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The Effects of Bioactive Nanoparticles on the Degradation of DLGA

Esperanza Díaz, Igor Puerto, Iban Sandonis

Departamento de Ingeniería Minera, Metalúrgica y Ciencia de Materiales.
Universidad del País Vasco (UPV/EHU).
María Díaz de Haro, 68, 48920 Portugalete, Spain.
E-mail: esperanza.diaz@ehu.es

Shortened version of the title: In-vitro Degradation of DLGA/nHA

Abstract

The effect of bioactive nanoparticles on the in-vitro degradation of DLGA composite scaffolds is investigated. Fabricated by thermally-induced phase separation, the scaffolds present a high porosity (> 90%). In-vitro degradation is performed by immersing the scaffolds in a phosphate buffered saline solution, to evaluate water absorption, pH change and weight loss. Chain scission by hydrolysis reduces the average molecular weight and increases the polydispersity index. The incorporation of modified hydroxyapatite nanoparticles significantly affects the DLGA degradation process, inducing appreciable changes in the morphology of the material, but not in its percentage of porosity. Nanohydroxyapatite blocks the entry of water, reducing the degradation rates.

Keywords: biodegradable polymer, nanohydroxyapatite, scaffolds, pH, in vitro, degradation.

1. Introduction

Organ and tissue loss or failure resulting from an injury or other type of damage is a major human health problem. Tissue engineering is an interdisciplinary research field that includes all processes involved in the development of biological substitutes that

restore, maintain, or improve tissue function. Basic tissue engineering requires a suitable cell source and an appropriate material structure (scaffold) onto which the cells may be seeded and grown (1,2). Scaffolds are three-dimensional porous biomaterials designed to provide an initial mechanical support and a three-dimensional niche for transplanted cells (3), which promote cell adhesion and extracellular matrix (ECM) deposition and permit sufficient transport of gases, nutrients, and regulatory factors to allow for cell survival, proliferation, and differentiation. Moreover, they are also expected to be mechanically strong, malleable, bioactive, and biodegradable (4-7).

The material used to fabricate these scaffolds must fulfil a series of physical and chemical requirements. Biodegradability is very critical factor and should be verified at a controllable rate that approximates the rate of tissue regeneration and provokes a minimal degree of inflammation or toxicity.

Polymers can offer unique properties such as a high surface to volume ratio, high porosity with very small pore size, controllable biodegradability and good mechanical behaviour. Moreover, they offer better chemical versatility than any other material, to match the physical and mechanical properties of various tissues or organs of the body (8).

There are two kinds of polymer materials, which may be derived from either natural sources or from synthetic organic processes. Synthetic polymers have the advantage of being produced in large uniform quantities and are normally cheaper and have a longer shelf life than natural polymers. Aliphatic polyesters, such as poly(L-lactic acid) and its copolymers with D-lactic acid and glycolic acid are widely used for scaffold fabrication.

Poly(lactide-co-glycolide) (DLGA) is a very popular biodegradable polymer, which has the approval of the US Food and Drug Administration for human clinical applications and combines good mechanical properties, toughness, excellent processability and adjustable degradation rate.

DLGA can be obtained via ring-opening copolymerization of lactide and glycolide. Copolymers can be fabricated using both L and D,L-lactide. D,L lactide copolymers are amorphous and offer good mechanical properties. The degradation kinetics of DLGA are influenced by its relative molecular mass, co-monomer ratio, specimen size, configuration, and environmental conditions, among other aspects; the intermediate copolymer being much more unstable than the homopolymers. DLGA has been widely used in the field of control drug release (8, 9).It is a biodegradable polymer and follows a bulk degradation mechanism when immersed in a neutral aqueous solution such as PBS solution. PLGA degrades via simple hydrolysis of ester bonds into its monomeric form, producing lactic and glycolic acids which are eliminated from the body by incorporation into the tricarboxylic acid cycle by normal metabolic processes. The degradation is crucial to scaffolds in tissue regeneration. The rate of degradation can affect some cellular processes such as cell growth, tissue regeneration, host response, and mechanical properties.

One of the limitations that have been found regarding the use of these polymers in tissue engineering applications is the decrease in pH that is observed in the surroundings of the implant, which is produced by the release of acidic degradation products. Several

studies have reported adverse effects caused by these processes such as inflammatory responses (10).

A way to reduce the acidity of the degradation products of these kinds of polymers could be by neutralizing them with an alkaline substance. In this approach, a bioactive ceramic such as HA (hydroxyapatite) may be of interest. Hydroxyapatite (HA) is a major component in bones and is widely used as a bioactive ceramic, because it shows good osteo-conductivity and forms strong chemical bonds with bone. It has an alkaline pH and shows poor mechanical properties, which counts as one of its disadvantages. HA has been successfully blended with poly-lactic polymers to form composite biomaterials, for the fabrication of porous scaffolds for bone replacement purposes. Recent research has found that the biocompatibility of these composite scaffolds is better, in addition the use of nano sized HA may have other special properties due to its small size and huge specific surface area (11, 12).

In this paper, thermal induced phase separation (lyophilisation) was employed to prepare DLGA scaffolds and DLGA/nHA composite scaffolds. The objective of this investigation is to study in-vitro degradation of DLGA scaffolds and DLGA/nHA composite scaffolds, which may not only lead to bioactive scaffolds, but may also present a potential to offset the decrease in pH in Phosphate Buffer Solution (PBS). The nanohydroxyapatite particles act as a physical barrier and block the entry of water causing a decrease in the scaffold degradation rates.

2. Materials and Methods

2.1. Raw Materials

Poly DL-lactide and Glycolide (DLGA) copolymers in a 47/53 molar ratio were supplied by PURAC (PURASORB PDLG500X, Netherlands) and purified by dissolution in chloroform. The weight-average relative molecular weight Mw= 94800, Mn= 65600 and polydispersity Mw/Mn =1.4452 of DLGA were determined using gel permeation chromatography (GPC, Perkin Elmer 200) in THF. GPC was performed with a tetrahydrofuran solvent using a reflective index detector of Perkin Elmer 200 as the detector. Calibration was done in accordance with polystyrene standards with a flow rate of 1ml/min. Nano hydroxyapatite (nHA) was supplied by Aldrich Chemistry (USA), with a particle size > 200 nm and Mw= 502.31 g ml^{-1}. 1,4 Dioxane purchased from Panreac p.a. (Barcelona, Spain) was used as solvent. Phosphate Buffer Solution in Water (PBS), supplied by Fluka Analytical (Sigma Adrich, USA) at a pH of 7.2, was used as the degradation fluid.

2.2. Fabrication of Porous Scaffolds

Pure DLGA and DLGA/nHA composite scaffolds were fabricated by TIPS (thermally induced phase separation) followed by a freeze-drying technique. Briefly, DLGA was dissolved in 1,4 dioxane in a proportion of 2.5% (w/v), by stirring for 2 hours at a temperature of 50 ºC. After its complete dissolution, the resultant solution was poured into aluminium moulds. At this step, nHA was blended by ultrasonic stirring for 5 minutes, in proportions of 10%, 30% and 50% of total polymer mass, to form the composite scaffolds. The solutions were frozen and freeze-dried for several days to extract the solvent completely. Foams such as porous scaffolds with porosity of up to 90% were obtained by this method.

2.3. In-vitro Degradation

Samples for degradation were cut into 0.5 cm^2 rectangular pieces and weighed. After that the specimens were placed in identical glass test tubes containing 10 ml of PBS, totally immersed and incubated in a thermostated oven at 37 ºC and under static conditions. After selected degradation times (1, 2, 4, 6 and 8 weeks), the specimens were recovered, carefully wiped to remove surface water and weighed to determine water absorption. The pH change in degradation medium was determined using a pH meter PCE 228 by PCE Instruments (Spain) and corrected by temperature. Finally, the samples were dried over 2 weeks to a constant weight that was recorded in order to determine the weight loss.

2.4. Characterization

2.4.1. Water Absorption and Weight Loss

Water absorption and weight loss were evaluated by weighing. The percentage of water absorption W_a% was calculated by the following equation:

$$W_a\% = \frac{W_w - W_r}{W_r} \times 100 \quad (1)$$

where, W_w is the weight of the wet/swallow specimen after removing surface water and W_r is the residual weight of a completely dry sample after degradation. Weight loss percentage (W_L%) was estimated with the following equation:

$$W_L\% = \frac{W_0 - W_r}{W_0} \times 100 \quad (2)$$

the original mass of the sample was designated as W_0.

2.4.2. Mercury Pycnometry

The porosity of the scaffolds was quantified by mercury pycnometry. To do so, the scaffolds were dipped one by one in a container of mercury, placed on electronic scales with the help of a metal device. Knowing the density of mercury (ρ_{HG} = 13.57 g cm^{-3}) and the mass indicated by the scale, we may calculate the volume of the mercury (Vol_{Hg}). The volume displaced by the mercury is equivalent to the volume of the sample in question. So, knowing the initial mass and Vol_{Hg} thereof (M_{sa}), the bulk density (ρ_a) may be calculated with the following equation:

$$\rho_a = M_{sa} / Vol_{Hg} \quad (3)$$

Using both bulk density and the density of the polymer (ρ_p), measured by pycnometry on the pulverized material, the percentage porosity was calculated by the following equation:

$$\% P = (1- \rho_a/\rho_p) \times 100 \quad (4)$$

where, P is the percentage porosity. Measurements were made for each material.

2.4.3. SEM Analysis

The bulk morphology of the scaffolds was examined using scanning electron microscopy (SEM) (HITACHI S-3400N, Tokyo, Japan). Prior to analysis, the samples were coated with a layer of gold, in a JEL Ion Sputter JFC-1100 at 1200 V and 5 mA., to avoid sample charging under the electron beam.

2.4.4. DSC Analysis

The thermal characteristics of the polymer were determined using differential scanning calorimeter (DSC TA Instruments) equipped with an intracooler. Approximately 10 mg of polymer was placed in a crimp-sealed DSC hermetic aluminum pan. A nitrogen purge gas was used to prevent oxidation of the samples during the experiments, which were subjected to temperature scans ranging between -20 ºC and 200 ºC at temperature/time ratios of 10 ºC/min.

3. Results and Discussion

3.1. Molar Masses and Polydispersity Changes

The temporal variation in the M_w, M_n and the polidispersity of DLGA and DLGA/nHA composite are shown in **Figure 1**. The M_w of the DLGA decreased during the entire degradation process. However, its rate of decrease differed in the case of each different scaffold. The molecular weight of the DLGA decreased more rapidly than those of the DLGA/nHA composites. The molecular weights (Mw) gradually decreased in both cases, indicating that the degradation occurred from the beginning of the period of immersion, although the composite scaffolds degraded very slowly. The fact that the composite material lost less molecular weight due to the presence of nanoparticles could be due to a well-adjusted buffer effect at the DLGA/nHA interface, the homogeneity of which prevented the penetration of PBS (10). This behavior differed greatly from that reported by other authors, which was probably due to the incorporation of nHA particles and the manufacturing process of the scaffolds (lyophilization). The degradation is a bulk mechanism, catalysed by carboxyl end groups that are formed by the rupture of polymer chains, which can therefore impede the autocatalytic nHA process. In

consequence, both the rate of degradation and the molecular weight loss values are lower (see Figure 2).

The polidispersity index of all DLGA and DLGA/nHA showed changes during the degradation process. The values of the DLGA scaffolds changed from 1.44 to 2.07 and those of the DLGA/nHA composite scaffolds varied from 1.44 to 3.30. Three peaks were observed in the chromatograms for these composites, at approximately 3 weeks into the degradation study, and the most prominent peak was selected for measurement. The polydispersity increase with in vitro degradation time was due to cleavage of the polymer chains as a result of hydrolysis (11-14).

3.2. Thermal Analysis

During heating, the polymer undergoes transition from a glassy to a rubbery state at the glass transition temperature (Tg). The poorly organized structure of the amorphous DLGA, required less heat to make this transition than the semi-crystalline or crystalline structures. In Figure 3a we can see the results obtained by Differential Scanning Calorimetry for a sample and for scaffolds DLGA, DLGA/nHA 10%, and DLGA/nHA composite scaffolds 50% without degrading. The DLGA used for this study is a Poly DL-lactide and glycolide (DLGA) amorphous copolymer in a 47/53 molar ratio. Looking more closely at this figure shows us that the introduction of nHA favors the rigidity of the polymer chains, which produces a considerable increase in the glassy transition temperature that rises from 18.2 °C for samples of scaffolds to about 30 °C for the composite (14, 15).

In an in-vitro degradation process, which we studied, a reduction of the molecular weight by chain cleavage in the hydrolysis process might be expected to occur. A given mass of polymer would imply higher fractions of chain-ends and hence increased free volume, ie the reduction in overall flexibility should increase the value of Tg. However, the stiffness caused by high amounts of nHA incorporated in the composites counteracted this effect and caused the Tg to go from 30 °C for samples with a higher content of nHA to 40 ºC for the same samples with a degradation time of 6 to 8 weeks (15) as seen in Figure 3b).

3.3. FTIR

The nHA were initially analyzed by IR. The characteristic bands of γ_2 (PO_4^{3-}) were observed at 566 and 601 $cm^{-1,}$, $\gamma_1(PO_4^{3-})$ at 954 cm^{-1} and γ_3 at 1087 and 1022 cm^{-1}. These reflections indicate the classification of the polyhedrons of PO_4^{3-} in the glass structure (see Figure 4) (12, 16). Looking at the spectra of the DLGA DLGA/nHA, we can see that there is apparently no absorption band that has altered their position or intensity, so that we can say that the polymer does not interact with nanohidroxyapatite DLGA hogoneamente but is dispersed in the dough, which will be confirmed in the SEM observation. This is contrary to the results obtained by other authors with PLLA/nHA composite scaffolds using the manufacturing process of electrospinning (12). In samples of scaffolds and DLGA scaffolds and DLGA/nHA composite scaffolds at different weeks of degradation, we can observe the appearance of a new band (very broad for the contribution of water absorption) at around 3350 cm^{-1} that can be attributed to alcohol groups formed during the excision of the polymer chains by the hydrolysis of DLGA into lactic acid and glycolic acid. Moreover, the peak

corresponding to 1750 cm^{-1} (C = O stretching) dims when degradation breaks up the chains over longer periods of time.

When the degradation process starts both for DLGA and for DLGA/nHA composite scaffolds, an absorption band appears that corresponds to COO^- asymmetric stretching at 1600 cm^{-1} that cannot be seen in the non-degraded scaffolds, which is due to the hydrolysis process of the polymer chains.

3.4. pH Variations

DLGA degradation is mainly achieved via chemical hydrolysis and a low pH or a very high pH causes significant catalysis of the hydrolysis of an ester bond. The pH of the buffer solution for the DLGA scaffolds continuously decreased and reached 6.78 after 6 weeks and the 6-8 weeks remained constant (see Figure 5). Any decrease in pH showed an increased degradation rate. In the case of the DLGA/nHAp composites, the pH also continuously decreased to values close to 6.91 for the sample with less nHAP (10%) and to 6.97 for samples with more nHAP (50%). But, in all of cases, the rates of degradation were less than the DLGA and the pH were higher. In-vitro investigations have shown that the microenvironment within the acid created during DLGA degradation, which is degraded by simple hydrolysis of ester bands, lactic acid and glycolic acid, in an accelerated process catalysed by the generation of carboxylic acid, results in a decrease in molecular weight (17, 18).

The addition of salts such as hydroxyapatite or sodium bicarbonate in the matrix caused neutralization of the acid released by DLGA. These particles are propitious to moderate

inflammation from the acid released by autocatalytical acceleration of DLGA, which could translate into an absence of live adverse response in tissue (12).

3.5. Water absorption

Figure 6 shows the percentage of absorbed water versus the degradation time (in weeks). The percentage of absorbed water increased over the degradation time in all samples. DLGA is a pure sample which absorbs more water. By comparing these results with those of Figure 1, we see that the more water the sample absorbs, the more rapidly it is degraded (accelerated weight loss). The sample with the larger content of nHA are unable to stabilize water consumption over the first three weeks of the degradation process.

The water absorption process is a balance between the dissolution of oligomers in solution and the material consumption PBS residue. An increase in water uptake reflects the degradation rate in the initial state (18). The accumulation of hydrophilic degradation products inside the scaffold leads to an increase in water absorption during the degradation process. When the absorption of water reaches a certain value, the speed of absorption is reduced as a result of the dissolution of degradation products. These products introduce nHA particles which slow down the rate of degradation of the PLGA scaffolds, because they are an alkaline solution which acts as a physical barrier that blocks the entry of water (12, 19) and causes a decrease in the rate of degradation.

Porous scaffolds made from amorphous poly-ester-like PLGA are often regarded as hydrophobic biomaterials, the hydrophobicity of which blocks the absorption of water and leads to degradation by cleavage of hydrolytically sensitive ester bonds. A higher

content of less hydrophobic GA units in the copolymers (as is our case) facilitates the absorption and diffusion of water and thus the hydrolysis. Not only could the copolymer composition affect the degradation behaviour, but so could the additives in the scaffolds. For example, the incorporation of nHAp nanoparticles in PLGA scaffolds adjusted the acidic degradation of PLGA.

3.6. Percentage weight loss

From a close look at Figure 7, we can see how the percentage weight loss increases almost exponentially until the fourth week of degradation for samples and DLGA and DLGA/nHA (10%), after which its increase is almost linear. This decrease in the degradation rate may correspond to a large accumulation of degradation products and their dissolution. In general the value of weight loss for all the samples under study increased as degradation increased over time. However, unlike the conclusions of other authors (11, 19) weight loss in the degradation process became smaller with increasing concentrations of nHA particles.

We can see how this loss is stabilized in the sample with the highest concentration of nHA at somewhere between 6 and 8 weeks, reaching a weight loss of about 34% compared to the weight loss of DLGA without nHA that was 78%. Some authors have proposed that degradation is faster and higher with increasing water consumption for the poly-Lactides and bulk copolymer degradation mechanism (13, 19).

The introduction of nHA particles slows the weight loss of DLGA scaffolds because they act as a physical barrier that blocks the entry of water (12) and causes a decrease in the rate of weight loss and consequently the degradation rate.

3.7. SEM

Morphological changes of the DLGA and DLGA/nHAp composite scaffold were determined by SEM microscopy observation. The addition may be seen in Figure 8 of particles that reduce the size of the pores, but do not appear to greatly affect the high percentage of porosity Figure 8a) and 8b). The particles are uniformly distributed in the polymer matrix and are included before the lyophilization process in which these porous supports are made. The highly porous scaffolds or those with a smaller pore size degrade more slowly than those with larger pore sizes or with fewer pores and thicker walls, which decrease diffusion of acidic degradation products and therefore improve acid hydrolysis. When comparing samples with DLGA and DLGA/nHA, we can see (Figure 8c) and 8d)) that the latter degrade more slowly than the DLGA (11, 18). Micropores were observed on the walls of the DLGA scaffolds over the first week of degradation (see Figure 8 e)), which is probably a morphological feature that increases the degradation rate. Over the first week of degradation, the surface morphology can be seen to change from a smooth to an abrupt surface. This may be due to degradation, because the porous surfaces of the degradation products are released and part of the nHA particles are exposed outside the scaffold walls (see Figure 8 f)) resulting in a rougher surface (18, 20). The formation of these precipitates on the composite surface may stimulate and enhance cell-material interactions and therefore their biological response, so that the filler material could be used where bone regeneration is needed.

4. Conclusions

The following conclusions may be drawn from the results that have been presented above. 3-dimensional DLGA and DLGA/nHA composite scaffolds with a porous structure and a porosity of over 90% were fabricated by thermally-induced phase

separation. nHA particles were uniformly distributed in the polymer matrix and did not appear to affect the percentage porosity although its morphology was affected. The effect of bioactive nanoparticles on the in-vitro degradation of these scaffolds translates into less variation in pH, and a decrease in the rate of degradation can be observed in the values obtained for M_w, and M_n, % weight loss. The nanohydroxyapatite particles acted as a physical barrier and blocked the entry of water, causing a decrease in the rate of degradation of the scaffolds.

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Keywords: biodegradable polymer, nanohydroxyapatite, scaffolds, in vitro, degradation.

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Figure Captions

Figure 1 a. Changes in M_w for DLGA and DLGA/nHA 30%, as a function of degradation time.

Figure 1 b) Changes in M_n for DLGA and DLGA/nHA 30%, as a function of degradation time.

Figure 1 c) Changes in polydispersity index, for DLGA and DLGA/nHA 30%, as a function of degradation time.

Figure 2. . Rupture of DLGA chains in the degradation.

Figure 3 a) DSC thermograms of DLGA, DLGA/nHA 10% and DLGA/nHA 50% after varius degradation times.

Figure 3 b) DSC thermograms of DLGA/nHA 50% after varius degradation times.

Figure 4 a) FTIR spectra of nHA, DLGA and DLGA/nHA.

Figure 4 b) FTIR spectra of DLGA/nHA 10% after various degradation times.

Figure 5. pH change of phosphate buffer solution against degradation time.

Figure 6. Water absorption, for DLGA and DLGA/nHA composite scaffolds, as a function of degradation time.

Figure 7. Weight loss of DLGA and DLGA/nHA composite scaffolds against degradation time.

Figure 8. SEM observation of surface morphology of PLGA a) PLGA before degradation. b) PLGA /nHA 10% before degradation. c) PLGA after degradation in vitro for 3 weeks. d) PLGA/nHA 10% after degradation in vitro for 3 weeks. e) PLGA

after degradation in vitro for 1 week. f) PLGA/nHA 10% after degradation in vitro for 6 weeks.

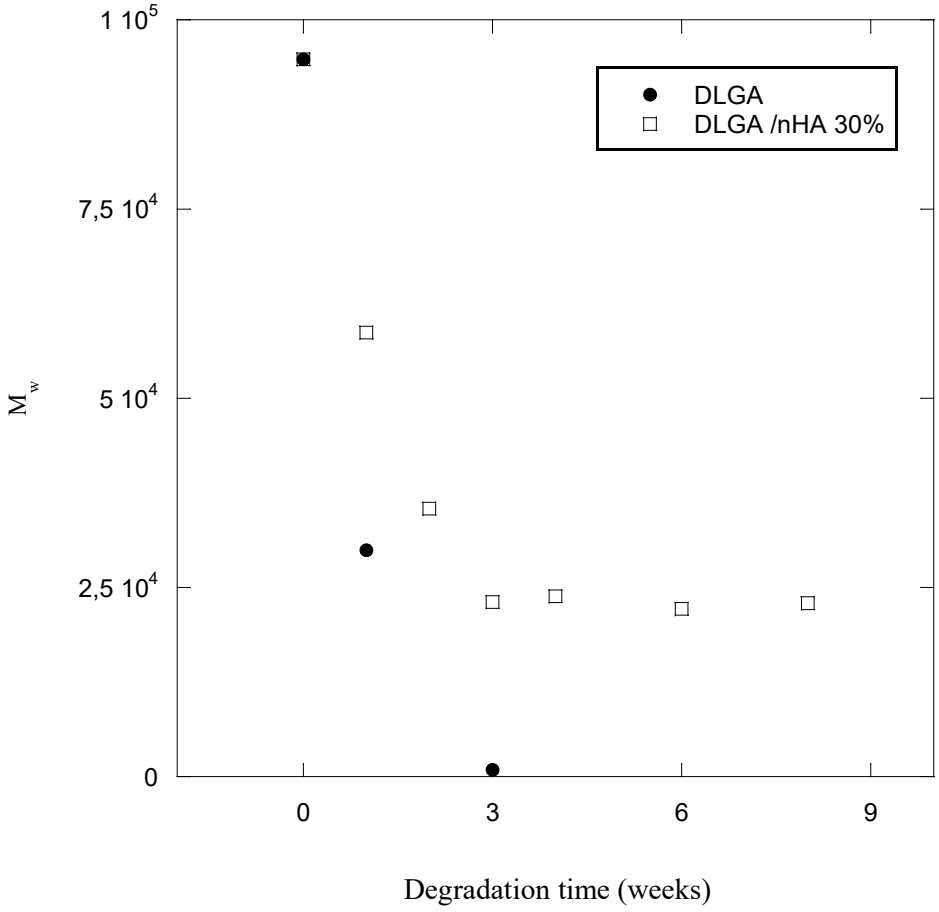
1 10^5
7,5 10^4
5 10^4
2,5 10^4
0
M_w
0
3
6
9
DLGA
DLGA /nHA 30%
Degradation time (weeks)

Figure 1a)

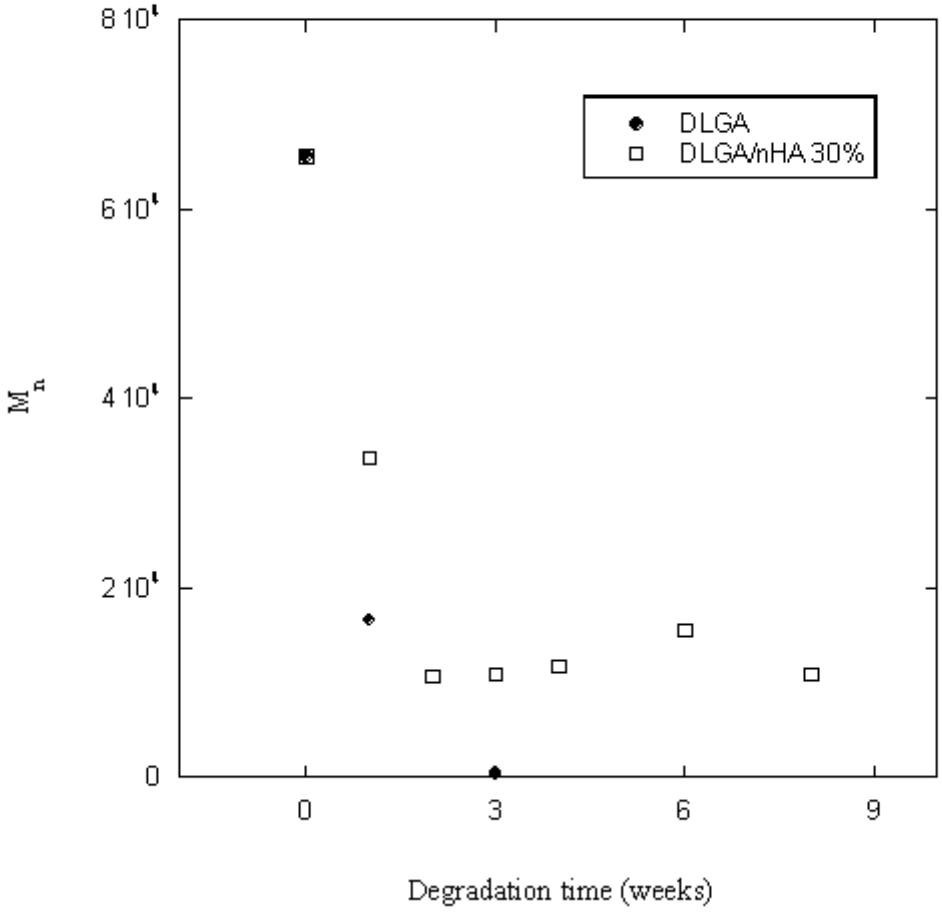
8 10⁴
6 10⁴
4 10⁴
2 10⁴
0
M_n
DLGA
DLGA/nHA 30%
0
3
6
9
Degradation time (weeks)

Figure 1 b)

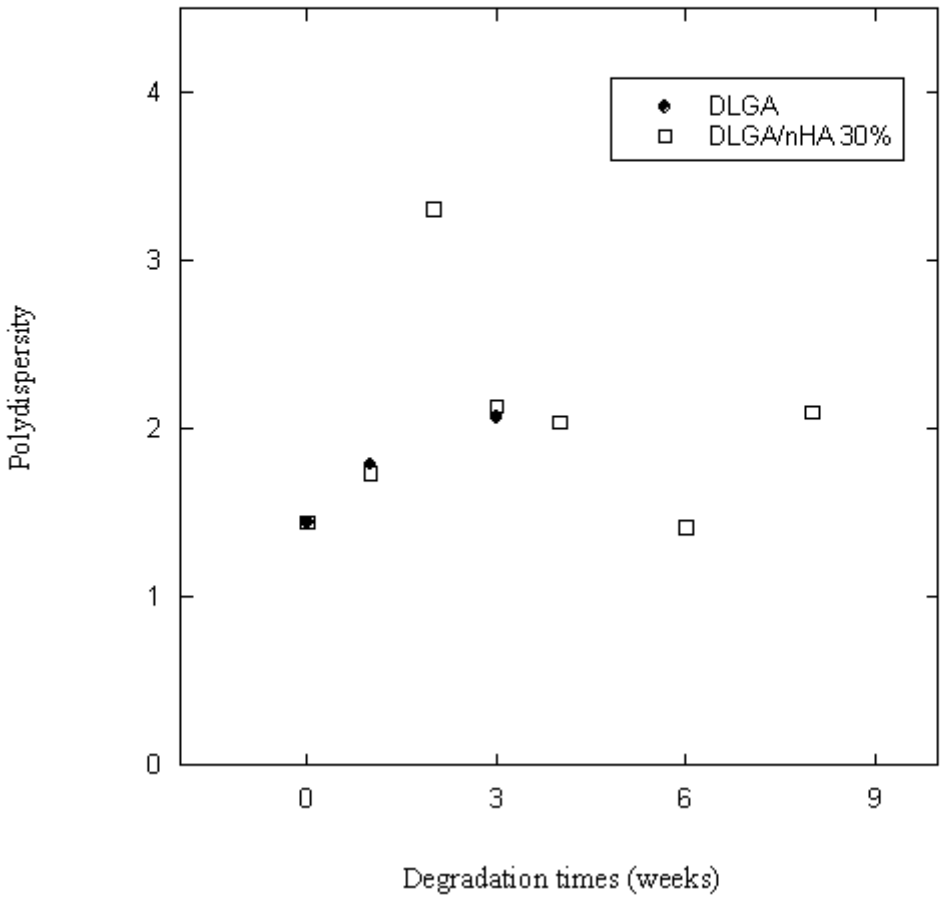
DLGA
DLGA/nHA 30%
Polydispersity
Degradation times (weeks)
0
1
2
3
4
0
3
6
9

Figure 1 c)

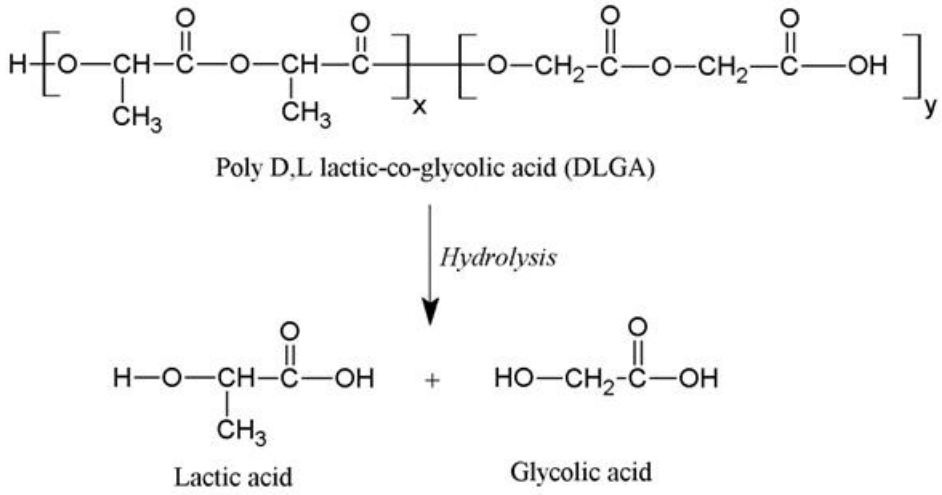
Poly D,L lactic-co-glycolic acid (DLGA)
Hydrolysis
Lactic acid
Glycolic acid

Figure 2. .

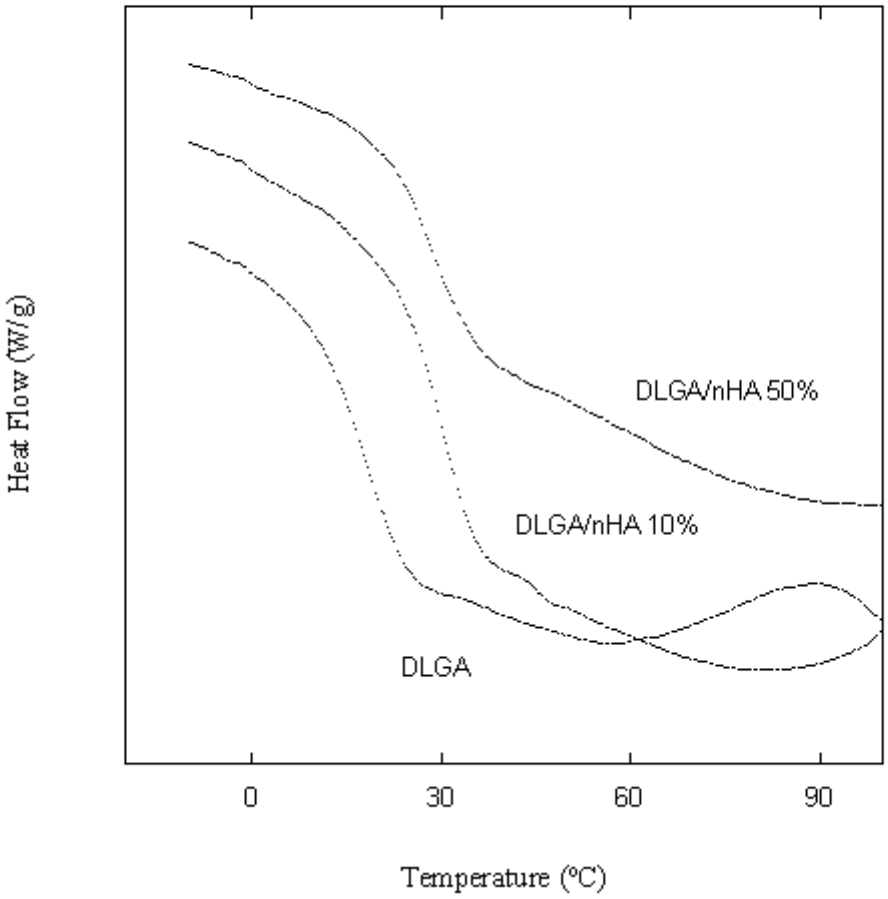
Heat Flow (W/g)
DLGA/nHA 50%
DLGA/nHA 10%
DLGA
0
30
60
90
Temperature (°C)

Figure 3 a)

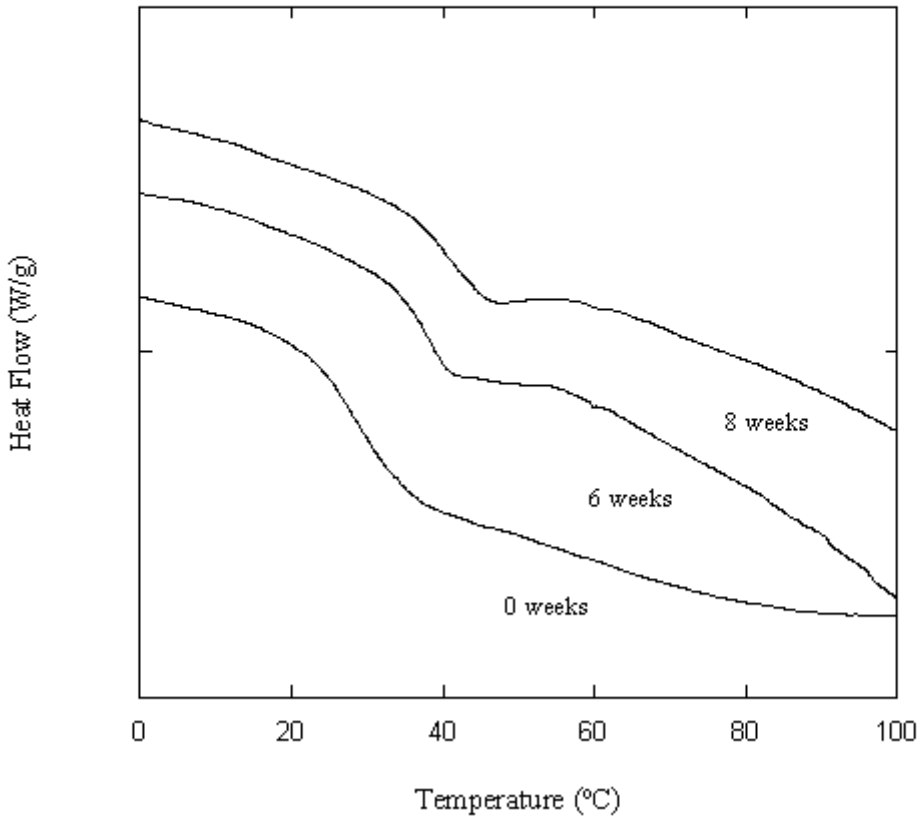
Heat Flow (W/g)
8 weeks
6 weeks
0 weeks
0
20
40
60
80
100
Temperature (°C)

Figure 3 b)

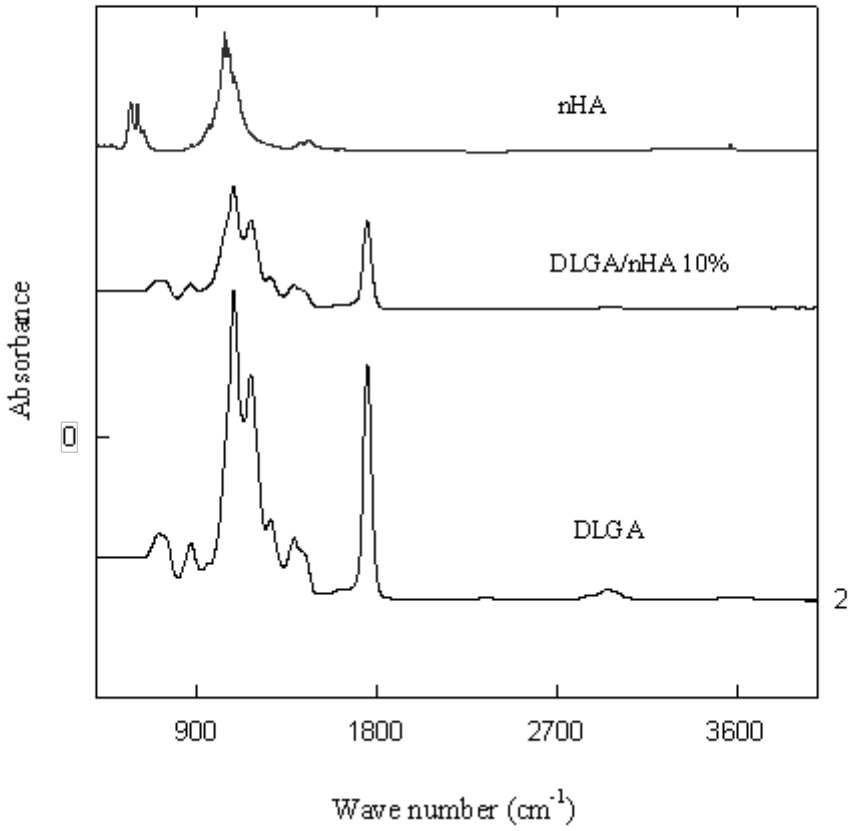
nHA
DLGA/nHA 10%
DLGA
Absorbance
0
2
900
1800
2700
3600
Wave number (cm-1)

Figure 4 a)

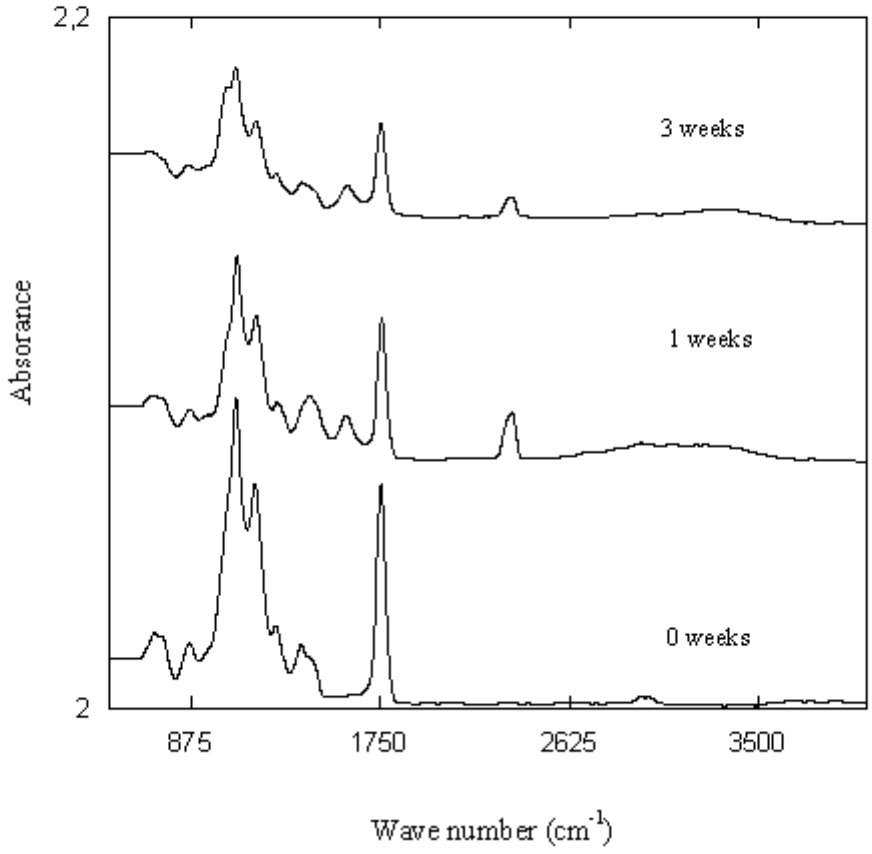
2,2
3 weeks
1 weeks
0 weeks
Absorance
2
875
1750
2625
3500
Wave number (cm-1)

Figure 4 b)

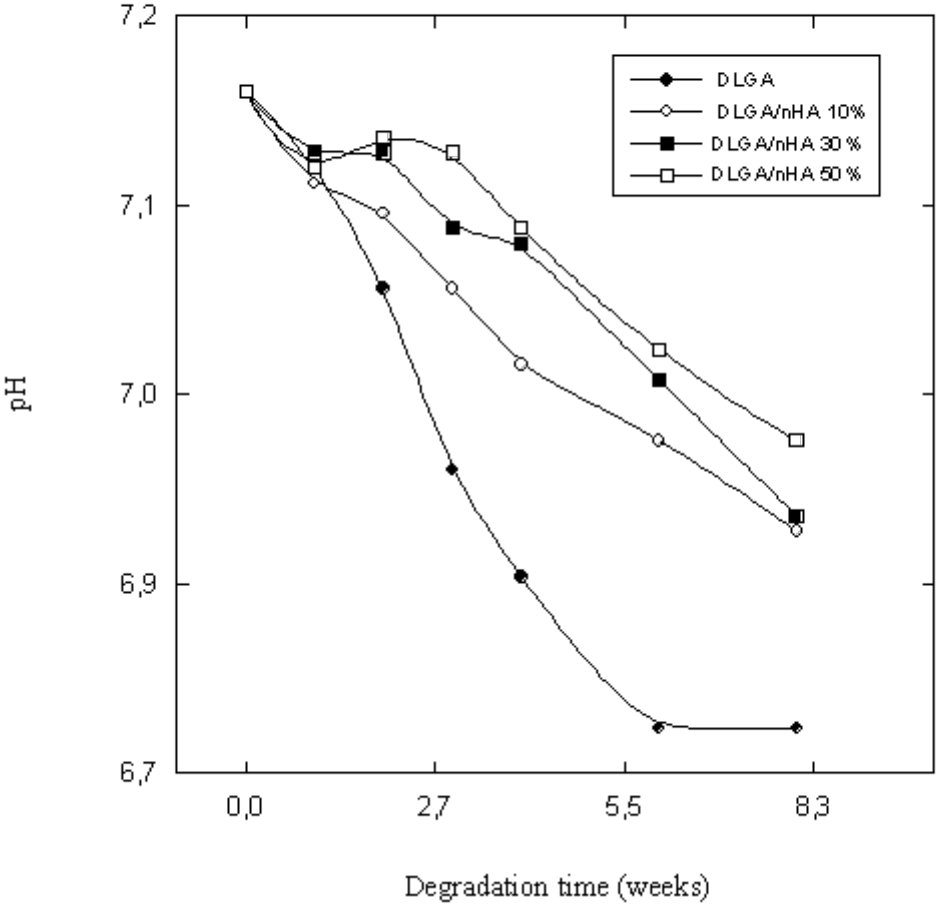
7,2
7,1
7,0
6,9
6,7
pH
0,0
2,7
5,5
8,3
Degradation time (weeks)
DLGA
DLGA/nHA 10%
DLGA/nHA 30 %
DLGA/nHA 50 %

Figure 5.

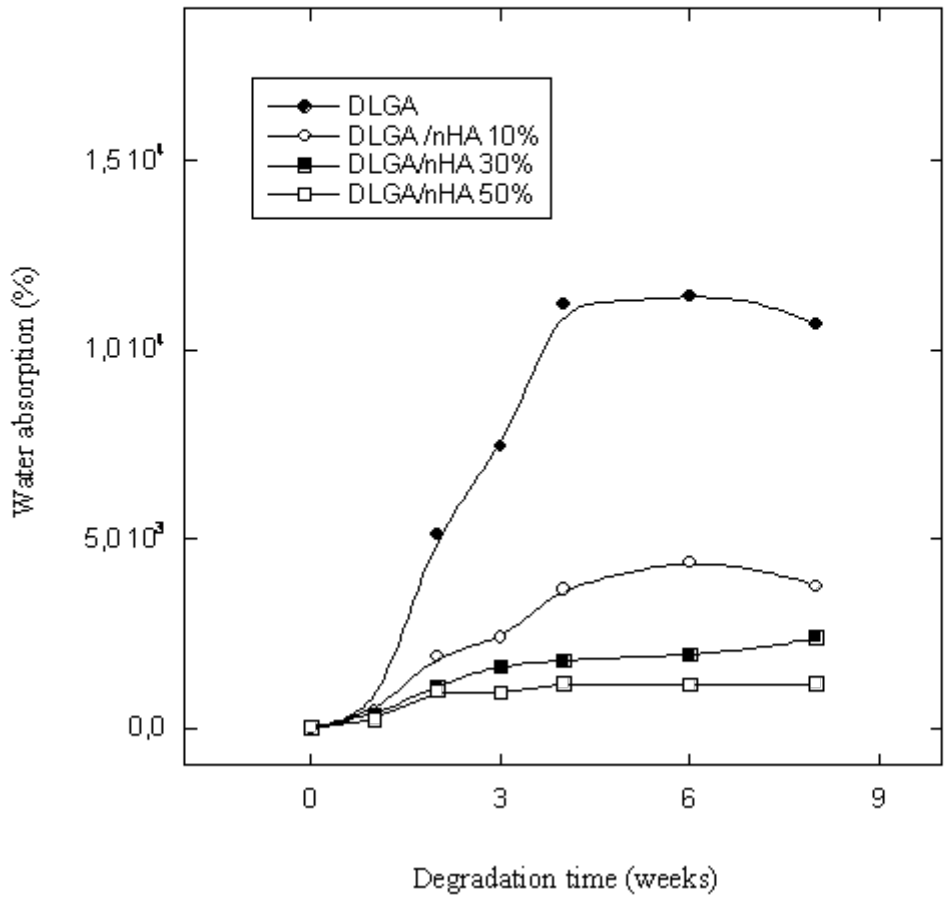
DLGA
DLGA /nHA 10%
DLGA/nHA 30%
DLGA/nHA 50%
Water absorption (%)
1,5 10⁴
1,0 10⁴
5,0 10³
0,0
0
3
6
9
Degradation time (weeks)

Figure 6.

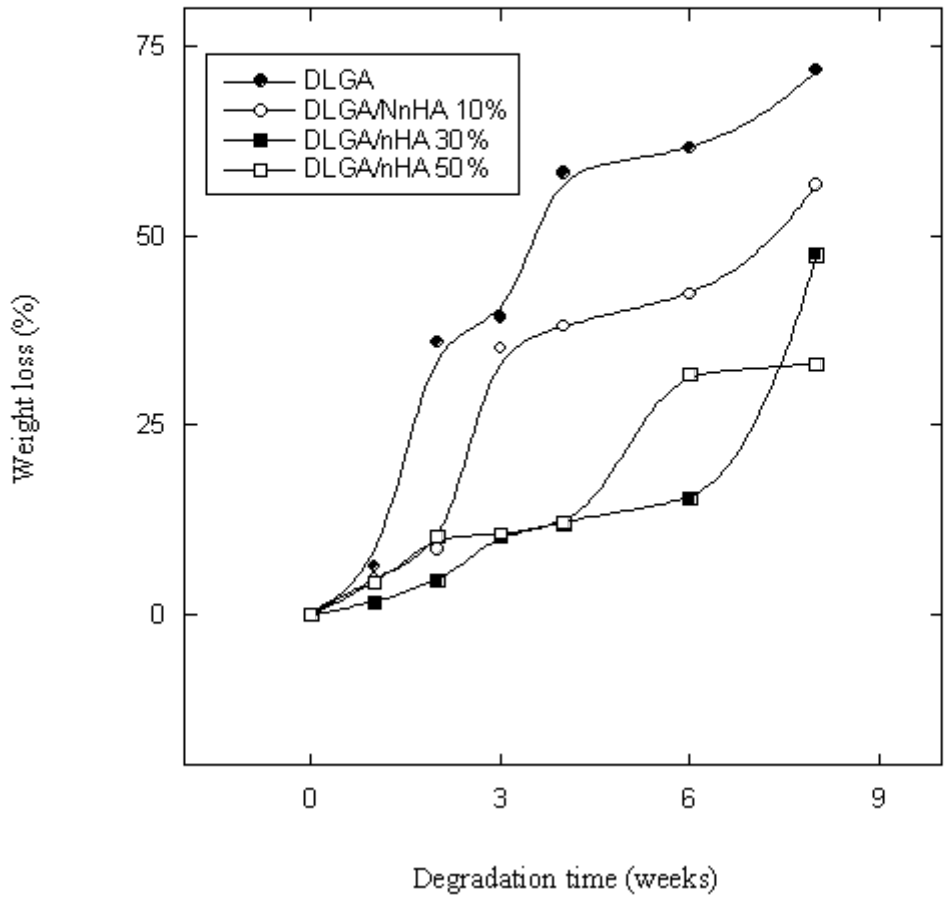
DLGA
DLGA/NnHA 10%
DLGA/nHA 30%
DLGA/nHA 50%
Weight loss (%)
75
50
25
0
0
3
6
9
Degradation time (weeks)

Figure 7.

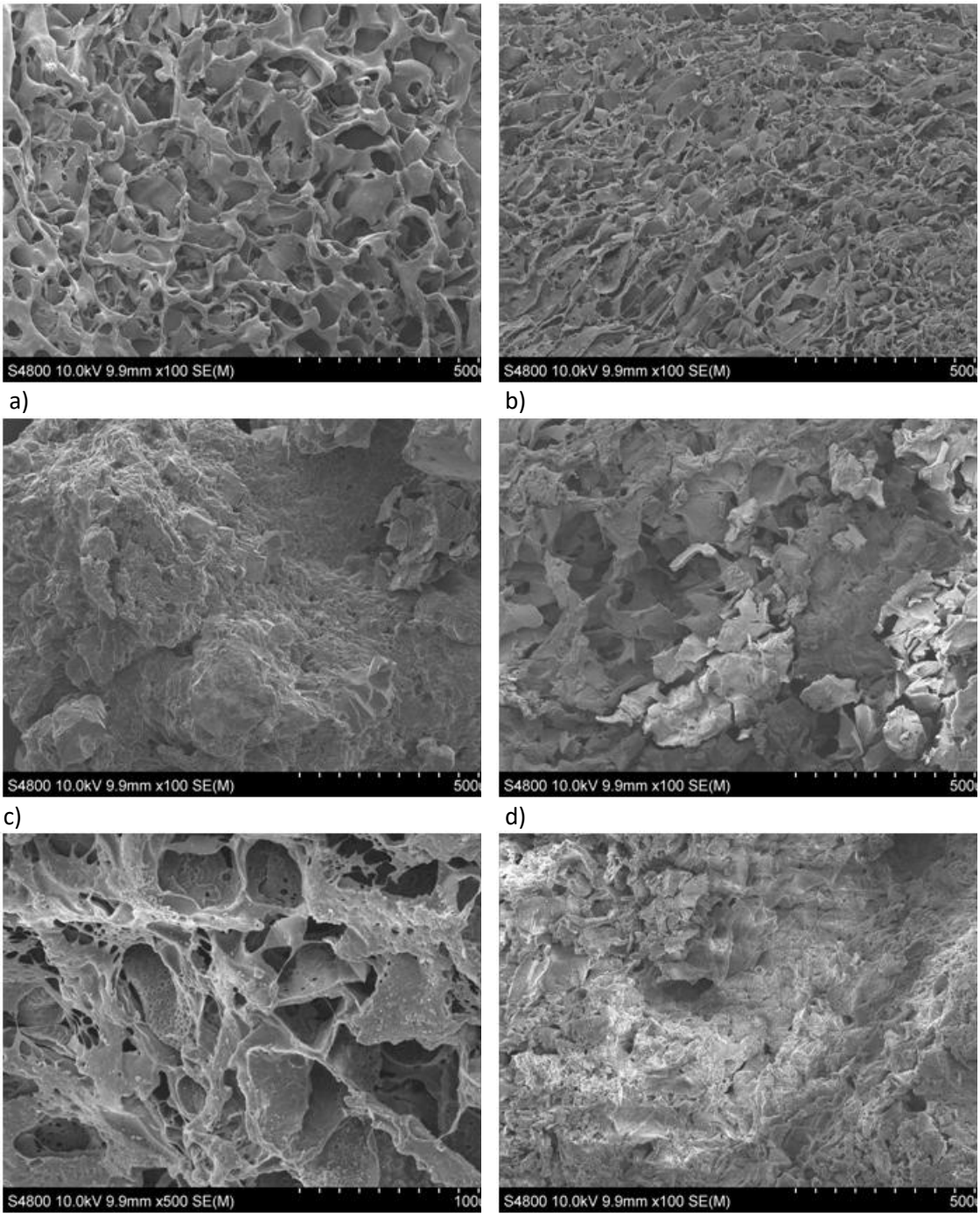
S4800 10.0kV 9.9mm x100 SE(M)
a)
S4800 10.0kV 9.9mm x100 SE(M)
b)
S4800 10.0kV 9.9mm x100 SE(M)
c)
S4800 10.0kV 9.9mm x100 SE(M)
d)
S4800 10.0kV 9.9mm x500 SE(M)
e)
S4800 10.0kV 9.9mm x100 SE(M)
f)

Figure 8.