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Osteoblast-like cell attachment to and calcification of novel phosphonate-containing polymeric substrates

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Abstract: In an attempt to interact natural bone and bone cells with biomaterials and to begin to develop modular tissue engineering scaffolds, substrates containing phosphonate groups were identified to mimic mineral–protein and natural polymer–protein interactions. In this study, we investigated poly(vinyl phosphonic acid) copolymer integration with existing materials as a graft-copolymer surface modification. Phosphonate-containing copolymer-modified surfaces were created and shown to have varying phosphate content within different polymeric surfaces. As the phosphonate content in the monomer feed approached 30% vinyl phosphonic acid, increased osteoblast-like cell adhesion (3- to 8-fold increase in adhesion) and proliferation (2- to10-fold increase in proliferation rate) was observed. Since surfaces modified with 30% vinyl phosphonic acid in the feed exhibited a maximal cell adhesion and proliferation (9.4×10^4 cells/cm^2/day), it was hypothesized that this copolymer composition was optimal for protein–polymer interactions. Osteoblast-like cells formed confluent layers and were able to differentiate on all surfaces that contained vinyl phosphonic acid. Most importantly, cells interacting with these surfaces were able to significantly mineralize the surface. These results suggest that phosphonate-containing polymers can be used to integrate biomaterials with natural bone and could be used for tissue engineering applications. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 78A: 433–440, 2006

Key words: osteoblast; surface interaction; tissue engineering; polymer

INTRODUCTION

Polymers, both natural and synthetic, are often utilized as matrices to initiate a repair or regenerative response, often referred to as tissue engineering.[1,2] To repair large tissues or defects, scaffolds need to be created that allow cells and signaling molecules to be properly localized and presented.[3] Materials currently used for scaffold design have not proven to be optimal in many settings,[4] suggesting the need for a new generation of materials designed specifically for biomedical use. The next generation scaffolds should mimic biologic matrices in a much better manner, specifically, natural biodegradability,[5] mineralization,[6] and protein interaction.[7] One way to create such systems is to form modular materials that may have different properties to allow different functions from various modules. Surfaces could be made to allow cell attachment and differentiation, while the structural basis of the scaffold may be made of a biodegradable material that allows strength over time. The structural material would then be remodeled as the cells integrate with the cell-adherent module. With this idea in mind, we have focused this study on creating graft copolymers on the surface of biomaterials, with the expectation

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that this layer will later be attached to a structural tissue engineering scaffold.

Extensive studies have been performed to render materials more cell adhesive utilizing biologic motifs and biologic mimicry. Surface modification of biomaterials with bioactive molecules is a simple way to increase cell adhesion, particularly for a specific cell type that expresses a given integrin.[8] Much work has also used ECM proteins, such as fibronectin, vitronectin, and laminin, for surface modification.[9] Biomaterials have been coated with these proteins, which usually have promoted cell adhesion and proliferation when the density of peptides or proteins was sufficient.[10] Native ECM proteins tend to be randomly folded upon adsorption to biomaterial surfaces, such that the receptor-binding domains are not always available or the proteins are irreversibly denatured.[11] To eliminate or minimize denaturation of cell-binding proteins, peptides have been coated or attached to surfaces.[12,13] However, single peptides and denatured proteins on surfaces interact with cells in an unnatural manner because of lack of appropriate three-dimensional binding domain presentation.[14] Alternately, natural ECM[15] and biomimetic protein adsorption[16] have been used to mimic natural protein presentation to cells.

Proteins are naturally sequestered in the extracellular matrix, specifically as polymer–protein complexes.[17] Heparin and other glycosaminoglycans interact with proteins through domains containing repeated basic (protein) and acidic (polymer) residues.[16] The overall charge of the protein influences the interaction, but basic and acidic proteins, for example, FGF-1 and FGF-2, both bind to heparin sulfate with high specificity due particularly to specialized and localized binding regions within the proteins. In addition to protein–polymer interactions, protein–mineral interactions are prevalent in biologic systems. Several proteins are now known to initiate nucleation of bone and tooth mineralized matrix.[18–20] These proteins generally interact with mineralized tissues through acidic residues, specifically phosphoserine. Because of the natural interactions of many bone-promoting proteins with acidic polymers, and the understanding that phosphate-rich proteins initiate bone growth, phosphonate-containing polymers were hypothesized to mimic natural polymers by interacting with serum proteins allowing natural-like adhesion and proliferation and initiating matrix mineralization.

Since first investigating this hypothesis,[7] we have become very interested in extending this work to allow (1) integrating the phosphonate-containing polymers with existing biomaterials and (2) determining if these materials allow cell-mediated mineralization. The goal of the present work was to understand osteoblast attachment, proliferation, and mineralized matrix production on polymeric materials having different compositions of poly(vinyl phosphonic acid). Phosphonate-containing, polymer-modified surfaces were created and shown to have varying phosphonate content within the polymeric surface. As the phosphonate content increases up to an optimal composition on the surfaces, increased osteoblast-like cell adhesion and proliferation was observed. Finally, we have shown that cells interacting with these surfaces are able to significantly mineralize the surface *in vitro* through cell-mediated mechanisms.

MATERIALS AND METHODS

Substrate preparation

All chemicals were purchased from Fisher Scientific or Sigma-Aldrich Chemical Company as chemical grade and used without purification unless otherwise noted. Circular glass coverslips (#1) or silicon surfaces (diameter 100 mm, thickness 500 μm, 10-Å silicon-oxide layer) were used for cell studies or surface analysis, respectively. Glass coverslips were cleaned and oxidized in chromic/sulfuric acid for 1 h. Following serial rinsing three times each in double distilled water, ethanol, and finally chloroform, each slide was dipped in a solution of 3% 3-acryloxypropyl trichlorosilane (AcPTS, United Chemical Industries, Bristol, PA) in chloroform[21] while being warmed to 60°C. All samples were subsequently washed sequentially in chloroform and ethanol baths three times for 1 min each before being dried and cured.

Graft polymer formation

Vinyl phosphonic acid (VPA) and acrylamide (AM) were each diluted in distilled deionized water to form stock solutions (50% w/v). In sterile 48-well tissue culture plates, monomer solutions, 250 μL total volume, were pipetted onto glass or the oxide surface of silicon substrates in individual wells. The total monomer concentration (VPA + AM) was maintained constant in all experiments, with only the relative feed ratio of VPA and AM changing by varying the volume of 50% VPA and 50% AM added. An aliquot (8 μL) of an aqueous solution of 9.0% 2,2-dimethoxy-2-phenylacetophenome (DMPA) in dimethyl sulfoxide (DMSO) was added to each of the monomer mixtures before photopolymerization. Following mixing with a pipette, samples were exposed to an ultraviolet light source (365 nm; 2.5 mW/cm^2) for 15 min. Using this system, surfaces grafted with poly(vinyl phosphonic acid-*co*-acrylamide) (pVPA-AM) of varying concentrations were created.[21] Throughout this article, the composition of the feed monomer is used to describe each grafted surface: pVPA40 refers to a surface that has 40% VPA monomer and 60% AM monomer in the monomer mixture.

Surface characterization

Silicon substrates were analyzed using a Kratos Axis ULTRA Imaging X-ray photoelectron spectrometer (XPS). XPS measurements were conducted at room temperature with a base pressure in the 1.5×10^{-9} Torr range with a monochromatic Al Kα X-ray source operated at 140 W (14 kV, 10 mA). The energy scale was calibrated with reference to the Cu_{2p} and Ag_{3d} peaks at binding energies of 932.7 and 368 eV. The area of analysis for high-resolution spectra was 2 mm wide by 1 mm long. Carbon (C_{1s}), nitrogen (N_{1s}), oxygen (O_{1s}), phosphate (P_{2p}), and silicon (Si_{2p}) spectra were acquired at a pass energy of 20 eV, a maximum acquisition time of 10 min per sample, and five passes per sample. Quantitative XPS analysis was computed with Kratos VISION software. The relative atomic concentrations were calculated using tabulated atomic sensitivity factors.

Osteoblast-like cell proliferation

MC3T3-E1 subclone 4 cells (CRL-2593, American Type Culture Collection)[22] represent a clonal osteogenic cell line commonly used to study the process of osteoblast maturation. MC3T3-E1 subclone 4 cells were maintained in 75-cm^2 tissue culture flasks in supplemented α-MEM, without ascorbic acid, supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 in air at 37°C. After reaching around 75% confluence, cells were passed by treatment with 0.05% trypsin. Cells were replated in 24-well tissue culture with the silanized pVPA-AM glass disks at the bottom of each well. The cells were plated at a density of $\sim 2 \times 10^4$ cells/cm^2 and grown in α-MEM supplemented with 10% fetal bovine serum for specific periods.

Hematoxylin and Eosin were used to stain the nucleus and cytoplasmic material of the cells at specific periods to visualize cells. At the specified times, cells were observed and counted using an inverted microscope (Olympus IX-70 microscope). Three different viewing areas of each coverslip were counted and an average of three surfaces was calculated and recorded. The total number of cells in each field was counted and used to determine the cell population.

Osteoblast-like cell matrix mineralization

Cells were plated on test substrates in 24-well tissue culture plates as described earlier. After reaching 75% confluence, supplemented α-MEM without ascorbic acid was replaced with supplemented α-MEM with ascorbic acid. Cells were kept in a humidified atmosphere of 5% CO_2 in air at 37°C, where the media was changed every 2–3 days and cells were observed by light microscopy. At various time points, cells were stained with Von Kossa stain and viewed for mineral formation. Briefly, cells were fixed in 4% paraformaldehyde for 30 min. The solution was aspirated and a 5% solution of silver nitrate was administered followed by exposure to a 60-Watt light source. The 24-well plate was wrapped with aluminum foil to reflect the light to all surfaces. This solution was carefully aspirated, then washed separately with 5% sodium thiosulfate and double distilled water.

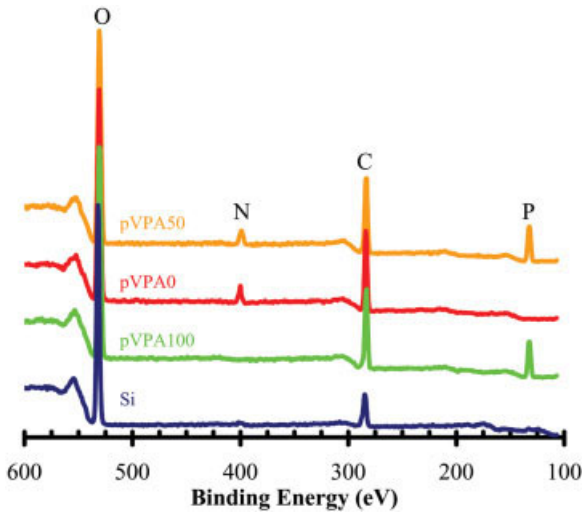

Figure 1. Survey X-ray photoelectron spectrographs of bare silicon substrate (Si), poly(vinyl phosphonic acid)-grafted surface (pVPA100), polyacrylamide-grafted surface (pVPA0), and surfaces grafted with equimolar VPA and AM in the monomer feed (pVPA50). Oxygen (~533 eV), nitrogen (~400 eV), carbon (~285 eV), and phosphorus (~132 eV) peaks are marked above the spectra. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Statistical analysis

All data are expressed as a mean ± standard deviation and was compared using two-way ANOVA with subsequent *post hoc* test. Differences at $p < 0.05$ were considered to be statistically significant. All samples were examined in triplicate at a minimum. Finally, all error bars are presented as standard deviations.

RESULTS

Chemical analysis

There was no presence of phosphate, P_{2p}, or nitrogen, N_{1s}, on the unmodified silicon surface (Fig. 1). Trace carbon was present on the surface because of atmospheric contamination, as expected. All pVPA surfaces had increased carbon and decreased silicon presence compared with that of unmodified surfaces. The pVPA100 (100% VPA in monomer feed) modified surfaces had significant phosphate, and no nitrogen detected, while the antithesis, pVPA0-modified surfaces, (100% AM in monomer feed) had significant nitrogen, and no phosphate detected. The pVPA50-

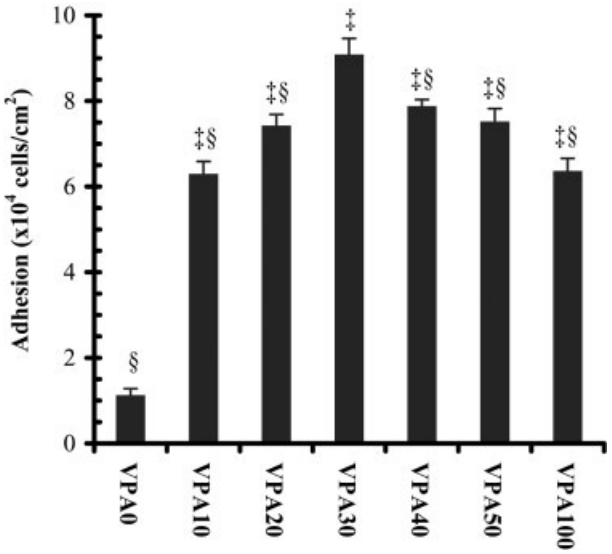

Figure 2. Number (adhesion) of MC3T3-E1 subclone 4 preosteoblast cells adhered to substrates modified with graft copolymers created from feed compositions of increasing VPA. Surfaces were modified with polyacrylamide (pVPA0), poly(vinyl phosphonic acid) (pVPA100), and 10% (pVPA10), 20% (pVPA20), 30% (pVPA30), 40% (pVPA40), and 50% (pVPA50) vinyl phopsphonic acid in the monomer mixture of VPA and AM. Symbols represent statistically significant ($p < 0.01$) difference from pVPA0 (‡) or pVPA30 (§).

modified surfaces showed traces of all the expected elements, phosphate, nitrogen, oxygen, and carbon at intermediate concentrations to those observed for pVPA100- and pVPA0-modified surfaces. Trace silicon was detected as part of all surfaces at nearly equal composition.

From high-resolution spectra, detected phosphate increased with a corresponding increase in the percentage of VPA present in the monomer feed. Conversely, detected nitrogen decreased with a corresponding increase in VPA. Also, as expected, detected carbon increased with a higher percentage of VPA in the monomer feed.

MC3T3-E1 subclone 4 adhesion and proliferation

Twenty-four hours after initial seeding of an equal number of cells on each sample, pVPA30-modified surfaces had the highest number of cells adherent and the pVPA0 samples had the lowest cell adherence (Fig. 2). All modified surfaces (above pVPA0) exhibited greater cell adhesion than pVPA0-modified surfaces. The number of adherent cells on modified surfaces exhibited a statistical maximum for pVPA30-modified surfaces, with fewer cells binding to pVPA-modified surfaces with greater or less VPA in the feed.

After 7 days, cells seeded on surfaces with pVPA continued to proliferate, with the pVPA30-modified surfaces having the highest cell count at all times (Fig. 3). Because of the cell repellant nature of AM, the pVPA0-modified surfaces showed proliferation at a much lower extent than that of the samples containing even a small amount of VPA in the monomer feed. Proliferation continued on all samples through the fourth day. All pVPA-modified surfaces reached confluence, with the pVPA30-modified surfaces still having the highest number of cells per area (Fig. 4). Only pVPA0- (no phosphonate groups present) modified surfaces never formed a confluent monolayer, but there was slight proliferation over the 7-day period.

The rate of proliferation on pVPA-modified surfaces ranged from 3.88×10^4 cells/cm^2/day to 9.4×10^4 cells/cm^2/day, while cells grown on pVPA0-modified surfaces proliferated at a rate of 0.29×10^4 cells/cm^2/day. The proliferation rate on all pVPA-modified surfaces was statistically greater than the proliferation rate on pVPA0-modified surface. There was no statistical difference in proliferation rate for cells grown on pVPA10-, pVPA20-, pVPA40-, and pVPA50-modified surfaces. Only pVPA30 surfaces exhibited a statistically increased proliferation rate from the other pVPA-modified surfaces.

MC3T3-E1 subclone 4 matrix production

On pVPA30-modified surfaces, MC3T3-E1 subclone 4 cells reached confluence between days 3 and 5 (Fig. 5). At this point, media was replaced with an ascorbic

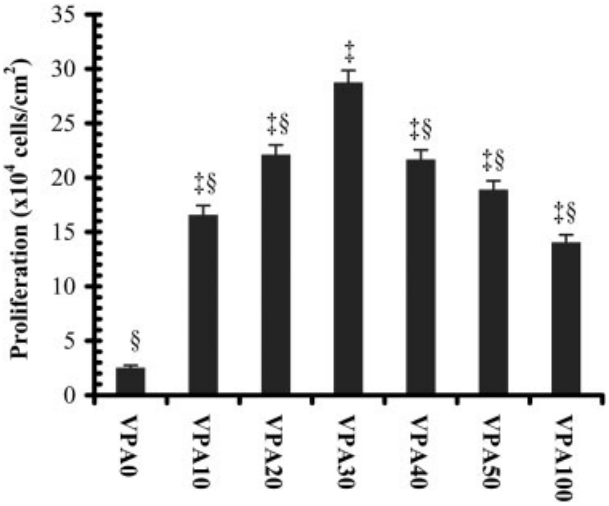

Figure 3. Number (proliferation) of MC3T3-E1 subclone 4 preosteoblast cells present after 7-day incubation on substrates modified with graft copolymers created from feed compositions of increasing VPA. Surfaces were modified with polyacrylamide (pVPA0), poly(vinyl phosphonic acid) (pVPA100), and 10% (pVPA10), 20% (pVPA20), 30% (pVPA30), 40% (pVPA40), and 50% (pVPA50) VPA in the monomer mixture of VPA and AM. Symbols represent statistically significant ($p < 0.01$) difference from pVPA0 (‡) or pVPA30 (§).

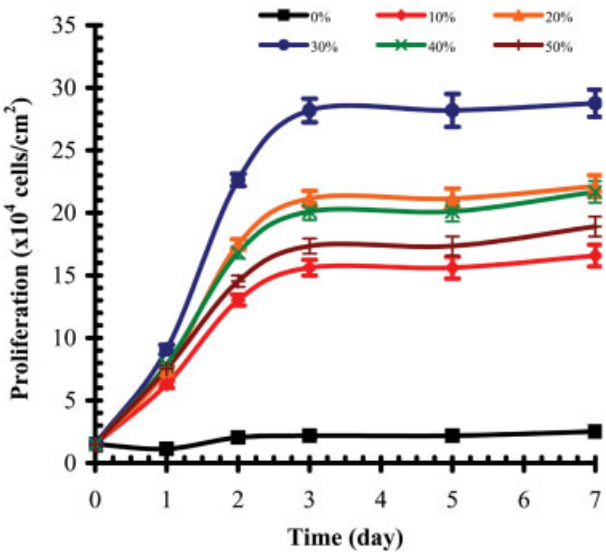

Figure 4. Number (proliferation) of MC3T3-E1 subclone 4 preosteoblast cells present on substrates modified with graft copolymers created from feed compositions of increasing VPA. Surfaces were modified with polyacrylamide (pVPA0), poly-(vinyl phosphonic acid) (pVPA100), and 10% (pVPA10), 20% (pVPA20), 30% (pVPA30), 40% (pVPA40), and 50% (pVPA50) vinyl phopsphonic acid in the monomer mixture of VPA and AM. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

acid supplemented media to initiate differentiation.[22,23] This allowed for differentiation of the cells into osteoblasts, and, in turn, matrix production. After 21 days of exposure to ascorbic acid supplemented media (25 days after seeding), the MC3T3-E1 subclone 4 cells were stained using the Von Kossa method for the visualization of mineralized matrix. Samples coated with pVPA30 showed significant areas of mineralization, black, while pVPA0 samples showed little mineralization (Fig. 6). This was partially due to lack of cell confluence for the pVPA0-modified surfaces. To confirm this, all created pVPA-modified surfaces were exposed to similar culture conditions without cells and these surfaces showed no observable mineralization at equivalent time points.

DISCUSSION

The ability to create surface coatings on biomaterials is well established with varying methods.[24–29] Regardless of the method, the surface must be confirmed to contain the chemical structure expected from the modification. Chemical characterization demonstrated the ability to create graft copolymers on glass substrates using conventional chemistries. As expected, the percentage of phosphate detected in a sample increased with increasing levels of VPA in the feed. Correspondingly, the percentage of nitrogen decreased with increasing levels of VPA. Further studies are necessary to elucidate the exact compositions of the polymers on the surface and the thickness of the polymer brush that it formed, but the fact that the composition of the

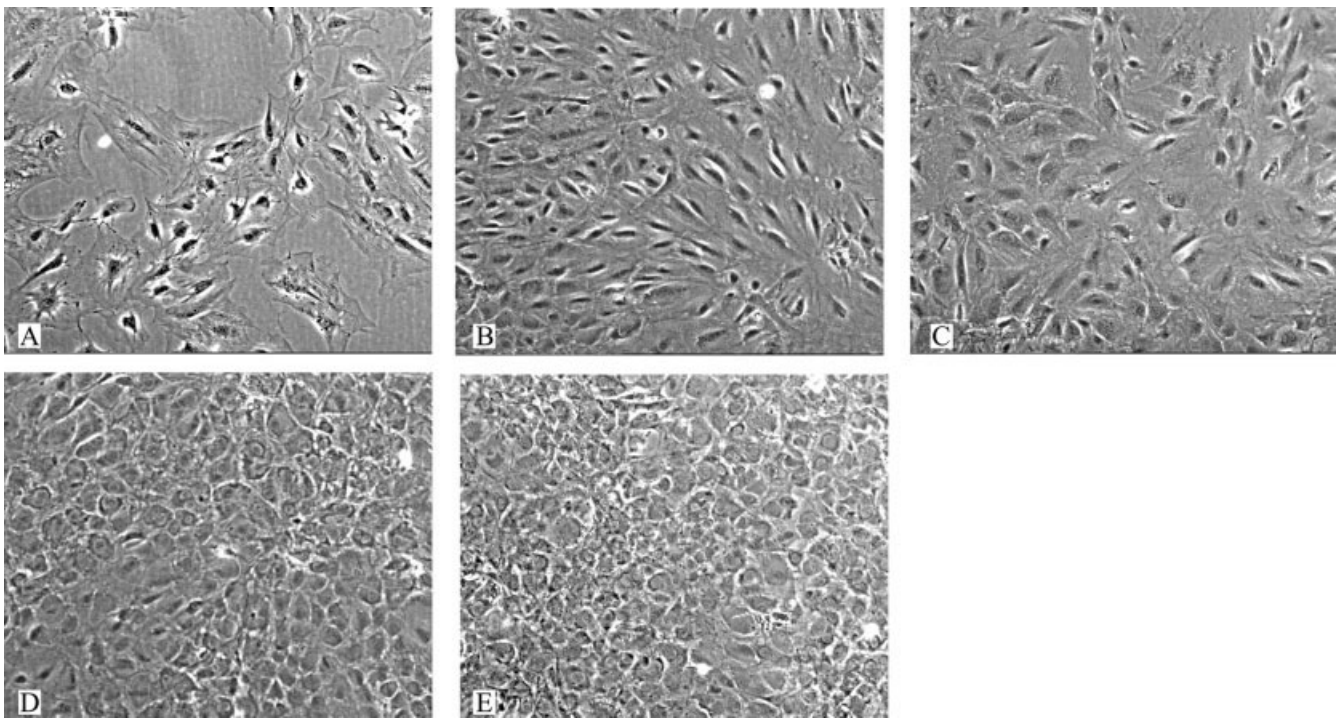

Figure 5. Micrographs of MC3T3-E1 subclone 4 preosteoblast cells grown on substrates with grafted (pVPA-AM) made with 30% VPA in the monomer feed on days (A) 1, (B) 2, (C) 3, (D) 5, and (E) 7 after seeding.

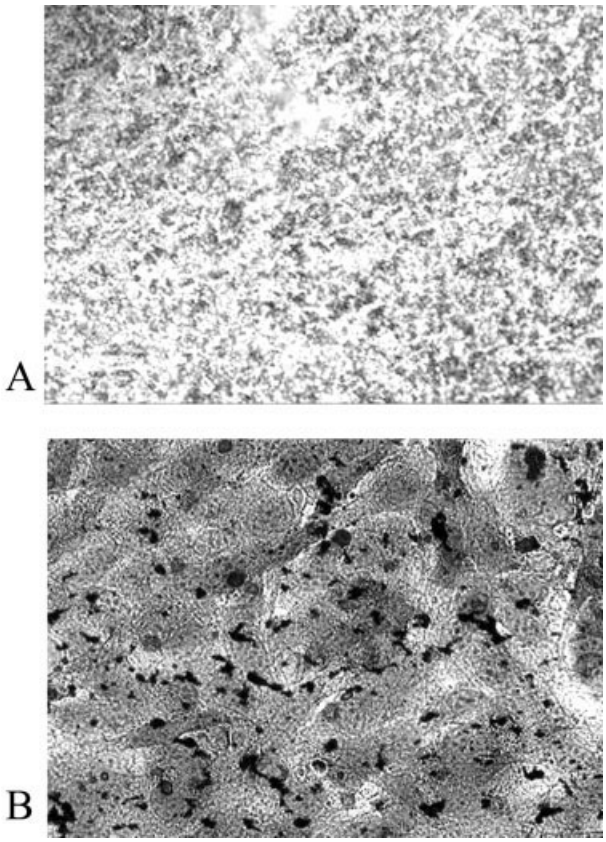

Figure 6. Micrographs of MC3T3-E1 subclone 4 cells grown on surfaces modified with (pVPA-AM) made with 100% AM (VPA0; (A)) and 30% VPA (pVPA30; (B)) in the monomer feed. Cells were stained with von Kossa stain following 4 days of culture and 21 days of differentiations with ascorbic acid.

graft copolymers has an impact on cell adhesion and proliferation is clear.

In similar studies, Tretinnikov et al. grafted polymer chains with phosphate functional groups onto argon plasma-pretreated films by ultraviolet light induced polymerization.[30,31] The macroscopic wetability of the grafted polymer was controlled by structure of the polymer, which was determined by the graft density. It was proposed that a high density of grafted polymer chains stretched normal to the surface and formed polymer "brushes". It is possible that the pVPA-AM grafted density was low enough that a randomized "mushroom-like" structure of polymer formed on the surface. This would lead to an unequal layer of thickness of pVPA-AM on the surface and potential cell adhesion to areas of minimal coverage. This is unlikely, since cells showed uniform adhesion on the pVPA-AM modified surfaces in our study.

Most noticeable was the lack of cell proliferation on surfaces grafted with just AM. If a patchwork of pAM was present, cells should still adhere, proliferate, and differentiate. Not only did pAM-grafted surfaces repel cells, but pVPA-grafted surfaces with VPA feed above and below pVPA30 had lower cell adhesion and proliferation than that of pVPA30. If VPA polymerization caused a substantial change to the polymer surface structure, this would not be expected. If the idea of nonuniform polymer surface is discarded, then pVPA-AM–protein and direct pVPA-AM–cell interactions must be present. The idea that phosphonate-containing polymers interact with cell-adhesive, naturally occurring proteins is becoming highly accepted.[7,32]

The adhesion and proliferation of cells further suggest that the polymers interact with serum proteins and subsequently allow natural attachment and differentiation. This is supported by the fact that the preosteoblasts not only adhere and proliferate on the surfaces, but also differentiate. Lee et al. previously demonstrated that the clonally derived, murine MC3T3-E1 cell line expresses factors of the osteoblast phenotype, including alkaline phosphatase, type I collagen, and nodular extracellular matrix mineralization.[23] It is unexpected that the cells will produce a mineralized matrix when they are placed in an environment that is not conducive to growth, as would be expected for cells with little cell–cell interactions and natural cell–matrix interactions. In this study, the final stage of differentiation (mineralization) was observed out of the three stages of osteoblast differentiation *in vitro*.[22] The first stage was characterized as cell proliferation and high-level type I collagen gene expression, synthesis, and secretion, but the cells remain mostly undifferentiated. Downregulation of replication and expression of the differentiated osteoblast factors was characterized during the second stage, which continued through approximately, the 10th day. Finally, MC3T3-E1 maturation began at about the 20th day and was defined by matrix calcification and increased alkaline phosphatase activity. In this study, based on the dramatic increase of cell proliferation occurring through the fifth day, this day was thought to be a transition point from the first stage to the second stage of differentiation. The earliest noticeable matrix began around day 20 in the experimental groups. This calcification progressed to where it was very apparent after day 24. Based on these results, the transition between stage two and stage three of MC3T3-E1 subclone 4 cells occurred around day 21. This is similar to that observed for tissue culture experiments where mineralization was cell mediated.[22]

The mineralization of pVPA-modified surfaces was potentially due to both cellular differentiation and also polymer-based calcification. Natural proteins and polymers promote osteogenesis *in vivo* and this was possible in our studies. It is expected that these surfaces, if placed in high calcium/phosphate solutions, would promote spontaneous calcification.[33] Modified surfaces incubated in media without cells did not show appreciable mineralization, but pVPA-modified surfaces with seeded cells did show mineralization. Kato et al. grafted polymer chains with phosphate side

groups onto substrates which, under physiological conditions, produced a thin hydroxyapatite layer on the surface of the substrate.[34] This process, like the pVPA-AM surface modification, mimics the biologic mineralization process and can serve as a microscopic lattice for osteoblasts and hydroxyapatite production. It is expected that the pVPA-modified surfaces will mineralize over time without cells, but the cell-mediated mineralization would more benefit osteointegration.

This is not the first attempt to incorporate pVPA-based and phosphate-based polymer systems for biomedical applications to initiate osteointegration. Tan et al. used the same polymers to form a hydrated copolymer hydrogel network which could be used as a bone tissue engineering scaffold.[7] Hydrogels have mechanical properties which can mimic soft tissues and the hydrogel's mesh sizes can be manipulated to allow ideal diffusion and transport of most biologic molecules. The pVPA hydrogels supported cell adhesion and proliferation, and it is expected that mineralization will follow (unpublished data). In addition, ethylene glycol methacrylate phosphate (EGMP) was incorporated into poly(ethylene glycol) hydrogel scaffolds by photopolymerization in the presence of cells. EGMP-containing hydrogels were shown to improve cell survival, to sequester osteopontin, and to spontaneously mineralize.[32] These hydrogels allowed cells to survive for short- and long-term better than poly(ethylene glycol) hydrogels, even though the EGMP monomer was toxic to cells in culture. It was not presented if cells increased mineralization beyond polymer-induced values, but it is expected that this would be the case. This further promotes the idea that phosphate- and phosphonate-containing polymers can increase the growth, proliferation, or adhesion of cells. Unfortunately, hydrogels are not appropriate for many tissue engineering applications because of the weak nature of the hydrogel. Thus, utilizing the protein-binding cell-adhesive polymers such as pVPA as a surface coating may benefit the tissue engineer in the future, specifically if these polymers can be integrated (as a module) into structural scaffolds, for example, poly(lactide-*co*-glycolide)[6,35] and poly(propylene fumarate).[36]

CONCLUSIONS

To study the proliferation and matrix production of osteoblast-like cells, we prepared an array of surfaces modified by grafting of different amounts of poly(vinyl phosphonic acid) copolymers. X-ray photoelectron spectroscopy was utilized to determine the precise concentration of phosphate, nitrogen, oxygen, and carbon on prepared samples of poly(vinyl phosphonic acid). The prepared poly(vinyl phosphonic acid) surfaces were not cytotoxic. In fact, poly(vinyl phosphonic acid)-modified surfaces promote cell adhesion and proliferation at all compositions created. The optimal concentration of poly(vinyl phosphonic acid) was determined to be around 30%, where the highest and fastest proliferation rate was observed. It was clear that the poly(vinyl phosphonic acid) modified surfaces enhanced both differentiation and mineralization of MC3T3-E1 subclone 4 cells *in vitro*. This study indicated that it is possible for preosteoblasts to adhere, proliferate, and mineralize on surfaces grafted with different compositions of poly(vinyl phosphonic acid). The results of this study further the potential use of poly(vinyl phosphonic acid) copolymers as polymers for use in bone tissue engineering scaffolds design.

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