Poly(β-hydroxybutyrate-co-β-hydroxyvalerate) Supports in Vitro Osteogenesis

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ABSTRACT

Studies have demonstrated that polymeric biomaterials have the potential to support osteoblast growth and development for bone tissue repair. Poly(β-hydroxybutyrate-co-β-hydroxyvalerate) (PHBV), a bioabsorbable, biocompatible polyhydroxy acid polymer, is an excellent candidate that, as yet, has not been extensively investigated for this purpose. As such, we examined the attachment characteristics, self-renewal capacity, and osteogenic potential of osteoblast-like cells (MC3T3-E1 S14) when cultured on PHBV films compared with tissue culture polystyrene (TCP). Cells were assayed over 2 weeks and examined for changes in morphology, attachment, number and proliferation status, alkaline phosphatase (ALP) activity, calcium accumulation, nodule formation, and the expression of osteogenic genes. We found that these spindle-shaped MC3T3-E1 S14 cells made cell–cell and cell–substrate contact. Time-dependent cell attachment was shown to be accelerated on PHBV compared with collagen and laminin, but delayed compared with TCP and fibronectin. Cell number and the expression of ALP, osteopontin, and pro-collagen α1(I) mRNA were comparable for cells grown on PHBV and TCP, with all these markers increasing over time. This demonstrates the ability of PHBV to support osteoblast cell function. However, a lag was observed for cells on PHBV in comparison with those on TCP for proliferation, ALP activity, and cbfa-1 mRNA expression. In addition, we observed a reduction in total calcium accumulation, nodule formation, and osteocalcin mRNA expression. It is possible that this cellular response is a consequence of the contrasting surface properties of PHBV and TCP. The PHBV substrate used was rougher and more hydrophobic than TCP. Although further substrate analysis is required, we conclude that this polymer is a suitable candidate for the continued development as a biomaterial for bone tissue engineering.

INTRODUCTION

Complications associated with the use of autografts and allografts in current bone repair strategies demonstrate the need for alternative implants that are capable of facilitating functional bone formation. Ideally, these implants would induce a low inflammatory response (biocompatible), be safely degraded in the body (biodegradable), support ostoblast-mediated bone deposition (osteoinductive), and support the ingrowth of vasculature to facilitate the newly formed tissue (osteocductive). Numerous biocompatible and biodegradable α-polyhydroxy acids have been examined for their effectiveness as replacements for autologous tissue grafts, on
the basis of their ability to support osteoblast cell development. Of these, polyactic acid (PLA),\textsuperscript{1–4} polyglycolic acid (PGA),\textsuperscript{5} and copolymers of PGA and PLA (PGA/PLA)\textsuperscript{6,7} have been shown to support osteoblast attachment, growth, and differentiation in vitro. In addition, these polymeric materials have also been shown to support bone formation in vivo.\textsuperscript{8,9}

An alternative polyhydroxy acid for bone tissue engineering is poly(\(\beta\)-hydroxybutyrate-co-\(\beta\)-hydroxyvalerate) (PHBV), a biodegradable polymer derived from Alcaligenes eutrophus.\textsuperscript{10} PHBV has been shown to be more stable in vivo than the more commonly used \(\alpha\)-polyhydroxy acids, as its degradation can be reduced\textsuperscript{11–13} by altering its molecular weight\textsuperscript{14,15} and valerate content.\textsuperscript{11,14–16} Therefore, the accumulation of degradation products at the site of implantation is less problematic for PHBV than for the faster degrading \(\alpha\)-polyhydroxy acids. In addition, the principal degradation product of PHBV, \(\beta\)-hydroxybutyric acid,\textsuperscript{17–19} is a normal constituent of blood that is safely metabolized by the liver.\textsuperscript{17,20} PHBV has also been shown to be biocompatible, as minimal inflammatory responses were observed in long-term studies of subcutaneous PHBV implants in mice\textsuperscript{12} and rats.\textsuperscript{21} PHBV can also be engineered to mimic the structure and mechanical properties of bone by combining it with reinforcing phases such as hydroxyapatite.\textsuperscript{15,22} Finally, PHBV can be manipulated to facilitate osteoconduction at the implant site, as the thermoplastic properties of PHBV allow for the appropriate shape, form, and porosity of the implant to be created.\textsuperscript{15} Taken together, PHBV may be a suitable candidate to support long-term bone regeneration in vivo.

Despite satisfying the criteria for an ideal bone regenerative implant, in vivo studies examining PHBV for this purpose are limited. A related polymer, poly(\(\beta\)-hydroxybutyrate) (PHB), has been shown to support bone tissue formation in vivo with minimal inflammation and fibrous tissue formation.\textsuperscript{23,24} Similar results have also been reported for PHB composites containing hydroxyapatite.\textsuperscript{25–27} More recently, in vitro growth of osteoblast cells on macroporous three-dimensional PHBV matrices has been reported,\textsuperscript{28} and increases in osteocalcin expression and alkaline phosphatase (ALP) activity over a 60-day growth period have also been shown for cells grown on PHBV.\textsuperscript{29} Although these findings support the use of PHBV for in vivo bone regeneration, a comprehensive characterization of osteoblast growth and differentiation on nonporous PHBV has yet to be conducted. This is particularly important, as it has been proposed that initial cell attachment and proliferation are dependent on the outermost functional groups of an implant surface rather than its bulk composition.\textsuperscript{30} Furthermore, an understanding of the initial cell response to an implant material, such as time-dependent cell attachment, is critical for determining the potential of an implant material to support accelerated wound repair. Such studies would also facilitate the determination of strategies to improve the implant surface if necessary.

To examine the interaction between the PHBV surface and osteoblast cells, this study assessed the ability of PHBV to support preosteoblast-like MC3T3-E1 S14 cell attachment, proliferation, and differentiation on the surface of solvent-cast PHBV films in vitro. We show that PHBV supports these aspects of cell behavior and therefore the osteogenic progression of MC3T3-E1 S14 cells.

**MATERIALS AND METHODS**

**Solvent casting of PHBV**

Amounts of 0.277 g of PHBV (8% valerate, Monsanto, St. Louis, MO) and 15 mL of chloroform (Biolab, Mulgrave, Australia) were stirred at 40°C for 15 min. The resulting polymer solution was poured into a 70-mm glass Petri dish, covered, and cooled to ambient temperature, and the solvent was allowed to evaporate slowly over a 3-day period. Samples of these casts were then subjected to X-ray photoelectron microscopy (PHI model 560 XPS/SAM/SIMS I multitechnique surface analysis system; PerkinElmer, Wellesley, MA) to confirm complete chloroform evaporation from the polymer. Disks 3 cm in diameter (herein referred to as “film”) were punched from the larger casts, washed three times in ultrapure water, dried, and sterilized in an autoclave.

**Cell culture**

MC3T3-E1 S14 cells (American Type Culture Collection [ATCC], Manassas, VA) were cultured in 25-cm\(^2\) flasks (Nunc, Roskilde, Denmark) in \(\alpha\)-minimum essential medium (\(\alpha\)-MEM; CSL, Parkville, Australia) supplemented with 10% fetal bovine serum (FCS, batch 097059401; JRH Biosciences, Brooklyn, Australia), 2 mM L-glutamine (CSL), 100 mM sodium pyruvate (Trace Biosciences, Brooklyn, Australia), gentamicin (25 \(\mu\)g/mL; Trace Biosciences), and penicillin (100 units/mL; Trace Biosciences) (herein referred to as “standard medium”) at 37°C, 5% CO\(_2\). Cells were subcultured every 3 days, using 0.25% trypsin–1 mM EDTA (Invitrogen Life Technologies, Mount Waverly, Australia) for 5 min at 37°C, 5% CO\(_2\).

**Cell culture on tissue culture polystyrene**

Before seeding, cells were serum starved in standard medium replaced with 0.25% FCS for 24 h at 37°C, 5% CO\(_2\). Cells were then trypsinized and seeded into 6-well plates (Nunc) at a density of 5 \times 10\(^4\) cells/cm\(^2\). Cultures were maintained until 1, 7, 14, or 21 days postseeding (herein referred to as the “endpoint”) in \(\alpha\)-MEM supplemented with 10% FCS, 2 mM L-glutamine, 0.05 mM
ascorbic acid 2-phosphate (Sigma, St. Louis, MO), and 25 mM β-glycerophosphate (Sigma) (herein referred to as “osteogenic medium”) with medium changes twice weekly.

Cell culture on PHBV films

To prevent cell attachment onto underlying tissue culture plastic (TCP), six-well plates (Nunc) were precoated with 150 μL of 12% poly(2-hydroxyethylmethacrylate) (poly-HEMA; Sigma) in 95% ethanol and allowed to dry. PHBV films were placed into poly-HEMA-coated six-well plates (Nunc) and cells were seeded as described for TCP. At assay endpoint, films were transferred to poly-HEMA-free conditions when conducting cell lysis procedures. All other aspects of cell culture were as for cells on TCP.

Cell attachment and morphology

To examine cell attachment to the films and TCP, 28.5 × 10^3 cells/cm² were seeded and grown for 7 days in osteogenic medium as described previously. The substrates were then washed with phosphate-buffered saline (PBS; Gibco, Grand Island, NY) and stained with acridine orange (0.5 μg/mL; USB, Cleveland, OH) according to the manufacturer’s instructions and viewed by confocal microscopy. For scanning electron microscopy (SEM), cells were grown as described for fluorescence microscopy and prepared for SEM by fixation in 3% glutaraldehyde in cacodylate buffer (Sigma, Thuringowa, Australia). Fixed cells were then incubated in 1% OsO₄ (ProSciTech) and dehydrated with ethanol. Substrates were then embedded in hexamethyldisilazane (HMDS; ProSciTech) and platinum coated with a sputter coater (Eiko, Ibaraki, Japan). SEM was conducted with the assistance of the Centre for Microscopy and Microanalysis (CMM, University of Queensland, St. Lucia, Australia), using a JEOL 6300 SEM (JEOL USA, Peabody, MA).

Time-dependent cell attachment

To compare cell attachment to PHBV with cell matrix proteins known to improve osteoblast attachment, cells were serum starved for 48 h and then seeded at a density of 28.5 × 10^3 cells/cm² in osteogenic medium onto PHBV films, TCP (positive control), TCP preblocked with bovine serum albumin (BSA) (negative control), and BD BioCoat plates (BD Biosciences Discovery Labware, Bedford, MA) coated in matrix proteins known to influence cell attachment: fibronectin, collagen, and laminin at ~10 μg/mL. To inhibit nonspecific binding, the BD BioCoat plates and negative control TCP were washed three times with PBS and then incubated with 2% BSA for 2 h at 37°C and washed again with PBS. Cells were then seeded and, after 5 min, the medium was collected and attached cells were trypsinized. Cells from both fractions were then counted on a Z series Coulter Counter (Beckman Coulter, Fullerton, CA) and the number of attached cells was expressed as a percentage of the total number of cells from both fractions (i.e., the total number of cells seeded). Triplicate readings were taken of each sample, and the assay was repeated six times for separate wells at intervals of 30, 45, 60, 90, and 120 min after cell seeding.

Cell proliferation: [³H]thymidine incorporation assay

To assess cell proliferation on PHBV and TCP, [³H]thymidine ([³H]Td) incorporation into replicating DNA was measured. Cells were incubated with 4 μCi of [³H]Td (Amersham Biosciences/GE Healthcare, Little Chalfont, UK) for 24 h before assay endpoint. They were then washed in PBS, trypsinized for 1 h, and scraped and the content of each well was transferred to separate filter papers (Whatman, Middlesex, UK), using a Millipore manifold system (Millipore, Bedford, MA). The filter papers were thoroughly washed on the manifold system with ultrapure water to completely lyse the cells and to remove nonspecific [³H]Td. Filters were then transferred to scintillation vials with 5 mL of Starclassic scintillation fluid (PerkinElmer Life and Analytical Sciences, Boston, MA) and counted in a Beta counter (PerkinElmer Life and Analytical Sciences).

Total DNA content

DNA content was measured by protocols based on previously described methods. At assay endpoint, growth medium was removed and cells were washed in PBS and stored at −20°C until required. Films were transferred to new poly-HEMA-free plates for freezing. Later, 500 μL of lysis buffer (25 mM Tris, 0.5% Triton X-100, pH 8) was added to each well and the plates were freeze–thawed four times. The matrix was disrupted with a cell scraper and the contents were transferred to 5-mL tubes. Three 20-μL samples were removed for the ALP activity assay described below. To the tubes, 1 mL of 10 mM EDTA (pH 12.3) was added to the samples and sonicated on ice, using a Sonifier cell disruptor B-30 (Branson Ultrasonics, Danbury, CT) set on a 60% pulse cycle for 10 min. 1 M KH₂PO₄ (200 μL) was added to neutralize the pH. A PicoGreen dsDNA quantification kit (Molecular Probes, Eugene, OR) was used to fluorescently label the DNA as per the manufacturer’s instructions. The samples were visualized at 700 nm with a dual-line laser system (Fluorimager 595; Molecular Dynamics, Sunnyvale, CA) and DNA was quantified with reference to a standard curve (in accordance with the manufacturer’s guidelines), using NIH Image software version 1.62 (http://rsb.info.nih.gov/nih-image/). Total cell number was calculated from measurements of DNA content on a basis of 8 pg of DNA per MC3T3-E1 cell.
ALP activity

Samples (20 μL) obtained during DNA extraction were combined with 20 μL of p-nitrophenyl phosphate (pNPP) substrate buffer (1% pNPP [Zymed, South San Francisco, CA], 10% substrate buffer [Zymed], 89% ultrapure water) and 20 μL of alkaline buffer solution (Sigma) in a 96-well plate and incubated for 20 min at 37°C. The reaction was stopped by the addition of 180 μL of 0.05 M NaOH and read at 405 nm (Bio-Rad microplate reader benchmark 10892; Bio-Rad, Hercules, CA). A second reading was taken after the addition of 8 μL of concentrated HCl (37%) to bleach the reaction. The second reading was subtracted from the first and converted into ALP activity units (1 unit of enzyme hydrolyzes 1 μmol of pNPP per minute at 37°C) as per the supplier’s instructions, using a standard curve. Calf intestinal ALP (Roche, Mannheim, Germany) was used as a positive control.

Matrix/intracellular calcium accumulation

To measure calcium accumulation, a 96-well plate assay was adapted from the Calcium C test kit (Wako Pure Chemical, Kyoto, Japan), in accordance with the manufacturer’s recommendations. At endpoint, cells were washed with PBS and incubated with 1 mL of 1% trichloroacetic acid for 5 min. A cell scraper was used to dissociate the monolayer and the lysate was transferred to a 1.5-mL Eppendorf, vortexed, and centrifuged for 20 min at 1600 × g at 25°C. Supernatant (100 μL) was combined with 10 μL of color solution and 100 μL of buffer solution in each well and incubated at room temperature for 5 min. The absorbance were read at 595 nm (Bio-Rad plate reader) and compared with a standard curve prepared with standard solutions provided in the kit.

von Kossa nodule staining

von Kossa staining was used to determine bone nodule formation in vitro. Cells were seeded onto films and TCP at a density of 1 × 10⁴ cells/cm² and cultured for 3 weeks in osteogenic medium. The monolayer was then washed with PBS, fixed with 4% paraformaldehyde (Sigma), and washed with ultrapure water. To stain nodules, the monolayer was treated with 1% silver nitrate (Sigma) for 45 min under ultraviolet (UV) and washed with ultrapure water. Monolayers were then treated with 5% sodium thiosulfate (Sigma) and photographed with a dissection microscope (Zeiss, Jena, Germany) equipped with a digital camera (AxioCam; Zeiss) using AxioVision software version 3.1 (Zeiss).

RNA extraction and cDNA synthesis

RNA was extracted for use in real-time polymerase chain reaction (PCR) analysis. Cells were washed in PBS and RNA was extracted with a NucleoSpin RNA II kit (Machery-Nagel, Düren, Germany) as per the manufacturer’s instructions. RNA concentrations were measured by spectrophotometer readings at 260/280 nm (GeneQuant pro, Amersham Biosciences/GE Healthcare). RNA (1 μg) was reverse transcribed with SuperScript II (Invitrogen, Carlsbad, CA) and oligo(dT) (Invitrogen) according to the manufacturer’s instructions.

Real-time PCR

The expression of selected osteogenic genes was quantified by real-time PCR, using an ABI PRISM 7000 sequence detection system and SYBR Green master mix (Applied Biosystems, Foster City, CA) and specific primers (Table 1) synthesized by GeneWorks (Hind-

<table>
<thead>
<tr>
<th>Gene</th>
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<th>Tm (°C)</th>
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*Abbreviations: F, forward; R, reverse; Tm, annealing temperature; accession no., GenBank accession number of primer target. aPCR product size.
marsh, Australia). The reaction cycle consisted of a first stage for 10 min at 95°C followed by 45 cycles of combined annealing and extension for 15 s at 95°C and for 1 min at 60°C. Results are expressed as a relative expression of hypoxanthine-guanine phosphoribosyltransferase (HPRT).

Water contact angle

Water contact angle measurements were performed on a custom-built apparatus consisting of a table-mounted Wild Heerbrugg/Leica longitudinal telescopic lens attached to a Cohu (San Diego, CA) high-performance charge-coupled device (CCD) camera. Samples were mounted on an adjustable Teflon mount and a drop of ultrapure water was delivered by a Hamilton syringe. The water drop on the material was observed using real-time video pictures captured via an LG-3 Scion frame wrapper operated by a PC. Contact angles were calculated with NIH Image software. Advancing contact angles were obtained by adding one water drop onto the material with the end of the needle above the water drop. A minimum of five repeats was conducted for each sample.

Scanning probe microscopy

An NT-MDT Solver P47 scanning probe microscope (SPM) (NT-MDT, Moscow, Russia) was used in contact mode (constant force) to obtain information on surface roughness of the samples. Contact “Golden” silicon tips (NT-MDT) with nominal tip curvature of about 10 nm were used for the scans. The contact cantilever had a force constant of 0.1 N/m. Scans were done over a nominal area of 7.5 × 7.5 μm. The scan speed was 0.8–1 Hz. A minimum of five areas on the surface was scanned.

Statistical analyses

All assays were conducted in triplicate for three separate repeats. The attachment assay was conducted six independent times, using triplicates for each sample Two-way analysis of variance (ANOVA) with Bonferroni post

FIG. 1. Cell morphology of MC3T3-E1 S14 cells on PHBV (A and B) and TCP (C and D) was similar after 7 days of culture in osteogenic medium. Original magnification for confocal microscopy (A and C) was ×40 and cells were stained with acridine orange. Original magnification for SEM (B and D) is indicated in the figure.
hoc testing was used to determine the effect of time and substrate on cell proliferation, cell attachment, ALP activity, calcium accumulation, and the expression of osteogenic genes. Statistical analysis for water contact angle and surface roughness was performed with an independent samples t test for a minimum of five independent repeats. All statistical analysis was conducted with SPSS software version 11 (SPSS, Chicago, IL).

RESULTS

Cell morphology and attachment

Both confocal microscopy (Fig. 1A and C) and SEM (Fig. 1B and D) demonstrated the morphology of cells cultured on PHBV and TCP to be the same. Irrespective of substrate, cells appeared flattened with clear substrate attachments and cellular processes extending to make contact with neighboring cells. Time-dependent cell attachment (Fig. 2) as determined by Coulter Counter measurements indicated that cell attachment to PHBV was comparable to other matrix proteins. After 30 min, cell attachment to TCP was significantly higher than attachment to all other substrates, with greater than 60% attachment achieved ($p < 0.001$ over all substrates). By 45 min, TCP and fibronectin had attained approximately 70% attachment, significantly higher than cell attachment on the other substrates ($p < 0.001$). After 60 min, cell attachment to PHBV was the same as to TCP and fibronectin, all three showing significantly more cell attachment than collagen and laminin. Minimal changes were observed after 60 min, except for fibronectin, which displayed maximal cell attachment (90% attachment) after 90 min over all other substrates. Cell attachment to laminin was the lowest, being comparable to the BSA-coated negative control wells, both of which showed minimal cell attachment of 10–15%.

Cell number

DNA content was the same for PHBV and TCP, indicating equal cell numbers across all time points (Fig. 3). However, irrespective of substrate, there was a time-dependent increase in cell number ($p < 0.001$), post hoc tests showing that this was due to a 38% increase in cell number from day 1 to day 7 ($p < 0.001$). No difference in cell numbers was observed in the last 7 days of growth.

![Figure 2](image-url)
Proliferation

Irrespective of the substrate, proliferation rates decreased over time \((p < 0.001)\) (Fig. 4), with the greatest decreases observed between days 7 and 14 for cells on PHBV \((p < 0.01)\) and between days 1 and 7 for cells on TCP \((p < 0.001)\). The highest rate of proliferation was observed on day 1 for cells on TCP \((6 \times 10^5 \text{ dpm per } 1 \times 10^5 \text{ cells})\) whereas the highest for cells on PHBV was on day 7 \((2.5 \times 10^5 \text{ dpm per } 1 \times 10^5 \text{ cells})\).

Alkaline phosphatase activity

Regardless of substrate, alkaline phosphatase (ALP) activity increased over time \((p < 0.001)\) (Fig. 5), with significant increases observed between days 7 and 14 for cells grown on TCP \((p < 0.01)\) and between days 7 and 14 for cells on PHBV \((p < 0.001)\). ALP activity for cells grown on PHBV was 62% lower on day 7 \((p < 0.01)\) but 50% higher on day 14 \((p < 0.01)\), indicating a delay in ALP activity for cells grown on PHBV compared with TCP.

Mineralization

Mineralization was measured by calcium accumulation and von Kossa staining. As observed with ALP activity, we saw a time-dependent increase in calcium accumulation regardless of substrate \((p < 0.001)\) (Fig. 6). A significant increase in calcium accumulation was observed between days 1 and 14 for cells on PHBV \((p < 0.01)\) whereas significant increases in calcium were detected between days 1 and 7 \((p < 0.001)\) and days 7 and 14 \((p < 0.001)\) for cells grown on TCP. Calcium accumulation was 52% lower for cells grown on PHBV than for those on TCP at day 14 \((p < 0.001)\). von Kossa staining indicated fewer positively stained bone nodules on PHBV compared with TCP after 3 weeks of culture in osteogenic medium (Fig. 7).

Real-time PCR

All osteogenic markers analyzed by real-time PCR were expressed by cells when grown on either substrate. Across all time points, osteopontin was the most abundantly expressed gene. Expression levels of ALP (Fig. 8A), osteopontin (Fig. 8B) and procollagen \(\alpha 1(I)\) (Fig. 8C) were comparable for cells grown on TCP and PHBV, with expression of all three factors increasing significantly over time \((p < 0.001, p < 0.001, \text{ and } p < 0.05\) respectively). cbfa-1 mRNA expression levels (Fig. 8D) also increased over time, irrespective of substrate, with increasing levels between days 1 and 7 \((p < 0.001)\) and between days 7 and 14 \((p < 0.001)\). On day 1, cbfa-1 expression was 73% lower for cells grown on PHBV than for those on TCP \((p < 0.05)\) whereas by day 14 cbfa-1 expression was increased by 68% for cells growing on PHBV compared with TCP \((p < 0.01)\). Osteocalcin mRNA expression (Fig. 8E) also increased over time, irrespective of substrate; however, its expression was significantly reduced for cells grown on PHBV than for those grown on TCP \((p < 0.001)\), especially on day 14 \((p < 0.001)\). These findings for cbfa-1 and osteocalcin also show a delay in osteogenic differentiation on PHBV compared with TCP.
PHBV and TCP surface hydrophobicity

PHBV solvent cast films were found to be more hydrophobic than TCP control samples. PHBV samples displayed an advancing contact angle of $\theta_A = 84^\circ$, indicating a hydrophobic surface, whereas TCP samples were found to be less hydrophobic, with an advancing contact angle of $\theta_A = 69^\circ$ ($p < 0.001$).

PHBV and TCP surface roughness

An average surface roughness of $R_a = 308$ nm was determined for PHBV whereas TCP yielded an average surface roughness of $R_a = 6.4$ nm, making it the smoother surface ($p < 0.001$). The topography of the PHBV samples showed a uniform surface punctuated with random pits and grooves (Fig. 9A and B). In contrast, TCP sam-

FIG. 4. Cell proliferation as measured by [3H]Td incorporation into replicating DNA standardized per $1 \times 10^5$ cells. Proliferation on PHBV (solid columns) was delayed compared with TCP (shaded columns), with lower proliferation on day 1 and higher proliferation on day 7. *1$p < 0.001$; *2$p < 0.01$.

FIG. 5. Alkaline phosphatase (ALP) activity, standardized per $1 \times 10^5$ cells, was measured in a colorimetric assay. Activity was lower on PHBV (solid columns) than on TCP (shaded columns) on day 7; the opposite was true on day 14. *1$p < 0.01$; *2$p < 0.01$. 
ples had random scratches that can be observed at angles to the scan direction (Fig. 9C and D).

DISCUSSION

Nonunion fracture repair depends on the ability of an implant to temporarily bear load at the site of injury while facilitating bone formation. Although it is possible to engineer PHBV to degrade at a rate that allows for the smooth transfer of the mechanical load to the developing tissue, little is known about the ability of the polymer surface to facilitate the cellular attachment, proliferation, and differentiation necessary for accelerated bone formation.

To address this, we evaluated these parameters by culturing MC3T3-E1 S14 preosteoblast-like cells on solvent cast PHBV films. We found that the attachment of cells to PHBV was comparable to various matrix proteins known to aid osteoblast attachment. Long-term culture over 2 weeks indicated cells on PHBV were comparable in cell number, morphology, and gene expression profiles for ALP, osteopontin, and procollagen α1(I) to those cultured on TCP; an in vitro surface optimized for non-

FIG. 6. Calcium accumulation (per $1 \times 10^5$ cells), as determined by colorimetric assay, increased over time for both substrates, with levels lower on PHBV (solid columns) than on TCP (shaded columns) on day 14. * $p < 0.001$.

FIG. 7. Silver nitrate staining of cell nodule formation on (A) PHBV and (B) TCP after 3 weeks of culture in osteogenic medium. Fewer nodules (black staining, indicated by white arrows) are evident on PHBV in comparison with TCP. Original magnification: $\times 20$. 
specific cell growth. Increases in both cell number and expression of these osteogenic factors over time indicate that PHBV is able to support osteoblast-like cell attachment, growth, and maturation.

However, differences in cell proliferation were observed between cells on PHBV and TCP. The lower proliferation rate of cells on PHBV on day 1 may be explained by the findings of the time-dependent cell attachment assays, which showed that cell attachment was lower on PHBV than on TCP in the first 120 min of culture. However, this cannot completely account for the observed trends in proliferation, as DNA content measurements indicated that cell numbers were equal on both substrates after 24 h of culture.

It is possible that cells cultured on PHBV remained in a longer lag phase of growth than cells grown on TCP. The lag phase is a period of cell adjustment to its substrate, and because it is often characterized by low levels of proliferation, it may have been the overriding factor contributing to the lower proliferative rates on PHBV on day 1. Furthermore, a longer lag phase would also explain the increased proliferation of cells on PHBV on day 7, while corresponding cells on TCP were in a reduced proliferative state. Presumably, the initially high proliferative rates of cells on TCP resulted in their early confluence and the associated contact inhibition of proliferation.

Given the differences in proliferation of cells on each substrate, cell numbers still remained equal on both substrates after 24 h of culture.

### FIG. 8

Real-time PCR was used to quantify gene expression of osteogenically related factors. Gene expression is expressed relative to HPRT mRNA expression (relative expression units). All factors increased with respect to time, with no significant difference between PHBV (solid columns) and TCP (shaded columns) for ALP (A), osteopontin (B), and procollagen α1(I) (C). cbfa-1 (D) and osteocalcin (E) expression on PHBV was delayed compared with TCP. *1p < 0.01; *2p < 0.001; *3p < 0.001; *4p < 0.01; *5p < 0.05; *6p < 0.05; *7p < 0.001; *8p < 0.001.
strates at all time points. It is possible that sustained proliferation of cells on PHBV until day 7, contrasting with a decrease in proliferation of cells on TCP during the same time period, would allow cell numbers on both substrates to be equal by day 7. A measurement of proliferation rates at earlier time points is needed to confirm this.

The MC3T3-E1 cell line follows a time-dependent developmental progression along the osteogenic lineage,34,35 and thus a longer lag phase would consequently cause a delay in the onset of cell differentiation when compared to a control culture. This hypothesis is supported by cbfa-1 mRNA expression. cbfa-1 is a transcription factor known to control the expression of osteogenic factors such as osteocalcin.36,37 Studies have shown that it is down-regulated during proliferation37 and increased during the onset of differentiation.36 Given that proliferation rates of cells on PHBV were lower than those on TCP at day 1, the lower levels of cbfa-1 mRNA in cells on PHBV at the same time point could be explained by a delay in the time-dependent expression of this factor. Although statistical analysis detected a significant difference in cbfa-1 mRNA levels between cells on each substrate on day 14, this may not necessarily be a biological effect, given that statistical analysis indicated that mRNA levels did not change over time for either substrate between day 7 and day 14. A lag in differentiation is also supported by ALP activity. ALP, an enzyme indicative of osteoblast differentiation,38 peak activity was delayed for cells on PHBV compared with those on TCP.

The delays observed in the expression of late stage bone markers further corroborate a lag in osteogenesis on PHBV. Osteocalcin, a protein expressed highly by mature cells during the later stages of osteogenesis,39,40

FIG. 9. Scanning probe micrographs of areas of 7.5 × 7.5 μm. (A) Three-dimensional image of PHBV solvent cast film displaying an average surface roughness of 308 nm (scale on y axis, 0–2000 nm). (B) Area scan of PHBV solvent cast film. (C) Three-dimensional image of TCP surface with an average surface roughness of 6.4 nm (scale on y axis, 0–80 nm). (D) Area scan of TCP surface. PHBV was rougher than TCP (p < 0.001).
demonstrated significantly reduced mRNA expression in cells on PHBV compared with those on TCP on day 14, indicating a less mature phenotype on PHBV. In accordance with this finding, calcium accumulation and in vitro bone nodule formation after 3 weeks in culture were also delayed for the cells grown on PHBV. Analysis of later time points is necessary to confirm lags in the expression of these late stage osteogenic markers.

A lag was not observed for the gene expression of ALP, osteopontin, and procollagen α1(I). However, given that these are all midstage markers of bone development and are expressed in both immature and mature cells, it is likely that mRNA expression levels may vary little over the course of differentiation. Alternatively, it may be that these factors are post-translationally controlled, which could explain why ALP gene expression did not correlate with protein activity. Similar findings have been reported by other studies that analyzed both gene and protein expression patterns for the same factor. To our knowledge, this is the first study to use real-time PCR as a tool to analyze cell responses to an implant surface. The discrepancy with ALP activity and mRNA expression illustrates the need to corroborate gene expression with protein expression to achieve a conclusive understanding of the cell phenotype.

The PHBV substrate used in this study was also evaluated by scanning probe micrographs and water contact angle measurements. Substrate surface properties affect the cellular response and therefore the overall performance of the implant in vivo. In particular, it has been shown that the chemical composition, surface morphology, surface energy, and hydrophobicity are key contributors to cell phenotype. It has been proposed that surface roughness affects both cell adhesion and cell–cell interactions. On rough surfaces, cells must realign themselves on the substrate and form focal adhesions that allow them to span across surface troughs. The resulting geometry of these adhesions affects the shape of the cell through cytoskeletal rearrangements, which in turn influence gene expression through integrins.

We showed that solvent cast films of PHBV had a much rougher surface topography than TCP. Other studies of MC3T3-E1 cell growth on titanium demonstrated that smooth surfaces enhance cell proliferation, whereas the expression of adhesion proteins such as fibronectin increases with increasing surface roughness. Increasing the surface roughness of titanium has also been shown to cause a decrease in MG-63 human osteosarcoma cell number and an increase in cellular osteocalcin, TGF-β1 and PGE2 production. Other studies using MG-63 cells have reported that titanium surface roughness prevents cell proliferation and ALP expression while increasing cellular RNA, protein, and matrix synthesis. These findings imply that rough titanium surfaces tend to select cell differentiation over proliferation. However, results of other studies suggest that cell response to surface roughness may be substrate specific. For example, studies examining cell response to TCP surface morphology found that rat bone marrow cells were sensitive to grooves on TCP, whereas MC3T3-E1 cells were not. Similarly, a study examining rat bone marrow cell growth on various polyhydroxy acids found that cell morphology, ALP, and cell proliferation rates were not different between cells on PLA and PHB surfaces, despite large differences in substrate surface morphology. As cell response to surface roughness appears to differ between substrate and cell types, it is likely that it is not the sole factor affecting MC3T3-E1 S14 cell growth on PHBV.

As mentioned, another important surface property of a biomaterial is hydrophobicity, measured by determining water contact angle, where a high contact angle indicates a hydrophobic surface. Hydrophobicity is related to the surface energy of a substrate, which in turn influences the type and orientation of proteins adsorbed onto the surface of a biomaterial. Protein adsorption directly affects cell attachment, and hence substrates with higher surface energy (more hydrophilic) are better at adsorbing cell adhesion proteins and inducing cell attachment. Supporting previous findings, we found PHBV to be more hydrophobic than TCP. As a result, the adsorption of adhesion proteins to PHBV from the culture medium may have been inferior to their adsorption on TCP. This in turn may explain the observed differences between the substrates in cell attachment. This notion is confirmed by previous studies, where the adsorption of collagen and fibronectin increased on PHBV after it was hydrolyzed (i.e., hydrophilicity increased). It was also demonstrated that PHBV substrates, treated with radio frequency-oxygen plasma to increase the hydrophilicity of the surface, were more effective in supporting rat stromal osteoblast cell growth, ALP activity, and osteocalcin expression than nontreated surfaces.

In summary, on the basis of other studies detailed above, it is possible that the hydrophobic PHBV surface did not adsorb proteins appropriate for cell attachment as well as TCP. In addition, the rougher surface morphology may have challenged cell spread and proliferation. The cells may have responded to these conditions by secreting proteins and matrix molecules to aid their attachment and growth. Previous studies showed an increase in RNA expression, protein synthesis (notably fibronectin), and proteoglycan synthesis for cells on rough titanium surfaces, which may reflect this phenomenon. Studies have also suggested that this readjustment of the microenvironment precedes the cells’ commitment to proliferation and developmental progression. As these processes take time, a lag may result, affecting the continued time-dependent development of the cells. Measurements of cell phenotypic changes over more frequent time points than presented here would con-
firm this lag in cell development. In addition, it would be valuable to compare cell behavior between PHBV and TCP, particularly actin polymerization and matrix protein expression, at early time points when many of the factors involved in microenvironment readjustment may be expressed.

Although this hypothesis may explain the results obtained in this study, it is important to consider that the MC3T3-E1 cell response to PHBV may not be mediated by surface roughness and hydrophobicity alone. The discrepancy in the literature surrounding the effects of surface properties on cell phenotype is most likely a reflection of the complex nature of a biomaterial surface and the synergistic influence that various surface parameters have on cell behavior. Surface parameters not measured in this study could also be influential and further experimentation is needed to elucidate this. Use of techniques to carefully alter a single surface property alone will allow better understanding of the influence of that particular property on cell phenotype.

Despite the lag observed for some phenotypic parameters, cell development was otherwise normal on PHBV, especially given that it was compared with a substrate optimized for in vitro cell growth. Considering the engineering advantages and bioabsorbable properties of PHBV, we conclude that it is an appropriate substrate to augment bone regeneration. This study supports previous findings, and has further illustrated the necessity of a comprehensive evaluation of the cell response to a biomaterial surface. It has led to an improved understanding of the complex interactions that occur at this interface, crucial to the success of the implant in osseointegration and wound healing. More importantly, these in vitro data will allow improvements to be made to the implant system to ensure greater success in vivo. One such improvement would be to change the surface properties of PHBV or couple factors to it such as matrix proteins that would increase the rate of cell attachment and decrease any lag in cell growth.

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