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Osteogenic Differentiation of Human Mesenchymal Stem Cells on Poly(ethylene glycol)-Variant Biomaterials

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Abstract

This study evaluated the osteogenic differentiation of human mesenchymal stem cells (MSCs) on tyrosine derived polycarbonates copolymerized with polyethylene glycol (PEG) to determine their potential as a scaffold for bone tissue engineering applications. The addition of PEG in the backbone of polycarbonates has been shown to alter mechanical properties, degradation rates, degree of protein adsorption, and subsequent, cell adhesion and motility in mature cell phenotypes. Its effect on MSC behavior is unknown. MSC morphology, motility, proliferation and osteogenic differentiation were evaluated on polycarbonates containing 0-5% PEG over a 14 day culture. MSCs on polycarbonates containing 0% or 3% PEG content upregulated the expression of osteogenic markers as demonstrated by alkaline phosphatase activity and osteocalcin expression although at different stages in the 14 day culture. Cells on polycarbonates containing no PEG were characterized as having early onset of cell spreading and osteogenic differentiation. Cells on 3% PEG surfaces were delayed in cell spreading and osteogenic differentiation, but had the highest motility as compared to cells on substrates containing no PEG and substrates containing 5% PEG at early time points. Throughout the culture, cells on polycarbonates containing 5% PEG had the lowest levels of osteogenic markers, displayed poor cell-substrate adhesion, and established cell-cell aggregates. Thus, designing substrates with minute variations in PEG may serve as a tool to guide MSC adhesion and motility accompanying osteogenic differentiation and may be beneficial for abundant bone tissue formation *in vivo*.

Introduction

Tissue engineered bone grafts are an alternative to autologous and allogeneic bone grafts in the treatment of critically sized bone defects caused by disease or trauma. Tissue engineered bone grafts are designed to promote osteoinduction, the process in which immature progenitor cells such as mesenchymal stem cells (MSCs) differentiate and produce bone tissue within a porous scaffold.¹⁻⁸ MSCs harvested from adult bone marrow are an ideal cell source for bone tissue engineering because of their potential to differentiate into osteoblasts, ease of *in vitro* maintenance and expansion, and the lack of antigenicity when used *in vivo* as an allogeneic cell source.¹⁷⁻²³

Bioresorbable polymers that act as scaffolds for bone tissue engineering should be designed to satisfy the necessary material properties to support, if not promote, MSC growth and differentiation, as well as achieve the required mechanical integrity and porous structure needed for bone growth and integration. These scaffolds need to provide the necessary structural support for laying down bone tissue, all while gradually resorbing to be fully

replaced by bone tissue. Tyrosine derived polycarbonates, an alternative to poly(α -esters) developed by Ertel and Kohn, are a library of structurally related bioresorbable polymers.⁷ Polymers of the poly(α -ester) family have been used in orthopedic applications for many years. However upon degradation, poly(α -esters) break down into acidic by-products which can be cytotoxic to surrounding cells. Since the monomer repeat unit of tyrosine derived polycarbonates is derived from a natural amino acid, L-tyrosine, there is minimal cytotoxicity to surrounding cells upon degradation.⁷ Furthermore, the bulk degradation of poly(α -esters) leads to micron-sized crystalline particles that are susceptible to phagocytosis by macrophages, enhancing the formation of a collagenous encapsulation.²⁹ Tyrosine derived polycarbonates are relatively amorphous, eliminating the concern of degraded crystalline particles.

The chemical structure of tyrosine derived polycarbonate is comprised of desaminotyrosyl-tyrosine alkyl ester (DTR), where R represents the modifiable alkyl ester pendent chain (Figure 1). For instance, poly(DTE carbonate) is desaminotyrosyl-tyrosine ethyl ester. By incorporating polyethylene glycol (PEG), a hydrophilic bioinert polymer, into the backbone of the poly(DTE carbonate) in step-wise increments, a new library of polycarbonates was developed in which the mechanical properties, degradation rates, degree of protein adsorption, and subsequent cell response could be tailored according to its potential application. These copolymers are represented by the standard nomenclature: poly(DTE-co- $x\%$ PEG_{MW} carbonate) where ($x\%$) is the mole percent fraction of PEG in the polymer backbone, and (MW) is the average molecular weight of PEG (Figure 2).

In terms of bulk mechanical properties, the addition of PEG into the backbone of the polycarbonates decreases the tensile strength and stiffness.³⁴ Furthermore, the addition of PEG increases the surface wettability, which has an adverse effect on the adsorption of proteins and its subsequent bioactivity and conformation. This leads to significant effects in cell adhesion and motility which can be modified through the addition of PEG in the backbone structure of polycarbonates at various molar concentrations. In a study completed by Tziampazis *et al.*, where human fibronectin was adsorbed onto PEG polycarbonates ranging from 0-10% molar fraction, the amount of fibronectin adsorbed and its bioactivity decreased at PEG molar fractions greater than 6%.³¹ Cell adhesion was also negatively affected at concentrations above 6%. Cell motility is an important parameter to study organogenesis, tissue remodeling, and pathologies. The incorporation of PEG in the backbone of polycarbonates had a pronounced effect on cell motility. It was found that PEG molar concentration had a biphasic effect on cell speed, the cell speed of L929 cells increased with increasing PEG molar concentrations up to an intermediate PEG concentration (3-4%), yet at higher PEG concentrations (>8%), motility decreased.²⁷

Due to its biocompatibility, mechanical strength, slow degradation as well as its ability for bone apposition, tyrosine derived polycarbonates have been successful as orthopedic implants in *in vivo* studies.^{12, 13} The goal of this study was to evaluate the potential of tyrosine derived polycarbonates copolymerized with polyethylene glycol as a scaffold for bone tissue engineering applications. The addition of PEG into the tyrosine derived polycarbonate backbone alters surface properties that may have a beneficial effect on MSC behavior. In this *in vitro* study, human MSCs in the presence of medium containing osteogenic factors were seeded on thin films of poly(DTE-co-3%PEG₁₀₀₀ carbonate), poly(DTE-co-5%PEG₁₀₀₀ carbonate) and compared to poly(DTE carbonate) to determine whether small changes in the PEG concentration influences cell adhesion and motility, and its effects on osteogenic differentiation. Cell morphology, cytoskeletal organization, proliferation, adhesion, and motility as well as the expression of osteogenic markers were characterized.

Materials and Methods

Polymer Substrate Preparation

The tyrosine derived polycarbonate PEG copolymers will be further referred to as: p(DTE-co-x%PEGc). P(DTE-co-3%PEGc) and p(DTE-co-5%PEGc) contain 3 or 5% polyethylene glycol (PEG), respectively, in which the molecular weight of PEG is 1000 g/mole. Polymers were synthesized and characterized according to methods previously published.^{21,34} Polymer films for cell culture and associated characterization were prepared by either solvent casting or spin-coating methods.³ For the solvent casting method, a 1% (w/v) polymer solution, dissolved in methylene chloride and filtered using a 0.45 μ m PTFE filter (Whatman, Floram Park, NJ), was cast into the wells of a 96-well polypropylene plate. Plates were allowed to air dry in a chemical hood for at least two days prior to cell culture use.

Spin-cast films on glass coverslips were prepared following a protocol developed by Brocchini.³ Briefly, a 2.5% (w/v) polymer solution was spin-coated using a photo-resist spinner (Headway, Garland, TX) at 2000 rpm for 20 seconds onto 15 mm glass coverslips (Fisher) previously coated with polystyrene silane. Spin-cast coverslips were fixed onto 24 well tissue culture polystyrene plates using methylene chloride. Air-water contact angle measurements were verified against previously reported data by Brocchini *et al.*³

To ensure the cells did not respond differently to the two processing methods, trends in proliferation and differentiation were evaluated and determined to be similar on films prepared by both methods.

Level of Protein Adsorption on Polymeric Surfaces by QCM-D

The adsorption of fetal bovine serum (FBS) (Calbiochem, San Diego, CA) on polymer surfaces was measured in real-time using the Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) technique described by Rodahl *et al.*²² Briefly, a quartz crystal is driven by applying an alternating electric field. Protein adsorption or desorption is measured by following changes in frequency (Δf) and dissipation (ΔD) of the quartz crystal at the fundamental frequency of 5 MHz and at one of its first six successive overtones: 15, 25, 35, 45, 55 and 65 MHz.

Thin films of polycarbonates (n=3) were prepared on gold-coated sensor crystals (5 MHz, QSX 301; Q-Sense AB, Goteborg, Sweden) by spin-coating. Using a 1% (w/v) polymer solution in methylene chloride, filtered using a 0.45 μ m PTFE syringe filter (Whatman), quartz sensors were coated at 4000 rpm for 30 seconds using a photo-resist spinner (Headway). Protein adsorption experiments were carried out at 37°C using a flow rate of 20 μ L/min. The adsorption of a 10% (w/v) solution of FBS in Dulbecco's Phosphate Buffered Saline (PBS; Sigma, St Louis, MO) was monitored in real-time until saturation. PBS was then allowed to flow removing any reversibly adsorbed protein.

Calculation of the adsorbed mass and viscoelastic properties of the protein layers were obtained applying a Voigt model using Q-Tools (Q-Sense AB, Goeteborg, Sweden).³² Assuming a homogenous layer of adsorbed protein on the polymer surface, values for the shear viscosity (η), shear elastic modulus (μ) and the layer thickness (δ) obtained by fitting the measured (Δf) and (ΔD) data for the 9th and 11th overtones.

Cell Culture

Human mesenchymal stem cells (MSCs) were isolated from commercially obtained bone marrow aspirates (Cambrex Bio Science, Walkersville, MD) collected from the superior

iliac crest of the pelvis of male donors of ages ranging from 18-36. The isolation method has been described in detail by Haynesworth.¹⁰ MSCs were plated into tissue culture polystyrene flasks with control medium: DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Hyclone, Logan, UT) and 1% antibiotic-antimycotic (Invitrogen), at 37°C, 5% CO₂ in a humidified incubator. At near confluency, in approximately 12-16 days, MSCs were detached from the substrate with 0.25% Trypsin-EDTA and resuspended in control medium. The cells were subcultured up to a maximum of two passages and then cryopreserved until use.

On day 0 of the experiment, polymer films were sterilized with UV prior to cell seeding. Cryopreserved MSCs were then thawed and seeded at $\sim 3 \times 10^4$ cells/cm² into 96 and 24 well plates. MSCs were maintained in control medium overnight. 24 hours after initial seeding, designated cells were induced with osteogenic medium (OS medium) which is control medium supplemented with: 10 mM beta glycerophosphate (Sigma), 50 μM L-ascorbic acid phosphate (Wako, Richmond, VA) and 100 nM of dexamethasone (Sigma), while the remaining cells were maintained in control medium. Cell culture medium was changed every 2-3 days. On day 12, MSCs maintained in OS medium, MSC/OS cells, were cultured with OS medium supplemented with 10 nM Vitamin D₃ to induce osteocalcin production. The cells were harvested for biochemical assays at days: 4, 7, 11 and 14. The proliferation and differentiation studies were performed on both MSCs and MSC/OS cells. Since MSCs did not undergo osteogenic differentiation in control medium based on the markers examined in this study, this data was not reported.

Cell Morphology and Cytoskeleton Arrangement

Cell morphology and cytoskeleton arrangement were observed at various points of the 14 day culture. Cells were observed using a phase contrast microscope (Nikon Eclipse TS 100, Melville, NY) at 10X, 20X and 40X magnifications. Images were taken with a digital camera (Nikon DXM 1200F) and were visualized with an image analysis software (Metavue, Downingtown, PA). Cytoskeletal organization was observed by fluorescently staining the f-actin filaments with Alexa Fluor® 488 phalloidin (Invitrogen). Fluorescent images were obtained using the 450 nm-490 nm excitation/505 nm emission filter using the epi-fluorescence attachment.

Cell Proliferation

Cell proliferation was determined with the PicoGreen® ds DNA quantitation kit (Invitrogen), in which DNA content is correlated to fluorescence intensity. MSCs of known cell number served as standards. Standards and samples (n=4 per group per time-point) were prepared by lysing cells with 0.1% Triton X-100. Fluorescence was detected with a microplate reader (FLX800, Biotek, Winooski, Vermont) at 480 nm excitation/520 nm emission.

Alkaline Phosphatase and Osteocalcin Production

The activity of alkaline phosphatase (AP) was determined by quantifying the conversion of para-nitrophenyl phosphate (p-Npp) (Sigma) to para-nitrophenol (p-Np). Samples (n=4 per group per time point) were prepared by lysing cells with 0.1% Triton X-100 and incubating them at 37°C for 30 minutes. The absorbance was read at 405 nm with a microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The AP activity was normalized to cell number determined from the cell proliferation data expressed as nmol of p-Np/min/cell.

Osteocalcin production was determined using harvested samples obtained at day 14. Samples (n=4) were prepared by lysing cells with 0.1% Triton X-100 and osteocalcin expression was measured using an ELISA assay (MetraBiosystems, MA).

Cell Motility

The motility of MSCs was observed and measured using confocal microscopy. On day 0 of the experiment, cryopreserved MSCs were seeded onto the spin-cast disks of p(DTE carbonate), p(DTE-co-3%PEGc) and p(DTE-co-5%PEGc) at a concentration of 3000 cells/cm². Cells were harvested at time-points: days 0, 4, 7, and 11. Data was not collected at day 14 because individual cells were difficult to discern due to cell aggregation, confluency or the extensive extracellular matrix. Cells were stained with Cell Tracker (Invitrogen) in control medium. The Cell Tracker solution was replaced with CO₂ independent medium (Invitrogen) and the cells were placed on a 37°C incubated microscope stage on a Leica TCS SP2 system (Leica Microsystems, Exton, PA) with an HC Plan APO CS 10x/0.4 air lens. 24 hours after initial cell seeding, cells on the remaining disks were induced with OS medium and placed in a 37° C, 5% CO₂ incubator until the date of harvest. On the date of harvest, the medium in the chamber was replaced with the Cell Tracker solution for 30 minutes at 37°C.

Ten sample areas which had approximately 20 cells per area, were taken of each film. Images were taken at 10 minute intervals over 18 hours. Only cells that translocated at least 1 pixel per time interval were tracked. Cell tracks of the cell centroids were determined utilizing Image Pro© software (Media Cybernetics, Silver Spring, MD). A Matlab© (The Math Works Inc., Natick, MA) script was written to compute individual mean-squared cell displacements and average velocities from the individual cell tracks. The mean squared displacement for the entire time interval is given by

$$\langle d^2(t=n\Delta t) \rangle = \frac{1}{(N-n+1)} \sum_{i=0}^{N-n} \left[[x((n+i)\Delta t) - x(i\Delta t)]^2 + [y((n+i)\Delta t) - y(i\Delta t)]^2 \right]. \quad (1)$$

The persistence time, cell speed, and Random Motility Coefficient (RMC) were calculated from the displacements by regression of the equation below, where μ represents the RMC, S represents the cell speed and P represents the persistence time. $16 \cdot 20 \cdot 30$

$$\langle d^2(t) \rangle = 2S^2P \left[t - P \left(1 - e^{-\frac{t}{P}} \right) \right] \quad (2)$$

$$\mu = \lim_{n_D \rightarrow \infty} \frac{\langle d^2(t) \rangle}{2n_D t} = \frac{1}{n_D} S^2 P \quad (3)$$

In equation (3), n_D is the number of dimensions over which the displacement is defined ($n_D = 2$ for 2D substrates).

Statistical Analysis

Multiple samples were collected for each measurement and results were expressed as mean $\pm$ standard deviation. Two-way ANOVA was performed on the entire data set. One-way ANOVA was performed to test statistical differences between groups and over time. Multiple comparisons were evaluated using the Tukey-Kramer test. $p < 0.05$ was considered significant in all statistical tests.

Results

Protein Adsorption on Polymeric Substrates

The QCM-D technique provides information on the adsorbed 'wet' mass of the adsorbed protein layer. In accordance with previous studies,³³ the adsorption of protein layers gives rise to the formation of a thin dissipative layer. In contrast to non-dissipative layers that can be modeled using the Sauerbrey relationship,²⁶ dissipative layers require a model that accounts for their viscoelastic nature. In order to obtain an accurate interpretation adsorbed layer, the Voigt model can be applied to the raw Δf and ΔD data. Values of the adsorbed mass for FBS adsorption using the Voigt model are reported in Figure 3 for p(DTE carbonate), p(DTE-co-3%PEGc) and p(DTE-co-5%PEGc). The incorporation of 5% PEG resulted in a significant decrease in the adsorbed mass compared to p(DTE carbonate) ($p < 0.05$).

Morphology of MSCs

Figure 4 reports the phase contrast images of MSC/OS cells at various time points of a 14 day culture. Cells on p(DTE carbonate) at 5 hours exhibited a fibroblastic morphology. However at 5 hours and through 24-48 hours, cells on p(DTE-co-3%PEGc) and p(DTE-co-5%PEGc) did not spread onto their respective surfaces, exhibited a rounded morphology and formed cell aggregates (Figures 4e, 4f and 4i). By day 7, MSC/OS cells on p(DTE-co-3%PEGc) displayed a cuboidal morphology, similar to cells on p(DTE carbonate) (Figure 4g). Darkened areas and cells on p(DTE-co-3%PEGc) and p(DTE-co-5%PEGc) may be attributed to the formation of a mineralized matrix (Figures 4h, 4k and 4l). By days 7 and 14, cells on p(DTE-co-5%PEGc) formed large aggregates but also some cells exhibited a spread morphology (Figures 4k and 4l).

MSC Cytoskeleton Organization

At 5 hours, there was a remarkable difference in the cytoskeleton organization of the cells on p(DTE carbonate) compared to the PEG containing polycarbonates. As early as 5 hours, MSC/OS cells exhibited prominent actin filaments on the p(DTE carbonate) (Figure 5a). However, cells had diffuse actin staining on p(DTE-co-3%PEGc) (Figure 5c). By day 7, MSC/OS cells exhibited prominent actin filaments on p(DTE-co-3%PEGc) and were comparable to the MSC/OS cells on p(DTE carbonate) (Figure 5b, 5d). Throughout the duration of the experiment, cells on p(DTE-co-5%PEGc) remained aggregated and exhibited diffuse actin staining (Figure 5e, 5f).

Cell Proliferation

Over the course of 14 days, cell proliferation on the various substrates cultured in OS medium (Figure 6) were determined. By days 4 and 7, the number of MSC/OS cells on p(DTE-co-3%PEGc) and p(DTE-co-5%PEGc) had decreased from the initial cell seeding number and were significantly less than MSC/OS cells on p(DTE carbonate) at day 7 ($p < 0.05$). Cell number increased significantly on p(DTE-co-3%PEGc) from day 7 to 14 ($p < 0.05$) and was comparable to p(DTE carbonate). However, cell number on p(DTE-co-5%PEGc) was significantly less than both p(DTE carbonate) and p(DTE-co-3%PEGc) at day 11 ($p < 0.05$). Throughout the 14 day culture, there was no cell proliferation on p(DTE-co-5%PEGc) and by day 14, the number of MSC/OS cells on p(DTE-co-5%PEGc) was significantly less than MSC/OS cells on p(DTE-co-3%PEGc) ($p < 0.05$).

Alkaline Phosphatase Activity and Osteocalcin expression

AP activity, an early marker for osteogenic differentiation was measured for MSC/OS cells (Figure 7). AP activity on undifferentiated MSCs was negligible. From days 4 to 11, the AP

activity of MSC/OS cells increased on p(DTE carbonate) and p(DTE-co-3%PEGc) ($p<0.05$) and a significant increase was also detected for cells on p(DTE-co-3%PEGc) from days 11 to 14. By day 14, cells on both p(DTE carbonate) and p(DTE-co-3%PEGc) had significantly higher levels of AP activity than p(DTE-co-5%PEGc). There was a PEG dependent concentration response in terms of AP activity, where the AP activity increased with decreasing PEG concentration. The AP activity of MSC/OS cells on p(DTE-co-5%PEGc) was negligible throughout the time course of the experiment.

Osteocalcin is a marker for mature osteoblasts and was measured for MSC/OS cells (Figure 8). Osteocalcin expression for undifferentiated MSCs was negligible. There were significantly lower levels of osteocalcin produced by MSC/OS cells on p(DTE-co-5%PEGc) as compared to both p(DTE carbonate) and p(DTE-co-3%PEGc) at day 14 ($p<0.05$).

MSC Motility on the PEG-variant Substrates

The motility of MSC/OS cells seeded on p(DTE carbonate), p(DTE-co-3%PEGc) and p(DTE-co-5%PEGc) was evaluated over 11 days. At the earliest time point of 18 hours post seeding (day 0), RMCs of 0.127, 0.370, and 0.148 μm^2 per minute were observed for p(DTE carbonate), p(DTE-co-3%PEGc), and p(DTE-co-5%PEGc), respectively (Figure 9). MSC/OS cells on p(DTE-co-3%PEGc) had a significantly higher initial RMC than on p(DTE carbonate) ($p<0.05$) and was higher than p(DTE-co-5%PEGc), but was not statistically significant ($p=0.06$). The relative RMC is obtained by measuring the RMC at a specific time-point and normalizing it to the initial RMC (Figure 10). Although MSCs on p(DTE-co-3%PEGc) had a higher initial RMC compared to p(DTE carbonate), the relative RMC decreases from day 4 and is significantly lower than the relative RMC of MSC/OS cells on p(DTE carbonate) from days 7 and 11 ($p<0.05$).

Discussion

In this study, the effect of modifying the molar fraction of PEG from 0-5% in tyrosine derived polycarbonates on MSC behavior was investigated. Due to the bioinert nature of PEG, it has been used in pharmaceuticals, coatings for biomaterials, and tissue engineering applications. PEG can be copolymerized with other polymers to create heterogeneous polymer surfaces with domains that either support or do not support cell attachment.^{5, 11} To enhance cell attachment and differentiation, copolymers containing PEG can be functionalized with peptides, growth factors or other soluble factors to create biomimetic materials for possible orthopedic^{2, 4, 19, 25} and vascular applications.^{14, 15}

The anti-cell attachment effect of PEG was reflected in the decrease in cell number from the initial cell seeding density on p(DTE-co-3%PEGc) and p(DTE-co-5%PEGc). Increasing the molar fraction of the PEG had a positive effect on cell-cell aggregation and a negative effect on cell proliferation as a function of time. For instance, cells on p(DTE-co-3%PEGc) and p(DTE-co-5%PEGc) were aggregated and did not exhibit comparable morphology to those on p(DTE carbonate) at the initial time-points (5 hours and 24 hours). At later time-points, the cell morphology of MSC/OS cells on p(DTE-co-3%PEGc) resembled that of p(DTE carbonate), while MSC/OS cells p(DTE-co-5%PEGc) remain aggregated. This was confirmed with the cell proliferation studies which indicated delayed cell proliferation on p(DTE-co-3%PEGc) at days 4 and 7, however approached the cell number values of p(DTE carbonate) by days 11 and 14. There was negligible cell proliferation on p(DTE-co-5%PEGc) throughout the duration of the experiment.

Cytoskeleton organization, as determined by actin-filament staining, indicates focal adhesion contacts between the cell and the substrate and is representative of the interactions between the membrane integrins and the protein ligands adsorbed on the substrates. Proteins

such as fibronectin present in serum can provide the ligand interactions with integrins.^{9, 28} The adsorbed mass of the serum protein layer decreases with increasing PEG concentration potentially providing fewer ligand binding interactions to integrins and therefore affecting the f-actin cytoskeleton organization. For instance, the lack of prominent actin/stress fibers in cells on p(DTE-co-5%PEGc) indicates limited focal adhesion contacts onto the substrate.

The phenomenon of cell aggregation versus cell-substrate adhesion and migration on polycarbonates containing PEG was reported by Ryan et al, in which L929 mouse fibroblasts were transfected with different cadherin clones and seeded on polycarbonates containing various concentrations of PEG. It was shown that decreasing the molar percent of PEG in polycarbonates increased the cell-substrate adhesion as well as increased the rate of emigration.²⁴ It has also been reported that there is a biphasic effect of PEG concentration on motility, wherein the greatest speed occurs at intermediate levels of pegylation (3% PEG).²⁷ We observed similar findings where the initial RMC of MSCs was significantly higher on p(DTE-co-3%PEGc) compared to p(DTE carbonate). This response was attributed to differential adhesion of cells where substrates that promoted intermediate adhesion strengths (3% PEG) were associated with a higher initial RMC, while substrates that promoted strong adhesion (0% PEG) and weak adhesion (5% PEG) were associated with reduced initial RMC's. The decline in the relative RMC of the MSC/OS cells on p(DTE-co-3%PEGc) could be attributed to the increased cell-substrate adhesion interactions over time. Conversely, the motility trends seen on p(DTE-co-5%PEGc) may be due the lower levels of adsorbed protein giving weak cell adhesion at the initial time point. The subsequent increase in cell motility may be attributed to substrate adhesion strengthening over time.

The commitment of MSCs towards a specific lineage can be easily attributed to soluble factors in medium or the extracellular matrix. However, recent studies on the effects of directed cell shape as well as substrate stiffness on MSC differentiation have been investigated. Previously it has been shown that cell shape and generation of cytoskeletal tension are influential components of stem cell differentiation.¹⁸ McBeath *et al.* reported that by dictating cell shape on fibronectin patterned substrates, lineage commitment of MSCs was observed for spread cells regardless of chemical cues. One finding indicated that MSCs favored osteogenesis when MSCs were cultured on large islands of fibronectin allowing for maximum cell spreading, while MSCs favored adipogenesis when cultured on smaller islands of fibronectin coated PDMS. In this study, where cell spreading was dictated by the chemistry of the substrate, similar findings of osteogenic differentiation were observed. The AP activity of cells on p(DTE carbonate) and p(DTE-co-3%PEGc) were comparable to each other, however was negligible on p(DTE-co-5%PEGc). The osteogenic markers were optimal for MSCs seeded on materials with maximal cell-substrate interactions.

Engler *et al.* reported that the stiffness of the substrate has been found to have an effect on MSC differentiation in the absence of inductive cues in the medium.⁶ MSCs that were seeded on soft matrices of collagen coated polyacrylamide gels representative of brain tissue, upregulated expression of neurogenic markers while MSCs seeded on stiffer matrices representative of bone tissue, upregulated the expression of osteogenic markers. The incorporation of PEG into the polycarbonate structure has been shown to decrease the tensile stiffness of the polymer in the wet state. The mechanical stiffness of the substrates presented here was not evaluated in this study however previous studies indicate that increasing the concentration of PEG decreases the mechanical stiffness in terms of tensile stiffness and strength.³⁴ Mechanical stiffness according to Engler, along with substrate wettability could attribute to the increased osteogenic differentiation on p(DTE carbonate). Unfortunately, the differentiation markers for other MSC lineages such as chondrocytes, adipocytes, or myocytes were not assayed in this study, so it is unknown whether markers for other

lineages were upregulated or downregulated in the presence of PEG. Further studies will need to be completed to understand the relationship between substrate composition and the resulting surface and physico-mechanical properties on MSC differentiation.

Conclusions

In this initial screening of tyrosine derived PEG copolymers, it can be concluded that the PEG concentration has a pronounced effect on the surface properties, which influence cell response. It was observed that the molar fraction of PEG in the backbone influences the relationship between cell-substrate attachment, cell-cell aggregation, cell motility and presence of osteogenic markers. In this initial evaluation of substrate suitability for a potential tissue engineered bone graft scaffold, it was shown that osteogenic differentiation was supported on p(DTE carbonate) and p(DTE-co-3%PEGc). Moreover, cell motility, an important parameter in terms of MSC infiltration within a scaffold, is initially greater on p(DTE-co-3%PEGc), making this substrate potentially the optimal option as a scaffold.

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Figure 2.
Chemical structure of poly(DTR-co-x%PEG₁₀₀₀ carbonate). R represents a modifiable ester pendent chain. X% represents the molar fraction of PEG.

Figure 3. Adsorbed mass of 10% FBS onto polymer substrates using QCM-D, Voigt model. Asterisk (*) indicate statistical significance between p(DTE carbonate) and p(DTE-co-5% PEG) ($p < 0.05$).

Figure 4.

Phase contrast images of cell morphology of MSC/OS cells at various time-points. The arrows on images e, f, i, k and l indicate areas of aggregation. (10X objective).

Figure 5.
Cytoskeleton organization, as indicated by f-actin filament staining using Alexa Fluor 488 phalloidin, of MSC/OS cells at various time-points (20X objective).

Figure 6.

Cell proliferation of MSC/OS cells on polymer substrates. Asterisk (*) indicate statistical significance between p(DTE carbonate) and p(DTE-co-5% PEGc) ($p < 0.05$). Symbol (+) indicate statistical significance between p(DTE carbonate) and p(DTE-co-3% PEGc) ($p < 0.05$). Symbol (#) indicate statistical significance between p(DTE-co-3% PEGc) and p(DTE-co-5% PEGc) ($p < 0.05$).

Figure 7.

Alkaline phosphatase activity of MSC/OS cells on polymer substrates. Asterisk (*) indicate statistical significance between p(DTE carbonate) and p(DTE-co-5% PEGc) ($p < 0.05$). Symbol (#) indicate statistical significance between p(DTE-co-3% PEGc) and p(DTE-co-5% PEGc) ($p < 0.05$).

Figure 8.

Osteocalcin production normalized to cell number at day 14 on polymer substrates. Asterisk (*) indicate statistical significance between p(DTE-co-3%PEGc) and p(DTE-co-5%PEGc) ($p < 0.05$).

Figure 9. Random motility coefficient of MSC/OS cells on polymer substrates after initial seeding (day 0). Asterisk (*) indicate statistical significance between p(DTE carbonate) and p(DTE-co-3%PEGc) ($p < 0.05$).

Figure 10. Relative random motility coefficient: random motility coefficient normalized to initial random motility coefficient of MSC/OS cells on polymer substrates as a function of time. Asterisk (*) indicate statistical significance between p(DTE carbonate) and p(DTE-co-3%PEGc) ($p < 0.05$).