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Lithium-end-capped polylactide thin films influence osteoblast progenitor cell differentiation and mineralization

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Abstract

End-capping by covalently binding functional groups to the ends of polymer chains offers potential advantages for tissue engineering scaffolds, but the ability of such polymers to influence cell behavior has not been studied. As a demonstration, polylactide (PLA) was end-capped with lithium carboxylate ionic groups (hPLA13kLi) and evaluated. Thin films of the hPLA13kLi and PLA homopolymer were prepared with and without surface texturing. Murine osteoblast progenitor cells from collagen 1 α 1 transgenic reporter mice were used to assess cell attachment, proliferation, differentiation, and mineralization. Measurement of green fluorescent protein expressed by these cells and xylenol orange staining for mineral allowed quantitative analysis. The hPLA13kLi was biologically active, increasing initial cell attachment and enhancing differentiation, while reducing proliferation and strongly suppressing mineralization, relative to PLA. These effects of bound lithium ions (Li⁺) had not been previously reported, and were generally consistent with the literature on soluble additions of lithium. The surface texturing generated here did not influence cell behavior. These results demonstrate that end-capping could be a useful approach in scaffold design, where a wide range of biologically active groups could be employed, while likely retaining the desirable characteristics associated with the unaltered homopolymer backbone.

Keywords

osteoblast differentiation; biomimetic scaffold; mineralization inhibition; end-capped polymer; functionalization

INTRODUCTION

Strategies to engineer healthy bone are a significant focal point of biomedical research; however, there are no options that yield engineered bones that remain viable and mechanically sound long-term.¹⁻⁴ To improve the success of bone tissue engineering methodologies, a more bioactive role for biomaterials has been deemed necessary and research is heavily directed toward developing biomimetic materials with osteoconductive, osteoinductive, and osteogenic properties.^{3,5,6}

It has been well established that cell behavior is strongly influenced by many aspects of a biomaterial's surface including surface chemistry, topography, hydrophilicity, surface charge, and stiffness, for example.⁷⁻¹³ Biodegradable materials are advantageous for tissue engineering applications because they provide structural support during the stages of cell attachment and growth, and may be replaced over time *in vivo* as new tissue forms, making way for tissue repair with healthy autologous tissue.^{2,5,14} Degradable synthetic materials, such as polylactic acid (PLA), have been widely investigated for tissue engineering strategies.^{15,16} However, due to its hydrophobic nature, PLA has a low affinity for cell attachment, which can result in minimal cell growth, limiting the success of PLA for tissue regeneration. Accordingly, there have been continued efforts to modify PLA to enhance cell-biomaterial interactions, while maintaining its favorable bulk properties.

A commonly used approach for PLA modification is copolymerization with hydrophilic or functionalized monomers. Although this method may successfully increase the hydrophilicity of the polymer to improve cell affinity, the degradation properties of PLA might be affected by incorporation of hydrophilic groups along the polymer backbone.¹⁶⁻¹⁸ Additionally, entrapment or sorption of functional molecules near the surface, or surface coating with amphiphilic copolymer oligomers^{17,19,20} have also been evaluated, however, application to complex 3-D scaffold structures is problematic because of difficulty ensuring homogenous, uniform coatings.

End-capping of biodegradable polyesters is being developed in the field of polymer recycling as a method to control the characteristics of PLA and its copolymers.²¹ In this approach, a permissive functional group, such as carboxyl, is covalently bound to the ends of the full polymer chains, resulting in a PLA polymer with available carboxylic acid end groups. Ro et al.²² used this method to produce PLA polymers end-capped with itaconic anhydride (ITA) and neutralized with metal acetates of different valences, such as sodium (Na^+), lithium (Li^+), calcium (Ca^{2+}), and zinc (Zn^{2+}), which resulted in functionalized PLA, referred to as telechelic ionomers, with improved thermal properties. This approach could be used for attachment of various bioactive atoms and molecules, such as metallic ions, creating polymer compositions effective for mediating cellular behavior, such as increased cell attachment and differentiation.^{14,23}

End-capping, however, has received little attention for tissue engineering, where potential advantages for this technology exist. For example, a wide range of ions or biomolecules that influence cell affinity and response could be covalently linked to polymers via end-capping, providing an opportunity to create tunable biomaterials for specific applications.

Importantly, it is expected that carboxylate salt end groups will not affect the degradation of the PLA, especially for the temperatures and environmental conditions used in cellular experiments such as described here, leaving desirable physical properties, such as degradation rates, intact. Further, with proper functional end-capping of PLA, end groups may agglomerate and function as physical crosslinks, allowing for manipulation of bulk polymer properties, if desired. The key question with end-capping is whether or not the functionalized polymer is biologically active and able to influence cell behavior.

Alteration of the surface texture, particularly the presence of nanoscale topographical features, has also been shown to influence cell behavior and this field has received considerable attention in tissue engineering.^{24–27} Annealing thin films at high temperatures can produce a textured surface with topographical features in the range of tens of nanometers, a scale size important in the extracellular matrix and cell signaling.²⁸

Accordingly, we evaluated the influence of end-capping, with and without texturing, on cell behavior. In selecting a neutralization agent for synthesis of the functionalized, end-capped PLA to be used for tissue engineering applications, it was necessary to select a metal ion that was also of biological interest. For our purposes of developing biomimetic polymers for bone tissue engineering, lithium was ideal.

Lithium acts as an activator of the β -catenin signaling pathway by inhibiting GSK-3 β enzyme, which has been identified as a key regulator of cellular response to Wnt signaling and associated with many cell processes such as cell cycle regulation and cell proliferation.²⁹ It has also been suggested that the Wnt/ β -catenin signaling pathway plays a role in the regulation of bone mass and in fracture repair.³⁰ Previous studies have shown decreased risk of fracture among patients treated with lithium,^{31,32} increased rat mesenchymal stem cell (MSC) proliferation and differentiation when cultured with lithium chloride (LiCl),³³ and increased mid-palatal bone growth in rats when administered LiCl *in vivo*.³⁴ On the other hand, other studies have shown that lithium has an inhibitory effect on bone mineralization and osteoid formation in rats³⁵ and inhibits osteogenesis of human and murine MSCs^{36–38} and murine preosteoblasts.³⁹

Taken together, these findings suggest that available lithium ions from a functionalized polymer would have an effect, either stimulatory or inhibitory, on osteogenic cell behavior. Specifically, here we hypothesized that a bioactive ion incorporated onto the ends of PLA in the form of topographically-textured thin films, would influence osteoblast progenitor cell attachment, proliferation, differentiation, and mineralization. The longer-term goal of this work is to create enhanced polymeric scaffolds for regeneration of mineralized tissues using novel end-functionalized PLA.

MATERIALS AND METHODS

Polymer synthesis

PLA (M_w 121,000) was obtained from Nature Works LLC. α -methacrylate- ω -hydroxy-PLA end capped with ITA was synthesized and neutralized with lithium acetate to produce ionomeric PLA (M_n 13,000, T_g 45°C, T_m 149°C) as previously described²² (Figure 1).

Briefly, PLA was melted under a N₂ purge at 190°C in a 250-mL flask equipped with a mechanical stirrer. 2-Hydroxyethyl methacrylate (HEMA), molar ratio (26:1 mol lactic acid) and a Tin(II) 2-ethylhexanoate (SnOct) catalyst (2%, w/w PLA) were added and the reaction mixture was heated and stirred at 190°C for 2 h to reduce the molecular weight of the PLA (by transesterification) and to functionalize one end with HEMA [Figure 1(a)]. The HEMA-functionalized polymer was dissolved in ethyl acetate and a two-fold molar excess of ITA and SnOct (1%, w/w PLA) were added to the flask and reacted (120°C for 6 h) to convert the terminal hydroxyl group to a carboxylic acid [Figure 1(b)]. The polymer (M_n 13,000) was precipitated with excess methanol, filtered, and dried at 70°C in a vacuum oven for 24 hr. The ω -(carboxylic acid) PLA was converted to the Li-ionomer by neutralization with excess lithium acetate [Figure 1(c)] and the product was washed with distilled water until the solution was neutral to remove the acetic acid side-product and then dried. A 0.25 M stock solution of lithium acetate was prepared using distilled, deionized water. The resulting polymer was dissolved in tetrahydrofuran in a round-bottom flask, and 1 equivalent of lithium acetate per equivalent of carboxylic acid was added to the reaction flask. After allowing the neutralization reaction to proceed for 0.5 h, the solvent was vacuum distilled and the precipitated polymer was dried at 70°C under reduced pressure for 24 h. The polymer was then washed with excess distilled, deionized water to remove any acetic acid and unreacted lithium acetate, filtered, and dried at 70°C under reduced pressure for 24 h. The telechelic ionomer is referred to here as hPLA13kLi

Thin film preparation and characterization

Thin films of PLA and hPLA13kLi were produced by spin coating polymer solutions onto the glass coverslips. Chloroform was purchased from Fisher Chemicals and was used as is. Glass coverslips of 18-mm diameter were purchased from Fisher Scientific. The glass coverslips were initially washed several times by immersing and sonicating in soap solution followed by washing in distilled water for 20 min. The discs were air dried in a clean environment and kept covered in polystyrene Petri dishes. A 1% (w/v) solution of each polymer in chloroform was prepared and spin coated for 80 s at 3000 rpm onto the clean coverslips. The solvent was allowed to evaporate, leaving behind a smooth glassy polymer film. All films were further dried in a vacuum oven at room temperature for 24 h. Texturing was produced by annealing PLA and hPLA13kLi thin films at 80 and 75°C, respectively, for 24 h in an oven. The four groups of films were used to evaluate the influence of end-capping and texturing on cell behavior. The films are referred to as PLA annealed, PLA non-annealed, hPLA13kLi annealed, and hPLA13kLi non-annealed.

The topography of the films was imaged with an atomic force microscope (AFM; MFP-3D, Asylum Research) using noncontact mode and silicon tips (radius of curvature $\approx$ 5–10 nm) with a spring constant of 2.5 N/m. Surface roughness values for each type of film were obtained from the MFP-3D software at five different positions on two discs of each type and the average value was determined.

In vitro cell cultures and analyses

As an alternative to traditional culture-terminating techniques for assessing differentiation of osteoblastic cell cultures (i.e., reverse transcription polymerase chain reaction) the use of

progenitor cells from transgenic mice that express green fluorescent protein (GFP) only upon differentiation to a mature matrix-producing osteoblast allows identification of osteoblasts in live cultures.⁴⁰ Through analysis of fluorescent images taken at various time points it is possible to observe and quantify osteogenic differentiation and this has been previously shown to be useful in the evaluating biomaterial-progenitor cell interactions.⁴¹ While other cell sources, that is, adipose-derived or bone marrow MSCs are widely used, the well-characterized osteoprogenitor cells from these transgenic mice are committed to the osteoblast lineage and therefore advantageous for elucidating the effects of a biomaterial on cell mineralization.

Primary murine osteoblast progenitor cells (Col2.3OB) were harvested from the calvaria of 4–6-day-old transgenic mice using a modified enzymatic digestion procedure.⁴⁰ Briefly, the calvaria from each mouse were dissected from the skull. The sutures of the calvaria were removed, the calvaria were collected, and then subjected to four 15-min digestions in an enzyme solution containing 0.25% trypsin–ethylenediaminetetraacetic acid (EDTA) and 2 U/mL collagenase P (Boehringer Mannheim) while incubated at 37°C on a rocking platform apparatus. Cell fractions from digestions 2 through 4 were pooled and centrifuged. The resulting cell pellet was re-suspended in culture medium consisting of Dulbecco's modified Eagle's medium (#11885, Gibco) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.1 mM nonessential amino acids (Gibco), then filtered through a 70-µm cell strainer, counted, and seeded for *in vitro* culture experiments.

The spin-coated disks were placed in wells of nontissue-culture treated 12-well plates (BD Falcon, Fisher Scientific) and sterilized under ultraviolet light for 1 h. Tissue culture treated 12-well plates (TCP, BD Falcon, Fisher Scientific) were used as control surfaces for these experiments to ensure that cell behavior was as expected for the duration of the experiment, not for direct comparison with the cells grown on the polymer films (data reported in Supporting Information). Cells were seeded onto the films or TCP (12 wells per condition) at a density of 1.5×10^4 cells/cm² with 1 mL per well of the culture medium previously described; and maintained for 7 days, with media changes every 2–3 days. After reaching confluence at Day 7, the medium was changed to differentiation medium consisting of alpha minimum essential medium (Gibco), 10% fetal bovine serum, 50 µg/mL L-ascorbic acid, 4 mM β-glycerophosphate, 100 U/mL penicillin, and 100 µg/mL streptomycin for two weeks with media changes every 2–3 days.

Adhesion and proliferation assays

Cellular attachment and proliferation on the 4 groups of thin films were assessed after 4 h and 48 h of culture, respectively. Phase contrast images of cells seeded on each type of film were captured at each time point using an inverted microscope equipped with a digital camera. Up to five images for six wells of each condition were captured. Cells in each image were counted using the Cell Counter Tool found in the Image J software package (National Institutes of Health) and the estimated total cell number for each well was calculated based on the number of cells found in the specified area of each image. The proliferation rate of cells cultured for each condition was calculated as: $(\# \text{ Cells}_{48\text{h}} - \# \text{ Cells}_{4\text{h}}) / \# \text{ Cells}_{4\text{h}}$.

DNA quantification

The total concentration of DNA within cell culture samples was determined, as a relative measure of cellular content, and used for data normalization. At Days 14 and 21, following aspiration of the culture medium, cells were rinsed with PBS. A DNA digestion solution consisting of 25 $\mu\text{L}/\text{mL}$ papain (#P-3125, Sigma), 2 mM *N*-acetyl-L-cysteine (#A-7250, Sigma), and 5 mM EDTA (BP120, Fisher) all dissolved in a 50 mM phosphate buffer solution was prepared. A volume of 1 mL of digestion solution was added to each well containing cell samples. The cells were scraped and then transferred with the solution to a microcentrifuge tube. The tubes containing samples were incubated overnight in a 65°C water bath.⁴² The total DNA content within the digested samples was measured using the Quant-iT™ PicoGreen® dsDNA Quantification kit (#P-7589, Invitrogen, Molecular Probes) according to the manufacturer's specifications.

Osteogenesis and mineralization quantification

Images obtained from fluorescence microscopy were analyzed and used for quantification of GFP expression of Col2.3OB cells differentiated to osteoblasts and xylene orange (XO) staining of mineralized calcium nodules within the cell cultures at days 14 and 21. As previously described, the expression of GFP is visible following differentiation of the progenitor cells to osteoblasts. To stain mineral nodules, XO, a nontoxic calcium-chelating fluorochrome, was used to nondestructively stain newly calcified tissue orange in color.⁴³ A volume of 1 μL of the 20 mM XO stock solution was added to the culture medium in each well of the 12-well plates at least 12 h before the specified time points for capturing images. After adding the XO, plates were gently swirled to distribute the dye throughout the culture medium, and the plates were incubated again at 37°C and 5% CO_2 . Before imaging, the culture medium containing XO was aspirated and replaced with fresh culture medium.

Images of GFP expression and XO staining were captured using a computerized inverted microscope (Zeiss SteREO Lumar V12, Carl Zeiss) equipped with a motorized X-Y-Z stage, motorized fluorescence cube, and color digital camera (AxioCam, Carl Zeiss). Images of GFP expression and XO staining were captured of the same area within each well using a user-defined computation program to specify usage of either the Topaz (yellow fluorescent protein) filter (Chroma Technology) or Cherry filter (Exciter ET577/20, Emitter ET640/40, Chroma Technology) for either GFP or XO, respectively, before moving to an adjacent area in the well. A series of 6×6 adjacent images with 5% overlap were captured of each culture well. Each series of images was compiled into one single image that was then used for quantification. The exposure time for either GFP or XO was determined by calibration measurement of control samples for each experiment. The determined exposure times were then used for capturing all images for the experiment.

Quantification of the percent area of GFP expression and XO staining for each sample was accomplished with image analysis software (ImageJ, National Institutes of Health). A reproducible method for determining the threshold level to exclude non-GFP and non-XO background fluorescence was previously determined and used in our laboratory.⁴¹ Following determination of the appropriate threshold values for both GFP and XO, the percent area of GFP expression and XO staining were determined for each sample image.

Percent area values were normalized to DNA content determined by PicoGreen[®] quantification.

Calcium assay

Cell culture samples from Days 14 and 21 were treated with a volume of 500 μ L of 5% trichloroacetic acid (TCA) per well of the 12-well plates for 30 min at room temperature to dissolve any calcifications for analysis. The TCA was collected and then an additional volume of 500 μ L of 5% TCA was added to the wells and the process repeated. A volume of 100 μ L of calcium determination reagent (Calcium CPC Reagent Set, #2400, Cima Scientific, De Soto, TX) was added to 5 μ L of each sample and standard. The sample absorbance was measured using a microplate reader and wavelength of 570 nm. Calcium content was determined based on a standard curve.

Statistical analysis

For all assays, two-way analysis of variance (ANOVA; Graph-Pad Prism 6[™]) was used to determine the effects of functionalization (PLA vs. hPLA13kLi) and texturing (annealed vs. non-annealed) on cell response. Tukey post-tests for multiple comparisons were performed to determine statistical significance between individual sample groups with significance set at $p < 0.05$. Data are expressed as mean and standard error of the mean.

RESULTS

Thin film characterization

Figure 2(a,c) shows the AFM topographic images of non-annealed and annealed PLA thin films, respectively. The non-annealed PLA films were smooth and flat (root mean square roughness < 2 nm) and did not show any distinctive morphological features. The PLA thin films annealed at 80°C for 24 h [Figure 2(c)] exhibited randomly arranged surface features resembling ridges and grooves with a roughness of 7.74 ± 0.25 nm. Needle-shaped structures were also observed throughout the film. Similarly, the non-annealed hPLA13kLi ionomer film did not exhibit any texturing, with any features being less than 3 nm [Figure 2(b)]. Annealing the hPLA13kLi ionomer films also gave rise to a randomly arranged morphology similar to the annealed PLA thin films, with the presence of needle-shaped features [Figure 2(d)] and a roughness of 14.27 ± 0.48 nm.

Adhesion and proliferation assays

Attachment values for the Col2.3OB cells to the four surfaces 4 h after seeding are shown in Figure 3(a). Two-way ANOVA showed statistically greater attachment on the hPLA13kLi films than on the PLA films ($p < 0.05$) and no effect associated with texturing ($p = 0.7519$). The two-way ANOVA interaction term was not statistically significant ($p = 0.1356$), meaning there was no inter-dependence of the composition and texturing variables. Tukey post-test analysis showed no significant difference observed between the individual sample groups.

The relative increase in cell number from 4 to 48 h in culture is shown in Figure 3(b). Again, there was a statistically significant effect of composition ($p < 0.001$) with less proliferation

on the hPLA13kLi films, and no differences associated with texture ($p = 0.7210$). The interaction was close to being statistically significant ($p = 0.0582$) and the opposite effects of texturing with PLA and hPLA13kLi are apparent in Figure 3(b). Comparison of individual sample groups with Tukey post-tests showed that cell proliferation was statistically higher on non-annealed PLA films than on the annealed ($p = 0.0151$) or non-annealed ($p = 0.0017$) hPLA13kLi films. Overall, the Li ionomer increased cell attachment and decreased proliferation relative to PLA.

Osteogenesis and mineralization quantification

Phase contrast images show the presence of Col2.3OB cells in confluent monolayer cultures on each surface at Days 14 and 21, in Figure 4(a–d) and (m–p), respectively. Expression of GFP, indicative of osteoblast cell differentiation, was observed in cells cultured on each surface at Days 14 and 21 as shown in Figure 4(e–h) and (q–t), respectively. The existence of mineralized nodules in cultures, if present, at Days 14 and 21, was confirmed with XO staining, as shown in Figure 4(i–l) and (u–x), respectively, with colocalization of mineral nodules to areas of GFP expression observed, as expected when cell-mediated mineralization is present.

Quantified results of GFP expression and XO staining at Days 14 and 21 are shown in Figure 5. The effects of end-capping and texturing are consistent across the four panels. There was greater GFP expression on the hPLA13kLi thin films than on the PLA ($p < 0.05$), but less XO staining ($p > 0.24$) at Day 21. The effects of end-capping and texturing were independent, meaning there were no significant interactions between the two factors in the 2-way ANOVA (all $p > 0.17$). Comparison of individual sample groups with Tukey post-tests showed that GFP expression was statistically higher on the annealed hPLA13kLi films PLA than on the annealed ($p = 0.0204$) or non-annealed ($p = 0.0147$) PLA films. Accordingly, the hPLA13kLi enhanced differentiation of the Col2.3OB progenitor cells, but suppressed mineralization. The texturing generated in the films did not influence either differentiation or mineralization.

Calcium assay

At Day 14 the calcium concentration per DNA content was very low for all surfaces, less than $0.10 \times 10^{-3} \mu\text{g/mL/ng/mL}$, with no statistical differences between the types of films. At Day 21 the results for PLA annealed and non-annealed were $0.4 \pm 0.4 \times 10^{-3}$ and $0.5 \pm 0.4 \times 10^{-3} \mu\text{g/mL/ng/mL}$, respectively. The calcium content at Day 21 on the hPLA13kLi surfaces was below the detection limit of the assay such that calculation of calcium content based on the obtained standard curve indicated that there was no measurable amount of calcium produced by cells cultured on the annealed or non-annealed hPLA13kLi surfaces. While these results are consistent with the XO staining mineralization data, the low values and large variances resulted in no statistically significant differences.

DISCUSSION

Lithium-functionalized PLA was successfully prepared using a previously described end-capping approach²² and used for fabrication of thin films in this study (Figure 2). The Li^+

moiety maintained its bioactivity; enhancing initial attachment and differentiation, but decreasing proliferation and mineralization of murine osteoprogenitor cells. While the present study was limited to one polymer and one functional group, the potential benefits of end-capping for polymer modification include simple incorporation of a wide range of functional molecules and ions, and distribution of the functional groups within the bulk polymer. The bioactive molecules are bound to the polymer, rather than being delivered in soluble forms, which could allow different aspects of control of cell response in 3-D scaffolds in culture or *in vivo*. Since the polymer backbone is unaltered, physical properties such as degradation characteristics are less likely to be affected at temperatures and environmental conditions used in cellular experiments such as in this study or *in vivo*. However, future studies evaluating the degradation of functionalized ionomeric polymers are needed to confirm the degradation profile for these materials.

For this initial proof-of-concept study, lithium acetate was selected for polymer neutralization because lithium has been determined to play a key role in the Wnt/ β -catenin signaling pathway, the pathway known to regulate progenitor cell differentiation to osteoblasts.^{29,30} Previous studies have shown that soluble lithium added to cell culture medium, lithium-supplemented dietary feed for animals, and lithium-based psychotropic drugs for humans are effectors of osteogenesis resulting in either stimulation or inhibition of osteogenic cell growth, differentiation, and mineralization.^{31–33,35–39} The deposition of lithium into calcium phosphate coatings has also been used to modify biomaterial properties to facilitate increased osteoblast cell attachment.⁴⁴ The effect of bound lithium ions within a functionalized polymer on osteoprogenitor behavior, has not previously been determined, and was demonstrated with the present experiments.

Both the PLA homopolymer and the ionomeric hPLA13-kLi supported Col2.3OB cellular attachment. We observed that the hPLA13kLi polymer films supported higher initial Col2.3OB cell attachment (Figure 3(a)) than the nonmodified PLA films; however, those cells proliferated at a slower rate [Figure 3(b)]. Previous studies have determined that supplementation of culture medium with lithium salts resulted in increased attachment and proliferation of MC3T3-E1 preosteoblasts.³⁹ A dosage-dependent response to lithium, however, has been demonstrated, where lower doses (4 mM) of lithium chloride resulted in increased proliferation of rat MSCs³³ and human MSCs, while proliferation was completely inhibited in human MSCs treated with higher doses (up to 40 mM) of lithium chloride.³⁷ Characterization of the synthesized ionomeric PLA with FTIR spectroscopy previously confirmed the presence of Li⁺ ions on the polymer chains at low ionic concentrations.²² While we did not measure the Li⁺ concentration in the present study, based on the molecular weight of the PLA and assuming complete conversion of the COOH groups to Li salt, the concentration would be in the micromolar range.

The presence of these Li⁺ ions in our functionalized polymer facilitated increased initial Col2.3OB cell attachment, demonstrating the capacity of end-capped polymers to present ionic groups to cells that provide an immediate response *in vitro*. The reduced proliferation rate on the hPLA13kLi films likely resulted from the continuous presentation of lithium ions to cells throughout the culture period. Similar outcomes were observed in the literature

where increased lithium concentrations in the media over time reduced cell proliferation.^{33,37}

All four groups of thin films supported Col2.3OB differentiation, with higher expression of GFP observed at both days 14 and 21 for the cells cultured on the hPLA13kLi films [Figure 5(a,c)]. While differentiated to osteoblasts, the Col2.3OB cells cultured on the hPLA13kLi films did not functionally produce mineral nodules, as evidenced by the absence of XO-stained mineral and calcium formation that is typically associated with mature osteoblasts. Higher XO staining [Figure 5(b,d)] was observed on the nonmodified PLA homopolymer films.

Numerous studies have been conducted to assess the effects of lithium on mineralization. Early evaluation of the effects of lithium on the direct precipitation of hydroxyapatite in solution showed that increased levels of lithium ions resulted in reduced rates of apatite crystal formation.^{45,46} Additional studies of lithium's effect on biological mineralization have shown that lithium has an inhibitory effect on bone mineralization and osteoid formation in rats³⁵ and inhibits osteogenesis of human and murine MSCs^{36–38} and murine pre-osteoblasts.³⁹ On the other hand, other studies have shown decreased risk of fracture among patients treated with lithium,^{31,32} and increased mid-palatal bone growth in rats administered LiCl *in vivo*.³⁴ Based on these conflicting reports in the literature, the suppression of mineralization in cells cultured on the hPLA13kLi films, caused by exposure to lithium ions, was not unexpected and may be attributed to several factors, including the lithium dosage, length of exposure to lithium, and the type and/or stage of differentiation of cells evaluated.

Work by de Boer et al.^{36,37} demonstrated that a lower level of lithium over time did not interfere with cell proliferation or differentiation, but did suppress mineralization, and high doses of lithium also resulted in inhibition of mineralization stemming from nonconfluent cultures. Shi et al.⁴⁷ used cultures of MC3T3-E1 cells treated with LiCl and/or vitamin D to show that the continuous administration of LiCl (from the onset of culture), with or without vitamin D resulted in complete inhibition of mineralization, while the addition of LiCl at later stages of culture (postconfluence and after the onset of mineralization) resulted in reduced mineral formation. Similarly, evaluation of primary mouse bone marrow cells in short and long-term culture showed that long-term exposure to LiCl resulted in decreased ALP and mineralized matrix formation.³⁸ Consistent with their findings, our Col2.3OB cells proliferated and differentiated normally on nonmodified and ionomeric surfaces, as demonstrated by confluent cell layers and expression of lineage-specific GFP, but mineralization was inhibited due to long-term lithium exposure over time. The end-capping approach allowed the lithium to remain active, resulting in cellular outcomes consistent with soluble lithium administration.

It is also important to note that although increased mineralization was not observed on lithium-functionalized surfaces, the use of lithium in this capacity still has potential in tissue engineering applications. Interface tissue engineering, where composite tissues or multiple tissue types are needed, such as mineralized bone to nonmineralized tendon, is a growing area of research interest. Polymers functionalized with lithium offer a biomaterial scaffold

that would prevent unwanted mineralization while permitting normal cell growth and differentiation, as demonstrated by our cultures of Col2.3OBs. Given the need to guide or direct progenitor cells with biomolecules to form particular tissue types, there is an array of potential uses for this technology in tissue engineering.

By annealing the spin-coated discs, we successfully created textured surfaces [Figure 2(c,d)] with roughness values on the nanometer scale that we used to evaluate the effects of texturing on Col2.3OB cell behavior. Each of the surfaces evaluated here, non-annealed and annealed films of both PLA and hPLA13kLi, were shown to support attachment, proliferation, and osteoblast differentiation as indicated by the GFP expressed by Col2.3OB cells on each surface. It was expected that the nanotextured surfaces would have mediated improved cellular responses characterized by increased cell attachment, differentiation, and mineralization^{48–50}; however, no effect due to texturing was observed in our experiments.

The morphology of polymer thin films has been widely investigated under various conditions.^{51–55} Texturing is governed by many factors including polymer chemistry, type of substrate, thermal and solvent annealing conditions, molecular weight, and film thickness. Dewetting of the polymer from the substrate or crystallization of the polymer are mechanisms typically responsible for texturing, however, the influence of these factors and the specific mechanism causing texturing were not evaluated in the present study since that was not within the scope of this work.

The lack of any effect with texturing may have been due to several reasons, including the means by which texturing was formed, the arrangement of the topographical features, the scale of surface features, the cell type evaluated, possible exposure of the glass substrate, or crystallinity. If the Col2.3OB cells were directly exposed to areas of the glass substrate, any effect due to texturing may have been decreased since cell mineralization may have been affected by the plain glass substrate.^{56,57} While the AFM results do not suggest an extensive effect, annealing could have caused some dewetting and exposure of the glass surface. Annealing likely caused crystallization, which may have also contributed.

Additionally, depending on the processing method, topographical features may be presented in either random or ordered patterns that have been shown to influence cell behavior even for materials with similar roughness values.^{49,58–60} The response of bone cells, in particular, to random or ordered features has been shown to be varied.^{25,26,60–63} This could have contributed to the lack of response to texturing in our experiments. The difference in scale of surface features between our annealed and non-annealed polymers may not have been large enough to influence behavior of the Col2.3OB cells. It has also been shown that various cell types have different levels of sensitivity to topographical features, where cell size in relation to the size of nanofeatures could negate an effect of texturing since large cells could form attachments across individual grooves or ridges, and still undergo normal processes as if growing on a nontextured, flat surface.

CONCLUSIONS

Here, we demonstrated the use of end-capping to synthesize lithium functionalized PLA. The results with thin films showed that the end-capped polymer was biologically active and the effects with murine osteoprogenitor cells were consistent with previously reported literature results for soluble lithium additions. Accordingly, end-capping could be an effective tool for the design of bioactive polymers, where it is desirable to retain the chemistry of the polymer backbone. While demonstrated here using lithium and PLA and evaluating the effects on mineral-producing cells, alternative ions or biomolecules could be incorporated into various polymers, to produce novel biomaterials for diverse tissue engineering applications.

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FIGURE 1.

(a) Synthesis of α -methacrylate- ω -hydroxy-PLA. (b) End-capping reaction with itaconic anhydride. (c) Preparation of Li-carboxylate-terminated PLA (hPLA13kLi) ionomer.

FIGURE 2.

AFM topographic images of (a) non-annealed PLA, (b) non-annealed hPLA13kLi, (c) PLA annealed at 80°C for 24 h, and (d) hPLA13kLi annealed at 75°C for 24 h.

FIGURE 3.

(a) Initial attachment of Col2.3OB cells following 4 h of culture; (b) the rate of cellular proliferation determined after 48 h of culture. Error bars represent the standard error of the mean.

FIGURE 4.

Representative phase contrast images of cells cultured on each surface at day 14 (a–d) and day 21 (m–p) of culture. Representative images of GFP expressed by cells cultured on each surface at day 14 (e–h) and day 21 (q–t) showed that each surface supported cell differentiation to osteoblasts, indicated by GFP expression. Representative images of mineral nodules stained with xylenol orange at day 14 (i–l) and day 21 (u–x) of culture showed mineral formed in cells cultured on both types of PLA films, with visibly less stained mineral on annealed and non-annealed hPLA13kLi films (scale bar = 100 μ m).

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FIGURE 5.

(a) GFP expression was observed for cells cultured on each surface at day 14, however, there was more GFP expressed in cells cultured on the hPLA13kLi films. (b) Quantification of XO staining at day 14 showed significantly more mineral in cells cultured on the PLA films. (c) GFP expression was observed for cells cultured on each surface at day 21, however, there was more GFP expressed in cells cultured on the hPLA13kLi films. (d) Quantification of XO staining at day 21 showed significantly more XO stained mineral in cells cultured on the PLA films. Error bars represent the standard error of the mean.