Chapter 2
Materials and Methods

2.1 Materials

Table 2.1 lists the polyesters used to fabricate scaffolds, specifying compositions, suppliers and polymer molecular weight distributions. It is pointed out that all copolymers employed possess a random distribution of their comonomers.

P(L)LA, $PLA_{75}GA_{25}$, P(LA-TMC), PCL and PEO were commercial polymers and they were used without further purifications.

P(D,L)LA and PLAGA copolymers, provided by the Institute of Polymers and Carbon Materials (Zabrze, Poland), were synthesized by ring opening polymerization using a zirconium-based initiator as previously described [1]. The use of these low toxicity initiators is particularly interested, as it was demonstrated that cell viability is higher when polymers are synthesized with zirconium compounds as catalysts instead of the widely used tin compounds [2]. These polymers were used after drying at 80 °C under vacuum in order to eliminate residual solvents employed during polymer purification steps.

Polymers supplied by the Centre for Biocatalysis and Bioprocessing of Macromolecules (New York, USA) (e.g. PPDL, P(PDL-CL) and P(PDL-DO)) were synthesized by ring opening polymerization catalyzed by Candida antartica Lipase B (CALB) as earlier described [3–5]. Enzyme catalyzed polymerizations allow to synthesize copolymers displaying a random distribution of monomeric units thanks to transesterification reactions promoted by CALB during the synthesis.

Chloroform (CLF), Dichloromethane (DCM), N,N-dimethylformamide (DMF), Dimethyl sulfoxide (DMSO), Methanol (MetOH), Tetrahydrofuran (THF), 2-Chloroethanol (CE), Acetone, 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) and Ethanol (EtOH) were purchased by Sigma–Aldrich Co. and they were used without further purification.

The cationic macroinitiator for the Atom Transfer Radical Polymerization, Poly(2-(*N*,*N*,*N*-trimethylammonium iodide) ethyl methacrylate-*co*-bis-2,3-(2-bromoisobutyl) glycerol monomethacrylate) (MI; $M_n = 21.2$ kg/mol, PDI = 1.34, by

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Table 2.1 Polymers used for scaffold fabrication

Polymer	Composition (molar ratio)	Supplier	Molecular weight distribution
Poly(L)lactide (Lacea H.100-E) [P(L)LA]	–	Mitsui fine chemicals (Dusseldorf, Germany)	$M_w = 172$ kg/mol $M_w/M_n = 3.2$[a]
Poly(D,L)lactide [P(D,L)LA]	D:L = 50:50	Institute of polymers and carbon materials, polish academy of science (Zabrze, Poland)	$M_w = 155$ kg/mol $M_w/M_n = 2.4$[a]
Poly((L)lactide-*co*-glycolide) [$PLA_{90}GA_{10}$]	LA:GA = 90:10	Institute of polymers and carbon materials, polish academy of science (Zabrze, Poland)	$M_w = 20$ kg/mol $M_w/M_n = 2.1$[b]
Poly((D,L)lactide-*co*-glycolide) (Resomer RG 756 S) [$PLA_{75}GA_{25}$]	LA:GA = 75:25	Boehringer (Ingelheim, Germany)	$M_w = 170$ kg/mol $M_w/M_n = 2.0$[a]
Poly((D,L)lactide-*co*-glycolide) [$PLA_{65}GA_{35}$]	LA:GA = 65:35	Institute of polymers and carbon materials, polish academy of science (Zabrze, Poland)	$M_w = 41$ kg/mol $M_w/M_n = 2.1$[a]
Poly((D,L)lactide-*co*-glycolide) [$PLA_{50}GA_{50}$]	LA:GA = 50:50	Institute of polymers and carbon materials, polish academy of science (Zabrze, Poland)	$M_w = 81$ kg/mol $M_w/M_n = 2.4$[a]
Poly((L)lactide-*co*-trimethylene carbonate) (Resomer LT 706) [P(LA-TMC)]	LA:TMC = 70:30[c]	Boehringer (Ingelheim, Germany)	[d]
Poly(ε-caprolactone) (787 Tone) [PCL]	–	Union carbide Co. (New Jersey, USA)	$M_w = 74$ kg/mol $M_w/M_n = 2.3$[a]
Poly(ω-pentadecalactone) [PPDL]	–	Centre for biocatalysis and bioprocessing of macromolecules, polytechnic university (New York, USA)	$M_w = 128$ kg/mol $M_w/M_n = 2.0$[a]
Poly(ω-pentadecalactone-*co*-ε-caprolactone) [P(PDL-CL)]	PDL:CL = 69:31	Centre for biocatalysis and bioprocessing of macromolecules, polytechnic university (New York, USA)	$M_w = 240$ kg/mol $M_w/M_n = 8$[e]
Poly(ω-pentadecalactone-*co*-*p*-dioxanone) [P(PDL-DO)]	PDL:DO = 47:53	Centre for biocatalysis and bioprocessing of macromolecules, polytechnic university (New York, USA)	$M_w = 70$ kg/mol $M_w/M_n = 2.3$[e]

(continued)

Table 2.1 (continued)

Polymer	Composition (molar ratio)	Supplier	Molecular weight distribution
Poly(ethylene oxide) [PEO]	–	Sigma–Aldrich (Milan, Italy)	$M_w \sim 1000$ kg/mol[f]

[a] measured by gel permeation chromatography (GPC) in CLF at 25 °C by using polystyrene standards
[b] measured by GPC in THF at 25 °C by using polystyrene standards
[c] mass ratio
[d] supplier provides inherent viscosity = 1.4 ± 0.2 dl/g, measured in CLF 0.1% w/V at 25 °C
[e] measured by GPC in ortho-dichlorobenzene at 135 °C by using polystyrene standards
[f] provided by the supplier

GPC in DMF at 70 °C, by using polymethylmetacrilate standards), was synthesized as previously described [6]. Glycerol Monomethacrylate (GMMA; Cognis, Southampton, UK), 2,2'-Bipyridine (2,2′-Bpy; Sigma–Aldrich), CuCl and $CuBr_2$ (Sigma–Aldrich) were used without further purification.

A six-arms star-branched oligo(D,L)lactic acid (PLA-T6; $M_n = 25$ kg/mol by ^{1}H-NMR), synthesized as described by Biela et al. [7], was kindly provided by Prof. G. Di Silvestro (Organic and Industrial Chemistry Dept., University of Milan). In brief, the PLA-T6 was obtained by polycondensation reaction of lactide and the exa-functional initiator di-pentaerythritol (T6) catalyzed by $Sn(Oct)_2$. In order to transform the carboxyl end groups of PLA-T6 into carboxilate terminal groups, the oligomer was subject to the following salification procedure prior to use: 400 mg of PLA-T6 were dissolved in 20 ml of THF with the addition of 0.6 ml NaOH 0.1 M. The solution was stirred for 2 h then oligomer was precipitated in cyclohexane, washed with deionized water and dried over P_2O_5 under vacuum for 2–3 days.

2.2 Scaffold Fabrication by $ScCO_2$ Foaming

$ScCO_2$ scaffold fabrication was carried out in a 60 mL stainless steel high-pressure autoclave (made in house) (Fig. 2.1a) connected with a high pressure PM101 pump (New Ways of Analytics, Lörrach, Germany) that was used to charge CO_2 into the autoclave. Temperature and CO_2 pressure inside the autoclave were accurately controlled during the foaming process by using: (1) a CAL 3300 temperature controller (Advanced Industrial Systems, Inc., Luisville, USA) connected to a thermocouple inserted into the autoclave (Fig. 2.1b) and (2) a backpressure regulator (Bronkhorst, the Netherlands) and a pressure transducer (Fig. 2.1c).

A polymer disc (200 ± 5 mg) inserted in a cylindrical Teflon mould (10 mm diameter and 10 mm height, Fig. 2.1d) was placed in the autoclave and the foaming process was carried out as follows: the autoclave was heated to the desired temperature and filled with CO_2 at 230 bar (pressurization stage). The system was maintained at constant temperature and pressure over a given period of time (soak time). The soak stage was followed by a depressurization stage during

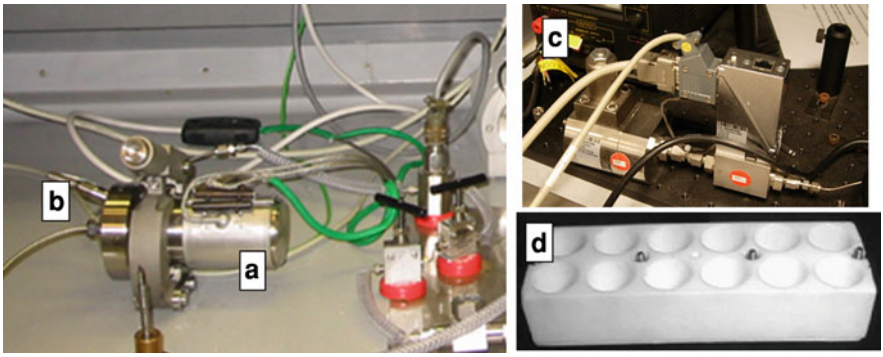

Fig. 2.1 $ScCO_2$ foaming apparatus **a** autoclave, **b** thermocouple, **c** back pressure regulator system and **d** Teflon mould with 12 wells for batch scaffold production. This mould was designed with a detachable base to allow easy removal of scaffolds after fabrication

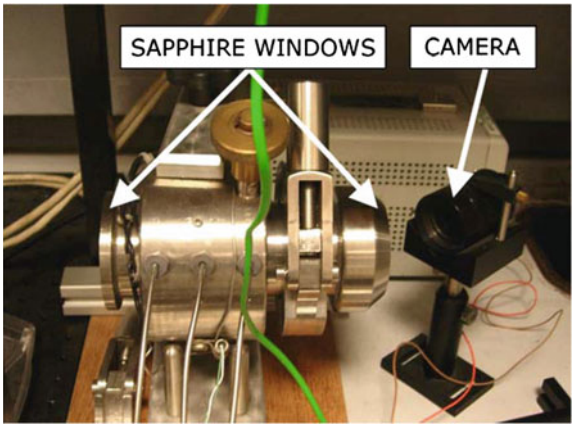

Fig. 2.2 View cell for real-time foaming process observations

which the pressure was decreased to ambient pressure at controlled depressurization rate (dP/dt). During this stage the temperature was either kept constant or lowered at a controlled cooling rate (dT/dt) down to a selected temperature. At the end of the process, after a spontaneous cooling to room temperature (RT), the Teflon mould containing the foamed sample was removed from the autoclave.

Alternatively, the foaming process was also carried out in a 100 ml stainless steel high-pressure autoclave equipped with two sapphire windows (view cell, Fig. 2.2) with the aim to visualize the macroscopic changes of sample aspect and shape during the foaming process. Sapphire windows were located at each end of the autoclave, one used for back illumination. A polymer disc (200 ± 5 mg) was placed in a Teflon mould ($10 \times 10 \times 2$ mm) inserted in the view cell, and foaming process was carried out as previously described. A CCD uEye camera (Firstsight Vision, UK) placed in front of the sapphire window was used to capture real time images of the sample subjected to the foaming process.

2.3 Scaffold Fabrication by Electrospinning

The electrospinning (ES) apparatus was placed in a glove box (Iteco Eng., Ravenna, Italy, 100 × 75 × 100 cm) equipped with a temperature and humidity control system (Fig. 2.3b). The ES apparatus (made in house) was composed of a SL 50 p 10/CE/230 high voltage power supplier (Spellman, New York, USA, Fig. 2.3c), a KDS-200 syringe pump (KDScientific Inc., Massachusetts, USA, Fig. 2.3d), a glass syringe containing the polymer solution, a stainless-steel blunt-ended N-P3-G18 needle (Hamilton, Bonaduz, Switzerland, Fig. 2.3e) connected with the power supply electrode and a grounded collector (Fig. 2.3f). The polymer

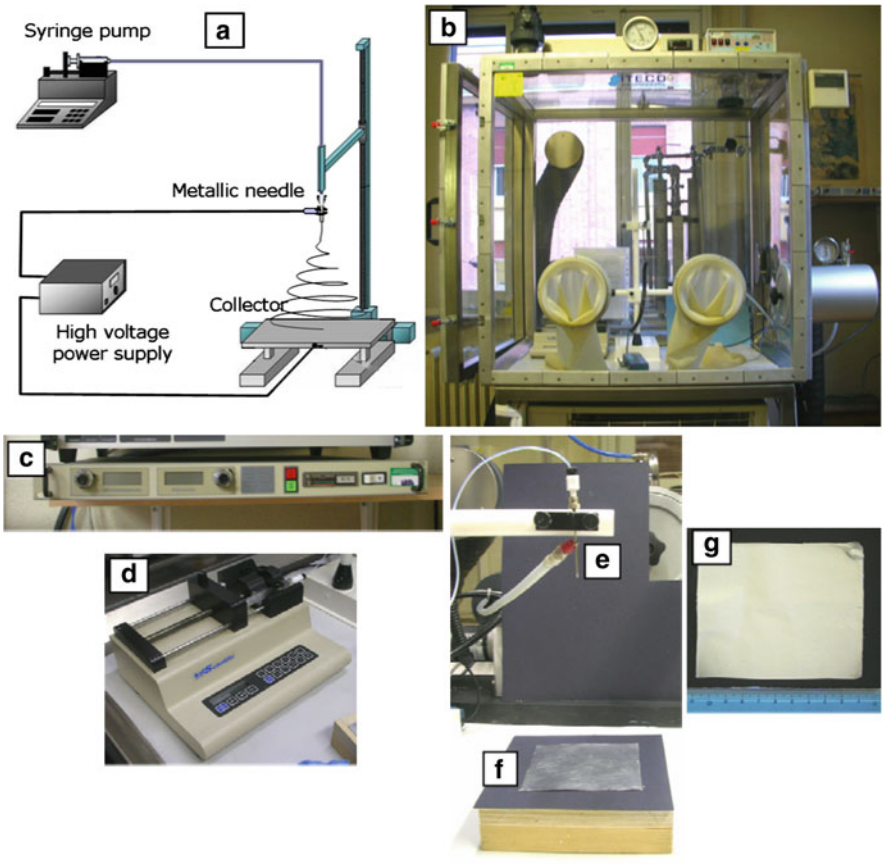

Fig. 2.3 **a** Scheme of the ES process, **b** glove box containing the ES apparatus composed of: **c** high voltage power supply, **d** syringe pump, **e** metallic needle and **f** collector. **g** representative picture of ES mat deposited on aluminium plate collector (10 × 10 mm)

solution was dispensed through a Teflon tube to the needle that was vertically placed on the target.

According to productivity and fibre deposition distribution requirements, collectors of different type and size were used. Alluminium plate collectors were employed for fabricating non-woven ES mats composed of randomly oriented fibres. Cylindrical rotating targets of different radius were used to collect ES fibres with different degree of spatial orientation. Finally, ad hoc developed targets, that allow to accurately control fibre deposition, were employed in order to fabricate patterned ES mats. Such collectors and the effect of their composition and geometry on mat morphology will be described in detail in Chap. 3.

ES polymer mats loaded with additives (i.e. Endothelial Cell Growth Factor Supplement and PLA-T6 oligomers) were obtained by simply electrospinning the polymeric solution containing the additional substance at the desired concentration.

In all cases, after fabrication, ES mats (Fig. 2.3g) were kept under vacuum over P_2O_5 at RT overnight in order to eliminate residual solvents.

2.3.1 Surface Modification

P(L)LA ES samples (3 ± 1 mg) containing 10% w/w of PLA-T6 oligomers were fixed on plastic rings (CellCrown^{TM}12, inner diameter = 15 mm, Scaffdex, Tampere, Finland, Fig. 2.4) and were immersed in EtOH for 15 min in order to ensure a fast and complete wetting of the intrinsically hydrophobic scaffold. EtOH was then replaced by deionized water through repeated rinses.

Each wet mat was placed in 10 ml of 0.1% w/V aqueous solution of the ATRP-macroinitiator (MI) and left at RT overnight under shaking to allow electrostatic adsorption to occur. Then, mats were thoroughly rinsed with deionized water and dried under nitrogen purge.

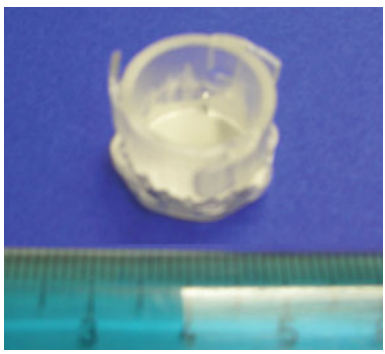

Fig. 2.4 ES mat fixed on a CellCrown^{TM}12 plastic ring (Scaffdex)

The obtained MI-coated mats were inserted in 50 ml Falcon tubes, placed in a parallel reactor and purged with nitrogen. Deionised water containing a mixture of GMMA, CuCl, $CuBr_2$ and 2,2′-Bpy (molar ratio = 60:1:0.3:2.8, GMMA concentration = 2.1 M) was bubbled with nitrogen for 45 min before addition of the required volume of MetOH (H_2O:MetOH = 1:1, by volume). Aliquots of this reactive mixture (22 ml) were then transferred to each Falcon tube containing ES mats to start polymerisation. The Surface-Initiated ATRP (SI-ATRP) of GMMA on nanofibers was carried out under nitrogen at RT for 16 h. The polymerization was interrupted by exposing mats to air. All samples were thoroughly washed with deionised water for one day.

2.3.2 In Vitro Degradation Experiments

Hydrolytic degradation studies were carried out on P(L)LA non-woven ES mats (25 ± 5 mg). Prior to degradation experiments specimens were dried over P_2O_5 under vacuum at RT for 2 days and they were weighted to yield the sample initial weight (m_0). Subsequently, samples were pre-wetted in EtOH for 15 min. EtOH was then replaced by deionized water through repeated rinses. Wet ES samples were immersed in phosphate buffered solution (0.1 M, pH = 7.4) and incubated in a shaking bath (SBS30 Stuart Scientific, Surrey, UK) at 37 °C and 50 revs/min. The buffer solution was periodically changed to keep the pH constant during the entire time scale of the degradation experiments. After selected exposure times, samples were recovered, repeatedly washed with deionized water to remove the buffer salt components and then dried over P_2O_5 under vacuum for 2 days to constant weight (m_x). The percentage weight remaining m(%) after buffer exposure was calculated according to Eq. 2.1:

$$m\,(\%\,) = 100 - \frac{m_0 - m_x}{m_0} \times 100 \tag{2.1}$$

Where m_0 is sample initial weight and m_x is sample weight after x days in buffer at 37 °C.

2.3.3 Scaffold Preparation for Cell Culture Experiments

Scaffold fixation on plastic rings (Fig. 2.5a, b) was adopted in order not only to avoid cell dispersion/outflow during cell culture experiments, but also to improve scaffold handling and to prevent scaffold shrinkage during the subsequent cell culture steps. Cell culture experiments were performed by inserting the ES samples, preliminarily mounted on plastic rings, into common TCPS culture wells (Fig. 2.5c). During the seeding step, cells were confined onto the upper scaffold surface by the walls of the ring. By this means, cell migration towards the TCPS well bottom was prevented (Fig. 2.5d).

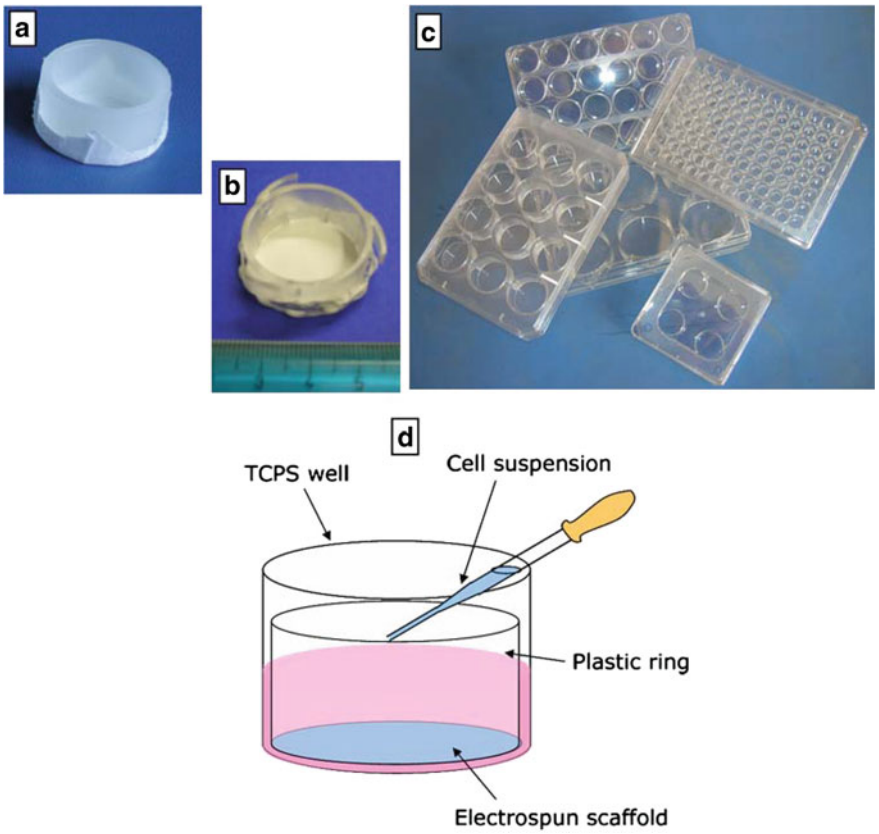

Fig. 2.5 **a** ES scaffold fixed on a Tecaflon plastic ring by using medical-grade silicon, **b** ES scaffold fixed on a CellCrown^{TM}6 plastic ring (Scaffdex), **c** common TCPS multiwell culture plates (different well dimensions are available) and **d** schematic representation of cell culture experiments: the ES sample fixed on the plastic ring is inserted into the TCPS well and cells are seeded on the upper surface of the scaffold

When cell culture experiments were performed in 12-multiwell TCPS plates (circular wells of 19 mm in diameter), ES scaffolds were fixed on Tecaflon (PVDF) plastic rings (internal diameter = 17 mm, external diameter = 18 mm) using silicone (GE Silicones Rubber, RTV 108Q, Fig. 2.5a). When cell culture experiments were performed in 6-multiwell TCPS plates (circular wells of 32 mm in diameter), CellCrown^{TM}6 plastic rings (inner diameter = 29 mm, Scaffdex, Tampere, Finland, Fig. 2.5) were used to fix the ES scaffolds without the need to use silicon glue.

Before exposure to culture medium, all scaffolds were subjected to a sterilization procedure using EtOH according to the following protocol: under a laminar flow, the scaffolds were immersed in 85% V/V EtOH for 15 min, followed by 70% V/V EtOH for 15 min, and then washed 3 times with phosphate buffered saline

(PBS, pH = 7.4) plus 2% Penicillin/Streptomycin (BioWhittaker-Lonza) and 0.2% Amphotericyn B (Sigma). Scaffolds were kept in this solution overnight under ultraviolet irradiation (TUV 30 W/G30 T8).

2.4 Characterization Methods

2.4.1 Thermogravimetric Analysis

Thermogravimetric analysis (TGA) measurements were carried out using a TGA2950 thermogravimetric analyzer (TA Instruments, New Castle, Delaware, USA). Analysis were performed on samples weighing 2–8 mg, from RT to 600 °C, at a heating rate of 10 °C/min, under N_2 flow.

2.4.2 Differential Scanning Calorimetry

Differential scanning calorimetry (DSC) measurements were carried out in helium atmosphere by using a Q100 DSC apparatus (TAInstruments, New Castle, Delaware, USA) equipped with a liquid nitrogen cooling system (LNCS) low-temperature accessory. Samples were placed in aluminum pans and subjected to heating scans at 20 °C/min from −80 °C to a temperature higher than glass transition temperature (T_g) for completely amorphous polymers, or higher than melting temperature (T_m) when semicrystalline polymers were analysed. Either quench cooling or controlled cooling at 10 °C/min were applied between heating scans. T_g values were taken at half-height of the glass transition heat capacity step while crystallization temperatures (T_c) and T_m were taken at the maximum of exotherm and endotherm peaks respectively. The degree of crystallinity, χ_c, was calculated using the following equation:

$$\chi_c = \frac{\Delta H_m}{\Delta H_m^0} \times 100 \tag{2.2}$$

Where ΔH_m is the experimental melting enthalpy obtained from the DSC scan and ΔH_m^0 is the melting enthalpy of 100% crystalline polymer.

2.4.3 Scanning Electron Microscopy

Samples were fixed with a conducting bi-adhesive tape on aluminium stubs and they were sputter coated with gold. Scanning electron microscopy (SEM) observations were carried out by using a Philips 515 microscope at an accelerating voltage of 15 kV. Images were acquired and analysed with EDAX Genesis software.

2.4.4 Micro X-Ray Computed Tomography

Micro X-ray Computed Tomography (μ-CT) images were acquired using a Skyscan 1174 Scanner (Skyscan, Aartselaar, Belgium). The scanner was set to a voltage of 50 kV and a current of 800 mA. By keeping constant the threshold range, the resulting 2D images were elaborated to obtain 3D reconstructions of the scaffolds, from which porosity and pore size were calculated, and pore interconnectivity was visually estimated.

2.4.5 Stress–Strain Analysis

Mechanical properties of foamed scaffolds were evaluated on 5 mm × 5 mm × 3 mm (thickness) specimens. Compression stress–strain measurements were performed with a TA.HDplus Texture Analyzer (Stable Micro Systems Ltd., Surrey, United Kingdom) at RT and at a cross head speed of 0.01 mm/s (load cell 750 N). Triplicate measurements were performed and average values (±standard deviation) are reported.

2.4.6 Wide Angle X-Ray Diffraction

Wide angle X-ray diffraction (WAXS) measurements were carried out at RT with a X'Pert PRO diffractometer (PANalytical, Almelo, the Netherlands) equipped with an XCelerator detector. Cu anode was used as X-ray source (K radiation at $\lambda = 0.15406$ nm, 40 kV, 40 mA) and 1/4 divergence slit was used to collect data in the range $2\theta = 2$–$60°$. After subtracting the diffractogram of an empty sample holder from the experimental diffraction curve, the amorphous and crystalline contributions were calculated by fitting method using the WinFit program. The degree of crystallinity (χ_c) was evaluated as the ratio of the crystalline peak areas to the total area under the scattering curve [8].

2.4.7 Gel Permeation Chromatography

Sample molar mass was evaluated by gel permeation chromatography (GPC) in chloroform (flow rate = 1 ml/min) at 35 °C by using a VE3580 solvent delivery system (Viscotek Corp., Texas, USA) with a set of two PLgel Mixed-C columns and a Shodex SE 61 refractive index detector. A volume of 100 µL of sample solution in chloroform (5% w/V) was injected. Polystyrene standards were used to generate a calibration curve.

2.4.8 ζ-Potential

Electrokinetic analyses were performed with a SurPASS electrokinetic analyzer (Anton Paar, Österreich, Austria) equipped with a cylindrical glass cell. ES samples pre-wetted in EtOH and thoroughly rinsed with deionized water were analysed. The wet sample was inserted into the cylindrical cell. The ζ-potential was determined from the measurement of streaming potential generated by the imposed movement of an electrolyte solution (KCl 1×10^{-3} M) through the sample. The ζ-potential, which is related to the charge density on sample surface, was determined at pH values in the range 5–9 by performing automatic titration.

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