QUANTITATIVE RESPONSE MEASUREMENT OF CELL SUBSTRATE INTERACTIONS *VIA* RT-PCR

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Introduction

New synthetic methodologies have enabled a remarkable advance in the rational design of polymeric materials that actively control cellular and physiologic responses for use in tissue engineering applications. These methods, which afford precise control over molecular architecture, mass, and composition, produce well-defined materials that are being incorporated into scaffolds capable of supporting and regulating the adhesion, growth, and function of target cells while being minimally detrimental to normal cellular processes and surrounding tissues. Recently, NIST has developed numerous platforms for characterizing the physical properties of sample libraries with orthogonal gradients in thickness,[1,2] composition,[3] temperature,[4] morphology,[5] and processing conditions[6] using combinatorial methodologies.

High-throughput metrologies for the rapid and systematic evaluation of synthetic materials, which would elucidate a candidate's potential biocompatibility, are needed. A model system to evaluate the biocompatibility of materials *in vitro* using real-time polymerase chain reaction (RT-PCR)[7,8] has been developed within the Biomaterials group.[9] Inflammatory responses play a prominent role in the biocompatibility of materials, as indicated by the induction of the cytokines interleukin-1 beta (IL-1β) and tumor necrosis factor-alpha (TNF-α). These responses have been characterized for several materials used in biomedical applications.[9,10] These RT-PCR studies have quantitatively documented the inflammatory response and yielded great insight regarding the initiation and propagation of the genetic cytokine profile of immune cells. The further development of improved methods for the quantification of cellular responses to biomaterials at the genetic level is of great importance. The extracellular matrix (ECM) has recently received considerable attention due to its importance in cell-cell signaling, wound repair, cell adhesion and tissue function.[11] ECM is one of the environmental factors (along with hormones) that communicate with a cell nucleus, modifying nuclear structures and leading to selective gene expression. RT-PCR is being used to investigate the genetic expression profiles of ECM components such as fibronectin, collagen, and actin in response to tissue-engineered scaffolds, copolymer blends, and functionalized nanoparticles. The quantification of critical cellular responses to interactions with synthetic substrates by way of measurement of the genetic regulatory profiles for inflammatory and ECM markers by RT-PCR will be described.

Experimental

Materials. Unless otherwise listed, all solvents and reagents were purchased from Sigma (St. Louis, MO) and used as received. QuantiTect SYBR Green RT-PCR Kit, and Rneasy Kit were obtained from Qiagen (Valencia, CA). Primer identification, isolation, and probe development was nearly identical to methods described previously.[9,10]

Cell lines. RAW 264.7 were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI (Life Technologies, Rockville, MD) supplemented with heat inactivated FBS (10 %, (mass fraction) Life Technologies, Rockville, MD) in 5 % CO_2: 95 % air (volume fraction) at 37 °C.

mRNA extraction. Cells were plated in sterile 150 mm x 25 mm non-pyrogenic polystyrene dishes (Daigger, Vernon Hills, IL). Specific cultures were incubated on 25 mm glass coverslips coated with the respective polymer film. Alternatively, nanoparticles were added to plated cells 24 h following seeding. The mRNA extraction protocol was followed according to the manufacturer's specification, except a 21-gauge needle was used to homogenize the sample. The RNA was treated with RNA Secure immediately following elution and stored at –20 °C. Standard spectrophotometric measurements were taken and a 2 % (mass fraction) agarose gel stained with 10 μg/mL ethidium bromide (Sigma, St. Louis, MO) was used to image the RNA. Densitometry was performed using the Versa Doc imaging system (Bio-Rad, Hercules, CA).

Measurements.

RT-PCR. 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 35 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.

Flow Cytometry. Apoptotic analysis of RAW 264.7 cells incubated on the thin films or in the presence of the nanoparticles was assessed using the Guava Nexin Kit (Guava Technologies, Hayward, CA). RAW 264.7 cells were plated in 24 well plates (50,000 to 100,000) cells per well and allowed to adhere on tyrosine-derived polycarbonate thin films or tissue culture polystyrene for 24 h prior to analysis by flow cytommetry. Full experimental details were described previously.[10]

Results and Discussion

RT-PCR and flow cytometry have afforded the characterization of several key cellular processes, including inflammatory cytokine production and apoptosis progression of macrophages in response to polymeric materials. Prof. Joachim Kohn and coworkers, of Rutgers University have developed a series of tyrosine-derived polycarbonates for use in orthopedic, tissue engineering and drug delivery applications.[12-15] The materials have undergone extensive study, are FDA approved, and we hope to use them as a starting point for the evaluation of biocompatible material response tolerances. The characterization data for the respective polymers, which differ only by the length of the alkyl side chain group, are listed in **Table 1**. PCL is ε-polycaprolactone, and the E, B, H, and O nomenclature on the tyrosine-derived polycarbonate correspond to the ethyl, butyl, hexyl, and octyl side chain alkyl groups, respectively.

Table 1. Characterization Data for Tyrosine Derived Polycarbonates

Sample	M_w (*10^3)	T_g (°C)	Contact angle
PCL	80.0		
DTE	131	98	73
DTB	79.1	72	77
DTH	57.3	62	86
DTO	61.6	51	90

Figure 1 depicts flow cytometry data for tyrosine-derived polycarbonates thin polymer films under identical solution conditions. Although the percentages detected by flow cytometry as a whole remain largely the same, the increases in the number of cell in early apoptosis cannot be ignored and suggest that simple "live-dead" analyses of materials do not accurately predict material performance. Further analyses of the way the genetic inflammatory and ECM profiles are affected by these materials using

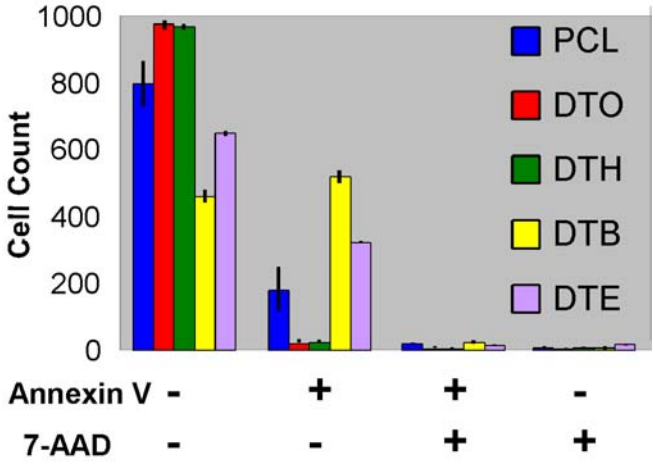

Figure 1. Four distinct populations of cells can be identified with flow cytometry: Viable cells: Annexin V (-) and 7-AAD (-), Early apoptotic cells: Annexin V (+) and 7-AAD (-), Late stage apoptotic: Annexin V (+) and 7-AAD (+), Necrotic: Annexin V (-) and 7-AAD (+).

RT-PCR are currently in progress. It is anticipated that thresholds established using FDA approved materials can used as a rapid and accurate biocompatibility screen to reduce the number of potential candidates, which are carried forward to further *in vitro* and animal testing.

Figure 2 contains data collected by RT-PCR, which measures gene copy numbers of the mRNA harvested from cells following exposure to a material. Below is the data acquired for the inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor- alpha (TNF-α) after 24 h of exposure to each of the tyrosine-derived polycarbonates, ε-polycaprolactone (PCL) and tissue culture polystyrene (TCPS).

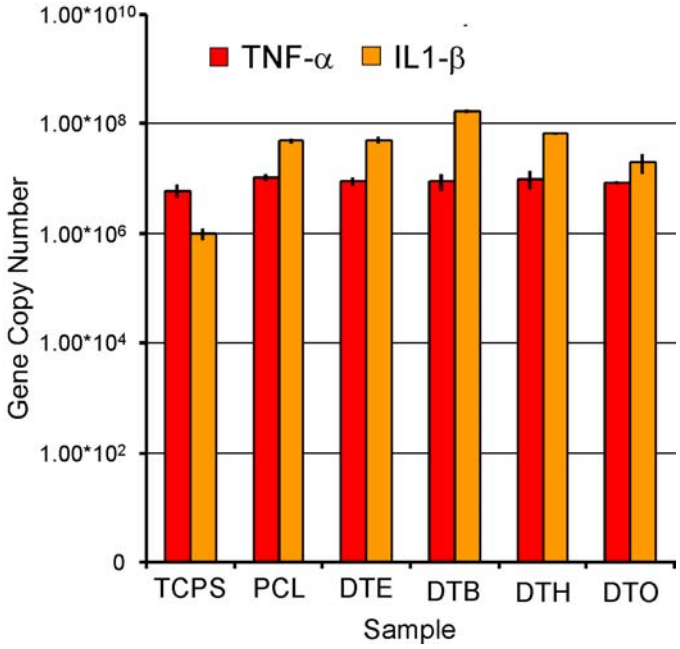

Figure 2. Gene copy numbers of interleukin-1β (IL-1β) and tumor necrosis factor- alpha (TNF-α) after 24 h of exposure. Error bars are representative of one standard deviation from the mean of triplicate samples harvested from a single population of Raw 264.7 cells, and is the estimate of the standard uncertainty.

The differences in TNF-α induction between the respective samples are negligible. Although small, statistically significant differences do exist between the induction properties for IL-1β between the respective samples and controls. While these increases would not prevent the use of these materials in vivo, we have demonstrated the ability to measure small response differences in biomaterials possessing very similar properties and chemical functionality. In addition, RT-PCR and its application are also relevant to other systems, including nanoparticles and peptide functionalized hydrogels, and research is currently underway in several areas.

Conclusions

The evaluation and identification of detrimental interactions between biological species and synthetic surfaces is a daunting challenge as the number of materials and control of physical variables increases. RT-PCR is a method for obtaining quantitative data that can provide valuable insight to the ways cells respone to the introduction of biomaterials and is an important tool that can be utilized when solving problems in tissue engineering.

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