Combinatorial growth of oxide nanoscaffolds and its influence in osteoblast cell adhesion

Claudia Y. Acevedo-Morantes,¹ Roberto A. Irizarry-Ortiz,² Pablo G. Caceres-Valencia,² Surinder P. Singh,¹ and Jaime E. Ramirez-Vick^{1,a)}

¹Department of Engineering Science & Materials, University of Puerto Rico, Puerto Rico 00680, USA

²Department of Mechanical Engineering, University of Puerto Rico, Puerto Rico 00680, USA

(Received 1 June 2011; accepted 26 August 2011; published online 17 May 2012)

We report a novel method for high-throughput investigations on cell-material interactions based on metal oxide nanoscaffolds. These scaffolds possess a continuous gradient of various titanium alloys allowing the compositional and morphological variation that could substantially improve the formation of an osseointegrative interface with bone. The model nanoscaffold has been fabricated on commercially pure titanium (cp-Ti) substrate with a compositional gradients of tin (Sn), chromium (Cr), and niobium (Nb) deposited using a combinatorial approach followed by annealing to create native oxide surface. As an *in vitro* test system, the human fetal osteoblastic cell line (hFOB 1.19) has been used. Cell-adhesion of hFOB 1.19 cells and the suitability of these alloys have been evaluated for cell-morphology, cell-number, and protein adsorption. Although, cell-morphology was not affected by surface composition, cell-proliferation rates varied significantly with surface metal oxide composition; with the Sn- and Nb-rich regions showing the highest proliferation rate and the Cr-rich regions presenting the lowest. The results suggest that Sn and Nb rich regions on surface seems to promote hFOB 1.19 cell proliferation and may therefore be considered as implant material candidates that deserve further analysis. © 2012 American Institute of Physics.

[<http://dx.doi.org/10.1063/1.4714727>]

I. INTRODUCTION

Combinatorial material science yields a large number of well-defined products by mixing a small number of starting chemical reagents in various proportions defined by a given reaction scheme and process conditions. This approach allows exploration of multidimensional chemical composition and process parameter space at a previously unavailable level of detail avoiding endless repetition of single experiments.¹

The lack of suitable high-throughput assays is particularly problematic in the field of biomaterials research where a wide range of specialized tests are required. Examples of key biomaterial properties include surface protein adsorption, rate and mechanism of degradation, specific biological responses of cells contacting the material surface, cytotoxicity, and degree of biocompatibility *in vivo*. Each of the above mentioned properties must be explored through rather tedious experiments. This is a critical bottleneck in the implementation of a combinatorial biomaterials discovery process.

The U.S. Prosthetic Implant market is about \$1 billion a year and growing at a rate of 10% per year; any improvement in osseointegration and lifetime of the prosthetic implant will have a considerable economic impact, especially if one takes into consideration that up to 30% of the 700 000 implants in procedures such as hip and knee replacement do not show proper osseointegration.² Osseointegration is a close structural and functional contact between living bone and the surface of an implant material.² Improvements in osseointegration requires focusing efforts in the bone-

implant interface, which is complex and involves numerous factors, e.g., implant material-related factors (composition, topography, and surface energy), mechanical loading, surgical technique, and patient variables, such as bone quantity and quality.³ Since osseointegration depends on the direct interaction between bone cells with the biomaterial, the understanding of the adhesion of osteoblasts to the bone-implant interface will pave the way to design new and efficient implant materials. An osteoblast culture model for *in vitro* investigation of the effect of surface structures and composition of implant materials on the different stages of osseointegration will suffice the different parameters.

Commercially pure titanium (cpTi) and its alloys (e.g., Ti-6Al-4V and Ti-6Al-7Nb) are widely used for the fabrication of prosthetic devices with the latter being commonly used for load bearing devices such as artificial hip joint stems. The attractive features of cpTi as a biomaterial include its excellent corrosion resistance, cytocompatibility, and good strength-to-weight ratio.⁴ The induction of corrosion resistance and cytocompatibility is related to the presence of an oxide (passive) layer that prevents the formation of fibrous tissue around the implant and creates direct contact to the bone tissue. Hanawa *et al.*^{5,6} have shown that presence of oxide layer also has a significant effect Ca/P ratio in the calcium phosphate (close to hydroxyapatite a bone material) film compared to pure titanium alloy during mineralization.

Physicochemical characteristics of surfaces have been found to influence cell attachment and proliferation, for example, the surface charge properties have been found to influence osteoblast cell attachment,¹¹ while investigations on the effect of surface energy of the materials¹² revealed that low osteoblast cell attachment and collagen production were related to

^{a)}Author to whom correspondence should be addressed. Electronic mail: jvick@ece.uprm.edu.

a low surface energy (hydrophobic) of substrates. It was suggested that an enhancement of polar components on the surface improves cell attachment and matrix synthesis.^{11–14} Lim *et al.*^{15–20} demonstrated that high surface energy (hydrophilic) surfaces supported homogeneous spatial osteoblast cell growth and mineral deposition, relative to low surface energy (hydrophobic) surfaces using a conditionally immortalized, human fetal-osteoblast cell line, hFOB 1.19.

In the case of Ti6Al4V, there is a formation of mixed oxide layer consisting of mainly TiO₂ with 6–8 at. % Al and less than 0.1 at. % V in the outermost atomic layers of the oxide.^{7–9} The percentage of area coverage by each of these oxides is strongly dependent on the surface composition and processing conditions. In comparison with cpTi, the calcium phosphates formed on Ti6Al4V had a Ca/P ratio less similar to that of hydroxyapatite. The dual character of the oxide film, i.e., the presence of areas covered by an aluminum oxide film on Ti6Al4V may have caused the changes in the Ca/P ratio.^{5,6,10} It is thus important to understand how the changes in surface composition, for example, by the local surface presence of Al-rich and V-rich oxides (in a matrix of TiO₂) influence cell adhesion and development and if this can be traced back to local contrast in protein adsorption. However, since the surface chemistry cannot be completely dissociated from the surface topography and energy, all of these need to be studied concomitantly. In addition, the presence of terraces, ledges kinks, and other nano and microstructures on the surface play a crucial role and are dictated by the processing conditions.

Although, all investigations indicate that surface chemistry plays a crucial role on osteoblast function, but the correlations and pathways between specific surface parameters and cell functions are not entirely clear. The present study aims to design a high throughput platform by depositing the different titanium alloys, with tin (Sn), chromium (Cr), and niobium (Nb) on a single titanium disk. A combinatorial physical vapor deposition technique has been used to create such alloys. The suitability for cell growth; including the evaluation of cell-morphology and cell-proliferation on designed alloys has been studied using the hFOB 1.19 osteoblast cell line.

II. MATERIALS AND METHODS

A. Substrate fabrication

Silicon carbide paper of grits 240, 320, 400, and 600 was used sequentially to grind the metallic surfaces. After grinding, a solution of Al₂O₃ (alumina particles 5 μ m and 3 μ m in size) was used as the polishing agent. The substrates were further polished by a polycrystalline diamond suspension with particle size of 3 μ m.

Systematic gradient of three different metals on the polished substrate surface (cpTi grade 1) was achieved by combinatorial deposition using magnetron sputtering (Figure 1). The thin-film thicknesses varied from a minimum value on one extreme of the disc to a maximum value of 20–40 nm on the other. Three elements: Tin (starting from point 1), Chromium (starting from point 7), and Niobium (starting from point 13) were deposited 120 degrees out phase between each other cre-

FIG. 1. Localization of the 28 points in the triangle inside of the CCS disc and the nine points on the cpTi controls (points A-I).

ating a continuous compositional spread (CCS) in the form of a triangle on the cpTi disc of 7.6 cm in diameter and 0.1 cm in thickness.

Thin film deposition was followed by low temperature annealing under protective atmosphere, using a plasma enhanced chemical vapor deposition (PECVD), where oxygen plasma was used to generate oxide scaffolds with large chemical composition spreads. The PECVD allows controlled oxidation on the surface of the substrate using a low temperature treatment that yields oxide films that are different in character than the ones obtained through high temperature processes or by electrolytic anodizing.

B. Substrate characterization

Scanning electron microscopy (SEM) was used to characterize the surface morphology of the oxide formed on the cpTi disks using a Field Emission Scanning Electron Microscope Supra 35 (Zeiss). The discs were analyzed in the “as-received” condition and without the use of coatings. The equipment allows analysis of features in the nanometer range using low accelerating voltage (3 kV). The metallic composition of the surface layers of the CCS disc was analyzed at Iowa State University (Prof. K. Rajan) using Auger Electron Spectroscopy (AES) using a JEOL JAMP-7830F equipped with a back-scatter electron detector (200 nm resolution) and EDS (~ 1 μ m resolution). The typical voltage employed in Auger and EDS was 15 kV.

C. Cell culture

Human osteoblast cell line hFOB 1.19 (CRL-11372, ATCC, Manassas, VA, USA) was cultured in 90% Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 Ham, 1:1 (DMEM) (Sigma-Aldrich, St. Louis, MO) with 2.5 mM L-Glutamine and 15 mM Hepes, without phenol red, supplemented with 0.3 mg/mL G418 (Sigma-Aldrich, St. Louis, MO) and 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich, St. Louis, MO). Cells were cultured at 33.5 °C and 5% CO₂. At confluence, cells were trypsinized and seeded at a final concentration of 1×10^4 cells/cm² onto the substrates.

D. Substrate preparation for cell culture experiments

CCS discs were used as experimental groups, and Ti6Al4V, were used as controls for cell growth. CCS discs were 7.6 cm in diameter and 0.1 cm in height (44.17 cm² area), Ti6Al4V was 0.8 cm in diameter and 0.1 cm in height (0.5 cm² area). Metal discs were incubated during 30 min with 0.53 mM EDTA/D-PBSA, washed with distilled deionized water, and the discs were scrapped using a plastic cell scraper, washed with absolute alcohol and washed again with distilled deionized water. Thereafter, the discs and controls were sterilized by autoclaving at 15 psi and 121 °C and placed in sterile Petri and 6-well plates (Corning, NY), respectively.

E. Cell culture experiments and imaging

The hFOB 1.19 cells were cultured on substrates at a density of 10⁴ cells/cm² and incubated for 3 days, at 33.5 °C and 5% CO₂. The following controls were used: a cell growth control (cells growing on Ti6Al4V) and a negative control (discs incubated with cell culture media without cells). Substrates were washed twice with serum-free medium and once with D-PBSA. Cells were fixed using methanol at 4 °C for 10 min and washed three times with D-PBSA. Non-specific staining was blocked by incubation in blocking solution (1% BSA in D-PBSA) for 1 h at room temperature. The blocking solution was removed, and the cells were permeabilized with 0.5% triton X-100 at 4 °C for 5 min, washed with D-PBSA, and incubated with Phalloidin-TRITC (Sigma-Aldrich, St. Louis, MO) at 10 µg/mL. After 1 h at room temperature of incubation, substrates were washed with D-PBSA and followed by the addition of 0.8 mg/mL DAPI (Research organics, Cleveland, OH), for 15 min at 37 °C. After washing again the substrates with D-PBSA, they were fixed with mounting media and stored at 4 °C in the dark. Images were captured using an inverted confocal laser scanning microscope (CLSM) (Fluo-View 300, Olympus, USA) equipped with 405LDnm, 543 nm Green HeNe, and 633 nm Red HeNe lasers. Phalloidin-TRITC has a peak excitation at 540–545 nm and a peak emission at 570–573 nm, while DAPI has a peak excitation at 345–350 nm and a peak emission at 455–470 nm.

Cell morphology was assessed via image analysis in which cell boundaries were outlined and analyzed with the Circularity plug-in for Image J (Wayne Rasband, NIH). The image was split into the different channels and calibrated using the scale bar. The method iterates using traditional maximum entropy-based thresholding methods for image segmentation. Morphology was then quantified using circularity values ($C = 4\pi \text{area}/\text{perimeter}^2$) averaged for cells in each frame ($n > 15$).

F. Proliferation rate of hFOB 1.19 cells

Cell-proliferation was measured at 1, 2, 3, 7, 14, and 21 days, on the 28 points on the CCS disc (1–28) and on nine cpTi points (A–I) (Figure 1). Cells were cultured on substrates at a density of 10⁴ cells/cm² and incubated at 33.5 °C and 5% CO₂, following labeling with Phalloidin-TRITC (Sigma-Aldrich, St. Louis, MO) at 10 µg/mL. Control groups were used at every stage of the process as described earlier.

Images were observed using a reflected-light fluorescence microscope (Model L2001, Microscopes Inc.) equipped with excitation filters 420–485 nm and 460–550 nm and emission filters 515 nm and 590 nm, and captured with a Coolpix 4500 digital camera (Nikon, NJ).

G. Cell viability

Viability of the cells cultured on the CCS substrates were assessed at prescribed intervals through the duration of the study (specifically, after 1, 7, 14, 21, and 28 days of culture) using the commercially available LIVE/DEAD viability/cytotoxicity assay (Invitrogen) and following manufacturer's instructions. Stained cells were visualized in situ using fluorescent microscopy. Live cells appear green (excitation: 494 nm; emission: 517 nm), while dead cells appear red (excitation: 528 nm; emission: 617 nm). Micrographs (taken at five random fields of view) were obtained and used to quantify the number of live and dead cells per substrate tested; percent cell viability on each substrate was thus obtained. These experiments were conducted in triplicate (per novel and pertinent control substrates) and repeated at three separate times.

H. Statistical analysis

Statistical analyses were carried out using PRISM software (Graphpad, USA). One way analysis of variance, followed by a Bonferroni post-test was used. In cases where a significant difference between sample variances was indicated (Bartlett's test), the Kruskal–Wallis non-parametric analysis of variance was used. Significance was determined at the 95% level in either case.

III. RESULTS AND DISCUSSION

A. Substrate characterization

AES measurements were taken at each vertex of triangle as well as in the midpoint of each side. The variation range in chemical composition for Sn was 8.23 to 19.42 at. %, for Cr 46.54 to 70.22 at. % and for Nb from 11.25 to 45.22 at. %. These data show that for a Ti substrate, the Sn, Cr, and Nb elements are distributed forming a continuous gradient of composition forming a compositional library, where each point in the triangle corresponds to specific combination of the metal species and the substrate.

The surface topography and grain size variation of the designed titanium oxide and combinatorial alloyed scaffolds substrates have been studied by FE-SEM. Figure 2 shows the topography at different points, i.e., 1 (Sn-rich area), 7 (Cr-rich area), 13 (Nb-rich area), and 24 at the center of the CCS triangle on titanium substrate. The Sn-rich area shows less discrete particles with high surface roughness consisting of terraces and ledges (Figure 2(a)). This morphological evolution could be attributed to the lower melting point of Sn compared to the other metal components. The Cr and Nb-rich areas show a larger number of particles with variations in size. Larger discrete particles of around 100 nm could be observed at the Cr rich area (Figure 2(b)). While regions of densely packed particles, a few nanometers in size, could be seen in the Nb-rich area (Figure 2(c)). The center region

FIG. 2. SEM of surface topography of the CSS disc in the points: (a) point 1 Sn-rich area, (b) point 7 Cr-rich-area, (c) point 13 Nb-rich area, and (d) center point 24. Pictures show variations in the surface topography of the substrate as well as variation in size of the titanium oxide scaffolds.

located at point 24 shows discrete particles about 300nm in size (Figure 2(d)).

B. Cell morphology on CCS discs

To understand how cell morphology is affected by surface properties we must focus on the osteoblast-biomaterial interaction process which involves attachment, adhesion, and spreading. Initial attachment requires the formation of an adsorbed protein layer on the biomaterial surface. The stabilization of this attachment depends on how accessible are the cell binding motifs (e.g., RGD) within these proteins. When the cells become stable, they will spread through the development of protrusions of their leading edge to form a lamellipodium. There are two types of adhesion structures focal adhesions and close contacts; the focal adhesion is much stronger bond and is associated with a low cellular motility.²¹ The strength of this adhesion will define their morphology, motility, and their capacity for proliferation and differentiation.²²

Cells were stained with Phalloidin-TRITC and DAPI to enable fluorescent imaging of the F-actin cytoskeleton and nuclei, respectively. CLSM micrographs obtained at 10X on the three corners and the center of the CCS triangle allowed the analysis of the hFOB 1.19 cell morphology as a function of surface composition, using cpTi, Ti6Al4V as a control (Figure 3). Cell morphology was quantified using the measure of circularity (C) as described in Sec. II, in which a value of 1 indicates a perfect circle while a value approach-

ing 0 describes spindle-type morphologies, thus providing a description of cell spreading and shape.

The number of attached cells per unit area was higher in the center and Nb-rich corner (point 13), where Nb and Cr were found to be in higher concentration (Figures 3(b) and 3(d), respectively). These values were comparable to the controls as seen in the case of Ti6Al4V ($p > 0.05$) (Figure 3(e)). In contrast, points 1 and 7 showed a much lower number of attached cells per unit area (Figures 3(a) and 3(c), respectively). Although these regions were rich in Cr, the amount of Nb was much lower than in the heavily populated regions. Analysis of these images showed that while cells presented pleomorphic shapes across the CCS triangle, the morphological distribution varied from region to region. Three types of cells are prominent across the CCS triangle and can be classified on the basis of circularity measure on different area, i.e., type 1: large, spread, multipolar (stellate) morphologies with highly organized F-actin stress fibers ($0.4 < C < 0.5$), type 2: bipolar, spindle-shaped with reduced area and lower expression of F-actin stress fibers ($0.02 < C < 0.3$) and, type 3: small round cells with minimal F-actin fibers ($0.6 < C < 0.9$). The number of cells with minimal F-actin fibers ($0.6 < C < 0.9$) were found to be much less and insignificant. In the regions where the cell density was low, circularity ranged between 0.06 and 0.27 in the case of point 1 (Figure 3(a)), and between 0.09 and 0.29 (type 2) with a couple of cells in the 0.42–0.75 range (type 1) in the case of point 7 (Figure 3(c)). Although the last region consisted mostly of bipolar spindle-shaped cells, it also included a few multipolar cells. In comparison,

FIG. 3. CLSM images of F-actin cytoskeleton (Phalloidin-TRITC labeled, red) and nuclei (DAPI labeled, blue) in hFOB 1.19 cells, growing on CCS disc after 3 days of culture. (a) Point 1; (b) Point 13; (c) Point 7; (d) center area; and (e) Ti6Al4V disc used as positive control group for cell-proliferation. Magnification: 10X.

the cells in areas with a medium cell density such as in the center of the CCS triangle (point 24), circularity found to be between 0.07 and 0.28 with a few cells in the 0.43–0.59 range (Figure 3(d)). Regions of high cell density (equivalent to the Ti6Al4V control) such as point 13, circularity found to be ranged between 0.02 and 0.31 for about 50% of the cells with the other half in the 0.40–0.70 range (Figure 3(e)).

Our results showed a correlation between topographical feature size and cell attachment. The SEM micrographs indicate the formation of microstructures in the form of terraces and ledges at point 1 (Figure 2(a)), as well as nanostructures with a grain sizes of 300 nm at the center (Figure 2(d)), 100 nm at point 7 (Figure 2(b)), and 10 nm at point 13 (Figure 2(c)). Overall, cell coverage was higher in the regions where smaller topographical features were found, i.e., Nb-rich region close to point 13, as seen in Figure 3(b). Two main cell morphologies were found across the CCS triangle, (1) large, spread, multipolar (stellate) morphologies with highly organized F-actin stress fibers, and (2) bipolar, spindle-shaped with reduced area and lower expression of F-actin stress fibers. The ratio of stellate to bipolar cell morphologies was found to increase on surface topographies with smaller feature size. It could be attributed to the fact that stellate cells prefer a larger surface area where they can firmly attach, allowing spreading and eventual proliferation. While, bipolar cells are still migrating, searching for an appropriate site where to attach to spread, proliferate and eventually differentiate.²³ It is also important to note that these topographical differences cannot be dissociated from varying metal oxide composition across the CCS triangle, giving an additional complexity to cell behavior on the surface.

Cell morphology varied across the CCS triangle as a response to both surface composition and topography. Variations in surface topography of an implant material from

micron size features to nanometer size features has been shown to enhance adhesion^{24–29} and subsequent cellular functions (e.g., calcium deposition). It has been demonstrated that nanometer size features on Ti (Ref. 30) can enhance osteoblast adhesion and other functions (e.g., alkaline phosphatase synthesis, calcium deposition, and collagen secretion) compared to their micron size features.³¹ In addition, increased protein adsorption on nanophased Ti surfaces has been correlated to improved osteoblast functions.^{32–34}

C. hFOB 1.19 cell proliferation on CCS discs

Cell proliferation was followed by counting cell numbers at 1, 2, 3, 7, 14, and 21 days, at 33.5°C and 5% CO₂, as described earlier. Figure 3 displays an arrangement of fluorescent micrographs of multiple locations over a Phalloidin-TRITC and DAPI stained CCS after a 3 day culture, revealing the existence of marked differences in proliferation levels as a function of surface composition. hFOB 1.19 cell proliferation was found equivalent to the Ti6Al4V control in the Nb/Cr-rich region of the CCS triangle (point 13, 45.22 at. % Nb, 46.54 at. % Cr, 8.23 at. % Sn), as quantified by calculating the cell proliferation ratio (proliferated cells to total number of cells) over the different regions on the CCS disk. It has been observed that point 7 (11.25 at. % Nb, 70.22 at. % Cr, 8.23 at. % Sn) and point 1 (11.25 at. % Nb, 46.54 at. % Cr, 19.42 at. % Sn) showed a lower number of cells, mostly bipolar spindle-shaped with reduced cell area and low amounts of F-actin stress fibers.

Figure 4 present contour maps showing hFOB 1.19 proliferations across the CCS triangle after 1, 2 and 3 weeks in culture. After 1 week, culture revealed high cell proliferation at the center of the CCS spread, specifically at point 27 (28.25 at. % Nb, 54.43 at. % Cr, 10.10 at. % Sn) (Figure 4(a)). This

FIG. 4. Contour maps showing cell proliferation after 7, 14, and 21 days of culture.

behavior extended to neighboring points towards higher Nb content after 2 weeks of culture (Figure 4(b)). Simultaneously, three additional regions form: one comprising of points 1, 18, and 17 within the CCS triangle (11.25–22.57 at. % Nb, 46.54 at. % Cr, 19.42–15.69 at. % Sn), point 3 (11.25 at. % Nb, 54.43 at. % Cr, 15.69 at. % Sn), and third at point 10 (28.24 at. % Nb, 58.38 at. % Cr, 8.23 at. % Sn). An interesting trend shown by these last three regions was that these were located at the edges of the CCS triangle at the boundary with the cpTi. Although high amounts of cells were initially expected at these cpTi regions, results (data not shown) demonstrated a lower number of cells that those found at these points within the CCS triangle, showing a preference for the regions where Nb, Cr, and Sn were found over cpTi alone. After 3 weeks of culture, the high proliferation center region was maintained along with skewing towards the Nb-rich corner of the CCS triangle (Figure 4(c)). The fourth region, i.e., point 10 seen after 2 weeks merged with the center region and expanded towards the Cr-rich region at point 7. The third region, i.e., point 3, formed after 2 weeks of culture, expanded both towards the center and the Cr-rich region of the CCS-triangle. The second region seen after 2 weeks showed a slight expansion but remained within the same cell count as the previous week. Another interesting result was that there was an increase in surface coverage of close to 9 times from week 1 to week 2, while there was only a 6% increase from week 2 to week 3. Cell viability ranged between 93% and 100% throughout the entire CCS surface (data not shown).

Proliferation of the hFOB 1.19 across the CCS triangle after 1 week in culture exhibited high cell proliferation at the center of the CCS spread, specifically at point 27 (Figure 4(a)). This behavior extended to neighboring points towards higher Nb content after 2 weeks of culture. At this time, three additional regions form: one made up by points 1, 18, and 17 within the CCS triangle, another at point 3, and another at point 10 (Figure 4(b)). After 3 weeks of culture, the high proliferation center region was maintained although skewed towards the Nb-rich corner of the CCS triangle (Figure 4(c)). The fourth region (i.e., point 10) seen after 2 weeks merged with the center region and expanded towards the Cr-rich region at point 7. The 3-week average composition of this region was 29.9 ± 3.2 at. % Nb (mean $\pm$ SD), 53.1 ± 3.2 at. % Cr, and 9.8 ± 0.6 at. % Sn and consistently showed a higher cell count compared to the other regions. Of the three metals, Nb has been shown to promote stronger osteoblast

attachment and spreading.⁵⁰ In addition, the SEM images of the Nb-rich region near point 13 showed a nanostructured surface with a feature size of about 10nm, known to improve osteoblast functions.

The third region formed after 2 weeks of culture (i.e., point 3) expanded both towards the center and the Cr-rich region (i.e., point 4) of the CCS-triangle. The 2-week average composition of this region was 11.0 ± 0.0 at. % Nb, 56.0 ± 2.8 at. % Cr, and 14.5 ± 2.1 at. % Sn. The second region (i.e., points 1, 18, and 17) seen after 2 weeks showed a slight expansion but remained within the same cell count as the previous week. The 2-week average composition of this region was 16.7 ± 0.0 at. % Nb, 46.0 ± 0.0 at. % Cr, and 17.7 ± 0.0 at. % Sn. All these highly proliferative regions had a consistently high Cr content which varied between 46 and 56 at. %, and a low Sn content which varied between 9.8 and 17.7 at. %.

Another interesting result was that there was an increase in surface coverage of 890% across the disk from week 1 to week 2, while there was only a 6% increase from week 2 to week 3. The CCS triangle cell coverage from the first to the third week was of 6, 30, and 31%, respectively. A possible explanation might involve osteoblast migration and proliferation in these compositional gradients. Osteoblast migration is believed to be the first step in bone formation,^{35,36} remodeling,^{37–39} and repair,^{40,41} usually in response to a chemotactic gradient.^{42–45} In addition, osteoblast migration can occur following a haptotactic gradient where the chemoattractants are bound to the surface instead of being in soluble form as in chemotaxis. Recently, surface density gradients of an integrin RGD ligand were created using gold nanodot patterns from diblock copolymer micellar solutions on glass coverslips, which could induce osteoblast polarization and initiate migration and signaling.^{46,47} The compositional gradients within the CCS triangle can also be seen as a haptotactic gradient expected to generate osteoblast migration, with osteoblasts migrating in the direction of a gradient leading to an optimal surface composition. In addition, osteoblasts actively migrating to new bone formation, remodeling or repair sites also show controlled proliferation.⁴⁸ When these cells reach their destination, they initiate the formation of bone matrix by stopping their migration and starting their proliferation and eventually differentiating into mature osteoblasts.⁴⁹ Osteoblast migrating over such a large surface area would require much time to reach their optimal location

to start proliferating and eventually differentiating. This is supported by the morphological data which showed a large proportion of migrating spindle-shaped cells in low proliferation regions, while large, spread stellate-shaped cells could be found on highly proliferative regions.

IV. CONCLUSION

We have successfully demonstrated the importance of combinatorial deposition of mixed oxide scaffolds on a titanium surface. The use of continuous combinatorial spreads as a biomaterial screening strategy allows for the simultaneous testing of multiple parameters such as surface composition and topography under identical culture conditions. However, isolating the effect of one parameter in this multi-dimensional system can be a challenge since many parameters affect each other. For instance, osteoblast attachment is a function of surface composition, morphology and roughness. But these parameters cannot be dissociated from each other as can be seen in the SEM micrographs at different areas of the CCS (Figure 3) where surface roughness is clearly a function of composition. It is thus a challenge to identify the effect on cell attachment by either parameter alone. The value offered by this approach is the ability to simultaneously assess cell behavior (i.e., morphology and proliferation) under multiple material properties combinations (i.e., topography and composition) in a way that is both fast and efficient, to screen for biomaterial candidates.

ACKNOWLEDGMENTS

This work was supported by a grant from the Bio Science and Engineering Initiative (BioSEI) from the University of Puerto Rico at Mayagüez, by NSF and NIH grant numbers 0420604 and 1SC2GM089642, respectively.

- ¹C. G. Simon, Jr., and S. Lin-Gibson, *Adv. Mater.* **23**, 369 (2011).
- ²T. Albrektsson and C. Johansson, *Eur. Spine J.* **10**, S96 (2001).
- ³D. M. Ehrenfest, P. G. Coelho, B. S. Kang, Y. T. Sul, and T. Albrektsson, *Trends Biotechnol.* **28**, 198 (2010).
- ⁴T. Albrektsson, P. I. Bränemark, H. A. Hansson, and J. Lindström, *Acta Orthop. Scand.* **52**, 155 (1981).
- ⁵T. Hanawa, in *The Bone-Biomaterial Interface*, edited by J. E. Davies (University of Toronto Press, Toronto, 1991), pp. 49–61.
- ⁶T. Hanawa, K. Asami, and K. Asaoka, *J. Biomed. Mater. Res.* **40**, 530 (1998).
- ⁷S. Roessler, R. Zimmermann, D. Scharnweber, C. Werner, and H. Worch, *Colloids Surf., B* **26**, 387 (2002).
- ⁸B. L. Gabriel, J. Gold, A. G. Gristina, B. Kasemo, J. Lausmaa, C. Harrer, and Q. N. Myrvik, *Biomaterials* **15**, 628 (1994).
- ⁹I. Milošev, T. Kosec, and H.-H. Strehlow, *Electrochim. Acta* **53**, 3547 (2008).
- ¹⁰T. Hanawa and M. Ota, *Biomaterials* **12**, 767 (1991).
- ¹¹J. D. Andrade, *Med. Instrum.* **7**, 110 (1973).
- ¹²L. Di Silvio, M. J. Dalby, and W. Bonfield, *Biomaterials* **23**, 101 (2002).
- ¹³H. Zreikaut and C. R. Howlett, *J. Biomed. Mater. Res.* **47**, 360 (1999).
- ¹⁴H. Matsuoka, H. Akiyama, Y. Okada, H. Ito, C. Shigeno, J. Konishi, T. Kokubo, and T. Nakamura, *J. Biomed. Mater. Res.* **47**, 176 (1999).
- ¹⁵J. Y. Lim, M. C. Shaughnessy, Z. Zhou, H. Noh, E. A. Vogler, and H. J. Donahue, *Biomaterials* **29**, 1776 (2008).
- ¹⁶J. Y. Lim, X. Liu, E. A. Vogler, and H. J. Donahue, *J. Biomed. Mater. Res.* **68A**, 504 (2004).
- ¹⁷C. Loty, J. M. Sautier, H. Boulekbache, T. Kokubo, H. M. Kim, and N. Forest, *J. Biomed. Mater. Res.* **15**, 423 (2000).
- ¹⁸Y. Wan, Y. Wang, Z. Liu, X. Qu, B. Han, J. Bei, and S. Wang, *Biomaterials* **26**, 4453 (2005).
- ¹⁹S. A. Harris, R. J. Enger, B. L. Riggs, and T. C. Spelsberg, *J. Bone Miner. Res.* **10**, 178 (1995).
- ²⁰C. A. Scotchford, M. Ball, M. Winkelmann, J. Vörös, C. Csucs, D. M. Brunette, G. Danuser, and M. Textor, *Biomaterials* **24**, 1147 (2003).
- ²¹J. C. Adams, *Cell. Mol. Life Sci.* **58**, 371 (2001).
- ²²K. Anselme, *Biomaterials* **21**, 667 (2000).
- ²³P. Zapata, J. Su, A. J. García, and J. C. Meredith, *Biomacromolecules* **8**, 1907 (2007).
- ²⁴T. J. Webster and J. U. Ejiofor, *Biomaterials* **25**, 4731 (2004).
- ²⁵T. J. Webster, R. W. Siegel, and R. Bizios, *Biomaterials* **20**, 1221 (1999).
- ²⁶T. J. Webster, C. Ergun, R. H. Doremus, R. W. Siegel, and R. Bizios, *Biomaterials* **21**, 1803 (2000).
- ²⁷T. J. Webster, C. Ergun, R. H. Doremus, R. W. Siegel, and R. Bizios, *Biomaterials* **22**, 1327 (2001).
- ²⁸J. U. Ejiofor and T. J. Webster, *Int. J. Powder Metall.* **40**, 43 (2004).
- ²⁹B. C. Ward and T. J. Webster, *Mater. Sci. Eng. C* **27**, 575 (2007).
- ³⁰S. Puckett, R. Pareta, and T. J. Webster, *Int. J. Nanomed.* **3**, 229 (2008).
- ³¹C. Yao, E. B. Slamovich, and T. J. Webster, *J. Biomed. Mater. Res. A* **85**, 157 (2008).
- ³²K. M. Woo, V. J. Chen, and P. X. Ma, *J. Biomed. Mater. Res. A* **67**, 531 (2003).
- ³³D. Khang, S. Y. Kim, P. Liu-Snyder, G. T. Palmore, S. M. Durbin, and T. J. Webster, *Biomaterials* **28**, 4756 (2007).
- ³⁴T. J. Webster, C. Ergun, R. H. Doremus, R. W. Siegel, and R. Bizios, *J. Biomed. Mater. Res.* **51**, 475 (2000).
- ³⁵A. H. Reddi, *Coll. Relat. Res.* **1**, 209 (1981).
- ³⁶M. J. Yaszemski, R. G. Payne, W. C. Hayes, R. Langer, and A. G. Mikos, *Biomaterials* **17**, 175 (1996).
- ³⁷J. Pfeilschifter, O. Wolf, A. Naumann, H. W. Minne, G. R. Mundy, and R. Ziegler, *J. Bone Miner. Res.* **5**, 825 (1990).
- ³⁸L. Rifas, S. L. Cheng, V. Shen, and W. A. Peck, *Connect. Tissue Res.* **23**, 163 (1989).
- ³⁹H. C. Tenenbaum, Cellular origins and theories of differentiation of bone-forming cells, in *Bone: The Osteoblast and Osteocyte*, edited by B. K. Hall (CRC Press, Boca Raton, FL, 1992), vol. 1, pp. 41–69.
- ⁴⁰L. J. Marden, A. H. Reddi, and J. O. Hollinger, *J. Craniofac. Surg.* **1**, 154 (1990).
- ⁴¹M. M. Sandberg, H. T. Aro, and E. I. Vuorio, *Clin. Orthop. Relat. Res.* **289**, 292 (1993).
- ⁴²L. S. Beck, L. Deguzman, W. P. Lee, Y. Xu, L. A. McFatrige, N. A. Gillett, and E. P. Amento, *J. Bone Miner. Res.* **6**, 1257 (1991).
- ⁴³A. H. Reddi, S. Ma, and N. S. Cunningham, *Ann. Chir. Gynaecol.* **77**, 189 (1988).
- ⁴⁴L. Rifas, S. L. Cheng, V. Shen, and W. A. Peck, *Connect. Tissue Res.* **23**, 163 (1989).
- ⁴⁵J. A. Yee, L. Yan, J. C. Dominguez, E. H. Allan, and T. J. Martin, *J. Cell. Physiol.* **157**, 528 (1993).
- ⁴⁶V. C. Hirschfeld-Warneke, M. Arnold, A. Cavalcanti-Adam, M. López-García, H. Kessler, and J. P. Spatz, *Eur. J. Cell Biol.* **87**, 743 (2008).
- ⁴⁷M. Arnold, V. C. Hirschfeld-Warneke, T. Lohmüller, P. Heil, J. Blümmel, E. A. Cavalcanti-Adam, M. López-García, P. Walther, H. Kessler, B. Geiger, and J. P. Spatz, *Nano Lett.* **8**, 2063 (2008).
- ⁴⁸L. R. Kark, J. M. Karp, and J. E. Davies, *Clin. Oral Implants Res.* **17**, 321 (2006).
- ⁴⁹M. O. Klein, C. Reichert, D. Koch, S. Horn, and B. Al-Nawas, *Clin. Oral Implants Res.* **18**, 40 (2007).
- ⁵⁰L. Shapira, A. Klinger, A. Tadir, A. Wilensky, and A. Halabi, *Clin. Oral Implants Res.* **20**, 578 (2009).