

Microstructure, degradation and in vitro MG63 cells interactions of a new poly(ϵ -caprolactone), zein, and hydroxyapatite composite for bone tissue engineering

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Abstract

Novel biodegradable biomaterials were investigated for potential application in bone tissue engineering. The biomaterials were prepared by blending poly(ϵ -caprolactone) and thermoplastic zein, a corn protein, with or without the incorporation of hydroxyapatite particles. The biomaterials were characterized in vitro to assess the degradation in phosphate buffer saline for 56 days by monitoring weight change, morphology, wettability, and tensile properties. The interaction between the biomaterials and MG63 was evaluated by proliferation, morphological characterization, and osteogenic differentiation assays up to 28 days in in vitro cultures. The incorporation of thermoplastic zein within poly(ϵ -caprolactone) enhanced the hydrophilicity and degradability, while minor effects were observed after the inclusion of the hydroxyapatite particles. Compared to the neat poly(ϵ -caprolactone), the multiphase poly(ϵ -caprolactone)/thermoplastic zein–hydroxyapatite composite improved the osteogenic differentiation of MG63 cells and is being considered a candidate material for bone tissue engineering applications.

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Introduction

In the past two decades, a great deal of attention has been directed toward creating bioactive and biodegradable polymeric composites to be used as a bone grafting material and suitable for the manufacture of porous scaffolds for bone tissue engineering (bTE) applications.^{1,2} These composites exploit the flexibility and processability of polymers with the stiffness, strength, and bioactive character of the inorganic fillers.²

Currently, natural-based biopolymers, mainly proteins (e.g. collagen, gelatin, zein, and silk) and saccharides (e.g. starch, alginate, and chitosan), are primary candidate materials for the design of bioactive composites for bTE applications, owing to their similarity with some of the most important organic components of the extracellular matrix (ECM) of native bone.³ The integration of calcium phosphate particles, such as hydroxyapatite (HA), within natural-based polymers may possibly be used for the fabrication of multiphase biomimetic composites with improved mechanical and biological response.^{1,2,4}

Recently, vegetable proteins, such as soybean and zein, are being explored as a new class of bone tissue-compatible materials.⁵⁻⁷ For example, Santin et al.⁵ have fabricated bioactive and biodegradable soybean-based bone fillers by thermosetting defatted soybean curd. They also showed that the final material could be processed into films, porous scaffolds, and granules for different surgical needs and was able to induce osteoblast cells differentiation *in vitro*.⁵

Also, zein, a major storage protein of corn, has been investigated for bTE purposes, particularly, zein and zein/inorganics composite porous scaffolds for bTE prepared via the salt leaching technique.^{6,7} The scaffolds were found to be able to support mesenchymal stem cell (MSC) adhesion, proliferation, and osteogenic differentiation *in vitro*.⁶ When implanted into the radius defects of rabbits, the zein scaffolds improved the ability of MSCs to promote the repair of critical-sized bone defects.⁷

To date, the nonthermoplastic behavior of natural polymers has significantly limited the melt-based processing of these materials, while the addition of gelation agents, such as water, and/or plasticizers, has reduced the melt viscosity to the level suitable for extrusion and injection molding processes.^{3,8} In addition, the physical properties of polymeric composites of natural origin, which are often rapidly degradable and excessively brittle, make these materials inadequate for *in vivo* bTE applications, where an appropriate prolonged mechanical support is required to withstand stresses and loading.^{9,10}

Blending natural and synthetic polymers may represent a suitable strategy to overcome the limitations mentioned. The unique biological, chemical, and physical functionalities of natural polymers can be matched with a wide variety of structures achievable for synthetic polymers, allowing the creation of multiphase biomaterials with improved performances. For instance, blending soy protein with poly(ϵ -caprolactone) (PCL) improved the processability and the mechanical properties of the soy protein.¹¹ Multiphase synthetic and natural polymeric blends have also been developed for the fabrication of porous scaffolds with improved biocompatibility.¹²⁻¹⁴

Our research group is currently investigating the use of PCL blends and thermoplasticized proteins in bTE.^{13,15,16} In particular, we have produced two novel thermoplastic proteins, thermoplastic gelatin (TG) and thermoplastic zein (TZ). We subsequently prepared incompatible PCL/TG and PCL/TZ cocontinuous blends by melt mixing process.^{15,16} PCL is also regarded as a bone tissue-compatible material, due to its ability to support *in vitro* and *in vivo* bone cell/tissue growth for

several months, without inducing a toxic response and preserving its mechanical function.^{2,13} The biocompatibility of these materials was also assessed *in vitro*, demonstrating that a multiphase system composed of PCL, TZ, and HA particles may improve the osteogenic differentiation of MSCs cultured in osteogenic medium.¹⁶ This result raises the questions of why this biomaterial enhanced cell behavior and how this behavior is correlated to its physical and chemical properties.

In this study, the *in vitro* degradation and biological response of multiphase TZ-based biomaterials with potential applications for bTE was assessed. MG63 osteoblasts were cultured on the biomaterials without osteogenic induction factors in the culture medium, and their adhesion, morphology, proliferation, and osteogenic differentiation were evaluated up to 28 days of culture.

Materials and methods

Materials

PCL ($M_w = 65$ kDa) and maize zein powder (Z3625, batch: 065K0110) were purchased from Sigma–Aldrich (Milan, Italy). Poly(ethylene glycol) (PEG) 400 was purchased from Fluka (Milan, Italy) and used as plasticizer for the preparation of the TZ. Microporous HA particles (ENGIpore, batch 071105, 250–355 μm size) were kindly supplied by Finceramica (Faenza, Italy).

Scaffolds preparation

Nonporous scaffolds for *in vitro* study were prepared by melt mixing/compression molding process, as described previously.¹⁶ Briefly, the TZ was prepared by mixing the zein powder with PEG 400 (4:1 mass ratio, respectively) in a twin counter rotating internal mixer (Rheomix 600 Haake, Thermo Electron, Karlsruhe, Germany) controlled by a measuring drive unit (Rheocord 9000 Haake, Thermo Electron, Karlsruhe, Germany) at 80°C and 50 rpm for 10 min. The multiphase biomaterials were prepared by using the same equipment at 70°C and 80 rpm for 6 min. The polymers weight ratios of the PCL/TZ blend and PCL/TZ–HA composite equal to 60/40 and 48/32/20, respectively, which were selected in order to allow for the formation of cocontinuous PCL/TZ systems and, therefore, to ensure the exposure of the two polymers even in the absence of a 3D pore structure. Neat PCL and a 80/20 (w/w) PCL–HA composite were also prepared by melt mixing for comparison. The biomaterials were finally compression molded at 80°C and 3 MPa into 1- or 2-mm-thick plates by a hot press (P300P, Collin, Germany).

In vitro degradation

The *in vitro* degradation tests were carried out on dog bone specimens to correlate the results of the gravimetric and morphological measurements with those of the tensile tests. At first, the samples were weighted and sterilized by γ -irradiation at a dose of 2.5 Mrad for 8 min and at room temperature (RT). Subsequently, the samples were placed in six-well culture plates (1 sample/well), immersed in 10 mL of phosphate buffer saline (PBS), and incubated at 37°C and 5% CO_2 up to 56 days, without refreshing media. At predetermined incubation intervals, three samples per group were dried under vacuum and weighted to assess the weight loss, as a consequence of possible dissolution of the polymeric phases into the PBS solution. The weight loss was evaluated as the percent weight loss with respect to the initial dry weight. Gravimetric measurements were performed by using a high-accuracy balance (AB104-S; Mettler Toledo, Milan, Italy). The incubation medium was also collected for pH measurement, and the effect of the degradation on the morphology,

wettability, and tensile properties of the biomaterials was investigated in a dry state by scanning electron microscope (SEM) analysis, contact angle (CA) measurement, and static tensile test, respectively.

SEM analysis. The surface and cross sections (obtained by cross-sectioning with a razor blade) of the samples were gold sputtered and analyzed by SEM (S440, LEICA, Wetzler, Germany) at an accelerating voltage of 20 kV, at various magnifications.

CA measurement. The tests were performed on a Contact Angle System OCA20 (Dataphysics, Milan, Italy), and the CA was determined using a tangent placed at the intersection of the liquid and solid phases by Software SCA20 (Dataphysics). For each sample, an ultrapure water droplet with a volume of 1 μL was dispensed over 10 different areas from which average and standard deviation of CA values were calculated.

Tensile testing. A 4204 Universal Testing Machine (Instron, Norwood, USA) equipped with a 1-kN load cell was used at RT according to ASTM standard D1708-02. The recorded data were analyzed in order to evaluate yielding stress (σ_Y) and strain (ϵ_Y) and stress (σ_B) and elongation (ϵ_B) at break. Three samples for each composition and degradation interval were tested.

Atomic force microscopy (AFM) imaging: The surface of the biomaterials was assayed with AFM imaging using contact mode at RT in wet with the BioAFM NanoWizard II (JPK, Berlin, Germany) apparatus. To simulate the effect of culture media on surface topography, the samples were treated in a serum-free medium for 2 h at 37°C, washed with PBS, and analyzed with AFM. Sharp silicon nitride microlevers (MSCT, Veeco, Cambridge, UK) with a 20 nm radius of curvature and nominal spring constant of 0.1 N/m were used for the analysis. The test was carried out at a scan rate and image resolution of 1 Hz and 512 \times 512 pixels, respectively. Different square areas (30 \times 30 μm) of the samples surface were examined, and the images were corrected for bow/tilt by a first-order plane fit correction, using JPK software.

Cell/scaffold interactions

Human osteoblast MG63 cells, kindly provided by Prof. Quarto, University of Genoa, Italy, were used to assess the biological response of the biomaterials. Cells were first cultured in a 75-cm³ flask at 37°C and 5% CO₂, washed with PBS (Sigma–Aldrich, Italy), and incubated with trypsin–ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin, 1 mM EDTA; Euroclone, Italy) for 5 min at 37°C. Disk-shaped nonporous scaffolds (d = 10 mm and h = 2 mm) were γ -sterilized, prewetted with medium for 2 h and, statically seeded with 1 \times 10⁴ cells/scaffold, resuspended in 50 μL of the medium, and placed in 24-well culture plates (1 scaffold/well), following incubation for 2 h to allow for cell adhesion. Subsequently, cell culture medium was added to each well to bring the total well volume to 1.5 mL. Culture medium used was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (Gibco-BRL Life Technologies, Milan, Italy) and antibiotics (penicillin G sodium 100 U/mL and streptomycin 100 $\mu\text{g}/\text{mL}$; Euroclone, Milan, Italy).

Cell viability and proliferation were evaluated by using AlmarBlue[®] assay. At predetermined time points, the cell/scaffold constructs were removed from the culture plates, washed with PBS, and placed into 24-well culture plates. DMEM medium (2 mL), containing 10% (v/v) of AlmarBlue, was added to each well; the constructs were then incubated for 4 h at 37°C and 5% CO₂. The final solution was collected and analyzed by a spectrophotometer at wavelengths 570 nm and 600 nm.

The number of viable cells per scaffold was assessed by comparing the absorbance to those on a calibration curve obtained by the correlation between a known cell number into the 24-well culture plates and the correspondent absorbance value. Five scaffolds for each composition were used for the proliferation test.

The cell adhesion and morphology were investigated by SEM analysis. The cell/scaffold constructs were extracted from the wells, washed with PBS, and fixed with 2.5% glutaraldehyde in 0.1 M Na-cacodylate at pH 7.4. Before SEM examination, the constructs were freeze-dried overnight. The functional activity of the proliferated cells was examined by measuring the alkaline phosphatase (ALP) activity, by alizarin red (AR) staining and immunofluorescent detection of osteopontin (OPN).

For the ALP activity test, at 7, 14, and 21 days of culture, the cell/scaffold constructs were washed twice with ice cold PBS, transferred to centrifuge tubes containing 300 μ L of cell lysis buffer (10 mM Tris-HCl, 10 mM $\text{NaH}_2\text{PO}_4/\text{NaHPO}_4$, 130 mM NaCl, 1% Triton X-100, and 10 mM sodium pyrophosphate), and lysed at -4°C for 45 min. After 5 min of centrifugation, the total amount of DNA was detected using PicoGreen[®] assay, and the ALP activity was measured using a biochemical assay. Four scaffolds for each composition were used for the ALP test.

The mineralized matrix was quantified by using alizarin red S (ARS) assay (Chemicon, Italy). At days 21 and 28 of culture, the cell/scaffold constructs were extracted from the wells, fixed with 4% paraformaldehyde solution for 20 min, rinsed three times with PBS, and incubated with ARS for 30 min. The constructs were rinsed three times with H_2O , incubated with 10% acetic acid for 30 min, and sonicated. After heating at 85°C for 10 min, the acidic supernatant pH was neutralized with 10% ammonium hydroxide. The optical density of the solution was analyzed by a spectrophotometer working at 405 nm, and the concentration of ARS was determined by comparing the absorbance values of the constructs with ARS standards. Four scaffolds for each composition were used for the ARS test.

Indirect immunofluorescent detection of OPN was performed on the cell/scaffold constructs at 21 and 28 days of culture. The constructs were removed from the wells and fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.2, for 30 min at RT. After washing with PBS, constructs were processed for immunofluorescence labeling. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min, washed with PBS, and treated with PBS-bovine serum albumin (BSA) 0.5% for 10 min to saturate nonspecific binding sites. Subsequently, the constructs were incubated for 1 h at RT with rabbit anti-human OPN and diluted (1:80 w/v) in PBS-BSA 0.5%. Finally, the constructs were rinsed twice with PBS for 5 min, and fluorescein-conjugated anti-rabbit IgG diluted 1:50 w/v in PBS-BSA 0.5% was used as a secondary antibody for 1 h at RT.

Statistical analysis

The statistical significance of the results was assessed by one-way analysis of variance (ANOVA). Tukey's post hoc test at the significance level $p < 0.01$ was used to identify statistically different groups by using Origin[®] software package.

Results and discussion

Tailoring the degradation rate of the scaffold biomaterial is a very important target of bTE.¹ The effect of degradation on the weight loss and pH change of the biomaterials up to 56 days is shown in Figure 1. The neat PCL and PCL-HA composite did not undergo any significant weight loss (Figure 1(a)) or morphological change (data not shown) over time, which indicated that longer

degradation times or more aggressive degradation media would be required to have an impact on the microstructural properties, as reported elsewhere.¹⁷ Conversely, the presence of TZ enhances the hydrophilicity (Table 1) and accelerates the degradation of PCL (Figure 1(a)). The weight loss of PCL/TZ and PCL/TZ–HA increased up to $12.1\% \pm 0.9\%$ and $10.7\% \pm 0.8\%$, respectively, at day 14, and remained almost unchanged during the following 6 weeks. The pH of the incubation media decreased progressively with time for all samples (Figure 1(c)). Consistent with the weight loss results, the decrease in pH was promoted by the TZ but was slightly retarded by the presence of HA particles. These results are in agreement with those of the morphological characterization of the PCL/TZ and PCL/TZ–HA samples before (Figure 2(a) and (b)) and after (Figure 2(c) to (h)) 28 days of in vitro degradation. Before degradation, both the systems exhibited nonporous surfaces, with the PCL/TZ–HA surface characterized by the presence of HA particles and enhanced roughness compared to the PCL/TZ surface. After 28 days of degradation, the PCL/TZ and PCL/TZ–HA surfaces showed extensive TZ protrusions over the smooth PCL phase (black arrows in Figure 2(c) and (d)), which were ascribed to the swelling of the TZ phase and, for the composite, also ascribed to the partial exposure of the HA particles (white arrows, Figure 2(d)). The analysis of the cross sections of the degraded samples showed that degradation occurred preferentially within the TZ phase (Figure 2(e)), with the formation of small pores of a few microns diameter (indicated by black arrows in Figure 2(g)).

Taking into account the high nonpolar amino acid content of zein,⁶ which renders this protein almost insoluble in aqueous environments, these results indicated that the degradation of the PCL/TZ and PCL/TZ–HA samples was mainly attributable to the PEG molecules, which increased the mobility of the polymeric chains. In addition, the cocontinuity of the polymeric network of the two polymers ensured the constant exposure of TZ to the external medium and, therefore, the continuous dissolution of the plasticizer in PBS. As a result, the final weight losses by the PCL/TZ and PCL/TZ–HA samples, 10% and 8%, respectively, were very close to the nominal PEG concentration, and we also observed the decrease of the pH of the solution to 6.5 (Figure 1(b)).

The wettability test data for the surface of the samples at different degradation times are in Table 1. The neat PCL and PCL–HA had CA values of $82.5^\circ \pm 5.5^\circ$ and $83.9^\circ \pm 6.5^\circ$, respectively, and minor differences were observed after the degradation process. The inclusion of TZ increased sample wettability, as indicated by the CA values of PCL/TZ and PCL/TZ–HA, which were $72^\circ \pm 1.6^\circ$ and $72.7^\circ \pm 3.1^\circ$, respectively, and in agreement with the more hydrophilic nature of zein,¹⁸ compared to PCL.¹⁹ For PCL/TZ and PCL/TZ–HA, a slight decrease of the CA to $67.3^\circ \pm 3.8^\circ$ and $69.1^\circ \pm 2.8^\circ$, respectively, was observed after 56 days of degradation, probably due to the enhanced exposure of the hydrophilic phases on the surface (Figure 2(c) and (d)). Wettability tests also indicated that the addition of HA particles alone was not sufficient to increase PCL hydrophilicity

Table 1. Contact angle values of the different biomaterials as a function of the degradation time, obtained by the wettability tests

Days	PCL	PCL–HA	PCL/TZ	PCL/TZ–HA
0	82.5 ± 5.5	83.9 ± 6.5	72 ± 1.6	72.7 ± 3.1
1	82.6 ± 5.9	81.9 ± 1.9	74.1 ± 2.7	72.8 ± 3.6
28	80.8 ± 3.9	84 ± 3.9	69.1 ± 1.5	70.5 ± 2.4
56	81.5 ± 4.3	82.3 ± 2.1	67.3 ± 3.8	69.1 ± 2.8

PCL: poly(ϵ -caprolactone); TZ: thermoplastic zein; HA: hydroxyapatite.

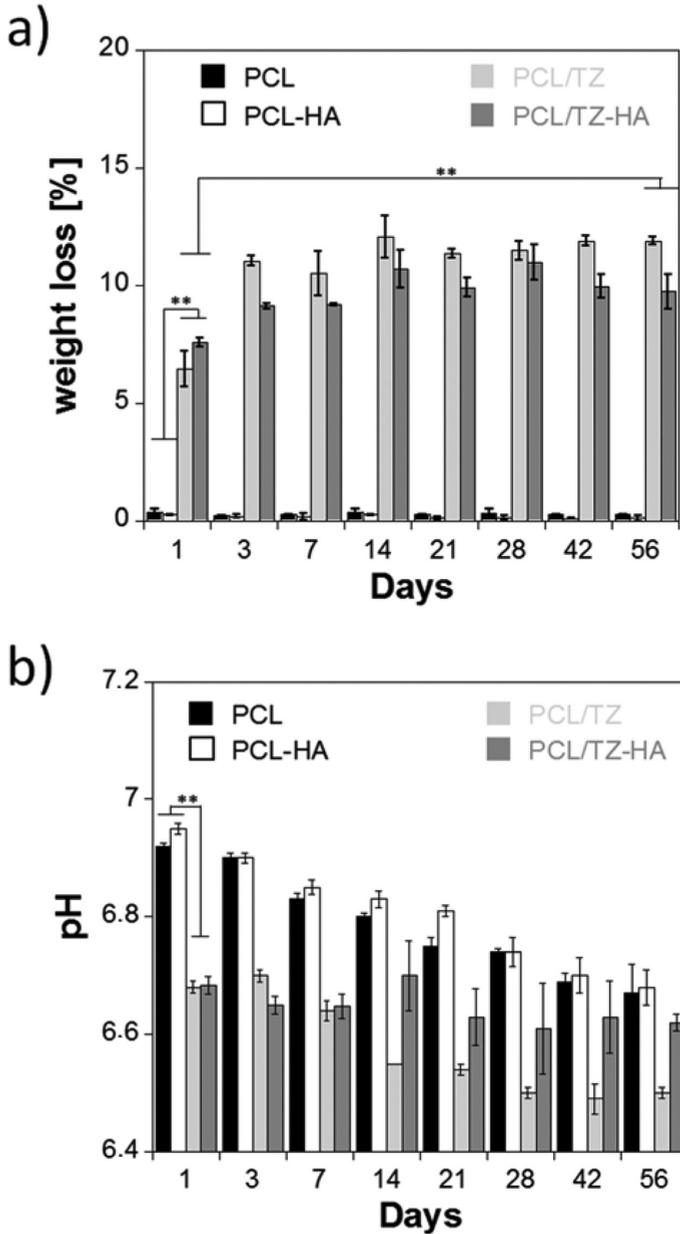


Figure I. (a) Weight loss and (b) pH as a function of degradation time.

PCL: poly (ϵ -caprolactone); TZ: thermoplastic zein; HA: hydroxyapatite.

(Table 1). This was probably due to the increase in surface roughness by the PCL-HA, which is reported to exert a negative influence on wettability.²⁰

Adequate mechanical support is a key requirement for bTE. The effect of the degradation process on mechanical properties of different biomaterials is shown in Figure 3. In agreement

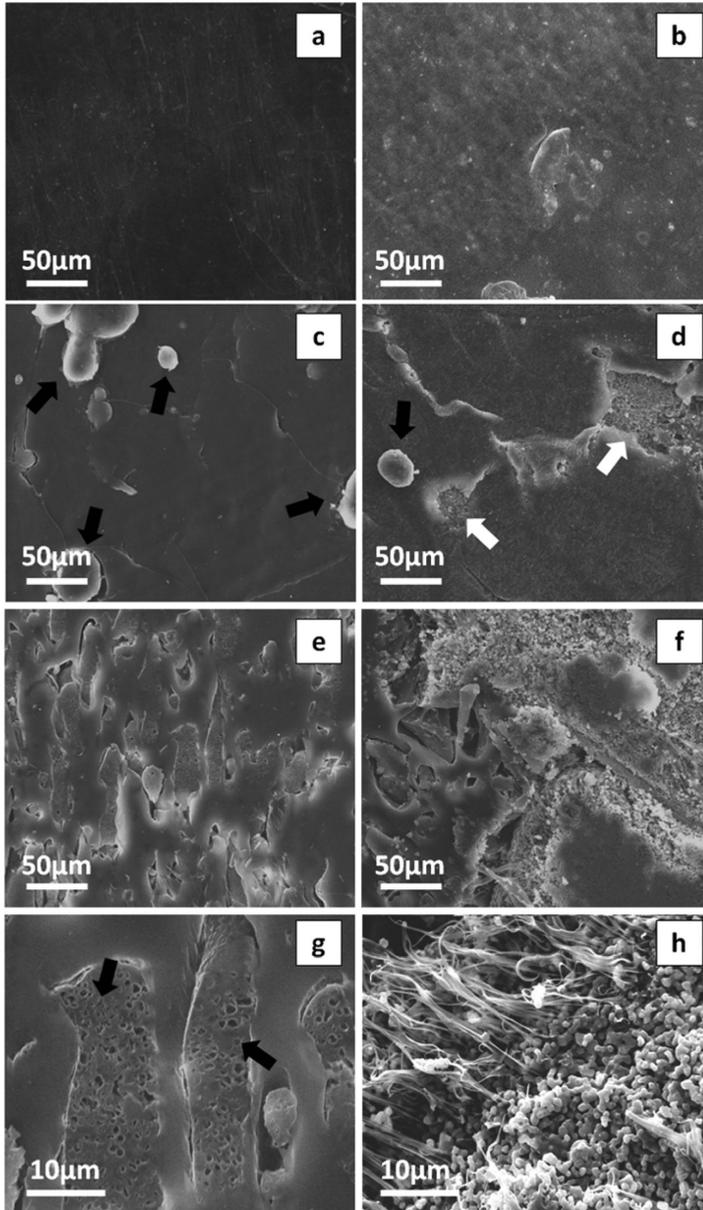


Figure 2. SEM micrographs of the surface of PCL/TZ (a) before and (c) after 28 days of in vitro degradation. SEM micrographs of the surface of PCL/TZ–HA (b) before and (d) after 28 days of in vitro degradation. SEM micrograph of the cross sections of (e and g) PCL/TZ and (f and h) PCL/TZ–HA after 28 days of in vitro degradation. The black arrows indicate the TZ phase, while the white arrows indicate the HA particles.

SEM: scanning electron microscope; PCL: poly(ϵ -caprolactone); TZ: thermoplastic zein; HA: hydroxyapatite.

with previous results, the mechanical properties of the PCL and PCL–HA samples were not dependent on degradation time, with neat PCL characterized by higher yield and break values. The ϵ_Y and σ_Y values of neat PCL were equal to 0.0990 ± 0.0500 mm/mm and 21.5 ± 1.2 MPa,

respectively, but 0.0510 ± 0.0045 mm/mm and 17.2 ± 0.7 MPa for the PCL–HA composite (Figure 3(a) and (c)). The degradation of TZ induced the decrease of the tensile properties of the biomaterials over time; for example, ϵ_Y and σ_Y values for the PCL/TZ decreased from 0.0440 ± 0.0100 mm/mm and 11.0 ± 0.5 MPa to 0.0300 ± 0.0070 mm/mm and 9.05 ± 1.2 MPa, respectively, after 7 days of degradation. Similar results were reported by Corradini et al.²¹ for PCL/zein blend and by Wan et al.²² for bacterial cellulose fiber–reinforced starch

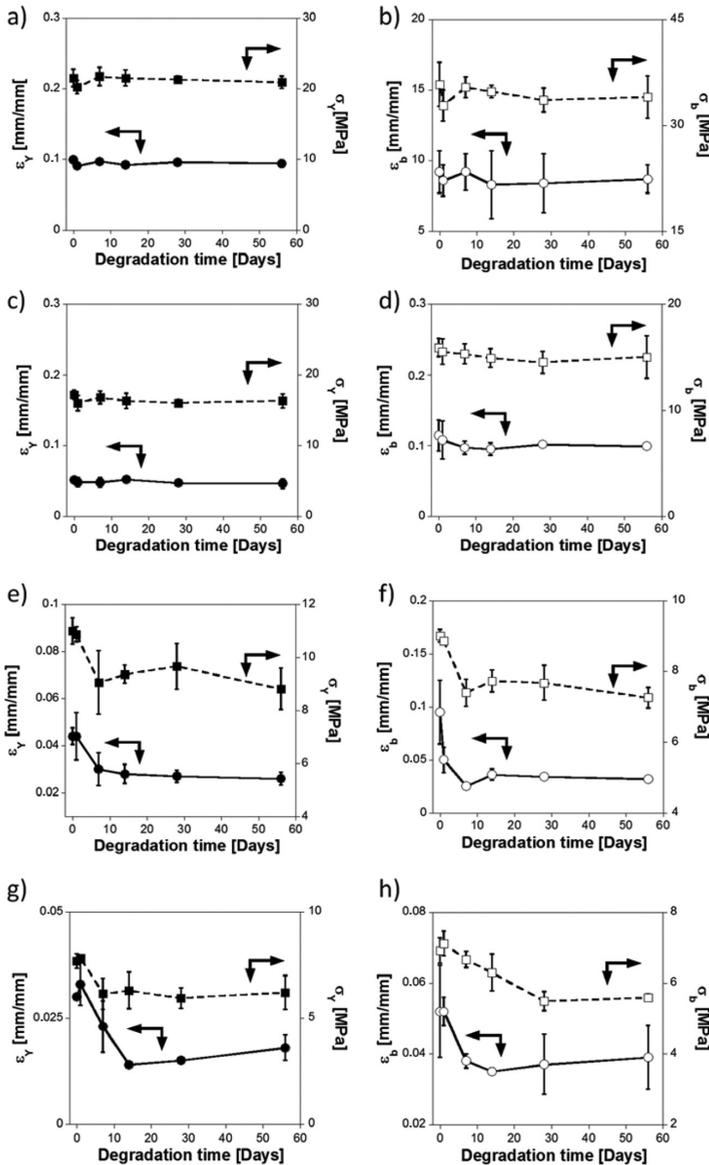


Figure 3. Evolution of the mechanical properties over the in vitro degradation of the scaffold biomaterials: (a and b) PCL, (c and d) PCL–HA, (e and f) PCL/TZ, and (g and h) PCL/TZ–HA. PCL: poly(ϵ -caprolactone); TZ: thermoplastic zein; HA: hydroxyapatite.

biocomposites, demonstrating that the incompatibility between the constituent materials and the accelerated degradation of the natural phase induced a faster decrease of the mechanical response of the biomaterials.

Cell interaction with a biomaterial scaffold starts at the surface. Therefore, surface topology is an extremely important factor, especially in the early stages of cell adhesion and proliferation.^{23,24} The AFM images of the wet surface of the samples are shown in Figure 4. The PCL was characterized by a rather smooth surface with uniformly distributed short spikes (Figure 4(a)). A similar result was achieved for the PCL–HA composite, although in this case, we observed a slight increase of the distribution of the spikes, and the presence of surface prominences due to HA particles (Figure 4(b)). Compared to PCL and PCL–HA samples, PCL/TZ and PCL/TZ–HA samples are characterized by greater surface irregularity, due to enhanced compositional heterogeneity and TZ and HA protrusions (Figure 4(c) and (d)). The increased irregularity of the surface of the PCL/TZ and PCL/TZ–HA samples in wet conditions, as well as the presence of TZ and HA phases exposed to the seeding surface, may affect cell/material interactions and therefore must be taken into account in further evaluation of the biocompatibility results.

Cell/scaffold interactions

For the design and characterization of biomaterials for bTE, the selection of the cell source and culture conditions is a critical parameter, as it greatly influences the seeded cell behavior and the

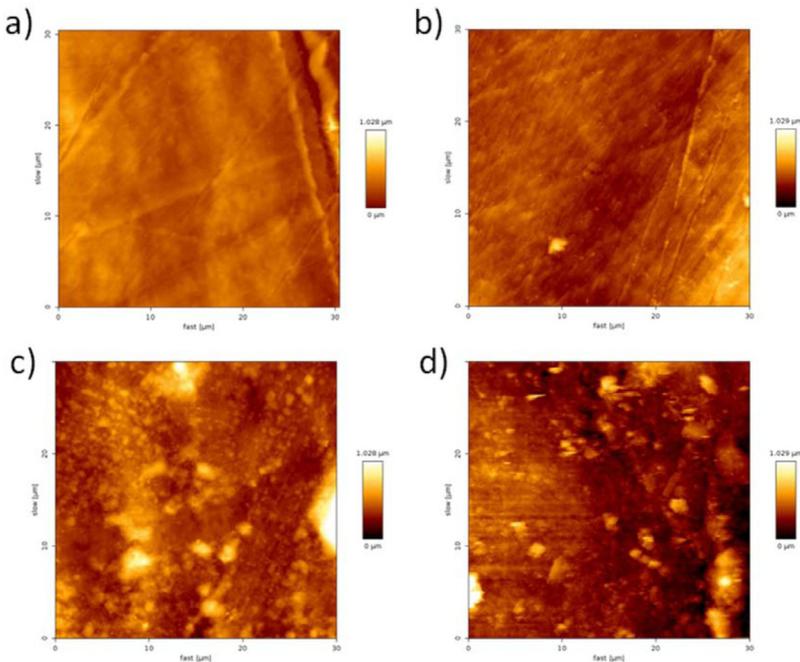


Figure 4. AFM images of the surface of the scaffold biomaterials after 2 h of soaking in the serum-free medium.

AFM: atomic force microscopy.

eventual structure and properties of the engineered tissue.^{24–26} The cell culture model used in this study is the human osteosarcoma cell line MG63. These cells are at a relatively early state in the osteoblastic lineage and, therefore, may represent a good model for examining the initial stages in cell osteogenic differentiation induced by biomaterial scaffolds.^{24,26,27} To underline the role of the biomaterial composition and microstructure on the biological response, and differently from our previous work,¹⁶ the *in vitro* biocompatibility tests were performed by seeding MG63 osteoblasts onto the scaffolds, and the cell/scaffold constructs were cultivated without the addition of soluble osteogenic differentiation factors.

The number of viable cells observed during culture time on the different scaffolds is shown in Figure 5. The number of viable cells increased over time for all of the samples, while different proliferation rates were observed as a function of the composition of the biomaterials. The number of viable cells that adhered to the multiphase PCL–HA, PCL/TZ, and PCL/TZ–HA, $\sim 5 \times 10^4$, was twice the number of cells on the neat PCL sample, namely, $2.8 \pm 1.5 \times 10^4$ (Figure 5). After 28 days of culture, the number of viable cells increased significantly on all samples, indicating the ability of the biomaterials to support cell proliferation. The highest MG63 cell number was observed on neat PCL scaffold, indicating the higher proliferative behavior of the osteoblasts on this scaffold at longer culture times. Although the observed differences at days 1 and 28 were not statistically significant, the AlmarBlue data indicated that cell adhesion was promoted on multiphase scaffolds which, conversely, had slower cell proliferation up to 28 days. These results were supported by SEM analysis of the surface of the cell/scaffold constructs over culture time, reported in Figure 6.

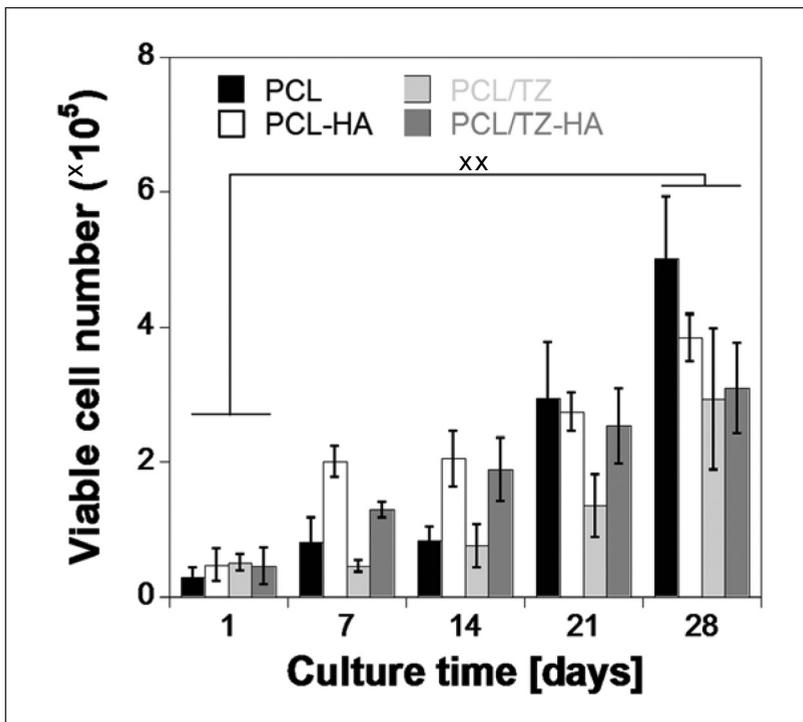


Figure 5. Cell proliferation measured by AlmarBlue test.

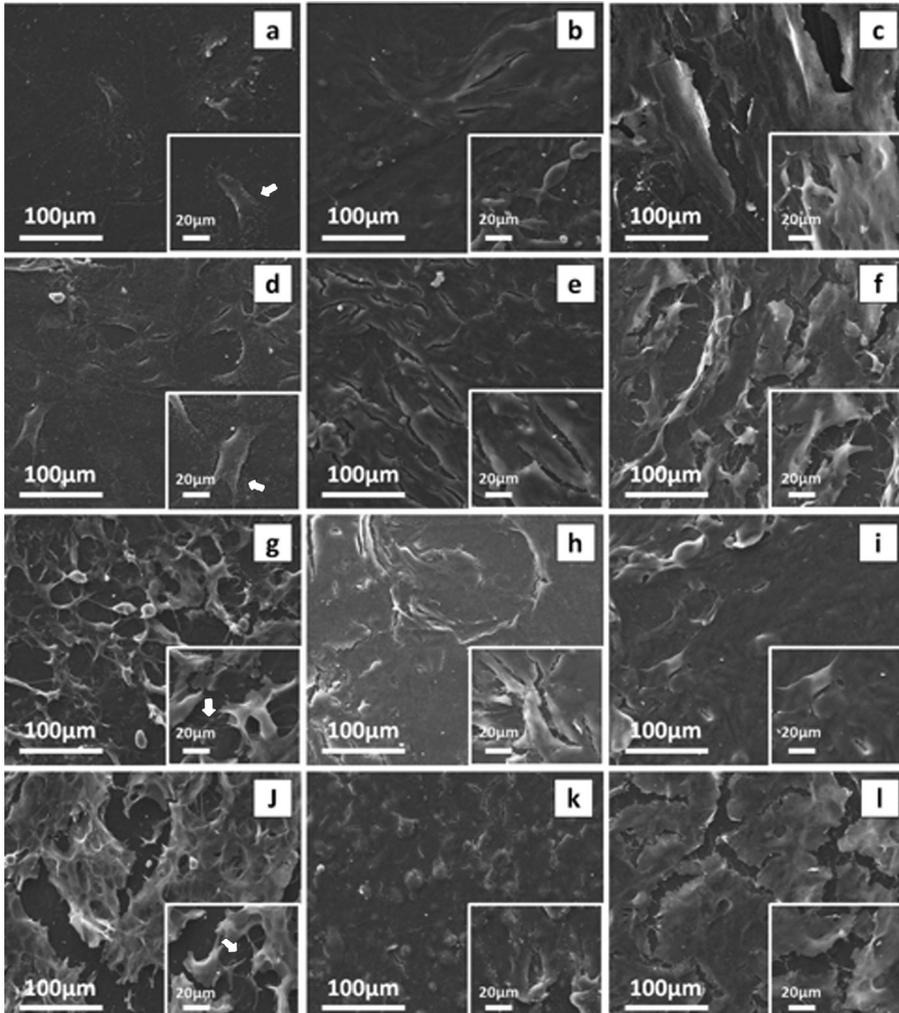


Figure 6. Morphology of the MG63 cells at day 1 (left column), day 14 (middle column), and day 28 (right column) of culture onto the different samples: (a–c) PCL, (d–f) PCL–HA, (g–i) PCL/TZ, and (l–n) PCL/TZ–HA.

PCL: poly(ϵ -caprolactone); TZ: thermoplastic zein; HA: hydroxyapatite.

At day 1, extensive cell distribution and colonization on the surface of the multiphase scaffolds was observed (Figure 6(d), (g), and (j)), while a clearly lower cell number was detected on neat PCL (Figure 6(a)). In agreement with the proliferation data of Figure 5, uniform dense cell sheets were observed on the seeding surface of all of the cell/scaffold constructs after 14 and 28 days of culture, indicating an almost complete colonization of the seeding surface by the cells (middle and right columns of Figure 6). The higher magnifications of the cell/scaffold constructs shown in the insets of Figure 6 highlighted the morphology of the cells, demonstrating that, especially for the multiphase scaffolds, the MG63 cells are well attached to the surface and displayed a flat and well-spread morphology already at day 1 (white arrows).

The osteogenic differentiation of the MG63 cells, evaluated by the ALP activity measurement, the ARS assay, and the immunofluorescent detection of OPN, is reported in Figures 7 and 8. As shown in Figure 7(a), the ALP activity of the cells cultured on neat PCL increased from 0.036 ± 0.045 at day 7 to 0.250 ± 0.131 at day 21 of culture. A similar trend was also observed for the PCL–HA composite, although for this sample the ALP values are significantly higher than the value obtained for the neat PCL, as shown by the value at day 21, 0.604 ± 0.215 . Interestingly, the MG63 cultured on the surface of the PCL/TZ and PCL/TZ–HA scaffolds are characterized by higher ALP expression already at day 7, equal to 0.219 ± 0.146 and 0.416 ± 0.211 , respectively, and the ALP activity remained almost constant over culture time. A slight increase of the ALP activity of MG63, from 0.229 ± 0.127 at day 7 to 0.416 ± 0.167 at day 21 of the culture, was also observed when the MG63 cells were cultured on culture plastics. The enhanced osteogenic expression of MG63 cells cultured on the multiphase systems was supported by the results of the ARS assay and immunofluorescent staining of OPN, shown in Figures 7(b) and 8, respectively. In the absence of osteogenic supplements, the cells seeded on the neat PCL did not undergo significant mineralization at days 21 and 28 of culture.¹³ Notably, both MG63 cultures on PCL–HA, PCL/TZ, and PCL/TZ–HA had one order of magnitude higher calcium deposition. A slight increase of mineralization from days 21 to 28 was also showed for the control cultures, although here ARS values were significantly lower than those achieved for the cells cultured on the multiphase scaffolds (Figure 7(b)). The osteogenic expression of the MG63 cultured on the scaffolds was finally assessed by the qualitative evaluation of the OPN (Figure 8). An increase of the OPN from days 21 to 28, for all of the different cell/scaffold constructs was observed. Nevertheless, the fluorescence intensity of OPN was significantly higher for the MG63 cells cultured on the multiphase scaffolds (Figure 8(c) to (h)), rather than the neat PCL (Figure 8(a) and (b)). The OPN staining also showed that the cells cultured on PCL/TZ and PCL/TZ–HA scaffolds were characterized by the formation of highly confluent cell layers and elongated and oriented morphologies (Figure 6).

The biocompatibility of PCL and HA with bone cells is well known,^{2,4,10,17,20,27} while few literature studies have been reported about the use of zein in bTE.^{6,7} Previous investigations of zein as a

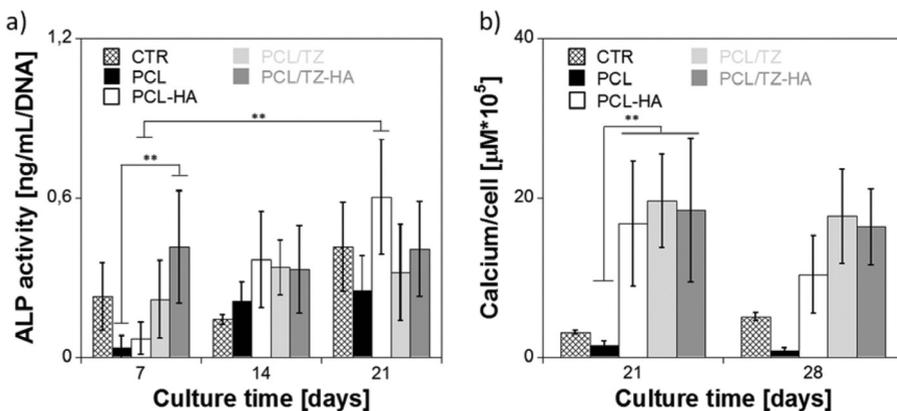


Figure 7. (a) ALP activity of MG63 cells cultured onto the scaffolds at days 7, 14, and 21 and (b) mineralized matrix measured by AR at days 21 and 28 of culture.

ALP: alkaline phosphatase; AR: alizarin red.

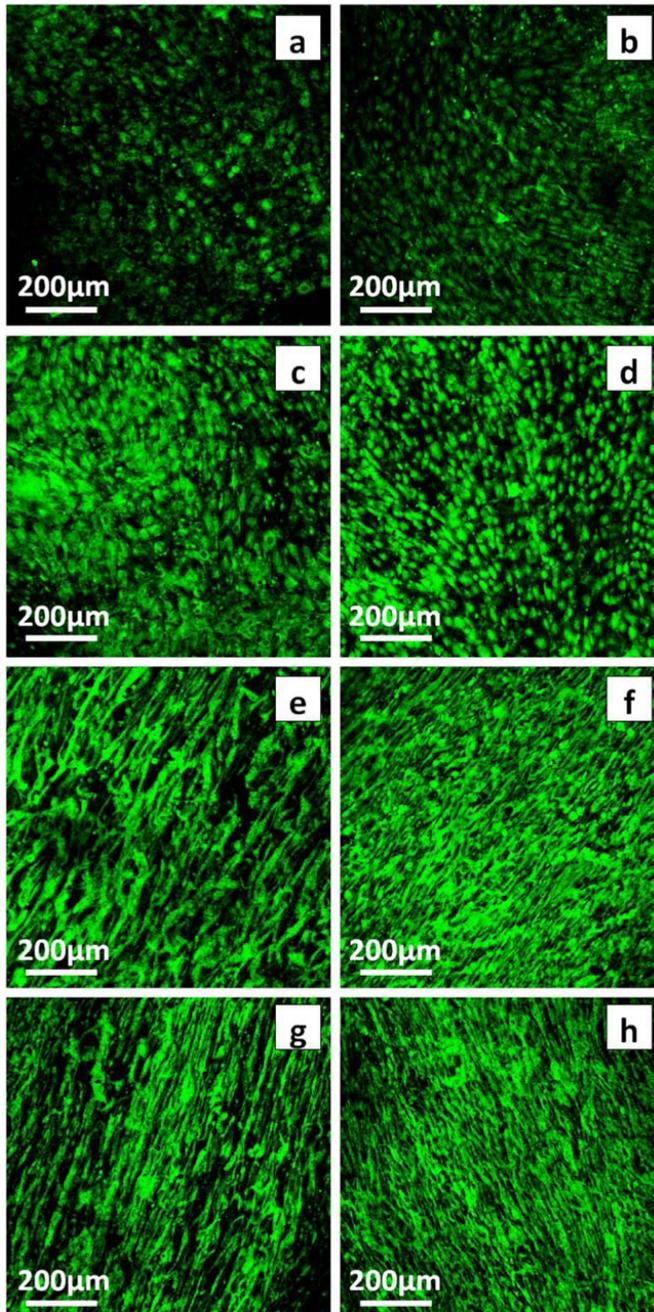


Figure 8. Immunofluorescence staining of OPN at (a, c, e, and g) days 21 and (b, d, f, and h) 28 of MG63 culture onto the scaffolds. (a and b) PCL, (c and d) PCL-HA, (e and f) PCL/TZ, and (g and h) PCL/TZ-HA.

OPN: osteopontin; PCL: poly(ϵ -caprolactone); TZ: thermoplastic zein; HA: hydroxyapatite.

biomaterial for TE proved that this vegetable protein reduces the blood pressure in hypertensive rats and has antioxidative activity and may be used for liver and fibroblast cells culture.²⁸ The porous zein and zein/HA composite scaffolds were found to support *in vitro* MSC adhesion, proliferation, and osteogenic differentiation.⁶ We recently investigated the preparation of multiphase biomaterials by blending PCL with TZ and/or HA particles for bTE with the aim of combining all the advantages of the individual components in a single one.¹⁶ The materials obtained were found to be biocompatible and found to improve the *in vitro* osteogenic differentiation of MSCs cultured in osteogenic conditions.¹⁶

In this study, we aimed to assess the osteogenic properties of TZ and HA when blended with PCL by culturing MG63 osteoblasts onto the biomaterials in the absence of osteogenic induction factors. As shown, the multiphase nonporous scaffolds decreased the proliferation rate of the osteoblasts and significantly accelerated osteogenic differentiation compared to neat PCL.

Cell adhesion, proliferation, and differentiation are interdependent events, which affect cell functionality and are guided by parameters including surface chemistry, wettability, and topography.^{18,24–26,29,30} From the point of view of scaffolds, topography and roughness, the results of the morphological characterization in dry (SEM images, Figure 2) and wet conditions (AFM images, Figure 4) indicated that neat PCL scaffolds were characterized by smoother surfaces if compared to the multiphase scaffolds. For PCL/TZ and PCL/TZ–HA scaffolds, hydrophilicity is significantly enhanced by the exposure of the TZ phase to the surface (Table 1).

Although the complex interrelations between topographical and physical and chemical properties of materials make the discrimination between the contribution of the different parameters very difficult, in the present study, the *in vitro* adhesion and differentiation of bone cells were promoted by rough and hydrophilic surfaces of the multiphase scaffolds. This is supported by literature accounts of the effect of surface properties on cell response. For example, Kunzler et al.²⁵ prepared biocompatible substrates characterized by a well-controlled surface roughness gradient and systematically investigated the *in vitro* osteoblast response to roughness. The results of their study indicated that cells preferred the rougher part of the roughness gradient, as evidenced by the enhanced cell adhesion and spreading observed on this portion.²⁵ Similar results were also achieved by Navarro et al.²⁶ for polylactic acid/glass composite that also highlighted the positive effect exerted by the inorganic phase on the adsorption of the proteins of the culture medium responsible for cell adhesion on the surface of hydrophobic polymers. This is consistent with our *in vitro* results, which indicated that the multiphase scaffolds improved MG63 cell adhesion, with earlier spreading and colonizing, than observed on the neat PCL scaffolds (Figures 5 and 6).

It is known that the downregulation of proliferation is associated with the expression of osteoblastic phenotype markers, such as ALP. The decreased MG63 proliferation on the multiphase scaffolds observed in the third and fourth weeks of culture, compared to the neat PCL (Figure 4), may be due to the increased tendency of cells to differentiate.²⁶ The results of the ALP, ARS, and OPN tests corroborated this assumption, since the cells cultured onto the multiphase composite scaffolds showed higher levels of ALP at day 7 and, compared to neat PCL scaffold, resulted in an impressively higher calcium deposition at days 21 and 28 of culture. Zhao et al.³¹ observed similar results for MG63 cells cultured on titanium implants and ascribed this effect to the fact that the increase of the surface roughness decreases cell proliferation and accelerates their differentiation and the release of factors that stimulate osteogenesis, as also observed in this study. The similar amounts of mineralization on the multiphase PCL–HA, PCL/TZ, and PCL/TZ–HA biomaterials observed at days 21 and 28 of culture

(Figure 7(b)) could not exclude the possibility of significant differences in calcium deposition for a longer culture time, which might also be due to different extents of degradation of the samples.

Conclusions

In this study, novel multiphase biomaterials for bTE were designed and fabricated by blending PCL, TZ, and HA. The materials were then characterized in order to assess their morphological and microstructural properties, wettability, in vitro degradation, and biological response. The TZ improved the wettability of PCL and accelerated the degradation of PCL/TZ blend and the three-phase PCL/TZ–HA composite after 56 days of incubation in PBS. However, the tensile properties of the biomaterials decreased significantly compared with PCL. The simultaneous addition of TZ and HA particles to PCL induced a significant increase of the osteogenic properties of the materials, by causing a magnitude higher calcium deposited by the MG63 cells, compared to PCL.

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References

1. El Kady AM, Mohamed KR and El-Bassyouni GT. Fabrication, characterization and bioactivity evaluation of calcium pyrophosphate/polymeric biocomposites. *Ceram Int* 2009; 35(7): 2933–2942.
2. Boccaccini AR, Erol M, Stark WJ, et al. Polymer/bioactive glass nanocomposites for biomedical applications: a review. *Comput Sci Technol* 2010; 70(13): 1764–1776.
3. Mano JF, Silva GA, Azevedo HS, et al. Natural origin biodegradable systems in tissue engineering and regenerative medicine: present status and some moving trends. *J R Soc Interface* 2007; 4(17): 999–1030.
4. Murugan R and Ramakrishna S. Development of nanocomposites for bone grafting. *Comput Sci Technol* 2005; 65: 2385–2406.
5. Santin M, Morris C, Standen G, et al. A new class of bioactive and biodegradable soybean-based bone fillers. *Biomacromolecules* 2007; 8(9): 2706–2711.
6. Qu Z, Wang H, Tang T, et al. Evaluation of the zein/inorganics composite on biocompatibility and osteoblastic differentiation. *Acta Biomater* 2008; 4: 1360–1368.
7. Tu J, Wang H, Li H, et al. The in vivo bone formation by mesenchymal stem cells in zein scaffolds. *Biomaterials* 2009; 30(26): 4369–4376.
8. Liu K and Hsieh F. Protein–protein interactions during high-moisture extrusion for fibrous meat analogues and comparison of protein solubility methods using different solvent systems. *J Agric Food Chem* 2008; 56(8): 2681–2687.
9. Zein I, Hutmacher DW, Tan KC, et al. Fused deposition modeling of novel scaffold architectures for tissue engineering applications. *Biomaterials* 2002; 23(4): 1169–1185.
10. Puppi D, Chiellini F, Piras AM, et al. Polymeric materials for bone and cartilage repair. *Prog Polym Sci* 2010; 35(4): 403–440.
11. Zhong Z and Sun XS. Properties of soy protein isolate/polycaprolactone blends compatibilized by methylene diphenyl diisocyanate. *Polymer* 2001; 42(16): 6961–6969.
12. Salgado AJ, Sousa RA, Fraga JS, et al. Effects of starch/polycaprolactone-based blends for spinal cord injury regeneration in neurons/glia cells viability and proliferation. *J Bioact Compat Pol* 2009; 24(3): 235–248.
13. Salerno A, Guarnieri D, Iannone M, et al. Effect of micro- and macroporosity of bone tissue three-dimensional-poly(ϵ -caprolactone) scaffold on human mesenchymal stem cells invasion, proliferation, and

- differentiation in vitro. *Tissue Eng Pt A* 2010; 16(8): 2661–2673.
14. Borzacchiello A, Gloria A, Mayol L, et al. Natural/synthetic porous scaffold designs and properties for fibro-cartilaginous tissue engineering. *J Bioact Compat Pol* 2011; 26(5): 437–451.
 15. Salerno A, Oliviero M, Di Maio E, et al. Design of porous polymeric scaffolds by gas foaming of heterogeneous blends. *J Mater Sci-Mater M* 2009; 20(10): 2043–2051.
 16. Salerno A, Oliviero M, Di Maio E, et al. Design of novel three-phase PCL/TZ–HA biomaterials for use in bone regeneration applications. *J Mater Sci-Mater M* 2010; 21(9): 2569–2581.
 17. Rezwani K, Chen QZ, Blaker JJ, et al. Biodegradable and bioactive porous polymer/inorganic composite scaffolds for bone tissue engineering. *Biomaterials* 2006; 27(18): 3413–3431.
 18. Tan PS and Teoh SH. Effect of stiffness of polycaprolactone (PCL) membrane on cell proliferation. *Mat Sci Eng C-Biomim* 2007; 27(2): 304–308.
 19. Shi K, Kokini JL and Huang Q. Engineering zein films with controlled surface morphology and hydrophilicity. *J Agric Food Chem* 2009; 57(6): 2186–2192.
 20. Lee JH, Rim NG, Jung HS, et al. Control of osteogenic differentiation and mineralization of human mesenchymal stem cells on composite nanofibers containing poly[lactic-co-(glycolic acid)] and hydroxyapatite. *Macromol Biosci* 2010; 10(2): 173–182.
 21. Corradini E, Mattoso LHC, Guedes CGF, et al. Mechanical, thermal and morphological properties of poly(ϵ -caprolactone)/zein blends. *Polym Advan Technol* 2004; 15(6): 340–345.
 22. Wan YZ, Luo H, He F, et al. Mechanical, moisture absorption, and biodegradation behaviours of bacterial cellulose fibre-reinforced starch biocomposites. *Comput Sci Technol* 2009; 69(7–8): 1212–1217.
 23. Zheng Z, Wei Y, Wang G, et al. Surface properties of chitosan films modified with polycations and their effects on the behavior of PC12 cells. *J Bioact Compat Pol* 2009; 24(1): 63–82.
 24. Meredith JC, Sormana J, Keselowsky BG, et al. Combinatorial characterization of cell interactions with polymer surfaces. *J Biomed Mater Res A* 2003; 66: 483–490.
 25. Kunzler TP, Drobek T, Schuler M, et al. Systematic study of osteoblast and fibroblast response to roughness by means of surface-morphology gradients. *Biomaterials* 2007; 28(13): 2175–2182.
 26. Navarro M, Engel E, Planell JA, et al. Surface characterization and cell response of a PLA/CaP glass biodegradable composite material. *J Biomed Mater Res A* 2008; 85(2): 477–486.
 27. Salerno A, Zeppetelli S, Di Maio E, et al. Processing/structure/property relationship of multi-scaled PCL and PCL–HA composite scaffolds prepared via gas foaming and NaCl reverse templating. *Biotechnol Bioeng* 2011; 108(4): 963–976.
 28. Dong J, Sun Q and Wang J. Basic study of corn protein, zein, as a biomaterial in tissue engineering, surface morphology and biocompatibility. *Biomaterials* 2004; 25(19): 4691–4697.
 29. Deligianni DD, Katsala ND, Koutsoukos PG, et al. Effect of surface roughness of hydroxyapatite on human bone marrow cell adhesion, proliferation, differentiation and detachment strength. *Biomaterials* 2001; 22(1): 87–96.
 30. Beşkardeş IG and Gümüşderelioglu M. Biomimetic apatite-coated PCL scaffolds: effect of surface nanotopography on cellular functions. *J Bioact Compat Pol* 2009; 24(1): 63–82.
 31. Zhao G, Raines AL, Wieland M, et al. Requirement for both micron- and submicron scale structure for synergistic responses of osteoblasts to substrate surface energy and topography. *Biomaterials* 2007; 28(18): 2821–2829.