Hydrolytic degradation and cytotoxicity of poly(lactic-co-glycolic acid)/multiwalled carbon nanotubes for bone regeneration

Esperanza Díaz[1, 2], Igor Puerto[1], Iban Sandonis[1], Sylvie Ribeiro[3,4], Senentxu Lanceros-Mendez[2, 5]

[1] Escuela de Ingeniería de Bilbao, Departamento de Ingeniería Minera, Metalúrgica y Ciencia de Materiales, Universidad del País Vasco (UPV/EHU), 48920 Portugalete, Spain

[2] BCMaterials, Basque Centre for Materials, Applications and Nanostructures, UPV/EHU Science Park, 48940 Leioa, Spain

[3] Centro/Departamento de Física, Universidade do Minho, 4710-057 Braga, Portugal;

[4]Centre of Molecular and Environmental Biology (CBMA), Universidade do Minho, Campus de Gualtar, 4710-057 Braga, Portugal

[5] IKERBASQUE, Basque Foundation for Science, 48013 Bilbao, Spain

***** Correspondence: esperanza.diaz@ehu.eus; Tel.: +34-66-678-7663

Abstract

Biodegradable poly(L-lactide–co-glycolide) (PLGA)/Multiwalled Carbon Nanotube (MWCNT) scaffolds produced by Thermally Induced Phase Separation (TIPS) are studied for bone regeneration. Their magnetic properties, cytotoxicity and *in-vitro* degradation are investigated. Certain properties are analyzed at 37 ºC over 16 weeks in phosphate buffer saline solution (PBS), as a function of degradation time: morphology, mass loss, pH value of PBS, and thermal behavior. The presence of small quantities of nanotubes in the scaffolds, ≤0.5 wt%, leads to a weak magnetic response although the PLGA was diamagnetic. The incorporation of MWCNTs in the scaffolds generated a morphology and a very different process of in-vitro degradation than might be expected in a PLGA scaffold. The in-vitro degradation process started on week 13 and rapidly advanced, although the structural integrity of the scaffolds was maintained and no

collapse of the structure occurred. Cytotoxicity tests on the samples showed cytotoxicity behavior at concentrations of over 0.3 wt% MWCNTs.

Keywords: PLGA, MWCNTs, scaffolds, cytotoxicity, magnetic, *in-vitro* degradation

1. Introduction

The rapid advance of tissue engineering has meant that certain biodegradable and biocompatible polymers such as PLLA (poly(L-lactide)), PCL (polycaprolactone), PLCL (poly(L-lactide-co-caprolactone)), PLGA (poly(L-lactide-co-glycolide)) are now used to produce scaffolds for the regeneration of bone tissue. Scaffold production is relatively easy with these polymers using different techniques: Thermally Induced Phase Separation (TIPS) (1), particulate-leaching techniques (2), and electrospinning (3), among others. However, the drawback is that their mechanical properties are not comparable with those of human bones. Some researchers added bioactive particles to the scaffolds, to remedy that incompatibility, which not only enhanced the mechanical properties of the scaffolds, but in addition promoted cellular activity such as HA (hydroxyapatite) (3-4), bioglass (5-6), and FeHA (nanoparticles of hydroxyapatite doped with iron) (7). Carbon nanotubes have only recently been added to these scaffolds.

Since their discovery by Lijima (8), carbon nanotubes have awakened increasing interest in the field of scientific research, because of the numerous properties that they add to the composition of various materials. MWCNTs possess a large variety of electrical, thermal and structural properties defined by their diameter, length, and chirality, hence the usefulness of their biomedical applications. There are various areas in which carbon nanotubes have been used: gene therapy, controlled drug release, imaging contrast agent for in vivo molecular imaging, and, since 2004, tissue engineering. A small quantity of carbon nanotubes composed of polymers (less than 0.5% by weight) can lead to impressive increases in their mechanical properties. Human bones have an average Young's Modulus (GPa) of 12-18, hydroxyapatite 95 (GPa), bioglass 35 (GPa) and the MWCNT (Multiwalled Carbon Nanotubes) between 200-1950 (GPa) (9). The facility of MWCNTs to enhance cellular adhesion and proliferation has meant that these materials are incorporated into other natural or synthetic materials to fabricate scaffolds for regenerative medicine (10-11).

One of the inconveniences of carbon nanotubes is that they are not biodegradables, however, it has been experimentally demonstrated in vivo that they can be excreted and therefore disappear from the human body when no longer necessary. Recent studies have centered on biocompatibility of these materials, improving their biodegradability, although maintaining their anti-tumoral

effects given that *in-vitro* tests demonstrated an anti-proliferative, anti-migratory and cytotoxic capability (12). Another of their inconveniences is their cytotoxicity, as they can contain remains of catalyzers and carbon deposits on the outside of the nanotube, due to their synthesis, although any such traces will disappear when they are purified and functionalized with various methods, such as refluxing in nitric acid (13-16)

The objective of this work is the development and characterization of PLGA and PLGA/MWCNTs scaffolds, using the TIPS technique, in order to analyze the influence of small additions of MWCNTs ≤ 0.5 % wt. In this research, our interest centers on the addition of small quantities of nanotubes in the nanocompounds that can modify the *in-vitro* degradation of the PLGA scaffolds, their magnetic properties and cytotoxicity, making their modulation possible, and allowing improved applicability in the field of tissue regeneration.

2.Experimental Section

2.1Materials

Poly(DL-lactide-co-glycolide) (PLGA) copolymers in molar ratios of 75/25, supplied by PURAC (PURASORB, Gorinchem, The Netherlands, PDLG7502), were purified by dissolution in chloroform. The weight-average relative molecular weight was Mw = 86.985 and Mn = 53.533 with a polydispersity of Mw/Mn = 1.6250. These values were determined using gel permeation chromatography (GPC, Perkin Elmer 200, Triad Scientific, Manasquan, NJ, USA) in tetrahydrofuran (THF). GPC was performed with a tetrahydrofuran solvent using a reflective index detector with a Perkin Elmer 200 (Triad Scientific, Manasquan, NJ, USA) as the detector. Calibration was done in accordance with polystyrene standards with a flow rate of 1 mL/min. Multiwalled Carbon Nanotubes 10-20 nm diameter, 10-30 μm length, were supplied by Nanostructured & Amorphous Materials Inc., Houston, USA. 1,4 Dioxane purchased from Panreac p.a. (Barcelona, Spain) was used as the solvent. Phosphate-buffered solution (PBS) in water, supplied by Fluka Analytical (Sigma Aldrich, St. Louis, MO, USA) at a pH of 7.2, was used as the degradation fluid.

2.2 Fabrication of scaffolds

PLGA/MWCNT composite scaffolds were prepared as follows: MWCNTs in different proportions of total polymer mass (0%, 0.1%, 0.3% and 0.5%) were added to a PLGA solution in 1,4 dioxane (2.5% (w/v)) and were homogeneously dispersed by ultrasonic stirring. The solution was then freeze-dried (LyoQuest of Telstar, Barcelona, Spain) for seven days. This process yielded foam scaffolds with porosities of up to 80%.

2.3 Magnetic analysis

Magnetic measurements were performed on a vibrating sample magnetometer (VSM) that has been developed at the University of the Basque Country (UPV/EHU). The magnetometer was calibrated with pure nickel (99.985%) and the available magnetic field range was ± 1.8 T (18Kg), with a resolution of ± 20 μT (0.2 G) and a moment sensitivity of 10^{-8} Am^2 (10^{-5} emu).

2.4 *In-vitro* degradation

The scaffolds were cut into rectangular pieces, immersed in test tubes containing 15 mL of PBS and the tubes were then placed in an incubator at 37 ºC. The rectangular pieces of scaffolds were recovered and wiped after 0, 8, 13, and 16 weeks and then weighed, in order to establish their water absorption. A pHmeter PCE 228 (PCE Instruments, Pons, Alicant, España) was used to determine the pH change in the PBS. The degraded samples were dried over 12 days to a constant weight.

The following equations were used to calculate the water absorption percentage, Wa%, and the weight loss percentage (WL%), equation 1 and 2 respectively:

$$Wa\% = \frac{Ww - Wr}{Wr} \times 100 \qquad (1)$$

$$WL\% = \frac{Wo - Wr}{Wo} \times 100 \qquad (2)$$

2.5 DSC analysis

Differential scanning calorimetry (DSC) was performed with a TA Instruments calorimeter. Approximately 4-10 mg amounts of the sample were encapsulated in aluminum pans and scanned under a dynamic N_2 atmosphere from -20 to 200 ºC at 10 ºC min^{-1} (first run) then cooled at 10 ºC min^{-1}, and finally heated at 10 ºC min^{-1} (third run). An empty aluminum pan was used as a reference.

2.6 SEM analysis

Scanning electron microscopy (SEM, HITACHI S-3400N, Tokyo, Japan) was used to analyze the scaffold morphology. Before the analysis, the scaffolds were coated with a layer of gold, in a JEL Ion Sputter JFC -1100 (Amiron Machinery, Oxnard, California) at 1200 V and 5 mA.

2.7 Cytotoxicity Assay

2.7.1 Material

Dulbbecco's Modified Eagle's Medium-High Glucose (DMEM-HG), Fetal Bovine Serum (FBS) and Penicillin/Streptomycin (P/S) were obtained from Biochrom. Phosphate-Buffered Saline (PBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Dimethylsulfo xide (DMSO) were purchased from Sigma-Aldrich.

2.7.2 Material Sterilization

For the *in vitro* assays, membranes with 5 $mg.mL^{-1}$ were cut and sterilized by UV for 2 h before cell seeding (1h each side). After that, the samples were washed 5 times with PBS solution for 5 min each.

2.7.3 Cytotoxicity process

The indirect cytotoxicity evaluation of the samples was conducted by adapting the ISO 10993-12 standard test method.

Briefly, the extraction media were prepared by immersing the samples in 24-well tissue culture polystyrene plate with DMEM containing 4.5 $g.L^{-1}$ glucose supplemented with 10% FBS and 1% P/S, at 37 ºC in a 95% humidified air containing 5% CO_2 and incubated for 24 h.

At the same time, the MC3T3-E1 cells were seeded in the 96-well tissue culture polystyrene plate at the density of 2×10^4 $cells.mL^{-1}$ and incubated for 24 h to allow the cell attachment on the plate. After this time, the culture medium from the 96-well tissue culture polystyrene plate was removed and the as-prepared extraction media (from the samples) were added to the wells (100 µL). Afterward, the cells were incubated for 72 h and after this time, the evaluation of the cell viability was quantified with MTT assay. A 20 % of DMSO was used as a positive control and the cell culture medium was employed as negative control. The MTT assay measures the mitochondrial activity of the cells, which reflects the viable cell number. At each time point, the medium of every well was removed and fresh medium containing 10% MTT solution (stock solution of 5 mg $MTT.mL^{-1}$ in PBS) was added. The viable cells with active metabolism convert MTT into a purple

colored formazan product. After 2 h of incubation, the MTT crystals were dissolved with DMSO and the optical density was measured at 570 nm.

All quantitative results were obtained from four replicate samples and controls, and were analyzed as the average of viability ± standard deviation (SD).

The percentage of cell viability was calculated with the equation 3.

$$\text{Cell viability}(\%) = \frac{\text{absorbance of sample}}{\text{absorbance of negative control}} \times 100 \qquad (3)$$

3. Results and Discussion

3.1 Magnetic analysis

Figure 1 shows the normalized magnetization curves of the PLGA/ MWCNTs scaffolds. The results were rescaled, in order to represent the moment against the Magnetic Field, assuming that all the samples would present the same slope for a high magnetic field associated with the diamagnetic signal of PLGA. The scaffolds that contained MWCNTs presented a very weak magnetic signal. Magnetization decreased, with the content of MWCNTs, due to the diamagnetic behavior of PLGA. The MWCNTs could be aligned in the PLLA scaffolds under a magnetic field and the magnetization and various thermal, electrical and optical properties will depend on the orientation of the MWCNTs in the polymer (17). Some authors (18) have found a superparamagnetic behavior with no coercivity and remanence to PLLA/m-MWCNTs composites, but with a much higher content of CNTs, at around 3 wt%, than the one used in this study to produce our samples. In addition, it must be taken into account that MWCNT dispersion into the polymer could play a key role in the magnetic properties of the composite.

3.2 *In-vitro* degradation

The follow up of the *in-vitro* hydrolytic degradation process was done through the study of pH variation, mass loss, PBS absorption, and using DSC and SEM

Figure 2 shows pH and mass loss. Figure 2a shows the variation of pH, which is maintained hardly without variations until week 13, from which point it fell very rapidly, reaching values at the end of week 16 of 6.92, 6.95, 6.97 and 7.03, respectively, for the following scaffolds PLGA, PLGA/ MWCNTs 0.5%, PLGA/ MWCNTs 0.3%n and PLGA/MWCNTs 0.1. This reduction was higher for the samples without nanotubes. The sample with the highest pH was PPLGA/MWCNTs 0.3%wt. Any decrease in pH showed an increase in the hydrolysis of ester bonds and therefore in the degradation rate. The addition of small quantities of MWCNTs to the scaffolds delayed the hydrolytic degradation process. These results were not coincident with the results of some authors (19) for other aliphatic polyesters such as poly(L-lactic acid) (PLLA) and with concentrations of CNTs at 3 wt%, which followed a continual descent of the pH and in addition the lowest pH values corresponded with those of the nanotube samples.

In figure 2b, we can see how the scaffold mass fell ever since the degradation process began and especially from week 13, up until when the loss of mass of all the samples was around 5%. From that point in time, up until the sixteenth week of *in-vitro* degradation, there was a loss of mass of 35%, 17%, 6%, and 60% for the PLGA, PLGA/ MWCNTs 0.1%, PLGA/ MWCNTs 0.3%, and PLGA/ MWCNTs 0.5% samples; in other words, the highest loss of weight was observed in the scaffolds prepared with PLGA and PLGA/MWCNTs 0.5%. These results coincided the pH changes, but not with the works of other authors (19, 20), in which the nanotube composition was observed to lose more weight in the PLLA, which might be because the PLLA is a crystalline polymer and the PLGA that we used was amorphous. In addition, in none of those works was degradation studied with such small quantities of nanotubes, except in the study by I. Olivas-Armendáriz et al. (21), in which the chitosan samples without nanotubes degraded more rapidly. We have no knowledge of any experimental work in which the *in-vitro* degradation process slowed down towards week 13 and as from that point speeded up considerably.

The addition of MWCNTs slowed down the loss of mass except for the sample with a higher content of nanotubes that had a morphology with some pores of a smaller size than pure PLGA, which could be caused by inadequate dispersion of the nanotubes in the polymeric matrix. This uneven dispersion could in turn mean that the acidic products resulting from the degradation process were freed from the scaffold with greater

difficulty, provoking an autocatalytic degradation. At the end of the degradation process under study and despite the high amount of mass loss, the scaffolds maintained their structure.

Water Absorption

If we observe figure 3, we can conclude that the addition of nanotubes reduces the % water absorption, yielding values of around 2000%, 1400%, 1000% and 700% for the PLGA, PLGA/MWCNTBs 0.1% wt, PLGA/MWCNTBs 0.3% wt and, PLGA/MWCNTBs 0.5% wt samples, respectively. This reduction is inversely proportional to the content of nanotubes and as is logical, scaffold degradation is slower as it absorbs less water. It should be highlighted that a small addition of nanotubes caused a large reduction in the percentage water absorption. C. Lin et al. (22) in their work with PLGA/MWCNT nanocomposite films, however, found the opposite behavior, in that the PLGA sample absorbed less than 11% and degraded less than the nanotube samples, by 13 and 15%. Their study, however, referred to films over seven weeks of *in-vitro* degradation. It is logical to suppose that the very porous scaffolds in our study absorbed much more water. In addition, degradation over 16 weeks released degradation products into the films that had no pores and were unable to filter into the interior of the material, provoking autocatalytic degradation. Some authors have explained this behavior because of the good dispersion of the nanotubes that improveds surface interaction with the polymeric matrix, so that the nanocomposite degradation can occur with greater rapidity (23)

The results obtained in this section are in accordance with those of pH and those of mass loss.

SEM

All of the scaffolds had a porosity that was higher than 80%. In the micrographs of Figure 4, a very porous morphology can be observed with interconnected cavities and a large enough volume to harbor a specific cell type, with cavity sizes of between 50 and 75 micrometers. It cannot be determined from the SEM images whether the dispersion in the MWCNTs is homogeneous or heterogeneous and we can see a random distribution in the polymeric matrix, where they are covered by PLGA, which appears to indicate good adhesion. In figures 4j, k and l, it can be seen that the scaffolds with a concentration of 0.5% MWCNT presented a more abrupt morphology, with much smaller pores, which are accentuated by the degradation process. The presence of smaller pores accelerated the autocatalytic degradation process by complicating the expulsion of the acidic products resulting from degradation from the scaffolds. These results can explain the

ones obtained from the variation of the pH, as the PBS solution of less acidity corresponding to the sample with the highest content of MWCNTs.

Thermal Characterization

In this section, the study of scaffold thermal behavior will be reported. To do so, two sweeps were conducted, one of -50 to 200 ºC with a heating speed of 10 ºC/min and a cooling speed of 20 ºC/min. The differential Scanning Calorimetry (DSC) results for PLGA and the PLGA/MWCNT scaffolds are shown in Table 1. The glass transition temperature (Tg) was determined from the DSC curve of the second run. Figure 5 shows the DSC of PLGA and PLGA/MWCNTs. The PLGA scaffolds had a Tg of around 50 ºC, which fell to approximately 44 ºC for the samples with nanotubes, regardless of their concentration. We believe that it is independent of the concentration, because the difference in the quantity of nanotubes added to the scaffolds was very small, in agreement with the results obtained by Nabipur et al. (24) for PLCL and a nanotube content of 0.5 wt%. This behavior differs from the one observed by other authors (25, 26), who noted that the addition of nanotubes increased the Tg considerably and accelerated crystallization processes in other polyesters such as PLLA. However, we observed no such behavior, certainly because the PLLA was semicrystalline and our PLGA was amorphous. In addition, the quantities of additional nanotubes were very low and the movement of the polymeric chains was not restricted in the heating process; on the contrary, the chains acted as plastifiers of the polymeric matrix (27).

As the weeks of degradation advanced, we observed a very significant reduction of Tg (see Table 1 and Figure 6), in other words, the addition of nanotubes increased the thermal range within which the behavior of the scaffolds remain ductile and therefore a much broader interval of use. This reduction in Tg could also be due in part to the absorption of PBS which provoked a plastification of the polymer with an increase in the free volume and therefore a reduction of the Tg in the system (28). In addition, a crystallization peak appeared in all the samples under study during the 16 week of *in-vitro* degradation, due to the separation of the polymeric chains in the hydrolytic degradation process and as they were shorter, they rearranged themselves more easily, giving rise to the crystallization process. The melting point of this peak (Tm ºC) was found between 120-130 ºC. The crystallinity (ΔHm) that appeared in week 16 of degradation diminished a great deal with the addition of larger quantities of CNT (see Table 1); in other words, the nanotubes appeared to be an obstacle to the crystallization process (24). The results of the thermal behavior agreed with those obtained by water absorption, pH, and mass loss, in which the degradation process began in the 13th week and was very rapidly terminated.

3.3 Cytotoxicity analysis

In order to assess the applicability of the developed materials for biomedical and tissue engineering applications, it is important to evaluate the cytotoxicity of the PLGA and PLGA composites with different amounts of MWCNTs. For that, an MTT assay method was used with the MC3T3-E1 pre-osteoblast cell line and the results after 72h are presented in figure 7.

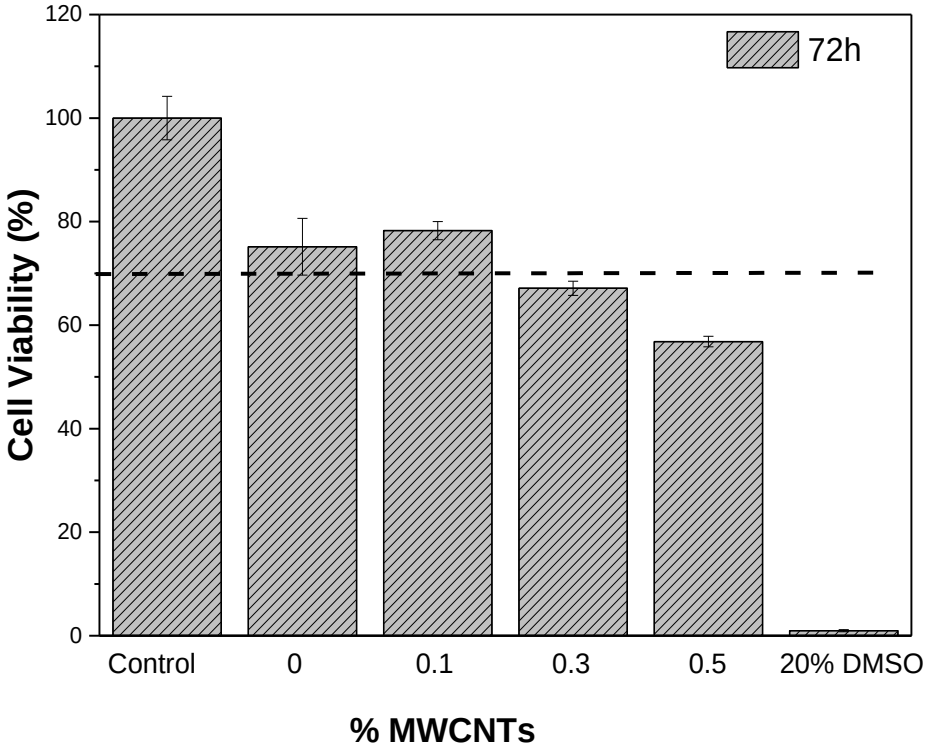

Figure 1- Cell viability of MC3T3-E1 pre-osteoblast cell line in contact with the as-prepared extraction media exposed with different samples up to 72h.

PLGA has attracted interest as a material for bone tissue engineering once it is a biocompatible polymer, as reported in the literature and also is approved for clinical use in human by the U.S Food and Drug Administration (FDA) [1]. Contrarily to the PLGA, the use of MWCNTs is controversy with their biocompatibility. However, due to their nanometer scale and extraordinary physicochemical properties, supporting the proliferation of different kinds of cells, MWCNTs have won popularity and have been used in tissue engineering applications [2], including in bone tissue [1, 3]. The concentration of MWCNTs were less than 0.5% (125 $\mu g.mL^{-1}$) because in the literature was referred that 100 $\mu g.mL^{-1}$ is already cytotoxic for the NIH-3T3 cells [4]. For these reason, it was decided to do two concentration below (0.1% - 25 $\mu g.mL^{-1}$ and 0.3% - 75 $\mu g.mL^{-1}$) and one above this value (0.5% - 125 $\mu g.mL^{-1}$) .

Analyzing the figure 7, it is observed that just neat PLGA and the PLGA/MWCNTs composite with 0.1% of MWCNTs are no cytotoxic (cell viability reduction is less than 30%). The presence of 0.3 and 0.5 % of MWCNTs lead to the cytotoxicity of the samples. With higher percentage of MWCNTs, high amount should stay in the surface of the materials leading to their cytotoxicity behavior.

Conclusions

In this work biodegradable and biocompatible PLGA/MWCNTs scaffolds have been produced using the thermally induced phase separation (TIPS) technique. The *in-vitro* degradation process was completed in PBS over 16 weeks at 37 ºC and, having started at week 13, the reaction was quickly completed. The addition of small quantities of nanotubes in the nanocompounds greatly modified the *in-vitro* degradation of the PLGA scaffolds, their magnetic properties and cytotoxicity, which could all be modulated in accordance with their application in the broad field of tissue engineering.

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