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Design of porous polymeric scaffolds by gas foaming of heterogeneous blends

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Abstract One of the challenges in tissue engineering scaffold design is the realization of structures with a predefined multi-scaled porous network. Along this line, this study aimed at the design of porous scaffolds with controlled porosity and pore size distribution from blends of poly(ε-caprolactone) (PCL) and thermoplastic gelatin (TG), a thermoplastic natural material obtained by de novo thermoplasticization of gelatin. PCL/TG blends with composition in the range from 40/60 to 60/40 (w/w) were prepared by melt mixing process. The multi-phase microstructures of these blends were analyzed by scanning electron microscopy and dynamic mechanical analysis. Furthermore, in order to prepare open porous scaffolds for cell culture and tissue replacement, the TG and PCL were selectively extracted from the blends by the appropriate combination of solvent and extraction parameters. Finally, with the proposed combination of gas foaming and selective polymer extraction technologies, PCL and TG porous materials with multi-scaled and highly interconnected porosities were designed as novel scaffolds for new-tissue regeneration.

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1 Introduction

Tissue engineering aims at the repair and reconstruction of biological tissues, overcoming the limitations of the traditional treatments, such as transplantation, that are inadequate for the large number of clinical needs [1]. One of the most efficient strategies developed to this aim was the design of 3D biocompatible and biodegradable porous materials suitable as scaffolds for cells and able to guide the process of new-tissue regeneration [2, 3]. With this ultimate goal, the scaffold must possess a three-dimensional and highly interconnected porous network with well defined porosity, pore size, shape and interconnectivity. These topological parameters may guide cell functions by regulating the interaction between the cells and the diffusion of nutrients and metabolic wastes in the whole 3D construct [3–8].

Several biodegradable thermoplastic materials, from both synthetic and natural origins have been investigated as suitable tissue engineering scaffold materials [3, 4, 9–15]. Materials of synthetic origin, such as polyesters, were found to be excellent biomaterials for the design of porous scaffolds with well controlled micro-structures [3, 16]. Synthetic thermoplastic materials may be easily processed with the technologies commonly used for the preparation of porous materials (e.g. gas foaming, reverse templating, phase separation) [9, 11, 12] and may allow for the design of scaffolds characterized by adequate functional and mechanical properties. However, these materials showed limited control over cell biosynthesis and new-tissue regeneration [12]. In order to overcome this limitation, great efforts are currently devoted to the design of porous scaffolds starting from materials of natural origin, such as collagen, gelatin, chitosan and starch [10, 13]. Indeed, the chemical and physical structures of these biopolymers,

similar to those of native biological tissues, may promote cell adhesion, proliferation and biosynthesis, finally enhancing the new-tissue regeneration ability of the natural-based scaffolds [12]. However, the preparation of porous biopolymer scaffolds is mainly limited to solvent-based processes [13].

Among the fabrication technologies that have been used to process biocompatible and bioresorbable materials into 3D porous scaffolds, the selective polymer extraction from co-continuous blends has steadily increased over the past years [14, 15]. In effect, detailed control over scaffolds microstructure may be achieved by the control of the morphology of the blends and therefore, scaffolds with open porosity and different pore size distributions may be designed by this technique [14, 15]. However, the optimization of the micro-architecture of the porous structure of the scaffolds requires a careful investigation of processing/structure/property relationships with respect to the specific system selected.

We recently reported the preparation of co-continuous blends of poly(ε-caprolactone) (PCL) and thermoplastic gelatin (TG) with the ultimate goal to design PCL scaffolds characterized by multi-scaled porosity distribution by the combination of gas foaming (GF) and selective polymer extraction (PE) processes [16].

In this study we investigated the effect of different solvents and extraction parameters on the selective extraction processes of PCL and TG from the blends, in order to obtain both synthetic and natural-based porous scaffolds with well controlled porosity and pore size distributions. Furthermore, the design of multi-scaled PCL and TG porous scaffolds by the combination of GF and PE technologies is presented in a comparative manner, in order to investigate the effect of materials and processing parameters on scaffold microstructures.

2 Materials and methods

2.1 Materials

PCL (M_W = 65 kDa, T_g = −60°C, T_m = 59–64°C) and gelatin powder (type B, M_W = 40–50 kDa) were purchased from Sigma-Aldrich (Italy). Glycerol anhydrous (99.5% purity grade) was purchased by Fluka (Italy) and used as plasticizer for the TG preparation. N_2/CO_2 mixture (80/20vol.%) (Air liquide, Italy) was used as blowing agent in the gas foaming process.

2.2 Blending and foaming

The PCL/TG blends were prepared by a melt mixing process, as described in [16]. Briefly, the TG was prepared by mixing 50 g of gelatin powder with 10 g of glycerol at 60°C, 60 rpm for 6 min in an internal mixer (Rheomix® 600 Haake + Haake Rheocord® 9000, Germany). The TG was subsequently melt mixed with PCL at 60°C, 80 rpm for 6 min in the same equipment and in compositions varying in the 60/40–40/60 (w/w) PCL/TG range. Finally, the blends were compression moulded at 80°C into 2 mm thick plates by using a hot press (P300P, Collin, Germany).

For the gas foaming experiments, disc-shaped samples (10 mm in diameter and 2 mm thick) were solubilized in a pressure vessel with 80/20 (v/v) N_2/CO_2 blowing mixture at 180 bar, 70°C for 4 h and subsequently cooled or heated to the desired foaming temperature (T_F). The pressure was then released to ambient pressure to allow the nucleation and growth of gas bubbles [9, 16].

2.3 Characterization

Dynamic-mechanical analysis (DMA) was used to evaluate the viscoelastic behaviour of the blended materials. Rectangular samples (length = 8 mm, width = 27 mm and thickness = 2 mm) were tested in a single cantilever bending mode, at an oscillatory frequency of 1 Hz and in the −90 to 60°C temperature range (2°C/min heating rate) by using a dynamic-mechanical analyzer Tritec 2000 (Triton Technology, Ltd. UK).

For the selective TG or PCL extraction, disc-shaped samples (10 mm in diameter and 2 mm thick) were soaked into the solvent and the weight evolution measured by using an AB104-S, (Mettler Toledo, Italy) balance. The selective TG extraction was performed by soaking the samples in water, while the selective PCL extraction was performed in chloroform. After the achievement of the equilibrium weight, samples were vacuum dried, weighted and analyzed by scanning electron microscopy (SEM) in order to characterize the polymer extraction efficiency and the micro-structural properties of the scaffolds. Three samples for each composition have been used for the analysis of the templating process. Furthermore, Image J® software was used to evaluate the pore size distributions of the scaffolds. SEM analysis has been performed on foamed blends, too, before and after the selective polymer extraction in order to investigate the effect of the combined processes on final scaffolds micro-structure.

An in vitro cell/scaffold interaction study has been performed in order to assess the ability of the designed scaffolds to be used for tissue engineering applications, following the same procedure described in [16]. Briefly, γ-sterilized disk-shaped PCL scaffolds (d = 10 mm and h = 4 mm) characterized by a multi-scaled porosity distribution were statically seeded with $4 * 10^5$ bone marrow derived human mesenchymal stem cells (hMSCs) (Clonetics, Italy). After incubation for 2 h in a humidified

atmosphere (37°C, 5% CO_2), 1.5 ml of culture medium was added to each cell/scaffold constructs, followed by a static in vitro culture for 4 weeks. In order to evaluate hMSCs adhesion, proliferation and colonization, at definite culture times the cell/scaffold constructs were fixed with 4% paraformaldehyde for 20 min at RT, rinsed twice with PBS buffer and stained with haematoxylin–eosin (H–E). As a control, PCL scaffolds without cells have been additionally analyzed following the same procedure.

3 Results and discussion

The design of porous scaffolds with interconnected porosity and well controlled pore size distribution is essential in tissue engineering to allow the regeneration of functional biological tissues in vitro and in vivo [3–8]. Indeed, the micro-architecture of the porous structure of the scaffold strongly affects the spatial organization and distribution of cells in 3D and therefore, the final properties of the new engineered tissue [17]. As a direct consequence, the development of process technologies able to achieve a fine control over the topological properties of the micro-architecture of the scaffolds is a key technological aspect in tissue engineering. To this aim, in this study we investigated the processing/structure/property relationships of PCL and TG porous scaffolds prepared by the selective extraction process, with or without the additional gas foaming step.

3.1 Co-continuous blends preparation

In Fig. 1 the time evolution of torque, mixing speed and melt temperature during the preparation of the 60/40 PCL/TG blend is reported. As evidenced in Fig. 1 and also reported in literature for other thermoplastic systems [18], torque evolution during blending started with a steep increase of the curve to a maximum followed by a continuous decrease to a rather stationary value, when also the melt temperature becomes constant.

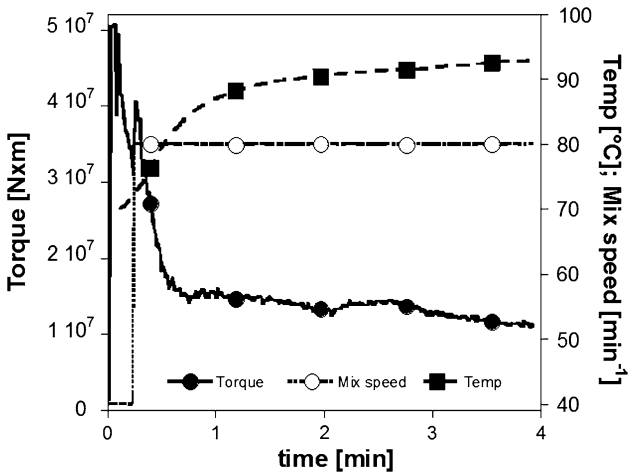

Fig. 1 Time evolution of torque (*filled circle*), mixing speed (*open circle*) and melt temperature (*filled square*) during 60/40 (w/w) PCL/TG blend preparation

The achievement of heterogeneous micro-structures was confirmed by the analysis of the SEM images of the fracture surfaces of the (a) 60/40 and (b) 40/60 PCL/TG blends reported in Fig. 2a, b, respectively and by the results of the DMA analysis, reported in Fig. 3. Indeed, the SEM analysis revealed the presence of two different phases, with the minor phase evidenced by the black arrows of Fig. 2.

Figure 3 shows the temperature dependence of the storage modulus (E') and damping factor (tan δ) of the 60/40 (w/w) PCL/TG blend. As expected, we observed a progressive decrease of E' with the temperature and the presence of two peaks in the tan δ curve at −60°C and 40°C ca. The first peak of tan δ may be ascribed to the glass transition (T_g) of the PCL (see Sect. 2), while the second peak may be ascribed to the T_g of the TG [19]. As also showed by the SEM analysis, the DMA results proved that, after blending, the two polymers, due to their different chemical nature, formed a multi-phase system.

3.2 Selective polymer extraction

In the selective polymer extraction process, one of the critical aspect is the selection of the optimal solvent and soaking parameters. By considering the high solubility of the gelatin in water [20, 21] and the no-citotoxic properties of this solvent, we investigated the selective TG extraction in water. In particular, two soaking temperatures, 30°C and 37°C, were selected and the weight evolution during soaking investigated for the different blends prepared. The results of this analysis are reported in Fig. 4, showing that the dissolution of the TG was strongly dependent on both blend composition and soaking temperature. Typical curves show an initial increase of weight, due to the sorption of water and corresponding swelling of the TG phase, followed by a weight reduction due to the dissolution of the TG. In particular, at 30°C we observed the increase of the water uptake with the increase of the concentration of the TG into the native blend (Fig. 4a). This effect may be ascribed to the increase of the TG amount and to the concomitant decrease of the stiffness of the PCL network, therefore promoting the water absorption and swelling of the TG phase. By increasing the temperature to 37°C, reduced water absorption occurred and we observed the decrease of the weight of the samples during soaking, as a consequence of the progressive TG dissolution (see Fig. 4b). These results may be explained by considering the effect of the temperature on the solubility of the pure

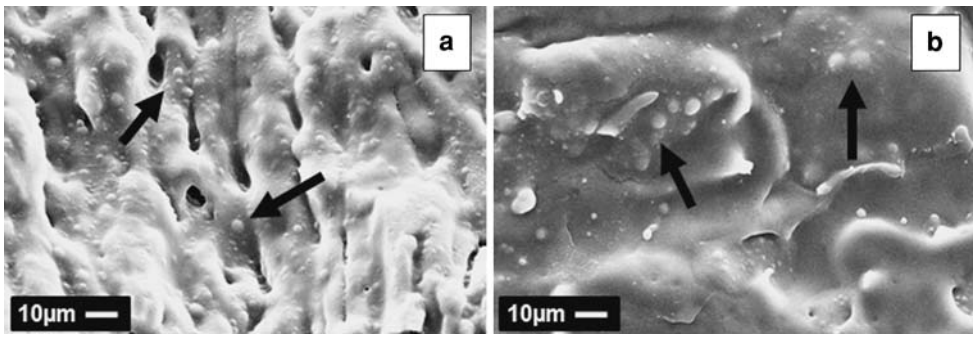

Fig. 2 Fracture surfaces of **a** 60/40 and **b** 40/60 (w/w) PCL/TG blends. The *black arrows* indicated the minor phase

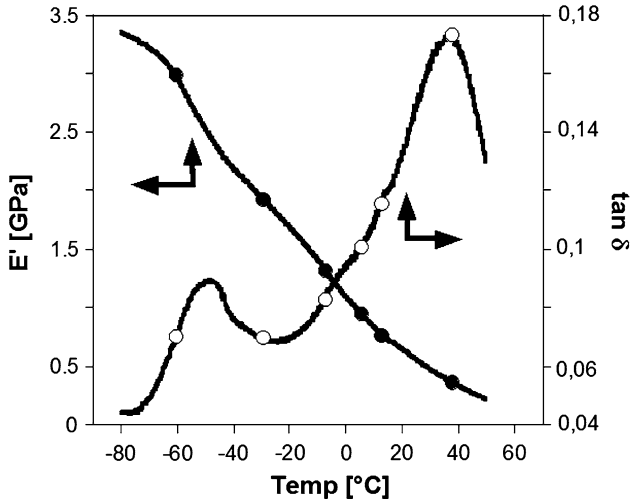

Fig. 3 *E'* (*filled circle*) and tan δ (*open circle*) curves of the 60/40 (w/w) PCL/TG blend

gelatin in water. Indeed, gelatin rapidly dissolves in aqueous environments at 37°C [20, 21], while the decrease of the temperature may enhance its water uptake and swelling. In order to explain these results, we may observe that the TG was prepared by the thermoplasticization of gelatin with glycerol. This process allowed the diffusion of the glycerol molecules into the protein network and the creation of an entangled gelatine/glycerol structure by the formation of weak hydrogen bridges between polymer and plasticizer molecules. Therefore, as reported in literature for pure gelatin [20, 21], the TG may swell when soaked in water and also, rapidly dissolved at 37°C (data not showed).

The final results of the TG extraction are reported in Fig. 5, showing the complete TG removing from the blends at 37°C, while TG residues may be observed at 30°C, with different efficiencies at different TG concentrations. The SEM micrographs of Fig. 6 confirmed the results of the TG extraction. In particular, the porous structure of the PCL scaffolds obtained at 37°C (Fig. 6a–c) well matched the blend composition and therefore, it was possible the enhancement of the pore volume of the scaffolds by increasing the TG concentration into the native blend. Differently, at 30°C (Fig. 6d) decreased pore volume may be observed (compare Fig. 6c, d) due to the presence of TG residues (see also Fig. 5). By considering these results, the soaking temperature of 37°C is required for the preparation of PCL scaffolds by the selective TG dissolution from the PCL/TG blends prepared.

Another important scaffold design advantage of this technique is the possibility of controlling the pore size of the scaffolds without affecting its overall porosity and pore interconnectivity. This may be achieved by performing a thermal annealing treatment before the selective polymer extraction step. In fact, the increase of the temperature increases the polymeric chains mobility and therefore, induces the increase of the mean dimension of the two

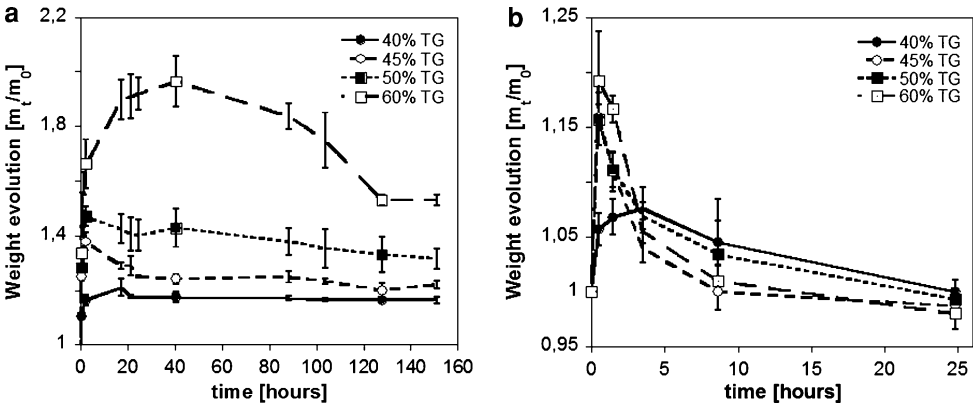

Fig. 4 Effect of PCL/TG blend composition on the weight evolution of the unfoamed PCL/TG blends at **a** 30°C and **b** 37°C (m_t = wet weight, m_0 = initial weight)

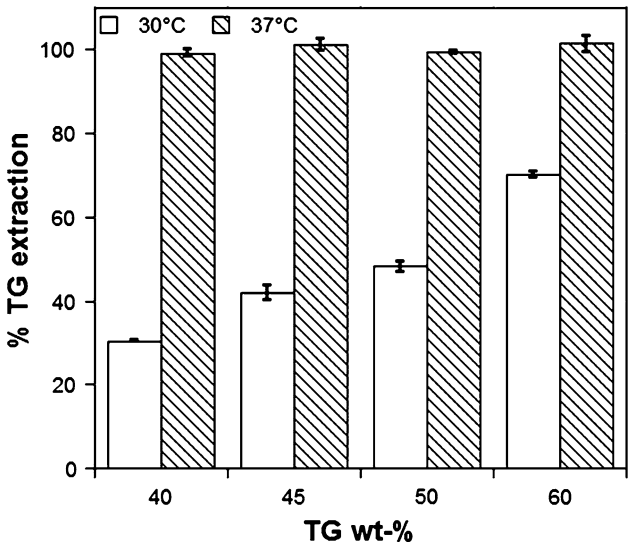

Fig. 5 Effect of blend composition and soaking temperature on the TG extraction from the unfoamed PCL/TG blends

immiscible phases by coalescing mechanism. As a direct consequence, the mean pores size of the scaffolds obtained after the selective polymer extraction increases, while maintaining the overall porosity unchanged [14, 15]. The microstructures of the PCL scaffolds obtained by performing the annealing process at 100°C for 4 h are reported in Fig. 7a, b, evidencing the increase of the pore size of the scaffolds with respect to those obtained without the annealing treatment (Fig. 6a, c). These results have been also confirmed by the pore size distribution analysis, with results reported in Fig. 8. In particular, the scaffolds obtained by the annealing process are characterized by greater mean pore size and wider pore size distributions if compared to those prepared without the thermal treatment.

Similar tests have been performed in order to prepare porous TG scaffolds by the selective PCL extraction process. To this aim, the PCL/TG blends have been soaked in chloroform at room temperature. The results of these tests (not reported) showed the possibility of extract selectively the PCL from all the blend compositions selected, therefore allowing the preparation of porous TG scaffolds with well controlled interconnected porosities.

3.3 Foaming and selective polymer extraction

One of the peculiarity of the PCL/TG co-continuous blends prepared is the possibility to be processed by gas foaming technology [16] before the selective polymer extraction, in order to prepare porous PCL and TG scaffolds with porosity distribution at different scales.

Figure 9 shows the SEM micrographs of foamed blends before (Fig. 9a, b) and after (Fig. 9c–f) the PCL and TG selective extraction processes. The microstructure of the PCL/TG foamed blends showed multi-phase morphologies. Furthermore, different porous micro-architectures may be achieved by controlling both blend composition and gas foaming parameters. In particular, the morphology of the 60/40 (w/w) PCL/TG blend foamed at $T_F = 40°C$ (Fig. 9a) was characterized by a foamed PCL phase and an almost unfoamed TG phase. In effect, the T_F selected was too close to the glass transition temperature of the TG (see Fig. 3) and therefore, limited pore nucleation and growth may be achieved into the TG phase [19]. Differently, the morphology of the 40/60 (w/w) PCL/TG blend foamed at

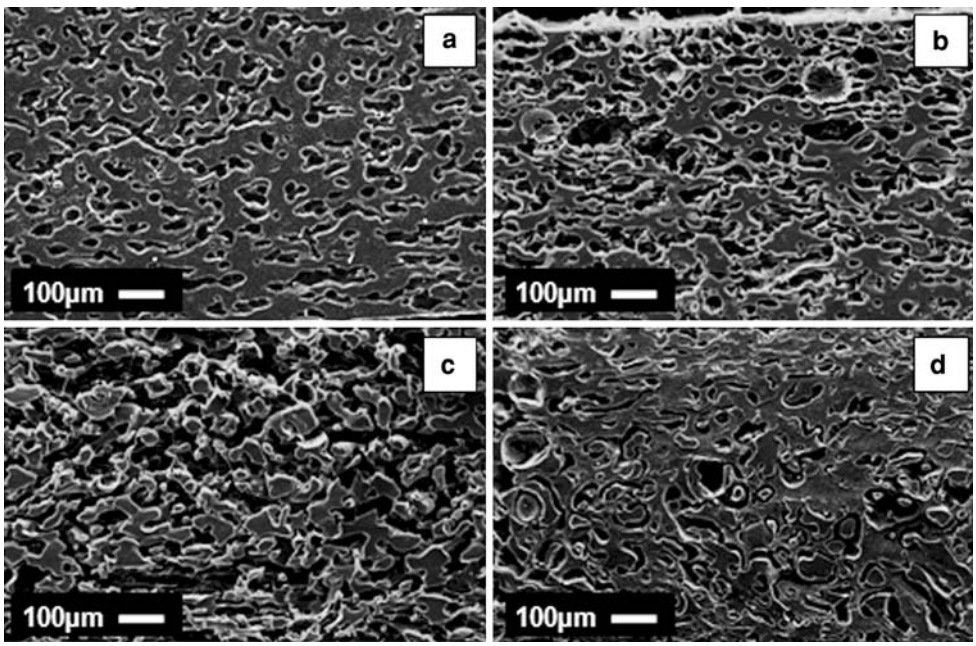

Fig. 6 SEM micrographs of porous PCL scaffolds prepared by using different PCL/TG composition and extraction temperature: **a** 60/40 (w/w) PCL/TG; **b** 50/50 (w/w) PCL/TG and **c** 40/60 (w/w) PCL/TG obtained at 37°C; **d** 40/60 (w/w) PCL/TG obtained at 30°C

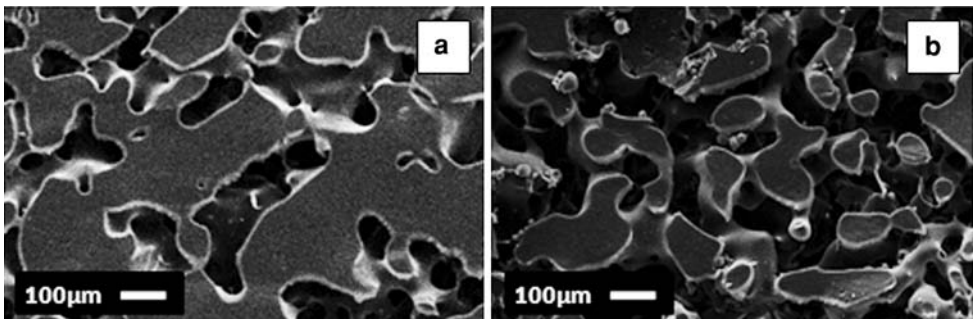

Fig. 7 SEM micrographs of porous PCL scaffolds after the annealing treatment: **a** 60/40 (w/w) PCL/TG and **b** 40/60 (w/w) PCL/TG

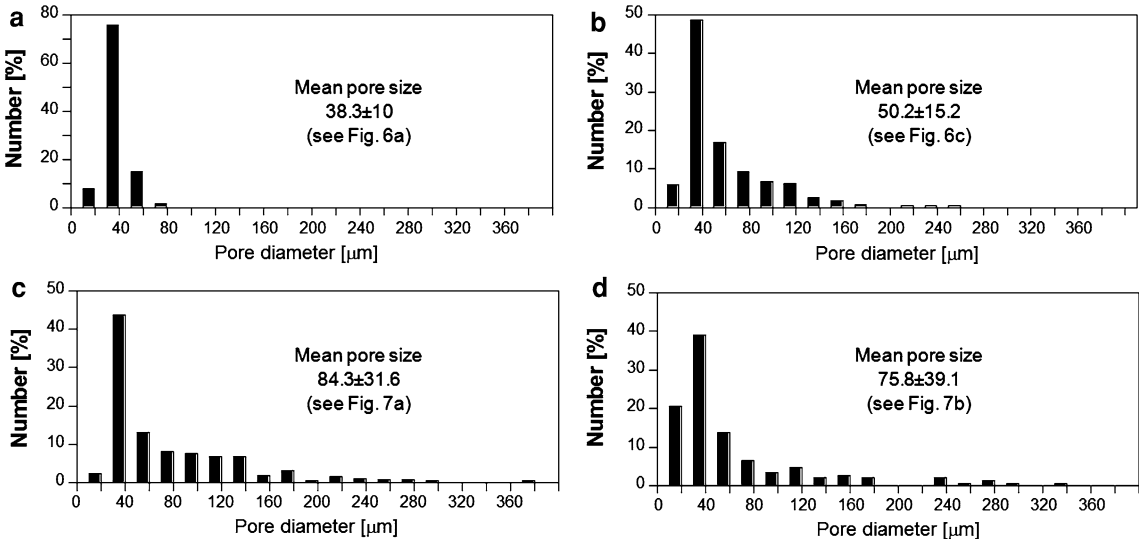

Fig. 8 Pore size distributions of the PCL scaffolds obtained from 60/40 (w/w) PCL/TG blends, before (**a**) and after (**c**) the annealing treatment at 100°C for 4 h; pore size distributions of the PCL scaffolds obtained from 40/60 (w/w) PCL/TG blends, before (**b**) and after (**d**) the annealing treatment at 100°C for 4 h

$T_F = 70$°C (Fig. 9b) evidenced a foamed TG structure and an unfoamed PCL phase. Indeed, when foaming was performed at too high temperatures, PCL does not crystallize and its porous structure collapsed [16].

The morphologies of the PCL and TG scaffolds obtained after the selective polymer extraction are reported in Fig. 9c–f, clearly showing multi-scaled pore size distributions. In particular, pores with mean diameters of the order of hundreds microns (macroporosity) were formed by the extraction of the polymeric phase, while smaller pores (microporosity), were induced by the gas foaming step. However, differences in the topological properties of the microporosity may be observed between the PCL and TG scaffolds prepared. In particular, the PCL scaffolds were characterized by enhanced macroporosity/microporosity interconnection with respect to the TG scaffolds (compare Fig. 9e, f). These differences may be mainly ascribed to the different foamability of the polymers and selected process parameters.

In order to further enhance the porosity interconnection of the gelatin-based scaffolds, we processed the foamed PCL/TG blends by a freeze-drying process before the selective PCL extraction step. This additional processing step consisted of soaking the samples in water at 30°C overnight, freezing at −20°C for 2 h and freeze-drying at 5°C for 1 day. The preliminary results of this test are reported in Fig. 10, showing an high magnification of the microporosity of the novel gelatin-based scaffolds. As shown, the additional freeze-drying step induced the formation of extensive interconnection within the microporosity (black arrows). By using this process we achieved: (i) the water uptake into the TG domains without extensive TG dissolution (compare results of Fig. 4) and (ii) the formation of interconnected pores by the subsequent sublimation of the crystal ices. This microstructure may, therefore, be preferable in view of the enhanced interconnectivity that may better support the diffusion of nutrients and metabolic wastes throughout the scaffold [8]. However, future investigations will be performed in order to investigate the effect of this additional treatment on the chemical–physical changes in the microstructure of the TG scaffolds.

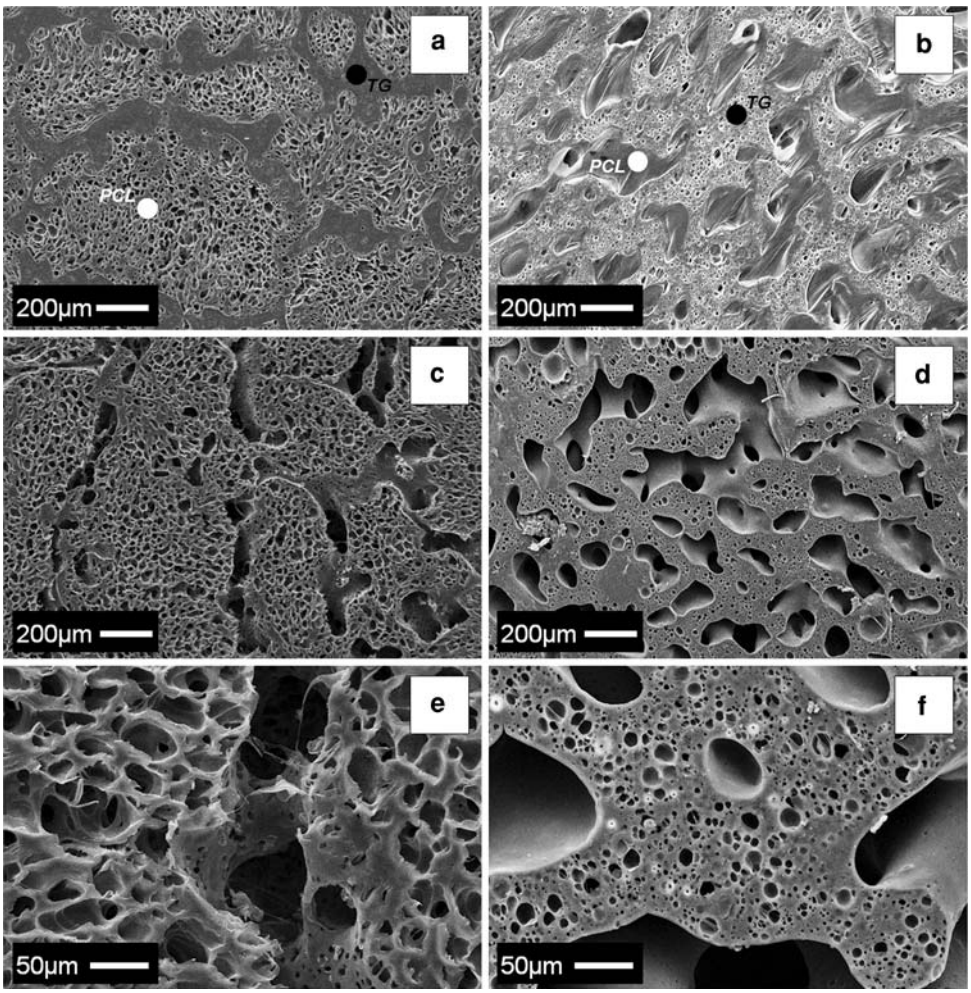

Fig. 9 SEM of 60/40 (w/w) PCL/TG foamed blend ($T_F = 40°C$) before (**a**) and after (**c**, **e**) the TG removal; SEM of 40/60 (w/w) PCL/TG foamed blend ($T_F = 70°C$) before (**b**) and after (**d**, **f**) the PCL removal

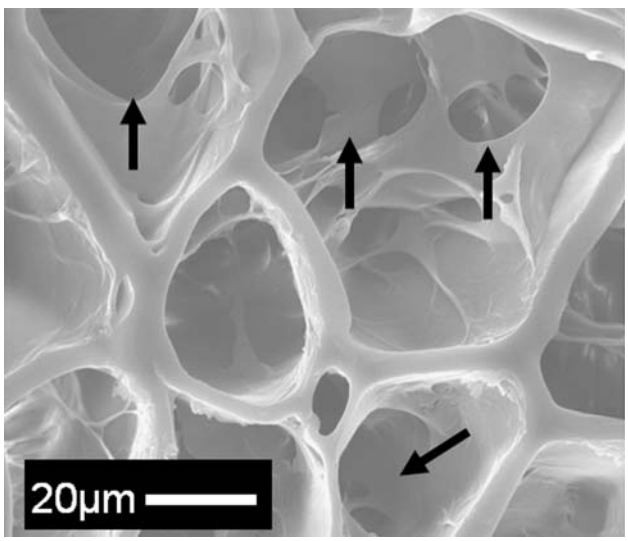

Fig. 10 SEM micrograph of gelatin-based scaffolds showing the interconnection of the microporosity induced by the additional freeze-drying step (evidenced by the *black arrows*)

In order to assess the effect of multi-scaled scaffold microstructures on new-tissue regeneration, we cultured hMSCs into the PCL porous scaffolds of Fig. 9c, e. In particular, hMSCs were statically seeded onto the scaffold surface and the cell/scaffold constructs were cultured in vitro for 4 weeks, by using the seeding/culturing procedures reported elsewhere [16]. Figure 11 reported the results of the histological analysis performed on the neat PCL scaffold (a) and hMSCs/PCL scaffold construct (b) after 4 weeks of culture. As clearly shown, when cultured into the multi-scaled PCL scaffolds the hMSCs were able to colonize the outer and inner regions of its porous structure, preferentially invading the macroporosity (see Fig. 11b). These results may be explained by considering the different size, shape and interconnectivity of the macroporosity, if compared to the microporosity induced by the gas foaming step. Indeed, the pores created by the selective extraction of the TG were characterized by reduced tortuosity and enhanced interconnectivity (see Fig. 9c, e) and therefore, may promote the diffusion of the

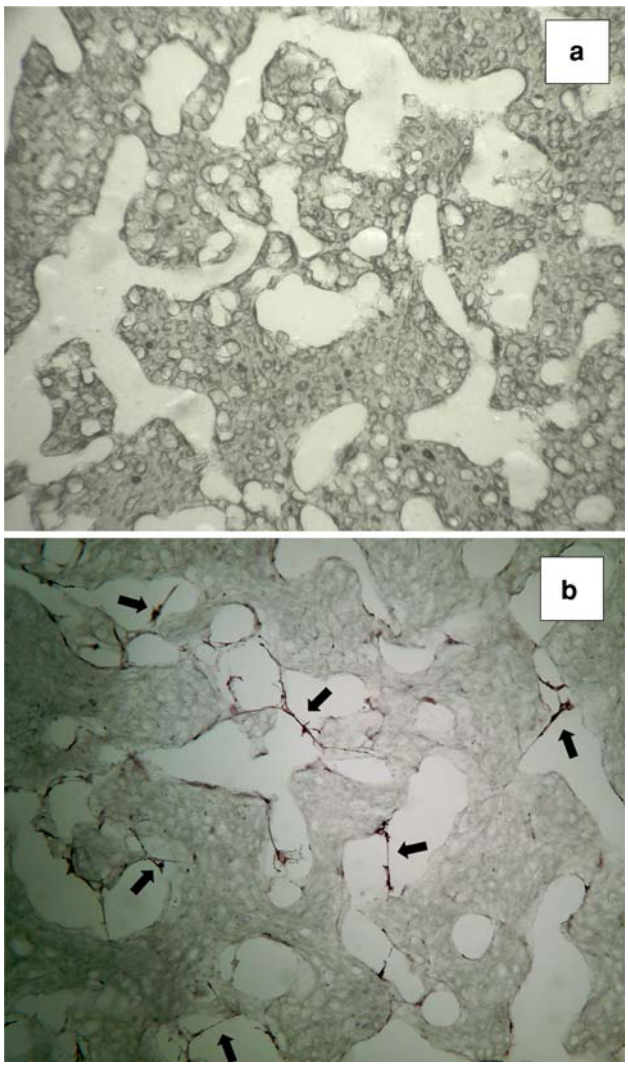

Fig. 11 Haematoxylin and eosin staining of the cross section of the PCL scaffold (**a**) and hMSCs/PCL scaffold construct (**b**) after 4 weeks of in vitro static culture. The *black arrows* indicated some representative cells into the macroporosity of the PCL scaffold

medium with cells during seeding. Consequently, the hMSCs colonized the macroporosity of the scaffolds, proliferate and created bridges between opposite pore walls (see black arrows of Fig. 11).

All of these results demonstrated the great advantages of the PCL/TG blended materials and the GF and PE combined technology in the design of porous scaffolds for tissue engineering.

4 Conclusions

In this study we prepared porous scaffolds with fine controlled porosity and pore size distributions by the selective polymer extraction from co-continuous PCL/TG blends, with or without the additional gas foaming process. The optimization of blends composition and selective polymer extraction parameters allowed an efficient removal of the templating polymeric phase and the preparation of porous scaffolds with different porosity architectures. Furthermore, by the additional gas foaming process we showed the possibility of preparing porous scaffolds with multi-scaled pore size distributions. Finally, the interconnectivity of the gelatin-based scaffolds has been improved further by the additional freeze-drying process, performed before the selective extraction of the PCL.

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