Mouse embryonic fibroblasts accumulate differentially on titanium surfaces treated with nanosecond laser pulses

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Mouse embryonic fibroblasts accumulate differentially on titanium surfaces treated with nanosecond laser pulses

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Biomaterial engineering, specifically in bone implant and osseointegration, is currently facing a critical challenge regarding the response of cells to foreign objects and general biocompatibility of the materials used in the production of these implants. Using the developing technology of the laser surface treatment, this study investigates the effects of the laser repetition rate (frequency) on cell distribution across the surface of the titanium substrates. The main objective of this research is building a fundamental understanding of how cells interact with treated titanium and how different treatments affect cell accumulation. Cells respond differently to surfaces treated with different frequency lasers. The results of this research identify the influence of frequency on surface topography properties and oxidation of titanium, and their subsequent effects on the pattern of cell accumulation on its surface. Despite increased oxidation in laser-treated regions, the authors observe that fibroblast cells prefer untreated titanium to laser-treated regions, except the regions treated with 25 kHz pulses, which become preferentially colonized after 72 h. © 2016 American Vacuum Society [http://dx.doi.org/10.1116/1.4962066]

I. INTRODUCTION

Cell behavior toward a foreign surface plays an important role in determining how the surrounding tissue will integrate or reject an implant, and thus in turn the quality, longevity, and functionality of a biomedical device. Once in the body, the biological interactions between the implant and the body, including the types of cells recruited and their activation, adhesion, matrix deposition, and migration across the surface of the implant, directly affect the performance of the implant. Understanding the mechanisms behind these interactions will allow greater control over implant functionality. Improvements in host integration, bone healing, and acceptance of the implant greatly benefit the patient.1,2

Cell interactions are highly affected by the surface characteristics of an implant, including the surface energy (hydrophobicity), topography, oxide phase composition, chemical properties, and charge of the surface. Due to the difficulty of controlling the conventional mechanical and chemical surface treatment methods, it is a common practice to investigate the effects of surface roughness and surface chemical properties on cell behavior separately. However, by introducing laser surface texturing, the surface chemistry and surface topography of biomaterials can be influenced simultaneously in micro/nanoscales,3–6 and their combinatorial effects may be beneficial.

This study concentrates on the effects of the laser frequency on the surface topography, surface chemistry, and oxide phase composition of titanium substrates, to investigate their effects on the enhancement of cell interactions on the titanium surface. This method can lead to a promising solution for the fabrication of better biomaterials and implants through commercialized nanosecond laser irradiation and have far-reaching applications in the medical industry.

II. MATERIALS AND METHODS

A. Laser processing of Ti sheets

Thin sheet titanium substrates and a Nd:YAG pulsed laser system (SOL-20 by Bright Solutions, Inc.) were used for all experiments conducted in this study. This laser system has a wavelength of 1064 nm, with a maximum output power of 25 W. The optical configuration offers a laser spot size of 20 μm on the substrate, with an absorption coefficient of 0.3. Figure 1 displays the general configuration used in this
study. The key parameter of frequency can be directly adjusted with this laser system.

As shown in the figure, the designed pattern was etched onto the surface of the material fixed in the sample holder, through the EZCAD designing software. This particular software allows for a number of different patterns and texts to be created. The scanning parameters, including scanning speed, dwell time, and scanning configurations, can be adjusted through this software. The combination of parameters was adjusted with the software, along with the power and frequency set for laser irradiation; the desired pattern was irradiated across the surface of the selected material.

The titanium sheets were ground finished to 1200-grit silicate-carbon papers to remove macrolevel surface defects and contaminations. Then, they were ultrasonically cleaned in distilled water and dried in desiccators. In this study, the titanium substrates were treated using frequencies of 25, 50, 70, and 100 kHz, constant scanning speed of 500 mm/s, and a constant average power of 10 W. The laser treated titanium samples were ultrasonically cleaned again in distilled water to remove debris from the surface.

B. Sample soaking in SBF for in vitro assessment

To assess the biocompatibility of the treated substrates, simulated body fluid (SBF) was used. This solution has a similar concentration of salts as human blood plasma and is supersaturated with hydroxy apatite (bonelike apatite). Immersing the substrates in SBF creates an in vitro environment for the substrates to interact with bonelike apatite, ergo display enhancement, or reduction of biocompatibility.

C. Scanning electron microscopy and energy-dispersive x-ray spectroscopy

The laser-irradiated areas, before and after SBF immersion, were examined using scanning electron microscopy (JEOL 6400 SEM) equipped with GELLER DPICT digital image acquisition software and a Gatan ChromaCL Cathodoluminescence imaging system allowing capturing if high resolution images. Further, an elemental analysis using energy dispersive x-ray (EDX) was conducted to observe the effects of laser treatment on oxidation and ablation level of the treated titanium surfaces.

D. Three dimensional optical microscopy

This study used the Zeta-20 Optical Profiler (Zeta Instruments) to scan the surface of the samples for quantitative topography measurements.

E. Cell maintenance—NIH/3T3 mouse fibroblast cell

Mouse embryonic fibroblasts (NIH/3T3, ATCC® CRL1658™) were maintained at 37°C with 5% CO₂ and 95% air in Dulbecco’s Modified Eagle’s Medium (DMEM)-supplemented with 10% heat-inactivated calf serum, 4.5 mg/ml glucose, 2 mM glutamine, and passaged every 2–4 days with trypsin. Titanium samples were washed thrice in 70% ethanol for 15 min followed by a 10 min wash in ddH₂O, then autoclaved and equilibrated for 5 min in DMEM before being incubated with freshly passaged cells. In order to measure the response of fibroblast cells to the treated titanium, NIH/3T3 cells were plated onto autoclaved, sterile treated titanium equilibrated in DMEM and incubated for 1–3 days. The titanium samples were then rinsed in phosphate buffered saline (PBS) to remove nonadherent cells and fixed in 4% formaldehyde in PBS overnight at 4°C.

F. Fluorescent staining

The actin cytoskeleton of cells adhering to the titanium samples was stained using Alexa-594-conjugated phalloidin (Thermo), and their nuclei were stained with DRAQ5 (Thermo). The samples were imaged using a Leica M205AF stereo microscope equipped for epifluorescent illumination and data capture via a Leica Microsystems CMS GmbH grayscale camera (DFC 360 FX).

Regions were selected for counting from within the treated area, 0–100 μm from the edge of the treated area and 100–200 μm from the treated area. The relative number of cells is measured by taking the ratio of the cell counts to the background count in the untreated area (100–200 μm).

G. Nondimensional temperature analysis

To verify the effects of laser irradiation upon the surface structure and topography, a thorough analysis of the temperature profile across the surface of the treated titanium was conducted. To develop the temperature history of the titanium substrates, the nondimensionalization method was used to track the changes due to frequency and calculate the nondimensional temperature across the surface. This method is a simple technique for tracking the trend of changes occurring in laser irradiation and is an effective step in investigating the effects of frequency upon the heat-affected profile.

To proceed with this method, a cylindrical symmetric flow without internal heat generation, and constant, isotropic
material properties was assumed; hence, a transient heat conduction equation and a proper boundary condition were defined\textsuperscript{9–11}
\begin{equation}
\frac{1}{r} \frac{\partial}{\partial r} \left( r \frac{\partial T}{\partial r} \right) + \frac{\partial^2 T}{\partial z^2} = \frac{1}{x} \frac{\partial T}{\partial t},
\end{equation}
where $x$ is the thermal diffusivity and is defined as $x = k/\rho C_p$, with $k$ being the thermal conductivity, $\rho$ the density, and $C_p$ the specific heat.

Using Eq. (4), the trend of change of surface temperature of temperature is the melting temperature of the material\textsuperscript{10,11} was tracked along the radius of the substrate. Equation (3) displays the simplified boundary equation
\begin{equation}
\frac{\partial T}{\partial z} \bigg|_{z=0} = -\frac{1}{\eta P(t)} k \pi r_0^2 \exp\left(-r^2/r_0^2\right),
\end{equation}
where $P$ is defined as the peak power delivered, $\eta$ is the fraction of energy absorbed by the surface, and $r_0$ is the beam radius (in this study the beam diameter is 20 $\mu$m).

To simplify the unit conversion, it is more convenient to define a set of nondimensional groups. The customized nondimensional variables that redefine radius, depth, temperature, and nondimensional time for $t \leq t_p$, respectively, are as follows. It must be noted that it is assumed that the reference temperature is the melting temperature of the material\textsuperscript{10,11}
\begin{equation}
R = r/r_0, \quad Z = z/r_0, \quad \theta = T/T_{ref}, \quad \tau = 4\pi t/r_0^2.
\end{equation}

These nondimensional variables are then used to simplify Eqs. (1) and (2). Equation (3) displays this simplified boundary equation
\begin{equation}
\frac{\partial \theta}{\partial Z} \bigg|_{Z=0} = -Q \exp\left(-R^2\right) [u(\tau) - u(\tau - \tau_p)],
\end{equation}
where $Q$ is the nondimensional power and is defined as $Q = \eta P/k \pi r_0^2 T_{ref}$.

As stated, the nondimensional temperature in this study was tracked along the radius of the substrate. Equation (4) defines this temperature profile\textsuperscript{10,11}
\begin{equation}
\theta(R, Z = 0, \tau_p) \approx \frac{Q}{\sqrt{\pi}} \arctan\left(\sqrt{\tau_p}\right) \exp\left(-R^2\right).
\end{equation}

Using Eq. (4), the trend of change of surface temperature of titanium substrates due to frequency is obtained. The results are displayed in details in Sec. III B.

H. Statistics

All experiments were out in Minitab\textsuperscript{5}, and the data points are averages unless otherwise mentioned. The error values indicate standard deviations of ten independent measurements.

III. RESULTS AND DISCUSSION

A. Effects of laser treatment on surface roughness

The surface of an implant is the contact point between the implant and the surrounding tissue. Hence, modifying the surface topography properties of an implant is an effective method for the enhancement of implant acceptance. Surface topography properties consist of three main characteristics: lay, roughness, and waviness.\textsuperscript{12–14}

The topographical properties of a material, particularly roughness, are very influential on cell attachment and cell behavior.\textsuperscript{2,3} Laser treatment influences all properties of topography, particularly the roughness, surface profile, and oxidation of the treated material, which are closely examined in Sec. III B.\textsuperscript{12–14}

Larger surface roughness exposes an area that is more readily available for cell attachment to take place. Figure 2 displays the treated titanium substrates with and without being previously immersed in SBF, to examine the effects of laser treatment on apatite inducing ability of substrates, as done elsewhere.\textsuperscript{5,14} In Fig. 2, the layers of apatite deposition across the surface of titanium are clearly distinct between (a) and (b), and deposited apatite is evident, presumably enhancing the biocompatibility of this titanium substrate.

Surface roughness can be easily controlled by the laser parameters. This study concentrates on the effects of laser frequency on the surface topography and subsequent cell growth and attachment. Figure 3 illustrates how influential frequency can be on the surface roughness. Different frequencies transfer various levels of energy to the irradiated substrate. This variation results in different effects on the surface topography of the material. Figure 3 shows that as the frequency increases, the heat affected zone decreases, and a deeper groove is ablated on the surface, which is in agreement with expectations.

A frequency of 25 kHz delivers a higher level of pulse energy, while the frequency of 100 kHz delivers the energy

**Fig. 2.** Deposition of apatite on treated titanium is evident when comparing scanning electron microscopy (SEM) of 100 kHz treated titanium from before application of SBF (a) and after a week’s incubation in SBF (b).
in a more concentrated manner across the surface of the titanium substrate, and hence, the irradiated area has a deeper groove and a narrower heat affected area compared to the substrate treated with a frequency of 25 kHz as shown in Figs. 3 and 4.

As seen in this figure, with an increase in frequency, a considerable deeper groove of ablation is created on the surface. However, looking around the area of the groove, more micro/nanoirregularities are observed with the 25 kHz frequency than with the higher frequencies, particularly at 100 kHz. This topological difference will affect the cell attachment, migration, and growth. Table I presents the depth and width of the irradiated zone measured from the surface profile. Lower frequencies have larger, shallower heat-affected zones, which result in a wider irradiated area.

Controlling for the increase in the surface area available with the lower frequencies (that have higher roughness), we expect that cells will prefer the shallower, rougher grooves produced at lower frequencies, resulting in a decrease in cell attachment as the frequency increases.15

### B. Effects of laser treatment—Oxidation level

Another factor in cell attachment is the oxidation level of the surface of the material. Oxidation directly affects the surface energy of the material, and hence with a higher oxidation, better cell adhesion is expected.5,12,16

During laser irradiation, a plume with radial surface tension is created surrounding the heat-affected zone due to the temperature gradient caused by energy transfer. This vaporized plume (plasma) consists of nanoparticles, ions, and clusters that are created through the ablation of the material during laser irradiation.16,17 Immediately following irradiation, an extreme temperature difference exists between the surface of the material and the plume, hence a high cooling rate results, which causes rapid resolidification of the ablated material. As a result, the tension within the laser plume shoots nanoparticles to the periphery of irradiated zones.18,19

### Table I. Surface profile measurement of frequencies.

<table>
<thead>
<tr>
<th>Frequency (kHz)</th>
<th>Width (µm)</th>
<th>Depth (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25</td>
<td>52.9 ± 5.0</td>
<td>6.38 ± 1.1</td>
</tr>
<tr>
<td>50</td>
<td>35.8 ± 5.0</td>
<td>11.6 ± 2.6</td>
</tr>
<tr>
<td>70</td>
<td>32.1 ± 3.1</td>
<td>13.0 ± 2.2</td>
</tr>
<tr>
<td>100</td>
<td>14.16 ± 4.1</td>
<td>15.7 ± 1.1</td>
</tr>
</tbody>
</table>

Fig. 3. Scanning electron microscopy (SEM) shows that the surface topography is distinct between laser frequency treatments. Micrographs showing laser induced line across the surface of titanium at different frequencies: (a) 25 kHz, (b) 50 kHz, (c) 70 kHz, and (d) 100 kHz.

Fig. 4. Surface roughness profiles of treated titanium show deeper groove with higher frequency treatment: (a) 25 kHz, (b) 50 kHz, (c) 70 kHz, and (d) 100 kHz.
Resolidification of the deposited nanoparticles creates nanotextured structures, which add to the nanoroughness of the treated surfaces. Figure 6 displays this process, along with the creation of titanium oxide, in detail.

Figure 5 shows the resulting titanium structure at the end of the laser irradiation. As shown, laser irradiation causes the surface of the substrate to undergo oxidation, and as a result, titanium oxide is observed on the surface of the treated substrates.18

Laser treatment of the titanium increases the surface temperature of the material up to oxidation temperature, which is in the range of 800–1000°C, and results in the creation of thin layers of titanium upon the surface of the substrates. To study the effects of frequency on surface topography and oxidation level of laser-treated titanium, the surface temperature profiles of the treated materials were examined. Using the nondimensionalization method explained in Sec. II, the trend of temperature change across the surface of the titanium substrate could be detected. Figure 7 displays these results.

The highest temperature occurs at the center of ablation, which according to Fig. 4 is the location with the most ablation. At 25 kHz, the surfaces are subjected to the highest temperatures, which should yield maximal oxidation.

An increase in the oxidation of the surfaces has a direct effect on increasing the wettability of the material. This consequently leads to an increase in cell attachment to the material, and may improve the biocompatibility of the implant surfaces. Having a layer of titanium across the surface provides a cushion-type template for the cells to attach to upon the surface, and therefore may result in a higher cell adhesion across the treated surface of the titanium.20–22 As expected, EDX analysis shows that the highest oxidation levels are present in the samples treated at 25 kHz (Fig. 8).

At a frequency of 25 kHz, the evaporation temperature of titanium (3287°C) is achieved along the irradiated area. Hence, a higher oxidation level is observed using this frequency. At 100 kHz, oxidation levels are barely above those observed in the untreated samples.

C. Effects of laser treatment on cell accumulation patterns

Mouse embryonic fibroblasts (NIH/3T3) were used to interrogate cell behavior on the surfaces of titanium substrates. Fibroblasts are the most common cells in animal connective tissue and play a critical role in wound healing as the first cells to migrate into a damaged area. At the wound site, they deposit extracellular matrix that lays the foundation for scarring and rebuilding tissue. Migration of local fibroblasts along the fibrin network laid down by the first invading fibroblasts and the beginning of re-epithelialization from the wound edges activates angiogenesis. The deposition of
collagen, fibronectin, and other matrix components during wound healing also forms the basis for the new matrix of connective tissue. Cells cultured on treated samples accumulate at a higher density on untreated titanium than on the laser treated grooves (Fig. 9). The increasing cell density with time demonstrates that these cells are proliferating on the titanium substrates, but somewhat surprisingly, some combination of differential migration, proliferation, adhesion, and/or apoptosis results in fewer cells having colonized the regions of the substrates treated with all laser frequencies at 24 h (Fig. 10).

Cell accumulation seems to be driven most highly by a combination of shallow grooves and high surface oxidation. At the 24 h time point, the cells seem relatively evenly distributed on each of the different treatments (Fig. 10, first panel), with perhaps a slight preference for the untreated titanium, although it should be noted that the variability in these data are high due to the low cell densities. At 48 and 72 h, however, there is a higher accumulation of cells on the titanium treated with the lower frequency lasers, especially at 25 kHz. This may be due to the cells being able to fill the shallower grooves with the extracellular matrix more rapidly, making the regions treated at the lower frequencies the first to be effectively colonized. This is consistent with the observation that the density of cells in the grooves treated at 50 kHz was catching up after 72 h; however, there was no apparent gain in the cell density within the 70 and 100 kHz grooves, suggesting that the higher oxidation levels of the titanium treated with the low frequency lasers is also a factor.

IV. CONCLUSION

Cell attachment, adhesion, and growth are important aspects of implant and biomedical device acceptance. Fibroblasts, being the first to colonize a wound site, are critical in understanding the first steps of interaction between the body and an implant. Using laser ablation, surfaced topology and oxidation can be better controlled. The frequency of laser treatment significantly affects the colonization of a titanium surface by fibroblasts. Cell accumulation is highest on the
untreated areas, which is surprising since these areas have a lower oxidation level than the laser treated sections and we would expect them to have unfavorable surface topography for cell adhesion. Of the laser textures, that are generated at 25 kHz, characterized by high oxidation and shallow nano-scale roughness, had the highest cell accumulation.

The laser frequency has an effect on the surface properties of treated titanium and an effect on cell accumulation. The laser treatment affects not only the topographical properties of the treated areas, but also the surface energy through oxidation of the surface of the material. This is a significant advantage of laser technology in comparison to conventional methods used for surface treatment, which involve several steps, low controllability and environmentally unfriendly acids and materials. Using this advantage in the production of biomedical devices could lead to a significant decrease in the production time, an increase in precision, and a decrease in cost to making titanium or titanium coated implants using commercialized and low-cost nanosecond laser systems.

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