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Imaging cells on polymer spherulites

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Summary

Polymers are commonly used to construct tissue-engineered medical products and the conditions used to process these polymeric materials can affect their biological performance. For semi-crystalline polymers, thermal processing conditions can induce crystallization of the polymer chains into spherulites, which cause the polymer surface to become roughened. This change in surface topology can influence cell behaviours such as adhesion, morphology and proliferation. Herein, a simple microscope technique is presented, which combines fluorescence microscopy with transmitted white light microscopy using crossed polarizers. This technique allows simultaneous imaging of cells and polymer spherulites, which enables the biomaterials researcher to observe the effects of spherulites on cell alignment and morphology.

Introduction

Polymers are one of the many materials used to fabricate tissue-engineered medical products (Ratner *et al.*, 1996). Polymer processing methods, such as how monomers are converted to polymers or how polymers are formed into products, can affect polymer properties, which in turn can affect how cells respond to the material. For instance, poly(L-lactide) (PLLA) is a crystalline, biodegradable polymer that is commonly used for tissue engineering applications. When PLLA is melted (heated above melting temperature, T_m) and annealed (held at temperature between glass transition, T_g, and T_m for several hours), the polymer chains crystallize into spherulites. Spherulites are spheroid bodies that form during polymer crystallization due to the packing of polymer chains. These spherulites roughen the polymer surface, which can affect cell proliferation (Park & Cima, 1996; Washburn *et al.*, 2004). Thus, cell behaviour on a polymer-derived tissue-engineered medical product can be influenced by heat-treating the product. In order to help characterize cell response to polymer crystallinity, epifluorescence microscopy and polarized light microscopy have been combined to yield a simple and unique method for imaging cells on spherulites. Polarized light microscopy was used to image polymer spherulites, which are birefringent, and fluorescence microscopy was used to image cells that had been fluorescently stained. To the author's knowledge, this is the first time that these techniques have been combined to observe cells cultured on a biomaterial.

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No benefit of any kind will be received either directly or indirectly by the author.

Materials and methods

Polymer films were made from poly(L-lactide) (PLLA) and poly(D,L-lactide) (PDLLA). PLLA is crystalline, whereas PDLLA is amorphous (glassy) and does not form spherulites when annealed. Films of PLLA (M_w = 300 000; Polysciences, Warrington, PA) or PDLLA (M_w = 330 000–600 000; Polysciences) were prepared by spreading pure polymer solutions (1% mass fraction in chloroform) onto 'Low-e' microscope slides (Kevley Technologies, Chesterland, OH) using a home-built flowcoater where the edge of a glass slide was used as the spreading knife. Films were approximately 25 mm × 20 mm. Films were melted at 200 °C for 5 min (above T_m) and then annealed at 120 °C for 8 h (above T_g and below T_m) under nitrogen to cause crystallization of the PLLA. The thickness of the films ranged from 200 to 400 nm, as determined by scraping the film and measuring scrape depth with tapping mode atomic force microscopy (Dimension 3100 Nanoscope IIIa, Veeco Instruments, Inc., Woodbury, NY). Atomic force microscopy was also used to characterize the topology of the polymer films.

MC3T3-E1 cells are a murine osteoblast-like cell line (Sudo *et al.*, 1983; Attawia *et al.*, 1996). Cells were obtained from Riken Cell Bank (Hirosaka, Japan) and cultured in flasks (75 cm^2 surface area) at 37 °C in a fully humidified atmosphere at 5% CO_2 (volume fraction) in alpha-modification of Eagle's minimum essential medium (BioWhittaker, Inc., Walkersville, MD)

supplemented with 10% (volume fraction) fetal bovine serum (Gibco, Rockville, MD) and kanamycin sulphate (0.06 mg mL^{-1}; Sigma Inc., St. Louis, MO). The medium was changed twice weekly and cultures were passaged with 2.5 g L^{-1} trypsin (0.25% mass fraction) containing 1 mmol L^{-1} ethylenediaminetetraacetic acid (EDTA) (Gibco) once a week. Cultures of 90% confluent MC3T3-E1 cells were used for the experiments.

Polymer films were sterilized in 70% ethanol (mass fraction) for 5 min, rinsed in media and seeded with 10^6 MC3T3-E1 cells in 150 cm^2 Petri dishes (7000 cells cm^{-2}) with 50 mL of media. Cells were incubated for 5 days, fixed for 5 min [0.5% Triton X-100 (mass fraction), 4% paraformaldehyde (mass fraction), 5% sucrose (mass fraction), 1 mmol L^{-1} $CaCl_2$, 2 mmol L^{-1} $MgCl_2$ in phosphate buffered saline, pH 7.4] and postfixed for another 20 min (same as fix but without Triton X-100). Fixed cells were stained for 1 h with 6 μmol L^{-1} DAPI (4′,6-diamidino-2-phenylindole, dihydrochloride) and 2 μmol L^{-1} Texas-red-C_2-maleimide (Molecular Probes, Eugene, OR) in phosphate buffered saline. DAPI stains cell nuclei to fluoresce blue and Texas-red-C_2-maleimide stains cell membranes to fluoresce red. Stained cells were mounted with a coverslip in Vectashield containing DAPI (Vector Laboratories, Inc., Burlingame, CA).

Imaging

Images of the cells on the polymer films were captured using a Leica DMR 1200 upright microscope using a 10× objective

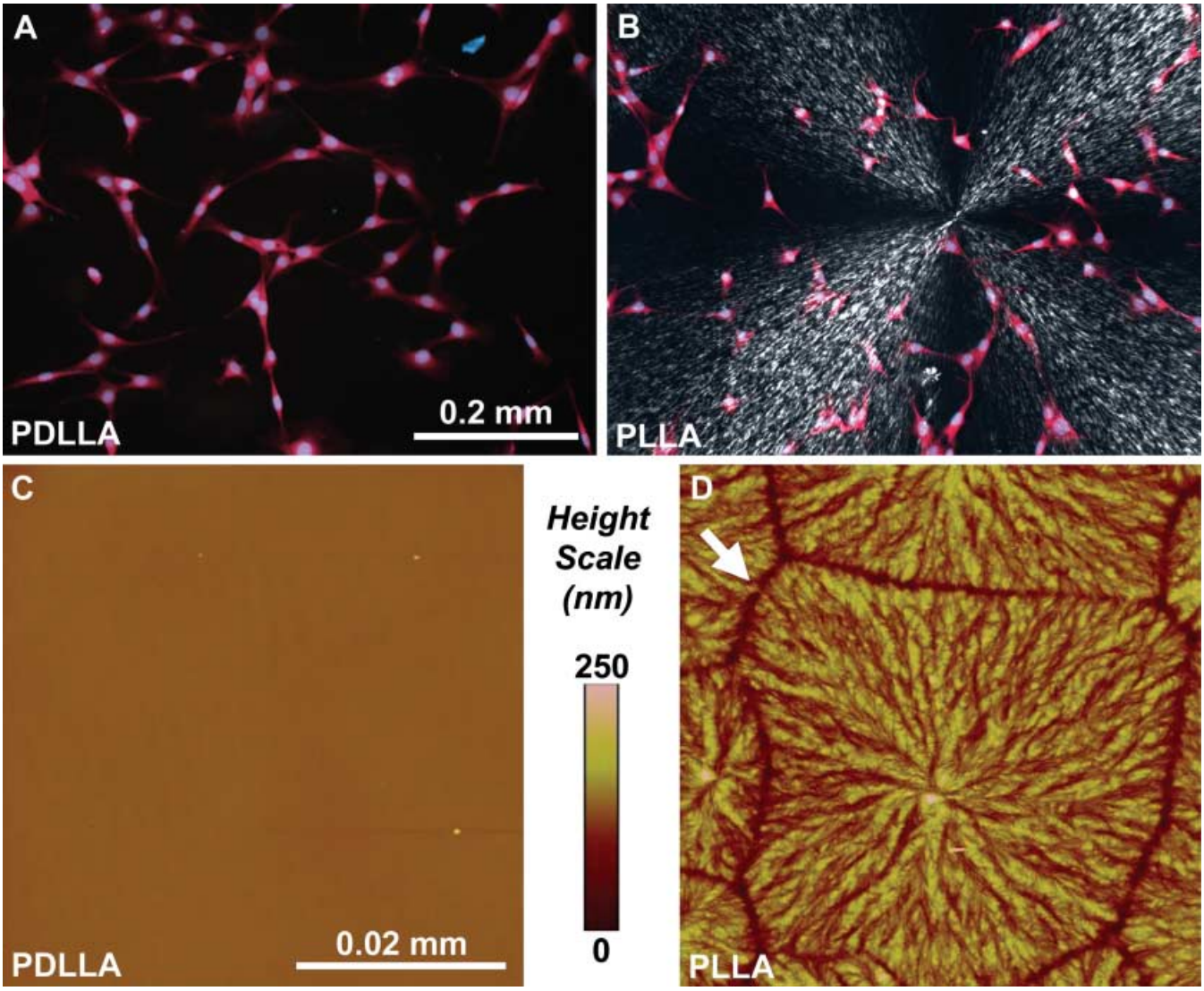

Fig. 1. A,B: Osteoblasts (MC3T3-E1 cells) cultured on an amorphous PDLLA film (A) or on a crystalline PLLA film (B). Both films were annealed. DAPI was used to stain cell nuclei to fluoresce blue and Texas-red-C_2-maleimide was used to stain cell membranes to fluoresce red. The large 'iron cross' in the background of (B) is birefringence from a spherulite seen with crossed polarizers. There is no spherulite in (A) because PDLLA is amorphous. C,D: Atomic force microscopy (height scale images) of annealed films of PDLLA (C) or PLLA (D). The arrow in (D) identifies the grain boundary between two spherulites.

and a Hamamatsu Orca ER digital camera (Vashaw Scientific, Inc., Frederick, MD). Once a field of view was chosen, it was photographed using three different microscope settings: epifluorescence with a blue filter cube for cell nuclei (DAPI), epifluorescence with a red filter cube for cell membranes (Texas-red-C_2-maleimide) and transmitted white light with crossed polarizers for the spherulites. The final figure was created by combining the three images into a colour composite using Image Pro software (Media Cybernetics, Carlsbad, CA).

Cells cultured on amorphous PDLLA are shown in Fig. 1(A). The cell nuclei are the small blue spheres and the red-stained polygonal cell membranes surround the nuclei. The background is flat black and no birefringent spherulites are visible because PDLLA is amorphous and does not crystallize. Cells cultured on crystalline PLLA are shown in Fig. 1(B). The radiating lamellae of a spherulite give rise to the Maltese cross visible under the crossed polars (birefringence) in the background of this image.

This new combination of fluorescence and polarized light microscopy allows direct observation of cells cultured on polymer spherulites, which can provide the biomaterials researcher with unique information. For instance, the surface of a PLLA film becomes roughened during spherulite formation as small ridges (height ~30 nm, width ~2 μm) are formed by the lamellae which radiate from the centre of each spherulite (Fig. 1D). Although cells are known to align on microgrooves (depth 0.5 μm, width 1–10 μm) in a process called 'contact guidance' (Walboomers *et al*., 2000), it can be observed in Fig. 1(B) that MC3T3-E1 cells do not align along the radiating ridges formed by the PLLA spherulites. In addition, deeper grooves form at the grain boundaries between PLLA spherulites (Fig. 1D: depth ~100 nm, width ~2 μm), and although a grain boundary is not visible in Fig. 1(B), we did not observe cells aligning along these grooves.

This new microscopy technique would also be useful for imaging cells on polymer specimens where there are amorphous regions between the individual spherulites such that the spherulites are discontinuous (not touching). This can occur after appropriate thermal treatments of crystalline polymers or in polymer blends (Tsuji & Ikada, 1996). The new microscopy technique would allow the observer to determine whether cells preferentially adhered or proliferated on spherulites vs. amorphous regions of the polymer specimen.

In summary, the images obtained by these methods (Fig. 1B) are a result of a novel combination of epifluorescence and polarized light microscopy and should be useful to tissue engineers for characterizing cell–material interactions. Processing conditions such as thermal treatments or blending can affect the properties of polymeric constructs. The new technique for imaging cells described in this paper can be used to observe directly cells on polymer spherulites, allowing the observer to determine the effects of polymer crystallization on cell behaviour. Thus, the unique information provided by this new microscopy technique could help tissue engineers to determine the appropriate processing conditions for constructing polymeric medical products.

Disclaimer

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