



Cell behaviour in new poly(l-lactic acid) films with crystallinity gradients

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ABSTRACT

We report a new facile method for the development of crystallinity gradients in poly(l-lactic acid) films, based on the gradual dipping of amorphous PLLA samples in a hot bath. Isothermal cold crystallization studies were performed using DSC and optical microscopy in order to find adequate conditions to generate crystallinity gradients in the PLLA films. Cellular response along PLLA samples with graded spherulitic developments was tested: cell adhesion is higher in more crystalline zones due to highly flattened appearance of the cells in regions densely populated with spherulites, but cell density was found to be higher in the more amorphous side.

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1. Introduction

Development of biomaterials exhibiting surfaces or interfaces with gradient properties have been inspired by the occurrence of natural tissues presenting continuous variations of properties along one direction, such as in the interfaces ligament-to-bone, tendon-to-bone and dentin-to-enamel [1].

Besides developing structural biomaterials exhibiting distinct mechanical properties at different positions, functionally graded biomaterials may be used to control in space cellular behaviour [2]. Surfaces with compositional gradients may be also used for screening cell response to biomaterials [3]. Most of the graded functional materials are produced by gradual changes in the chemical or biochemical composition that change in the surface or in the bulk, typically along one direction of the system. Much less work has been presented on the preparation of such materials using pure physical modifications. A possibility in this context would be to control the gradual crystallinity, degree of a semi-crystalline polymer, by means of adequate thermal treatments.

Poly(l-lactic acid), PLLA, is a widely used biodegradable polymer in different biomedical fields. PLLA can be classified as a macromolecule with low crystallization kinetics [4], which allows the user to control the spherulitic growth and the degree of final crystallinity. It is known that crystallinity affects the mechanical properties [5,6], the biological response [7,8] and the biodegradation profile of PLLA [9].

PLLA films presenting crystallinity gradients have been reported previously prepared by simply exposing the extremity of samples to different temperatures [10]; such method is based on pure heat transport offering a poor control on the variation in crystallinity along the sample. In this work we intend to induce for the first time the development of a crystallization gradient using a methodology based on a gradual dipping treatment of amorphous PLLA samples into a bath at constant temperature, and to study the influence of the resulting crystallinity gradient on cell behaviour. This method also allows to create a variety of controlled crystallinity gradients by changing the thermal history of the submerged films.

2. Materials and methods

2.1. PLLA films preparation

PLLA ($\langle M_n \rangle = 86,000$, $\langle M_w \rangle = 151,000$) was from Purac Biochem. Amorphous PLLA films, with thickness and length of ca. 0.95 mm and 6.5 cm, respectively, were prepared by hot pressing at 200 °C followed by rapid cooling in cold water. A heating plate was used to maintain silicon oil in a vessel at a constant temperature. A claw with adjustable vertical position was used to submerge the PLLA films into the oil at a controlled rate.

2.2. Materials characterization

DSC measurements were carried out in Q100TA Instruments. Temperature and heat flow calibrations were carried out using a pure indium standard. Isothermal cold crystallization experiments

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were carried out at distinct temperatures after erasing the thermal history of the samples at 200 °C and quickly cooling down to room temperature.

Microscopy observations were carried out using an Olympus BH-2 polarized light microscope. Two types of observations were performed: (i) monitoring of the evolution of the spherulitic structure at constant temperature using a hot-stage (Linkam THMS 600) and (ii) observation of 20 μm thickness samples extracted along the PLLA film length cut using a microtome with a glass blade.

2.3. Biological tests

L929 mouse fibroblasts line (European collection of cell culture—UK) was used to test the cellular adhesion on the samples cut from the gradient films. The samples (1 × 1 cm²) were sterilized by immersion in 70% (v/v) ethanol overnight and then washed twice with PBS. The cells were seeded in the samples and nourished with Dulbecco's modified minimum essential medium (MEM) supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic. The cultures were incubated for 18 h and the cellular density was 25,000 cells/cm². For the fluorescent images, calcein AM was used to stain the cells. 2 μl of calcein AM and 1 ml of D-MEM culture medium without phenol red or FBS were added to each sample, followed by 10 min of incubation at 37 °C. Cell counting was performed with ImageJ following an automated route for counting cells of single color images. The mean area of each individual cell was calculated based on the number of cells and total fraction area. For each condition, 4 fields of 5 × magnification and 6.2 mm² of total area were evaluated and a *P* value of <0.05 was considered to be statistically significant.

3. Results and discussion

Isothermal cold crystallization results obtained by DSC at distinct temperatures are shown in Fig. 1A. A slower crystallization rate was

found at 90 °C, which increased until 130 °C. These results are in agreement with the ones found elsewhere [11,12]. For temperatures higher than 130 °C the crystallization kinetics began to slow down again. The change in the crystallization kinetics of PLLA at around 120 °C could be also related to a transition from the crystallization regime from III to II [13]. The kinetics of the cold crystallization process also appears to be faster than that of the melt crystallization process for the same temperature [14]. This should be the result of a more extended production of crystallization nuclei that can even be produced below *T_g* [15].

The half-time (*t*_{1/2}) of crystallization and the crystallinity degree (*X_c*), obtained from the DSC scans, are presented in Fig. 1B. The enthalpy of fusion of pure crystalline PLLA (93 J/g) [16] was used to calculate *X_c*. *X_c* tends to increase with increasing temperature up to 140 °C, followed by a slight decrease [17]. Note that it is expected to observe the same kind of evolution if *X_c* would be obtained using other techniques, such as X-ray diffraction. The gradient samples were prepared at the temperature leading to the slowest crystallization (90 °C). The images obtained at 90 °C using optimal microscopy for different crystallization times are presented in Fig. 1C, where the densification of the spherulitic structure with increasing time is consistent with the DSC data.

The dipping methodology used to generate the gradients in crystallinity in the amorphous PLLA films is schematically illustrated in Fig. 2. The sample is immersed along the vertical direction (*z*) with a rate of *z* = *dz*/*dt*. In the end one obtains a sample in which the bottom side accumulated more crystallization time and in the other extreme (*z* = 0) the crystallization time was zero. The immersion time at any given point *z* of the sample can be obtained by integrating *dt* = *dz*/*ż*

$$t(z) = \int_0^z \frac{dz}{\dot{z}} \quad (1)$$

For a constant dipping rate *t*(*z*) = *z*/*ż*. We opted for an average *ż* = 0.15 cm/min that allowed to cover a crystallization time scale

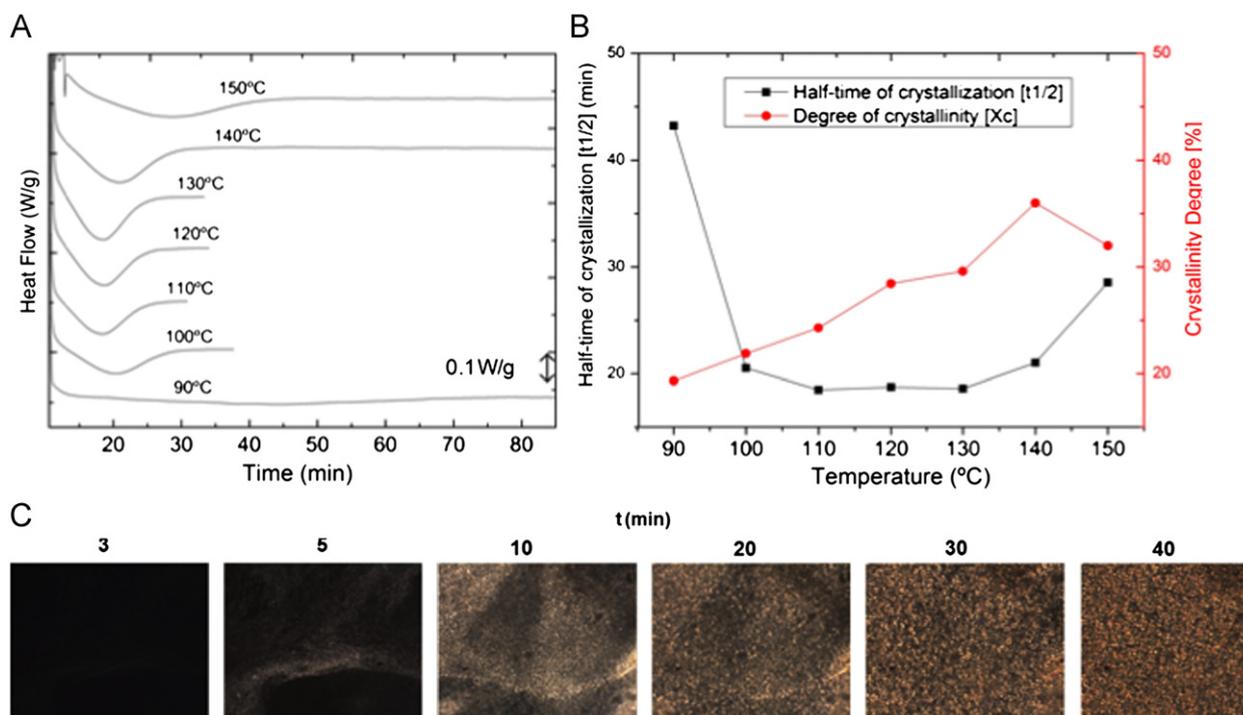


Fig. 1. (A) Isothermal crystallization curves for different temperatures. (B) Half time of crystallization and crystallinity degree as function of crystallization temperature. (C) Hot stage polarized light observations monitoring the development of spherulites during cold crystallization of PLLA at 90 °C at different times; each panel has a width of 800 μm.

compatible with the spherulitic development observed by DSC and optical microscopy along the length of the prepared PLLA films at 90 °C. Thin samples were collected at different distances of the gradient film corresponding to different crystallization times and observed by polarized light microscopy (Fig. 3A). We can observe a constant increase in the density of small spherulites

with increasing immersion time at 90 °C. Such evolution in crystallinity could have an influence on how cells interact with the surface at different positions along the sample.

Calcein AM staining of L929 cells previously cultured on the sample are also shown in Fig. 3A. It is possible to see an evolution in the cell distribution along the gradient. In the less crystalline regions the cells are more homogeneously distributed, while for the regions highly populated with spherulites the cells tend to agglomerate in clusters. Higher magnification images allow to evidence the differences in the less and more crystalline regions—see bottom images in Fig. 3A. Cells seem more spread in the more crystalline zones, indicating a higher degree of adhesion strength. Moreover, in the more crystalline region it is visible that the fluorescent fingerprint of the cells left on the surface upon migration evidencing a spherulitic morphology, which suggests that the cells tend to adhere in the spherulites-rich regions. By analysing the images obtained in the two extremes (7 min and 42 min of immersion at 90 °C) it was possible to estimate cell density and cell contact area—see Fig. 3B. Significant decrease in cell number and significant increase in cell area was observed from the less to the more crystalline regions. The difference in topography between amorphous and crystalline regions may have an influence on the different cellular behaviour [10]. It was shown before that for surfaces populated with large spherulites, cells tend to orient along the front lines between spherulites [18]. Such anisotropy is lost when the spherulites are small, being consistent with the

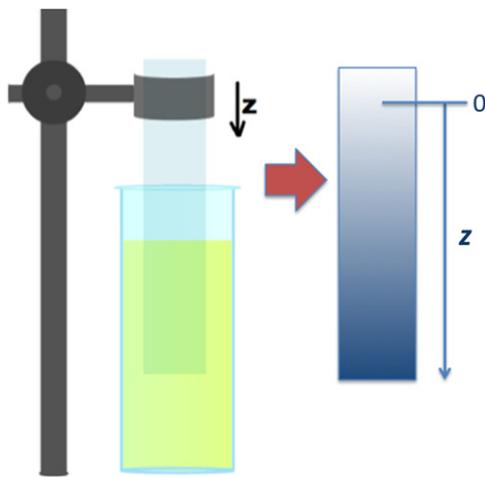


Fig. 2. Schematic of the sample dipping in heated oil (left) and of the graded film (right).

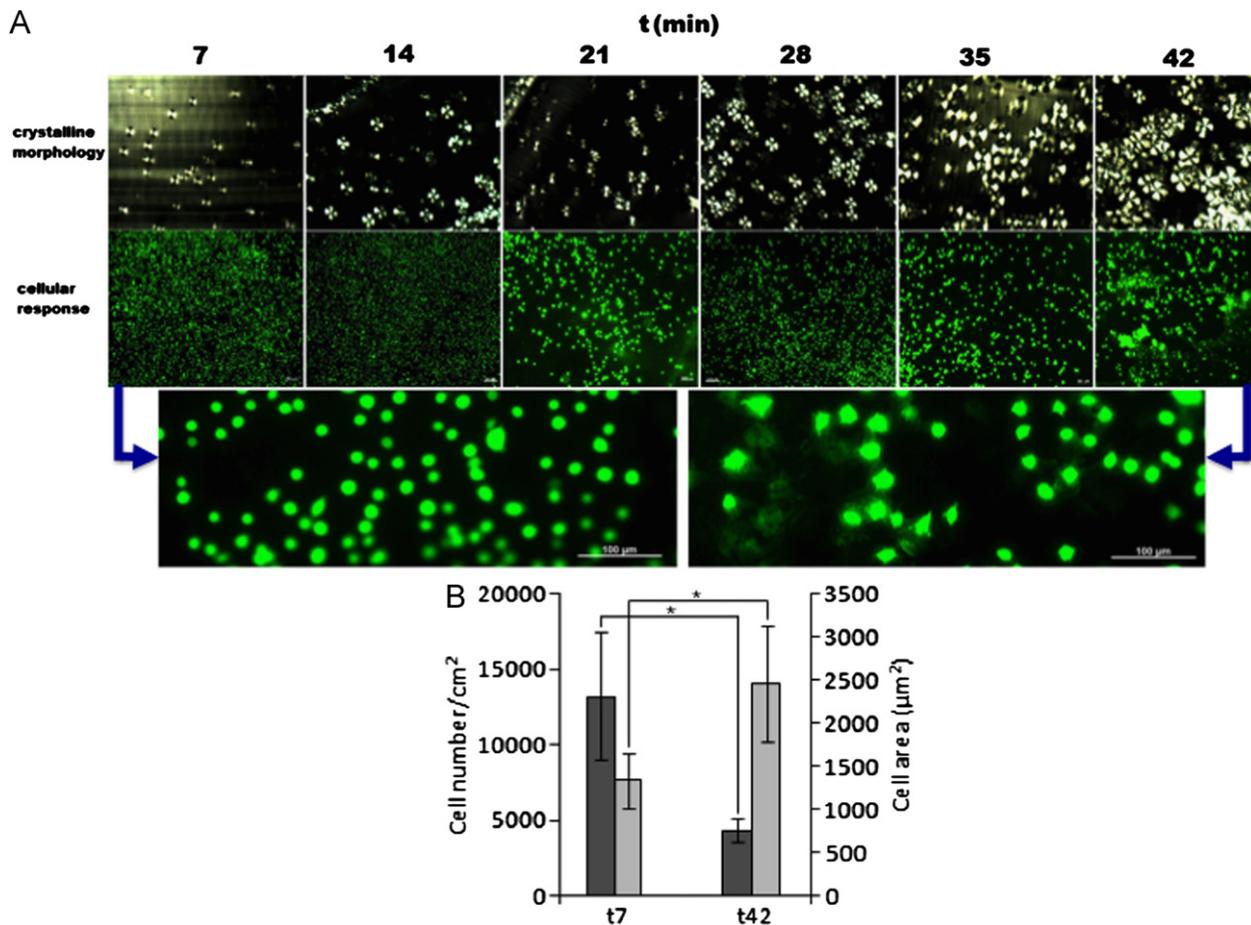


Fig. 3. (A) Crystallinity gradient along the sample's length obtained at 90 °C (top images) and cellular response to this gradient (bottom series of images). Each panel represents 220 μm in the upper montage and 2700 μm in the bottom montage. The two magnified images in the bottom depict cells in the surface of less (left) and more (right) crystalline regions of the gradient surface. (B) Quantification of cell density (dark bars) and cell area (light bars) on the more less (t7) and more (t42) crystalline sides of the gradient surface.

result of this work, because cold crystallization leads to small spherulites around 30 μm , which is on the same order of the size of the cells.

4. Conclusions

PLLA films with a controlled crystallinity gradient have been successfully developed using a simple dipping methodology. Further biological tests were performed *in vitro* using L929 mouse fibroblast cells to assess the effect of the crystallinity gradient on cell behaviour. Differences were observed in the cellular response to this gradient, where the distribution of the cells seems to be influenced by the spatial organization of the spherulites.

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