MATERIALS
SCIENCE &
ENGINEERING

Photocrosslinkable polyaspartamide/polylactide copolymer and its porous scaffolds for chondrocytes

CrossMark

Calogero Fiorica [a,*], Fabio Salvatore Palumbo [a], Giovanna Pitarresi [a], Gaetano Giammona [a,b]

[a] Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche, Sezione di Chimica e Tecnologie Farmaceutiche, Università degli Studi di Palermo, Via Archirafi 32, 90123 Palermo, Italy
[b] Mediterranean Center for Human Advanced Biotechnologies (Med-Chab), Viale delle Scienze Ed.18, 90128 Palermo, Italy

ARTICLE INFO

ABSTRACT

With the aim to produce, by a simple and reproducible technique, porous scaffolds potentially employable for tissue engineering purposes, in this work, we have synthesized a methacrylate (MA) copolymer of α,β-poly(N-2-hydroxyethyl)-DL-aspartamide (PHEA) and polylactic acid (PLA). PHEA-PLA-MA has been dissolved in organic solvent at different concentrations in the presence of NaCl particles with different granulometry, and through UV irradiation and further salt leaching technique, various porous scaffolds have been prepared. Obtained samples have been characterized by scanning electron microscopy and their porosity has been evaluated as well as their degradation profile in aqueous medium in the absence or in the presence of esterase from porcine liver. PHEA-PLA-MA scaffold that has shown homogeneous porosity and the best degradation profile has been further characterized to study its mechanical properties along with its capacity to incorporate and to control the release of dexamethasone. Finally, the ability to allow a three-dimensional culture of bovine articular chondrocytes have been also investigated.

© 2017 Elsevier B.V. All rights reserved.

1. Introduction

The morphology of a scaffold for tissue engineering is of crucial importance for a correct spatial distribution of cells, for their anchoring and spreading and also to make the construct itself able to resist to mechanical stimulations of the body area which should be regenerated.

In principle, an ideal scaffold should represent a temporary substitute of extracellular matrix (ECM) that can be colonized in vitro or in vivo by cells able to produce new ECM components, essential for the restoration of physiological conditions.

In addition, the physicochemical and morphological properties of the scaffold, such as its swelling behavior and its porosity, could facilitate the exchange of nutrients/oxygen with the surrounding tissues and the removal of waste products.

The biomaterials should be processed to give an enough porous structure for efficient nutrient and metabolite transport without significantly compromising the mechanical stability of the scaffold [1].

In fact, even if a substantial scaffold porosity is necessary to allow the cell distribution, mechanical properties are often inversely proportional to the degree of porosity [2].

An ideal scaffold should show mechanical properties similar to those of natural tissues, biocompatibility and a controlled degradation rate together with the ability to entrap and to release specific bioactive factors for improving regeneration process [3,4].

Many biomaterial processing techniques, such as sonication [5], electrospinning [6,7], thermally induced phase separations (TIPS) [8], freeze drying [9], salt leaching [10], gas foaming [11] and 3D printing [12], have been employed in recent years to obtain porous structures for various biomedical applications.

If on one hand the preformation of a porous scaffold allows to finely control its morphological characteristic in terms for example of pores dimension and shape, on the other hand the use of these devices needs often an invasive surgical implantation that, could lead to development of infections and involves longer recovery times.

However, preformed porous scaffolds are often desirable to guarantee a uniform three dimensional distribution of the implanted cells and their high surface area can be exploited for the release of different drugs [13].

For example, Saber-Samandari et al., developed very recently chitosan-graft-poly(acrylic acid-co-acrylamide) based porous scaffolds for bone tissue engineering through the freeze drying process. They

* Corresponding author at: Lab of Biocompatible Polymers, Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche (STEBICEF), Sezione di Chimica e Tecnologie Farmaceutiche, Via Archirafi, 32, 90123 Palermo, Italy.
E-mail address: calogero.fiorica@unipa.it (C. Fiorica).

demonstrated that these scaffolds, beside to be biocompatible, sho optimal characteristics as drug delivery systems [14].

Still, Fereshteh et al. developed porous scaffolds for tissue engineering purposes from poly(ε-caprolactone) and zein and they demonstrated that these devices can control the release of tetracycline hydrochloride [15].

In this last case, authors demonstrated also that the presence of zein was crucial to control the degradation profile of the scaffold. This is a crucial aspect because, beside the morphology of the scaffold, also the structure of starting biomaterial is important since it influences the capacity of the construct to interact with body fluids, its mechanical properties and its biodegradability.

Here our aim was to propose a facile and reproducible technique to obtain porous scaffolds for potential cartilage tissue engineering purposes, exploiting the combination between UV crosslinking and salt leaching procedures and employing, as a starting biomaterial, an amphiphilic copolymer obtained by grafting polylactic acid (PLA) to α,β poly-(N-2-hydroxyethyl)-DL-aspartamide (PHEA).

Generally, polyesters, promote cell adhesion, give optimal mechanical properties to scaffolds but they show poor affinity towards biological fluids, due to their low hydrophilic character [16]. On the contrary, PHEA is a biocompatible and water soluble polyaminoacid, widely employed for the production of several devices for tissue engineering [17–19].

In previous papers it has been demonstrated that the presence of synthetic polyaminoacids appropriately allowed to tune cell adhesiveness together with chemical, physical and mechanical properties [20, 21].

Therefore, the combination of the physico-chemical properties of PLA and PHEA could be useful to produce scaffolds with appropriate mechanical properties together with a good affinity towards the cells and the physiological medium.

In this paper, for the first time, PHEA-PLA was functionalized with methacrylic anhydride (MA) to obtain a UV photocrosslinkable copolymer (named PHEA-PLA-MA) employable as a starting material for the production of porous scaffolds.

By changing the production parameters, a series of different scaffolds was obtained.

The scaffold that has shown homogeneous porosity and the best degradation profile has been chosen to incorporate and to culture bovine articular chondrocytes as well as to incorporate and to control the release of dexamethasone, a drug employed in the treatment of osteoarthritis [22] for its anti-inflammatory properties and for its capacity to stimulate the differentiation of stem cells into chondrocytes and/or osteoblasts [23,24].

A scaffold that controls the release of dexamethasone should be potentially able to reduce the chronic inflammation process and, at the same time, to stimulate the mesenchymal stem cells of the surrounding tissues to differentiate in order to restore the tissue homeostasis.

2. Experimental section

2.1. Materials and methods

Dimethyl sulfoxide (DMSO), N,N-dimethyl formamide (DMF), dichloromethane, (DCM), hexamethyldisilazane, 1,1′-Carbonyldiimidazole (CDI), 4-(Dimethylamino)pyridine (DMAP), dexamethasone, esterase from porcine liver, Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's phosphate buffered saline (DPBS), trypsin–EDTA solution, amphotericin B solution, penicillin-streptomycin and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (Italy). Diethylamine (DEA) and methacrylic anhydride (MA) were purchased from Fluka (Italy).

CellTiter 96® AQueous One Solution Cell Proliferation Assay (for MTS assay) was purchased from Promega.

RESOMER R 202 (D,L polylactic acid, PLA 14 kDa) was purchased from PURAC.

α,β-Poly(N-2-hydroxyethyl)-D,L-aspartamide (PHEA) was prepared and purified according to a procedure reported elsewhere [25]. PHEA weight-average molecular weight was 41 kDa (Mw/Mn = 1.78) as determined by size exclusion chromatography (SEC).

^{1}H NMR spectra were obtained with a Brucker AC-300 instrument.

Freeze drying procedures were conducted employing a LABCONCO freezone 6 freeze drying system.

Scanning electron microscopy (SEM) was performed using a Scanning Electron Microscope (ESEM QUANTA FEI 200F) with an accelerating voltage of 15 kV. For SEM analysis of cell containing scaffolds, samples were fixed in formaldehyde, dehydrated by hexamethyldisilazane (Sigma-Aldrich) and dried under vacuum prior to be gold sputtered and analyzed.

UV irradiation was performed using a Rayonet reactor equipped with a Rayonet Carousel motor assembly and 16 mercury lamps of 8 W at medium pressure with an emission at 366 nm.

UV analysis was performed with a UV–VIS 2401 PC spectrophotometer produced by Shimadsu.

Atomic force microscopy (AFM) was performed using a Bruker FastScan atomic force microscope.

Compression tests were performed using an Instron apparatus (Model 3345, Instron, Norwood, MA).

2.2. Synthesis of PHEA-PLA and PHEA-PLA-MA graft copolymers

Derivatization of PHEA with acid terminated PLA to obtain the PHEA-PLA graft copolymer was carried out by using CDI as a coupling agent to activate the terminal carboxyl group of PLA by using a procedure already reported with minor modifications [26].

Briefly, CDI dissolved in DMF was added to a PLA solution in the same solvent (molar ratio CDI/PLA equal to 1.5); after 4 h of activation at 40 °C, the PLA activated solution was added to a PHEA solution (molar ratio PLA/PHEA repetitive units equal to 0.06) in the presence of DEA as a catalyst (fivefold molar excess respect to PHEA repetitive units). The reaction was left under argon atmosphere and continuous stirring at 40 °C for 24 h and then precipitated in pure diethyl ether. The obtained suspension was centrifuged (9800 rpm for 20 min at 4 °C) and the product was washed in diethyl ether:dichloromethane mixture (15:1 v/v) for five times (30 min per washing), then dried under vacuum at room temperature and recovered with a yield of 200 wt% based on the starting PHEA.

For the insertion of methacrylic moieties, 500 mg of PHEA-PLA was dissolved in 10 ml of DMF in the presence of 167 mg of DMAP (molar ratio DMAP/PHEA repetitive units equal to 1.2) and 1.7 ml of MA (molar ratio MA/PHEA repetitive units equal to 10). The reaction was left under argon atmosphere and continuous stirring for 24 h at 25 °C.

The product was isolated by precipitation in diethyl ether, washed 5 times with the same solvent and dried under vacuum at room temperature. PHEA-PLA-MA copolymer was obtained with a yield of 80 wt% based on the starting PHEA-PLA.

2.3. ^{1}H NMR analysis

^{1}H NMR analysis was performed by dissolving PHEA-PLA or PHEA-PLA-MA in DMF-d7 at a concentration of 5 mg/ml. Spectra were recorded at 300.12 MHz performing 16 scans per sample with an acquisition time of 1.36 s per scan.

2.4. Production of PHEA-PLA-MA porous scaffolds

PHEA-PLA-MA was dissolved in DMSO at concentrations of 60 or 80% (w/v).

NaCl with different granulometry (between 60 and 90 μm and between 90 and 125 μm) was added to the polymeric solutions, setting a

weight ratio between PHEA-PLA-MA and NaCl equal to 30:70, to obtain viscous pastes that were irradiated at 366 nm for 1 h by using a Rayonet reactor. Scaffolds were washed extensively with double distilled water in order to eliminate the salt. Porous scaffolds thus obtained (named as samples A, B, C, D, see Table 1), were freeze dried, gold sputtered and analyzed by SEM in order to investigate if a uniform porous structure was obtained for all the produced scaffolds and to choose scaffolds suitable for further physicochemical and biological characterization.

Table 1 shows the composition of pastes employed for UV irradiation.

Only the scaffolds for which SEM analysis showed the presence of a uniform porous structure have been further studied (scaffolds A and C).

2.5. Porosity determination

The determination of porosity was performed as reported by Hutmacher et al. [27].

Briefly, each freeze dried scaffold was immersed into a known volume of ethylene glycol (V1) in a graduated cylinder and the volume of the solvent with the scaffold was measured (V2).

The system has been degassed under vacuum until no more bubbles formation was observed, the scaffold was then removed from the cylinder and the remaining volume of ethylene glycol was measured again (V3).

The % of porosity has been determined by using the following equation:

$$\% \text{ of porosity} = (V1-V3/V2-V3)*100$$

Each experiment was performed in triplicate.

Scaffold porosity was also studied using a pycnometer (Pycnomatic ATC, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The porosity was measured adopting helium as a penetrating agent. Cubic specimens were used to accurately evaluate the bulk volume. Knowing the bulk and the skeletal volume, it is possible to obtain the porosity of the samples by the following formula:

$$\% \text{ of porosity} = (Vb-Vp)/Vb*100$$

where Vb is the bulk volume and Vp is the volume calculated by the pycnometer.

2.6. Studies of hydrolytic and enzymatic degradation

Scaffolds were weighed, immersed into DPBS pH 7.4 alone or in the presence of esterase (50 U/ml) and incubated in an orbital shaker (35 rpm) at 37 °C. After scheduled times, samples were washed three times with double distilled water and freeze-dried. The degradation was expressed as recovered weight % respect to the starting weight of each sample calculated as:

$$\text{Recovered weight}\% = (\text{final weight/initial weight}) \times 100$$

Each experiment was performed in triplicate.

Only the scaffold with higher porosity and better degradation profile was further studied (scaffold C).

Table 1
Composition of pastes containing PHEA-PLA-MA and NaCl in DMSO employed for scaffold production by their UV irradiation.

Sample	PHEA-PLA-MA concentration w/v	Grain size μm
A	80%	60–90
B	80%	90–125
C	60%	60–90
D	60%	90–125

2.7. Atomic force microscopy (AFM) analysis and compression mechanical studies

The elastic modulus of the scaffold was determined by AFM microscope using a Bruker TESPA wafer A05615 probe having an optical sensitivity of 80 nm/V and a constant force of 14.65 N/m (calculated after calibration with sapphire surface).

The cantilever of the probe has a rectangular geometry and a thickness of 4 μm, the tip has a standard geometry with a radius of 8 nm.

Measurements were taken in four different regions of the sample and loading and unloading curves were obtained with a trig threshold of 2 nm.

To determine the elastic modulus from the force/separation curves, the Hertz model has been used, considering, for all the obtained curves, force boundary between 10 and 70%.

Equine decellularized bone was used as a comparison sample.

Compression mechanical studies were performed on scaffold swollen in DPBS pH 7.4, shaped as disks with 10 mm of diameter and 1.5 mm of height. Samples were tested in compression until failure with a uniform strain rate of 1 mm min^{-1} and with a 1 kN load cell.

2.8. Dexamethasone incorporation and release studies

Dexamethasone was dissolved in acetone (10 mg/ml) and the obtained solution was employed to wet the scaffold. The weight ratio between scaffold and drug was 10:1. Sample was then dried at 37 °C in orbital shaker for 24 h.

The encapsulation efficiency % (EE%) was calculated by comparing the employed initial amount of dexamethasone with that extracted from the film.

To determine the value EE%, dexamethasone containing scaffold was treated with acetone to dissolve entrapped drug, the organic solvent was then filtered and the evaporated under vacuum. The obtained residue was dissolved in DPBS pH 7.4 and the concentration of drug was determined by using a UV spectrophotometer. A calibration curve was made by diluting a solution of dexamethasone in DPBS pH 7.4 with an initial concentration of 0.04 mg/ml.

For the release studies, dexamethasone containing scaffold was immersed into DPBS pH 7.4 (50 ml) and at scheduled times, 2 ml of medium were withdrawn for UV analysis and replaced with the same volume of fresh medium. Each experiment was performed in triplicate.

2.9. Chondrocyte incorporation

Bovine articular chondrocytes were isolated as reported in a previous work [28].

Cylindrical scaffolds of 5 mm diameter and height were sterilized by UV irradiation for 1 h using a 125 W UV-lamp and inserted into each well of 96 well plate.

Chondrocytes were harvested and suspended (10×10^6 cells in 1 ml) in DMEM supplemented with 10% v/v of FBS, 1% v/v of penicillin–streptomycin solution, 1% v/v of glutamine solution and 0.1% v/v amphotericin B solution.

50 μl of obtained cell suspension were poured into each scaffold, plates were incubated for 30 min and then each scaffold was transferred into a 48 well plate with 500 μl of culture medium (supplemented DMEM).

Samples were cultured for 7 and 14 days by refreshing the culture medium every two days. At scheduled times, the metabolic activity of incorporated chondrocytes was evaluated by MTS assay by using supplier instructions and results were expressed as % of cell viability compared to that of chondrocytes cultured in 48 well plate employed as a positive control. Chondrocytes containing scaffolds were investigated by scanning electron microscopy (SEM). For this study, at scheduled times, samples were fixed with 10% v/v formaldehyde, rinsed with water and dehydrated with ethanol series (30%, 50%, 70%, 90% v/v and

pure ethanol), then treated with hexamethyldisilazane and dried in a flow hood.

Dried scaffolds were freeze fractured, situated on the stubs by using an appropriate adhesive tape, gold coated (thickness of gold coating of about 20 nm) and analyzed by SEM in order to observe cells inside scaffolds. Each experiment was performed in triplicate.

2.10. Statistical analysis

Data are presented as means ± standard deviation, SD. t-Test was employed for the statistical analysis, p values littler than 0.05 were considered as statistically significant.

3. Results

3.1. Synthesis of PHEA-PLA and PHEA-PLA-MA graft copolymers

Synthesis of PHEA-PLA was performed in organic solvent, in the presence of DEA as a catalyst, by using a molar ratio between PLA and PHEA repetitive units equal to 0.06.

PHEA-PLA ^{1}H NMR spectrum shows peaks at δ 1.3 and 1.7 (582H of PLA, 2d: O—CO—CH(CH_3)—O—); 2.9 (2H of PHEA, m: —CO—CH—CH_2—CO—NH—); 3.1, (2H of PHEA, t: —NH—CH_2—CH_2—O—); 5.1 (194H of PLA, m: —O—CO—CH(CH_3)—), 4.8 (1 of PHEA, m: —NH—CH(CO)CH$_2$—).

Molar functionalization in PLA for PHEA-PLA copolymer was 2 ± 0.5% as calculated by comparing integrals of the peaks at δ 1.3 and 1.7, attributed to methyl groups of PLA repetitive units, with signals at δ 3.1 and 5.1 attributed to protons of PHEA backbone.

The reaction between PHEA-PLA and methacrylic anhydride (MA) has been performed in DMF in the presence of DMAP as a catalyst, under argon and continuous stirring for 24 h. Molar ratio between MA and PHEA repetitive units was 10.

PHEA-PLA-MA ^{1}H NMR spectrum shows, in addition to the characteristic peaks of PHEA-PLA, also peaks between δ 5.4 to 6.1 attributable to the protons of the methacrylic moieties.

Molar functionalization in MA in final PHEA-PLA-MA copolymer was 20 ± 2% as calculated by comparing integrals of the peaks between δ 5.4 and 6.1 due to protons of methacrylic moieties, with the signals at δ 3.1 and 5.1 attributed to protons of PHEA backbone. Fig. 1 shows the chemical structure and the ^{1}H NMR spectrum of PHEA-PLA-MA copolymer.

3.2. Production of PHEA-PLA-MA porous scaffolds

The high solubility of PHEA-PLA-MA in DMSO allows to produce viscous pastes composed of concentrated solutions of the copolymer containing undissolved particles of NaCl, chosen as a porogen. By changing the concentration of the copolymer (60 and 80% w/v) and the granulometry of salt (60–90 μm and 90–125 μm), four different pastes have been produced. In all cases, the weight ratio between PHEA-PLA-MA and NaCl was 30:70.

Table 1 (see Experimental section) shows the composition of pastes employed for UV irradiation.

For each sample, the crosslinking of PHEA-PLA-MA copolymer was performed by UV irradiation at 366 nm until each paste shifts from a semi-solid to a solid form. Under these conditions, the crosslinking process start to takes place already after 30 min; no significant difference in solidification time has been observed for all samples. The photocrosslinking reaction is showed in Scheme 1.

How it is possible to observe from the Fig. 2, only samples A and C, namely those obtained using NaCl with lower granulometry, show a uniform porous structure.

In addition, these scaffolds show an average pore diameter near to size of NaCl employed in the starting paste and also their inner structure shows a uniform porosity (see Fig. 3).

Taking into account these results, only scaffolds A and C have been employed for further characterization.

3.3. Porosity determination

The determination of porosity has been performed by using the liquid displacement method [27]. Results obtained through this analysis showed that the porosity of sample A and C was 50 ± 4% and 70 ± 6%, respectively.

These results were confirmed by the analysis also performed with the helium pycnometer (49.3 ± 1.9% and 70.5 ± 2.4, respectively for sample A and C). The gas pycnometer allows the knowledge of the real volume of a porous sample, excluding the volume of the pores.

3.4. Studies of hydrolytic and enzymatic degradation

In order to have information about the potential biodegradability of chosen scaffolds (samples A and C) in physiological medium, their weight loss after incubation at 37 °C in DPBS pH 7.4 has been evaluated.
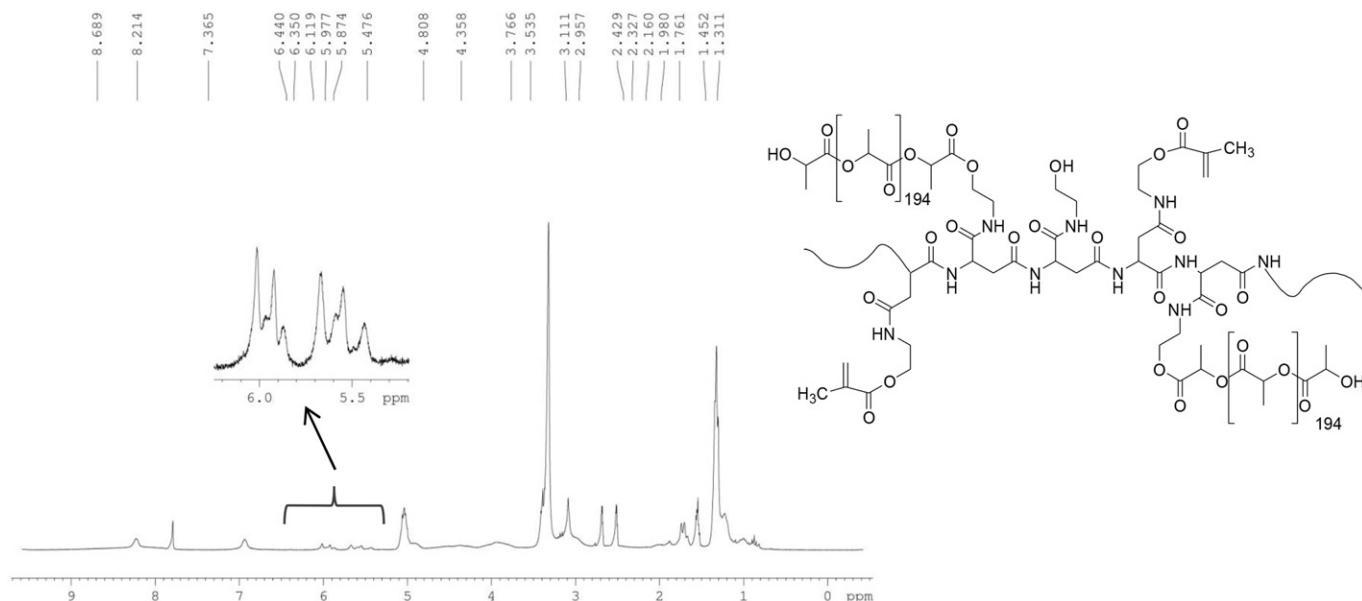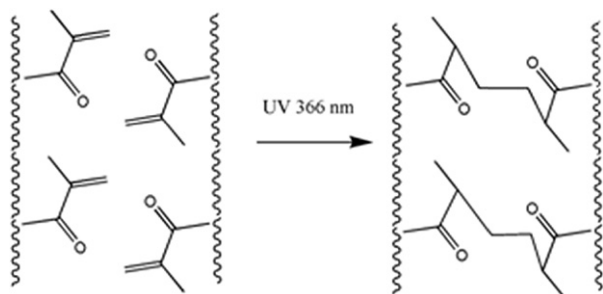

Fig. 1. Chemical structure and ^{1}H NMR spectrum of PHEA-PLA-MA copolymer.

Scheme 1. UV triggered crosslinking reaction of PHEA-PLA-MA.

Experiments were performed both in the absence and in the presence of esterase, ubiquitous enzymes that in vivo could hydrolyze ester bonds of PLA portions in PHEA-PLA-MA.

As shown in Fig. 4a, the hydrolytic degradation profiles of scaffolds A and C are similar over a period of 7 days, where both samples lose 15% about of their initial weight. After 14 days, the degradation is significantly faster for sample C (p < 0.05) and also after 21 days, its weight loss is higher than that of sample A (60% against 30% about) (p < 0.05).

Sample C shows a linear trend throughout the period of analysis (y $= -2.8272x + 102.64 \, R^2 = 0.9943$) while sample A shows a less linear profile (y $= -1.2368x + 93.755 \, R^2 = 0.9622$).

Different research groups have demonstrated in the past years that primary chondrocytes as well as chondro stimulated mesenchymal cell, when cultured in 3D scaffold, if healthy, produce abundant new ECM after 28 days [29,30]. Taking into account these information and considering our obtained results, we believe that sample C shows better hydrolytic degradation profile compared to sample A. In fact, thanks to

its faster degradation, the elimination of the implanted material from the body may occur in tandem with the formation of new tissue [31].

The enzymatic degradation was followed over a period of 7 days to demonstrate if the investigated scaffolds are susceptible to the action of physiological enzymes, like esterase.

As shown in Fig. 4b, for each scheduled time, the weight loss of both scaffolds in the presence of esterase is higher than that observed in DPBS pH 7.4 alone (30% against 15% about), thus demonstrating that the presence of enzyme increases the degradation rate.

No significant (p > 0.05) differences were observed between the investigated scaffolds.

According to what reported in literature, an ideal porous scaffold for tissue engineering purposes must has, among all the other features, a uniform porous structure with highly interconnected pores and must shows an optimal degradation profile [13,32,33].

Both porosity and degradation results, clearly indicate that the scaffold C shows, among the obtained scaffolds, physicochemical features potentially suitable for tissue engineering applications. For this reason, only this scaffold was further investigated.

3.5. Atomic force microscopy (AFM) analysis and compression mechanical studies

Nanoindentation experiment (performed with atomic force microscopy) of the local elastic modulus of PHEA-PLA-MA scaffold (sample C) has showed a value of 1.25 ± 0.5 GPa that is higher than that reported in the literature (0.1–1 MPa) for soft materials, like hydrogels, produced with various natural or synthetic polymers, whose major drawback is the lack of mechanical resistance [34–36].

On the other hand, the elastic modulus of PHEA-PLA-MA scaffold is lower than that of equine decellularized bone (used as a comparison
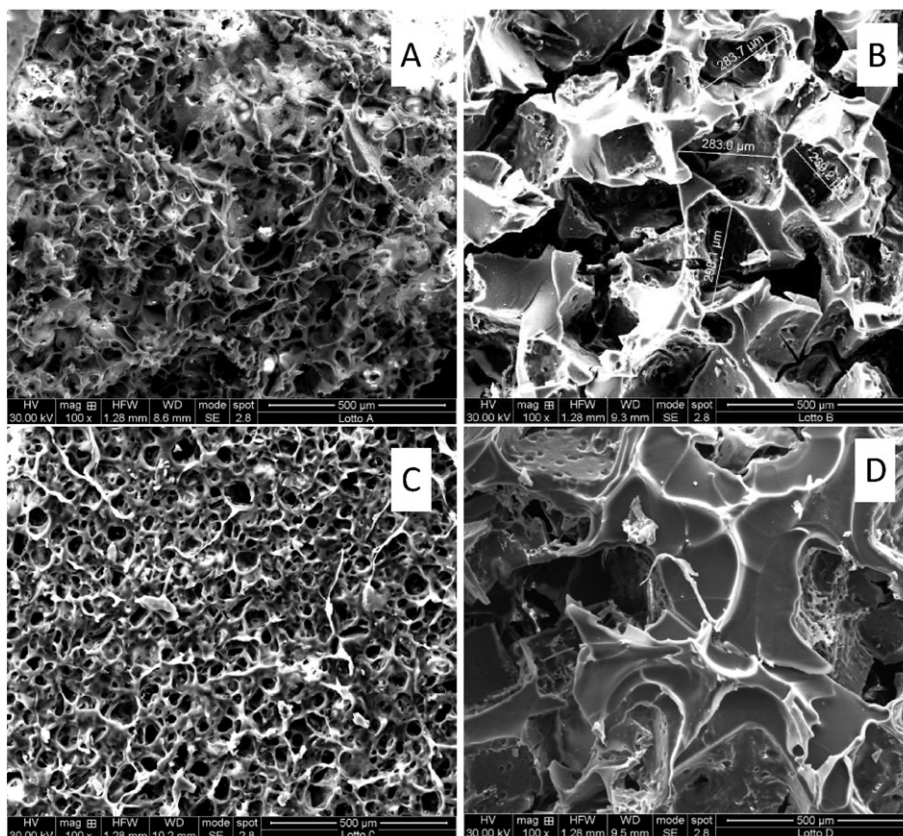

Fig. 2. SEM images of porous scaffolds (samples A, B, C, D) obtained by UV irradiation of pastes containing different concentrations of PHEA-PLA-MA and NaCl with different grain size, after their washing with double distilled water and freeze drying.
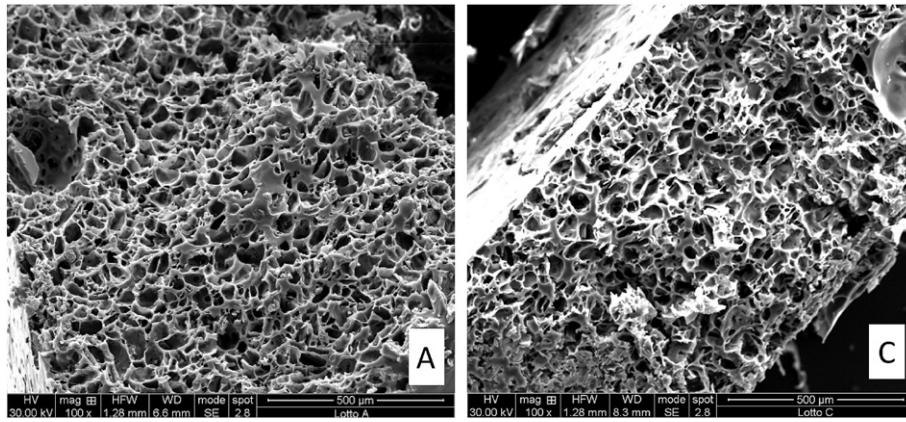
Fig. 3. SEM images of sections of porous scaffold (samples A and C).

sample) that showed a value higher than 5 GPa (out of the cantilever sensitivity).

The bulk elastic modulus, calculated as the slope of the stress/strain curve, resulted to be 48.33 ± 12.11 MPa.

3.6. Dexamethasone incorporation and release studies

The EE% of dexamethasone resulted to be $60 \pm 5\%$.

The release profile of dexamethasone from PHEA-PLA-MA scaffold C in DPBS pH 7.4 is reported in Fig. 5.

As it can be observed, there is only an initial and slight burst effect (lower than 10%) and then the release of the anti-inflammatory drug continues until 150 h.

3.7. Chondrocyte incorporation

In order to evaluate if scaffold C is able to host chondrocytes for a potential cartilage regeneration, bovine articular chondrocytes have been seeded onto the scaffold, both with and without dexamethasone, and their metabolic activity has been determined by MTS assay after 7 and 14 days of culture. Fig. 6 shows that the metabolic activity of the cells cultured in the presence of scaffold is always comparable to that of positive control (about 80%), i.e. cells cultured in bidimensional conditions in the culture plate. In addition, since not significant difference ($p > 0.05$) has been observed for drug loaded and drug unloaded scaffolds, the presence of dexamethasone does not influence viability of chondrocytes.

The increase in cell viability between 7 and 14 days of culture was significant only for the control ($p < 0.05$) thus meaning that probably

cells are not proliferating into the scaffolds due to the high seeding density.

In fact, in Fig. 7 it is possible to observe that many cells are present into the scaffolds and that they take contact with the biomaterial through several protrusions, thus meaning that chondrocytes are recognizing it as a substrate suitable for their adhesion.

These results confirm the cytocompatibility of PHEA-PLA-MA porous scaffold along with its cell adhesiveness.

4. Discussions

The grafting of PLA to PHEA backbone allows the obtainment of the PHEA-PLA copolymer which is insoluble in water and shows still hydroxyl groups exploitable for further chemical derivatization.

Indeed, in our previous works, free primary hydroxyl groups of PHEA in PHEA-PLA copolymer have been already exploited to covalently bind bioactive molecules such as heparin or anti-inflammatory drugs [6, 18]. In addition, high solubility of PHEA-PLA in cytocompatible organic solvents, like dimethyl sulfoxide or N-methyl-2-pyrrolidone, allows to prepare injectable in situ gel forming solutions able to release drugs in a controlled manner [37].

To obtain a photo-crosslinkable copolymer, PHEA-PLA was functionalized with methacrylic anhydride (MA) and the obtained copolymer was named PHEA-PLA-MA.

We have demonstrated that it is possible to obtain chemical networks after UV exposure of PHEA-PLA-MA organic solutions containing NaCl particles with controlled granulometry. These networks give rise to the formation of porous scaffolds when washed with water.

NaCl has been used as a porogen since it is not soluble in DMSO and after the crosslinking process, it remains entrapped into the polymer
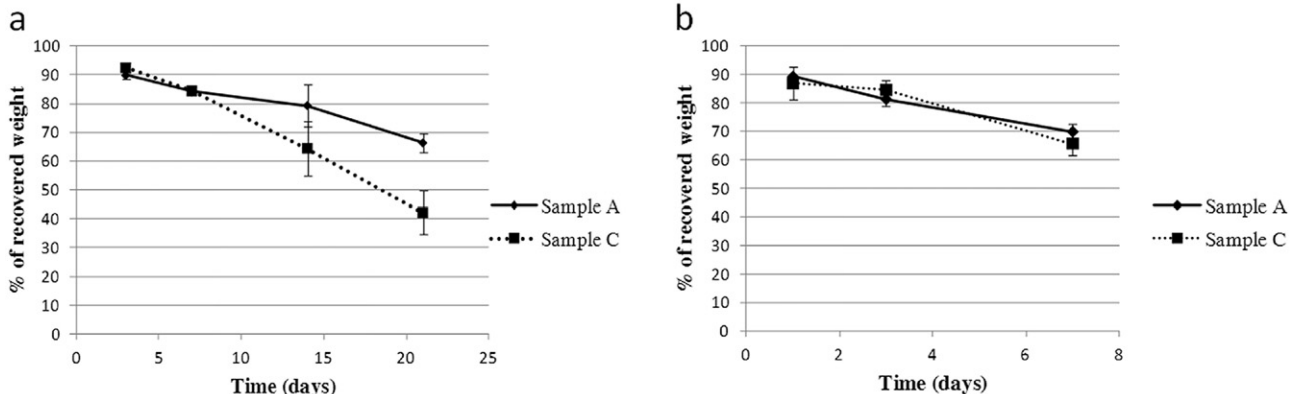

Fig. 4. Percent of weight recovered after treatment of scaffolds A and C with DPBS alone (a) or in the presence of esterase (50 U/ml) (b) as a function of time and respect to starting weight.
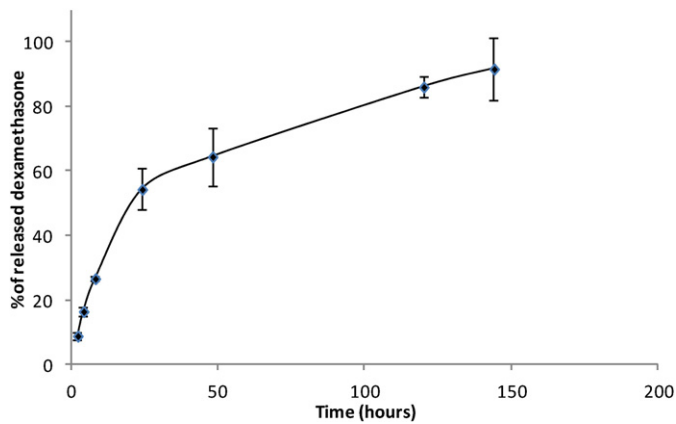
Fig. 5. Release of dexamethasone in DPBS pH 7.4 from PHEA-PLA-MA scaffold (sample C).

network as particles. The washing of each scaffold with double distilled water allows the dissolution of salt and the formation of a highly porous structure.

By changing the copolymer concentration and the NaCl grain size we obtained a series of scaffolds with different morphological characteristics.

However, as already stated, only scaffolds obtained with NaCl at lower grain size (sample A and C) show a uniform and reproducible porosity.

Probably, during washing, the removal of coarse particles of salt (in samples B and D) causes a deformation of the polymer network and a collapse of the porous structure.

For the porosity analysis, scaffolds are immersed into a liquid able to penetrate into the porous three-dimensional structure without causing swelling of the scaffold itself.

Indeed, the rationale of the method is that the liquid should fill the void spaces (pores) when the scaffold is immersed into it, in such a way that the difference of volume of the liquid before and after the scaffold immersion, is directly proportional to the porosity of the scaffold itself. If a swelling process occurs, the amount of liquid into the scaffold is greater than that necessary to occupy the voids and the analysis is not correct. In our experiment, the liquid employed was ethylene glycol, since this liquid does not swell the scaffolds. Moreover, results obtained with the liquid displacement method were also confirmed by analyzing the samples with helium pycnometer by using the method reported by Ho et al. [27].

This analysis gives information not only on the entity of the porosity but also on the interconnection of the pores which is of crucial importance for the diffusion of both oxygen and nutrients.

The difference in porosity between sample A and C could be due to the different concentration of PHEA-PLA-MA in the paste employed for UV irradiation (see Table 1). Probably, the lower polymer concentration in sample C allows the formation of a less compact polymeric network and a more porous structure after salt removal.

The differences in the degradation profile between sample A and C are due probably to a greater clearance of the degradation products caused by higher porosity of sample C. In fact, after an initial lag time for this scaffold, there is an increase in the degradation rate. This hypothesis seems to be confirmed by the analysis of the curves.

For the sample A, the process of elimination of degradation products is not completely concomitant with the degradation process itself and the loss on weight is lower.

Porosity data and degradation profile of scaffold C suggest that this sample is suitable for a potential application in cartilage regeneration. For this reason, scaffold C has been chosen to evaluate its mechanical properties, ability to incorporate and release dexamethasone, and to allow the viability of articular bovine chondrocytes.

We have demonstrated that simply by taking in contact the sample with an organic solution of dexamethasone it is possible to obtain high efficiency of incorporation of the drug. The presence of acetone in dexamethasone solution allows the swelling of PHEA-PLA-MA scaffold thus determining an efficient drug incorporation.

Since the release profile shows only a moderate burst effect and a prolonged dexamethasone release, it reasonable to suppose that the drug establishes hydrophobic interactions with the PLA chains and is retained in the polymeric network once the scaffold is submerged in DPBS pH 7.4.

Nanoindentation experiments performed on a biomaterial by using atomic force microscopy (AFM), give information mainly on mechanical properties of the microenvironment encountered by the cells after their seeding and shed light on possible interactions between the cells and the material itself.

Results obtained suggest that PHEA-PLA-MA scaffold (sample C) could be recognized by chondrocytes as a suitable substrate for their adhesion.

To evaluate if this scaffold could be implanted in the osteochondral region for the treatment of osteoarthritis, it is also important to study its brittleness in bulk.

Indeed, as above reported, AFM gives information concerning only the micrometric elastic modulus, which is important to understand the interactions between cells and scaffold, but it is not suitable to obtain information about bulk mechanical properties of a biomaterial.

For this reason, compression mechanical studies have been performed on scaffold C swollen in DPBS pH 7.4.

Obtained results agree with those reported in the literature for similar scaffolds [38,39] (obtained by employing polyesters as starting materials) and confirm a potential use of this scaffold as substitute of damaged cartilage.

Dexamethasone, chosen as an example of drug employed in the treatment of osteoarthritis, has been loaded into sample C by incubating the scaffold with a solution of drug in acetone (10 mg/ml). Thanks to the amphiphilic properties of PHEA-PLA-MA, the scaffold, when treated with the organic solution of drug, swells thus allowing a penetration of drug molecules into the polymeric network. The amount of incorporated dexamethasone was 85% w/w respect to the initial amount incubated with the scaffold.

The release profile shows that there is only an initial and slight burst effect (<10%), thus demonstrating that only a little amount of dexamethasone migrates on the scaffold surface during the drying process and then it is ready for a fast release.

Probably this phenomenon could be due to PLA lipophilic chains that compete with the acetone in the retention of drug during the removal of organic solvent.
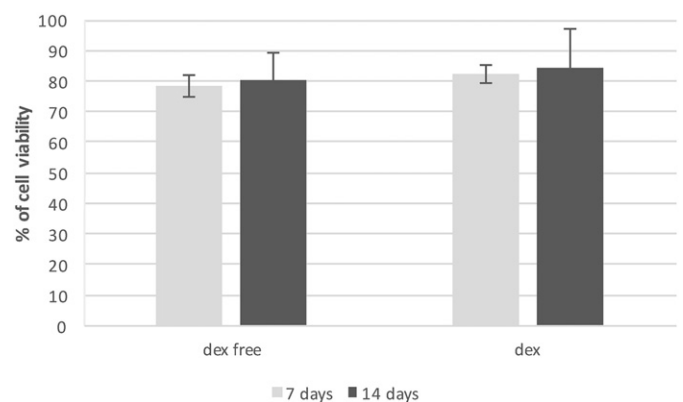

Fig. 6. Cell viability (expressed as % of control viability) of chondrocytes cultured into PHEA-PLA-MA scaffolds in the presence or not of dexamethasone after 7 and 14 days of culture.
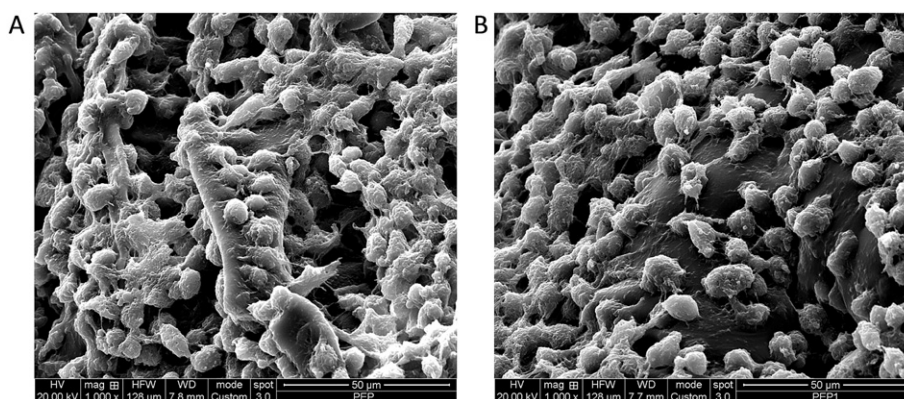
Fig. 7. SEM images of bovine articular chondrocytes cultured for 14 days in PHEA-PLA-MA scaffolds without (A) and with dexamethasone (B).

The release of the anti-inflammatory drug continues until 150 h; this release profile appears particularly interesting since it could guarantee the presence of the drug in the target site in the first days after the scaffold implantation.

Three-dimensional culture study demonstrated that bovine chondrocytes can grow into the scaffold with a good spreading and viability profile as demonstrated by MTS and SEM analysis.

5. Conclusions

By a simple and reproducible chemistry, it is possible to obtain a photocrosslinkable copolymer with a polyaminoacid/polyester structure, named PHEA-PLA-MA, that has been employed to produce a variety of porous scaffolds.

It has been demonstrated that the physicochemical properties of these scaffolds can be simply tailored by varying some parameters such as polymer concentration and granulometry of porogen. In particular, it has been shown that it is possible to obtain a scaffold having optimal degradation profile, porosity and mechanical properties, able to incorporate and to control the release of dexamethasone and to act as a three dimensional support for the culture of bovine articular chondrocytes. In light of these results, PHEA-PLA-MA scaffold seems an optimal biomaterial for potential cartilage regeneration.

References

[1] B.P. Chan, K.W. Leong, Eur. Spine J. 4 (Suppl. 4) (2008) 467–479 17 Suppl.
[2] N. Annabi, J.W. Nichol, X. Zhong, C. Ji, S. Koshy, A. Khademhosseini, F. Dehghani, Tissue Eng. Part B Rev. 16 (4) (2010) 371–383.
[3] M. Biondi, F. Ungaro, F. Quaglia, P.A. Netti, Adv. Drug Deliv. Rev. 60 (2) (2008) 229–242.
[4] Y. Tabata, Drug Discov. Today 10 (23–24) (2005) 1639–1646.
[5] Z. Ma, P. Zhang, X. Yu, H. Lan, Y. Li, D. Xie, J. Li, T. Yi, J. Mater. Chem. B 3 (37) (2015) 7366–7371.
[6] G. Pitarresi, C. Fiorica, F.S. Palumbo, F. Calascibetta, G. Giammona, J. Biomed. Mater. Res. A 100 (6) (2012) 1565–1572.
[7] I. Cantón, R. Mckean, M. Charnley, K.A. Blackwood, C. Fiorica, A.J. Ryan, S. MacNeil, Biotechnol. Bioeng. 105 (2) (2010) 396–408.
[8] F. Carfì Pavia, V. La Carrubba, V. Brucato, F.S. Palumbo, G. Giammona, Mater. Sci. Eng. C 41 (2014) 301–308.
[9] X. Wu, Y. Liu, X. Li, P. Wen, Y. Zhang, Y. Long, X. Wang, Y. Guo, F. Xing, J. Gao, Acta Biomater. 6 (3) (2010) 1167–1177.
[10] M. Flaibani, N. Elvassore, Mater. Sci. Eng. C 32 (6) (2012) 1632–1639.
[11] S.A. Poursamar, J. Hatami, A.N. Lehner, C.L. da Silva, F.C. Ferreira, A.P.M. Antunes, Mater. Sci. Eng. C 48 (2015) 63–70.
[12] S.C. Cox, J.A. Thornby, G.J. Gibbons, M.A. Williams, K.K. Mallick, Mater. Sci. Eng. C 47 (2015) 237–247.
[13] Q.L. Loh, C. Choong, Tissue Eng. Part B Rev. 19 (6) (2013) 485–502.
[14] S. Saber-Samandari, S. Saber-Samandari, Mater. Sci. Eng. C 75 (2017) 721–732.
[15] Z. Fereshteh, M. Fathi, A. Bagri, A.R. Boccaccini, Mater. Sci. Eng. C 68 (2016) 613–622.
[16] Poly(Lactic Acid): Synthesis, Structures, Properties, Processing, and Applications - Wiley Online Library, 2010.
[17] G. Pitarresi, F.S. Palumbo, C. Fiorica, F. Calascibetta, G. Giammona, Eur. Polym. J. 46 (2) (2010) 181–184.
[18] G. Pitarresi, C. Fiorica, F.S. Palumbo, S. Rigoglioso, G. Ghersi, G. Giammona, J. Biomed. Mater. Res. A 102 (5) (2014) 1334–1341.
[19] C. Fiorica, F.S. Palumbo, G. Pitarresi, A. Gulino, S. Agnello, G. Giammona, RSC Adv. 5 (25) (2015) 19715–19723.
[20] C. Fiorica, R.A. Senior, G. Pitarresi, F.S. Palumbo, G. Giammona, P. Deshpande, S. MacNeil, Int. J. Pharm. 414 (1–2) (2011) 104–111.
[21] 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 (9) (2007) 3953–3962.
[22] Y. Li, Y. Wang, S. Chubinskaya, B. Schoeberl, E. Florine, P. Kopesky, A.J. Grodzinsky, Osteoarthr. Cartil. 23 (2) (2015) 266–274.
[23] H. Kim, H.W. Kim, H. Suh, Biomaterials 24 (25) (2003) 4671–4679.
[24] H.A. Awad, Y.-D.C. Halvorsen, J.M. Gimble, F. Guilak, Tissue Eng. 9 (6) (2003) 1301–1312.
[25] R. Mendichi, A. Giacometti Schieroni, G. Cavallaro, M. Licciardi, G. Giammona, Polymer (Guildf) 44 (17) (2003) 4871–4879.
[26] E.F. Craparo, G. Teresi, M.C. Ognibene, M.P. Casaletto, M.L. Bondì, G. Cavallaro, J. Nanopart. Res. 12 (7) (2010) 2629–2644.
[27] S.T. Ho, D.W. Hutmacher, Biomaterials 27 (8) (2006) 1362–1376.
[28] F.S. Palumbo, C. Fiorica, M. Di Stefano, G. Pitarresi, A. Gulino, S. Agnello, G. Giammona, Carbohydr. Polym. 122 (2014) 408–416.
[29] N.-C. Cheng, B.T. Estes, T.-H. Young, F. Guilak, Regen. Med. 6 (1) (2011) 81–93.
[30] M. Leyh, A. Seitz, L. Dürselen, H.-R. Springorum, P. Angele, A. Ignatius, J. Grifka, S. Grässel, Stem Cell Res. Ther. 5 (3) (2014) 77.
[31] F.J. O'Brien, Mater. Today 14 (3) (2011) 88–95.
[32] S. Mohanty, L.B. Larsen, J. Trifol, P. Szabo, H.V.R. Burri, C. Canali, M. Dufva, J. Emnéus, A. Wolff, Mater. Sci. Eng. C 55 (2015) 569–578.
[33] H. Janik, M. Marzec, Mater. Sci. Eng. C 48 (2015) 586–591.
[34] C.D. Markert, X. Guo, A. Skardal, Z. Wang, S. Bharadwaj, Y. Zhang, K. Bonin, M. Guthold, J. Mech. Behav. Biomed. Mater. 27 (2013) 115–127.
[35] Z. Drira, V.K. Yadavalli, J. Mech. Behav. Biomed. Mater. 18 (2013) 20–28.
[36] Q.T. Nguyen, Y. Hwang, A.C. Chen, S. Varghese, R.L. Sah, Biomaterials 33 (28) (2012) 6682–6690.
[37] C. Fiorica, F.S. Palumbo, G. Pitarresi, M. Giorgi, F. Calascibetta, G. Giammona, J. Pharm. Pharmacol. 67 (1) (2015) 78–86.
[38] R. Scaffaro, F. Lopresti, L. Botta, S. Rigoglioso, G. Ghersi, J. Mech. Behav. Biomed. Mater. 54 (2015) 8–20.
[39] R. Scaffaro, F. Lopresti, L. Botta, S. Rigoglioso, G. Ghersi, Macromol. Mater. Eng. (2015) (n/a-n/a).