desirable hybrid non-woven membrane. Fibre morphology and size distribution was assessed, thermal and chemical properties of the microcomposite membranes were investigated and addressed to the processing technique parameters. PLLA and Bonelike[®] - PLLA membranes were also evaluated for their performance regarding the cell viability/proliferation of osteoblastic cells.

Materials and Methods

Bonelike[®] synthesis: Ceramic powder was synthesized according to the method described elsewhere [19, 20]. Briefly, P₂O₅-CaO based glass with the chemical composition of 65P₂O₅-15CaO-10CaF₂-10Na₂O (mol %) was prepared by mixing the appropriate quantities of high purity (>98%) grade reagents (sodium carbonate (Na₂CO₃, Sigma Aldrich), calcium hydrogenphosphate (CaHPO₄, Sigma Aldrich), calcium fluoride (CaF₂, Sigma Aldrich) and di-phosphorus penta-oxide (P₂O₅, Sigma Aldrich) in a platinum crucible, and then heating it at 1450 °C for 90 min in a furnace. The prepared glass was crushed in an agate mortar and sieved to a granule size below 50 µm. Bonelike[®] was obtained by adding 2.5 %wt of bioglass to the previous prepared HA. The Bonelike[®] powder was mixed with the microcrystalline cellulose and PVA and the resulting suspension was poured into Alumina (Al₂O₃) plates, dried in a woven at 60 °C for two days and then the samples were sintered at 1300 °C using a heating rate of 4 °C.min⁻¹, followed by natural cooling inside the furnace. Finally, using standard milling and sieving techniques, Bonelike[®] granules with particle size ≤150 µm were obtained.

Electrospun membrane preparation: Poly(L-lactic acid) (PLLA, Purasorb PL18, M_w = 217 – 225 kDa) from Corbion (Netherlands) was dissolved in a mixed solvent of N,N-dimethylformamide (DMF, from Merck) and dichloromethane (MC, from Sigma-Aldrich) (3/7 v/v) to achieve a polymer concentration of 10 wt% of the solution. Composite solutions were prepared by dispersing the ceramic powder in a solution of

DMF/DCM (3/7 v/v) in an ultrasound bath (Bandelin, Model Sonorex Super RK106) during 6 h, to promote a good dispersion of the microparticles. After this stage, the polymer was added to the solution and stirred at room temperature until complete dissolution. The concentration of ceramic filler related to polymer (w/w) was 80 %.

The polymer solution was placed in a commercial glass syringe (10 mL) fitted with a steel needle with 500 μm of inner diameter. Electrospinning was conducted at 1.25 kV.cm^{-1} with a high voltage power supply from Gamma High Voltage. A syringe pump (from KDSscientific) was used to feed the polymer solutions into the needle tip at 0.5 mL.h^{-1} . The electrospun fibres were collected in ground collecting plate placed at 20 cm apart from the needle (random aligned fibres). All experiments were conducted at $21 \pm 2 \text{ }^{\circ}\text{C}$ and a relative humidity of $43 \pm 5\%$.

Membranes Characterization: Electrospun fibre membranes were coated with a thin gold layer using a sputter coater (Polaron, SC502) and their morphology was analyzed using a scanning electron microscope (NanoSEM FEI Nova200, from FEI) with an accelerating voltage of 10 kV. Fibers average diameter and its distribution was calculated over approximately 50 fibres using the Image J [21]. From the average fibre diameter (d), the surface area to volume ratio can be estimated [22]:

$$\text{surface area to volume ratio} = \frac{4}{d} \quad (1)$$

Sample porosity was obtained through the pycnometer method described elsewhere [23], and equation 2:

$$\varepsilon = \frac{W_2 - W_3 - W_s}{W_1 - W_3} \quad (1)$$

where W_1 , W_2 , W_3 and W_s , are the weight of the pycnometer filled with ethanol, the weight of the pycnometer filled with ethanol and the sample immersed inside, the residual weight of the pycnometer when the saturated samples is removed and the sample weight in the dry state, respectively.

Infrared measurements (FTIR) were performed at room temperature in a IRAffinity-1S (Shimadzu) in ATR mode from 4000 to 650 cm^{-1} . FTIR spectra were collected with 32 scans and a resolution of 2 cm^{-1} , at room temperature. The thermal behaviour of the electrospun fibre mats were analysed by differential scanning calorimetry measurements (DSC) with a Q100 (TAinstruments) apparatus. The samples were cut into small pieces from the middle region of the electrospun membranes and placed into 50 μl aluminium pans and heated between 30 and 200 $^{\circ}\text{C}$ at a heating rate of 10 $^{\circ}\text{C}.\text{min}^{-1}$. The thermal degradation kinetics of the samples was characterized by thermogravimetric analysis (TGA) in a Q500 apparatus from TAinstruments at heating rate scans from 10 $^{\circ}\text{C}.\text{min}^{-1}$ up to 40 $^{\circ}\text{C}.\text{min}^{-1}$. All measurements were performed under a nitrogen atmosphere.

Stress-strain measurements were performed at room temperature, using a Shimadzu Universal Testing Machine (AG-IS with a 10 N load cell) in tensile mode, at a strain rate of 0.5 $\text{mm}.\text{min}^{-1}$. Rectangular stripes of 10x40 mm^2 were measured with a calliper (Mitutoyo) and a thickness of ~ 250 μm thickness was measured with a DUALSCOPE® MPOR (Fischer). From the stress-strain data, elasticity modulus was calculated in the linear zone, between 0 and 2 % of strain, for all the samples. The ultimate tensile strength and the strain-at-failure were also determined. Measurements were performed on five specimens of each sample and the values presented are calculated through the average and standard deviation.

In Vitro Cell Culture Studies: Cell culture studies were performed in PLLA and Bonelike® - PLLA membranes. Nonwovens membranes were cut out with punch ($\phi = 15$ mm), sterilised by UV exposure (254 nm) for 30 minutes, each side, and held at the bottom of the 24-well culture by using Teflon inserts (inner diameter of 10.5 mm). All the experiments were performed in triplicate.

MG63 osteoblastic-like cells (ATCC number CRL-1427TM, passage 25) were cultured in MEM- α supplemented with fetal bovine serum (FBS, 10% v/v), 50 $\mu\text{g}.\text{ml}^{-1}$ ascorbic acid, 100 IU. ml^{-1} penicillin, 2.5 $\mu\text{g}.\text{ml}^{-1}$ streptomycin and 2.5 $\mu\text{g}.\text{ml}^{-1}$ amphotericin B, at 37 $^{\circ}\text{C}$ in a 5% CO_2 humidified atmosphere. Cells were cultured until 70 – 80% confluence is reached. Detachment of adherent cells was achieved by a 5 min incubation in 0.05% trypsin – 0.25% EDTA solution, at 37 $^{\circ}\text{C}$. After, the cell suspension was centrifuged and the pellet suspended in fresh culture medium. For cell counting, a cell suspension/Trypan Blue (1:1 proportion) was prepared, and the colourless (viable) cells

were counted in a Neubauer haemocytometer chamber. Then, cells were seeded over the fibre mats at a density of 1.5×10^4 cells/well. Seeded membranes were cultured for 1, 3 and 7 days, and evaluated for the cell viability/proliferation. Cells cultured on standard polystyrene culture plates were used as control.

Cell viability/proliferation was analyzed by the MTT assay. This is based in the reduction of the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) by viable cells to a dark blue formazan product that accumulates in the cytoplasm [24]. At each time-point, the medium was removed from the wells to eliminate any dead cells and was replaced by fresh one. Then, MTT (0.5 mg.ml^{-1}) was added to each well, and cultures were incubated for 3 hours at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . After the incubation period, the samples were transferred to new wells, observed under the microscope and an image was recorded. Finally, the formazan salts were dissolved in dimethylsulphoxide (DMSO) and the absorbance was measured at $\lambda=550 \text{ nm}$ on a plate reader (Power Wave XS2 spectrophotometer, Biotek).

Statistical Analysis: Experiments were performed in triplicate. Quantitative data are presented as mean $\pm$ SEM. Significant differences in statistical analysis were determined using one-way ANOVA test with Tukey's HSD post hoc analysis.

Results and Discussion

Electrospun fibre mats morphology

Electrospinning processing parameters such applied electric field, solution viscosity and sample collecting procedure have influence on fibre morphology and average diameter. Smooth and defect free electrospun fibres were obtained for PLLA with an average diameter of $510 \pm 150 \text{ nm}$ (figure 1a and b). The same experimental parameters were used to process electrospun fibre mats from PLLA with microparticles of Bonelike[®], figure 1c. Composite membranes showed that the ceramic filler particles were randomly entrapped among the fibrous polymer matrix, rather than wrapped, as commonly observed when nanoparticles are used, such HA or TCP [18] . Thus, the achieved Bonelike[®] - PLLA mats organization enhances surface exposure of the bioactive ceramic filler, because they preferentially occupy the porous space among the cylindrical fibres (figure 1). The PLLA fibres in the composite mats have the same

general appearance that the ones observed for the pristine PLLA (figure 1c), however, a reduction of the average fibre diameter to 440 ± 170 nm was noticed (figure 1d). In addition, for pristine PLLA electrospun mats, a higher percentage of the fibre is in the range between 400 – 800 nm, and for the microcomposite membranes, the fibre size was located between 300 – 400 nm, processed under same experimental conditions. Although, the Bonelike[®] microparticles do not affect the fibres surface morphology, the changes in the fibre average diameter and distribution obtained for the microcomposite membranes, is possibly since the inclusion of the ceramic microfiller can affect charge density of the solutions. This is probably because Bonelike[®] can be ionized and carry more chargers, stretching the droplet even further, leading to a decrease of the polymer average fibre diameter. Similar behavior was reported by Zhou et al. [25] for PLLA-nanoclays electrospun fibres. Finally, the reduction of the average fibre diameter led to an increase of the surface area to volume ratio from 7.8 up to $9.1 \mu\text{m}^{-1}$, calculated from equation 1. It is believed that higher surface area to volume ratio favors cell attachment and growth [22].

Figure 1 – Morphology of electrospun fibre mats: a) PLLA membrane, b) distribution of PLLA fibre diameter, c) Bonelike[®] - PLLA membrane (Insets shows the morphology of a synthesized Bonelike[®] particle), d) Bonelike[®] - PLLA fibre diameter distribution and e) a detail of a Bonelike[®] particle entrapped between the polymeric electrospun fibres.

Fourier transform infrared spectroscopy in attenuated total reflectance (FTIR-ATR) mode was performed to the filler, the matrix and the composite membrane, to monitor variations at a molecular level that might occur due to the electrospinning conditions. Figure 2 shows the characteristic infrared spectra observed for the PLLA, Bonelike[®] and Bonelike[®] - PLLA membranes. The broad absorption band located in the region between $1300 - 900 \text{ cm}^{-1}$ was assigned to the ν_3 vibrational mode and the band at 960 cm^{-1} is due to the ν_1 vibrational mode of the phosphate ions [26].

The FTIR spectrum of the polymer matrix presents the characteristic absorption band of amorphous α and α' phase at 955, 1044, 1134 and 1183 cm^{-1} , and no absorption bands related to the crystalline phases α (921 cm^{-1}) and β -PLLA (908 cm^{-1}) phase were detected, suggesting that the electrospinning process yields amorphous PLLA fibre membranes [23, 27]. The spectrum collected for the microcomposite membrane presents

the absorption bands characteristic of the PLLA matrix, and no new modes seems to appear or are totally suppressed due to the presence of the microceramic filler in the polymer solution during electrospinning, which suggests that the polymer matrix remains in the amorphous phase after processing.

Figure 2 – Infrared spectra of PLLA, Bonelike[®] and Bonelike[®] - PLLA membranes. Arrows represent the characteristic PLLA α and α' vibrational bands (955 (a), 1044 (b), 1134 (c) and 1183 cm^{-1} (d)) and ν_3 vibrational band of Bonelike[®] phosphate ions.

The mechanical properties of the developed microcomposite membranes were through quasi-static measurements in tensile mode. It was observed that the incorporation of the Bonelike[®] particles leads to a decrease of the Young modulus and stress at break, without compromising the overall deformation of the material (table 1). This is probably due to the increase of the porosity from 79 ± 3 % up to 88 ± 5 %, and to the presence of larges pores on the microcomposite membrane (figure 1a and d).

Table 1 – Mechanical properties of the PLLA and Bonelike[®] - PLLA electrospun membranes.

Sample	<i>E</i> <i>MPa</i>	σ_{break} <i>MPa</i>	ϵ_{break} %
PLLA	18 ± 6	8 ± 2	38 ± 7
Bonelike [®] - PLLA	5.4 ± 1.3	0.57 ± 0.2	41 ± 8

Thermal properties

Differential scanning calorimetry was performed to the electrospun fibrous mats to study the materials thermal properties (figure 3). Electrospun neat PLLA fibres present a large overshoot in the glass transition (T_g) region, around 60 °C, reported to be related to the recovery of the enthalpy of the sample stored at room temperature, around 30 °C below the T_g , and thus subjected to physical aging [23, 28]. As the temperature

increases, a cold-crystallization process was observed immediately after the glass transition, shown as a broad exothermic peak between 70 – 110 °C with a maximum (T_c) around 81°C. Melting starts at 130 °C and ends at 160 °C, represented as a broad endothermic process in figure 3. Microcomposite membranes present a polymer glass transition shifted towards low temperatures, with maxima around 51 °C, and the cold-crystallization process that occurs immediately after, T_g , presents a peak ~73 °C. This behaviour is probably related to the presence of the microparticles that hinders the crystallization of PLLA chains during solvent evaporation, phenomena also observed by Deng et al. [29].

The melting of Bonelike[®] - PLLA membranes occur in the same range of the neat polymer mats, nevertheless a shoulder can be detected at lower temperatures. When the solvent evaporates during the electrospinning process, amorphous polymer chains are frozen in conformations that facilitate additional crystallization when the temperature was increased above T_g and segmental diffusion was possible [28]. These regions probably have higher structural defects, especially near interfacial regions such rough surface Bonelike[®] microparticles (figure 1c), giving rise to the lower temperature shoulder observed for the polymer composite membranes melting transition (figure 3).

Figure 3 – Differential scanning calorimetry of PLLA and Bonelike[®] - PLLA membranes.

The degree of crystallinity (ΔX) of electrospun membranes was calculated following equation:

$$\Delta X = \frac{\Delta H_m - \Delta H_{cc}}{\Delta H_m^0} \quad (2)$$

where, ΔH_m , ΔH_{cc} are the melting and cold crystallization enthalpy, respectively, and ΔH_m^0 the enthalpy for a 100 % crystalline sample (93 J.g⁻¹ [27, 28]). PLLA electrospun fibre mats seem to be amorphous [23, 28], regardless the presence of Bonelike[®] particles in the polymer solution and during the polymer crystallization during processing.

Thermogravimetric analysis was performed to the electrospun neat PLLA to the Bonelike[®] - PLLA microcomposite membranes at several heating rates (figure 4). A major weight loss process was observed in the range between 300 – 400 °C and no dehydration process around 100 °C was observed. From the thermogravimetric data, the amount of Bonelike[®] microparticles present in the electrospun membrane was between 60 – 80% (table 1), and such broad distribution of the amount of the microparticles present in the electrospun mat is probably due to the small amount of sample present in the sample holder, typically between 12 - 15 mg in this work, and to the large size of Bonelike[®] particles used (figure 1). PLLA onset temperature (T_{onset}) of degradation, that is calculated by extending the pre-degradation portion of the curve to the point of the interception with a line draw as a tangent to the steepest portion of the mass curve occurring during degradation [28], was found to be similar between neat PLA and hybrid Bonelike[®] - PLLA membranes (table 1).

Polymer stability was characterized by the kinetic parameters of the thermal degradation according to the Kissinger's mathematical model. The activation energy (E_{act}) can be obtained from the plots of the logarithm of the heating rate vs reciprocal temperature at the maximum reaction rate, in constant heating rate experiments [30, 31]. In the Kissinger's model, the E_{act} can be obtained without the knowledge of the reaction mechanism, according to the equation 2:

$$\ln\left(\frac{\beta}{T_p^2}\right) = \frac{\ln(AE_{act})}{T} + \ln[n(1 - \alpha_p)^n] - \frac{E_{act}}{RT_p} \quad (3)$$

where T_p and α_p are the absolute temperature and the conversion at the maximum mass loss rate, respectively. The activation energy can be calculated by plotting $\ln \beta/T_p^2$ vs $1000/T_p$, being the slope of the straight line proportional to E_{act} (figure 4d) and the results for the PLLA and Bonelike[®] - PLLA particles microcomposite membranes are reported in table 1. The linear fittings were obtained with $R > 0.98$.

The activation energy obtained for the neat PLLA membrane was 134 kJ.mol⁻¹, which is in the range of the reported values for this polymer, 80 – 176 kJ.mol⁻¹ [32-36], while the composite membranes showed a decrease of the E_{act} down to 71 kJ.mol⁻¹ (table 1). Thermal degradation of PLLA is a complex phenomenon and involves the generation of significant amounts of volatile decomposition products during pyrolysis, e.g., cyclic oligomers, L-lactide, carbon dioxide, acetaldehyde, ketene and carbon monoxide [36].

PLA tends to follow the dominant reaction pathway of intermolecular transesterification to form cyclic oligomers, usually residues of carbon dioxides and acetaldehyde from fragmentation reaction. The presence of residual metals used as catalysts during polymer synthesis and the molecular weight of the material are also reported to affect the polymer degradation kinetics and activation energy [37].

Figure 4 – Thermogravimetric results obtained for: a) neat PLLA membrane, b) Bonelike[®] - PLLA membrane, c) comparison between the PLLA and Bonelike[®] - PLLA membrane thermogravimetric data recorded at 20 °C.min⁻¹ and d) Kissinger's plot for the PLLA and Bonelike[®] - PLLA mats.

The polymer degree of crystallinity does not influence the thermal degradation process, as the melting of the material occurs at ~150°C, which is more than 100 °C below the polymer T_{onset} of degradation (figures 3 and 4). In this way, the differences observed for the activation energy should be related to chemical reactions with the ceramic fillers within the polymer-ceramic interfacial region. Zhou and Xanthos [38] studied the effect of nanosized and micro-sized clays in PLLA thermal degradation kinetics and they reported that a decrease of the activation energy was due to a complex reaction between the filler and the matrix. The thermal stability of PLA with 5 wt% zeolite showed that incorporation of nanofillers lead to a decrease of the polymer thermal degradation activation energy [39].

In this work, there is higher amount of ceramic filler with a heating accumulation capability different of the polymer matrix, leading to an increase of the temperature at the interfacial regions between the polymer and the ceramic filler, and this is probably the main reason for the decrease of the activation energy observed between the neat and the microcomposite electrospun membranes.

Table 2 – Residue obtained at 450 °C and E_{act} calculated by Kissinger's model for the neat PLLA and Bonelike[®] - PLLA mats.

Sample	T_{onset} (@ 20°C.min ⁻¹) °C	Residue at 450°C %	E_{act} kJ.mol ⁻¹
PLLA	338	< 2	134

Cytocompatibility

Cell viability/proliferation was evaluated by the MTT reduction assay, which is widely used to assess cell metabolic activity and short-term proliferation on biomaterials [40]. Figure 5 presents the results for the behavior of MG63 osteoblastic cells cultured on PLLA and hybrid PLLA/Bonelike[®] fibre membranes for 1, 3 and 7 days. Cell viability/proliferation increased over the time up to 7 days on the two membranes. After 1 and 3 days, no significant differences were found between PLLA and hybrid Bonelike[®] - PLLA membranes, with respect to cell proliferation. However, after 7 days, significantly higher values ($p < 0.05$) were noticed on the composite membrane compared to the neat PLLA. Differences between control (cells cultured on standard culture plates) and membrane groups could be addressed to a lower cell adhesion to the substrates, as suggested by the significantly decreased MTT reduction values at day 1; an initial effect of cell cycle arrest may also play a role. Both have been reported for several biomaterials [41-44]. Despite the initial lower values, metabolic activity increase from day 1 to day 7 was higher for PLLA-Bonelike[®] ($582 \pm 185\%$), followed by PLLA ($321 \pm 36\%$) and Control ($177 \pm 16\%$), indicating that the cell growth rate during the culture time was significantly higher on the membranes, compared to control. Additionally, the ceramic-polymer composite membrane herein proved to have improved biological properties in terms of human osteoblastic cell response, in comparison with the PLLA mats.

Figure 5 – a) Cell viability/proliferation of MG63 osteoblastic cells cultured over PLLA and PLLA/Bonelike[®] hybrid microcomposite membranes for 1, 3 and 7 days, estimated by MTT assay. Values are presented as the mean $\pm$ SEM of three independent experiments; *statistical differences were reported as* * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (* - compared to control; # - compared between membranes). Microscopic photographs of b) PLLA and c) PLLA/Bonelike[®] membranes showing an abundant layer of cells, seen as purple dots after the reduction of the MTT to the insoluble formazan product, at 7 days of culture. The circles in c) represent spots where the ceramic filler (BL - Bonelike[®]) can be observed.

Microscopic observation of the samples after 7 days of cell culture showed that the membranes did not degrade for the entire cell culture time. MG63 cells (seen as purple dots, after the reduction of the MTT to the insoluble formazan product) were homogeneously dispersed throughout the membranes. After 7 days, an abundant cell layer covered the entire membrane. The presence of random distributed ceramic microfiller between the polymer fibres, establishing direct cell contact, was clearly observed.

The improved behavior observed for the microcomposite PLLA/Bonelike[®] membranes when compared to the pristine PLLA needs to be further investigated but some of the possible reasons could be the excellent biocompatibility of the glass reinforced hydroxyapatite, because its chemical composition is similar to the natural bone mineral [19, 20], the morphology and size of the particles and the overall roughness of the hybrid microcomposite electrospun mats with different hard spots randomly distributed among the soft polymeric matrix fibres, that was addressed as a beneficial stimuli for cell adhesion, growth and even differentiation [7, 45].

Conclusions

PLLA solutions with and without glass reinforced-HA microparticles were electrospun to produce hybrid composite fibre mats with potential applications for bone tissue regeneration. The polymer matrix in the microcomposite fibrous membrane presents smooth and beadless fibres, like the one obtained for the neat PLLA. Further, Bonelike[®] microparticles were successfully entrapped outside the individual fibres in a random fashion, which creates a novel scaffold with different stiffness gradients. When the microparticles are added to the solution, the average fibre diameter of the polymer matrix decreases from 510 ± 150 nm down to 440 ± 170 nm, leading to an increase of the surface area to volume ratio from 7.8 up to $9.1 \mu\text{m}^{-1}$. After processing, the obtained neat and microcomposite membranes are nearly amorphous. TGA analysis showed that the Bonelike[®] - PLLA membranes presented 60 to 80 % of glass reinforced hydroxyapatite, and both membranes had a single thermal degradation process in the range between 300 – 400 °C. The presence of the ceramic microparticles played an important role in polymer matrix thermal degradation, and a decrease of the thermal

degradation activation energy from 134 kJ.mol^{-1} , obtained for the neat PLLA membranes, down to 71 kJ.mol^{-1} , calculated for the hybrid microcomposite membranes, was noticed.

Finally, *in vitro* results suggest that the developed processing method does not induce cytotoxic effects in MG63 osteoblastic cells, and the presence of exposed Bonelike[®] microparticles among the polymeric fibres creates an environment that enhances cell proliferation rate, when compared to the neat PLLA membrane. These results are suggestive of the potential of microcomposite membranes with an active stiff bioceramic immobilized on the surface of the polymeric fibres. Furthermore, the simplicity and scalability of the developed processing method suggests a large application potential of this novel hybrid polymer-microparticles fibre membranes for bone regenerative medicine.

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Figure 6 – Morphology of electrospun fibre mats: a) PLLA membrane, b) distribution of PLLA fibre diameter, c) Bonelike[®] - PLLA membrane (Insets shows the morphology of a synthesized Bonelike[®] particle), d) Bonelike[®] - PLLA fibre diameter distribution and e) a detail of a Bonelike[®] particle entrapped between the polymeric electrospun fibres.

Figure 7 – Infrared spectra of PLLA, Bonelike[®] and Bonelike[®] - PLLA membranes. Arrows represent the characteristic PLLA α and α' vibrational bands (955 (a), 1044 (b), 1134 (c) and 1183 cm^{-1} (d)) and ν_3 vibrational band of Bonelike[®] phosphate ions.

Figure 8 – Differential scanning calorimetry of PLLA and Bonelike[®] - PLLA membranes.

Figure 9 – Thermogravimetric results obtained for: a) neat PLLA membrane, b) Bonelike[®]- PLLA membrane, c) comparison between the PLLA and Bonelike[®]- PLLA membrane thermogravimetric data recorded at 20 °C.min⁻¹ and d) Kissinger's plot for the PLLA and and Bonelike[®]- PLLA mats.

Figure 10 – a) Cell viability/proliferation of MG63 osteoblastic cells cultured over PLLA and PLLA/Bonelike[®] hybrid microcomposite membranes for 1, 3 and 7 days, estimated by MTT assay. Values are presented as the mean±SEM of three independent experiments; statistical differences were reported as * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (* - compared to control; # - compared between membranes). Microscopic photographs of b) PLLA and c) PLLA/Bonelike[®] membranes showing an abundant layer of cells, seen as purple dots after the reduction of the MTT to the insoluble formazan product, at 7 days of culture. The circles in c) represent spots where the ceramic filler (BL - Bonelike[®]) can be observed.