DEVELOPMENT OF NANOSTRUCTURED SURFACES BASED ON BIORESORBABLE POLYMERS AS CELL SUBSTRATES

***B. Atxa*[1], *A. Larrañaga*[2], *J.M. Ugartemendia*[2]**

[1]Graduate in Industrial Engineering by the University of the Basque Country (UPV-EHU), batxa001@ikasle.ehu.eus
[2] Department of Mining-Metallurgy Engineering and Material Science in the Faculty of Engineering of Bilbao, UPV-EHU
Plaza Ingeniero Torres Quevedo, 1, 48013 Bilbao, Biscay

Abstract: The surface topography of the scaffolding devices is a key parameter in the interaction between the cells and the material and can modify cell behavior. Poly(ι-lactide-co-ε-caprolactone) (PLCL) has been applied in many tissue engineering applications thanks to its biodegradability, biocompatibility and the possibility to finely control its final properties. In the present project, the crystal morphologies and surface topographies of PLCL systems with different co-monomer compositions and chain microstructures were analyzed by means of differential scanning calorimetry (DSC) and atomic force microscopy (AFM). Results show that these parameters permit the tailoring of surface microtopographies, with morphologies ranging from spherulitic to amorphous.

Keywords: Poly(ι-lactide-co-ε-caprolactone), crystallization behavior, surface topography, atomic force microscopy.

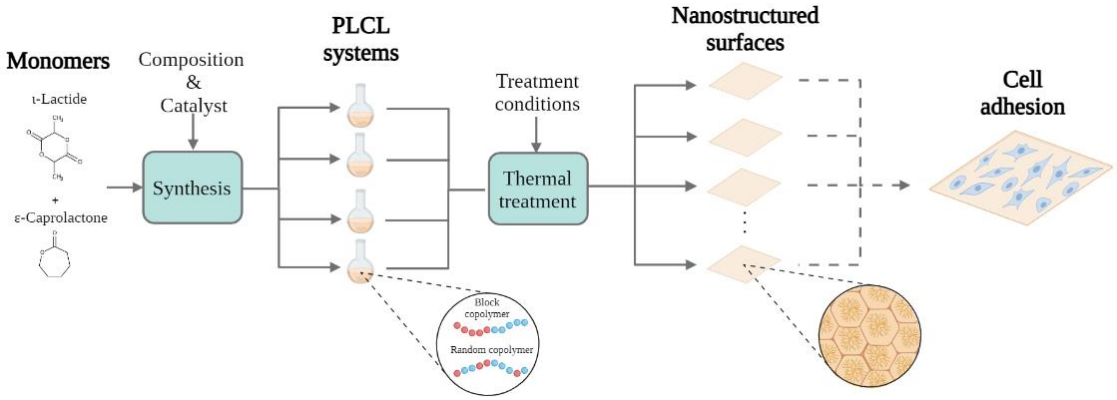

Figure 1. Schematic representation of the project.

1. INTRODUCTION.

The scaffolds employed in tissue regeneration must mimic the extracellular matrix (ECM), the solid matrix in which cells reside. In this sense, biodegradable materials with physical, chemical, mechanical and morphological properties that resemble those of the original ECM are required for scaffolding elements in tissue engineering [1]. The surface topography of the scaffolding devices is a key parameter in the interaction between the cells and the material and can modulate the response of the cells independently of the chemical composition of the substrate. Cell differentiation, adhesion patterns, orientations and cell shape are influenced by the surface topographies [2].

Polyesters, such as polylactide (PLA), polycaprolactone (PCL) or polyglycolide (PGA) and their copolymers, have been widely used in tissue engineering scaffolding devices thanks to their biocompatibility and biodegradability [3]. A good control of the synthesis and processing conditions of the (co)polymers allow for variations in their properties. In this context, copolymerization is one of the best ways of tuning properties to obtain tailor-made materials.

Particularly, copolymers based on ι-Lactide and ε-caprolactone can present a variety of mechanical properties, degradation kinetics, shape-memory behavior and controlled drug-release properties depending on their composition and average length of their repeating units [4], [5].

In tailoring the surface topography of the scaffolding devices, the crystal behavior of the PLCL copolymers is vital. Thermal treatments, co-monomer ratios and ι-lactide number average sequence lengths determine the supramolecular arrangements which lead to different crystallization morphologies, ranging from spherulites and axialite-like crystal aggregates to amorphous forms [6], [7].

The control of the chain microstructure is particularly relevant to tailor the properties of copolymers and their

crystallization behavior. It has been demonstrated that PLCLs with shorter average length of ι-lactide unit sequences have limited crystallization capability than PLCLs showing a more blocky character [8]. Chain microstructure varies depending on the conditions of the synthesis process, the employed catalysts and the rates of incorporation of the co-monomers [8].

In the case of PLCLs, different factors affect the chain microstructure of the copolymers. Grijpma et al. [9] reported the effect of the polymerization temperature on chain microstructure, concluding that higher temperatures lead to higher randomness character. Fernandez et al. [8] studied the influence of reaction time, showing that as the polymerization process is extended over time the randomness character of the polymer decreases. In this same study the effect of the monomer to catalyst ratio was studied, with a higher amount of catalyst resulted in copolymers with a higher randomness caracter.

The use of different catalyst/initiators has also been studied as a strategy to tune the chain microstructure of PLCL copolymers [10]. Stannous octoate ($SnOct_2$) is the most commonly used catalyst for ring opening polymerization due to its good performance. This catalyst has been reported to lead to blocky PLCLs [8], [10]. Different bismuth salts, on the other hand, have been successfully employed to synthesize PLCL systems with more random nature [8]. Triphenyl bismuth (Ph_3Bi), in particular, has been used in the synthesis of other random copolyesters [11].

In this work, several statistical poly(ι-lactide-co-ε-caprolactone) copolymers are synthesized at different mass feed ratios and employing either $SnOct_2$ or Ph_3Bi as the catalyst/initiator to obtain block and random PLCL copolymers with different compositions. Copolymer composition and chain microstructure of the PLCLs are investigated by means of proton and carbon nuclear magnetic resonance spectroscopy (^{1}H NMR and ^{13}C NMR), their molecular weight distribution is determined using gel permeation chromatography (GPC) measurements and the phase structure is studied by analysis of thermal transitions determined by differential scanning calorimetry (DSC).

A study of the crystallization capability of the PLCL systems is performed employing DSC and atomic force microscopy (AFM) to analyze the different crystalline morphologies.

For the preliminary cell adhesion studies, human pulmonary fibroblasts (MRC-5) are used following routine seeding, staining and microscopy procedures.

2. METHODOLOGY.

Synthesis

Four poly(ι-lactide-co-ε-caprolactone) (PLCL) copolymers were synthesized by ring opening polymerization employing predetermined amounts of ι-LA and ε-CL and $SnOct_2$ or Ph_3Bi as a catalyst.

The synthesized PLCL copolymers were analyzed to evaluate their characteristics. Proton and carbon nuclear magnetic resonance spectroscopy (^{1}H NMR and ^{13}C NMR) tests were performed to determine the compositions and the chain microstructures of the polymers through the lengths and distribution of the repetitive monomer sequences. Gel permeation chromatography (GPC) tests were performed to determine the molecular weight distribution of the resulting polymers. The copolymer systems were thermally characterized by means of differential scanning calorimetry (DSC) and the thermal transitions were determined. A first scan was used to determine the melting temperature (T_m) and the heat of fusion (ΔH_m) of the precipitated copolymers and erase their thermal history. The degree of crystallinity (X_c) was calculated assuming that only the lactide units crystallize. Then, a second scan was made to determine the glass transition temperatures (T_g).

Crystallization analysis

A set of thermal treatments was determined for the copolymers, which were first conducted by means of DSC. Three different procedures were applied: isothermal crystallization treatments at 50 ºC and 70 ºC and a quenching treatment.

From the synthesized copolymers, surfaces with different microtopographies were prepared by making use of the semicrystalline character of PLCL. Films were prepared by solvent casting and they were thermally treated.

Finally, the surfaces were analyzed using atomic force microscopy. To obtain a comprehensive view of the morphology of the samples, several locations were scanned for each sample, progressively decreasing the scan size to be able to observe the smaller structures. Images were then and measurements of the sizes of crystalline formations were performed analyzing the cross-sections of the phase and height images.

Cell adhesion study, preliminary work

Samples were sterilized by ethanol washing and UV irradiation. For the cell seeding, different cell densities were tested and the cells were incubated for 24 h. Then the cells were fixed and stained employing DAPI and rhodamine-phalloidin. The samples were observed by fluorescence microscopy.

3. RESULTS AND DISCUSSION.

Synthesis

The results of the synthesis and characterization processes can be seen in Table 1. The yield of the reactions was in all cases above 85% and the obtained compositions were close to the feed compositions

Table 1. NMR characterization, thermal properties and GPC measurements of PLCLs.

	Catalyst	M/C	Yield (wt. %)	Composition (ι-LA wt. %)	M_w (kg mol^{-1})	Microstructural magnitudes			1st DSC scan			2nd DSC scan
						l_{LA}	l_{LC}	R	T_m (ºC)	ΔH_m (J/g)	X_c (%)	T_g (ºC)
PLCL 80-20 Bi	Ph3Bi	1000:1	87%	78.9	139.6	3.65	1.24	1.08	105.5	13.3	16%	28.2
PLCL 80-20 Sn	SnOct2	1000:1	90%	80.0	101.6	5.38	1.70	0.77	144.6	25.4	31%	27.3
PLCL 70-30 Bi	Ph3Bi	1000:1	85%	70.7	121.4	2.45	1.28	1.19	127.6	1.5	2%	14.0
PLCL 70-30 Sn	SnOct2	1000:1	89%	69.3	112.3	4.31	2.42	0.65	135.0	21.0	29%	18.3

The results from the gel permeation chromatography show weight average molecular weights above 100 kg mol^{-1} in all cases, being the values higher in the cases of the copolymers synthesized using Ph_3Bi as the catalyst.

With regard to the chain microstructure parameters, the average values of the results of the ^1H NMR and ^{13}C NMR tests have been included. The use of Ph_3Bi as the catalyst favored the formation of LA-CL dyads and alternating LA-CL-LA triads, shortening the repeat unit length values of LA and CL and leading to copolymers with a randomness character close to 1. The values or the randomness character (R) were even above 1, suggesting the presence of alternating monomer sequences (-LA-CL-LA-CL-LA-CL-) in the copolymer chains. The systems synthesized using $SnOct_2$ showed a blockier tendency, with longer repeat unit lengths, especially in the case of LA, and lower values of R.

As can be seen from the results of the first DSC scan, a higher randomness character and shorter ι-LA repeat unit length, as in the copolymers synthesized with Ph_3Bi, limits the capability of crystallization of the ι-LA units, causing the melting temperature to be lower and reducing the crystallinity fraction greatly. For the systems synthesized using $SnOct_2$, a much higher crystallinity degree was obtained, with fractions around 30% in both cases. Both the crystallinity fraction and the melting temperature were slightly higher in the case of the PLCL 8020 Sn system. This can be explained by both the presence of higher amounts and the longer repeat unit lengths of ι-LA.

In the second scan, all the PLCLs showed a homogeneous amorphous phase except for the 8020 Sn system, which shows a very minimal melting peak.

Crystallization analysis

According to the DSC analysis, the crystallization capability of the copolymers increases with the length of the ι-lactide repeat sequences, as well as with increasing isothermal crystallization temperature. According to the DSC traces, none of the systems were capable of forming crystals in the quench except for the PLCL 8020 Sn system, in which a subtle endothermic peak was detected.

In the Bi systems, PLCL acts as a single phase amorphous copolymer showing a single hybrid Tg. In the case of the Sn systems, however, two glass transitions are registered. The lower Tg is associated to the hybrid amorphous miscible LA-CL phase, composed mostly of ε-CL units and the higher Tg suggests the presence of amorphous polylactide domains which are phase separated [7].

The crystalline morphology of the PLCL systems was analyzed by means of atomic force microscopy. Figure 2 shows AFM height images corresponding to the systems. It is possible to distinguish spherulitic morphologies for the samples treated at 70 ºC, excepting the 7030 Bi system. The Sn systems, when treated isothermally at 50 ºC led to lamellar morphologies in which it is possible to identify small axialites. In the quench, these systems show an amorphous phase with some disperse lamellae.

The variations in the crystal morphologies lead to different topographies which are associated to varying roughness values. A summary of the characteristics of the surfaces can be seen in Table 2. The samples with spherulitic morphologies present microcavities between the spherulites and characteristic topographies, leading to much rougher surfaces.

Table 2. Morphologies and roughness of quenched and isothermally crystallized PLCL.

	Treatment	Morphology	Size	R_a (nm)	R_{max} (nm)
PLCL 80-20 Bi	quenching	amorphous	-	0.223	3.38
	iso. 50ºC	amorphous	-	1.76	20
	iso. 70ºC	spherulitic	7 µm	39.2	356
PLCL 80-20 Sn	quenching	lamellar	150 nm x 25 nm	0.882	20.2
	iso. 50ºC	lamellar/ axialitic	300 nm x 32 nm 800 nm	8.11	95.4
	iso. 70ºC	spherulitic	12 µm	136	2311
PLCL 70-30 Bi	quenching	amorphous	-	0.215	2.67
	iso. 50ºC	amorphous	-	0.302	5.74
	iso. 70ºC	amorphous	-	0.203	10.0
PLCL 70-30 Sn	quenching	lamellar	380 nm x 16 nm	1.70	21.0
	iso. 50ºC	lamellar	285 nm x 25 nm	14.3	133
	iso. 70ºC	spherulitic	14 µm	110	1551

Cell adhesion study, preliminary work

In the cell adhesion studies nuclei staining (blue channel) and cytoskeleton actin staining (red channel) were performed. The images show that the cell adhesion was possible, an important step for the viability of the developed materials for their use as cell substrates.

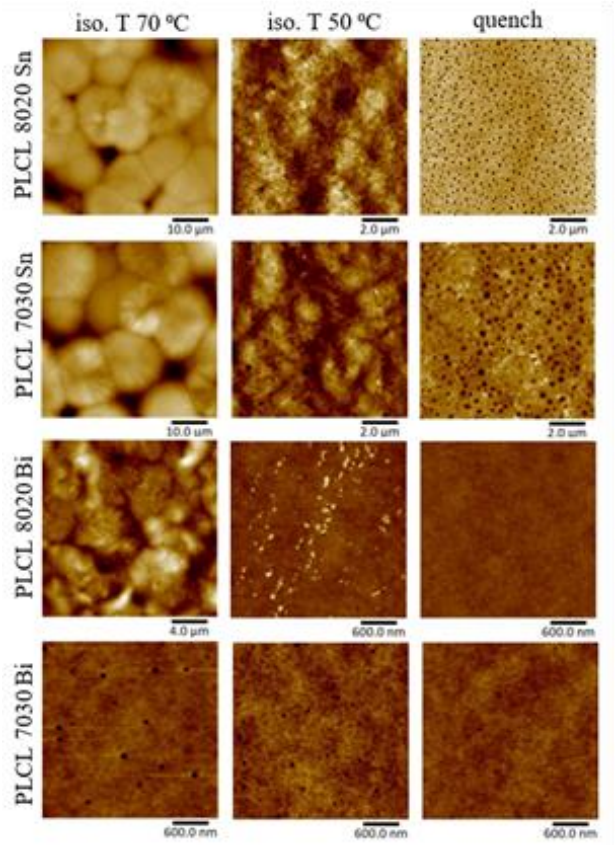

Figure 2. Atomic force microscopy height images of PLCLs.

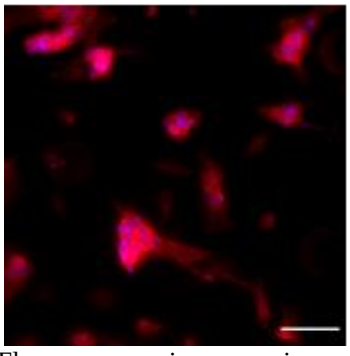

Figure 3. Fluorescence microscopy images of MRC-5 cells on PLCL. Scale bar lenght: 200 µm.

4.- CONCLUSIONS.

The crystallization morphologies of poly(ι-lactide-co-ε-caprolactone) (PLCL) systems with different co-monomer compositions and chain microstructures were studied for various thermal treatment conditions, exploring surface topographies capable of modulating cell growth and adhesion behaviors.

A ring opening polymerization method was used to synthesize PLCL copolymers. Ph_3Bi proved to be a suitable catalyst to obtain copolymers presenting random morphologies, while $SnOct_2$ led to copolymers of blockier nature.

The number average sequence length of ι-lactide was determinant in the crystallization capability of the obtained PLCL copolymers, with increasing values of l_{LA} leading to higher crystal fractions.

The crystallization analysis showed a plethora of morphologies depending on the thermal treatment and the composition and chain microstructure of the PLCL.

Finally, the preliminary work of the cell adhesion study permitted the verification of the cells' ability to adhere to the developed material.

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