Fabrication and characterization of porous PHBV scaffolds for tissue engineering

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1. Introduction

Tissue engineering is a fast-moving interdisciplinary pursuit that combines life sciences, medicine, materials science and bioengineering. It intends to overcome the lack of available organs and donor shortages for transplantation as well as to enhance self-regeneration of damaged tissue. Novel tissue engineering technique uses three dimensional (3D) scaffolds as structural templates for cell adhesion and subsequent tissue formation. They must be biodegradable, biocompatible, non-toxic and with high interconnected porosity to allow ingrowths of a significant number of cells, maintained in a viable state by proper diffusion of nutrients and passage of waste. Polyhydroxyalkanoates (PHAs) — polyesters produced by bacterial fermentation — satisfy those requirements and are being tested in different biomedical applications: controlled drug delivery membranes, sutures, wound dressing, bone scaffolds, artificial heart valves, etc [1,2].

PHAs scaffolds with a great surface area for cellular adhesion can be achieved by different techniques; salt leaching [3,4], thermal induced phase separation [5], electrospinning [6] and emulsion solvent evaporation [7-9] are some of them. A great deal of research was devoted to control the...
parameters of each of these processes in order to get the porous size and interconnected structure suitable for each cell. Furthermore, the process employed to get the scaffolds must be chosen according to the physico-chemical characteristics of the surface, since the biochemical reactions and physiological processes, that enable a proper proliferation of cells, depend on the cell-substrate interaction [10]. Finally, γ or UV irradiation, plasma treatment, chemical functionalization [11], chemical or biochemical hydrolysis [12,13] and coatings are some of the several physical, chemical and biochemical techniques that could be applied to enhance the cell-substrate interaction.

Therefore, the aim of this work is to apply three different methods to produce PHBV scaffolds: salt leaching (SL), emulsion solvent evaporation (ESE) and thermal induced phase separation (TIPS), remarking the parameters to be controlled in order to get different porous structure. Furthermore, the effect of chemical and enzymatic treatments to enhance the hydrophilic behavior of the scaffolds and the chemical changes due to the γ-ray sterilization will be discussed in detail.

2. Materials and Methods

2.1. Materials
Poly(3-hydroxybutyrate-co-3 hydroxyvalerate) (PHBV) 12% wt HV M_w = 185397 Da, M_w/M_n = 1.47, was purchased from Sigma Aldrich and was used as received. All the chemicals used in this work such as chloroform, absolute ethanol, dioxane, Tween 80, sodium chloride and sodium hydroxide were analytical grade. Lipase enzymatic preparation Stick Away® with an activity of 15 KLU¹/g was provided by Novozymes.

2.2. Methods

2.2.1. Salt leaching (SL)
Porous scaffolds were prepared by solvent casting/particulate leaching process combined with salt fusion technique using NaCl as particulate porogen. Sieved NaCl crystals (106-355 µm) were dispersed in a Petri dish (100 mm diameter) at 90% relative humidity for partial fusion of the salt crystals during 18 h. Then the fused salt crystals were dried in a vacuum desiccator for 24 h. Chloroform solution of PHBV was poured over the bed of the porogen crystals and let the solvent evaporate at room temperature. The NaCl was leached out three times with aqueous solution of ethanol 10% vol. and then with distilled water. The scaffolds were dried over a filter paper and then under vacuum to eliminate water completely.

2.2.2. Emulsion solvent evaporation (ESE)
Emulsions have been used as templates to make not only scaffolds for tissue engineering but also devices for controlled drug delivery [14,15]; it is produced by a homogenized mixture of a non-polar solvent solution of the polymer and water, stabilized with a surfactant.

In this work the emulsion was generated by a homogenized mixture of a 5% w/v chloroform solution of PHBV mixed with absolute ethanol. Polysorbate 80 (or Tween 80), a synthetic non-ionic surfactant approved for paranteral use [16], was chosen as a suitable surfactant. Because of the spontaneous diffusion of the ethanol, an interfacial turbulence was promoted between the two phases by sonicaton, leading to the formation of smaller particles. Once sonicated, the emulsion was extended onto a PTFE plate (using a knife separated 600 µm from the plate) and left at room temperature to evaporate the solvents [17]. Then, the membranes were immersed in an aqueous ethanol 10% v/v, renewing the solution three times in 24 h in order to leach the surfactant, Finally, they were rinsed with a large amount of distilled water, dried first over a filter paper and then under vacuum to eliminate water completely.

¹ KLU: kilo lipase unit correspond to 1µm butyric acid produced from tributyrin per minute
2.2.3. Thermal induced phase separation (TIPS)

PHBV was dissolved in dioxane at 70 °C with agitation during 2 h to give a 6%w/v solution. 5 ml aliquots of the solution were poured into borosilicate flasks; these flasks were divided in four groups, each suddenly cooled down in air to the following quenching temperatures: 8°C (fridge), 0 °C (water-ice bath), -5°C (freezer) and -196°C (liquid nitrogen) during 30 minutes. Each group of samples was freeze-dried at -86°C during 25 hs in a Labconco Free Zone Plus lyophilizer.

2.2.4. Enzymatic and alkaline hydrolysis

The enzymatic hydrolysis was performed using a lipase (Stick Away®, Novozymes) solution in phosphate buffered saline characterized by pH=7.3 and an activity of 33 KLU/l. Salt leached scaffolds samples of 1x1 cm$^2$ were immersed into the enzymatic solution for 20 h at 37°C. Afterward, the samples were washed with aqueous solution of ethanol 70 %v/v and then with a large amount of distilled water. Finally they were dried over a filter paper and then under vacuum to eliminate water completely.

For the alkaline hydrolysis the samples (1x1 cm$^2$) were sunk in a 0.5 N NaOH aqueous solution for 45 minutes at 55°C. The samples were washed and dried as explained above.

2.2.5. Sterilization

Samples were sterilized with γ irradiation in air at 10 kGy/h, up to a dose of 25 kGy, at the semi-industrial $^{60}$Co pilot plant of the Ezeiza Atomic Centre (Buenos Aires, Argentina).

2.3. Characterization

2.3.1. Scanning electron microscopy

In order to preserve the inner porous structure of the scaffolds, samples were prepared via freeze-cutting. Surfaces were coated with gold and observed under high vacuum in a FEI, Quanta 200 SEM. The pore size was measured from the micrographs using the image analyzer software ImageJ®.

2.3.2. Contact angle measurement

Static water contact angles of the modified samples were measured by an ad-hoc device to get digital images of 5 µl drops onto the surface. ImageJ® was used to process the images.

2.3.3. GPC analysis

Molecular weight distribution was determined by gel permeation chromatography (GPC) in a Waters system equipped with a 5µm Phenogel column. Tests were performed at 30°C and chloroform was the eluent and sample solvent. The set-up was calibrated at 1ml/min with narrow molecular weight polystyrene standards.

2.3.4. Spectroscopic analysis

Fourier transform infrared (FTIR) spectra were collected in the range of 400 to 4000 cm$^{-1}$. The analyses were performed over a solution casted onto a KBr window. $^1$H Nuclear Magnetic Resonance (NMR) spectra were performed for solutions of the sample in deuterated chloroform.

2.3.5. Dyeing of the treated surface

The surface density of carboxyl groups can be determined by technique based on dyeing the sample with 0.01 g/ml of Toluidine Blue O (C.I. Basic Blue) at pH 10 and 30°C for 5 h [17]. After dyeing, the films were rinsed with distilled water, followed by soaking in 0.1 mM NaOH to remove adsorbed dye molecules. Finally, the associated dye molecules were desorbed in 50 %v/v acetic acid.

The dye concentration was determined at 633 nm using a spectrophotometer; then the carboxyl group density was calculated from the calibration curve.
3. Results and Discussion

3.1. Morphological characterization

3.1.1. SL scaffolds

The main advantage of this processing technique is the ease of fabrication without the need of specialized equipment. Porous substrates 80 mm in diameter were obtained. The mean size of the pores on the surface was \((110\pm20)\) µm; pores observed in the transversal cuts showed the same size. The thickness of the substrates could be tailored by changing the thickness of the salt bed inside the Petri dish; it ranged from \((0.50 \pm 0.02)\) to \((1.50 \pm 0.02)\) mm. These samples could be compressed easily, however, the manual bending without breaking decreased as the thickness increases.

The surface of the scaffold, showed in figure 1, shows pores with regular shape, resembling a replica of the salt grains. On the other hand, the transverse cross-section, pictured in figure 2, reveals the interconnected pores that extend uniformly along the whole thickness of the scaffolds. Furthermore, the partial fusion of the salt grains leads to the interconnection of the inner pores through small voids, as illustrated in figure 3. Figure 4 (enlargement of figure 1) remarks the rounded and rather thick walls of the open pores (on the surface). However, inside the SL scaffolds the interconnected pores have thinner walls, as follows from figure 5.

![Figure 1](image1.png) **Figure 1** SL scaffold surface; the square porous structure resembles a replica of the salt grains.  
![Figure 2](image2.png) **Figure 2** Interconnected pores observed in a cross-section of a SL scaffold.
3.1.2. ESE scaffolds

It is well known that emulsion solvent evaporation is useful for microparticle preparation, where changes in the emulsion stirring speed and the polymer or surfactant concentration may enable to control the particle size [16]. Even when in this work the stirring speed and the polymer concentration were kept constant, changes in the surfactant concentration led to different porous structures as shown in figure 6.
The first interesting feature is that even a small amount of surfactant (2.8%) stabilizes the emulsion, leading to the formation of microparticles, which size increases with the volume fraction of the surfactant, as observed in figures 6 b to d. The spreading of the emulsion onto the PTFE surface distorts the shape of the microspheres and sticks them in a fairly closed packed structure. Consequently, the pores formed between the close packed structure follows a fairly linear increase with the volume fraction of surfactant, as shown in table 1. However, the emulsion without surfactant has an irregular structure as shown in figure 6a with bigger pores.

**Table 1** Dependence of the pore size of ESE scaffolds on the surfactant content.

| Surfactant (% v/v) | Pore size (µm) |
|--------------------|----------------|
| 0                  | 7 ±2           |
| 2.8                | 3 ± 1          |
| 4.0                | 4 ± 1          |
| 7.5                | 7 ±2           |

3.1.3. **TIPS scaffolds**

Porous cylindrical samples ca. 10 mm in diameter and 15 mm in height were obtained. Different orientation cuts were made in order to determine the pore sizes and orientations. The morphology of the samples were analysed by SEM and pore size were measured from the micrographs using ImageJ®. Results, summarized in table 2, showed that the pore size tend to decrease as the quenching temperature decreases.
Table 2 Pore size of TIPS scaffolds

| Quenching temperature (°C) | Pore size (µm) | Transversal cut | Longitudinal cut | On the surface |
|---------------------------|----------------|-----------------|------------------|---------------|
|                           |                |                 |                  |               |
| 8                         | 52 ± 8         | 50 ± 17         | 44 ± 9           |               |
| 0                         | 47 ± 13        | 58 ± 20         | 17 ± 3           |               |
| -5                        | 41 ± 13        | 46 ± 20         | 19 ± 4           |               |
| -196                      | 9 ± 2          | 8 ± 3           | 4 ± 1            |               |

At each quenching temperature the pore structure was homogeneous in size and no preferred orientation was observed, as illustrated in figure 7.

In figure 7a, the structure obtained at 8°C has a thin wall and a highly fractured morphology. On the other side the sample quenched in a water-ice bath (0°C) shows a similar morphology with thin walls (figure 7b) and the same magnitude in the pore size. The spongy structure obtained by freezer quenching has small pore up to 50 µm and open spaces up to 150 µm, as it can be seen in figure 7c. Detailed micrographs of the sample walls show a rounded platelet structure that forms the smaller pores. The sample quenched in liquid nitrogen (figure 7d) allowed getting the smaller pore sizes. The dendritic structure of pores oriented toward the center of the sample, could be attributed to the fast cooling rate.

3.2. Hydrophilic enhancement and sterilization

The hydrophilic behavior of the surfaces was quantified by measuring the contact angle of a sessile drop of distilled water. The first row of table 3 points out that both the porous scaffolds fabricated by SL as those produced by solvent casting were hydrophobic before any surface treatment.

Both enzymatic and alkaline hydrolysis were effective to enhance the hydrophilic behavior of the scaffolds. The effect is more noticeable in the porous surface because it has a higher effective area for hydrolysis. However, neither FTIR nor 1H NMR spectral analyses were suitable to determine any change in the chemical structure of PHBV due to the alkaline or enzymatic hydrolysis.

Table 3 Contact angle of a sessile drop of water on the original and treated scaffolds.

| Porous Scaffolds | Solvent Casting |
|------------------|-----------------|
| Original         | 103±5°          |
| Alkaline Hydrolysis | 0°             |
| Enzymatic Hydrolysis | 0°             |

Neither GPC showed major differences between the molecular weight of the untreated SL scaffolds nor the enzymatic hydrolyzed substrates (table 4). However, the GPC analysis revealed a decrease in the molecular weight after the alkaline hydrolysis and even a greater reduction in the after the gamma ray sterilization, as summarized in table 4.
Figure 7 SEM images of scaffolds produced by TIPS under different quenching temperatures: a) 8°C, b) 0°C, c) -5°C and d) -196°C; white arrow indicates the cooling direction towards the centre of the sample.

Table 4 Changes in the molecular weight distribution of a PHBV SL scaffold due to hydrophilic enhancement and \( \gamma \) irradiation (25 kGy @ 10 kGy/h)

|                  | Mn (Da) | Mw (Da) | Mw/Mn |
|------------------|---------|---------|--------|
| Original         | 130709  | 187990  | 1.44   |
| Alkaline Hydrolysis | 94794   | 168176  | 1.76   |
| Enzymatic Hydrolysis | 130953  | 187228  | 1.43   |
| Original + \( \gamma \) | 69439   | 116964  | 1.68   |
| Alkaline Hydrolysis + \( \gamma \) | 74427   | 117085  | 1.57   |
| Enzymatic Hydrolysis + \( \gamma \) | 67877   | 111849  | 1.64   |

Finally, in order to establish whether the alkaline hydrolysis produces carboxyl groups mainly on the surface of the scaffold, they were dyed with Toluidine Blue O; this technique allows quantifying carboxyl groups in a certain surface area. Since it could have been very difficult to determine the effective area of a porous scaffold, flat scaffolds were prepared by solvent casting (PHBV dissolved in chloroform, poured onto a Petri dish followed by evaporation of the solvent under fume hood and finally under vacuum). The dyeing procedure was applied to cast films subjected to alkaline hydrolysis during different periods of time. The results, summarized in figure 8, showed an increment of carboxyl groups...
surface density with the hydrolysis time. These results demonstrated that the hydrophilic enhancement is due to hydrolysis just on the polymer surface.

![Figure 8 Alkaline hydrolysis: carboxyl group density vs. hydrolysis time.](image)

4. Conclusions
The three methods presented in this work are suitable to produce 3D porous scaffolds. Nevertheless, each has advantages and drawbacks, to be considered in order to get the proper morphology according to the cells to be harvested.

Salt leaching is a simple technique with little equipment requirements, where the pore size is easily controlled by the grain size of the porogen. However, this technique is not adequate for large scale scaffold production because of the great number of steps. Furthermore, SL substrates more than 1 mm thick are rather brittle so they can be applied only onto flat surfaces.

Emulsion solvent evaporation is more suitable for applications in which flexibility is a requirement, for instance, to adapt the scaffold to anatomic curvatures. Tween 80 proved to be a proper surfactant to stabilize the emulsions. Furthermore, a linear correlation between the surfactant content and the pore size allows producing an interconnected network of pores according to the cells to be harvested.

Thermal induced phase separation is a versatile technique to get different pore sizes and change its orientation by controlling the quenching temperature and the thermal gradient. The ability to tailor the scaffold morphology, make TIPS suitable for different tissue engineering applications with wide different pore sizes and structural requirements.

Both the enzymatic and alkaline treatments lead to the hydrophilic enhancement of the surface. This is mainly due to the increase in the surface density of carboxylic groups, which could be quantified by the Toluidine Blue O dyeing. GPC, FTIR or RMN are not suitable techniques to evaluate the hydrolysis because it only affects the molecules on the surface while the spectral analysis considers changes in the bulk. It is expected that a highly hydrolyzed surface would enhance the biodegradation rate; in vitro biodegradation experiments are being run to evaluate this statement.

Finally, it was shown that alkaline treatment and sterilization by gamma irradiation partially reduces the molecular weight.

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