Cellular Response to Phase-separated Blends of Tyrosine-derived Polycarbonates

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INTRODUCTION

New combinatorial approaches to material synthesis and characterization are affording opportunities to address complex biological hypotheses, including cell-biomaterial interactions, and cover large variants in the physico-chemical parameter space simultaneously.[1-7] Surface characteristics such as hydrophobicity, morphology, surface charge, and chemical functionality each play key roles in governing cell adhesion and proliferation.[8] These factors, not withstanding new chemical and processing methodologies, are continually increasing the physical parameter space, making gradient sample fabrication methods very attractive. Orthogonal gradient methods developed in the NIST Combinatorial Materials Center (NCMC) produce well-defined materials that afford simultaneous coverage of multidimensional chemical, composition, and physical property parameter space. Our initial efforts have focused on developing *in vitro* assays for the measurement of inflammation and extracellular matrix (ECM) gene regulation, which we anticipate will provide preliminary assessments of *in vivo* material performance.

EXPERIMENTAL[#]

Abbreviations. ATCC: American Type Culture Collection, FN: fibronectin, IL-1β: interleukin-1 beta, RT-PCR: real-time polymerase chain reaction, TCPS: tissue culture polystyrene.

Materials. ε-Polycaprolactone was obtained from Sigma (M_w = 80,000). QuantiTect SYBR Green RT-PCR and RNeasy kits were obtained from Qiagen. Unless otherwise listed, all solvents and reagents were purchased from Sigma and used as received. Cells were cultured in sterile 150 mm × 25-mm nonpyrogenic tissue culture polystyrene (TCPS) flasks (Daigger). Tyrosine-derived polycarbonates were synthesized as described previously.[9] In these studies, the alkyl ester pendent chains (R) of the poly(desaminotyrosyl tyrosine ester carbonate) are either an ethyl (E) or octyl (O) chain. The weight average molecular mass and PDI for each of the polymers used in these studies are listed. DTE: M_w = 131,000, M_w/M_n = 3.0; DTO: M_w = 61,500, M_w/M_n = 2.7.

Thin Film Preparation. Round glass coverslips (22 mm, Fisher, No 1½) were precleaned using a 1 h ultrasonication bath in a 50:50 (volume %) methanol/water solution. Tyrosine-derived polycarbonate homopolymers and blends were spun coat onto the coverslips at speeds of 209.4 rad/sec from dichloromethane solutions 10 mg/mL in concentration. Blends were mixed in various compositions using DTE and DTO tyrosine-derived polycarbonates in 70/30, 50/50, 30/70 ratios (DTE/DTO, by mass). Following the spin coating process, the polymer coated glass disks were annealed at 105 °C for 16 h under vacuum.

Contact Angle Measurements. The static contact angle of water on the prepared surfaces was measured at 25 °C using water as the probe fluid by operating a drop shape analysis system of DSA 10 Mr2 (Krüss, Germany). The standard uncertainty of contact angle measurements for each composition was determined by the standard deviation between six independent measurements on each of two sample discs prepared under identical conditions.

Atomic Force Microscopy (AFM). Tapping-mode atomic force microscopy measurements were conducted in air with a Nanoscope IV system (Digital Instruments) operated under ambient conditions with standard silicon tips (Nanodevices; L, 125 μm; normal spring constant, 40 N/m; resonance frequency, (300 to 360) kHz).

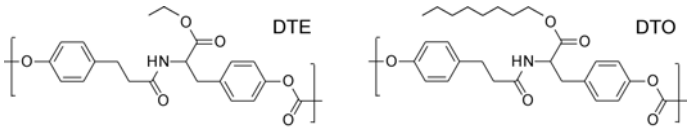
Differential Scanning Calorimetry (DSC). The thermal behavior was studied by differential scanning calorimetry using a Perkin Elmer DSC 7 operated under nitrogen and calibrated with indium. The DSC scanning curves were collected at a heating rate of 10 °C/min.

Cell Lines. MC3T3-E1 osteoblast cells (subclone 4) were obtained from ATCC and cultured in flasks (75-cm^2 surface area) at 37 °C in a fully humidified atmosphere at 5 % CO_2 (volume) in Eagle's minimum essential medium (α-modified, BioWhittaker, Inc). The medium was supplemented with fetal bovine serum (10 %, volume fraction, Gibco). Established protocols for the culture and passage of MC3T3-E1 cells were followed,[10] including passage with trypsin (2.5 g/L) containing 1 mmol/L ethylenediamine tetraacetic acid once per week.

RT-PCR. RT-PCR was carried out using the QuantiTect SYBR Green RT-PCR Kit and protocol (Qiagen). All RT-PCR experiments were performed using the iCycler (Bio-Rad). The protocol utilizes the following thermal parameters: Reverse Transcription: 30 min at 50 °C. Activation step: 15 min at 95 °C. 3 Step Cycling: denaturation for 30 s at 95 °C, annealing for 2 min at 57 °C, extension for 2 min at 72 °C for 45 cycles. A melt curve was subsequently performed to analyze the products generated, which began at 50 °C and increased to 95 °C in 1 °C increments. Using a reverse transcriptase enzyme, mRNA was converted to the cDNA template of the specific marker. Then gene specific primers, a DNA polymerase, and a fluorescent moiety were utilized to amplify and label the amplicon generated. The gene product accumulation was then measured during the exponential phase of the amplification reaction.[11] The copy number from each of the samples was obtained by extrapolating to a standard gene curve of known concentration and copy number to yield quantitative data. The assay also includes the analysis of mRNA that does not change in relative abundance (18S) during the course of treatment to serve as an internal control.[12] The details concerning RNA harvest, standards development, and primer design are detailed elsewhere.[13]

RESULTS AND DISCUSSION

The Rutgers group has developed a library of tyrosine-derived polycarbonates for use in orthopedic, tissue engineering and drug delivery applications.[9,14-17] These materials share a structurally identical backbone with a rich chemical and structural diversity, from which the physical and chemical nature of the pendant ester substituent affects significantly the mechanical properties, degradation rates, and cellular responses.



Scheme 1 Synthetic scheme and chemical structure desaminotyrosyl–tyrosine alkyl esters and the resulting polycarbonates. The pendent R groups of the polycarbonates reported in this article consist of ethyl and octyl esters, respectively. The corresponding polymers are referred to as poly(DTE carbonate) and poly(DTO carbonate).

In this instance our goal is to define how the surface topology and surface energy of structurally related but immiscible blends affect biological response. Thin films of polymer mixtures macroscopically phase separate with domain sizes typically on the micron length scale and generally lead to changes in the film properties including topography, roughness, and surface energy. Recent studies have also shown that cells respond to topographic features on the nanometer and micron length scales.[18,19] What remains unclear is how the surface characteristics, including the extent of phase separation, within this series of tyrosine-derived polycarbonates and blend materials will influence the acute inflammatory response and extracellular matrix production. The extent of phase separation of binary polymer mixtures is dictated by a range of variables including temperature, film thickness, and the chemical nature of both the boundaries and individual polymers, respectively. The AFM images of the DTE and

DTO homopolymers in Figure 1 depict smooth and featureless surface morphologies. However in each of the blends, two distinct phases, which correspond predominately to DTE and DTO are seen. In the 70/30 DTE/DTO blend, a two-phase bicontinuous network was observed while the 50/50 and 30/70 blends exhibit isolated domains of various size. The darker phase features in Figure 1 decrease qualitatively with increasing DTO content. AFM micrographs indicated that 16 h at 105 °C was required to complete the annealing process.

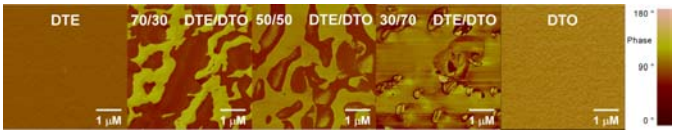


Figure 1. From left to right are five 5 μm × 5 μm AFM images in which the amount of DTO is increasing. The discrete DTE/DTO blends form phase-separated domains under *in vacuo*, 105 °C annealing conditions.

Two-dimensional thin films consisting of homopolymer and discrete compositional blends of tyrosine-derived polycarbonates were prepared and the surfaces were characterized in an effort to elucidate the nature of the corresponding cell responses *in vitro*. Genetic expression profiles of IL-1β and fibronectin (FN) in MC3T3-E1 osteoblasts were measured using real-time reverse transcriptase polymerase chain reaction (RT-PCR) and the results are depicted in Figure 2.

The osteoblasts demonstrated significant changes in IL-1β mRNA levels on the blends relative to the homopolymers, but the exhibited trends measured in these experiments were very different. The homopolymers DTE and DTO demonstrated ≈5.1×10^3-fold and 1.4×10^3-fold increases in cytokine induction in relationship to TCPS control levels. The gene copy numbers produced by each of the homopolymers, and the blend samples were expected to exhibit similar expression levels. The 50/50 and 30/70 (DTE/DTO) blends measured ≈2.7×10^4 fold and 1.0×10^4 fold increases over control levels. However, the 70/30 DTE/DTO blend only registered at 2.9 fold increase over TCPS at the 24 h time point. The underlying physico-chemical parameter causing the significant and reproducible decrease in IL-1β gene expression is unknown at this point and is currently being investigated further.

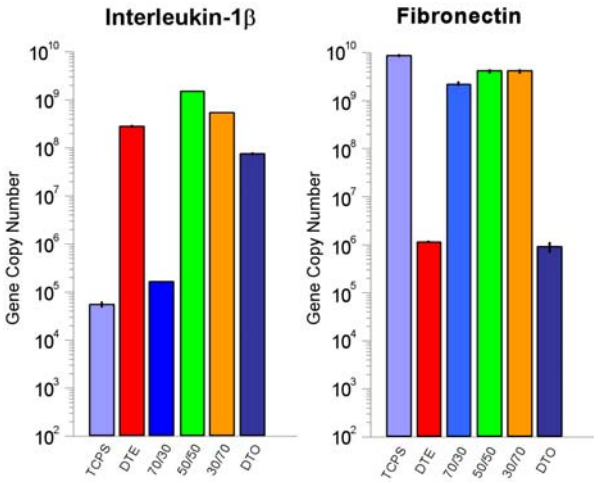


Figure 2 depicts gene copy numbers of interleukin-1β and fibronectin after 24 h of surface exposure on the respective homopolymers and blends for MC3T3 E1 bone osteoblasts. Error bars are representative of one standard deviation from the mean of triplicate samples harvested from a single population of cells, and are the estimate of the standard uncertainties.

The osteoblast mRNA expression levels of FN on the homopolymer samples were 4.8×10^3-fold and 9.6×10^3-fold lower than the TCPS population. However, the osteoblasts levels of FN on the blend samples were comparable to TCPS levels. These findings provided data that was complementary, meaning that the DTE/DTO polymer blends stimulated the up-regulation of FN relative to each of the homopolymers singly in both cell types.

CONCLUSIONS

The results of the discrete blend analyses provide the foundation for examination of 1-D and 2-D gradients of DTE and DTO to examine further the optimal composition and processing conditions that maximize extracellular matrix production and minimize IL-1β response. These studies are currently in progress. The preliminary results described in this paper suggest strongly that there are numerous opportunities for combinatorial approaches to polymeric formulations in biomaterial applications.

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Certain commercial materials and equipment are identified in this paper in order to specify adequately the experimental procedure. In no case does such identification imply recommendation by the National Institute of Standards and Technology nor does it imply that the

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