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MC3T3-E1 osteoblast adhesion to laser induced hydroxyapatite coating on Ti alloy

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Abstract. An *in vitro* cell study evaluating cell adhesion to hydroxyapatite (HA) coated prosthetic Ti-6Al-4V alloy via laser treatment is presented in comparison with uncoated alloy. Based on our previous *in vitro* biocompatibility study, which demonstrated higher cell attachment and proliferation with MC3T3-E1 pre-osteoblast cells, the present investigation aims to reveal the effect of laser coating Ti alloy with HA on the adhesion strength of bone-forming cells against centrifugal forces. Remaining cells on different substrates after centrifugation were visualized using fluorescent staining. Semi-quantifications on the numbers of cells were conducted based on fluorescent images, which demonstrated higher numbers of cells retained on HA laser treated substrates post centrifugation. The results indicate potential increase in the normalized maximum force required to displace cells from HA coated surfaces versus uncoated control surface. The possible mechanisms that govern the enhancing effect were discussed, including surface roughness, chemistry, wettability, and protein adsorption. The improvement in cell adhesion through laser treatment with a biomimetic coating could be useful in reducing tissue damage at the prosthetic to bone junction and minimizing the loosening of prosthetics over time.

Keywords: Laser coating; hydroxyapatite; centrifugation; adhesion; osteoblast

1. Introduction

Ti alloys are undisputedly the most widely employed metal for long-term load bearing bone implantation in the current market (Okazaki *et al*. 1998, Geetha *et al*. 2009). These alloys offer advantages of high strength-to-weight ratio, good mechanical properties, excellent corrosion resistance, and non-ferromagnetic properties that offer favorable compatibility with magnetic imaging (Okazaki *et al*. 1998, Geetha *et al*. 2009). Despite of their successful translation into biomedical materials since 1950s, Ti alloys are subjected to continuous modifications to further improve their in vivo performance. Imparting bioactivity to these bio-inert alloys is amongst those strategies, which can promote the bone integration of the load bearing implants. Various naturally

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occurring and synthetic materials have been evaluated for the precise combination of mechanical and chemical properties to mimic and assimilate with healthy bone, among which calcium phosphate bio-ceramics (e.g., hydroxyapatite, HA) are widely recognized to promote favorable biological responses, owing to their similarity to the minerals present in natural bone, their excellent bioactivity, and osteoconduction. HA bio-ceramics are rarely used alone for load-bearing applications due to their brittleness. Instead, they are applied as coatings to metallic implants, which takes advantages of the mechanical properties of metal/alloys and the bioactivity of bio-ceramics (Liu *et al.* 2004).

In the past decades, many novel surface processing techniques have been attempted to combine the favorable chemical properties of HA bio-ceramics with the excellent mechanical properties of Ti alloys in order to achieve an integration of properties that are suitable for bone implants (Tsui *et al.* 1998a, b, Hanawa 1999, Pham *et al.* 2000, Kurella and Dahotre 2005, Nayab *et al.* 2005, Nayab *et al.* 2007, Cui *et al.* 2008, Paital and Dahotre 2009, Variola *et al.* 2009). The surface engineering techniques that have been employed to integrate an HA surface layer to Ti alloy substrates include spray coating, ion implantation, and so forth (Tsui *et al.* 1998 a, b, Hanawa 1999, Pham *et al.* 2000, Nayab *et al.* 2005, Nayab *et al.* 2007, Cui *et al.* 2008). However, these techniques are usually associated with at least one of the following limitations, including weak binding strength between the surface layer and the substrate, lack of uniformity, and high costs (Cui *et al.* 1999, Wen *et al.* 2000, Yang *et al.* 2003, Jagielski *et al.* 2006). On the contrary, laser treatment has been accepted to be a potent surface modification method to deposit HA layer on Ti substrates (Kurella and Dahotre 2005, Paital and Dahotre 2009). By the laser melting based coating, a strong, uniform surface layer can be synthesized. Moreover, surface patterns can be produced by laser treatment, which can introduce topographical cues that in turn influence cell behaviors (Paital *et al.* 2010a, 2011, Yang *et al.* 2010a, 2011, Nag *et al.* 2013). The incorporation of surface features on the implant material is a facile yet effective method to direct the subsequent cell responses, which has been studied in many previous researches (Ball *et al.* 2008, Puleo *et al.* 1999, Boyan *et al.* 2001, Yang *et al.* 2012). Therefore, laser coating with HA could affect the bioactivity of the substrates by means of both chemical and physical interventions. A number of investigations have been made to fabricate micro-textured HA coating and validate the effectiveness of laser treatment to improve bioactivity (Paital *et al.* 2010b, Yang *et al.* 2011).

Despite of the growing number of investigations on the cellular behaviors of laser synthesized HA coatings, research focusing on the cell adhesion to the laser treated substrates has been scarce. A good initial cell adhesion can affect cell morphology, migration, and the downstream proliferation and differentiation (Reyes and García 2003, Pendegrass *et al.* 2010), which is considered to play a paramount role in the success of implant-tissue integration. Typically, the cell adhesion is examined by fluorescent staining of focal adhesion (FA) plaques, which is a viable method to visualized and quantify FA formation. However, fluorescent staining cannot provide information regarding the extent of physical bonding generated by the cell-substrate interaction. Adhesion assays have been, therefore, developed to evaluate the adhesion strength of cells, which also characterizes the resistance of adherent cells to possible detaching forces *in vivo*. The techniques to measure cell adhesion strength usually make use of laminar flow, gravity, or other approaches to induce shear stress to a population of cells (Marcotte and Tabrizian 2008). Microscopic characterization using atomic force microscopy (AFM) was also adopted to examine the adhesion of individual cells (Marcotte and Tabrizian 2008). Among these characterization techniques, centrifugation stands out since that centrifuge is common lab equipment with convenient accessibility and the experimental procedures are straightforward. The centrifugal

force generated by a lab centrifuge addresses a major concern of population-based testing methods by applying nearly equivalent force along the sample surface (Reyes and García 2003, Robinson *et al*. 2011). In the meantime, the tedious and problematic method of individually compressing or shearing cells with micro-scale tools can also be avoided using centrifugation method.

The present study builds on our previous work, which reported the bioactivity of laser micro-textured HA coating through *in vitro* cell culture study (Paital *et al*. 2010a). A quantitative assessment of cell adhesion strength is conducted with HA coated Ti alloys by laser melting. Direct evidence on the improved adhesion of pre-osteoblast cells to the implant material surface is provided through a centrifuging technique.

2. Materials and methods

2.1 Sample preparation and laser processing

Commercially available Ti-6Al-4V alloy (50 mm×50 mm×3 mm) was used as substrate for coating. The samples were polished with 400-grit SiC paper to remove oxidation followed by cleaning with ethanol in ultrasonic bath for about 10 minuets. Two sets of samples were prepared. One set of samples was used for HA coating and the other set was reserved as uncoated samples as control samples for comparison with coated samples during cell culture studies. HA [$Ca_{10}(PO_4)_6(OH)_2$] powder of a spherical morphology with a unimodal distribution in the range of 10 - 30 μm obtained from Fisher Scientific was taken as the precursor material. The precursor powder was mixed in water-based organic binder (LISI W 15833) and reducer (LISI W 15853, Warren Paint and Color Company) at a volumetric ratio of 2:1:1.5. The mixed slurry was then sprayed onto the substrate coupons using an air pressurized spray gun to obtain a uniform thickness of 80 μm in the coated precursor deposit. The sprayed samples were air dried for 24 hours to remove the moisture. The precursor deposited samples were subjected to laser surface treatment to fuse HA with Ti-6Al-4V substrate. A diode-pumped, continuous wave (1064 nm wavelength) ytterbium (IPG YLS-3000) fiber laser with Gaussian power distribution within the beam and beam focal spot diameter of 0.6 mm on the sample surface was employed for surface treatment. The laser processing parameters included 500W beam power and 300 mm/s linear beam scanning speed. Combination of these parameters provided laser beam input energy (laser fluence) of 14.15 J/mm^2. The linear laser tracks were indexed in lateral direction with the lateral spacings of 100 and 200 μm (distance between the centers of two consecutive tracks). Such lateral displacement of the beam allowed treatment of entire surface and lateral spacings of 100 and 200 μm were selected to generate surface (physical) texture to possibly match with the length scales of the naturally occurring three dimensional extracellular matrix (ECM) present in the human bone (Stevens and George 2005). Further, as extensively described in our previous work (Paital *et al*. 2010a), such lateral laser beam spacings are also expected to provide thermodynamic conditions suitable for generation of desired chemical and microstructural transformations in the coated region.

Both uncoated and HA coated samples were further machined to conduct studies for assessment of cell adhesion strength via centrifugation assay. The samples were machined into trapezoidal coupons (7.3 mm short side×12.0 mm long side×15.5 mm edge×3.0 mm thick) using a wire-cut electrically-discharged machine (EDM) as schematically shown in Fig. 1. These dimensions were chosen to produce coupons of the size and shape suitable to match dimensions

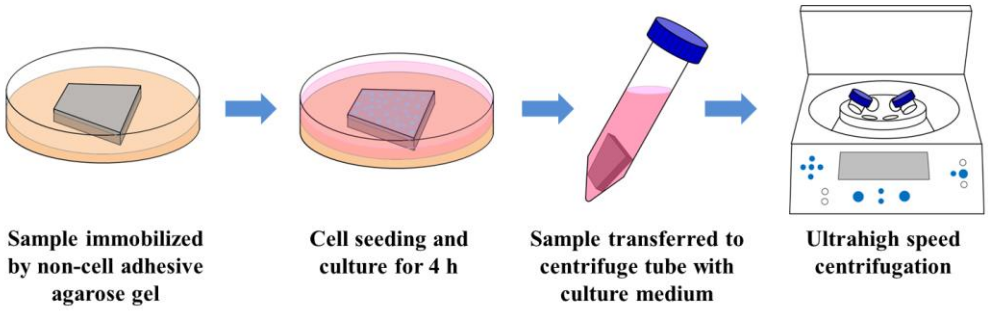

Fig. 1 Illustration of cell seeding on a Ti sample immobilized by non-adhesive agarose gel and the subsequent centrifugation process

and place coupons at the bottom of a centrifuge tube immersed in a cell culture medium to experience minimum or no vibration during centrifuge acceleration.

2.2 Cell culture

Two types of HA coated Ti-6Al-4V samples were tested, including samples processed with the laser tracks of lateral spacings of 100 μm and 200 μm, respectively. Each sample was rinsed thoroughly with sterile water prepared via 0.22 μm filtration. Both sides of each sample were exposed to UV light for 1 h to ensure sterility. Samples were then placed into sterile 35-mm petri-dishes for cell culture studies. To confine cell attachment to the samples, non-adhesive SeaPlaque agarose gel (Lonza) was prepared to 1.5 wt.% in phosphate buffered saline (PBS), sterilized with a 0.22 μm filter, and used to immobilize the samples in the culture dish (Fig. 1).

Mouse MC3T3-E1 pre-osteoblast cells (ATCC CRL-2593) were used in this study. Cells (passages 9-12) were maintained in culture medium composed of alpha-minimum essential medium (α-MEM) supplemented with 10% fetal bovine serum, and 1% penicillin-streptomycin (Invitrogen). Upon confluency, cells were detached from the flask using trypsin, centrifuged, re-suspended, and counted using a trypan-blue dye and a hemocytometer. Samples were seeded at a density of 1.5×10^4 cells/cm^2. Cells were allowed to attach to the sample over a 4 h period at 37 ℃ under a 5.0% CO_2 atmosphere.

2.3 Centrifugation assay

To assess the attachment strength, samples from each treatment group were exposed to three different peak centrifugal forces of 0, 2,000, and 4,000×g (relative centrifugal force), in a fixed angle centrifuge (Eppendorf) at maximal ramp acceleration for five minutes. After centrifugation, samples were gently washed with PBS and the adherent cells on each sample were stained with Ethidium Homodimer-1 and calcein-AM for 20 min at 37℃ under 5% CO_2 atmosphere. Subsequently, each set of samples was viewed on a Zeiss Axio Observer A1 inverted fluorescent microscope (Zeiss). Images were recorded using an AixoCam MRm. The fluorescent images obtained were converted to grayscale images to improve the contrast and reveal more morphological details. The density of adherent cells on each substrate was determined via image analysis. Student's *t*-test was performed to statistically compare the results. The significance level

was defined as $p<0.05$. Data were reported in the form of mean ± standard deviation.

3. Results

Previously the authors have conducted extensive studies on laser based HA coating on Ti-6Al-4V (Cui *et al*. 1999, Wen *et al*. 2000, Boyan *et al*. 2001, Reyes and García 2003, Yang *et al*. 2003, Jagielski *et al*. 2006, Paital and Dahotre 2009, Paital *et al*. 2010b). In these efforts, the effects of laser processing parameters have been explored on the coating microstructure, surface morphology (physical texture), chemical composition, surface energy, as well as their effects on biocompatibility (e.g., cell adhesion, proliferation and mineralization). The laser processing parameters employed in the present work were adopted from previous studies, hence it is expected that all these physical, chemical, and biocompatibility effects would be nearly similar to those extensively presented and explained in these studies. However, the key observations from these studies relevant to the present efforts include 1) samples processed at 100 μm lateral line spacing has reduced surface roughness, higher surface energy, increased wettability (hydrophilicity) and enhanced mineralization (bioactivity) in simulated body fluid (SBF) compared to samples processed at 200 μm lateral line spacing, and 2) samples with 100 μm lateral line spacing demonstrated a higher number of initial attachment of mouse MC3T3-E1 osteoblast-like cells at 1day and increased cell proliferation compared to 200 μm lateral line (Paital *et al*. 2010a).

Since the focus of the present study is to evaluate the effect of HA coatings produced under two different lateral line spacings on cell adhesion (attachment) strength, no further observations and explanation/discussion regarding coating microstructure, morphology, chemical composition, surface energy, and wettability are presented here. Representative images and the average cell counts of adherent cells remained on the untreated and laser treated substrates following 0, 2,000, and 4,000 ×g centrifugation treatment are presented in Figs. 2-4.

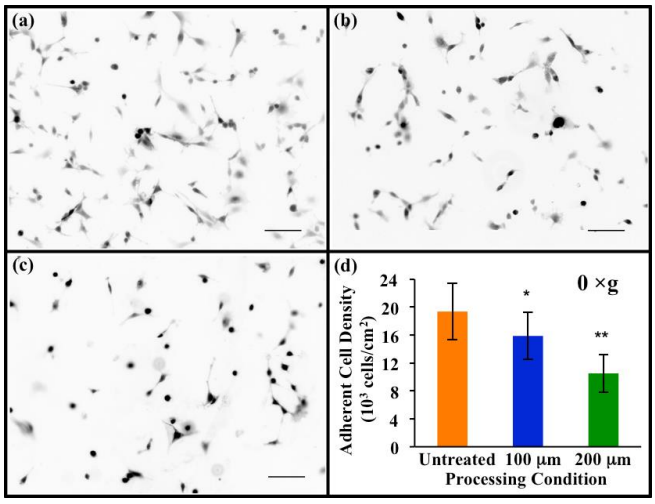

Fig. 2 Representative images of adherent cells on (a) untreated, (b) 100 μm laser treated, and (c) 200 μm treated Ti samples without centrifugation (scale bar: 100 μm); (d) the density of adherent cells on different substrates without centrifugation. (* and ** denote statistical differences at $p<0.05$ compared to untreated and 100-µm-spacing treated substrates, respectively)

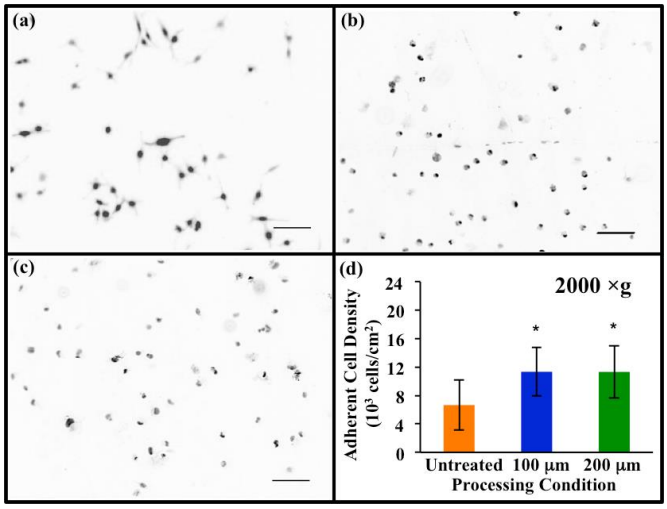

Fig. 3 Representative images of adherent cells on (a) untreated, (b) 100 μm laser treated, and (c) 200 μm treated Ti samples after 2,000 ×g force centrifugation (scale bar: 100 μm); (d) the density of adherent cells on different substrates after centrifugation at 2,000×g. (* denotes statistical differences at $p < 0.05$ compared to untreated substrates)

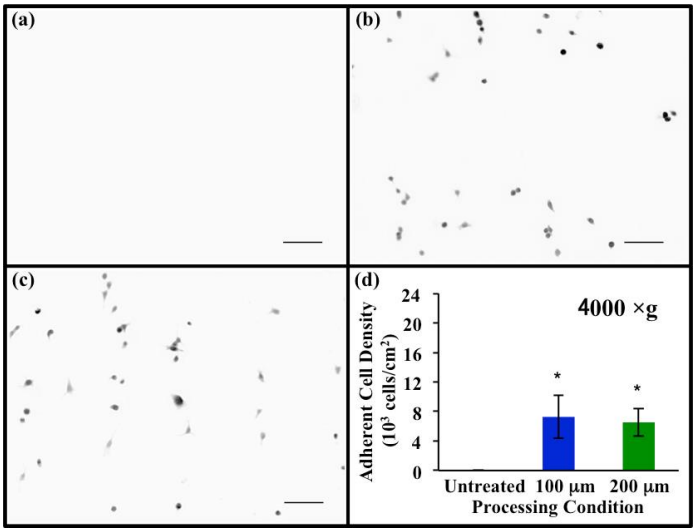

Fig. 4 Representative images of adherent cells on (a) untreated, (b) 100 μm laser treated, and (c) 200 μm treated Ti samples after 4,000 ×g force centrifugation (scale bar: 100 μm); (d) the density of adherent cells on different substrates after centrifugation at 4,000×g. (* denotes statistical differences at $p < 0.05$ compared to untreated substrates)

As shown in Fig. 2, cells exhibited various levels of initial attachment on different substrates under non-centrifugation condition. On the untreated Ti alloy samples (Fig. 2(a)), a relatively higher number of cells were attached after 4 h of culture, which exhibited triangular shaped morphology similar to the representative cell shape in earlier observations (Paital *et al*. 2011). In contrast, the numbers of cells attached on the laser treated HA coated samples (Figs. 2(b) and 2(c))

were lower than that on the uncoated control, and the cells were spread to a less extent exhibiting more elongated or round morphology. The quantified results were presented in the histogram in Fig. 2(d). In particular, the lowest number of average cell attachment was observed on 200-μm-spacing condition.

For the moderately centrifuged samples subjected to 2,000×g detaching centrifugal force, a drastic decrease in adherent cell numbers was observed for the untreated Ti alloy sample (Fig. 3(a)). The average adherent cell density on the untreated sample dropped to 6,650 cells/cm^2, which is greatly lower than that on the non-centrifuged sample (approximately 20,000 cells/cm^2). Such a transition suggested that the applied centrifugal force was able to disrupt cell-substrate bonding on bare Ti alloy. For HA coated samples, the average numbers of adherent cells post centrifugation treatments (Figs. 3(b) and 3(c)) were comparable to the cell number on the non-centrifuged groups (Figs. 2(b) and 2(c)), suggesting that osteoblast cell adhesion to the substrates was improved as a result of HA coating. Cell morphology after 2,000×g centrifugation was also modified, which was dependent on the coating conditions. A closer examination on the morphology of remaining adherent cells in Fig. 3(a) showed a reduction in cell size, and the cell shape transformed from polygonal spread morphology to a less extended, round or elongated bipolar morphology.

The significance of HA coating in improving osteoblast adhesion to the titanium substrate became more pronounced when the centrifugal force was increased to 4,000×g. As shown in Fig. 4, no adherent cells can be observed on the untreated titanium sample after centrifugation, whereas both HA coated samples were capable to retain a good population of attached cells. Similar to the cell morphology after 2,000×g centrifugation, the round and less extending appearance of cells was observed on HA coated samples subjected to 4,000×g centrifugal force. Moreover, a preferential orientation/alignment of the cells can be identified on HA coated Ti alloys post centrifugations, especially in Fig. 4(c).

The reduction in density of adherent cells on different substrates as a function of centrifugal forces is summarized in Fig. 5. The results presented were normalized to the density of adherent cells on non-centrifuged samples within each group. The density of adherent cells on the untreated Ti alloy sample decreased with the increase in centrifugal force. After centrifugation at 2,000×g, the external force was found to have detached approximately two thirds of the initial adherent cells. A total removal of cells on the untreated Ti alloy was observed after centrifugation at 4,000×g. A gradual decrease in cell density was also observed on 100 μm HA treated surfaces, with fractions of remaining cells being 71% and 45% after centrifugations at 2,000 and 4,000×g, respectively. The cell adhesion strength on 200 μm HA treated substrates was found even stronger than on 100 μm HA treated substrates after 2,000×g centrifugation, on which the cell density was comparable with the non-centrifuged sample. When the debonding force was increased to 4,000 ×g, there were still more than 60% of cells maintained on the 200 μm HA treated substrates. Improved adhesion was further defined by comparing the centrifugation force at which 50% of cells remain on treated samples versus comparison with non-centrifuged sample (Fig. 5). For the uncoated Ti, the centrifugal force to introduce a 50% loss of cells was estimated to be 1,500×g based on interpolation. The 100 μm treated samples showed 50% of loss at approximately 4,000 ×g, whilst the results of the 200 μm treated samples indicated 50% of loss at even higher debonding force. Therefore, the adherent cells exhibited stronger bonding forces on both 100 μm and 200 μm HA treated Ti substrates, as indicated by the higher detaching forces than those on untreated Ti substrates. When comparing between the two laser treated conditions, the 200 μm HA treated group displayed stronger cell adhesion than the 100 μm HA treated group.

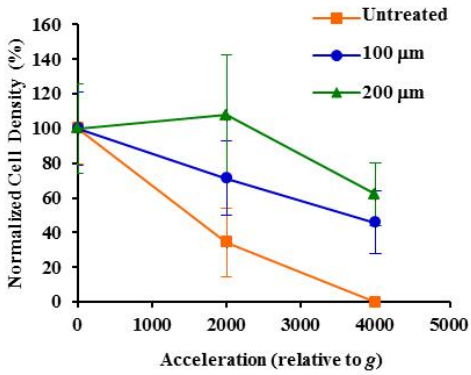

Fig. 5 Remaining fraction of adherent cells on different substrates as a function of centrifugation forces (results presented as the percentages to the cell density on non-centrifuged sample for each group)

4. Discussion

The adhesive strength of living cells cultured on biomaterials surfaces can be gauged by applying an external force, such as the centrifugal force. Commonly the centrifugal force can be imposed at various time points following initial cell seeding to biomaterials, ranging from 4 h to 24 or 48 h of culture periods (Paital *et al*. 2011, Robinson *et al*. 2011). In the present study, the attachment window was set to 4 h to assess the effects of coating on the initial cell adhesive strength. Furthermore, choosing such a short time point could minimize the concern of loss of sensitivity due to the limitation of generating sufficient centrifugal detachment force for cells cultured for extended periods of time (Reyes and García 2003). Another parameter that is worth mentioning here is the initial cell seeding density. In this study a moderate cell density was used to ensure accurate cell number quantification after centrifugation while avoiding excess interference arising from intercellular interactions that might skew the investigation of cell-material interaction with this centrifugation assay. The upper value of forces under which adhesion was tested with our current cell culture system has been chosen following a pilot investigation which successfully detached (greater than 99%) the cell population on uncoated Ti samples and was set to 4,000×g. The efficacy of a coating, which improved cell adhesion, has been evaluated at the force necessary to remove 50% of the cells attached.

Understanding the relative cell populations prior to force exposure allows evaluation of relative improvement of the adhesion strength on HA coated samples. It is noted that a slightly lower number of adherent cell was found on HA coated substrate at 4 h post seeding as compared to the untreated surface. This difference could be a result of multiple factors, including surface roughness (Ball *et al*. 2008, Deligianni *et al*. 2001), grain size and crystallinity of the deposited HA (Balasundaram *et al*. 2006, Ball *et al*. 2001), residual stress within the coating (Ball *et al*. 2001), and the duration of incubation prior to characterizations (Deligianni *et al*. 2001). After laser impingement, both surface chemistry and topography of the Ti alloy substrates were modified (Paital *et al*. 2010a, Dahotre *et al*. 2014). The higher initial attachment on the uncoated Ti could be a subsequent result of lower surface roughness, which leads to a faster rate of attachment and flatter cell shape. In addition, the laser energy used in the current study is much higher than those used in our previous investigations (Paital *et al*. 2010a, 2011, Yang *et al*. 2011), which may yield

coarser grain size and higher crystallinity of the HA coating, whereas finer grain size and lower crystallinity were found to enhance cell adhesion (Balasundaram *et al*. 2006). The higher energy may also result in higher residual stress within the coating that can cause a reduced cell adhesion as compared to the annealed equivalents, which bear lower residual stresses (Ball *et al*. 2001). The lower number of adherent cells on HA coated surfaces can also be partially attributed to the shorter attachment time allowed in this evaluation, as compared with the 24 h incubation in the previous study (Paital *et al*. 2010a, 2011), since a high bone-forming cell proliferation rate on coarse HA coating was reported (Deligianni *et al*. 2001). Based on the above discussion, it is established that the selection of laser parameters is essential in determining the subsequent bioactivity of the coating surfaces, the effects of which were elaborated in some of our previous work (Paital and Dahotre 2009, Paital *et al*. 2010a, b, c, 2011, Yang *et al*. 2010a, b, 2011, Dahotre *et al*. 2010, 2014). By tuning the laser parameters, it is feasible to obtain higher initial cell attachment on HA laser coated substrates, which will be pursued in our future study.

The results from both remaining adherent cell counts and cell morphology observations indicated that laser coating of HA is proficient in promoting cell adhesion to the Ti substrates. Moreover, the cell adhesion on 200-μm-spacing treated HA surface was stronger than that on the 100-μm-spacing treated condition, demonstrated by the highest resistance to the debonding centrifugal force. The underlying mechanisms of these improvements may be attributed to many different factors, such as surface chemistry and surface roughness. In terms of uncoated and coated Ti alloys, the chemical presence of HA could have introduced a different adhesion scheme with stronger binding affinity between cells and the surface, which agrees with the previous observations that HA coated Ti alloys can better support cell adhesion (Chang *et al*. 1999, Balasundaram *et al*. 2006). Okamoto *et al*. have reported tight adhesions of osteoblasts to HA coated Ti alloy and proposed that the enhanced adhesion of osteoblasts was promoted by Arg-Gly-Asp (RGD) containing serum proteins adsorbed to the HA coating, which facilitates the formation of focal adhesion; whereas the cell adhesion on bare Ti is governed by different factors other than RGD-containing protein (Okamoto *et al*. 1998). In the comparison between 100 and 200 μm spaced HA coating, the surface chemistry is the same and the major influence may be a result of change in surface roughness. The finding from the current study is in accordance with the findings reported by Deligianni and co-workers that a higher surface roughness of the HA coating correlates with a higher shear stress resistance of bone marrow cell adhesion (Deligianni *et al*. 2001).

The promoting effect of the HA coating may be further dependent on surface wettability or free energy, which is also closely related with surface topography and chemistry. In many investigations, it was assumed that wettability dominates not only the materials compatibility but also cell attachment strength (Ochsenbein *et al*. 2008, Yang *et al*. 2010a). In our previous study, the wetting behavior of HA coated Ti alloy has been characterized, which revealed different wettability of uncoated, 100-μm-spacing coated, and 200-μm-spacing coated substrates (Paital *et al*. 2010a). It was found that surface treatment with HA coating has altered the surface to be more hydrophilic than the uncoated Ti surface, with the 100-μm spaced HA coating showing greater increase in hydrophilicity than the 200-μm spaced coating (Paital *et al*. 2010a). The different wetting behavior on the HA coating can affect subsequent protein adsorption, the type and concentration of which subsequently influence cell adhesion behavior. For example, the presence of fibronectin was found to encourage and strengthen cell adhesion (Garcia *et al*. 1997). Therefore, a surface that boosts fibronectin adsorption is beneficial for the strengthening of cell adhesion. It was reported that, a more hydrophobic surface is more preferable for fibronectin adsorption when

the concentration of fibronectin in the medium is low and especially with the presence of serum albumin (Grinnell and Feld 1982). The more hydrophobic 200-μm spaced HA coating may favor more fibronectin adsorption, which accounts for the enhanced adhesion. The precise underlying adhesion strengthening mechanisms of these laser treated, HA coated substrates would require further in-depth investigations.

The changes in cell morphology and alignment also implicate the enhancement of cell adhesion on HA laser treated samples. The morphological changes induced by centrifugation on the samples were mainly characterized by reduction in cell area and the extent of cell spreading. On the untreated surface, the cells extended to polygonal shapes after 4 h incubation. It is considered that the largely spread cells on the untreated or treated substrates may be more prone to detaching by external force than those elongated or rounded cells on treated samples. On the untreated Ti samples, the focal adhesions were usually spotted at the periphery of cytoplasma with a dispersive distribution, according to our previous studies after short-term incubations (Huang *et al*. 2011, Dahotre *et al*. 2014). The cells were strained to thinner and more polarized shape due to the detachment at some of the focal adhesion sites along the circumferential of the cell. More significant changes in morphology were observed on HA treated samples, on which cells changed from elongated to rounded morphology. This morphological transformation could be a result of partial detaching at the periphery and strong adhesion on the center of the cell body. At even higher centrifugal forces (>4,000×g), a complete detachment was observed due to the thorough removal of peripheral focal adhesion plaques. On the HA coated surfaces, the cell morphology before centrifugation was found to be elongated, which is comparable to the cell morphology on rough laser treated surface found in our previous study (Dahotre *et al*. 2014). Similar to the detaching course on the untreated surfaces, the peripheral focal adhesion plaques were removed first, leading to the loss of cell polarity. We postulate that the focal adhesions may be more concentrated to the center of the cell bodies on HA coated specimens, which was capable of maintaining the adhesion of the cell on the surface after the detachment of peripheral adhesion and resulted in the rounded cell morphology. This postulation can also be supported by the observations of the strong green fluorescence, representing the high concentration of focal adhesion associated protein (vinculin) in the center of the cell body in our previous report (Dahotre *et al*. 2014). The adhesion strength of individual focal adhesion plaques on HA coated samples may also differ from that on untreated samples due to the different chemical affinity as discussed above. Moreover, images of cell populations on non-centrifuged samples (Fig. 2) did not reveal any significant attachment preference on any of Ti samples, with cells adhered in a random and asymmetrical fashion. Yet, a pattern emerged on force exposed, HA coated samples, which was particularly visible in the 4,000 ×g test group. It can be observed that cells remaining attached after centrifugation demonstrate a ‘row’ orientation on HA coated samples. The row like orientation is more pronounced on the 200-μm spaced samples. The cells remaining after force exposure are thought to locate within the laser tracks, since the preferential attachment of cells was found to be along the laser track in our other report (Dahotre *et al*. 2014), which may be due to the comparable track size with the size of cells. Thus, the physical presence of laser tracks could have exhibited higher affinity for cell alignment and encourage greater adhesion strength. Further investigations will be conducted in our follow-up research, including fluorescent staining of focal adhesion and SEM examinations, in order to provide experimental evidences for our postulations.

Finally, the increase in the ultimate force attainable before a certain degree of detachment is of particular interest for future study. Results of this *in vitro* study indicate that the highest centrifugation force achievable before 50% detachment has been increased after laser coating with

HA. It will be further helpful to evaluate the debonding forces exerted on individual cells and the subsequent effect of laser treatment, the results of which can be compared with the individual cell adhesion strength reported in the literature. The individual testing offers an interesting point-of-view but introduces additional variation in experimental controls due to the inherent heterogeneity of cell populations. In the meantime, centrifugation testing provides an overall inspection of the cell behavior. Therefore, combining both centrifugal testing and individual cell testing will provide a comprehensive understanding of cell behavior in terms of adhesion strength and cell/material interactions.

5. Conclusions

This paper presents a continuous effort to complement our previous work on the laser coating effect on materials properties of and cell responses to the Ti-6Al-4V alloy. In particular, the adhesion strength of bone-forming MC3T3-E1 osteoblast-like cells was evaluated using a centrifugation technique. Enhanced cell adhesion to laser induced HA coating on Ti alloy was found in this study, which was evidenced by the increase in the centrifugal force required to remove osteoblasts attached on treated Ti alloy surface. The underlying enhancing mechanisms were proposed to be related to the laser induced surface morphology changes, the presence of HA, surface wetting, and protein adsorption. The improvement in cell adhesion through laser treatment with a biomimetic coating could be useful in reducing tissue damage at the prosthetic to bone junction and minimizing the loosening of prosthetics over time. Future study will be performed to further investigate the cell adhesion strength on HA laser coated Ti-6Al-4V alloys with enhanced initial attachment in a wider range of applied force to examine the debonding forces, and the mechanisms governing improved cell adhesion will be exploited and discussed by analyzing cell adhesion related proteins.

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