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Integration of photo-crosslinking and breath figures to fabricate biodegradable polymer substrates with tunable pores that regulate cellular behavior

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ABSTRACT

Honeycomb films of crosslinked poly(ε-caprolactone) triacrylate were fabricated by incorporating the breath-figure method with photo-curing. Two different pore sizes of 5.6 and 3.0 μm were achieved by modulating the airflow rate at 0 and 50 mL min^{-1}, respectively. Mouse pre-osteoblastic MC3T3-E1 cell adhesion, spreading, proliferation, mineralization, and expression of integrin subunits of α_1, α_2, β_1, and gene markers of osteocalcin, osteopontin, alkaline phosphatase (ALP), and collagen type I were investigated on these two series of honeycomb films and their flat control. The honeycomb films, especially those with smaller pores, could significantly promote MC3T3-E1 cell adhesion, spreading, proliferation, ALP activity, mineralization, and gene expression of osteoblastic markers, via fostering expression of integrin subunits.

1. Introduction

Cells can recognize and respond to surface features via inter- and intracellular signaling events [1–5]. These signaling events are of critical importance for subsequent cell proliferation, differentiation, and development of endogenous extracellular matrix (ECM) [1–5]. Among many topographical features at the micro- and nanoscale to which cells react, substrates with ordered pore/pit distribution have been used to mimic the basement membrane with hierarchical porous structures [1–5]. Unlike methods such as phase separation, colloidal templating, and lithography [1–3], the breath-figure method is very convenient to prepare honeycomb polymer films as condensed water droplets on the surface of polymer solution work as template during the evaporation of volatile solvent in a humid condition [5–9]. For example, honeycomb poly(ε-caprolactone) (PCL) films with pore sizes (diameters) of 3–20 μm were prepared in the presence of a surfactant to regulate cellbehavior [5,10–12]. Although great effort has been focused on developing porous substrates/scaffolds for enhancing bone cell functions [1–3,5,13–15], there are not many studies on how to use honeycomb films of biodegradable polymers with tunable micropores to regulate osteoblastic adhesion, differentiation, and gene expression. In addition, the honeycomb polymer films with tunable pores for cell studies here were fabricated by incorporating photo-curing with the breath figure method without the need of a surfactant.

Photo-curable and biodegradable PCL triacrylate (PCLTA) synthesized using a facile method in our research group has proved to be a promising candidate biomaterial for diverse tissue-engineering applications [16,17]. A semi-crystalline PCLTA network was used in this study because it could better support mouse pre-osteoblastic MC3T3-E1 cell proliferation and mineralization than more compliant amorphous counterparts [16,17]. There were three steps in preparing these honeycomb films (Scheme 1). First, the temperature on the polymer solution surface decreased when the moist air was blew over it and the volatile, water-miscible, relatively non-toxic solvent tetrahydrofuran (THF) evaporated, which consequently triggered nucleation and growth of water droplets. Second, water droplets with similar diameters were arranged into a hexagonal array, followed by exposure to UV light to initiate photo-curing of PCLTA. At last, polymer precipitated around the water droplets and then THF and water were completely removed. As summarized in recent reviews [7,8], photo-cured polymer honeycomb structures with enhanced stability indeed exist but they were not used for regulating cell behavior [18,19]. In this study, we obtained honeycomb films of photo-cured PCLTA and further studied mouse pre-osteoblastic MC3T3-E1 cell adhesion, spreading, proliferation, differentiation, and gene expression on them.

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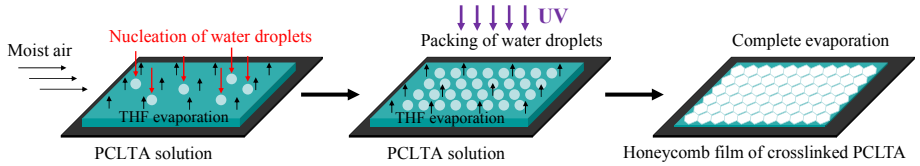

Scheme 1. Preparation of honeycomb films of photo-cured PCLTA.

2. Experimental section

2.1. Preparation of honeycomb photo-cured PCLTA films

All chemicals used in this study were purchased from Sigma–Aldrich unless noted otherwise. PCLTA (M_n = 20020 g mol^{-1}, M_w = 22670 g mol^{-1}) synthesized in our lab and photo-initiator, phenyl bis(2,4,6-trimethyl benzoyl) phosphine oxide (BAPO, IRGACURE 819, Ciba Specialty Chemicals) were dissolved in distilled THF at concentrations of 50 and 0.5 mg mL^{-1}, respectively [16,17]. Then the PCLTA/BAPO solution in THF was cast onto clean glass slides and put in a chamber. At a relative humidity of 60%, honeycomb polymer films with pore sizes of 5.6 and 3.0 μm were fabricated by controlling the airflow rate at 0 and 50 mL min^{-1}, respectively. Polymer films became opaque gradually during solvent evaporation for 5 min, followed by exposure to UV light (λ = 315–380 nm) generated from a Spectroline lamp (SB-100P; intensity: 4800 μw cm^{-2}) for 30 min. For comparison, flat films were prepared by casting the same PCLTA/BAPO solution on glass slides and photo-curing at the same condition.

2.2. Physical characterizations

To obtain their gel fractions, photo-cured PCLTA films with the original weight (W_o) were soaked in excessive methylene chloride for 2 days. Then the films were dried in vacuum for 2 h and weighed as W_d. The gel fraction was then calculated using $W_d/W_o \times 100\%$. The measurement was performed in triplicate. The surface morphology of both flat and honeycomb films was observed using Scanning Electron Microscopy (SEM; S-3500, Hitachi Instruments, Tokyo, Japan) at an accelerating voltage of 10 kV. Differential Scanning Calorimetrical (DSC) measurements were carried on a Perkin Elmer Diamond differential scanning calorimeter in a N_2 atmosphere. The photo-cured PCLTA films were first heated from 25 to 100 °C at a heating rate of 10 °C/min, then cooled to −80 °C at a cooling rate of 10 °C/min, and heated to 100 °C again at 10 °C/min. The melting temperature was determined from the exothermal peak in the DSC curve obtained in the last heating run. The crystallinity was calculated by dividing the heat of fusion, which was measured from the exothermal peak, by the value for 100% crystalline PCL, according to our previous report [16].

2.3. Water contact angle and protein adsorption

Prior to measurements, all photo-cured PCLTA films were dried completely in vacuum overnight. Water contact angles were determined at room temperature using a Ramé-Hart NRC C. A. goniometer (model 100-00-230) and the images of water droplets on the polymer films were taken using a 3.0 megapixel camera (Moticam 2300, Motic). For protein adsorption, the polymer films were immersed in the culture media for MC3T3-E1 cells for 4 h at 37 °C. The films were washed with PBS five times to remove unattached proteins and soaked in 300 μL of 1% sodium dodecyl sulfate (SDS) solution 5 times for 1 h each to collect proteins adsorbed on the surface. A microplate reader (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA) and a MicroBCA protein assay kit (Pierce, Rockford, IL) were used to detect the protein concentrations in the collected SDS solutions. A standard curve for calibration was plotted using albumin in the kit.

2.4. Cell studies

MC3T3-E1 cells (ATCC, Manassas, VA) were cultured as previously reported [15–17,20,21]. Prior to cell studies, all polymer films were immersed in a mixture of 70% alcohol solution and acetone (75/25, v/v) overnight to remove excessive unreacted polymer chains and BAPO. The films were dried in vacuum and further sterilized by 70% alcohol solution for 3 × 1 h followed by complete drying in vacuum. MC3T3-E1 cells were seeded on all the samples at a density of ~15,000 cells per cm^2 and cultured in α-Minimum Essential Media (α-MEM; Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS, Gibco) and 1% penicillin/streptomycin (Gibco) for 4 h, 1, 2, 4, and 14 days in an incubator at 37 °C with 95% humidity and 5% CO_2. To count cell number and observe F-actin cytoskeleton, the attached cells on the films were fixed with 16% paraformaldehyde (PFA) solution for 20 min. After PFA was removed, cells were washed twice with phosphate buffered saline (PBS), permeabilised with 0.2% Triton X-100, and stained using rhodamine-phalloidin (RP) for more than 1 h at 37 °C and 4′,6-diamidino-2-phenylindole (DAPI) at room temperature. Cell images were taken on an Axiovert 25 light microscope (Carl Zeiss, Germany). Cells on each film were counted using DAPI-stained images and the mean number of nuclei per field (n > 15) was calculated. Average cell area was obtained from more than 20 non-overlapping cells at day 1 post-seeding using ImageJ software (National Institutes of Health, Bethesda, MD). Focal adhesions (FAs) were stained with mouse monoclonal anti-vinculin antibody (V9264, Sigma–Aldrich) for more than 1 h at 37 °C followed by five rinses with PBS. Then the FAs were labeled by anti-mouse IgG-FITC antibody (F0257, Sigma) at 37 °C for 5 h and observed on a confocal microscope (SP2, Leica). To observe the morphology of both cells and films using SEM, all samples were dehydrated with graded ethanol solutions (25%, 50%, 70%, 95%, and 100%), dried in vacuum overnight, and sputter-coated with gold-palladium.

2.5. Alkaline phosphatase (ALP) activity, calcium content, and gene expression

After MC3T3-E1 cells were cultured on the polymer films for 14 days, all samples were rinsed with PBS twice, trypsinized, washed again, and the cell suspension was centrifuged for 4 min at 1000 rpm. The obtained cell pellet was re-suspended in 1 mL 0.2% Nonidet P-40 solution and sonicated in ice water for 2 min. Fluorescence-based ALP detection kit (Sigma, St. Louis, MO) and QuantiChrom calcium assay kit (BioAssay Systems, Hayward, CA) were used to determine the ALP activity and calcium content of the cell lysate, respectively [16,20,21]. After MC3T3-E1 cells were cultured for 14 days, RNA was isolated from the cell pellet using a RNeasy Mini Kit (Qiagen, Valencia, CA), from which cDNA was then synthesized using a cDNA synthesis kit (Thermo Scientific).