Development of biocompatible and fully bioabsorbable PLA/Mg films for tissue regeneration applications

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Abstract

During recent years, Mg reinforced polylactic acid (PLA) composites have emerged as potential biocompatible and bioabsorbable materials for biomedical applications. It has been shown that Mg particles added to a matrix based on a biodegradable polymer can address the lack of bioactivity and the low mechanical properties of the polymers and, furthermore, it can counteract the detrimental effects associated to the high degradation rate of Mg, as alcalinization and elevated H₂ release. Additionally, the polymer can protect the Mg particles, by tailoring their degradation rate. Former processing of these composites performed by extrusion, compression and injection molding employed Mg contents up to 10 wt.%. Higher amounts of Mg resulted in heterogeneous materials and thermally degraded matrices, with the corresponding higher degradation rate. In the present work, Mg reinforced PLA films with Mg content as high as 50 wt.% were obtained without compromising the thermal stability of the polymer. Firstly, a successful dispersion of Mg microparticles was achieved by a breakthrough in processing introducing a colloidal step where organic additives were added to modify the Mg particle surface and promote a chemically stable suspension. The resulting

colloidal suspension was then used as feedstock to obtain composite films by tape casting. The films show advantageous *in vitro* behaviour in terms of degradation, hydrogen release and oxygen permeability. In addition, the viability with fibroblastic cells (MEF) opens a window of opportunity for these composite films as bioabsorbable material for tissue engineering and wound dressing applications.

Introduction

During recent years, there has been an increasing interest in the development of different structural materials for tissue engineering such as skin, tendon, cartilage and muscle. These structural tissues, have proved challenging due to difficulties in their manufacturing with analogous mechanical properties [1,2]. Current natural polymers such as collagen, chitosan, alginate, present good biocompatibility but offer inadequate mechanical performance [2]. Regarding tissue regeneration in a damaged area, a recent proposal reported on the use of material that releases bioactive ions such as Zn^{2+} , Si^{4+} and Mg^{2+} [3–5]. Mg^{2+} ions are of special interest because they are essential in the metabolism at a biochemical level, such as protein synthesis, muscle function, nervous and immune system [6,7]. Several authors have reported that Mg^{2+} ions promote skin tissue renewal, improve skin barrier function, enhance skin hydration, and regulate epidermal proliferation and differentiation [8–10]. Additionally, the released Mg^{2+} ions present an extraordinary biological response, promoting the formation of collagen in skin wounds [4]. These advantages offer a promising opportunity for the applicability of Mg as biomaterial for tissue regeneration or more specifically in wound healing applications.

In spite of the aforementioned benefits, before proposing Mg as a suitable temporary implant material, its degradation rate should be properly controlled, as Mg is one of the most reactive structural materials, presenting a wide range of degradation types such as

local corrosion or cracking by stress corrosion [11]. Moreover, its fast degradation produces an increase in the local pH that is accompanied with hydrogen release, which can compromise the biofunctionality and viability of the injured tissue.

Conversely, poly(lactic acid) (PLA) is a biocompatible and biodegradable polymeric biomaterial, approved by the FDA, and it is already used for wound dressing [12–14] due to its wide processing techniques, mechanical durability and elasticity [15].

However, PLA has some technical limitations such as poor hardness, low degradation rate, poor cellular interaction and high hydrophobicity, which limits its water uptake capacity [16,17].

Recently, the feasibility of introducing Mg particles within a PLA matrix has been demonstrated [18]. With regards to the neat polymer, Mg enhances its mechanical properties, particularly the creep strength [19] and compression modulus [20]. The degradation of these compounds *in vitro* has also been studied by several authors in several simulated media, such as Cifuentes *et al.* [18] in PBS (Phosphate buffer solution), Zhao *et al.* [21] in SBF (Simulated body fluid) and Li *et al.* [22] in KBM (Keratinocyte Basal media). In these articles, it is demonstrated that the incorporation of Mg particles into a PLA matrix prevents the acidification of the medium due to the degradation of the polymer. As in the case of dense materials [18], PLLA/Mg scaffolds [23] have already demonstrated an improved degradation rate. According to *in vitro* tests, the PLA/Mg composites are not cytotoxic and produce an increase of the cell proliferation and the initial induction of osteogenesis [20,24].

Related to tissue engineering applications, PLA/Mg composites combine the extraordinary properties associated with Mg release and a polymeric support. In these materials, the addition of Mg gives the opportunity to have a close control of material

degradation rate. The aim of this study was to process PLA/Mg film-shape composites and to characterize them in terms of hydrophobicity, permeability, in vitro degradation response and cell proliferation. Materials processing was performed by including a completely novel step for the mixing of Mg particles with PLA, which allows incorporating Mg in amounts as high as 50 wt.% in the final composite. Since Mg is the component that accelerates the composite degradation, it is important to explore the limits of this route, which will determine the capacity of truly tailoring the film degradation rate. To produce the films, a suspension of Mg particles in a PLA solution was prepared, and films were shaped by tape casting, avoiding the drawbacks associated with a mixing step that involves melting. This new method prevents thermal degradation of the polymer during processing and the poor metal particle dispersion in the polymeric matrix, as well as enhances a matrix/particle interaction. Although Mg particles are in the micrometric range, PLA is a hydrophobic polymer while Mg particles are hydrophilic, and thus, improving dispersion by filler surface modification results in numerous advantages. The successful dispersion of Mg microparticles was achieved by a breakthrough in processing by introducing a previous colloidal step incorporating a dispersant [25]. Polyethylenimine (PEI) was selected as additive since it has been demonstrated that this component maintains the chemical and colloidal stability of metal particles in a water medium. Even at pH values higher than 11, the amino groups of branched PEI are slightly deprotonated, and they can still be effectively adsorbed onto the surface of Mg particles. Thus PEI modifies the surface of Mg and promotes a chemically stable suspension.

Experimental procedure

Processing of PLA/Mg films

Colloidal suspensions with a high solid content (35 vol.%) were formulated in deionized water. Spherical Mg particles ($\text{Mg} > 99.8\%$) of less than $50\ \mu\text{m}$ ($d_{50} = 29.22\ \mu\text{m}$) in diameter, obtained by atomization of cast ingots (supplied by Nitroparis, Spain), were used. Mg suspension was prepared as follows: i) TMAH (Tetramethylammonium hydroxide) was added to deionized water to adjust pH value in the range of 11 to 12; ii) by mechanical stirring, PEI (Polyethylenimine - 0.2 wt.% respect to Mg) was added as particle modifier; iii) Mg particles were added to the solution; and iv) the suspension was milled during 45 min with nylon balls to fully disperse the Mg particles. Once the suspension was prepared, it was centrifuged to eliminate the water and TMAH in order to segregate the Mg modified particles.

PLA solutions were prepared using pellets of PLA polymer 2003D with D-isomer content of 4.25 % provided by Natureworks® (USA). Tetrahydrofuran (THF), supplied by Panreac (Germany), was used as solvent. Mg modified particles were mixed with the PLA solution under vigorous mechanical stirring. More experimental procedure information is described in [26].

Figure 1. Tape casting process schedule

The composite films were prepared by the tape casting technique. The PLA/Mg solutions were cast on a glass plate using a moving tape casting device with two doctor blades (Figure 1). The casting parameters were $10\ \text{mm s}^{-1}$ of casting velocity and $100\ \mu\text{m}$ of gap height between the blades and the carrier tape. After drying in air at room

temperature overnight, the films were further dried at 60 °C for 24 h. Films with four Mg contents were produced, together with an unreinforced PLA film for comparison purposes (Table 1).

Table 1. Film compositions (nominal values)

Film	Mg (wt.%)	PLA (wt.%)	PEI (wt.%)
PLA	-	100	-
F5	5	95	0.2
F10	10	90	0.2
F30	30	70	0.2
F50	50	50	0.2

While the side of the film that was in contact with the glass plate is flat, the free side is rough. Unless otherwise stated, all experiments were performed on the rough surface.

Microstructural characterization and roughness

The film cross sections were observed by scanning electron microscope (SEM-FEG HITACHI S-4800, Japan) equipped with energy dispersive X-ray spectroscopy (EDS) and INCA energy software (Oxford Instrument). After embedded in epoxy resin, cross sections were mechanically polished with 9 µm diamond paste. A thin graphite layer was deposited on all samples prior to SEM examination.

The roughness of the samples was studied by an optical profilometer. Different parameters have been evaluated: (i) the superficial average roughness, S_a , (ii) the average height of the peaks, R_h , and (iii) the S_{ku} kurtosis parameter.

Physico-chemical characterization

Thermogravimetric analysis (TGA, model TAQ500, EEUU) was used to study the thermal stability of PLA/Mg composites and to check the real amount of Mg against the nominal one. This experiment was carried out in dynamic conditions at 10 °C/min from 20 °C to 500 °C under argon atmosphere (40 ml/min). The onset temperature (T_0), the temperature of highest rate of change on the weight loss curve (T_p), and the final

temperature (T_e) were determined. T_0 and T_e were calculated by finding the intersection of the baseline and the extrapolated tangent at the inflection point of the weight loss curve. T_p , also known as the inflection point, was calculated from the first derivative of the weight loss curve. $\%_R$ was calculated from the residual $\%_{\text{weight}}$ of PLA at 500 °C and $\%_{Mg}$ was determined from $\%_{\text{weight}}$ of composite at 500 °C subtracting the previous $\%_R$ of as-received PLA.

The contact angle measurements of ultrapure water on films were obtained using an EasyDrop standard equipment (KRÜSS), by drop method after 10 s. The values were estimated by the software using the Young-Laplace model. At least three measurements on different areas of each film specimen were averaged.

Permeability of PLA films towards oxygen, nitrogen and carbon dioxide was determined using the time-lag method [27]. The experimental setup was composed of two tanks, feed and permeate (31 cm³), separated by the test membrane (effective area of 1385 mm²). The whole unit was inserted in a thermostatic cabinet. All experiments were performed at 30 °C, with a feed pressure of 1 bar (100 kPa). Permeability through dense membranes is usually expressed in terms of permeability coefficient (P) and the diffusion (D), which are related by apparent solubility (S) by the following expressions:

$$P = D \cdot S$$

P is a variable expressed in barrers (1 barrer = 10⁻¹⁰ cm³(STP)·cm/cm²·s·cm Hg) and is calculated from the slope of the linear section of pressure vs time plot according to the equation:

$$P = \frac{273}{76} \left(\frac{Vl}{ATp_a} \right) \frac{dp_b}{dt}$$

where V is the volume of the lower chamber, l the film thickness, A is the effective area of the film, p_a the pressure of the upper chamber and T the temperature. D , Apparent diffusion coefficient was obtained using the time lag model:

$$\theta = \frac{l^2}{6D}$$

θ value is the intersection of linear section of pressure vs time plot with the time axis as Figure 2 shows.

Figure 2. Example of pressure vs time curve, and the intersection of linear section with x axis (θ).

In vitro evaluation

In vitro degradation behaviour was studied by immersion of the specimens in Phosphate Buffered Saline solution (PBS) at 37 ± 1 °C. Although PBS does not accurately simulate body conditions, it is suitable for comparative studies on the hydrolytic degradation of PLA-based materials [24]. The amount of H_2 released was recorded daily for up to 90 days. A ratio of volume of media (ml) to specimen surface (cm^2) of 20:1 was used and the solution was renewed once a week. Details of the experimental setup are found in [18].

In order to study the cytocompatibility of the PLA/Mg films, circular discs of 1 cm in diameter were obtained. All sample surfaces were disinfected by applying UV light

during 30 min to both sides. Mouse embryotic fibroblast cells (MEF cells) were grown in cell culture flask containing DMEM, 10 % FCS, 1 % non-essential amino acids and 1 % antibiotic (penicillin and streptomycin) for 48 h. Afterwards, the cells were harvested by trypsin and 100,000 cells were seeded per each sample and incubated in a humidified atmosphere of 95 % relative humidity and 7.5 % CO₂, at 37 °C for 24 h.

Cytotoxicity tests were performed by the indirect method. PLA/Mg discs were cut from the films and immersed in the DMEM medium during 24 h, 1, 2, 3, and 4 weeks. Fibroblast cells were cultured in 96-well plates and incubated for 24 h to allow initial cell adhesion. The medium was then replaced with 0.5 mL of experimental film extract at the different times of immersion. After incubating the cells for 24 h, cell viability was measured through the enzymatic conversion of tetrazolium salt (WST-8 assay kit, Sigma Aldrich, Germany). Culture medium was completely removed from the samples and a new one, freshly prepared, was added containing 1 vol.% WST-8 solution, followed by incubation for 2-4 h. Subsequently, 100 mL of supernatant from each sample was transferred into a 96 well-plate and the absorbance was measured at 450 nm with a microplate reader (PHOmO, autobio labtec instruments co. Ltd, China). Cell viability was defined as:

$$\text{cell viability (\%)} = \frac{\text{abs(films)} - \text{abs(blank)}}{\text{abs(+control)} - \text{abs(blank)}}$$

To assess the viability and morphology of cells, live staining was performed with Vybrant (Vybrant®) and nuclei were counterstained with 4–6-diamidino-2-phenylindole (DAPI). Cells were fixed with paraformaldehyde solution. The images of calcein stained cells were taken by using a fluorescence microscope (FM) (Axio Scope A.1, Carl Zeiss Microimaging GmbH, Germany).

During the direct tests, fibroblasts were cultured for 24 h on the films. Once the cells had been cultured on the materials, the cell viability was evaluated by WST-8 and the cells were stained with the Vybrant / DAPI system.

Finally, the morphology and the cellular distribution on the surface were evaluated by means of images with a fluorescence microscope.

Statistical Analysis

Statistical analysis of cell viability of MEF cells was obtained by an analysis of one-way variance (ANOVA) to evaluate the statistical significance of differences. Cell viability measurements were given as a mean value $\pm$ standard deviation ($n = 6$), and difference at $P 0.05$ was considered to be statistically significant.

Results and discussion

Microstructural characterization of the films

Through the study of the films by optical microscopy, a change in the colour and an increase in the materials' opacity were observed when increasing the Mg content, Figure 3a and d. In the composites with lower amount of Mg (F10), a successful Mg particle dispersion in the matrix was achieved together with a high homogeneity. However, in F50 composites, although the homogeneity was still high, some agglomeration of particles was observed (Figure 3d), which is associated with a poor dispersion due to the higher particle concentration. Figure 3b and c and Figure 3e and f show SEM images of composites F10 and F50 at two magnifications, which show the same trend. Agglomeration of particles is clearly observed in Figure 3e, for F50 film.

Figure 3. Optical microscopy images (a,d) and SEM surface images (b, c, e, f) of films: a, b, c) F10, d, e ,f)F50.

Morphology investigation of the film cross sections by SEM reveals the Mg particle distribution as well as the particle-polymer interface, as shown in Figure 4. In all cases, Mg particles were fully embedded in the polymer matrix, so that they were always covered by a PLA layer. This polymer distribution around the particles reflects that Mg particles act as nucleation agents on the PLA during the solvent evaporation [28,29]. Regarding the homogeneity, in the films with low load (F10-Figure 4a and b) a high dispersion was achieved. When the weight content increases by up to 50 wt.% (F50-Figure 4c and d), although a good Mg dispersion is still observed, a few particle agglomerates can be encountered. In the cross section, the phenomenon of agglomeration is observed by particle stacking in the perpendicular axis to the tape casting plane, which affects the roughness of the film.

Figure 4. SEM images of a,b) F10 and c,d) F50 cross sections

The film thickness was evaluated by images SEM analysis. Results show an increase from 25 μm for PLA to 30 μm for film composite. The main change observed is that the Mg addition increases film roughness in terms of S_a and R_h . The increase in S_a is associated with the fact that as the load increases, a greater number of irregularities appear on the film surface. The increase in R_h is mainly determined by an increase in the number of agglomerates with a higher amount of Mg particles, as shown in Figure 3c and f. These agglomerates tend to grow in the perpendicular axis to the film surface increasing the R_h as well as film thickness. In the case of Kurtosis factor, a decrease is detected as the amount of film load increases. Since S_{ku} is a measure of the sharpness of the roughness profile, this indicates that Mg particles tend to agglomerate in the film plane rather than vertically. That phenomenon indicates that, although the roughness is greater in the films with higher Mg content, F30 and F50, it is less abrupt than in the F5 and F10 cases.

Table 2. Roughness parameter summary for F5, F10, F30 and F50 composite films

Roughness parameters			
Film	S _a	R _h (μm)	S _{ku}
F5	1.0	6.4	0.2385
F10	2.5	9.9	0.2069
F30	3.8	13.4	0.1397
F50	4.7	18.9	0.0923

Physic-chemical characterization of the films

Thermal degradation behaviour of as-received PLA pellets, and PLA and PLA/Mg films was determined by TGA analysis over the temperature range of 20–500 °C under argon atmosphere. TGA measurements provide mainly information about PLA modification as a consequence of Mg addition and also the actual inorganic fraction of the composites. Furthermore, this technique evidences the fact that there are no detectable solvent traces or impurities in the composite samples. Figure 5 illustrates the TGA (Figure 5a) and their respective derivative thermogram (DTGA) curves (Figure 5b). For the pure polymer and its composites, the initial degradation temperature (T_0), the maximum degradation temperature (T_{max}) and the final degradation temperature (T_e), the loss of mass at the first degradation peak (Δ_{w1}) and the percentage of magnesium ($\%_{Mg}$) are summarized in Table 3. The peaks in the DTGA curves represent the temperature at maximum degradation rates (T_{max}).

All composite films have a pronounced peak of thermal degradation, which is attributed to the decomposition of polymers (PLA and PEI) whose temperatures (246 - 289 °C) decrease with the increase of Mg particle content in the composites. Additionally, the content increase also produces longer degradation intervals from 13 °C in the F5 to 23 °C in the F50 (Figure 5b). These results show that Mg induces the degradation of the PLA. This behaviour has been reported in the literature, associated to the MgO (present in the surface of the Mg particles) that acts as a catalyst for the PLA depolymerization

reaction when the polymer is subjected to elevated temperatures [30]. Additionally, TGA also shows that no solvent has been retained in the films, as no weight loss is observed at low temperatures.

The weight loss in the main peak oscillates between 43 % - 90 % because films present a second degradation at 400°C, which is more evident in F30 and F50 composites. This degradation is related to the dehydration of $\text{Mg}(\text{OH})_2$ phase precipitated on the Mg particles and, therefore, the formation of crystalline MgO [31].

Table 3. Degradation parameters of PLA and PLA/Mg films.

PLA	T_0	T_p	T_e	Δw	% _R
	265	346	359	97	0.6
PLA/Mg	T_0	T_p	T_e	Δw	% _{Mg}
F5	279	289	293	90	4.9
F10	264	276	282	85	10.0
F30	245	261	267	64	27.5
F50	234	246	254	43	45.1

Table 3 also shows the amount of residual Mg (at 500 °C) of the composites analysed by TGA. The minor difference between the actual and nominal Mg content indicates that the processing method used in this work is sufficiently accurate, especially at low weight fractions of Mg. The materials only have a small load defect at 30 and 50 wt.% Mg, likely derived from the handling of the suspensions. (See experimental method).

Figure 5. a) PLA and F5, F10, Y30 and F50 composite thermograms and b) TGA derivate.

The contact angle was measured to evaluate the hydrophobicity of the films. Figure 6a shows the contact angle results of the composites compared with PLA. As can be seen, the composite films are slightly hydrophilic, which can be attributed to the roughness of the film. The hydrophilic character of the Mg particles also helps to this hydrophilicity. This result also indicates that these two factors, roughness and the presence of Mg, weight more than the hydrophobic character of PLA, even though a PLA layer covers every Mg particle. The better hydrophilicity of PLA/Mg films could be relevant for the intended applications, according to previous results with PLA implants [16].

The structural characteristics of the composite matrix have been evaluated indirectly by determining the transport properties of the PLA films as a function of the Mg content. Gas permeability measurements were carried out for CO₂, O₂ and N₂ using the time-lag method. The permeability coefficient (P) and the diffusion (D) are summarized in Figure 6b and c. The addition of Mg produces a decrease in the gas diffusion and permeability, greater in the case of composites with 10 and 30 %. This is directly related to the crystallinity fraction of the composites, which were 45 and 31 % for F10 and F30, respectively [26]. Therefore, the gas permeability reduction with the increase of load is

likely associated with a barrier effect created by the Mg particles [32], as they obstruct the gases molecules flow through the polymer [33]. Regarding wound dressings applications, oxygen plays a fundamental role in healing the injury [14]. Although the films studied do not have a high permeability to this gas, some authors demonstrate that oxygen-impermeable dressings can be useful in the early stages of healing [34]. In addition, the initial films' permeability is not high, but the reactivity of Mg and the degradation of PLA over time might be enough to increase its permeability with time and make the use of these composites a feasible application.

Figure 6. a) Contact angle for PLA and composite films, example of a water drop on film material b and c) diffusion and permeability parameters of PLA, F10 and F30 composites.

In vitro degradation behaviour

Figure 7 presents the volume of hydrogen released as a function of immersion time for the F5, F10, F30 and F50 films. This test was performed for 3 months in PBS, except for the sample F50 which was completely degraded after 2 months of immersion. As

expected, it is observed that hydrogen evolves much more slowly from samples with a low amount of Mg, but the differences are not only appreciated in the greater amount of accumulated hydrogen, also in the corrosion rate. An increase in the slope of the release is observed in the first 20 days of immersion with the load amount. The pH value of the PBS solution was also monitored, and in no case was the buffer capacity of PBS exceeded, for this reason pH evolution is not shown in this study. According to the literature, pure magnesium at 37 °C releases an average of 40 ml/cm² per day [35], which is much higher than that of 0.025 ml /cm²/day established for film with the highest Mg content (F50). The tolerated amount of hydrogen by the human body is 2.25 ml/cm²/day, so in terms of hydrogen accumulation, these films would be suitable for biomedical use.

Additionally, the concentration of Mg²⁺ ions released into the medium was measured by Inductively Coupled Plasma (ICP) at different immersion times. The results presented in Figure 7b are not cumulative because solution was replaced every week. In the first immersion week, films released the highest amount of ions which increases with the Mg content of the film.

Figure 7. a) Hydrogen evolution as a function of immersion time for F5, F10, F30 and F50 composite. b) Mg²⁺ ions released to the medium at different immersion times.

Figure 8 shows the morphology of the degraded films (F10, F30 and F50) after 1 week of immersion. The main signal of degradation is the growth of a new phase surrounding the embedded Mg particles. EDX results obtained at different points of the cross section of F10 are gathered in Figure 8g. Spectra in 3 areas around Mg particles reveal the presence of O, Na, P and Cl, in addition to Mg. In spectrum 1, only Mg is detected. However, in spectrum 2 there is a notable increase in the amount of oxygen at the position of the new phase, associated with $\text{Mg}(\text{OH})_2$, which is formed by the reaction of Mg with the medium. It is assumed that the PBS medium reaches and corrodes the Mg particles due to the permeability of the PLA matrix. In contrast, it can be seen that there are also elements that correspond to the PBS (Na, P, and Cl), indicating the deposition of salts in the degradation front and in the cracks (Spectrum 3). Cifuentes *et al.* [18] have already observed the deposition of the salts contained in the degradation medium (PBS) on the degraded Mg particles.

Figure 8 . SEM images of a,d) F10, b,e) F30, c,f) F50 after 1 week of immersion. g) Elements

concentration according to EDX results of F10 after 1 week of immersion.

In the case of Figure 8 d and f, it is observed that Mg particle degradation does not always happen radially, rather, it sometimes progresses from preferential reaction centres such as defects or cracks formed in the matrix. These defects make the contact of the medium with the Mg particle easier. $\text{Mg}(\text{OH})_2$ is a fragile hydroxide that cracks easily, as seen in Figure 8d and e. When a Mg particle begins to degrade, the $\text{Mg}(\text{OH})_2$ contributes to degrade the matrix which accelerates its own films degradation. $\text{Mg}(\text{OH})_2$ in the first week of immersion is present in the PLA cracks (Figure 8a and d), and this behaviour happens because the hydroxide begins to dissolve in the medium.

In Figure 9, micrographs of the test pieces are presented after 1 month of immersion. In the case of F10 composite (Figure 9a and d), partially unreacted particles are detected, as well as particles that have reacted completely and were dissolved in the medium. An increase in number and size of cracks is also observed. This phenomenon is again likely due to the precipitation of the hydroxide in the defects, which forces the growth of cracks through the acceleration of polymer degradation by the local pH increase [36].

The degradation degree is higher in the case of F30 and F50 composites, where a majority of particles have already been dissolved in the medium. For the F30 films, some particles maintain a core of Mg that continues reacting and releasing hydrogen as is illustrated in Figure 7a. However, the film with the highest amount of magnesium (F50) presents a degradation state considerably more advanced, as neither particles nor traces of hydroxide are found. Also a great amount of cracks are present and even some of them have advanced to practically break the film, exposing more polymer surface to the medium and making the depolymerization of the PLA by hydrolysis easier.

In conclusion, composite films present promising *in vitro* behaviour, which indicates that the thin PLA layer that surrounds the Mg particles is sufficient to protect them. In

addition, there is a release of Mg^{2+} , which is beneficial in the formation of collagen and angiogenesis for tissues growth [4] and the H_2 release does not compromise the implant biocompatibility.

Figure 9. SEM images of a,d)F10, b,e)F30, c,f)F50 after 1 month of immersion.

Cell culture tests

Both indirect and direct assays have been performed to evaluate the MEF cells response in contact with films or its degradation extracts. Cell viability was carried out using MEF cells (Mouse Embryonic Fibroblasts) due to the potential application of these materials in the replacement of epithelial tissues as dressings [37]. Indirect method provides information about any cytotoxic component present in a sample, such as solvents or impurities. Figure 10 shows that that composite films do not exhibit cytotoxicity (cell viability > 75 % [38]), and that their degradation products promote cell proliferation. A significant increased cell viability of up to 125 % ($P < 0.01$) can be seen with Mg addition for F10, F30 and F50 after 24 h and 1 week of immersion, however after 2 weeks, this increase with respect to the PLA is observed only for F30 and F50. For an immersion time of 3 weeks, the cell viability is similar for all the samples. After the fourth week, cell viability of PLA decreases significantly with respect to the composites. F5 shows a different trend. It exhibits no significant

difference with PLA during the first 3 weeks, however, in the fourth week, F5 extract shows higher cell viability than PLA ($P < 0.01$) due to the release of Mg^{2+} .

The results of the cell viability measurements in contact with the degradation extracts indicate that the combination of two effects takes place: the increasing of cell viability due to the presence of Mg^{2+} and its decreasing due to the degradation of PLA. The first phenomenon has already been reported in the literature, Mg ions promote the adhesion and proliferation of osteoblasts *in vitro* [39] as well as fibroblasts [40]. However, Gu *et al.* [41] show that after 2 days of degradation of pure Mg, the fibroblasts cell viability of the extracts is lower than that of the control, which is associated with a high concentration of Mg^{2+} ions that can precipitate as $Mg(OH)_2$ and increase the pH of the medium. These factors explain why the cell viability increases in the extract of F10, F30 and F50 in the first weeks and decreases in the following weeks, while in the F5 films cell viability increase is produced in the last degradation week.

These results have a correlation with the films degradation behaviour. It was observed that the hydrogen release rate is slower for samples with a low amount of Mg (F5). Thus cell viability increases in F5 for longer immersion times than in the other films since cell viability can be associated with the composite degradation of the composite. In the case of F10, F30 and F50 films, a significant degradation occurs in the first week of immersion that yields an evident increase in cell viability. For the longer periods of immersion, however, a slight decrease in cell viability is observed, probably associated to the precipitation of $Mg(OH)_2$.

In addition, in the first weeks, the cell viability that decreases in PLA, associated with the decrease of pH during PLA degradation, is counterbalanced by the increase of pH due to $Mg(OH)_2$ precipitation. Consistently, in pure polymer extracts a slight decrease of cell viability is observed which is attributed to the pH decrease.

Figure 10. Cell viability of MEF cells in composite films extracts at different degradation times.

Once the cellular behaviour was analysed in the degradation extracts of the films and toxicity was not detected, a direct assay of cell viability was carried out. In this case, cell proliferation depends on the same factors as in indirect tests, but also on the surface properties, such as roughness or contact angle. Figure 11 shows the cell viability of MEF cells measured by the WST-8 assay after 24h of cell culture on the PLA and PLA/Mg films. For all composite films, a significant increase in viability was observed compared to pure PLA, which reflects the effect of Mg on the increase in cell growth observed in indirect tests (Figure 10). Some differences were observed, when varying the Mg contents of the films. The addition of 5, 10, 30 wt.% of Mg particles in the PLA (F5, F10, F30) produces a clear increase in the viability of cells, while 50 wt.% (F50) does not present significant differences with respect to the neat PLA film. Finally, the morphology and cell adhesion on the different films was evaluated by Vybrant and DAPI staining. The fluorescence images are shown in Figure 11 at two magnifications to study how the cells have been distributed on the surface and how they are adhered to the PLA, F5, F10, F30 and F50 films.

In Figure 11b the cell growth on the PLA films is observed. Cells cover practically the whole sample surface, except the corners because the film there was deformed causing an agglomeration of dead cells in these areas. The fibroblasts present their common physiological morphology, compared to the control (Figure 11). Cells grow in a monolayer without signs of toxic components that would decrease the fibroblasts density. F5 presents a homogeneous cell distribution on the surface which means that the film is a stable surface for cell adhesion. The same behaviour is observed for samples with a content of 10 and 30 wt.% Mg (F10, F30), however in this case, a significant increase in the fibroblast amount is detected with respect to the films of lower Mg content. This phenomenon is associated with an increase in the Mg content of the films and consequently, to an increase in the films degradation rate (Figure 7a) that, as observed in Figure 11a, produces an increase in cell viability. Another phenomenon associated with the increase of cell adhesion is roughness [42]. Consequently, the increase in roughness associated with the increase of Mg load in the PLA films is also related to the improvement in cell viability. Finally, the films with 50 wt.% Mg (F50) were analysed observing a decrease in cell growth and cell adhesion. This phenomenon is related to the high amount of Mg in the film and to a possible particles agglomeration. Although the high amount of Mg incorporated to the composite does not lead to cytotoxicity, it produces a larger local H_2 release (as observed in Figure 7a - 0.08 ml/cm²) in the first 24 h of immersion, in comparison to samples with lower Mg contents, making difficult the cell adhesion.

Summarizing the results described above, cell proliferation in PLA/Mg films in direct assays depends on several factors: (i) the amount of Mg particles, whose degradation products increase the cell viability of the composites (Figure 10), (ii) the roughness and (iii) the hydrogen release. Mg alloys have already been studied for applications in

osseointegration and bone repair, obtaining successful results in cell assays with osteoblasts [43,44]. Khalajabadi *et al.* [45] observed in Mg/HA/MgO composites a viability of 60-70 % with osteoblast cells. The good proliferation of fibroblasts in the PLA/Mg films reinforces the good results obtained in previous works with osteoblastic cells on bulk samples of similar materials processed by extrusion molding [24]. It should be noted that in the first case, the films contain up to 50 wt.% Mg, while in the second case, Mg contents greater than 15 wt.% Mg did not offer competitive advantages due to an increase in thermal degradation of the polymer during processing. This fact highlights the advantages of the use of colloidal techniques for the preparation of PLA/Mg composites.

It is relevant for this study that the release of Mg^{2+} promotes the formation of collagen and the angiogenesis in skin wounds [4], which offers a promising opportunity for the applicability of PLA/Mg films in this field.

Figure 11. a) Cell viability of MEF on PLA and composite films. Fluorescent staining of MEF cells on film, b) PLA, c)F5, d)F10, e)F30, and f)F50 after 24h of culture. (**P < 0.01)

Conclusions

PLA/Mg films (25-30 μm in thickness) with Mg content as high as 50 wt.% processed in this work offer great potential as bioabsorbable material for tissue engineering and wound dressing applications. The incorporation of Mg particles produces an improvement in PLA hydrophilicity also associated with the roughness increase (1-4.7 μm) and produces a decrease of gas permeability, convenient in the early stages of healing. The Mg particles are embedded homogeneously in the polymer matrix and protected by a PLA layer, as drawn from the microstructural characterization that permits to control the hydrogen release rate, so that no composition exceeds the tolerable hydrogen release amount set for the human body (2.25 ml/cm²/day). Furthermore, the cell viability assay by an indirect method demonstrated that the

presence of Mg^{2+} ions has the ability to promote proliferation of MEF cells and compensates the pH decrease associated with PLA degradation. Finally, by direct methods the Mg addition promotes cell adhesion on films.

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Statement of Significance

Magnesium materials have extraordinary biodegradable properties and bioactive behavior due to release of Mg^{2+} ions, which offer a promising opportunity for their applicability as biomaterials for tissue regeneration. However, Mg is one of the most reactive metals with a high degradation rate. In contact with water produces H_2 , associated with a risk of failure of the implant. One alternative to minimize this drawback is the use of Mg particles surrounded by a biodegradable biocompatible polymer such as polylactic acid (PLA) to obtain PLA/Mg composites. In this work we processed Mg reinforced PLA in the shape of films that would be suitable for tissue regeneration. In vitro behavior of PLA/Mg films demonstrated that Mg^{2+} ions increase the fibroblastic cells growth.