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Modulation of physical and biological properties of a composite PLLA and polyaspartamide derivative obtained via thermally induced phase separation (TIPS) technique

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ABSTRACT

In the present study, blend of poly L-lactic acid (PLLA) with a graft copolymer based on α,β-poly(*N*-hydroxyethyl)-DL-aspartamide and PLA named PHEA-PLA, has been used to design porous scaffold by using Thermally Induced Phase Separation (TIPS) technique.
Starting from a homogeneous ternary solution of polymers, dioxane and deionised water, PLLA/PHEA-PLA porous foams have been produced by varying the polymers concentration and de-mixing temperature in metastable region. Results have shown that scaffolds prepared with a polymer concentration of 4% and de-mixing temperature of 22.5 °C are the best among those assessed, due to their optimal pore size and interconnection. SEM and DSC analysis have been carried out respectively to study scaffold morphology and the influence of PHEA-PLA on PLLA crystallization, while DMF extraction has been carried out in order to quantify PHEA-PLA into the final scaffolds.
To evaluate scaffold biodegradability, a hydrolysis study has been performed until 56 days by incubating systems in a media mimicking physiological environment (pH 7.4). Results obtained have highlighted a progressive increase in weight loss with time in PLLA/PHEA-PLA scaffolds, conceivably due to the presence of PHEA-PLA and polymers interpenetration. Viability and adhesion of bovine chondrocytes seeded on the scaffolds have been studied by MTS test and SEM analysis. From results achieved it appears that the presence of PHEA-PLA increases cells affinity, allowing a faster adhesion and proliferation inside the scaffold.

1. Introduction

Bone and cartilage engineered tissues have been developed as alternatives to autografts and allografts to repair and reconstruct osteochondral defects. Tissue engineering involves the in vitro seeding of cells onto scaffolds supporting their adhesion, migration, proliferation and differentiation, and defines the three dimensional (3D) shape of the tissue to be engineered [1]. The optimal scaffold should be biocompatible, biodegradable and cell-conductive to stimulate new tissue formation [2]. To secure a high density of colonizing cells and to promote neovascularization when implanted in vivo, the scaffolds should have optimal mechanical properties, large surface area and pore size distribution as well as a highly interconnected porous structure [3].

Thermally induced phase separation (TIPS) can be employed in order to produce a well interconnected porous structure as tissue engineering scaffold. In fact, TIPS is a very convenient methodology for fabricating porous materials as scaffold architectures that can be obtained by means of the manipulation of processing parameters and system properties. TIPS technique is based on thermodynamic demixing of a homogeneous polymer-solvent solution into a polymer-rich phase and a polymer-lean phase, usually by either exposure of the solution to another immiscible solvent or cooling the solution below a binodal solubility curve [4]. A variety of polymers such as polyesters have been employed as well as blends and composite of polymers with nanohydroxyapatite [5,6]. Poly(L-lactide) (PLLA) has been widely used as a scaffold material for bone and cartilage tissue engineering because of its superior mechanical properties, well-known biodegradability and biocompatibility [7,8,9]. PLLA is derived from 100% renewable resources and could be eventually degraded into CO_2 and H_2O under natural conditions [10,11]. However, the acid degradation products and mostly poor cell affinities limit the scope in tissue engineering applications [12]. In contrast, different polysaccharides such as chitosan have been commonly used with polylactide for improving the cytocompatibility and neutralizing the acid degradation products of polylactides in biomedical application [5,13].

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In the past decades, functionalized biodegradable polymers have attracted much attention. In particular, R-amino acid have been used as a building blocks for constructing biodegradable polymers to mainly impart chemical functionality, such as hydroxyl, amine, carboxyl or and thiol groups. These functionalized biodegradable polymers not only result in improved hydrophilicity and possible interactions with proteins and genes, but also facilitates further modification with bioactive molecules [14]. In this context, α,β-poly(*N*-hydroxyethyl)-DL-aspartamide (PHEA) is a biocompatible protein-like copolymer showing water solubility, absence of toxicity, antigenicity and teratogenicity. PHEA has been successfully used as starting material to generate different graft copolymers for several biomedical and pharmaceutical applications [15]. In previous papers has been demonstrated that the presence of the synthetic polyaminoacids appropriately allowed to tune cell adhesiveness together with chemical, physical and mechanical properties [16,17,18].

The aim of this paper is to prepare porous biodegradable scaffolds via TIPS starting from a blend of PLLA with a graft copolymer based on PHEA and PLA named PHEA-PLA [19], in the proportion of 75:25 (wt/wt). The goal is to develop new composite scaffolds exhibiting processability, workability stiffness and biocompatibility proper of PLLA together with chemical versatility, higher water affinity and faster rate of hydrolysis of PHEA in physiologic media, moreover improving the scaffold cellular affinity [19]. In fact, adhesion of tissue cells to biomaterials is a prerequisite of paramount importance for the successful incorporation of implants and the colonization of scaffolds in tissue engineering applications. First, PLLA/PHEA-PLA porous foams have been produced via TIPS by varying the polymers concentration and resident temperature in metastable region to control the final structure in terms of complex morphology, average pore size and degree of interconnection, according to a protocol already developed by the authors for PLLA [20,21]. Second, an investigation regarding the in vitro degradation rate of the blends has been carried out. As a preliminary assay short term viability and above all adhesion of bovine chondrocytes seeded in the scaffolds have been confirmed by MTS test and SEM analysis.

2. Materials and Methods

2.1. Materials

All reagents were of analytical grade unless otherwise stated. Dimethyl sulfoxide (DMSO), dichloromethane (CH_2Cl_2), ethanol, *N*,*N*^{1}-dimethylformamide (DMF), diethyl ether, *N*-*N*^{1}-dicyclohexylcarbodiimide (DCC), *N*-hydroxysuccinimide (NHS), Dulbecco's Phosphate Buffered Saline (DPBS) were obtained from Sigma–Aldrich (Italy). Diethylamine (DEA) was purchased from Fluka (Italy).

α,β-Poly(*N*-2-hydroxyethyl)-D,L-aspartamide (PHEA) was prepared and purified according to a procedure reported elsewhere [22]. Spectroscopic data (FT-IR and ^{1}H NMR) were in agreement with previous results [23]. PHEA weight-average molecular weight was 38 kDa (Mw/Mn = 1.78) as determined by size exclusion chromatography.

PURASORB® PDL 02 A (acid terminated GMP grade copolymer of DL-lactide 14 kDa) was purchased from Corbion Purac. PLLA (RESOMERTN L 209S) supplied by Boehringer Ingelheim Pharma KG, 1,4 dioxane (Sigma) and double distilled water were utilized to prepare the ternary solution. Chondrocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and 1% penicillin/streptomycin and 0.25% amphotericin B, purchased from Euroclone (Milan, Italy).

2.2. Apparatus

FT-IR spectra were carried out by using a Brucker Alpha instrument. Proton nuclear magnetic resonance (^{1}H NMR) was performed using a Brucker AC-300 instrument of 300 MHz.

Size exclusion chromatography (SEC) was performed using an Agilent 1260 Infinity Multi-Detector Bio-SEC solution. SEC system was equipped with a pump system, two Phenogel columns from Phenomenex (5 μm particle size, 10^3 Å and 10^4 Å of pores size), and Bio-Dual Angle LS/DLS and RI Detector. Analyses were performed with 0.1 M LiBr DMF solution as eluent with a flow of 0.6 ml/min and poly(ethylene oxide) standards (range 145 and 1.5 kDa) to obtain the calibration curve. The column temperature was set at 60 °C (±0.1 °C).

The morphology of the samples was analysed by Scanning Electron Microscopy (SEM) using a SEM-FEI QUANTA 200F on sample cross section fractured in liquid nitrogen and gold sputtered (Sputtering Scancoat Six, Edwards) for 40 s under Argon atmosphere before imaging.

A Setaram 131 evo Differential Scanning Calorimeter was used to attain scaffold melting thermograms. The samples were heated from 60 to 210 °C at a rate of 10 °C/min, under a nitrogen gas flow of 1 ml/min.

2.3. Synthesis and characterization of PHEA-PLA graft copolymer

The synthesis of PHEA-PLA graft copolymer was performed similarly to how reported elsewhere [19]. A solution of 600 mg of PHEA in 12 ml of anhydrous DMSO containing 576 μl of DEA as a catalyst was prepared. PLA *N*-hydroxysuccinimide (NHS) derivative (i.e. PLA-NHS) was prepared and purified according to a procedure reported elsewhere [19,24]; then a suitable amount of PLA–NHS in 12 ml of anhydrous DMSO was added to obtain a ratio (X) between moles of PLA–NHS and moles of PHEA repeating units equal to 0.1. The reaction was carried out under argon at 40 °C for 24 h. PHEA–PLA copolymer was purified following a different procedure. First the reaction solution was dialyzed by using dialysis tubes (Visking) with a cutoff of 25,000 Da against DMSO for three days, then it was dialyzed by using dialysis tubes with a cutoff of 12,000–14,000 Da against water to remove the organic solvent. The obtained suspension was freeze-dried, and then the product was dissolved in DMF and precipitated in diethyl ether. The recovered solid was washed twice in diethyl ether dichloromethane mixture (15:1 vol/vol) and in diethyl ether, then dried under vacuum. The purified product was finally characterized by FT-IR, ^{1}H NMR.

FT-IR spectra (KBr) of PHEA–PLA graft copolymer showed a broad band centred at 3450 cm^{-1} (ν_{as} OH + ν_{as} NH of PHEA), bands at 1760 (ν_{as} COO of PLA), 1655 (amide I of PHEA), 1542 (amide II of PHEA), 1457 (δ_{as} CH3 of PLA), 1383 (δ_s CH3 of PLA), 1183 (ν_s C–O–C ester group of PLA), 1089 (ν C—O alcoholic of PHEA) cm^{-1}.

^{1}H NMR spectra of PHEA–PLA graft copolymer (in DMSO-d_6) showed: δ 1.25 and δ 1.45 (2d, –O–CO–CH(**CH$_3$**)–O– of PLA), δ 3.1 (m, 2H –NH–**CH$_2$**–CH$_2$–OH of PHEA), δ 4.6 (m, 1H, NH–**CH**(CO)CH$_2$– of PHEA), δ 4.1 and δ 5.1 (m, 1H, –O–CO–**CH**(CH$_3$)–OH and m, 1H, –O–CO–**CH**(CH$_3$)–O– of PLA). The degree of derivatization percentage (DD%) in PLA was expressed as:

$$DD\% = (\text{moles PLA chains/moles of repeating units of PHEA})\ 100 \quad (1)$$

This ratio was calculated by comparing the integral of two peaks related to protons attributable to methyl groups of PLA chain at δ 1.25 and 1.45, with the integral of protons at δ 3.1 attributable to –NH–CH$_2$–CH$_2$–OH belonging to the ethanolamine groups of PHEA.

PHEA-PLA copolymer was obtained with a yield of 260% (w/w) based on the starting PHEA.

To confirm that PHEA-PLA copolymer maintains its stability after 120 min at the temperature of 65 °C (process temperature), Size Exclusion Chromatographic (SEC) analysis was performed. Two aliquots of PHEA-PLA copolymer were dispersed in dioxane-deionised water mixture 87/13 (wt/wt) at 65 °C for defined time points (5 and 120 min after complete solubilisation). Both samples were treated to remove solvents and the solid recovered was solubilized in 0.1 M LiBr DMF solution. SEC analysis was performed and peak max retention time (min) and peak area (mV·s) have been evaluated.

Table 1
PHEA-PLA SEC data recovered after 5 and 120 min at 65 °C.

Treatment time (min)	M_W (Daltons)	Peak max RT (min)	Peak area (mV·s)
5	116,431	19.55	9033.044
120	111,907	19.57	9204.447

2.4. Scaffold preparation and characterization

A homogeneous ternary solution composed of PLLA/PHEA–PLA (75/25), 1,4 dioxane and deionised water was prepared with a constant dioxane to water weight ratio of 87/13 (wt/wt) based on previous literature studies on the same system [25,26]. Polymer concentration was chosen to be 4%, 5% and 6% (wt/wt). The solution, initially kept at 65 °C, was hot poured into a disc-shaped aluminium sample holder (diameter 10 mm, thickness 2 mm). The temperature was then suddenly lowered to 22.5, 25, 30 or 33 °C (demixing temperature) for a well-defined demixing time (30 min), by pool immersion of the sample holder into a thermostatic water bath, according to a protocol developed by the authors and described in detail elsewhere [26]. Then a quench by pool immersion in an ethyl alcohol bath at a temperature of −20 °C for 15 min was performed in order to freeze the as-obtained structure. Finally, the as-obtained scaffolds were subjected to washings in deionised water for 24 h and drying at 20 °C under vacuum overnight, in order to completely remove any remaining solvent trace.

The morphology of the samples was analysed by SEM and obtained images were exported as 24-bit image files using the tagged image file format (tiff). The melting thermograms, temperatures and enthalpies of the samples were determined with a DSC analysis.

In order to quantify the presence of PHEA-PLA into the blend scaffolds, they were processed with DMF under stirring over night to extract PHEA-PLA fraction. After this time, extracted PHEA-PLA was precipitated in deionised water and freeze-dried, while residual scaffolds were washed several time with deionised water, then with ethanol and dried under vacuum. FT-IR, DSC and SEM analysis were performed on residual scaffolds; scaffolds recovered weight was compared to the starting dry weight of the samples. As comparison, the same treatment was performed on PLLA scaffolds obtained at polymer concentration of 4% wt/wt, demixing temperature equal to 30 °C and demixing time of 45 min.

2.5. Scaffold hydrolysis and weight loss studies

To evaluate rate of scaffold hydrolysis under body fluid-like condition, PLLA/PHEA-PLA scaffolds (10 mm large and wide, 2 mm thick) were weighed (W_i) and kept in a 24 well plate filled by 1 ml of DPBS pH 7.4. Samples were left under stirring (100 rpm) in an orbital shaker at 37 °C until 56 days. The liquid into the wells was changed every two days. After each incubation time, the samples were weighted (W_S) and were washed several time with deionised water to extract salts and

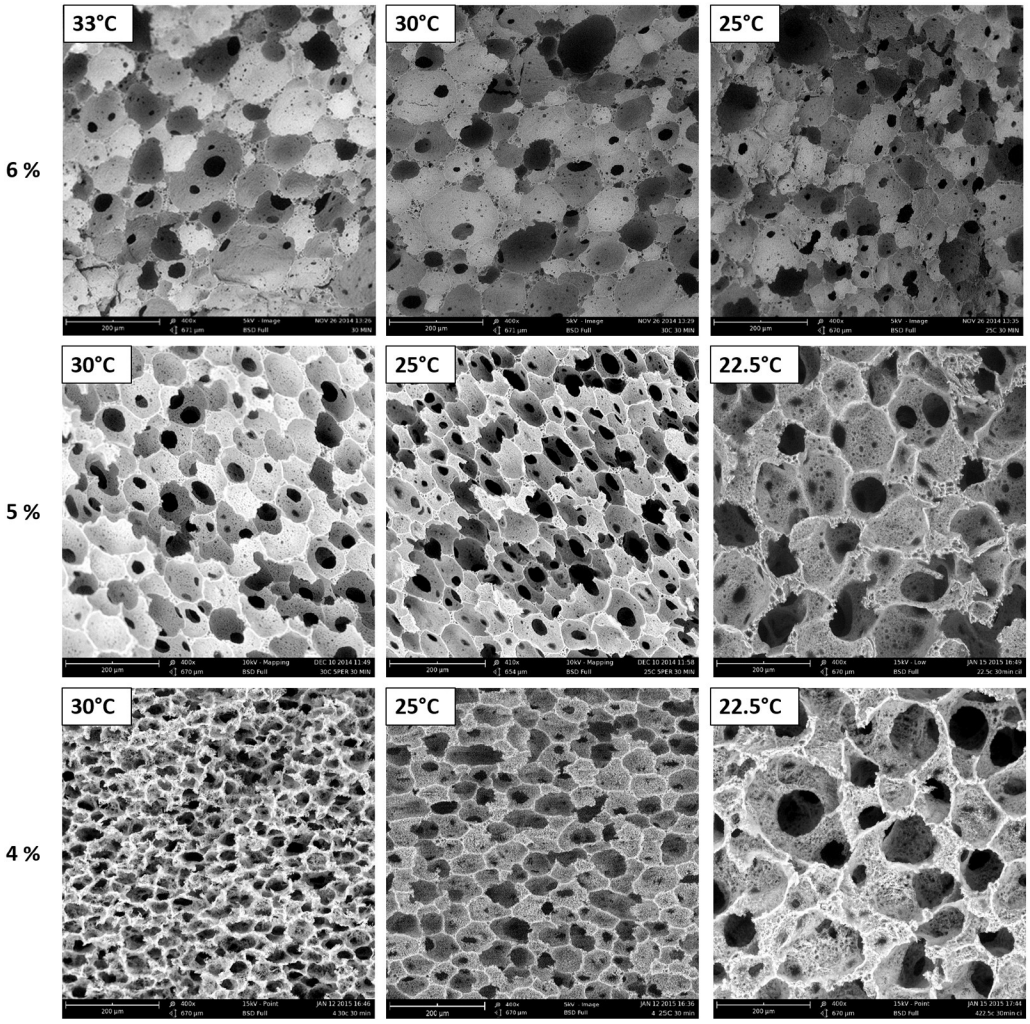

Fig. 1. SEM images of PLLA/PHEA-PLA scaffolds obtained at different polymer concentrations (6%, 5% and 4%) and demixing temperature (33, 30, 25 or 22.5 °C).

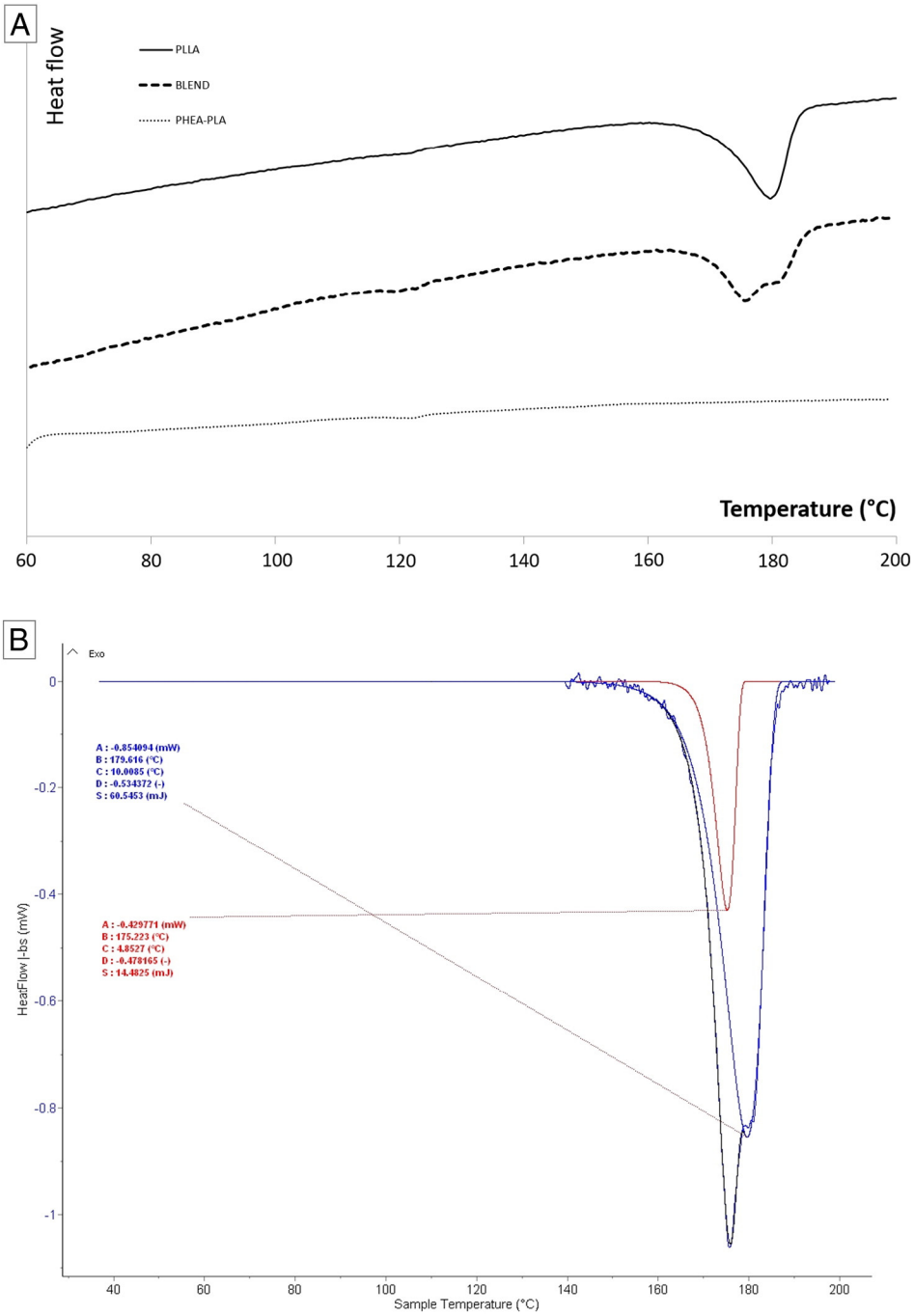

Fig. 2. A: DSC thermograms of virgin PLLA, virgin PHEA-PLA and PLLA/PHEA–PLA scaffold; **B**: Peak deconvolution of the thermogram of PLLA/PHEA-PLA scaffold.

water soluble hydrolysis products, then washed with ethanol and dried under vacuum. Finally, the dried samples were weighed (W_T). The swelling behaviour was expressed as the swelling weight ratio (q) between W_S and W_i. The weight loss (%) of the residues after the hydrolysis was expressed as mass washed out from the scaffolds respect to the starting dry weight of the sample. Also, IR analysis on residual scaffolds was performed. For this study, PLLA scaffolds have been used as comparison.

2.6. Isolation of bovine chondrocytes

Articular cartilage was cut from the metacarpophalangeal joint of bovine feet obtained from a local abattoir and used within 12 h. The cartilage was dissected, reduced in small pieces with a scalpel and treated for 3 min with a cold ethanol solution (70% v/v in sterile DPBS pH 7.4). Pieces were then washed several times with sterile DPBS pH 7.4 and incubated at 37 °C for 1 h with a solution containing 20 U/ml of Dispase (Sigma-Aldrich) in complete DMEM, i.e. DMEM supplemented with 10% v/v of fetal bovine serum (FBS), 1% v/v of penicillin/streptomycin solution (100 U ml-1 penicillin and 100 μg ml-1 streptomycin), 1% v/v of glutamine and 0.1% v/v amphotericin B solution [27,28]. After, cartilage pieces were incubated for further 18 h with a solution of type I collagenase (64 U/ml) (from clostridium histolyticum) (Sigma-Aldrich) in complete DMEM. After the enzymatic digestion, the medium was filtered through a sterile filter (120 μm), chondrocytes were collected by centrifugation (1500 rpm for 5 min), suspended in fresh complete DMEM and seeded in T-75 culture flasks. Isolated chondrocytes were used between passages 3 and 4 [28].

2.7. In vitro cell compatibility study

Chondrocytes were treated with trypsin and their number was determined by using a Bürker chamber, then they were suspended in complete DMEM. Blend scaffolds were sterilized by ethanol 70% (v/v) solution so they were washed with sterile DPBS. Therefore an appropriate volume of cell suspension was added on the top of scaffolds, at density of $1 \cdot 10^5$ cells/scaffold. After 1 h, scaffolds were placed into 24 well plate and 1 ml of complete DMEM was added. Incubation was left at 37 °C and 5% of CO_2. PLLA scaffolds as comparison model have been used.

Viability of chondrocytes seeded into PLLA/PHEA-PLA and PLLA scaffolds was investigated at established time points (after 24, 48 and 72 h) by MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4sulfophenyl)-2H-tetrazolium assay (CellTiter 96® AQueous One Solution Cell Proliferation, PROMEGA) following the manufacturer instructions. Each cell proliferation assay was performed in triplicate and results were reported as absolute optical density measured at 490 nm.

A SEM analysis was performed to observe chondrocytes after 24 h of incubation into PLLA/PHEA-PLA and PLLA scaffolds. Samples were washed with fresh DPBS pH 7.4 and fixed with formalin 4% (v/v) at 4 °C for 2 h, then were rinsed with water and dehydrated with ethanol series (25%, 50%, 75% v/v and pure ethanol). Finally samples were treated with hexamethyldisilazane (Sigma-Aldrich), dried under vacuum prior to be gold sputtered and analysed by SEM [24].

2.8. Statistical analysis

All the experiments were repeated at least three times. All results were reported as means ± standard deviation (S.D.) and, when applicable, statistical analysis for significance was performed by means of the Student's *t*-test, using Microsoft Excel statistical function for t tests, assuming unequal variance and two-tailed distribution; values of $p < 0.05$ were considered statistically significant while a p-value < 0.01 was considered as highly significant.

3. Results and discussion

3.1. Scaffolds preparation and characterization

Aim of this work was to use a blend of high molecular weight L-polylactic acid (PLLA) with PHEA-PLA graft copolymer to create a hybrid porous scaffold combining favourable properties of crystalline PLLA for TIPS processing with favourable properties of the amorphous PHEA-PLA in terms of water affinity and time of reabsorption. Interpenetration between the two polymers could give peculiar physical characteristics suitable to improve scaffold water affinity and rate of hydrolysis of blended scaffold if compared to PLLA scaffold alone. In our previous work a graft derivative of PHEA and crystalline PLLA was successfully employed for the production of scaffold through the TIPS technique [29]. Here instead an amorphous derivative of PHEA obtained grafting PLA of low molecular weight, named PHEA-PLA [15,30] has been physically mixed with PLLA to test if TIPS technique could allow polymers interpenetration. In particular, it has been investigated how blending influenced TIPS with respect to the possibility to control porosity. Then, scaffold physical properties such as wettability and hydrolytic reabsorption into buffered physiologic medium have been evaluated. PHEA-PLA and PLA (12 kDa) have been characterized via FT-IR, ^{1}HNMR and DSC analysis. The results compared as reported into the supplementary material. For experiments performed in this work the degree of derivatization percentage in PLA (DD%) onto the PHEA-PLA derivative was 5 ± 2 mol%.

Different PLLA/PHEA-PLA scaffolds have been obtained from 87/13 (v/v) dioxane/water solution, at different polymers concentration (4, 5 and 6% wt/wt) and demixing temperature (22.5, 25, 30 or 33 °C). The solutions have been heated at 65 °C while continuously stirred in order to achieve a homogeneous clear solution. PHEA-PLA stability, at the condition of temperature and time for TIPS procedure, was assessed via SEC. This experiment was carried out for 120 min (which corresponds to the time needed for scaffolds production) at 65 °C. Molecular weight, retention time and area peaks were registered and reported in Table 1.

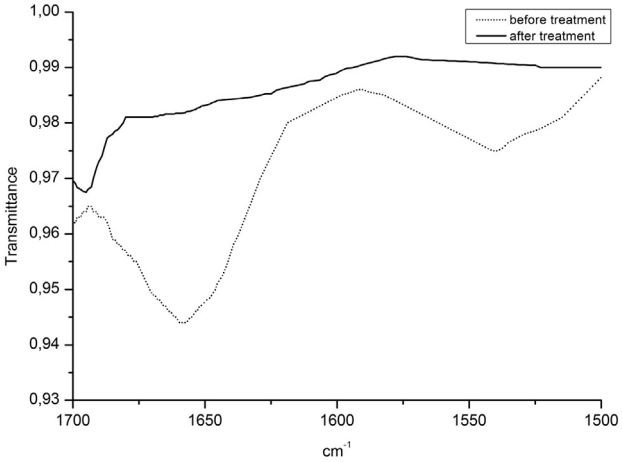

Fig. 3. FT-IR overlay spectra of PLLA/PHEA-PLA scaffold before (dash line) and after (continuous line) DMF treatment. Peaks at 1665 cm^{-1} and 1542 cm^{-1} are attributable to the amide I and II of PHEA.

No significant alterations on PHEA-PLA M_w from 5 to 120 min were found, thus suggesting that copolymer stability is maintained with these operative conditions.

TIPS method offers the possibility to control pore size by varying the experimental condition and provides means to control pore structure. The porosity of the scaffolds can be increased by decreasing polymer concentration and final size of pore is the result of balancing between nucleation and growth processes [31]. Fig. 1 reports SEM images of PLLA/PHEA-PLA scaffolds obtained under different polymer concentrations and demixing temperature, for a well-defined demixing time (30 min). A polymer concentration of 6% has led to scaffolds with few pores, poorly interconnected, independently of demixing temperature used (33, 30 or 25 °C). In the samples prepared using a polymer concentration of 5% wt/wt, it was possible to observe an increase of pore size by decreasing demixing temperature from 30 °C to 22.5 °C. Even if a sensible modification in terms of pore size has been obtained, the level of pore interconnection remains still unsatisfactory. Largest pores and homogeneous porosity have been obtained when setting up the polymer concentration at 4% and the demixing temperature at 22.5 °C. A high level of interconnection has been achieved as well as the formation of micropores (about 1–2 μm diameter) in the macropores wall.

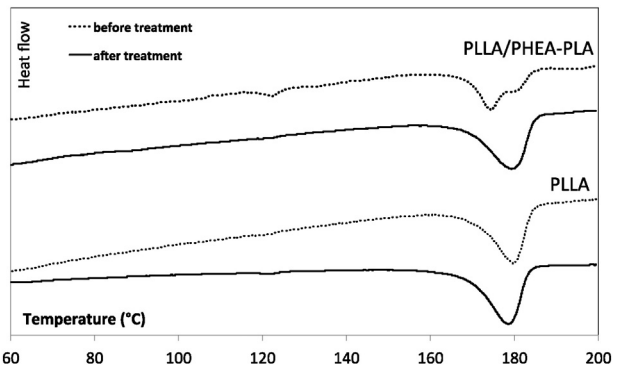

Fig. 4. DSC thermograms of pure PLLA and PLLA/PHEA-PLA scaffolds before and after the DMF treatment.

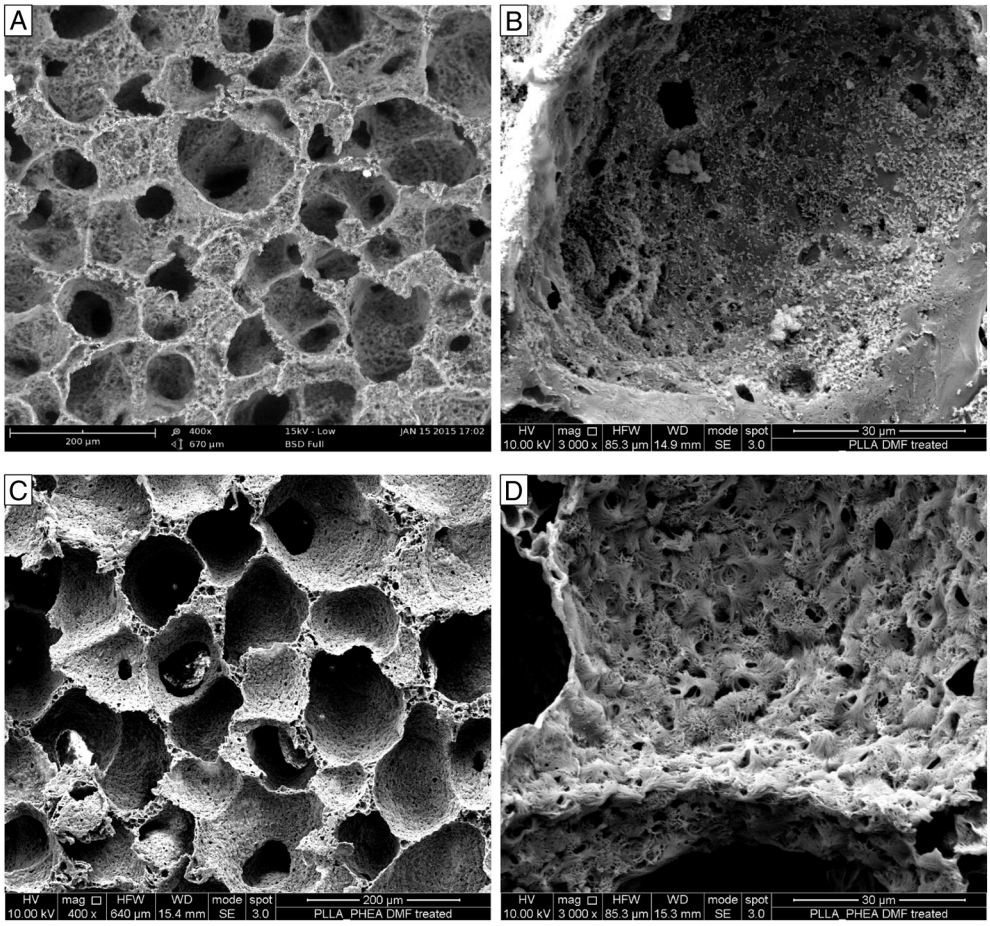

Fig. 5. SEM images of PLLA (**A**–**B**) and PLLA/PHEA-PLA (**C**–**D**) scaffold after DMF treatment. Scale bar corresponds to 200 μm for **A** and **C** panels and 30 μm for **B** and **D** panels.

Scaffolds prepared with a polymer concentration of 4% and demixing temperature of 22.5 °C were selected for further experiments because their pore size and interconnection seems to be optimal for cell seeding, cell penetration and migration during the in vitro cell culture [32].

In Fig. 2 A and B the thermograms of pure PHEA-PLA powder, PLLA and PLLA/PHEA-PLA scaffolds are shown (the PHEA-PLA was employed as unprocessed powder and not as porous scaffold because of inefficient TIPS processing). The PLLA thermogram presents the typical profile of the semi-crystalline polymer with the melting point at 180 °C and a melting enthalpy of about 56 J/g. As expected, PHEA-PLA trace confirms that the polymer is amorphous. The thermogram of the blended scaffold is partially different from that of pure PLLA in the melting region. As a matter of fact, the DSC trace exhibits a double peak, determined by the superposition of melting of two populations of crystals. This can be clearly understood by looking at the deconvolution of the melting peaks reported in Fig. 2B. The first peak is characterized by a 5 °C width, a relatively lower enthalpy and a lower melting point (located at a temperature around 175 °C). The second peak presents a width of about 10 °C with a relatively larger enthalpy and a melting point superimposed to that of pure PLLA. All things considered, the major effect of the presence of PHEA-PLA moieties in the blend, during polymer crystallization, consists of inducing the formation of a secondary population of small and defective PLLA crystals (having a lower melting point with respect to PLLA). This secondary population accompanies the primary population of PLLA crystals, having a melting point superimposable to that of pure PLLA. On the other hand, the change in melting enthalpy is of minor relevance.

In order to determine the effective amount of PHEA-PLA incorporated during the phase separation process, blend scaffolds were treated with DMF. After DMF treatment, a loss weight equal to 18.43% ± 0.47 has been quantified. FT-IR analysis on residual scaffold highlighted the absence of the peaks at 1665 cm^{-1} and 1542 cm^{-1} attributable to the amide I and II of PHEA (Fig. 3). This result conceivably suggests that PHEA-PLA copolymer was completely removed from scaffold. Moreover, from the quantitative data, it can be assumed that a part of the copolymer has been lost during scaffold preparation considering that the starting concentration of PHEA-PLA was equal to the 25 wt%.

Pure PLLA scaffold were subjected to the same treatment and used as control. DSC analyses (Fig. 4) have evidenced that the trace of PLLA does not change before and after the DMF treatment. Beside, an evident modification occurs in the profile of blended scaffold after the treatment. It is easy to notice that the profile of PHEA-PLA/PLLA blended scaffold after treatment is very similar to that pure PLLA, since the double peak disappears and the melting temperature increases until about 180 °C. This result led us to suppose that during the treatment only the PHEA-PLA was removed by DMF and confirms the efficiency of the method.

Table 2
% weight loss of the hydrolysed scaffolds.

	Day 1	Day 7	Day 14	Day 28	Day 56
PLLA	$0.16\% \pm 3.22 \cdot 10^{-5}$	$0.18\% \pm 4.4 \cdot 10^{-5}$	$0.55\% \pm 7.2 \cdot 10^{-3}$	$1.06 \pm 2.2 \cdot 10^{-3}$	$2.02\% \pm 5.3 \cdot 10^{-3}$
PLLA/PHEA-PLA	$2.70\% \pm 2.9 \cdot 10^{-3}$	$4.51\% \pm 3.1 \cdot 10^{-3}$	$8.24\% \pm 2.9 \cdot 10^{-3}$	$9.74\% \pm 2.9 \cdot 10^{-3}$	$18.98\% \pm 3.5 \cdot 10^{-3}$

Furthermore, it has been demonstrated that the extraction of PHEA-PLA changes the scaffold structure. SEM analysis on PLLA/PHEA-PLA scaffold after DMF treatment, reported in Fig. 6c and 6d, showed that although a homogenous porosity has been maintained, the superficial structure of single pores appeared indented. Conversely, the surface of the PLLA scaffolds remains unchanged after the same treatment (Fig. 5 A and B). Thus, it is possible to hypothesize that PHEA-PLA copolymer is distributed uniformly into the scaffold thank to an interpenetration occurred between the two polymers during the phase separation.

3.2. Swelling and weight loss studies

PLLA/PHEA-PLA scaffolds have been incubated with DPBS pH 7.4 until 56 days using PLLA scaffolds as comparison (see experimental section). Swelling behaviour has been determined as a function of time and expressed as the swelling weight ratio (q) between W_S and Wi. It has been obtained a q value equal to 16.30 ± 0.13 for PLLA/PHEA-PLA scaffolds and equal to 12.42 ± 0.02 for PLLA scaffolds. Both values do not undergo statistically significant changes during the 56 days. In Table 2 the weight loss percent of samples after 1, 7, 14, 28 and 56 days of incubation has been reported. In PLLA scaffolds a modest weight loss after 56 days has been confirmed, as were described in a previous work [26]. Instead, in PLLA/PHEA-PLA scaffolds a statistically significant ($p < 0.05$ just after the first day of incubation) progressive increase in weight loss with time was observed.

These results confirm that the presence of PHEA-PLA on composite scaffold, increased affinity for biological fluids if compared to PLLA scaffold. Beside network compactness, water penetration and swelling ratio remained unchanged indicating of hydrophobic PLLA structure remained the same. FT-IR spectra of PLLA/PHEA-PLA scaffolds after 1 and 56 days of incubation were compared (Fig. 6). Although the weight loss was statistically significant, it was demonstrated that PHEA-PLA copolymer is still present in the scaffold after 56 days of incubation.

All these results suggest that PHEA-PLA and PLLA interpenetrated, giving rise to stable and durable scaffolds. This interpenetration causes a scaffold degradation involving both PLLA and PHEA-PLA and significantly improves the rate of hydrolysis if compared to the scaffold of pure PLLA.

3.3. In vitro cells compatibility study

A preliminary biological evaluation of scaffolds was performed using bovine chondrocytes. Chondrocytes were seeded at low density (10^5 cells for construct) and proliferation followed for 3 days just to assay cell loading, viability and initial growth, and attachment of cells. Viability of seeded chondrocytes has been evaluated by MTS. Obtained results have been compared to those found using PLLA scaffolds.

Fig. 7 shows the growth of the cells into the two different scaffolds as a function of time. An evident and statistically significant cell growth ($p \leq 0.05$) inside the PLLA scaffold was observed just after 24 h, followed by a slight and not statistically significant increase in the further 48 h. In the case of PLLA/PHEA-PLA scaffold, a continuous growth was observed during the whole culture time being statistically significant from time 0 to 24 h and from 48 to 72 h ($p \leq 0.05$).

The SEM micrographs in Fig. 8 show cells morphologies seeded into the two scaffolds. Immediately after the seeding, the cells growing into the PLLA/PHEA-PLA scaffold appear spread and well adhered to the surface, whereas those growing into pure PLLA scaffold appears with pseudo-spherical morphology. Moreover, the cells on blend scaffold start to take contact each other through protrusions detectable at high magnification. Conversely, the cells seeded into the PLLA scaffold present few protrusions or in some cases did not present them. The described morphologies could indicate a higher level of cell-substrate interaction in the PLLA/PHEA-PLA scaffolds.

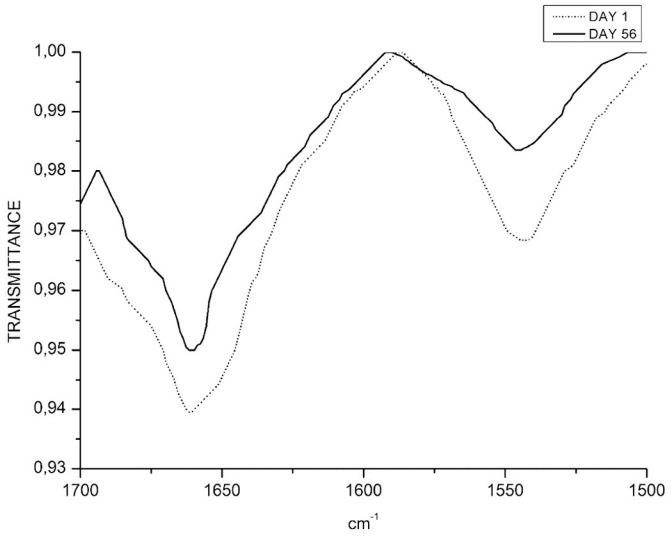

Fig. 6. FT-IR overlay spectra of PLLA/PHEA-PLA scaffold after 1 day (dash line) and 56 days (continuous line) DPBS incubation.

The higher cellular affinity towards the composite scaffold with respect to PLLA scaffold could be associated, in addition to the chemical structure of PHEA, to the fast hydrolysis rate detected in the composite scaffold. As a matter of fact, the increased affinity for biological fluids given by the presence of PHEA, could promote a higher level of cell adhesion.

Taken together SEM images and MTS data let us to suppose that the presence of PHEA increases material cells affinity.

4. Conclusion

Porous scaffolds for tissue engineering applications have been realized via TIPS by using blends of PLLA with PHEA-PLA. These new composite structures offer both the opportune crystallinity of PLLA, necessary for the production of scaffolds trough a TIPS method, and the higher water affinity and faster rate of hydrolysis of PHEA-PLA.

Various PLLA/PHEA-PLA scaffolds with variable porosity have been obtained by modifying polymers concentration and demixing temperature. Obtained results have confirmed that it is possible to achieve structures with an open porosity and a good level of interconnection. DSC results allowed to assume that PHEA-PLA copolymer, during polymer crystallization, would lead to the formation of small and defective PLLA crystals in addition to the primary population of PLLA crystals. The reached progressive increase in weight loss with time in PLLA/PHEA-PLA scaffolds could be conceivably due to the polymers interpenetration, the control of PLLA crystallinity and the enhanced water affinity.

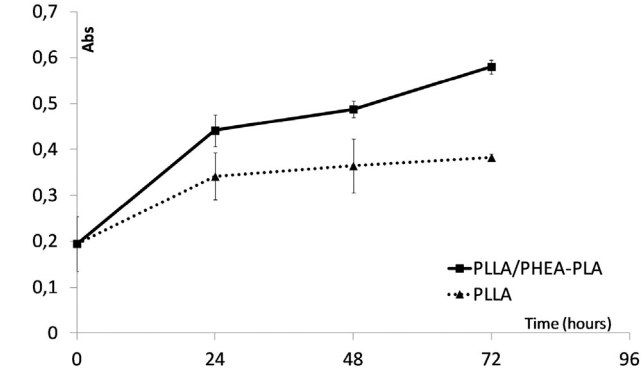

Fig. 7. Chondrocytes viability results from MTS assay after 0, 24, 48 and 72 h from seeding.

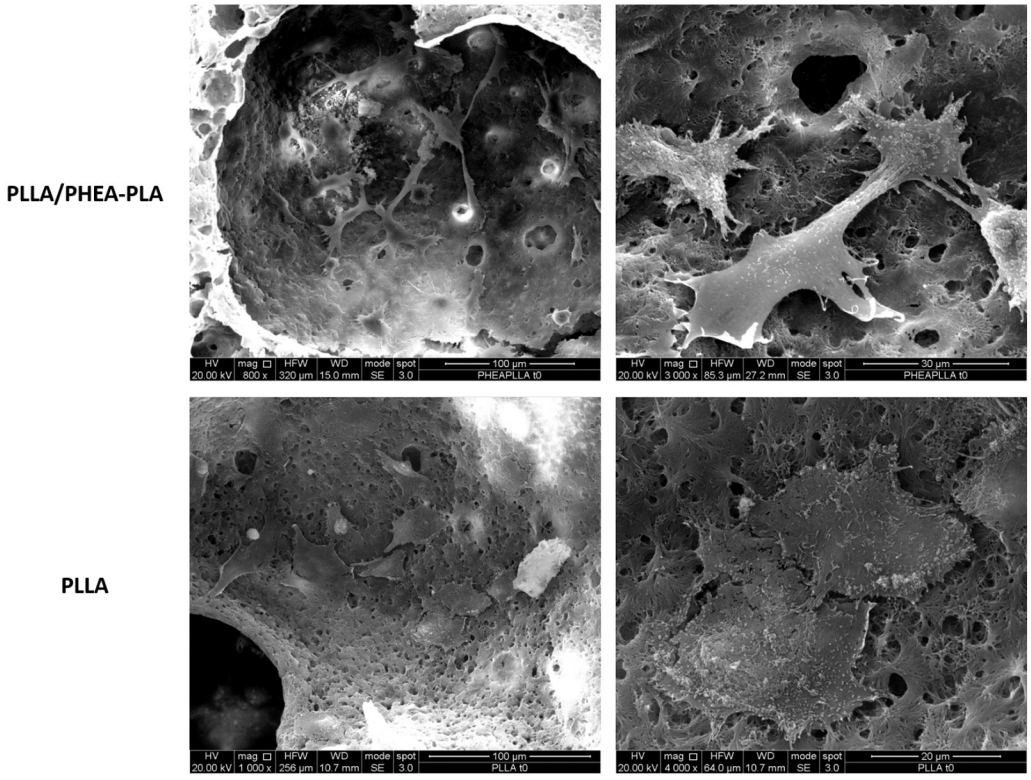

Fig. 8. SEM images of PLLA/PHEA-PLA and PLLA scaffold after 24 h of cells seeding.

The faster rate of hydrolysis of PLLA/PHEA-PLA scaffolds in physiologic media seems not to interfere with the scaffold cellular affinity. From the achieved in vitro results achieved in fact appears that the presence of PHEA improved the material affinity towards the cells inside the scaffold.

All things considered, the results presented in this paper candidate PLLA/PHEA–PLLA scaffolds as a new construct with significant potential in tissue engineering applications.

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References

[1] M. Dahl, N.R. Jørgensen, M. Hørberg, E.M. Pinholt, Carriers in mesenchymal stem cell osteoblast mineralization - state-of-the-art, J. of Cranio-Maxillo-Facial Surg. 42 (2014) 41–47.
[2] D. Ben-David, T. Kizhner, E. Livne, S. Srouji, A tissue-like construct of human bonemarrow MSCs composite scaffold support in vivo ectopic bone formation, J. Tissue Eng. Regen. Med. 4 (2010) 30–37.
[3] P. Schumann, F. Tavassol, D. Lindhorst, C. Stuehmer, K.H. Bormann, A. Kampmann, R. Mülhaupt, M.W. Laschke, M.D. Menger, N.C. Gellrich, M. Rücker, Consequences of seeded cell type on vascularization of tissue engineering constructs in vivo, Microvasc. Res. 78 (2009) 180–190.
[4] Y.S. Nam, T.G. Park, Porous biodegradable polymeric scaffolds prepared by thermally induced phase separation, J. Biomed. Mater. Res. 47 (1999) 8–17.
[5] S.A. Martel-Estrada, C.A. Martínez-Pérez, J.G. Chacón-Nava, P.E. García-Casillas, I. Olivas-Armendáriz, In vitro bioactivity of chitosan/poly (D,L-lactide-co-glycolide) composites, Mater. Lett. 65 (2011) 137–141.
[6] X. Liu, L. Smith, J. Hu, P. Ma, Biomimetic nanofibrous gelatin/apatite composite scaffolds for bone tissue engineering, Biomater. 30 (2009) 2252–2258.
[7] F. Peng, X.H. Yu, M. Wei, In vitro cell performance on hydroxyapatite particles/poly(L-lactic acid) nanofibrous scaffolds with an excellent particle along nanofiber orientation, Acta Biomater. 7 (2011) 2585–2592.
[8] J. Hu, X. Sun, H.Y. Ma, C.Q. Xie, Y.E. Chen, P.X. Ma, Porous nanofibrous PLLA scaffolds for vascular tissue engineering, Biomater. 31 (2010) 7971–7977.
[9] J.E. Park, M. Todo, Development and characterization of reinforced poly(L-lactide) scaffolds for bone tissue engineering, J. Mater. Sci. Mater. Med. 22 (2011) 1171–1182.
[10] T. Maharana, B. Mohanty, Y.S. Negi, Melt-solid polycondensation of lactic acid and its biodegradability, Prog. Polym. Sci. 34 (2009) 99–124.
[11] P.A. Gunatillake, R. Adhikari, Biodegradable synthetic polymers for tissue engineering, Eur. Cells and Mater. 5 (2003) 1–16.
[12] J. Ren, P. Zhao, T.B. Ren, S.Y. Gu, K.F. Pan, Poly(D,L-lactide)/nanohydroxyapatite composite scaffolds for bone tissue engineering and biocompatibility evaluation, J. Mater. Sci. Mater. Med. 19 (2008) 1075–1082.
[13] C. Chen, L. Dong, M.K. Cheung, Preparation and characterization of biodegradable poly(L-lactide)/chitosan blends, Eur. Polym. J. 41 (2005) 958–966.
[14] H. Sun, F. Meng, A.A. Dias, M. Hendriks, J. Feijen, Z. Zhong, Alpha-amino acid containing degradable polymers as functional biomaterials: rational design, synthetic pathway, and biomedical applications, Biomacromolecules 12 (2011) 1937–1955.
[15] G. Pitarresi, F.S. Palumbo, R. Calabrese, E.F. Craparo, G. Giammona, Crosslinked hyaluronan with a protein-like polymer: novel bioresorbable films for biomedical applications, J. of Biomed. Mater. Res. Part A. 84A (2008) 413–424.
[16] C. Fiorica, R.A. Senior, G. Pitarresi, F.S. Palumbo, G. Giammona, P. Deshpande, S. MacNeil, Biocompatible hydrogels based on hyaluronic acid cross-linked with a polyaspartamide derivative as delivery systems for epithelial limbal cells, Int. J. Pharm. 414 (2011) 104–111.
[17] F.S. Palumbo, G. Pitarresi, C. Fiorica, S. Rigogliuso, G. Ghersi, G. Giammona, Chemical hydrogels based on a hyaluronic acid-graft-a-elastin derivative as potential scaffolds for tissue engineering, Mater. Sci. Eng. C 33 (2013) 2541–2549.
[18] G. Pitarresi, F.S. Palumbo, G. Tripodo, G. Cavallaro, G. Giammona, Preparation and characterization of new hydrogels based on hyaluronic acid and α,β-polyaspartylhydrazide, Eur. Polym. J. 43 (2007) 3953–3962.
[19] G. Pitarresi, F.S. Palumbo, A. Albanese, M. Licciardi, F. Calascibetta, G. Giammona, In situ gel forming graft copolymers of a polyaspartamide and polylactic acid: preparation and characterization, Eur. Polym. J. 44 (2008) 3764–3775.
[20] V. La Carrubba, F.C.`. Pavia, V. Brucato, S. Piccarolo, PLLA/PLA scaffolds prepared via thermally induced phase separation (TIPS): tuning of properties and biodegradability, Int. J. Mater. Form. 1 (2008) 619–622.
[21] F.C.`. Pavia, V. La Carrubba, V. Brucato, Tuning of biodegradation rate of PLLA scaffolds via blending with PLA, Int. J. Mater. Form. 2 (2009) 713–716.
[22] G. Giammona, B. Carlisi, S. Palazzo, Reaction of a,b-poly(Nhydroxyethyl)-DL-aspartamide with derivatives of carboxylic acids, J. Polym. Sci.: Polym. Chem. Ed. 25 (1987) 2813–2818.
[23] R. Mendichi, G. Giammona, G. Cavallaro, A. Giacometti Schieroni, Molecular characterization of a,b-poly(N-hydroxyethyl)-DLaspartamide by light scattering and viscometry studies, Polym. 24 (2000) 8649–8657.
[24] G. Pitarresi, F.S. Palumbo, F. Calascibetta, C. Fiorica, M. Di Stefano, G. Giammona, Medicated hydrogels of hyaluronic acid derivatives for use in orthopedic field, Int. J. Pharm. 449 (2013) 84–94.

[25] F.J. Hua, G.E. Kim, J.D. Lee, Y.K. Son, D.S. Lee, Macroporous poly(L-lactide) scaffold 1. Preparation of a macroporous scaffold by liquid-liquid phase separation of a PLLA-dioxane-water system, J. Biomed. Mater. Res. 63 (2002) 161–167.
[26] F.C.`. Pavia, V. La Carrubba, S. Piccarolo, V. Brucato, Polymeric scaffolds prepared via thermally induced phase separation: Tuning of structure and morphology, J. Biomed. Mater. Res. A 86A (2007) 459–466.
[27] J. Liebman, R.L. Goldberg, Chondrocyte culture and assay, Current protocols in pharmacology, UNIT 12.2, J. Wiley & Sons (Inc.), 2001.
[28] F.S. Palumbo, C. Fiorica, M. Di Stefano, G. Pitarresi, A. Gulino, S. Agnello, G. Giammona, In situ forming hydrogels of hyaluronic acid and inulin derivatives for cartilage regeneration, Carbohydr. Polym. 122 (2015) 408–416.
[29] F. Carfì Pavia, V. La Carrubba, V. Brucato, F.S. Palumbo, G. Giammona, Synthesis, characterization and foaming of PHEA–PLLA, a new graft copolymer for biomedical engineering, Mater. Sci. Eng. C41 (2014) 301–308.
[30] G. Pitarresi, C. Fiorica, F.S. Palumbo, F. Calascibetta, G. Giammona, Polyaspartamidepolylactide electrospun scaffolds for potential topical release of ibuprofen, J. Biomed. Mater. Res. A 100A (2012) 1565–1572.
[31] C.A. Martínez-Pérez, I. Olivas-Armendariz, J.S. Castro-Carmona, P.E. García-Casillas, Scaffolds for tissue engineering via thermally induced phase separation, Adv. in Regen. Med, In, 2011 Chapter 13.
[32] V. Karageorgiou, D. Kaplan, Porosity of 3D biomaterial scaffolds and osteogenesis, Biomater. 26 (2005) 5474–5491.