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Impact of Polymer-bound Iodine on Fibronectin Adsorption and Osteoblast Cell Morphology Radiopaque Medical Polymers: Tyrosine-derived Polycarbonate Blends as a Model System

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Abstract

Imaging of polymer implants during surgical implantations is challenging in that most materials lack sufficient X-ray contrast. Synthetic derivatization with iodine serves to increase the scattering contrast but results in distinct physico-chemical properties in the material which influence subsequent protein adsorption and cell morphology behavior. Herein we report the impact of increasing iodine inclusion on the cell morphology (cell area and shape) of MC3T3-E1 osteoblasts on a series of homopolymers and discrete blend thin films of poly(desaminotyrosyl tyrosine ethyl ester carbonate), poly(DTE carbonate) and an iodinated analogue poly(I₂-DTE carbonate). Cell morphology is correlated to film chemical composition via measuring Fibronectin (FN) adhesion protein adsorption profile on these films. FN exhibits up to 2 fold greater adsorption affinity for poly(I₂-DTE carbonate) than (poly(DTE carbonate)). A correlation was established between cell area, roundness and the measured FN adsorption profile on the blend films up to 75 % by mass poly(I₂-DTE carbonate). Data suggest that incorporation of iodine within the polymer backbone has a distinct impact on the way FN proteins adsorb to the surface and within the studied blend systems; the effect is composition dependent.

Keywords

tyrosine-derived polycarbonates; biomaterials; MC3T3-E1; cellular morphology; imaging; Fibronectin; protein adsorption

Introduction

X-Ray based fluoroscopy is the preferred imaging modality for monitoring the surgical implantation of biomedical devices. However, most polymeric materials lack sufficient X-ray contrast to be seen under clinically relevant imaging conditions. A widely employed synthetic solution to increase the scattering cross section of a material has been to incorporate heavy atoms, such as iodine.¹ Variations of this strategy including physical

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Certain equipment and instruments or materials are identified in the paper to adequately specify the experimental details. Such identification does not imply recommendation by the National Institute of Standards and Technology, nor does it imply the materials are necessarily the best available for the purpose.

mixing and covalent linkages have been used successfully in poly(methyl methacrylate)², poly(D,L-lactic acid)³ and tyrosine-derived polycarbonates⁴ to increase the visibility of these materials in clinical X-ray imaging conditions.

Tyrosine-derived polycarbonates have found increased utility *in vitro* and *in vivo* in numerous biomedical applications including deployable cardiovascular stents, tissue engineering scaffolds for orthopedic applications, nanospheres for drug delivery, and thin film coatings where X-ray based imaging could be useful for monitoring implantation and integrity.⁵⁻¹¹ The recent FDA approval of a single component material for hernia mesh applications and the ongoing clinical trials of several other library members highlights their utility and potential.¹² In addition poly(desaminotyrosyl tyrosine ethyl ester carbonate), poly(DTE carbonate) has demonstrated great potential in several bone in growth studies.¹³⁻¹⁵ However, due to its amorphous nature and atomistic composition of H, C, N and O atoms, poly(DTE carbonate) possesses low x-ray contrast. To increase the x-ray scattering cross-section and clinical utility, a derivative poly(I₂-DTE carbonate) was developed by incorporating iodine atoms into the polymer's aromatic backbone.^{6,16-20} As the inclusion of a radiocontrast agent often influences the physicochemical properties of a material,⁵⁻⁸ we felt it necessary to determine how the incorporation of radiocontrast agent influences the protein adsorption profile of fibronectin, FN, a common mediator of cell adhesion and the resultant cellular morphologies on thin films of various compositions. Previously, we identified the optimal poly(I₂-DTE carbonate) composition for several imaging techniques using a 3D gradient scaffold approach and identified that 9 to 46 % of poly(I₂-DTE carbonate) incorporation was required for robust imaging-based quantification depending on the technique.^{21,22} Several factors are convoluted with iodine impact and may contribute to the cellular responses including material composition, film thickness for 2D film coatings, surface characteristics including roughness, morphology, surface energy, mechanical properties, and protein adsorption profile.²³⁻²⁵ In order to discern any morphological changes induced by poly(I₂-DTE carbonate) component within the blend and decouple it from other factors, all the aforementioned factors should be taken into account. Following that line, thin films of poly(DTE carbonate), poly(I₂-DTE carbonate) and several discrete blends were fabricated and characterized prior to cell culture studies which aimed to correlate cell morphological attributes to physical properties. In addition, we assess the intermediary role that the adsorbed protein layer modulates the cell-material interactions. The morphological data generated is discussed within the framework of a common adhesion protein, fibronectin (FN), affinity profile that was measured for each of the polymeric films.

In this contribution, we report and evaluate the impact of iodine incorporation in poly(DTE carbonate), its iodinated derivatives poly(I₂-DTE carbonate), and their blends on FN adsorption and MC3T3-E1 cell morphology. In addition, as the differences were expected to be subtle, we present an approach to thorough data treatment including collection, modeling, and analysis in order to discern the statistical differences between the respective polymer compositions.

Experimental

Materials

Solvents and reagents were purchased from Sigma (St. Louis, MO) and used as received unless otherwise stated. poly(desaminotyrosyl tyrosine ethyl ester carbonate) (poly(DTE carbonate)) (mass average molecular mass, $M_w = 105$ KDa, polydispersity, (PDI), $M_w/M_n = 1.76$, glass transition temperature T_g (Differential Scanning Calorimetry (DSC), bulk, 10 °C/min) = 100 °C) and poly(2,6 iodo-desaminotyrosyl tyrosine ethyl ester carbonate) (poly(I₂-DTE carbonate)) ($M_w = 294$ KDa, PDI = 1.86, T_g (DSC, bulk, 10 °C/min) = 145 °C), used in this contribution were reported previously.²⁶ 1,4-dioxane, acetone, chloroform, ethanol

and sodium dodecyl sulfate were purchased in HPLC grades and used as received. MC3T3-E1 cells were cultured in 75 cm² non-pyrogenic sterile polystyrene flasks (Corning, NY). Human plasma fibronectin, FN, (Gibco, Invitrogen cell culture, Carlsbad, CA) was obtained as (1 mg) dry lyophilized powder containing buffer and salt components. A FN stock solution was prepared (1mg/1mL) in deionized water (18 MΩ cm⁻¹). The FN concentration was measured using a Nanodrop spectrophotometer (ND-1000, Wilmington, DE) by measuring the absorption at 280 nm with extinction coefficient ($E_{1\text{cm},280\text{nm}}^{1\%}$) = 12.8. A FN solution (2.5 μg/mL) that mimics the FN concentration in cell culture media was prepared by freshly diluting the stock solution prior to every experiment with phosphate buffered saline solution (PBS, Dulbecco, 1×) containing Ca⁺² and Mg⁺² ions to match the conditions in the cell culture media.

Thin Film Preparation

Pre-cleaned glass slides (25×75 mm, 50×75 mm, Fisher Scientific, Pittsburgh, PA) were thoroughly rinsed with toluene, ethanol, and acetone and dried under a stream of N₂. Briefly two dimensional films (18×50 mm) of uniform thicknesses were flow coated using 2 % (by mass in 1,4-dioxane) solutions of the respective polymers as described previously.²⁷ Solutions of poly(DTE carbonate), poly(I₂-DTE carbonate) and their discrete composition of 25:75, 50:50, 75:25, 90:10 % (by mass) poly(I₂-DTE carbonate): poly(DTE carbonate) were the flow coating method. The method generates thin films of desired thickness by holding polymer solution between a glass slide blade and a substrate attached to a moving stage. The substrate is then moved with a constant velocity and acceleration to spread a thin layer of polymer solution on the substrate. Solvent evaporation leaves a thin polymer layer of homogenous thickness and smooth surface. Typical flow coating conditions include: velocity = 25 mm/sec, acceleration = 100 mm/sec², deceleration = 200 mm/sec², solution volume = 50 μl, and gap height of 200 μm. Films were annealed at 170 °C for 2 days. A parallel set of uniform thickness thin films were prepared on silicon substrate for x-ray photoelectron spectroscopy (XPS) film characterization using identical conditions. Thin film thicknesses based on at least six points were measured using a spot interferometer (Filmetrics F20, San Diego, CA) coupled with a computer-controlled x-y translation stage. Films possessed typical thicknesses of 100 ± 10 nm. All reported standard uncertainty in thickness is one standard deviation based on the pooled variance of at least 5 measurements.

Contact-angle measurements

Contact angle measurements on the thin films of uniform thickness of various compositions were conducted at 25 °C employing water and methylene iodide as probing fluid using a Krüss system (Krüss G2-MK4, Krüss GmbH, Germany). Operating on a 2 μL liquid droplet placed on the film's surface, contact angles were averaged over five independent measurements on each film. Measurements uncertainty was determined from the data and reported as one standard deviation. All reported standard uncertainty is one standard deviation based on the pooled variance of at least five measurements. Thin films' surface energy was calculated based on Owens, Wendt, Rabel and Kaelble method and employing contact angle from polar solvent (water and nonpolar solvent (methylene iodide)).

Atomic Force Microscopy (AFM)

AFM surface imaging of the annealed thin films was performed in tapping mode using a Nanoscope IV (Digital Instruments, Woodbury, NY) with phosphorus doped silicon tips. Root mean square (RMS) roughness data were collected from each sample using standard methods. Three 5×5 μm sized regions were scanned for each sample to calculate the RMS surface roughness and to investigate surface morphology. All reported standard uncertainty is one standard deviation based on the pooled variance of at least 5 measurements.

X-Ray Photoelectron Spectrometry

XPS measurements were performed using a Kratos Axis Ultra Spectrometer (Kratos Analytical, Manchester, UK) with a monochromatic Al K X-ray source (1486.6 eV) operating at 150 W under 1.0×10^{-9} Torr. Measurements were performed in hybrid mode using electrostatic and magnetic lenses and the pass energy of the analyzer was set at 160 eV, with an energy resolution 0.5 eV using an acquisition time of 440 s to obtain survey spectra. All XPS spectra were recorded using the Kratos VISION II software; data files were translated to VAMAS format and processed using CasaXPS software package (v. 2.3.12). Binding energies were calibrated with respect to C 1s at 285 eV. Elemental compositions were determined after subtraction of a universal Tougaard background and tabulated relative sensitivity factors derived from Scofield cross-sections. The fitting parameters were peak position, full width at half-maximum, intensity, and the Gaussian fraction. Spectra were fitted using a multiple region optimization of the I3d, C1s and N1s envelopes to maintain the correct stoichiometry for the poly(DTE carbonate) and poly(I₂-DTE carbonate) components. Average atomic compositions and standard deviations are from triplicate measurements.

Quartz Crystal Microbalance (QCM)

FN adsorption profiles were measured on thin films of poly(DTE carbonate), poly(I₂-DTE carbonate) and their 75:25, 50:50, 25:75, 10:90 % (by mass) blends using quartz crystal microbalance (Q-Sense D300, QCM-D) at 25 °C employing $2.5 \mu\text{g mL}^{-1}$ FN concentration. The progress of FN adsorption was monitored in real time by measuring the change in QCM frequency at 5.0 MHz base frequency and its overtones at 15, 25, and 35 MHz. The frequency change relative to the buffer base line, Δf , was taken as probe for the relative increase of FN adsorbed on the film surface. The QCM crystal was cleaned by immersion in dichloromethane solvent for 10 min followed by sonication in DCM for 1 minute, rinse with deionized water ($18 \text{ M}\Omega \text{ cm}^{-1}$), the crystal was further sonicated in a sodium dodecyl sulfate (SDS, 2 % in $18 \text{ M}\Omega \text{ cm}^{-1}$ water) solution for 1 minute and rinsed. Finally the crystal was treated for 10 minutes in UV-ozone system to remove organic contaminant on the surface and rinsed with water, acetone and water (3× each) and dried with a jet of N₂ dry gas. Thin films of different polymer compositions were spun coated on QCM crystal using 1 % (by mass) of the corresponding polymer composition (210 rad/sec, 60 sec). Consistent results were obtained by using the same QCM crystal as well as following the same cleaning procedure and thin film preparation techniques.

Cell Morphology

Low passage (10 and 14) mouse osteoblast cell line MC3T3-E1 cells (Riken Cell Bank, Hirosaka, Japan) were cultured in expansion medium containing α -minimum essential medium (MEM) plus 10 % (by volume) fetal bovine serum (FBS) and 60 $\mu\text{g/mL}$ kanamycin sulfate in all experiments. For examination of cell spreading and morphology, cells were seeded at low density (25 cells/ mm^2) on annealed uniform thin films: 6 compositions (100:0, 75:25, 50:50, 25:75, 10:90, 0:100 %; poly(DTE carbonate): poly(I₂-DTE carbonate) respectively by mass) in duplicate $25 \times 75 \text{ mm}$ specimen dimension. In addition, two clean glass cover slips and two tissue culture grade polystyrene (Nunc international, Naperville, IL) with $25 \times 75 \text{ mm}$ dimensions were tested for each time point as controls. Seeded cells were incubated on thin films and controls for 4 h at 37 °C, in a humidified 5 % CO₂ atmosphere. Cells were then fixed for 5 min (0.5 % by volume Triton X-100, 4 % by volume paraformaldehyde, 5 % by mass sucrose, 1 mmol/L CaCl₂, 2 mmol/L MgCl₂ in PBS, pH 7.4) then rinsed and post-fixed for 20 min (in the same fixative as before without Triton X-100). The cover slips were PBS-rinsed prior to staining with Alexa fluor 488 C₅ maleimide (1 ng/mL) and 4',6-diamidino-2-phenylindole (DAPI, 10 ng/mL) in PBS (1 h, in the dark). Following serial rinses in PBS and deionized water, the cover slips were air-dried and

mounted on glass slides with Vectashield containing DAPI. All reported standard uncertainty for the raw data in cell area, roundness and aspect ratio is one standard deviation based on the pooled variance of all cells measured (200 to 2200 cells). The standard uncertainty of cell data transformed after log-normal transformation is one standard deviation based on the pooled variance of all cell data.

Automated Fluorescence Microscopy

Cell number, cell area, and cell shape parameters were determined by automated fluorescence microscopy with a Leica DMR 1200 upright microscope equipped with a computer-controlled translation stage (Vashaw Scientific, Inc., Frederick, MD). Image Pro software (Media Cybernetics, Carlsbad, CA) controlled the stage and image acquisition modules. Images were collected on each film in a 40×5 grid. Two fluorescence images were captured at each grid position: (1) a green channel ($\lambda_{ex} = 494$ nm, $\lambda_{em} = 517$ nm) image for Alexa fluor 488-stained cell bodies and (2) a blue channel ($\lambda_{ex} = 345$ nm, $\lambda_{em} = 458$ nm) image for DAPI-stained cell nuclei. The green cell body images were used for quantifying cell area and the blue cell nuclei images were used to determine cell number. Two hundred images were collected on each of the 16 films/controls for a total of 3200 images. Each captured image had an area of 0.347 mm^2 ($10\times$ eyepiece, $10\times$ objective, $40\times$ magnification), and a total area of 69.4 mm^2 was imaged on each film. Image ProTM software (v. 4.5.1) was used to automate the image analysis. Cell morphology data collected from the automated fluorescence microscope include cell count, cell spread area, and shape parameters (roundness and aspect ratio) as a function of polymer chemical composition. Two films per composition were imaged, each of which was represented in a separate sub-distribution. Prior to analysis, all aggregated cells and objects with more than one cell nucleus were removed as they skew the morphometric parameters. We have incorporated both an exploratory data analysis method (EDA) that relies mostly on graphical plots and classical quantitative data analysis to quantitatively assess statistical differences. Classical quantitative analysis requires that data sets satisfy several requirements, including normalized data distributions, prior to analysis. As cellular data sets generally tail to the right, the data were log transformed into normally distributed data and modeled using a general linear model and tested using two-way analysis of variance (ANOVA) to assess the impact of different parameters on the cell response.

Data Analysis

The reported morphometric data descriptors and the associated uncertainty are based on the mean, in the form μ_i^*/σ_i , with the standard deviation, σ , of the anti-log of the transformed data and its standard deviation. Box-Whisker plots of the collected cell data were constructed for cell area, roundness, and aspect ratio and compared to transformed data μ_i^*/σ_i plots for both cell area and roundness data. Outliers were removed from cell data for Box-Whisker plots construction with exclusion of cell data with more than $12000 \text{ }\mu\text{m}^2$ area, 60 roundness ratio, or 10 aspect ratio. The transformation procedure and the data treatment were conducted by a customized in house developed package called *icell* that runs under Igor Pro (v 5.05) scientific data handling software (WaveMetrics Inc.). Box-Whisker plots were constructed using OriginProTM 7 software package.

Results

Thin Film Surface Morphology

In seeking to measure the impact of mixing different compositions of poly(DTE carbonate) and poly(I₂-DTE carbonate), Figure 1, on surface morphology, AFM was used, Figure 2, to reveal their phase-behavior as a function of poly(I₂-DTE carbonate) mass content. While dynamic mechanical analysis (DMA) reported previously²⁵ indicated that the respective

compositions are not entirely miscible, AFM indicated that the polymer blends are partially phase mixed at the observable length scales on the surface as no sizable distinct phase separated domains were observed. The films are relatively smooth with few defects and have an average RMS roughness of 0.3 ± 0.02 nm within the probed area ($5 \times 5 \mu\text{m}$) across all compositions.

Thin Films Surface Energy

In addition to morphology, surface energy is an additional property that is affected by roughness and chemical surface heterogeneity.²⁸⁻³⁰ The surface energy was calculated from contact angle measurements employing water and methylene iodide as probing fluids. Taking into consideration both the dispersive and the polar contributions of the probing solvents, the films free surface energy was calculated using Owens, Wendt, Rabel and Kaelble method.^{28,29} Figure 3 plots water advancing contact angles and the free surface energy as a function of thin film composition. The films have an average advancing water contact angle of 79.0 ± 1.7 degrees and an average surface energy of 44.7 ± 1.1 mN/m. The plot in Figure 3 suggests that both variables are insensitive to the incorporation of iodine in the poly(DTE carbonate) polymer backbone at the length scales of the measurements.³¹ The films have surface energies that are comparable to other polymers possessing amide, carbonate, and ester functional groups, examples include polyamide-6,6 (PA-66), polyethylacrylate (PEA), and Bis-phenol A polycarbonate (PC) with surface energies of 46.5, 37.0, 45.0 mN/m respectively.³²

Thin Film Surface Chemical Composition

XPS scans of the films surface were conducted to reveal the surface chemical composition (up to 10 nm surface depth) and discern if the two blend components have self assembled into an organized versus random pattern on the surface. Both homopolymers surface energies show little differences and the data suggests that iodine atoms have no preference to reside at the polymer air interface which presumably will boost the interface polarity and hence the contact angle. The measured surface composition found in the blend films matched the bulk “as-mixed” composition, Figure 4.

FN Adsorption Profile

Due to the key role FN plays in cell adhesion and its abundance in the media (FBS contains an average of $25 \mu\text{g mL}^{-1}$),³³ we decided to probe the relative affinity of FN adsorption on the thin film series by QCM. Although FN adsorption on thin polymer film surfaces is an evolving non reversible process that does not reach equilibrium, the relative magnitude of the frequency shift in QCM curves long adsorption time may be used as a probe for the relative FN affinity to adsorb. The real time frequency shift (7th overtone) of FN adsorption ($2.5 \mu\text{g mL}^{-1}$ in PBS buffer) as a function of film composition is shown in Figure 5.

Shown in Figure 5, FN has the highest affinity toward poly(I₂-DTE carbonate) and lowest affinity toward 25 % by mass containing poly(I₂-DTE carbonate) thin films. Annealing the poly(I₂-DTE carbonate) containing thin film sample (48 h at 170 °C under vacuum above its T_g) slightly increases the amount of adsorbed FN when compared to the as cast sample. The relative affinity of FN to the discrete blends did not trend directly with composition of poly(I₂-DTE carbonate) up to 75 % by mass. The FN affinity has increased in films with 90 % by mass poly(I₂-DTE carbonate). This data is qualitatively supported by a recent report that demonstrates that inclusion of iodine within the polymer backbone increases the protein adsorption on the material surface, an effect that contracts the effect of poly(ethylene glycol) on reducing protein adsorption.³⁴

Statistical Approach to Cell Morphology Parameters

Data collected from automated fluorescence microscopy include cell count, cell spread area, and shape parameters (roundness and aspect ratio) as a function of polymer chemical composition. The cell count characterizes the total number of cells adhered to a specific thin film and is an indication of relative cell adhesion to this surface. Cell count is reported as collected without additional statistical treatment. Cell spread area, and shape parameters on the other hand were collected for each cell on the film surface giving rise to histogram distributions of values in two sub-distributions for each of the two replicas. In order to satisfy the underlying assumptions and correctly apply a univariate model, $y_i = \mu + e_i$, that includes both a deterministic component (mean) and a probabilistic component (error), cell data should be normally distributed. If the data are not normally distributed, data transformation can be applied to render the data normal in distribution which can be statistically analyzed.

Transformation of Non-normal Distributed Data

The conformity to the required normality criteria is checked by EDA plots. The EDA plots are shown for cell spread area data of poly(DTE carbonate), Figure 6. Both the histogram and normal probability plots show the data with skewed distribution and curved nonlinear (non-normally distributed) behavior. The cell area and shape factor data for other polymer blends were also tested and exhibit the same non-normal behavior. The ability to process and analyze the collected cell data requires that the cell data conform to the normality criteria. Additionally, non-normal data does not qualify the use of the univariate model and the subsequent representation of the data using the mean and the standard deviation. One way to solve this dilemma is to transform non-normal data into normally distributed data. A plethora of transformations exist for treating non-normal data, and the trial and error process for finding the “best” treatment can become very tedious.

Alternatively, a method developed by Box and Cox in 1964 specifies a procedure for estimating the best transformation to normality within a family of power transformations.^{35,36} The transformation follows the formula in Equation 1.

$$y(\lambda) = \begin{cases} \frac{y^\lambda - 1}{\lambda} & \lambda \neq 0 \\ \ln y & \lambda = 0 \end{cases} \quad (1)$$

Where λ is a parameter that gives the best transformation scheme and it can take a range of values. The “best” transformation the cell data to normality is the λ value that maximizes a function called the log-likelihood function, Equation 2.

$$f(y, \lambda) = -\frac{n}{2} \ln \left[\sum_{i=1}^n \frac{(y_i(\lambda) - \bar{y}(\lambda))^2}{n} \right] + (\lambda - 1) \sum_{i=1}^n \ln(y_i) \quad (2)$$

Here we realize that an iterative procedure of computing the value of $f(y, \lambda)$ for a range of λ values has to be conducted followed by finding the value of λ value that corresponds to maximum of $f(y, \lambda)$. This was achieved by a customized computer program that we developed in house. The respective cell area data sets were tested to find the maximum λ value that transforms each of the data into normal distributions. The optimal λ value for each film composition was found to be different and located within the range [0.11, -0.44]. Per each composition, there are two sub-distributions and a λ value exists for each respective sub-distribution. It is important to use the same λ value for transformations when comparing data sets as the location (represented by the data mean) is remote from the

untransformed data mean and the transformed data give numerical outputs with no physical meaning. To solve this problem, we elected to use $\lambda = 0.0$ value and use it for all the data sub-distributions.

Figure 7 illustrates the combined sub-distribution data histograms for all thin compositions for raw and normally distributed data after transformation with $\lambda = 0.0$, in addition to glass and tissue culture polystyrene controls. The overlaid histograms for both sets of data show the power of data transformation and how the log-normal distribution can be used to statistically differentiate closely related data sets.

Discerning the somewhat subtle statistical differences between film compositions and their impact on cell spread area requires the use of quantitative analysis. We employed Levene test and Bartlett to check for homogeneity of variances followed by two-way multilevel ANOVA test and finally a pair wise comparison between films of different compositions. The combined cell spread area for the six film compositions are homogenous in variances as tested by both Levene and Bartlett tests at 95 % confidence ($p = 0.506$, $p = 0.649$ respectively). Comparing homogeneity of variances of the film data with the glass and polystyrene film controls indicate homogeneity of variances at 95 % confidence ($p=0.586$ and $p=0.663$, respectively). Homogenous variances are a prerequisite for the ANOVA analysis.

Cell Count and Area

Poly(DTE carbonate) possessed more adherent cells on its surface than poly(I²-DTE carbonate) followed by 50 % $\approx$ 25 % 2248, 75 % and lowest in case of 90 % poly(I₂-DTE carbonate) film for both the total count and the total single adhered cells. For the cell area, we conducted the ANOVA analysis on the transformed cell data to assess the impact that the film compositions have on the cell area. The impact was tested further using pair wise analysis employing Tukey HSD test.³⁷ Figure 8a shows the cell spread area as a function of film composition. The data is presented in the form of μ_t^*/σ_t rather than the form $\mu \pm \sigma$ as a result of data transformation treatment with the log-normal and the back transformation of the cell area into the normal form. The Tukey HSD pair wise test result is marked on the cell data graph and it gives insight about the statistical differences between every film compositions at 95 % confidence level ($P < 0.05$).

The behavior of cell spread area on the polymer films can be outlined as follows. All polymer films are statistically different from PS substrate. The poly(DTE carbonate) homopolymer film is the only data that is different from glass substrate. The 90 % poly(I₂-DTE carbonate) blend is the only blend that is different in cell area from poly(DTE carbonate) film. The 50 % poly(I₂-DTE carbonate) blend showed an increase in cell area compared to 25 % poly(I₂-DTE carbonate) blend. Another way to qualitatively assess the cell area data is through construction of Box-Whisker plot as shown Figure 8b. The plot illustrates the 1st to 99th percentiles Whisker bounded data range with 25th to 75th percentile Box bounded. Qualitatively, both the mean and median for cell area data, except the controls, follow the same trend as the mean of the transformed data plot (Figure 8a). The data do not suffer from large spread among film composition as well as the controls with data range between 1st to 99th percentiles are found to be widely spread for 50% and poly(DTE carbonate) samples and narrowly spread for 25 % sample.

Cell Shape

The cell shape is characterized by both cell roundness and aspect ratio parameters. The roundness is defined as the ratio of cell square perimeter to 4π times the cell area for the cell 3D shape projected as 2D object (a sphere would have value of 1 while straight line would

have value of ∞). The aspect ratio is defined as the ratio of the cell longest axis length to the length of its shortest axis as its shape is approximated to that of an ellipse. For both the roundness and the aspect ratio data, the data distribution looks skewed to higher value more than their cell area counterpart, Figures 9a and 10. The aspect ratio data, per all film compositions, tends to be skewed to higher values than roundness data (50 % of the data sets have roundness: aspect skew ratio of 1:1.1 to 1:1.2). Treating cell roundness data the same way as the cell area data was appropriate to yield normalized data sets after transformation. Although the transformed data do not show homogeneity in their variances under Levene's test, conducting oneway multilevel ANOVA analysis would give qualitative assessment of the differences within the data. The ANOVA test indicated that the cell roundness data contain significant differences at 95 % confidence level. Pair wise comparison within the data with Tukey HSD test revealed that within the film compositions there is significant differences between the blends containing 25 % by mass and both 50 % and 75 % by mass poly(I₂-DTE carbonate). Comparing the film blends data sets to the controls indicate statistical differences with the PS film but no differences with glass substrate. Cell roundness data are shown in Figure 9a. The Box and Whisker plot for the cell non-normal roundness data is constructed and the data trend is compared to the transformed data as shown in Figure 9b. The plot illustrates that the data mean, but not the median, follow a very close trend as that of the transformed data except for the glass control, which has a higher value for cell roundness.

For the cell aspect ratio data the, treating the data with log-normal transformation was not appropriate since the data belongs to a skewed distribution. Several other transformations recommended for highly skewed data such as transforming y_i to $1/y_i$, y_i^2 , or $1/y_i^2$ were applied to the cell aspect ratio without generating appropriate normalized distributions.³⁶ For highly skewed data such as cell aspect ratio, we use the Box-Whisker plot to report and compare aspect ratio data for all film compositions including data for the controls.

Figure 10 illustrates the Box-Whisker plot for the cell aspect ratio. Qualitatively and based on the mean and the median, cells have highest aspect ratio on the thin film blend with 25 % by mass poly(I₂-DTE carbonate) with greatest data spread and smallest aspect ratio on thin film blend with 75 % by mass poly(I₂-DTE carbonate) with very narrow data spread

Discussion

The impact of poly(DTE carbonate), poly(I₂-DTE carbonate), and several discrete thin film blends on osteoblast cell morphology can be discussed in terms of pertinent parameters including surface morphology, chemical composition, surface energy, film stiffness, and the nature of surface adsorbed protein layer that is formed upon interaction of cell culture media with film surface and contributed by the cell to the extracellular matrix. As illustrated by AFM images, the film surface morphologies are relatively smooth without the distinct phase-separated domains that have been shown previously to affect cell morphology and gene expression.⁸ While the mechanical properties (stiffness) of the substrates have been shown to play an important role on directing cell phenotype,³⁸ the elastic modulus of poly(DTE carbonate), poly(I₂-DTE carbonate) and their blends were previously characterized by wrinkling based metrology (1.0 to 2.0 GPa) and this moduli range imparts glassy solid behavior and exists outside the moduli range (1 to 100 kPa) that was reported to impact cell morphological and phenotypical behavior.^{38,39} Composition remains the only variable that changes in which the amount of poly(I₂-DTE carbonate) polymer was progressively increased in the blend series. As XPS data indicated, the films surface compositions (≈ 10 nm depth) agree with the starting solution bulk composition and indicate proportional increases in iodine content. While the films are hydrophobic with consistent surface energy, there is a distinct difference in the relative FN adsorption profiles of the

homopolymers (up to 2 fold) and their blends that must be occurring on a length scale smaller than the drop used to assess the fluid contact angles. The increasing amounts of poly(I₂-DTE carbonate) polymer within the blend is likely to interact with the cells indirectly by changing the nature of this adsorbed adhesion proteins layer. Multiple changes in protein adsorption can occur by altering protein conformation and orientation of specific protein chain modules on the polymer substrate. These changes would be reflected on the cell adhesion and spreading due to cell interactions with these proteins through their surface receptors, such as the integrin receptor RGD sequence.⁴⁰⁻⁴² The adsorption of different serum proteins and subsequent cell adhesion studies have shown that the packing density and strength of adsorption strongly influence the conformation and epitopes available for integrin binding.^{38,39} While cells were cultured in a medium containing 10 % (by volume) FBS, the serum contains a plethora of protein, including FN, as well as other nutrients essential for cell survival. FN was considered as a representative adhesion protein probe. Studying FN adsorption profiles of various concentrations is a particularly important first step to correlate polymer composition and cell morphology. The adsorption profile was found to correlate with the cell data up to 75 % by mass poly(I₂-DTE carbonate) containing films. High FN adsorption affinity was observed in conjunction with large cell spread area while low FN adsorption affinity was observed with less cell area. Subsequently, FN affinity also correlates with cell roundness. Polymer films in which cells with more spread area are generally less round. This is illustrated by the 50 % by mass poly(I₂-DTE carbonate) blend with highest cell area and lowest cell roundness, Figure 11. Beyond the 75 % by mass poly(I₂-DTE carbonate) threshold, higher FN adsorption affinity did not correlate to higher cell spread area or roundness. Compared to controls, the glass substrate shows similar cell response as thin films while the PS substrate shows both higher cell spread area and low cell roundness. The PS surface is hydrophilic due to the negative charges induced by plasma modification that is designed to promote cell attachment and spread to maximize the cell culture yield.

Conclusions

In this work, the impact of iodinated surfaces on osteoblast cell morphological response was investigated using 2D thin films of poly(desaminotyrosyl tyrosine ethyl ester carbonate), poly(DTE carbonate), its iodinated form, poly(I₂-DTE carbonate) and several discrete blends. While the surface energy was minimally different, poly(I₂-DTE carbonate) exhibited an approximately 2 fold increase in FN adsorption relative to poly(DTE carbonate) the morphology of the MC3T3-E1 cells on each of the homopolymers were not statistically different. This is clearly indicative of a packing and/or conformational difference of the FN that occurs during the adsorption process and related to the molecular effects of iodine present in the poly(I₂-DTE carbonate). We also found a correlation between cell area, roundness and the FN adsorption profile on the blended films up to 75 % by mass poly(I₂-DTE carbonate). In addition, cells on the 50 % by mass poly(I₂-DTE carbonate) films possessed larger areas and less roundness and films on 25 % by mass poly(I₂-DTE carbonate) yielded lower area and roundness. While subtle, these differences could not be accounted for previously in the absence of a rigorous automated approach combined with statistical data treatment. These results show that the poly(I₂-DTE carbonate) can be incorporated into biomaterials applications and while FN adsorption profiles may be composition dependent, the resulting effects on cell morphology are relatively small indicating a variable accessibility in the bioavailability of cells binding receptors.

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Poly(DTE carbonate)

poly(I₂-DTE carbonate)**Figure 1.**

Chemical Structure of poly(DTE carbonate) and poly(I₂-DTE carbonate).

Figure 2.

AFM images of poly(DTE carbonate) **A**, poly(I₂-DTE carbonate) **B** and several discrete blends as a function of increasing poly(I₂-DTE carbonate) mass % content. The images demonstrate the smooth nature and lack of macrophase separation within the blends at the surface. Image size: 5×5 μm.

Figure 3. Advancing water contact angle measurements and calculated free surface energy based on Owens, Wendt, Rabel and Kaelble method as a function of thin film chemical compositions.

Sample	Expected % Poly(I ₂ -DTE carbonate)	Actual Surface % Poly(I ₂ -DTE carbonate)
Poly(DTE carbonate)	0.0	0.0
75:25	25.0	23.6 ± 3.4
50:50	50.0	48.3 ± 4.1
25:75	75.0	74.0 ± 1.3
Poly(I₂-DTE carbonate)	100.0	100.0

Figure 4.

Normalized XPS Iodine peaks for poly(DTE carbonate), poly(I₂-DTE carbonate) and their blend. Inset: N1s peak used to normalize all other signals. The table indicates that the amount of poly(I₂-DTE carbonate) detected at the surface via XPS for each of the respective formulations match the expected values.

Figure 5.

Real-time QCM data of normalized frequency shift (7th overtone) for FN adsorption at 2.5 $\mu\text{g mL}^{-1}$ concentration in PBS buffer on poly(DTE carbonate), poly(I₂-DTE carbonate) and each of the discrete blends. Inset: Frequency shift taken as the average of Δf between 15 and 35 min as a function of thin film composition, data uncertainty represents $\pm$ standard deviation.

Figure 6.

The EDA plots used to examine the cell spread area conformity to the univariate, $y_i = \mu + e_i$ model. (a) Data histogram, (b) Normal probability plot.

Figure 7.

Histograms of raw and log-transformed cell spread area data of poly(DTE carbonate) and poly(I₂-DTE carbonate) and each of their respective blends in addition to glass and polystyrene film controls. Left column graphs: raw data, right column graphs: the same data after transformation overlapped with Gaussian red curve fit.

Figure 8.

(a) Cell spread area of MC3T3-E1 cells cultured on different film composition as a function of increasing poly(I₂-DTE carbonate) mass % content as well as glass and polystyrene controls. Data represent μ_t^*/σ_t Symbol $\times$ marks statistical differences vs. PS substrate, 99 % confidence ($p < 0.05$). At 95 % confidence ($p < 0.05$), $\dagger$ marks statistical differences vs. 50 % poly(I₂-DTE carbonate), ++ marks statistical differences vs. poly(DTE carbonate), and $\S$ marks statistical differences vs. glass substrate. (b) Box-Whisker plot of non-normal cell area data. Diamond symbols represent data outliers outside 1st to 99th data percentiles.

Figure 9.

(a) Roundness of MC3T3-E1 cells cultured on different film composition as a function of increasing poly(I₂-DTE carbonate) content as well as glass and polystyrene controls. Data values represent μ_t^*/σ_t . Symbols + marks statistical differences vs 25 %, $p < 0.05$ while * mark marks statistical differences vs PS, $p < 0.05$. (b) Box-Whisker plot for cell roundness. Diamond symbols represent data outliers outside 1st to 99th data percentile.

Figure 10.

Box-Whisker plot for non-normal cell aspect ratio data as a function of film composition including glass and polystyrene controls. Diamond symbols represent data outliers outside 1st to 99th data percentile.

Figure 11.

Optical fluorescence images of MC3T3-E1 nuclei (blue) and cell bodies (green) on homopolymers of poly(DTE carbonate), poly(I₂-DTE carbonate) and several discrete blends showing very subtle differences in cell morphology found among the various blends and further highlighting the value of an objective, automated approach to discerning differences among closely related materials. Image size 200×200 μm