Plasma modification of PCL porous scaffolds fabricated by Solvent Casting/Particulate Leaching for Tissue Engineering

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Abstract: This study points out how the plasma modification of PCL porous scaffolds, produced by Solvent Casting/Particulate Leaching, may enhance their biocompatibility. A C_2H_4/N_2 plasma deposition followed by a H_2 plasma treatment was used to increase the hydrophilicity of the whole scaffold to support osteoblast cell proliferation, both outside and inside the scaffold. A better cell growth was obtained on plasma modified scaffolds.

Keywords: plasma, surface modification, scaffold, tissue engineering, porosity.

1. Introduction

In recent years, Tissue Engineering has become of great interest and an excellent alternative to artificial prosthesis and organ transplant to replace diseased or damaged organs. This new emerging field is based on the use of cells seeded in a three-dimensional (3D) scaffold in order to reconstruct, replace, or repair living tissues and organs [1]. The scaffold provides the initial structural integrity and organizational backbone for cells to assemble into a functioning tissue [2]. Thus, chemical, biological and mechanical characteristics of a scaffold are of outmost importance.

Porous, biodegradable polymers have been found to play a vital role to create a suitable scaffold. It supplies the necessary support for cell attachment, proliferation and differentiation that allow a correct tissue regeneration. Parallel to the formation of the new tissue, the scaffold must undergo a biocompatible degradation [3]. Nowadays, there are many techniques that realize scaffolds with suitable biocompatibility [4-6]. Among them, Solvent Casting/Particulate Leaching allows the control of micro-structural characteristics such as porosity, pore size and pore interconnection degree [7]. In this work poly(ε-caprolactone) (PCL) scaffolds were produced with this technique.

PCL is an optimum polymer in Tissue Engineering applications for its good mechanical properties and biodegradability but its low hydrophilicity does not make PCL very much biocompatible and suitable for cell adhesion and proliferation. Thus, by controlling scaffold surface chemistry by a surface modification tool, it should be possible to control adsorption of extracellular matrix proteins, and, in turn, to enhance cell adhesion and motility [8]. Generally, when cells are seeded *in vitro* into 3D scaffolds, cell adhesion is favoured at the peripheries, resulting in poorly populated interiors, since the external surfaces of the scaffolds are more accessible than the inner ones.

At present, a range of biological, physical, and chemical methods are used to affect surface modifications on biomedical devices and biomaterials. These approaches can be used to tune a range of properties, including wettability, permeability, biostability and/or chemical inertness, adhesion, biocompatibility, topography, electrical, optical and frictional characteristics.

Cold plasma processes can be used to tailor the surface composition of scaffolds. Recent advances in radiofrequency plasma processes (RF, 13.56 MHz) Glow Discharges for biomedical applications, include the achievement of functional surfaces for direct cell growth and biomolecule immobilization, the deposition of non-fouling coatings, the deposition of nano-composite bacterial resistant coatings and the synthesis of nano-structured surfaces [9]. Even though many approaches for the modification of the surface chemistry of polymers have been described, it remains an interesting challenge to understand their efficiency when applied to complex 3D structures.

Recent papers describe surface modification processes applied to scaffolds, using O_2 and N_2 low pressure plasmas [10-12] and Dielectric Barrier Discharge (DBD) plasmas [13,14], to promote the penetration of active species in the inner part of a scaffold. The use of plasma processes in the modification of 3D materials is a very interesting approach to produce cell/scaffold constructs, where cells are homogeneously distributed, possibly suitable for the reconstruction of tissues as cartilage or bone [15,16].

The aim of this work is to show the ability of active species generated in low pressure RF (13.56MHz) Glow Discharges to modify the inside of a scaffold to render it a suitable support for cell seeding, albeit physical limitations like high Debye length and gas density, do not

allow the ignition of low pressure glow discharges inside small pores.

2. Experimental part

- Solvent Casting/Particulate Leaching process

PCL (Mw ~ 65,000, pellets, Aldrich) scaffolds were produced by means of Solvent Casting/Particulate Leaching technique. PCL pellets were first dissolved in chloroform (Fluka, 20/80 wt/wt), by stirring for about three hours at room temperature. To create the pores into the scaffold, Sodium Chloride crystals (NaCl, SigmaUltra) were employed as porogen, sieved to a specific size range (150-300 μm), added and homogeneously mixed to the polymer solution at different weight ratios (10/90, 8/92, 5/95 wt/wt). The viscous polymer solution was then cast in Teflon molds (10 mm diameter, 3 mm thickness) and dipped in ethanol (Carlo Erba Reagents) for about two hours at room temperature, to separate chloroform in a phase inversion process. The polymer/salt composite was then leached in bidistilled water for five days to remove the porogen and create voids into the scaffold. The resulting porosity can be varied in a controlled way by changing the amount of salt added, while the pore size is dependent on the size of the salt crystals. The resulting porous scaffolds were dried into an oven for 30 minutes and stored in a desiccator up to plasma treatments and/or characterization.

- Surface Modification by Plasma Deposition

PCL scaffolds were plasma modified in a stainless steel parallel-plate plasma reactor. A first 30 minutes deposition process was performed in a mixture of Ethylene/Nitrogen (C_2H_4/N_2, 7 sccm / 21 sccm, Air Liquid). The pressure was set at 300 mTorr. RF discharges were ignited between the upper (stainless steel, shielded, RF connected) and the lower (grounded, sample holder) electrode. A RF (13.56 MHz) generator was utilized at 50 W of RF power in continuous (CW) mode. Immediately after this deposition process, scaffolds were plasma treated in H_2 (HG 200 Hydrogen Generator) at the following experimental conditions: 10 sccm H_2, 400 mTorr pressure, 10 W power, 30 s time.

- SEM, XPS and WCA surface characterization

After the drying procedure, the scaffolds (10 mm diameter, 5 mm thickness) were frozen in liquid nitrogen and cut, in order to obtain different sections to be analysed. For each sample, the surface and a horizontal section, cut at 3 mm of depth from the scaffold surface, were examined.

A Stereoscan 360 Cambridge Scanning Electron (60 - 130 Pa), magnification of 70x and 250x, was used to examine the surface morphology (pore distribution, size and interconnectivity degree) of the scaffolds.

X-ray Photoelectron Spectroscopy (XPS) measurements were performed with an AXIS ULTRA Spectrometer (KRATOS Analytical, UK). The samples were irradiated with monochromatic AlKα X-rays (hν=1486.6 eV). The area of analysis was 400x700 μm^2 and the take off angle (TOA) was 90° with respect to the sample surface. The peak fitting of the C1s spectra was done considering the four components of pure PCL, as shown in Figure 1, with the add of two other components for the plasma treated scaffolds: the N-C=O peak (BE 288.1 eV) and the C-N peak (BE 286.0 eV).

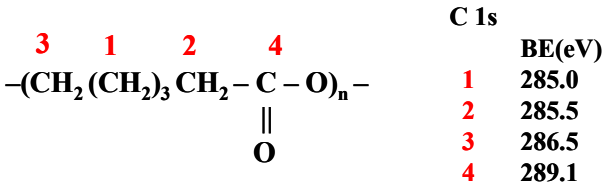

Fig.1: PCL chemical structure and the corresponding C1s fitting components.

Static Water Contact Angle (WCA) measurements were carried out at room temperature with a CAM200 digital goniometer (KSV instruments) equipped with a BASLER A60f camera, by using the sessile drop (2 μl) method. Five measures per sample were performed. WCAs were measured by using a micrometric syringe, perpendicular to the measuring plane.

- Cell culture

Cell culture experiments on untreated and plasma modified 3D scaffolds were performed with the human Saos2 osteoblastic cell line (ICLC). Cells were routinely grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma Chemical Co.) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 50 IU/ml penicillin, 50 IU/ml streptomycin and 200 mM glutamine, and kept at 37 ºC in a saturated humid atmosphere containing 95% air and 5% CO_2 in 75 cm^2 flasks (Iwaki, UK). Cells were detached with a trypsin/EDTA solution (Sigma), suspended in the correct medium and seeded at a concentration of $3x10^4$ cells into the scaffolds placed in 1 cm wide well of a 48 cell culture well plate (Iwaki).

- Cell proliferation assay

The mitochondrial activity of seeded Saos2 cells was determined by the MTT colorimetric assay. This test can detect the conversion of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (Sigma) to formazan. Cells, after each time point, were incubated with a tenth of the medium of the bromide in 5% CO_2 (37 ºC, 3 h) to allow the formation of formazan crystals. They were then dissolved in 10% Triton X-100 and acidic isopropanol (HCl 0.1N). The optical densities (O.D.) of the solutions were read by a spectrophotometer (Jenway 6505), at the wavelength of 570 nm with respect to a reference wavelength of 690 nm. Statistical analyses were assessed by the Student's T test between samples. Differences were considered statistically significant for $p< 0.01$.

3. Results and Discussion

- Morphological and structural properties of PCL scaffolds

Solvent Casting/Particulate Leaching, among the various scaffolding techniques, stands out for its simplicity of operation, for the possibility of producing scaffolds with a good pore interconnectivity degree, controlled composition, porosity and pore size. High porosity and pore interconnectivity degree and a suitable pore size are key parameters that permit cells to penetrate in depth into the scaffold, and to produce an homogeneous cell/scaffold construct to be used as a tissue substitute in a biomedical application.

In this study, scaffolds with 150-300 µm pore size were produced by using suitable sieved salt crystals. Three different PCL/NaCl compositions were realized (10/90, 8/92, 5/95 wt/wt) in order to obtain scaffolds with different degrees of porosity.

According to SEM micrographs, for each scaffold typology, the surface and the section faces were topologically similar; the section was more porous and a better pore interconnectivity degree is visible (Figure 2). By varying the NaCl %, from 90 to 95%, more porous scaffolds were obtained.

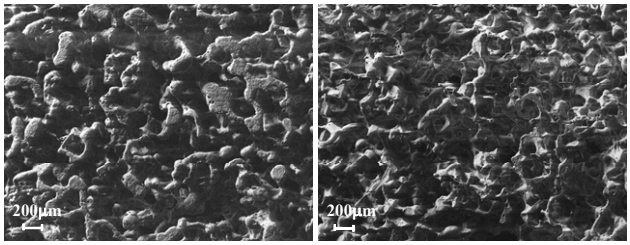

Fig.2: SEM images (70x) of a scaffold (95% NaCl, 150-300 µm pore size). Surface (left) and section (right).

- Effect of the Plasma process

Scaffolds with a PCL/NaCl composition of 5/95 wt/wt, chosen for their higher porosity among the others, were coated with a $C_xH_yN_z$ film, 548±2 nm thick, as described above.

XPS analysis attested that the scaffolds before the plasma treatment had a chemical composition typical of PCL, demonstrating that during the scaffolding process phases there were not any solvent and porogen residuals (Table 1 and Figure 3, left).

In order to evaluate the penetration of the plasma treatment in the inside of the scaffold, a XPS study in depth was performed. Table 1 summarizes the chemical composition of an untreated scaffold (A) and a plasma treated scaffold: surface (B) and section (C), 3 mm below the surface.

Plasma treated scaffolds exhibited a chemical composition completely different from the untreated ones, attesting for a successful modification of the material. In fact, Table 1 indicates the presence of nitrogen on the scaffold surface and section, and an increase of the C% and a decrease of O% after the plasma treatment. As shown in Table 2 and Figure 3 (left), in the C1s spectrum fitting of the plasma modified scaffold, two new contributions appear with respect to pure PCL one (Figure 3, right): the N-C=O peak (BE 288.1 eV) and the C-N peak (BE 286.0 eV), according to the presence of the N1s peak (6,0±0,5 N%).

	C%	O%	N%	N/C	O/C	N/O
A	77,5 (±0,8)	22,5 (±0,8)	\	\	0,3	\
B	82,4 (±1,5)	11,6 (±1,2)	6,0 (±0,5)	0,1	0,1	0,5
C	84,7 (±0,7)	9,7 (±1,1)	5,6 (±0,7)	0,1	0,1	0,6

Table1: C%, O% and N% of XPS wide spectra and N/C, O/C and N/O ratios of an untreated PCL scaffold, 95% NaCl, 150-300 µm pore size range (A), a plasma treated PCL scaffold surface (B) and a plasma treated PCL scaffold section, 3 mm below the surface (C).

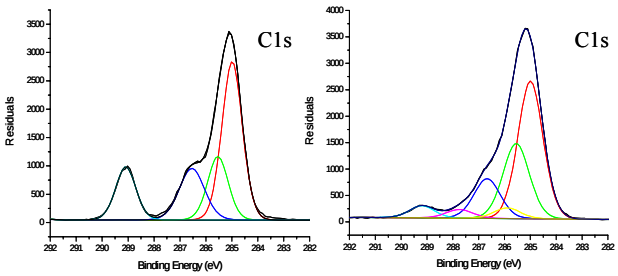

Fig.3: C1s spectra of a PCL scaffold surface (95% NaCl, 150-300 µm pore size range), the untreated (left) and the plasma modified one (right).

C1s						
	C1	C2	C3	C4	C5	C6
A	47,6 (±1,4)	17,9 (±1,1)	19,1 (±0,4)	15,4 (±0,3)	/	/
B	52,8 (±5,7)	22,5 (±5,1)	13,5 (±1,4)	4,8 (±0,6)	3,0 (±0,5)	3,3 (±0,9)

Table 2: C1s fittings of the untreated (A) and the plasma modified (B) PCL scaffold surface (95% NaCl, 150-300 µm pore size range).

Observing Figure 3, there is an evident alteration of the C1s of PCL after the plasma treatment: the appearance of two nitrogen-containing peaks, the high reduction of the O=C-O contribution and the little increase in the components C1, C2 and C3 mean the formation of a hydrocarbon coating with C-N and amino groups on its surface.

Exciting results pointed out that the chemical composition, both in the external part of the scaffold and in its body, was very similar up to a thickness of 3 mm, maintaining a quite constant density of N atoms.

Static WCA value on PCL flat substrates was 85±2°, it lowered to 70±3° after the plasma deposition process. Further lowering of the WCA was recorded to 41±1° after the H_2 plasma treatment, probably due to the reduction of N-groups to $–NH_2$ ones in the coating, and to its exposure to air. WCA measures on PCL scaffolds clearly showed an increase in the hydrophilicity after the two plasma processes, obtaining a value of 77±1° with respect to 115±1° for the untreated scaffold. These values were quite higher than the WCAs of flat PCL because of the scaffold micro-roughness.

- *In vitro* cell behaviour on untreated and plasma modified scaffolds.

A preliminary cell adhesion experiment was performed with Saos2 osteoblast cell line, a type of cells that are usually employed to test the biocompatibility of 3D scaffolds used as bone substitutes.

The MTT proliferation assay (Figure 4) showed that, after 48 hours of cell culture, osteoblast cells adhered to plasma modified 3D scaffolds much better than the untreated ones. These results confirm how the plasma treated scaffolds are quite promising materials for Tissue Engineering applications.

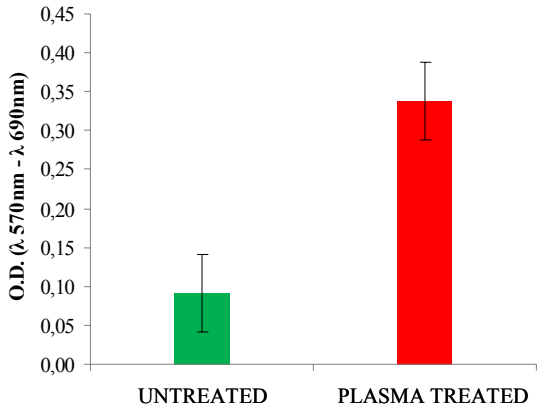

Fig.4: MTT proliferation assay on untreated (left) and plasma treated PCL scaffolds (right).

4. Conclusion

In this study, a successfully plasma modification process was proposed to chemically modifying PCL scaffolds, produced by means of Solvent Casting/Particulate Leaching. Scaffolds with controlled chemical composition, porosity, pore size and a good pore interconnectivity degree, were obtained by this technique. C_2H_4/N_2 plasma deposition followed by a H_2 plasma treatment were used to introduce hydrophilic N-containing groups into the PCL scaffolds, leading to increased wettability and better cell growth with respect to the untreated ones.

The new and surprising outcome was the excellent ability of the plasma treatment to penetrate into the inside of the scaffolds, testified by a quite constant N% in the chemical composition of the whole scaffold and an improved hydrophilicity compared to the untreated scaffolds. The chemical composition and wettability of plasma treated scaffolds evidently stimulate Saos2 osteoblast cell growth into the plasma modified scaffolds compared to the untreated material.

Further studies are in progress to better evaluate the scaffold biodegradability and the cell growth/proliferation into the scaffolds, as a function of the plasma parameters.

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