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Tuning the microstructure and biodegradation of three-phase scaffolds for bone regeneration made of PCL, Zein, and HA

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Abstract

The aim of this study has been the design of novel multi-phase porous scaffolds with bi-modal pore size distributions and controlled biodegradation rate for bone tissue engineering (bTE), via a gas foaming–leaching approach. Poly(ε-caprolactone) (PCL) has been melt mixed with thermoplastic zein (TZ) and hydroxyapatite particle, to prepare multi-phase PCL–TZ and PCL–TZ–HA composites suitable to be further processed for the fabrication of 3D porous scaffolds. To this aim, these systems have been gas foamed by using CO_2 as blowing agent and, subsequently, soaked in H_2O to leach out the plasticizer from the TZ. This combined process allows the formation of an interpenetrated micro- and macro-porosity network within the samples. The effect of the different formulations on the micro-structural properties and *in vitro* biodegradation of the scaffolds has been investigated, and the results correlated to the mechanisms involved in the formation of the bi-modal pore structure. Results demonstrated that the multi-phase nature of the biomaterials prepared as well as their composition significantly affect the micro-structural properties and biodegradation rate of the scaffolds. The optimal selection of the processing conditions may allow for the design of multi-phase 3D porous scaffolds suitable for bTE.

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degradation, gas foaming, PCL, scaffold, zein

Introduction

To date, biodegradable porous scaffolds are widely used in tissue engineering (TE) for the repair/regeneration of biological tissues.[1–5] Scaffolds for TE are open-pore biocompatible and biodegradable foams that provide a temporary substrate for transplanted cells to growth and proliferate in three dimensions and that, simultaneously, ensure the correct structural and biological functions during implantation *in vivo*.[1–5]

The design of biodegradable porous scaffolds requires the appropriate selection of materials and process technologies. Biodegradable scaffolds should be resorbable at a predetermined rate to be progressively replaced by the regenerated tissue.[5] The optimal balance between scaffold degradation and 3-D new-tissue regeneration may be achieved by controlling scaffold composition and pore structure.

For bone TE (bTE), several studies have been reported about the role of the topology of the pore structure of the scaffold on the ability of transplanted cells to allow for the regeneration of a functional 3-D new tissue.[6,7] For instance, it has been reported that scaffolds with a 100–500 μm porosity (macro-porosity) may well accommodate bone cells and also, may allow for their optimal migration and colonization in three dimensions.[3,5–7] Furthermore, advances in bTE demonstrate that bi-modal pore size scaffolds characterized by interpenetrated micro- and macro-porosity network enhance the regeneration ability of a porous scaffold.[3,8,9] Nevertheless, the selection of the bone scaffold material and pore structure is synergic to the achievement of the structural and mechanical functionality required for hard tissues regeneration.[5,9,10]

Scaffold composition and pore structure have also a key role on the degradation process.[5,6,10,11] In principle, an ideal scaffold for bTE must degrade with a degradation rate matching the rate of new bone produced by the cells and the degradation bi-products must be well tolerated and opportunely metabolized by the host.[5,6,10,11] It is required that the degradation and resorption kinetics allow the scaffold to retain its physical properties for several months,[5] for this is, typically, the characteristic tissue regeneration time scales.

Polymeric materials have been used widely for the design of bTE scaffolds due to their optimal biocompatibility and feasibility to be processed into porous scaffolds with well-controlled architectures.[3–7,9–12] Among the class of the poly(a-hydroxy acids), poly(ε-caprolactone) (PCL) has been widely used.[5,9,10] Indeed, PCL is highly biocompatible with bone cell and tissue and due to its low hydrophilicity and high crystallinity, it is characterized by slow degradation and resorption rates, therefore providing a structural support for long times.[5] However, PCL alone does not allow for the design of porous scaffolds able to simultaneously provide the

micro-structural properties, degradation rate, and biological response required for load-bearing applications.

Blending PCL with natural polymers and/or bioactive inorganic fillers has been a successful approach for the design of biomaterial scaffolds with improved performances.[9,13–15] Indeed, when mixed with PCL, polymers from natural resources such as starch and chitosan may allow for a fine tune of its wettability and degradation rate.[13,14] Similarly, calcium phosphates, such as hydroxyapatite (HA), have been also widely used in order to enhance the mechanical and biological responses of PCL scaffolds and to modulate their biodegradation rate.[15]

Our group has recently investigated the processing of natural biocompatible proteins, such as zein, in order to prepare a thermoplastic material suitable for the design of polymer foams via the gas foaming technology.[16] The thermoplastic zein (TZ), has been also used for the design of multi-phase PCL–TZ and PCL–TZ–HA composite biomaterials for bTE applications.[17] Along with these research lines, this study aims to fabricate porous scaffolds for bTE starting from PCL–TZ and PCL–TZ–HA composite materials via the gas foaming technology. In particular, in this study, we describe a novel approach to imprint a bi-modal pore size distribution within these materials by the combination of gas foaming and leaching in water. The obtained multi-phase porous scaffolds have been analyzed in terms of thermal degradation, morphology, mechanical properties, and *in vitro* degradation.

Experimental part

Materials

PCL ($M_W = 65$ kDa, $T_m = 59$–64°C) and maize zein powder (cod.: Z3625, batch: 065K0110) have been purchased from Sigma–Aldrich (Italy). Poly(ethylene glycol) (PEG) 400 has been purchased by Fluka and used as plasticizer for the preparation of the TZ. HA particles (Fin-Granule, Finceramica, Faenza, Italy) with size in the 70–105 μm range, have been used as bio-ceramic filler. CO_2 (Air liquide, Italy) has been used as physical blowing agents for gas foaming experiments.

Methods

Porous multi-phase PCL–TZ and PCL–TZ–HA composite scaffolds have been prepared by a three-step process, as shown in the scheme of Figure 1. First, PCL, TZ, and HA have been blended in an internal mixer (Rheomix® 600 Haake, Germany), as described in Salerno et al.[17] Briefly, PCL pellets have been melted at 70°C, 20 rpm for 2 min and subsequently TZ and HA granules have been added into the mixing chamber. Table 1 reported the three different compositions used. The blended materials have been compression molded at 80°C and 30 MPa into 2 mm-thick plates by a hot press (P300P, Collin, Germany) and subsequently, disc-shaped samples ($d = 10$ mm and $h = 2$ mm, Figure 2) have been prepared by machining. The samples have been subsequently saturated in an autoclave with

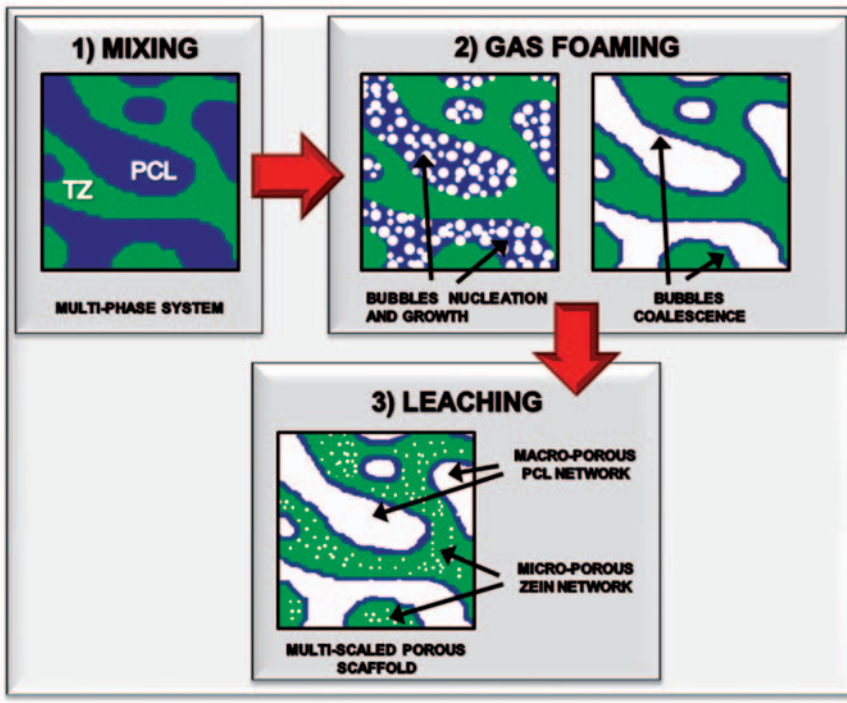

Figure 1. Scheme of the gas foaming–leaching combined process used for the design of the multi-phase PCL–TZ and PCL–TZ–HA composite scaffolds with bi-modal pore size distributions.

Table 1. Formulation of the different scaffolds before (B) and after (A) the leaching step

	PCL (wt%)		TZ (wt%)		HA (wt%)	
Scaffold formulations	Before leaching	After leaching	Before leaching	After leaching	Before leaching	After leaching
PCL–TZ	60	68.4	40	31.6	–	–
PCL–TZ–HA_{10}	54	59.6	36	29.4	10	11
PCL–TZ–HA_{20}	48	52.2	32	26.1	20	21.7

CO_2 at 150 bar, 70°C for 4 h. The autoclave temperature has been increased to 100°C and the pressure quenched to the ambient to induce the formation of an open macro-porosity within the PCL phase. The final scaffold pore structure has been achieved by soaking the obtained foams (Figure 2) in dH_2O for 2 days at 37°C. This additional step promotes the leaching of the plasticizer (PEG) from the

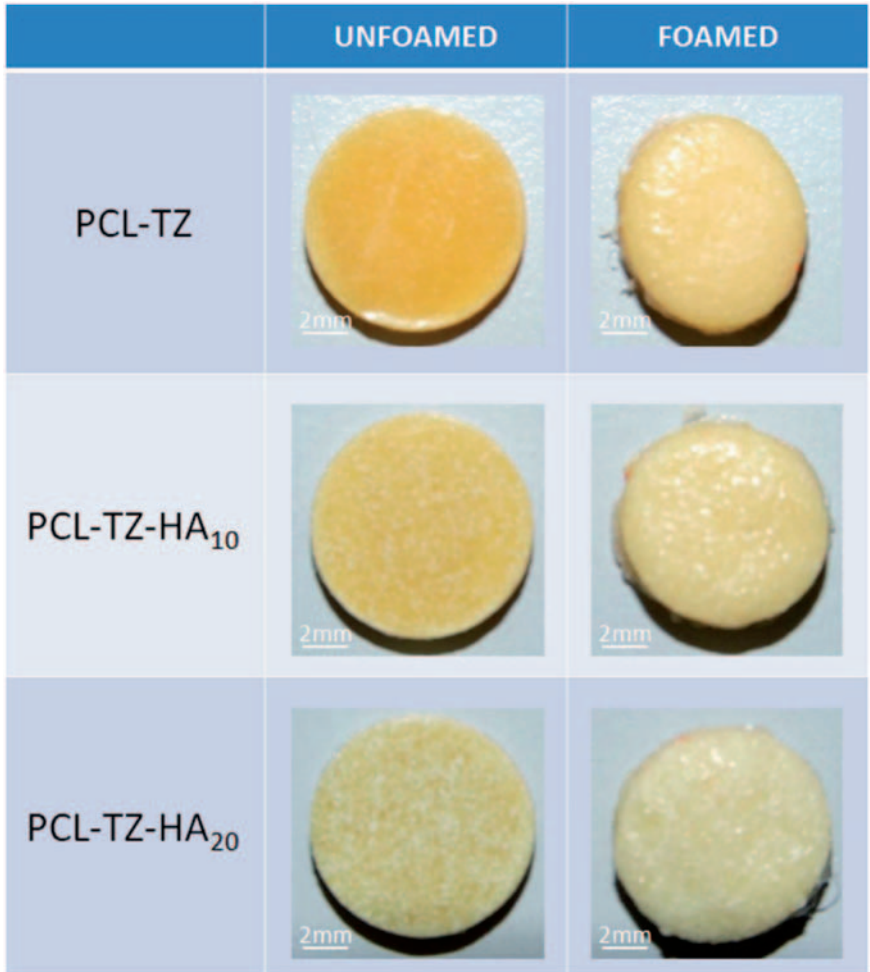

Figure 2. Macroscopic views of the multi-phase biomaterials before and after the gas foaming step.

TZ phase and induces the formation of a micro-porosity network. These scaffolds have been finally vacuum-dried for characterization.

Characterization

The effect of the leaching step on the final scaffold composition and thermal degradation has been assessed by gravimetric and thermogravimetric analysis (TGA) measurements, respectively. For gravimetric measurements, the samples have been weighted by an high accuracy balance (10^{-3} g, AB104-S, Mettler Toledo, Italy), before and after the leaching step. The final scaffold composition has been

determined by considering the amount of PCL and HA unchanged and ascribing the weight loss to the TZ phase. TGA has been carried out on a TGA2950 (TA Instruments, USA) over a 30–600°C temperature range at 10°C/min under inert atmosphere. The scaffolds have been tested before and after the leaching step and the results compared to those achieved for neat PCL and TZ.

The morphology of the scaffolds before and after leaching has been evaluated by scanning electron microscope (SEM) analysis. The scaffolds have been cross-sectioned by a razor blade, gold sputtered, and analyzed by a S440 (LEICA, Germany) SEM. The pore size distributions of the scaffolds have been determined by image (Image J®) analysis. To this purpose, the SEM micrographs have been converted to 8-bit digital images and the resulting images analyzed in order to evaluate the pore size distributions and the mean pore size of the scaffolds (ASTM D3576). At least 100 pores for each scaffold were selected.

The compressive mechanical properties of the scaffolds have been evaluated by static compression tests. Five cylindrical samples ($d = 5$ mm and $h = 3$ mm) for each scaffold type have been analyzed on an Instron 4204 (Instron, Italy) at a cross-head speed of 1 mm/min and with a 1-kN loading cell. The stress versus strain curves have been recorded and the elastic compressive modulus calculated as the slope of the initial linear portion of the curves.

Scaffold degradation has been assessed *in vitro* for 21 days. Disc-shaped scaffolds ($d = 5$ mm and $h = 3$ mm) have been γ-sterilized at a dose of 2.5 Mrad for 8 min, weighted, and incubated in PBS at 37°C and 5% CO_2. At pre-determined time points, the scaffolds have been washed with fresh water, vacuum-dried and weighted to assess the overall weight loss. The amount of degraded TZ has been assessed by dividing the obtained values for the nominal zein fraction within the scaffolds (Table 1). Three scaffolds have been used for each time point. The effect of the degradation on the morphology of the scaffolds has been evaluated by SEM analysis of the cross-sections of the scaffolds.

Results

Figure 2 shows the macroscopic views of the multi-phase samples before and after the foaming process. The images of zein-based materials, typical yellowish in color, clearly evidence the increase of sample volume after foaming, although, especially for the PCL–TZ–HA composites, the samples retained their disc-shaped geometry.

Table 1 and Figure 3 report the composition and the results of TGA conducted on the scaffolds before and after the leaching step. As it can be observed, after leaching, the overall weight loss of all the scaffolds is close to the amount of plasticizer within the TZ (Table 1). Due to the unchanged amount of PCL within the scaffolds, the PCL/TZ ratio increases from 1.5, for the initial scaffold composition, to about 2 after leaching. The thermal behavior of the multi-phase materials well replicate those of the neat polymers with the presence of three main thermal degradation process (Figure 3). Peaks 1 and 2, at 110°C and 320°C, respectively, are related to the evaporation of water absorbed mainly within zein (peak 1) and to the

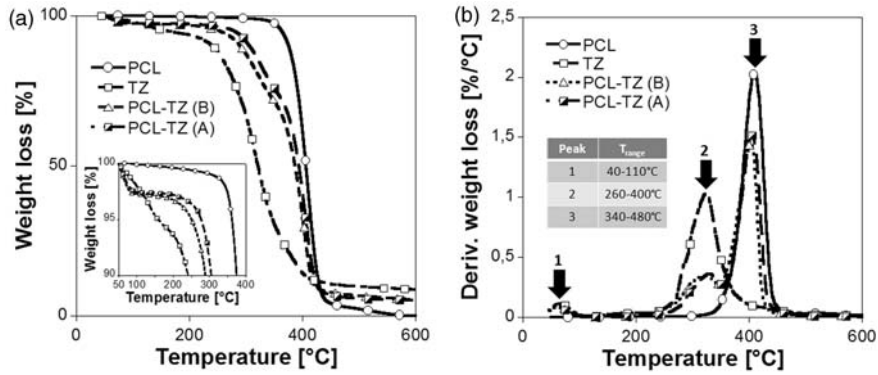

Figure 3. (a) TGA and (b) DTGA curves of the PCL–TZ scaffold before (B) and after (A) the leaching step. TGA/DTGA curves of neat PCL and TZ are also reported for comparison.

thermal degradation of TZ (peak 2). Peak 3, at 410°C, is ascribed to the thermal degradation of PCL. Similar thermal degradation curves have been observed on the scaffolds before and after leaching. However, as shown in Figure 3 for the PCL–TZ scaffold, after leaching, peak 2 shifted to higher temperatures.

The results of the morphological characterization of the scaffolds are shown in Figure 4. As shown in Figure 4(a)–(c) and also reported in the scheme of Figure 1, the selection of a foaming temperature of 100°C, higher than PCL melting temperature, allows the formation of a macro-porosity within the samples. These large pores are induced by the foaming and subsequently, collapse of the PCL at the interface with the TZ phase (Figure 1). The morphological characterization also shows a significant decrease of the expansion degree of the PCL–TZ–HA composites if compared to the PCL–TZ, as evidenced by the decrease of both macro-pores number and size (compare Figures 4(a)–(c)). Figure 4(d)–(n) report the morphology of the scaffolds after leaching. By comparing the low magnifications of the cross-section of the scaffolds before (a–c) and after (d–f) leaching, the increase of the void fraction of the scaffolds is evident. In particular, the higher magnifications of Figures 4(g)–(i) evidence the multi-phase nature of the scaffolds with the presence of the zein phase (white arrows) and HA particles (black arrows) interdispersed within the PCL porous network. Furthermore, the higher magnification SEM micrographs reported in Figures 4(l)–(n) clearly show the micro-porosity of the zein phase induced by the extraction of the plasticizer during the leaching step. The obtained bi-modal scaffolds have been selected for further pore size distribution analysis, mechanical characterization, and *in vitro* degradation test.

Figure 5 reports the results of the pore size distribution analysis of the scaffolds after leaching. As shown, all the scaffolds are characterized by a micro-porosity in the 1–15 μm range (Figure 5(a)) with a mean pore size equal to 5 μm (Figure 5(c)). Similarly, the three scaffolds also evidence a 20–300 μm range macro-porosity but,

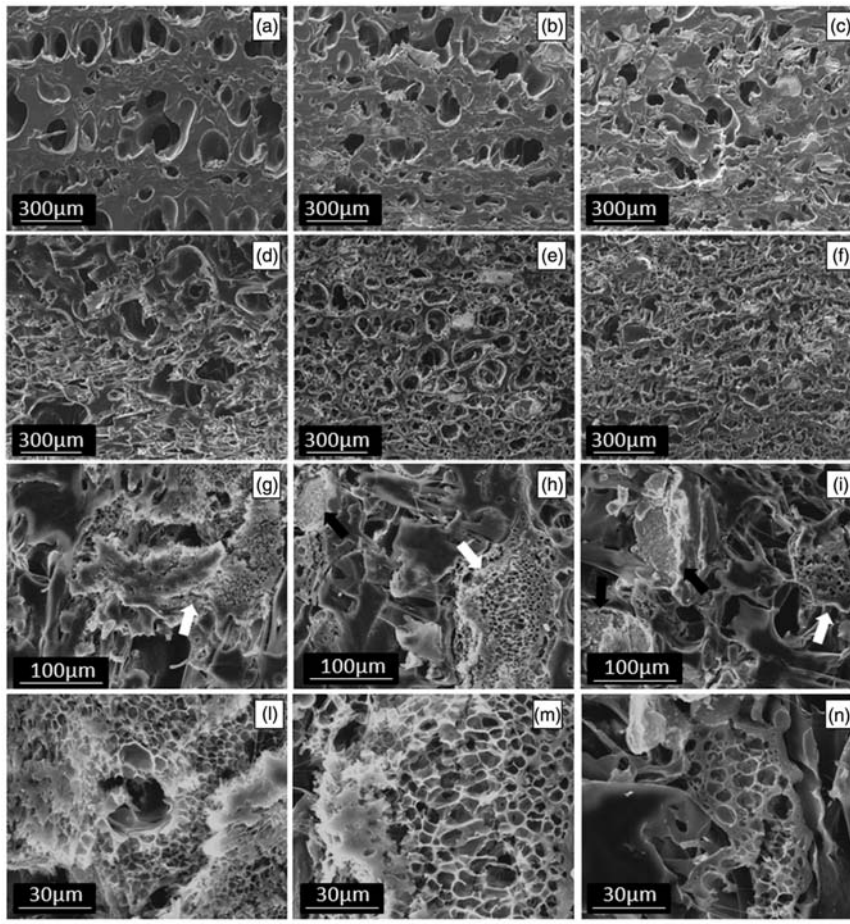

Figure 4. Morphology of the multi-phase scaffolds before (a–c) and after (d–n) the leaching step. (a, d, g, l) PCL–TZ; (b, e, h, m) PCL–TZ–HA_{10} and (c, f, i, n) PCL–TZ–HA_{20} scaffolds.

in this case, the mean pore size decreases from 150 μm to 110 and 100 μm ca. when the HA concentration increases from 0 to 10 wt% and 20 wt%, respectively.

Figure 6 shows the results of the static compression tests performed on the different scaffolds. All the scaffolds evidence similar stress versus strain curves, characterized by the almost linear increase of σ with the increase of ε in the first stage, followed by a steep increase of the slope of the curves as a consequence of the densification, with the absence of a plateau.[4,9] The compressive modulus, evaluated in the first linear portion of the curves, increases from 20.1 ± 2.4 MPa for the PCL–TZ to 26 ± 4.4 MPa for the PCL–TZ–HA_{20}.

The results of the *in vitro* degradation test are reported in Figures 7 and 8. As shown in Figure 7(a), the weight loss of all of the scaffolds increases over

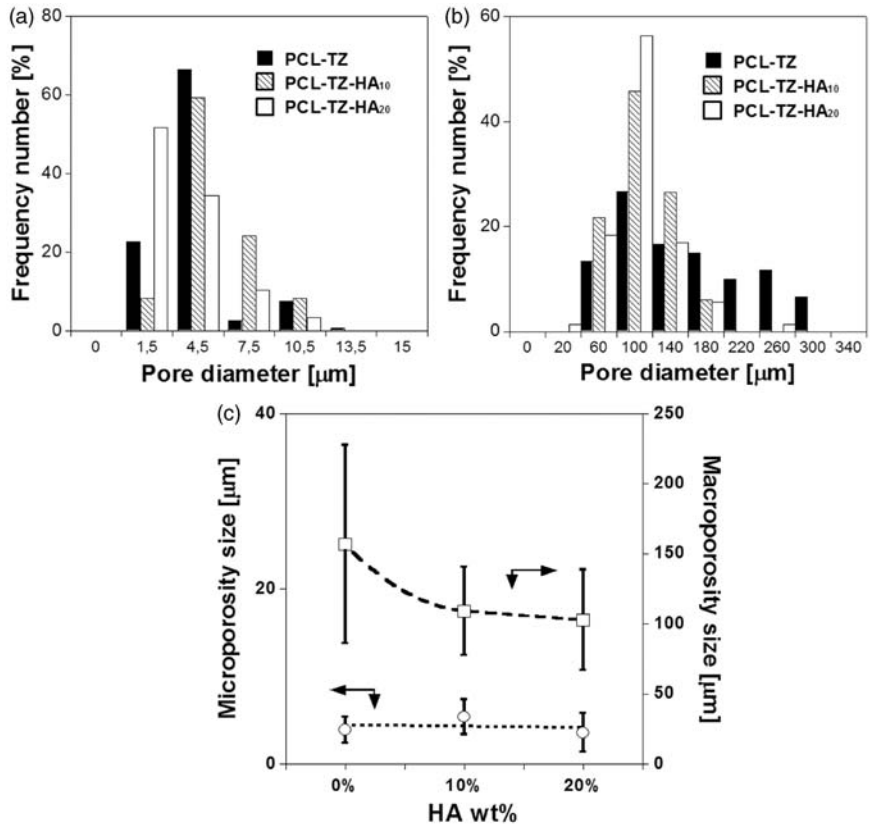

Figure 5. Pore size distributions of the multi-phase scaffolds prepared by the gas foaming–leaching combined process: (a) micro-porosity and (b) macro-porosity distributions; (c) Effect of biomaterials formulation on the mean pore size of the scaffolds.

time from day 1 to day 21. However, differences in the rate of weight loss have been observed with respect to the different scaffold formulations. In particular, the overall weight loss decreases with the increase of the HA concentration within the scaffolds, resulting in a weight loss for PCL–TZ after 21 days three times higher than the PCL–TZ–HA_{20}. Similar trends have been observed for the zein weight loss, with a 60% value for the PCL–TZ scaffold after 21 days of incubation. The degradation results of Figure 7 have been confirmed by the analysis of the morphology of the cross-sections of the scaffolds after 14 days of incubation, reported in Figure 8. Indeed, as evidenced by the white arrows of Figure 8(a) and (b) and by the higher magnification of Figure 8(c), a more irregular and discontinuous zein structure is observed after incubation in PBS (compare Figure 4(d)–(f) with Figure 8(a)–(c).

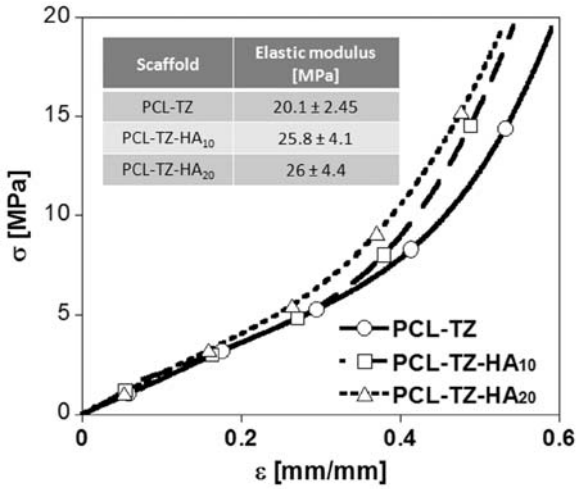

Figure 6. Stress vs strain curves of the different scaffolds prepared. The inset reports the values of the mean value ± standard deviation of the elastic compression modulus of the scaffolds.

Discussion

To date, the majority of *in vitro* and *in vivo* bTE strategies use scaffolds seeded with cells.[1–15] Despite the great advantages offered by this approach, the design of porous biodegradable scaffolds is often difficult because of opposed micro-structural and biological requirements. For instance, scaffold with high pore volume may allow for improved cell colonization and fluids transport but, on the other hand, may not insure the mechanical response required for load-bearing applications.[4,9,10] Therefore, the correct design of bTE scaffolds requires approaches capable of simultaneously satisfying key biological and micro-structural requirements.

PCL is a biodegradable and biocompatible polymer characterized by slow *in vitro* and *in vivo* degradation and resorption rates, therefore matching the rate of bone tissue growth well.[3,5,9,10] However, PCL scaffolds with high overall porosities do not ensure the mechanical response necessary for load-bearing applications.[9,10] On the other hand, the decrease of its overall porosity to 50–60% may allow the matching of the structural requirements but, in contrast, may hinder fluid transport and cell/tissue infiltration within the scaffold.[18] Zein is corn protein recently investigated and successfully implemented as the biomaterial for bTE applications.[19,20] Compared to PCL, zein is characterized by higher hydrophilicity and faster *in vitro* and *in vivo* degradation and resorption rates.[11,19–21] Composites prepared by mixing HA particles within PCL or zein have been also reported to have enhanced mechanical properties and biological response if compared to neat polymers.[15,20]

We recently investigated the preparation of multi-phase PCL–TZ and PCL–TZ–HA composite biomaterials for bTE.[17] The *in vitro* biological characterization

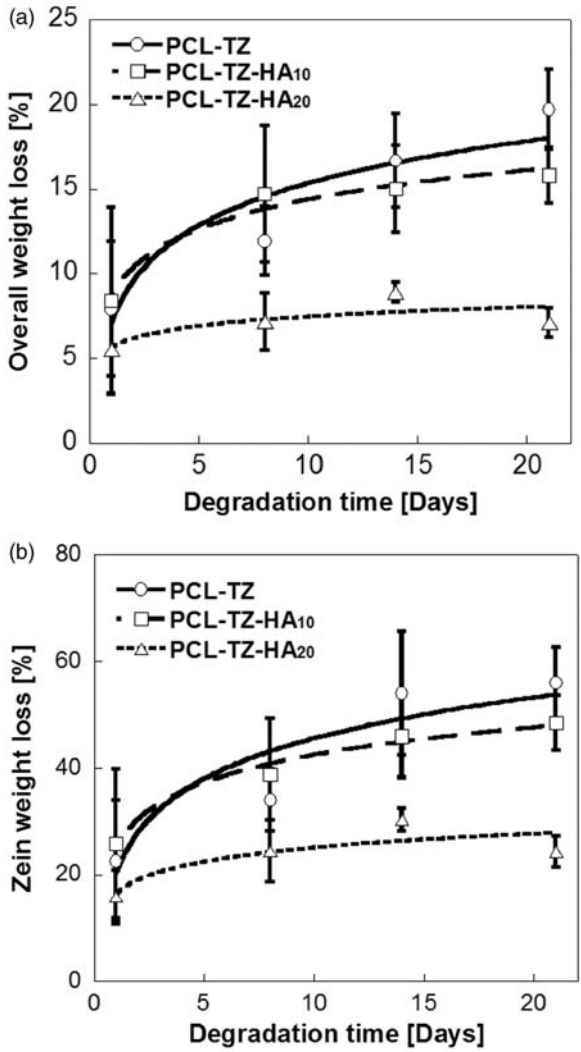

Figure 7. Effect of degradation time and scaffold formulation on the (a) overall weight loss and (b) zein weight loss.

performed on these systems also demonstrated that they are able to support the adhesion, proliferation, and osteogenic differentiation of mesenchymal stem cells.[17] Along this research line, in this study, we designed novel multi-phase bTE porous scaffolds with ab-initio micro-structural properties that match the micro-structural requirements for hard TE application and, on the other hand, are also characterized by a well-controlled biodegradation rate. As shown in the scheme of Figure 1, the multi-phase PCL–TZ and PCL–TZ–HA composite porous scaffolds have been

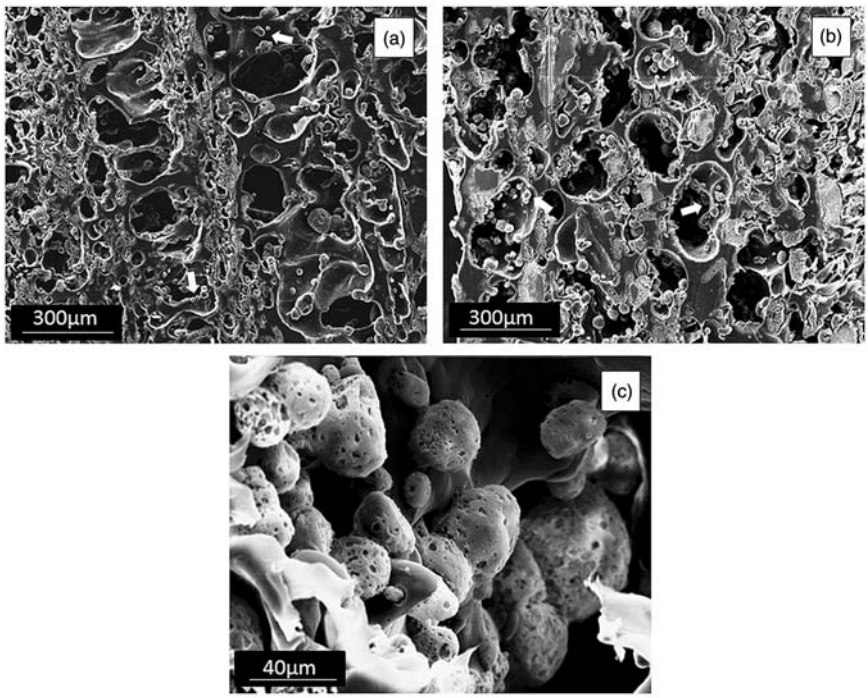

Figure 8. Morphology of (a and c) PCL–TZ and (b) PCL–TZ–HA scaffolds after 14 days of degradation in PBS.

prepared by a gas foaming–leaching combined process. In particular, the gas foaming process parameters have been appropriately selected for the creation of the macro-porosity within the PCL phase, while the leaching step performed for the formation of the micro-porosity within the TZ phase (Figures 4(d)–(n)).

A large number of studies highlight the importance of the macro-porosity to achieve an optimal adhesion, proliferation, and infiltration of cells in 3D.[3–10] Furthermore, a micro-porosity with pore at sub-cellular size scale may promote the transport of fluids and may also positively affect cell adhesion and bone-inducing protein adsorption, therefore enhancing the regeneration ability of porous scaffolds.[3,8,9] In a previously reported work, we have also demonstrated that, when combined in a unique porous PCL scaffolds, the micro- and macro-porosities promote and guide the three-dimensional colonization and proliferation of mesenchymal stem cells *in vitro*.[9] In this context, the scaffolds designed in this study are characterized by bi-modal pore size distribution (Figures 4 and 5) matching the optimal pore size for bone cells adesion and colonization in 3D.

It is important to point out that, the formation of an interpenetrated PCL–TZ network together with the different foaming windows of PCL and TZ[9,16,22] is essential for the optimal design of these multi-phase scaffolds. Indeed, foaming temperatures higher than PCL melting temperature are suitable to allow for the

formation of an open macro-porosity within the PCL phase (Figure 4(a)–(c)). Nevertheless, the interpenetrated TZ network, which is more stable than PCL at higher temperatures,[16] provide the necessary structure to avoid the complete collapse of the samples during foaming (Figures 2 and 4). Concomitantly, the selection of a scaffold composition in the co-continuity range (Table 1) may allow for an efficient leaching process of the plasticizer from the TZ and therefore, insures the creation of an homogeneous micro-porosity within the scaffolds (Figure 4(g)–(n)).

Because of the different mechanisms involved in the formation of the micro- and macro-porosities, we observed different dependences of the micro- and macro-porosit distributions on the HA concentration (Figure 5). In particular, the macro-porosity distribution shifts to lower values with the increase of the HA from 0 to 20 wt% (Figure 5(b)) and accordingly, the mean pore size decreases from 150 to 100 μm, respectively (Figure 5(c)). Conversely, we did not observe differences in the micro-porosity distribution and size with respect to the HA amount (Figure 5(a)). These results may be explained by considering that the macro-porosity originated during foaming depends on the size and morphology of the PCL phase and, also, on the stiffness of the system. The dispersion of inorganic particles, such as HA, within the blend may have a strong impact on all these parameters[22,23] and therefore on the final pore structure of the scaffolds. Conversely, the micro-porosity depends only on the initial composition of the TZ and therefore, is almost unaffected by the HA.

Providing adequate mechanical support is a critical issue for the design of bTE scaffold.[9,10] In principle, the scaffold must sustain cells and new-growing tissue and, simultaneously, avoid excessive new-tissue deformation under the *in vivo* mechanical loads.[10] Figure 6 reports the results of the compressive tests of the multi-phase scaffolds prepared. No significant differences have been observed in their elastic behavior, but a slight increase in the elastic modulus has been observed from the PCL–TZ to the PCL–TZ–HA_{20}. It is however important to point out that all the scaffolds may provide an initial mechanical response adequate for hard tissue repair/regeneration.[10]

The degradation properties of a scaffold are of crucial importance for biomaterial selection and design but also for the long-term success of a tissue-engineered construct.[11–14] A scaffold for bTE must be characterized by a degradation rate matching the production rate of the new tissue by the cells.[11–14] Furthermore, the degradation products have to be well-tolerated and easily metabolized by the host.[11–14] There are several papers dealing with the biodegradation of PCL and zein, both *in vitro* and *in vivo*.[11,19,21,24] The degradation of PCL, which involves the hydrolytic chain scission of the ester linkage followed by the loss of mass, is affected by several factors, mainly molecular weight and crystallinity of the polymer, sample geometry, and degradation medium.[11,24] Nevertheless, PCL usually requires at least 6 months to appreciate significant weight loss under non-aggressive degradation media.[11,24] In general, zein degrades faster than PCL and depending on the degradation medium, a 30–90% weight loss has been reported for zein

scaffolds in the first 2 weeks of incubation *in vitro*.[21] The results of the *in vitro* degradation test performed on our multi-phase scaffolds are reported in Figures 7 and 8. As shown in Figure 7(a), the weight loss of all of the scaffolds increases over time from day 1 to day 21. However, differences in the weight loss curves have been observed with respect to the different scaffold formulations, with the decrease of the weight loss of the scaffolds with the increase of the HA concentration (Figure 7(a)). By considering a negligible PCL and HA weight loss in the first 21 days of incubation in PBS,[11,24] the weight loss may be totally ascribed to the protein phase and therefore, we obtained the trends of the TZ, reported in Figure 7(b). These results evidence a weight loss of zein for the PCL–TZ three times higher than the PCL–TZ–HA_{20}, after 21 days of incubation in PBS. Although it is out of the aim of this study to investigate the mechanisms involved during degradation of zein within our scaffolds, these results seemed to indicate that this process is significantly affected by the micro-architecture of the pore structure of the scaffold. Indeed, the degradation process of zein depends on the penetration of the medium within the scaffold and therefore, on the architecture of the pore structure. High pore volume and size may allow for a faster fluid diffusion and therefore for a more efficient zein degradation. As previously described, the HA particles reduce the size of the macro-porosity (Figure 4) and may also directly hamper the diffusion of the PBS within the scaffolds, therefore reducing the weight loss of zein (Figure 7). The SEM micrographs of the cross-section of the scaffolds after 14 days of incubation reported in Figure 8 support these considerations. Indeed, differences in the morphology of the scaffolds are clearly evident before (Figure 4) and after incubation (Figure 8) as well as we observed the fragmentation of the zein network and the presence of segregated protein phases (Figure 8(c)). These results corroborate the concept that multi-phase scaffolds obtained by blending polymers with different degradation rates may provide micro-structural properties matching, ab-initio, the bTE requirements, and may allow for a more efficient design of the degradation rate profile of the scaffold.

Conclusions

In this study, we investigated the design of novel multi-phase PCL–TZ and PCL–TZ–HA composite porous scaffolds for bTE with well-controlled micro-structural properties and degradation rate produced via a gas foaming–leaching process.

The as-prepared scaffolds are characterized by bi-modal pore size distribution and static compression properties matching the requirements for hard tissue repair/regeneration. The combination of PCL and TZ also allowed a fine control over the degradation of the scaffold. Furthermore, the incorporation of the HA particles enhanced the mechanical properties and retarded the zein weight loss.

All these results are very promising for the design of novel multi-functional porous scaffolds for bTE with the desired micro-structural properties and biodegradation via the gas foaming process.

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