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Fabrication of Honeycomb-Structured Poly(DL-lactide) and Poly[(DL-lactide)-*co*-glycolide)] Films and their Use as Scaffolds for Osteoblast-Like Cell Culture

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Summary: Surfactant-free honeycomb-structured Poly(DLlactide) (PDLLA) and Poly-(DLlactide-co-glycolide) PDLLGA thin films were fabricated by water droplet templating methods. Thin films with uniform pore structure were obtained after controlled evaporation of solvents in a humid atmosphere. Solvent, polymer concentration and humidity were found to be important factors in the formation of honeycomb-structured thin films. Preliminary cell culture studies with MG-63 osteoblast-like cell lines showed promising degrees of cell attachment and proliferation on these films, suggesting that they are applicable as scaffolds for tissue engineering.

Keywords: biodegradable; honeycomb; scaffold; thin film; tissue engineering

Introduction

Polymeric materials with highly porous structure have attracted significant attention in recent years.[1] They are used as microfiltration devices,[2] for catalytic templating,[3,4] as chemosensors[5] and, most pertinant to this study, as scaffolds for tissue engineering.[1,6–8] A number of processing techniques have been developed to generate such materials with controlled and uniform pore structures including: 3-D printing,[9] stereolithography,[10] templating of silica spheres,[11] and emulsion templating.[12] However, these techniques usually involve multiple processing steps and/or complex equipment. Francois *et al.* pioneered a one-step water-templating method to generate polymer films with honeycomb patterns in which a solution of star-shaped polystyrene in carbon disulphide was cast onto a substrate and the volatile solvent was evaporated under humid gas flow, leading to the spontaneous formation of hexagonally ordered pores.[13] Subsequently, Shimomura *et al.* further extended the applicability of this technique to a wider range of substrates including block copolymers, inorganic materials and amphiphilic polyion complexes.[14]

Biocompatible polyesters such as polylactide (PLA), polyglycolide (PGA), and poly(lacide-*co*-glycolide) (PLGA) are some of the most widely used synthetic polymeric materials for bone tissue engineering,[15–17] and recently water-templating has been applied to these polymers. For example, Chen *et al.* reported that a honeycomb-patterned film could be formed after blending PLGA9010 ($n_{LA}/n_{GA} = 90{:}10$) or PLGA7030 ($n_{LA}/n_{GA} = 70{:}30$) with PE-*block*-PEG;[18] Li *et al.* fabricated honeycomb-structured films by evaporating a poly(L-lactide) (P_LLA) solution in water miscible terahydrofuran (THF) under humid conditions;[19] and Shimomura *et al.* reported cell adhesion to honeycomb-patterned thin films of PLA containing biocompatible surfactants.[7] However,

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to our knowledge, surfactant-free honeycomb-patterned films of PLA or PLGA have not previously been evaluated for cell attachment or proliferation.

In this study we demonstrate that honeycomb-structured films can be fabricated from $P_{DL}LA$ and a series of $P_{DL}LGA$ polymers without the aid of surfactants, and that these films are viable scaffolds for culture of osteoblast-like MG-63 cell lines.

Experimental Part

Materials

$P_{DL}LA$ was prepared using a previously published method, which yields predominantly heterotactic polymer.[20] GMP grade $P_{DL}LGA8515$, $P_{DL}LGA7525$, and $P_{DL}LGA5050$, with nLA/nGA ratios of 85:15, 75:25 and 50:50, respectively, were obtained from Alkermes, Inc. or Aldrich and used as received. The molecular weights and molecular weight distributions of all polymers used in this study were analysed by Gel Permeation Chromatography (GPC, Polymer Laboratories PL-GPC50) (Table 1). Chloroform ($CHCl_3$, HPLC grade), tetrahydrofuran (THF, GPC grade) and ethyl acetate (HPLC grade) were all purchased from Fisher Scientific and used as received.

Film Preparation and Characterization

The polymers were dissolved in an appropiate solvent overnight prior to film preparation. Thin films were prepared by casting polymer solutions (100 μL spreading volume) on glass cover slips (13 mm diameter) in a glovebox whose environment was maintained at 26 °C and 70%, 80% or 90% relative humidity. The morphologies of thin films were characterized by scanning electron microscope (SEM, JEOL JSM 6480LV) with 10–15 kV accelerating voltage. Pore sizes were analysed using the ImageJ software package.[21]

Table 1.
Characterization of $P_{DL}LA$ and $P_{DL}LGA$ samples used to prepare honeycomb films.

Sample	M_n	M_w	M_w/M_n (PDI)
$P_{DL}LA$	58000	87000	1.49
$P_{DL}LGA8515$	57000	120000	1.79
$P_{DL}LGA7525$	190000	290000	1.54
$P_{DL}LGA5050$	34000	53000	1.56

Cell Culture on $P_{DL}LA$ and $P_{DL}LGA$ Thin Films

MG-63 osteoblast-like cells (European Collection of Cell Cultures) were used to assess cell attachment and proliferation on the honeycomb-structured films. Thin films were first sterilised by submerging them in 70% ethanol for 30 min and washing twice with an excess of phosphate buffered saline (PBS). The cells were seeded onto the thin film-coated glass cover slips with an initial cell density of 20,000/cm^2 in 24-well culture dishes, and were maintained in a humidified environment at 37 °C and 5% CO_2. Cells were maintained in a cell culture medium comprising Dulbecco's Modified Eagles Medium (DMEM, Invitrogen), supplemented with 10% (v/v) heat inactivated foetal calf serum (FCS, Sigma-Aldrich), 1mM sodium pyruvate (Sigma) and 1% (v/v) non-essential amino acid (NEAA, Sigma-Aldrich). Media were changed on the second day for the cell proliferation assessment. Tissue culture plates (TCP) were also used as positive control substrates.

Cell Proliferation

Cell proliferation was determined after 6 and 72 hr by MTT assay (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, MTT, Aldrich). The method used in this study was modified from Chiba *et al.*[22] In brief, 2 mg/ml MTT stock solution was prepared in PBS and kept in the dark. A 400 μg/ml working solution was prepared by diluting the stock solution with culture medium in a 1:4 ratio. 1 ml of MTT working solution was added to each well (24 wells) and incubated for 3 hr at 37 °C. The blue formazan crystals were then dissolved by adding 1 ml dimethyl sulfoxide (Sigma) to each well. The absorbance was analysed at 570 nm using a plate reader (VersaMax™, Molecular Devices).

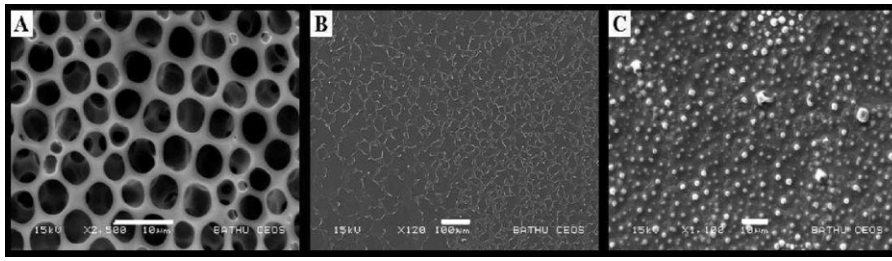

Figure 1.
SEM images of $P_{DL}LGA5050$ ($M_n = 34{,}000$) films from different solvents: (A) $CHCl_3$ (scale bar 10 μm); (B) ethyl acetate (scale bar 100 μm); (C) THF (scale bar 10 μm). Concentration: 1 g/L, temperature: 26 °C, relative humidity: 90%, spreading volume: 100 μL.

Cell Morphology

Cell morphology was examined by scanning electron microscope (SEM, JEOL JSM 6480LV) after 72 hr. At harvest, the culture media were removed and the samples were fixed in 2.5% glutaraldehyde for 2 hr. The sample was then washed with serum free culture medium and post-fixed in 1% osmium tetroxide in Zetterqvist's buffer for 1 hr. After being thoroughly washed with serum free culture medium, the sample was stained with 2% aqueous uranyl acetate for 1 hr in dark and then freeze-dried overnight. A thin layer of gold was sputter-coated onto the sample prior to SEM examination.

Results and Discussion

Solvent Selection

The nature of the solvent is one of the key factors which governs the formation of the honeycomb-structured films. For instance, honeycomb-structured linear polystyrene could be fabricated from $CHCl_3$ or toluene but not from carbon disulphide (CS_2) or THF.[23] In our study, $CHCl_3$, ethyl acetate and THF were selected to investigate solvents effects for the formation of honeycomb films of $P_{DL}LA$ and $P_{DL}LGA$.

Initially, fabrication of $P_{DL}LGA5050$ films was investigated using a range of solvents (Figure 1). Under the conditions employed, films with a regular pattern could be produced from $CHCl_3$ [Figure 1(A)] but not from ethyl acetate or THF [Figure 1(B) and 1(C), respectively]. Following the successful preparation of patterned $P_{DL}LGA5050$ films from $CHCl_3$, this solvent was also investigated for $P_{DL}LGA8515$ and $P_{DL}LGA7525$. Figure 2 shows that thin films fabricated from $P_{DL}LGA7525$ [Figure 2(B)] possess a 3D-honeycomb structure of interconnected pores. In contrast, evaporation of

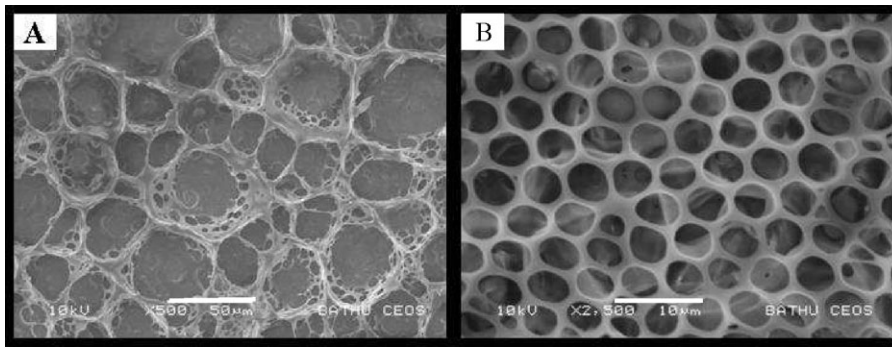

Figure 2.
SEM images of film surfaces from different polymers: (A) $P_{DL}LGA8515$ (scale bar 50 μm); (B) $P_{DL}LGA7525$ (scale bar 10 μm). Solvent: $CHCl_3$, concentration: 1 g/L, temperature: 26 °C, relative humidity: 90%, spreading volume: 100 μL.

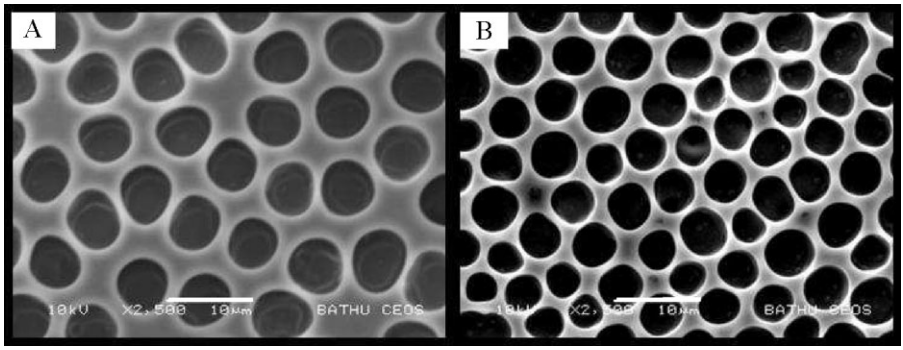

Figure 3.
SEM images of film surfaces (scale bar 10 μm): (A) $P_{DL}LGA8515$; (B) $P_{DL}LA$. Solvent: THF, concentration: 20 g/L, temperature: 26°C, relative humidity: 90%, spreading volume: 100 μL. (scale bars 10 μm).

$P_{DL}LGA8515$ solutions under the same conditions [Figure 2(A)] resulted in an irregular structure with a wide range of pore size and morphology. These observations are consistent with increasing glycolide content leading to an increase in polymer hydrophilicity, which has previously been reported to favour the formation of honeycomb-structured films of PLGA from $CHCl_3$ solution.[18]

The failure to form ordered structures for $P_{DL}LA$ and $P_{DL}LGA8515$ from $CHCl_3$ led us investigate THF as a solvent, since it has been reported that honeycomb-structured thin films of poly(L-lactide)[19] and cellulose acetate butyrate[24] could be prepared from this solvent. As shown in Figure 3, both $P_{DL}LGA8515$ and $P_{DL}LA$ films fabricated from THF possess regular patterned structures. In contrast, all attempts to form ordered structures using $P_{DL}LGA7525$ and $P_{DL}LGA5050$ in THF were unsuccessful.

Influence of Humidity on Pore Size

To investigate the effect of humidity on pattern formation and pore size, thin films of $P_{DL}LA$ were fabricated from THF at a range of relative humidities. As shown in Figure 4, patterned $P_{DL}LA$ films could be prepared at relative humidities of 90, 80 and 70%, with pore diameter increasing with humidity (approximate pore size of 3.5, 4.5, and 5.2 μm for 70, 80 and 90% relative humidity, respectively). A similar trend of increasing pore size with increasing humidity was previously observed for a range of amphiphilic polymers.[25] In a control experiment, thin films were also prepared under ambient conditions (30% relative

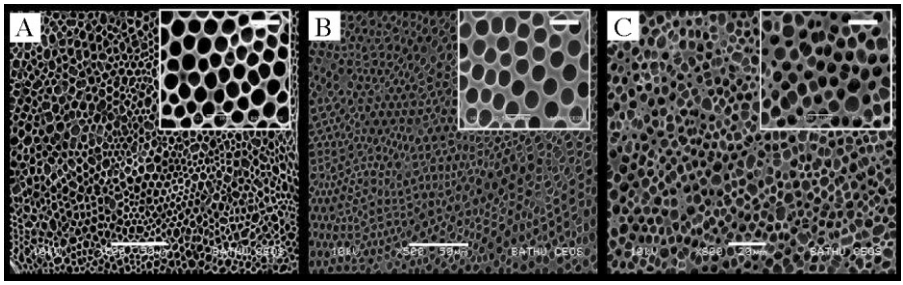

Figure 4.
SEM images of $P_{DL}LA$ films prepared at different relative humidities: (A) 90% (scale bars 50 μm and 10 μm); (B) 80% (scale bars 50 μm and 10 μm); (C) 70% (scale bars 20 μm and 10 μm). Solvent: THF, concentration: 20 g/L, temperature: 26°C, spreading volume: 100 μL.

Table 2.
Cell attachment and proliferation determined by MTT assay.

Substrate	Structure (Solvent)	6 hr (Cell/cm^2)	72 hr (Cell/cm^2)
$P_{DL}LA$	3.55 μm (THF)	13300 ± 4000	29300 ± 4500
$P_{DL}LGA5050$	2.95 μm ($CHCl_3$)	13500 ± 2400	29400 ± 5600
TCP	flat film	15000 ± 6000	55000 ± 6500

humidity), and no pores were formed after complete evaporation of the solvent, confirming that water-templating is a crucial factor in honeycomb-patterned film formation.

Cell Attachment, Proliferation and Morphology

Honeycomb-patterned films prepared from $P_{DL}LA$ and $P_{DL}LGA5050$ were investigated as scaffolds for tissue engineering. MG-63 osteoblast-like cell cultures were grown on these two films and on a standard polystyrene tissue culture plate (TCP) for comparison. After 6 and 72 hr, cell attachment and proliferation was evaluated by MTT assay (Table 2). After 6 hr, both patterned films showed similar levels of cell attachment to TCP, and after 72 hr high levels of cell proliferation were maintained (approximately 55% of TCP) indicating that honeycomb-patterned PLA and PLGA films are viable scaffolds for the growth of osteoblast-like cells. An SEM image of the $P_{DL}LA$ film taken after 72 hr (Figure 5) reveals a typical morphology of the MG-63 cells attached to the film, which suggests that these osteoblast-type cells can maintain their phenotype on the films. Examination of the cell morphology also highlights a possible role played by the porous structure of the film in promoting cell attachment since cell fibrils appear to penetrate the surface structure through the pores.

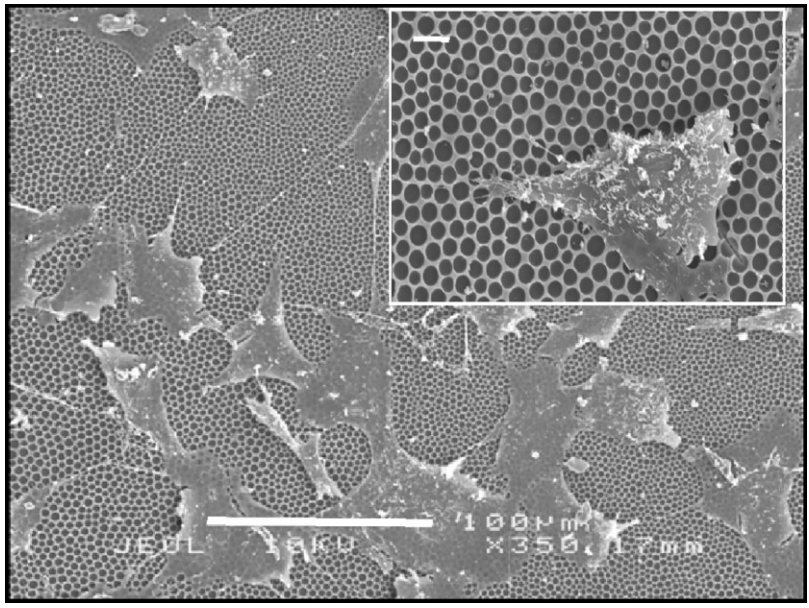

Figure 5.
SEM image of MG-63 osteoblast-like cells grown on $P_{DL}LA$, insert shows a magnification of part of the same image (scale bars 100 μm and 10 μm).

Conclusion

We have shown that a water-templating technique can be applied to the fabrication of honeycomb-patterned thin films of $P_{DL}LA$ and $P_{DL}LGA$, and that choice of solvent, polymer concentration and humidity are all important factors in controlling the porous mesostructure of the film. A preliminary study of cell attachment and proliferation shows that PLA and PLGA films prepared in this manner, from either THF or $CHCl_3$ solution have potential as use for scaffolds for osteoblast-like cell culture.

Having established the feasibility of this approach to the preparation of well-controlled porous scaffolds for tissue engineering, we are currently investigating in more detail the effects of polymer composition and microstructure, and the influence of scaffold mesostructure on the attachment and proliferation of cells on honeycomb-structured thin films of biocompatible and resorbable polymers.

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