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Electrospinning of poly(β -hydroxybutyrate) scaffolds: morphology and aging

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Q8 Fibrous polymeric scaffolds which comprise capabilities of biomimicry to the native tissue architecture are promising for achieving functional tissue-engineered products with minimal surgical implantation. This research focused on the conditions in producing fibrous scaffolds of biodegradable microbial poly(β -hydroxybutyrate) (PHB) by electrospinning. Such conditions were found to influence the morphology of the scaffolds, which, in turn, appeared to control the wettability and the aging behavior, both of outmost importance for medical applications. Commercial and biosynthesized PHB, from a mutant *Azotobacter vinelandii* OP strain, were investigated. The morphology, wettability and thermal properties of the fibrous scaffolds were characterized using optical and electron microscopy, thermogravimetric analysis and scanning calorimetry. Tuning the voltage and the solution concentration (at constant flow rate) produced either a filamentous morphology or aggregates of filaments. The wettability was strongly correlated with the degree of porosity, and predominantly a hydrophobic behavior was attained in an otherwise hydrophilic material. Aging, monitored at room temperature up to 11 months by degree of crystallinity, α , showed that scaffolds with homogeneous filamentous morphology exhibited an increase in crystallinity leading to brittleness. However, scaffolds with aggregated filaments were quite stable as the degree of crystallinity remained nearly constant over the 11-month period. Applications are envisioned for PHB biomimetic scaffolds in bone tissue repair involving bone defects and as membranes for guided bone regeneration.

Q9 Notation

2θ	scattering angle
hkl	indices of the crystallographic plane
I	X-ray scattered intensity
K	constant in Scherrer's equation (≈ 1)
L_{hkl}	lateral crystal size along the hkl direction
M_v	viscosity-average molecular weight
p	porosity
q	scattering vector
T_c	crystallization temperature
T_{dec}	temperature of thermal decomposition
T_m	melting temperature
α	Mark-Houwink's exponent
α_c	degree of crystallinity
β_o	full width at half maximum
ΔH_c	crystallization enthalpy
ΔH_m	melting enthalpy
$[\eta]$	intrinsic viscosity
λ	radiation wavelength

1. Introduction

The development of biocompatible polymeric materials with tuned physical properties and added functionality has recently surged due to significantly potential biomedical applications. Furthermore, adding biodegradability just broadens the scope of these applications.^{1–4} Although materials are an essential component for a given application, the processing route can also define the properties and behavior of these materials. Moreover,

for any material to have commercial viability, it must perform as designed; must withstand processing, handling and sterilization procedures; and must have a lifetime appropriate to particular applications and needs of users. Furthermore, during shelf life storage, the polymers must retain their physical properties. Therefore, the development of polymeric materials for potential biomedical applications goes from the synthesis to processing and understanding the influence of sterilization and storage time on their physical properties. Examples of close coupling between synthesis and processing to produce smart, functional polymers are shape memory polymers,^{5–7} functional hydrogels,⁸ smart coatings⁹ and electrospun scaffolds.^{10,11}

The processing of polymers by electrospinning is a technique that can produce micrometer- to nanometer-scale fibrous morphologies and greatly modify the physical properties of polymers. These morphologies are ideal to constructing biomimetic scaffolds useful for diverse biomedical applications. This usefulness arises from closely mimicking the fibrillar components (e.g. collagen) of the extracellular matrix (ECM). The ECM surrounds the cells of any biological tissue.¹² Thus, fibrous scaffolds with high porosity and specific surface area and topography are ideal candidates for engineering ECM, promoting naturally the cell functions. However, in order to assess the feasibility of an electrospun polymer as scaffold, it is essential to perform biocompatibility tests and functionality evaluation in vitro and in vivo by seeding cells and determining the cell viability.

Electrospinning occurs when the electrical forces at the surface of a polymer solution or melt overcome the surface tension and cause an electrically charged jet to be ejected. When the jet dries or solidifies, an electrically charged fiber remains. This charged fiber can be directed or accelerated by electrical forces and then collected in sheets or other useful geometrical forms. Important factors in electrospinning processing are the applied voltage (V), the solution flow rate (Q), polymer concentration (c), polymer molecular weight (M_w) and nozzle-to-collector distance (H).¹³

The proper choice of electrospinning parameters enables the production of a thin fiber web constituted by continuous smooth fibers of homogenous diameter to be deposited onto a substrate. The filament diameters can range from the nanometer to micrometer scale.^{13–15} Since the pioneering work of Reneker at Akron, polymer electrospinning has proved to be effective in modifying the properties of bulk polymers and is finding diverse industrial, medical, automotive and aerospace applications.¹³ Electrospun polymeric fibers are being utilized to produce polymer nanocomposites.^{14,16–18}

The application of electrospinning to biodegradable and/or biocompatible polymers is elucidating novel applications in tissue engineering, antibacterial membranes and hydrogels, metal and biomolecule immobilization systems and shape memory composites. Song *et al.* (2012) electrospun poly(vinyl alcohol)–collagen–hydroxyapatite to produce biomimetic bone-like ECM for the modification of orthopedic prosthetic surfaces. A similar approach was utilized to incorporate hydroxyapatite and alumina into a polycaprolactone (PCL) scaffold.¹⁹ Furthermore, cross-linked electrospun collagen scaffolds proved effective for osteoblast viability.²⁰ Electrospun fibrous scaffolds of PCL were investigated for biomolecule immobilization and tissue engineering.²¹ On the other hand, electrospinning poly(lactic acid) (PLA) and amino-functionalized polyhedral oligomeric silsesquioxane (Poss) was effective in immobilizing palladium nanoclusters to produce a novel catalytic system.²²

Wound-dressing materials with antibacterial properties have also been produced by electrospinning polymer/silver nitride solutions²³ and PLA–silver nanoparticle solutions.^{24,25} On the other hand, poly(ethylene glycol)/Poss-based polyurethanes were electrospun and silver nitride incorporated to produce hydrogel mats with antibacterial properties.²⁶ Robertson *et al.*²⁷ applied dual electrospinning of PCL and a polyether-based thermoplastic polyurethane to produce shape memory elastomeric composites. Furthermore, Tumbic *et al.*¹⁵ recently described nanoscale blending of PCL and PLA by utilizing dual electrospinning, thus tuning the micro/nanostructure and thermomechanical properties of the blends.

Therefore, biomimicking electrospun fibrous polymeric structures is of great potential for scaffold-assisted tissue and bone repair and regeneration applications. Moreover, the addition of other functionalities such as shape memory and biodegradability expands their use.

Poly(β -hydroxybutyrate) (PHB) is a polyester produced by numerous bacteria, from the polyhydroxyalkanoate (PHA) family,^{28–31} which are biodegradable. PHB has mechanical properties similar to those of conventional plastics such as polypropylene, although it exhibits higher rates of crystallization and higher degrees of crystallinity, leading to brittleness and low elongation to break.^{32–35} Due to properties of biocompatibility and biodegradability, medical applications for PHB are being proposed, where the chemical composition and product purity are critical. The degradation properties of PHB and PHA in general are desirable for the absorption of these materials in biomedical applications. Furthermore, the degradation product of PHB, D-(–)-3-hydroxybutyrate, is a common intermediate metabolite present in animal cells and was also detected in relatively large amounts in human blood plasma.³⁶ Therefore, there are high expectations for PHA in the medical field, for instance, in surgical sutures, drug delivery, coatings for cardiovascular implants and scaffolds in tissue engineering, among others.^{37–39}

Wang *et al.*⁴⁰ reported the suitability of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) scaffolds to promote in vitro adhesion, proliferation and differentiation of bone marrow cells. Wang *et al.*⁴¹ extended this investigation into different compositions of the copolyester PHBHHx and determined the cell viability of fibroblast and osteoblast cells. The utilization of PHBHHx in tissue engineering has been recently reviewed by Chang *et al.*⁴² PHAs have also been utilized in neural regeneration and nerve tissue engineering, thanks to their high biocompatibility and low cytotoxicity.⁴³ Sevastianov *et al.*⁴⁴ studied the hemocompatibility properties of the PHAs. On the other hand, Shishatskaya *et al.*⁴⁵ reported the growth and differentiation of murine marrow osteoblast cells in poly(3-hydroxybutyrate)–hydroxyapatite.

In the present paper, fibrous scaffolds of PHB from commercial and bacterial sources^{30,35} were manufactured using electrospinning and the correlation between processing conditions and the morphology of the scaffolds is discussed. The aging behavior of PHB scaffolds held at room temperature up to 11 months was characterized by changes in crystallinity. The results of this investigation may be valuable for tissue engineering approaches aimed at designing and implementing prevascularized scaffolds and functional blood vessel networks.

2. Experimental section

2.1 Materials

A commercial poly(3-hydroxybutyrate) (denoted PHB50) from Sigma-Aldrich (St Louis, Missouri, USA) and a specially PHB produced from a mutant strain of *Azotobacter vinelandii* (OP), denoted PHB230, were studied.^{30,35} The biosynthesis procedure of PHB230 consisted of the bacteria being cultured in Burk's medium, and the strain was cultured in 500 ml Erlenmeyer flasks for 72 h in an orbital incubator shaker with a shaking diameter of 2.5 cm and a shaking frequency of 200 revolutions/min at 29°C

Table 1. Physical chemical and thermal properties of PHB homopolymers

Sample	$\overline{M}_v$: ^a kDa	T_m : ^b °C	ΔH_m : ^b J/g	T_c : ^c °C	ΔH_c : ^c J/g	T_{dec} : ^d °C
PHB50	50	159	60	103	61	237
PHB230	230	178	67	106	66	238

^a $\overline{M}_v$ determined by dilute solution viscosimetry, using the Mark–Houwink equation⁴⁶
^b T_m determined by differential scanning calorimetry from the second heating, scanning at 20°C/min
^c T_c determined by differential scanning calorimetry from the first cooling, scanning at 20°C/min
^d Determined by thermogravimetric analysis under dried nitrogen, scanning at 20°C/min^{35,47}

and pH of 7.2. The aeration conditions were monitored through changes in the oxygen transfer rate (see the paper by Peña *et al.*³⁰ for details of the biosynthesis). The physical chemical and thermal properties of the specimens are summarized in Table 1.

The average molecular weight was determined by capillary viscometry using an Ubbelohde-type viscometer (manufactured by Cannon Instrument Company Inc., State College, Pennsylvania, USA). Relative viscosity measurements were carried out on diluted PHB/chloroform solutions at 30°C. Viscosity-average molecular weight, M_v , was determined from intrinsic viscosity values, $[\eta]$, using the Mark–Houwink equation (Equation 1) and the coefficients $k = 0.77 \times 10^{-4}$ and $\alpha = 0.82$ reported by Marchessault *et al.*⁴⁶

1. $[\eta] = k\overline{M}_v^\alpha$

As-cast PHB films were prepared dissolving the polymers in chloroform, pouring in a 25 mm dia. petri dish and drying first at room temperature overnight in a hood and then under vacuum at 40°C for 6 h. The final films' average thickness was c. 35 μ m.

2.2 Electrospinning

The fibrous scaffolds were prepared by electrospinning from a chloroform solution utilizing an in-house built instrument encased in an acrylic box (see Figure 1). The polymers were dissolved in chloroform (analytical grade, purchased from Sigma-Aldrich Chemicals) at 60°C under continuous gentle magnetic stirring. Solution concentrations varied from 12 to 14% weight/volume (w/v), and the applied voltages ranged from 8 to 12 kV. The distance between needle and target (aluminum foil screen) was kept constant at 6 cm. Mats were electrospun for 2 h at a rate of 0.2 ml/h utilizing an NE-1000 programmable single syringe pump (New Era Pump Systems Inc., Farmingdale, New York, USA). A CZE 1000R high-voltage source model manufactured by Spellman High Voltage Electronics Corp. (Hauppauge, New York, USA) was utilized.

2.3 Wide-angle X-ray scattering

Q14 Two-dimensional wide-angle X-ray scattering (Waxs) patterns were obtained using a Microstar rotating anode generator with a copper target manufactured by Bruker (Germany). Waxs patterns were recorded using a mar345 image plate with a resolution of 3450 $\times$ 3450 pixels and 100 μ m/pixel; a sample-to-detector distance of 20 cm was used. The patterns were analyzed using the X-ray scattering software Polar v2.6 (Stonybrook Technology and Applied

Research Inc., New York, USA). The degree of crystallinity, α_c , was calculated from the ratio of the area under the crystalline peaks to that of the total diffraction curve. The amorphous halo was approximated to a Gaussian curve, and the crystalline peaks were fitted to Gaussian functions, utilizing the software PowderX.⁴⁸ (PowderX is software for X-ray scattering analysis freely available for academic and non-commercial use from Professor Chen Dong (chengdon@aphy.iphy.ac.cn), Institute of Physics, Chinese Academy of Sciences, PO Box 603, Beijing 100080, China.) The expression for the degree of crystallinity is⁴⁹

2.
$$\alpha_c = \frac{\int_0^\infty I_c(q)q^2dq}{\int_0^\infty I(q)q^2dq}$$

where I_c is the part of the intensity associated with the crystalline phase, I is the total diffracted intensity and q is the magnitude of the scattering vector defined as

Figure 1. Experimental setup for polymer solution electrospinning: A, pump; B, syringe; C, needle connected to high voltage source; D, grounded collector screen; E, scanning electron micrograph of non-woven polymer filaments; F, high voltage source; G, acrylic box

$$3. \quad q = \frac{4\pi \sin \theta}{\lambda}$$

On the other hand, the crystallite size, L_{hkl} , was determined from the full width at half maximum (FWHM) of the 020 reflection, using Scherrer's equation⁴⁹

$$4. \quad L_{hkl} = \frac{K\lambda}{\beta_o \cos \theta}$$

where L_{hkl} is the mean dimension of the crystallites perpendicular to the planes hkl , λ is the radiation wavelength, β_o is the FWHM in radians of the hkl reflection at 2θ and K is a constant that is commonly assigned a value of unity.⁴⁹

2.4 Contact angle

The contact angle of a water droplet on the scaffolds was determined by the sessile drop technique. A distilled water drop (obtained by purification) was placed on the substrates using a Pasteur pipette and acquired by a MicroView 800X digital optical microscope. The contact angles were determined using the ImageJ software (software freely available from the National Institutes of Health, USA).⁵⁰

2.5 Polarized optical microscopy

Films cast from the chloroform solution were prepared and heated up to 200°C, held for 3 min and then cooled to 30°C at 10°C/min using the FP82HT hot stage and an FP90 controller manufactured by Mettler Toledo (Columbus, Ohio, USA). The morphology of the as-cast films was investigated by polarized optical microscopy (POM) using an Eclipse E600 microscope manufactured by Nikon Instruments Inc. (Melville, New York, USA) equipped with a Motic 2.0 digital camera (Motic, China).

2.6 Scanning electron microscopy

The samples were mounted on a sample holder using a carbon tape and coated with gold for 60 s using an Evex gold sputter coater to yield a coating thickness of approximately 200 Å. The scanning electron microscopy (SEM) micrographs were obtained under vacuum conditions with a Hitachi SU1510 scanning electron microscope with secondary electrons at 15 kV. The filaments' diameter and porosity of electrospun scaffolds were measured from SEM micrographs using the ImageJ software.

3. Results and discussion

The influence of electrospinning parameters, solution concentration and applied voltage on the morphology of the non-woven scaffolds was studied. The flow rate and sample to collector distance were kept constant at 0.2 ml/h and 6 cm, respectively. The average filament diameter, degree of porosity and water contact angle were determined to assess the influence of the processing parameters on final morphology and physical properties of the scaffolds.

3.1 Scaffold morphology

Fibrous non-woven scaffolds with micrometer-scale pores and random orientation of filaments were obtained by electrospinning, as demonstrated by SEM analysis. Furthermore, the results demonstrated that the solution concentration and applied voltage significantly influenced the morphology of the scaffolds. Firstly, the influence of solution concentration and applied voltage was extensively investigated for the smaller-molecular-weight specimen, PHB50. The findings were then applied to the higher-molecular-weight specimen, PHB230. Figure 2 corresponds to a scaffold of PHB50 produced from 12% (Figure 2(a)) and 13% w/v solution concentration (Figure 2(b)) and applying 9 kV. The 12% solution (Figure 2(a)) exhibits morphology of relatively smooth cylindrical filaments and with no beads present. However, at a relatively larger scale, the morphology is relatively inhomogeneous as the filaments are arranged into large, *c.* 100 μ m agglomerates. The mean diameter of the filaments is $3.7 \pm 0.7 \mu$ m. On the other hand, the solution of 13% w/v produced very homogeneous, randomly oriented, cylindrical filament morphology with uniform filament diameter, as shown in Figure 2(b). Note the smoothness of the filaments and the absence of bead morphology as well as uniform spatial distribution of filaments. The mean diameter of the filaments was found to be $2.8 \pm 0.4 \mu$ m – that is, a slight increase in solution concentration produced more homogeneous fibrous scaffolds and with reduced filament diameter.

However, keeping the solution concentration of PHB50 at 13% w/v but increasing the voltage to 12 kV now produced scaffolds with non-homogeneous morphology, as shown in Figure 3(a). Note that the SEM micrograph shows a morphology consisting mostly of thicker filaments and large aggregates of filaments. There is no evidence of bead morphology, and the large aggregates appear to consist of fused filaments. It is believed that at a higher acceleration voltage, there is not enough time for complete solvent evaporation, and the residual solvent plasticized the filaments. Therefore, there is propensity for the filaments to fuse with each other. The mean diameter of the filaments was found to be $6.7 \pm 1.7 \mu$ m. The solution concentration of PHB50 was further increased up to 14% w/v, and in order to enable solvent evaporation, the acceleration voltage was reduced to 10 kV; the micrograph of the scaffold thus obtained is shown in Figure 3(b), and a higher-magnification micrograph is shown in

Figure 2. SEM micrographs of non-woven scaffolds of PHB50 electrospun from (a) 12 and (b) 13% w/v solutions in chloroform applying a voltage of 9 kV

Figure 3. SEM micrographs of non-woven scaffolds of PHB50 electrospun from (a) 13% w/v solution at 12 kV and (b, c) 14% w/v solution at 10 kV

Figure 3(c). The morphology now consists of relatively few thick filaments ($c. 10 \pm 1.1 \mu\text{m}$ thick) and predominantly thinner, fused filaments. The mean diameter of the thinner filaments was found to be $4.8 \pm 0.7 \mu\text{m}$. The micrograph exhibits a honeycomb-like morphology, better appreciated in Figure 3(c). It is suggested that the higher viscosity of the polymer solution slowed down the rate of evaporation of the solvent, thus plasticizing the filaments. Therefore, after deposition on the collector, the filaments in close contact would weld to each other, giving rise to the honeycomb-like morphology.

These results then suggest that electrospinning of the higher-molecular-weight PHB230 should require a lower-solution concentration and smaller acceleration voltage. Indeed, Figure 4 shows the SEM micrograph of a scaffold electrospun from a 12.5% w/v solution at 8 kV. The morphology consists of relatively thick and randomly oriented filaments as well as fused filaments forming a porous network. Note that this scaffold morphology resembles that obtained with PHB50 solutions of 14% w/v and electrospinning at 10 kV. The mean diameter of the filaments was found to be $4.5 \pm 0.7 \mu\text{m}$. In this case, the larger molecular weight produces relatively viscous solutions at this concentration. Therefore, solvent evaporation was slowed down and electrospun filaments were partially plasticized, thus promoting filament fusion.

A detailed investigation was carried out on the agglomerates found in the fibrous electrospun scaffolds. Figure 5(a) shows an SEM micrograph of PHB50 scaffold electrospun from the 13% w/v solution at 12 kV. The morphology consists of randomly oriented and entangled cylindrical filaments and relatively large

aggregates/bundles of filaments. Examination of these microaggregates at higher magnifications revealed a porous morphology in the filaments, as shown in Figure 5(b). These micrographs clearly exhibit highly porous filaments fused to each other. It is suggested that the porosity is due to incomplete evaporation of the solvent when the filaments were ejected from the needle and before depositing on the ground collector. These results also suggest that the incomplete evaporation of solvent gave rise to the aggregation/fusion of filaments.

This hypothesis is supported by closely examining the scaffolds with homogeneous fibrous morphology. Figure 6(a) shows an SEM micrograph of a scaffold of PHB50 electrospun from the 13% w/v solution but lowering the applied voltage down to 9 kV. Note that the morphology consists of smooth, cylindrical and randomly oriented filaments. Furthermore, the higher-magnification micrograph shown in Figure 6(b) shows no sign of porosity in the filaments.

Figure 5. (a) SEM micrograph of non-woven PHB50 scaffold electrospun from 13% w/v solution at 12 kV. (b) Higher-magnification micrograph exhibits porous threads

Figure 4. (a) SEM micrograph of non-woven scaffold of PHB230 electrospun from 12.5% w/v solution at 8 kV; (b) higher magnification of the micrograph

Figure 6. (a) SEM micrograph of non-woven PHB50 scaffold electrospun from 13% w/v solution at 9 kV; (b) higher magnification of the micrograph

Figure 7. Crossed POM and AFM micrographs of PHB230: (a–c) non-woven scaffold electrospun from 12.5% w/v solution at 8 kV; (d–f) solution-cast film

The morphology and topography of polymer scaffolds influence adhesion and cell viability as these closely mimic the fibrillary components (e.g. collagen) of the ECM.⁵¹ The topography and roughness of the electrospun scaffolds and a solution-cast film were investigated by atomic force microscopy (AFM). Figure 7 shows crossed POM and AFM micrographs of a scaffold electrospun from PHB230, 12.5% w/v and 8 kV (Figures 7(a)–7(c)), and a PHB230 film cast from the chloroform solution (Figures 7(d)–7(f)). Figures 7(a) and 7(b) show that the morphology and topography of PHB230 reflect the fibrous morphology and aggregates and are highly non-homogeneous, with pronounced tops and valleys (note the pronounced z-scale in Figure 7(c)). The scaffold exhibited a roughness of 44 nm. Not surprisingly, the solution-cast film exhibits a relatively uniform thickness and the roughness (Figures 7(d)–7(f)) was determined to be 1.8 nm (note the z-scale in Figure 7(f)). As stated earlier,

the degree of roughness is an important property that influences cell adhesion.^{52,53} It is well documented that PHB exhibits fast crystallization kinetics, even under rapid quenching.^{32–35} The polarized optical micrographs of Figures 7(a) and 7(d) show that electrospun scaffolds and cast films exhibit considerable birefringence due to crystallization of PHB. The pronounced degree of crystallinity already present in as-cast films and as-spun scaffolds was found to be a good indication of the influence of storage time (aging) on their physical properties – that is, a higher degree of crystallinity produces more brittleness. Aging was therefore correlated with degree of crystallinity, and this will be discussed below.

3.2 Wetting behavior

The wetting ability of the electrospun non-woven scaffolds was determined by measuring the contact angle of water drops, and the behavior was compared to that obtained from solution-cast films. Moffa *et al.*⁵² and Zhang *et al.*⁵³ have pointed out on the critical role that wettability plays in the adhesion, proliferation and viability of cells. Figure 8 shows images of water drops on electrospun scaffolds of PHB50 electrospun from 13% w/v solution, 9 kV (Figure 8(a)); 13% w/v solution, 12 kV (Figure 8(b)); 14% w/v solution, 9 kV (Figure 8(c)); and 14% w/v, 10 kV (Figure 8(d)). Figure 8(e) shows a water drop on a scaffold of PHB230 electrospun from 12.5% w/v solution, 8 kV. The image of a water drop on a cast film of PHB230 is shown in Figure 8(f). The PHB50 scaffold with uniform filamentous morphology (i.e. no agglomerates), electrospun from a 13% w/v solution concentration and 9 kV, exhibited only a slightly larger contact angle than as-cast films did (see Figures 8(a) and 8(f)). Strikingly, water contact angles were significantly larger for scaffolds with inhomogeneous fibrous morphologies as shown in Figures 8(b)–8(e). At first sight, these limited data suggest that the contact angle is independent of the molecular weight of PHB. However, more research needs to be

Figure 8. Images of water drops on surfaces of (a) PHB50 scaffold electrospun from 13% solution, 9 kV; (b) PHB50 scaffold electrospun from 13% solution, 12 kV; (c) PHB50 scaffold electrospun from 14% solution, 9 kV; (d) PHB50 scaffold electrospun from 14% solution, 10 kV; (e) PHB230, electrospun from 12.5% solution, 8 kV; and (f) PHB230 solution-cast film

carried out before conclusions are drawn. It is interesting to note that these results agree with those reported by Zhu *et al.*⁵⁴ where a superhydrophobic behavior was attained in quite inhomogeneous morphologies of electrospun PHA scaffolds, which exhibited a mixture of filaments, beads and aggregates. The inhomogeneous morphology of electrospun scaffolds and the associated porosity appear to be the driving force for high water contact angles, regardless of the polymer utilized, as shown by Vargas-Villagrn *et al.*^{24,25} for PLA scaffolds.

In order to rationalize the wetting behavior of the scaffolds, it is suggested that air trapped in the rough morphology is the cause for the high contact angle (i.e. hydrophobic behavior) observed in the electrospun scaffolds.⁵⁴ Therefore, the degree of porosity (an intrinsic property of the morphology) in the scaffolds was correlated with the water contact angle. As stated in the experimental section, the porosity was determined using image analysis of the corresponding SEM micrographs. The values at 0% porosity correspond to the solution-cast films, and the results are shown in Figure 9. It can be seen that the contact angle (CA) increases exponentially with the degree of porosity; the best fit to the experimental data gives

$$5. \quad CA = 59.8^\circ + 1.9e^{P/22.7}$$

These results also show that the scaffold with the most uniform filament morphology – that is, smooth cylindrical filaments with nearly constant diameters (see Figure 2(b)) – exhibit a low degree of porosity, and therefore, the contact angle is quite close to that

obtained for cast films. Furthermore, Figure 9 also shows that for a similar degree of porosity, the hydrophobic behavior of PHB230 is only slightly larger than that of PHB50, thus reinforcing the notion that scaffold morphology – that is, degree of porosity – drives the contact angle on the scaffolds.

These findings are important in the context of scaffolds for tissue engineering because they suggest that the conditions of electrospinning that control the scaffold morphology determine the adhesion to the surface of the scaffolds.

Figure 9. Plot of water contact angle as a function of porosity of electrospun scaffolds of PHB. Line corresponds to an exponential fit to experimental data. ■, PHB50; ●, PHB230

(a)

Figure 10. (a) Waxes pattern of a PHB scaffold electrospun from 13% solution and 9 kV; (b) azimuthally averaged intensity scan

3.3 Aging

The influence of storage time – that is, aging – of materials needs to be addressed as physical or chemical changes may occur affecting their performance. PHB is prone to becoming brittle, and this has been attributed to an increase in crystallinity.^{32–34} In order to address the influence of storage time, the electrospun scaffolds were stored at room temperature under dry conditions for up to 11 months. The degree of crystallinity, α , and crystal size, L_{hkl} , were monitored during the storage time. Figure 10 shows a typical Waxes pattern of a PHB50 electrospun scaffold. The processing conditions were 13% w/v concentration and at 9 kV. The Waxes pattern was obtained with the incident beam perpendicular to the scaffold surface. The crystalline isotropic rings indicate a random orientation within the plane of the scaffold, denoting the lack of preferred macromolecular orientation.⁴⁹ Similar results were obtained for all scaffolds investigated – that is, no macromolecular alignment was detected regardless of electrospinning conditions. The intensity peaks are in agreement with the α -form of the orthorhombic unit cell of PHB.⁵⁵

Figure 11 shows the evolution of the degree of crystallinity within the scaffolds, and the lateral crystal size values along the 020 direction as a function of storage time at room temperature for all the scaffolds were investigated. It is well known that PHB becomes brittle on storage time at room temperature.^{55–58} The deterioration of the mechanical properties has been associated with the progressive development of secondary crystallization that has been found to extend over months; physical aging of the amorphous regions seems to be subsidiary in this phenomenon. The data of Figure 11 show that for all the PHB scaffolds, only PHB13&9 exhibits a clear increase in the degree of crystallinity with increasing storage time. The concurrent variation of α and L_{020} for the PHB13&9 scaffold suggests that the observed crystallinity enhancement is associated with an improvement in the lamellar morphology due to secondary crystallization and not, for example, to the destruction of amorphous regions during an incipient degradation process. The fact that the crystallinity α (and crystal size L_{020}) remains constant with storage time for the other PHB scaffolds indicates that the morphology plays a significant role on the development of crystallinity on storage.

It is noteworthy that the PHB12&9 and PHB13&12 scaffolds exhibit high levels of porosity (above 50%) with respect to PHB13&9 ($\approx 22\%$) (see Figure 9). The fact that secondary crystallization cannot be detected for PHB12&9 and PHB13&12 could be associated with the large surface area characteristic of porous morphologies that would favor a more efficient degradation under ambient conditions that could result in destruction of lamellar stacks. On the other hand, it may be that some solvent from the electrospinning process could still be occluded within the polymer filaments, preventing the proper development of interlamellar stacks.

4. Conclusion

This investigation addressed important factors pertaining to the possible biomedical applications of biodegradable and biocompatible polyhydroxybutyrate scaffolds by analyzing the relationships between

Figure 11. Degree of crystallinity, α , and lateral crystal size along the (020) direction, L_{020} , for the electrospun PHB50 scaffolds, as a function of storage time at room temperature. Concentration and voltage for electrospinning conditions: $\bullet$, 12%, 9 kV; $\blacksquare$, 13%, 9 kV; $\blacklozenge$, 13%, 12 kV

electrospinning parameters, morphology and aging. It was shown that the solution concentration and applied voltage determined the morphology of electrospun scaffolds, including fiber diameter, porosity and homogeneity of the scaffold. Water contact angle studies revealed that the morphology controls the wettability properties that are of utmost importance for tissue engineering applications. It was found that the degree of porosity of the scaffold drives their hydrophobic behavior. Aging for up to 11 months at room temperature conditions showed that the PHB scaffolds with uniform filamentous morphology were not stable, and the degree of crystallinity and crystal size gradually raised. On the other hand, scaffolds with aggregated filaments were stable for this period of time, suggesting that secondary crystallization was hindered. This work offers a route to producing biodegradable scaffolds with stable mechanical properties and limited hydrophobicity. Furthermore, these biomimetic scaffolds would enable the possibility of implantation for cell growth and tissue remodeling in a well-controlled manner.

Investigations on the cell viability of HEK293 cells and normal human osteoblasts seeded on biosynthesized PHB scaffolds have been carried out,⁵⁹ and detailed results will be published elsewhere.

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