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Bone Cell–materials Interactions and Ni Ion Release of Anodized Equiatomic NiTi Alloy

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Abstract

Laser processed NiTi alloy was anodized for different durations in H₂SO₄ electrolyte with varying pH to create biocompatible surfaces with low Ni ion release as well as bioactive surfaces to enhance biocompatibility and bone cell–materials interactions. The anodized surfaces were assessed for their *in vitro* cell–materials interactions using human fetal osteoblast (*hFOB*) cells for 3, 7 and 11 days, and Ni ion release up to 8 weeks in simulated body fluids. The results were correlated with surface morphologies of anodized surfaces characterized using field-emission scanning electron microscopy (FESEM). The results show that the anodization creates a surface with nano/micro roughness depending on anodization conditions. The hydrophilicity of NiTi surface was found to improve after anodization due to lower contact angles in cell media, which dropped from 32° to < 5°. The improved wettability of anodized surfaces is further corroborated by their high surface energy comparable to that of cp Ti. Relatively high surface energy, especially polar component, and nano/micro surface features of anodized surfaces significantly increased the number of living cells and their adherence and growth on these surfaces. Finally, a significant drop in Ni ion release from 268 ± 11 to 136 ± 15 ppb was observed for NiTi surfaces after anodization. This work indicates that anodization of NiTi alloy has a positive influence on the surface energy and surface morphology, which in turn improve bone cell–materials interactions and reduce Ni ion release *in vitro*.

Keywords

Nitinol; Surface modification; Anodization; Surface energy; Cell–material interactions; Ni release

1. Introduction

Nitinol, an equal-atomic Ni and Ti alloy (NiTi), can be classified as a new biomaterial. Among many metallic materials, Nitinol is gaining more attention due to its excellent biocompatibility comparable to or better than Ti in combination with shape memory effect and superelasticity. Nitinol alloy has a low elastic modulus between 20 to 90 GPa, which is

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closer to that of dense (cortical) bone (between 15 to 25 GPa) [1]. The biocompatibility of NiTi and its unusual mechanical properties make it an ideal engineering material for variety of bone implant applications including maxillofacial and dental implants, cervical and lumbar vertebral replacements, joint replacements, bone plates, bone tissue engineering, spine fracture fixation, anchorage and repair [1–4]. However, the release of Ni in to the human body is considered as a serious problem with NiTi alloy implants, as Ni is known to be allergenic and carcinogenic, though essential for the human body. Therefore, great deal of research efforts has been made on Nitinol surface modifications and coatings [5].

Various surface modification techniques such as electrochemical treatments [6–9], chemical etching [1,10–12], heat treatments [6,10–13], ion implantation [6,14], laser and electron-beam irradiation [15], and variety of coatings [16–19] have been used to reduce the Ni release and improve biocompatibility/bioactivity of NiTi alloy. These techniques were aimed to create a uniform, homogeneous, and thick TiO_{2-x} films, up to $\sim 30 \mu\text{m}$, on NiTi surfaces, which not only act as a barrier for Ni release but also enhance biological performance. However, thick surfaces developed via coating methods can potentially crack at low strains ($<1\%$), which is detrimental to their effectiveness as Ni release barrier [20]. Further, Nitinol surfaces modified using various processes that involve temperatures between 160 and 600°C (heat treatments, laser/electron beam melting and ion implantation) usually interfere with the heat treatments of Nitinol associated with alloy/device production to achieve optimal shape memory and superelastic properties [5]. As a consequence of above treatments, the phase composition of the bulk and sub-surface layers will change leading to significant changes in corrosion and Ni release characteristics of nitinol implants [5]. Therefore, alteration/segregation of Ni in NiTi alloy surface or sub-layers should be avoided during surface modification processes to minimize toxicity. In addition, long-term exposures to low temperatures in the range of 100 to 160°C developed originally for pure Ti are not suitable for Nitinol because of the presence of Ni. It is clear from previously published investigations that among various surface modification techniques, chemical or electrochemical approaches can provide Nitinol surfaces with a variety of chemistries, topographies and thickness $< 1 \mu\text{m}$, which can ensure negligible Ni release and good biological responses.

In this study, the surface of Laser Engineered Net Shaping (LENS) processed NiTi alloy has been modified using anodization in H_2SO_4 electrolyte with varying pH at 20V. The influence of anodization on *in vitro* cell-materials interactions and Ni release of NiTi alloy is discussed in detail. In the recent years, we have extensively used LENS to create fully dense, porous, compositionally and structurally graded materials for load-bearing metal implants [21–30]. Specific advantages of using LENS to create NiTi alloy implants include (i) alloys with novel microstructures and high purity can be obtained due to high cooling rates, (ii) as the fabrication is carried out in protective atmosphere (oxygen $< 10 \text{ ppm}$), LENS processed Nitinol will retain the desired phases of initial powder, (iii) tailorable microstructure, porosity, shape, and size of the part in one operation, (iv) being a computer-aided design (CAD) based manufacturing process, LENS can be used to make net shape, functional patient specific implants, and (v) the process is reliable and has the potential for direct low volume manufacturing. Therefore, in the present paper, we report the evaluation of *in vitro* biological properties of anodized NiTi alloy surfaces, processed via LENS, using human fetal osteoblast cell line (hFOB) and Ni ion release up to 8 weeks in simulated body fluids.

2. Materials and Methods

2.1. Preparation and Anodization of NiTi Alloy

Equiatomic NiTi alloy powder (Crucible Research, Pittsburg, PA) with particle size in the range of 50 to 150 μm was used in this study. The nominal composition (wt %) of the powder was 55.2% Ni, 0.007% C, 0.05% O, 0.006% N, and balance Ti. Fully dense bulk alloy samples were fabricated using a LENS-750 unit with a 0.5 kW continuous wave Nd-YAG laser system, on a substrate of rolled Ti plates with 3 mm thickness. LENS is a layered manufacturing technology, which involves complete melting and layer-by-layer deposition of metal/alloy powders (Fig. 1) in an additive fashion directly from a 3D CAD model, using a focused high-power laser beam as the heating source, for fabrication of near-net shape functional parts without using special tooling, molds or dies. Detailed description of LENS operation and capabilities can be found elsewhere [23,26,27,29]. Samples were fabricated in a controlled atmosphere with O_2 content less than 10 ppm to limit oxidation of the NiTi alloy during processing. Dense cylindrical NiTi alloy samples with 7 mm diameter and 40 mm height were made using 350 W laser power, 13 mm/s scan speed and a powder feed rate of 5.5 g/min. Constituent phases of laser processed NiTi alloy samples were identified using a Siemens D 500 Kristalloflex diffractometer (Madison, WI, USA) with Cu K α radiation and a Ni filter. The samples were scanned over the 2θ range 30 to 80° at a step size of 0.02° and a count time of 0.5 s/step. Further to identify the presence of secondary precipitates/compounds, which can change one step austenite $\leftrightarrow$ martensite transformation to multi-step transformation [31], a differential scanning calorimetry (DSC) was done using a simultaneous thermal analyzer (Netzsch, STA 409 PC, Burlington, MA) with heating and cooling rates of 1°C/min.

Cylindrical samples were removed from the substrate and disc samples of 2 mm thick were then cut from the cylindrical samples using a water jet. The disc samples were abraded by silicon carbide paper in successive grades of 400, 600, and 1200 grit then ultrasonically cleaned in distilled water and dried at room temperature. Final polishing was done using a cotton polishing cloth with a 0.5 μm alumina suspension and the samples were cleaned and used for further testing. Electrolytic cell for anodization consisted of a 100 mL beaker atop a magnetic stirrer. A NiTi alloy anode was suspended on one side of the cell and a platinum cathode (1cm²) was suspended on the other side of the cell. Both anode and cathode were connected to the power supply by platinum wires and a thermometer was also placed in the beaker. A DC power supply (Hewlett Packard 0–60 V/0–50 A, 1000 W) was used for the applied voltage. 1 (N) sulfuric acid (H_2SO_4) was used as electrolytic solution was prepared by dissolving 0.42 g of citric acid and 0.42 g of NaF in a solution containing 5.5 mL H_2SO_4 and 94.5 mL distilled H_2O . Electrolyte with three different pHs (4.5, 2.0 and 1.5) was achieved by adding either HCl or NaOH. All anodization experiments were performed at 20 V for different durations at 15 minute increments up to 1h. Temperature of the electrolyte was maintained at 30 °C. The temperature rise due to exothermic anodic reaction was controlled by using relatively large amounts of electrolyte (40–60ml) and changing the solution after 30 minutes if needed. NiTi surfaces after anodization were characterized using a field emission scanning electron microscope (FESEM; FEI, SIRION, OR). The surface roughness of each test samples was measured using a surface profilometer (SPN Technology, Goleta, CA).

2.2. Contact Angle Measurements

To evaluate the influence of microtexturing of Nitinol surface, achieved via anodization, on surface wettability, contact angles were measured using a sessile drop method on a face contact angle set-up equipped with a microscope and a camera. For comparison, contact angles were also measured on un-anodized samples, which were ground using a series of

SiC grinding papers with various sizes up to 1200 grit and then polished on a velvet cloth using a series of Al₂O₃ powders up to 0.05µm suspended in distilled water. Finally, just before testing, all the samples were ultrasonically cleaned in an alcohol bath. A 0.5–1.0 µl droplet of distilled water and Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham (DMEM/F12, D2906, Sigma, St. Louis, MO) at pH 7.4 (cell media) was suspended from the tip of the micro-liter syringe. The syringe tip was advanced toward the disk surface until the droplets made contact with the sample surface. Images were collected using the camera and the contact angle between the drop and the substrate was measured by the computer software. Two samples for each anodized condition were used to collect the contact angle data in water and cell media. For each sample 10 data points were used.

2.3. Surface Energy Calculation

Surface energy of NiTi and Ti control sample surfaces was calculated based on contact angle measurements, which were conducted with two liquids with polar and non-polar characteristics: distilled water and diiodomethane. In this study, Fowkes' equation was used to calculate the energy:

$$\gamma_L(1+\cos\theta)=2\left(\sqrt{\gamma_L^d\gamma_s^d}+\sqrt{\gamma_L^p\gamma_s^p}\right) \quad (1)$$

where θ is the contact angle, of liquid L and solid S, γ^d is the dispersion component of the surface energy, γ^p is the polar component.

2.4. Nickel Ion Release in Simulated Body Fluid

Two samples per anodization/un-anodization condition were immersed in cell plates containing 2 ml of simulated body fluids (SBF) and were kept under thermostatic conditions inside a biologically controlled environment at 37°C for 8 weeks. The ionic concentrations of the SBF used in present study are as follows: 2.5mM of Ca²⁺, 1.5mM of Mg²⁺, 142.0mM of Na⁺, 5.0 mM of K⁺, 147.8 mM of Cl⁻, 4.2 mM of HCO₃⁻, 1.0 mM of HPO₄²⁻, 0.5 mM of SO₄²⁻. The samples tested include un-anodized and as-polished fully dense NiTi, anodized NiTi with contact angle of 20, 10 and 5°. SBF was changed every 4 days and the old solution was stored and refrigerated for subsequent analysis. A Shimadzu Atomic Absorption spectrometer (AA-6800 Shimadzu Scientific Instruments, Columbia, MD) in the graphite furnace mode was used to determine the Ni concentration in the SBF as a function of time. Integrations and data collection were carried out using Shimadzu Wizard software (Shimadzu Scientific Instruments, Columbia, MD). Ni stock solutions were freshly prepared in ionization buffer to obtain a final concentration of 1µg/mL. Ni and ionization buffer standards were purchased from High-Purity Standards (Charleston, SC, USA) and standard curves were prepared at determined concentrations of 0 to 200 ng/L (ppb). Each of the stored solutions per composition was diluted with deionized water from 2ml to 10ml in order to produce an adequate sample size for the spectrometer. Calculations were made according to these dilutions. Three reading per sample were taken and recorded.

2.5. In vitro Bone Cell-materials Interactions

For the cell-materials interaction studies, human fetal osteoblast (hFOB) cells, CRL-11372 (ATCC, VA, USA) were used. Cells were plated at a density of 10,000 cells/ml in standard 24-well tissue culture plates. The base medium for this cell line was a 1:1 mixture of Ham's F12 medium and DMEM (DMEM/F12, Sigma, St. Louis, MO) with 2.5 mL-glutamine (without phenol red). The medium was supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 0.3 mg ml⁻¹ G418 (Sigma, St. Louis, MO). hFOB cells were seeded onto the sterilized sample surface. All samples were sterilized by autoclaving

(Amerex Instruments Ltd., CA) for 20 min at 121°C before cell culture. Cultures were maintained at 34°C under a 5% CO₂ and 95% air atmosphere. Culture medium was changed every 3 days for the duration of experiment. Samples were then removed from culture at 3, 7 and 11 days of incubation to study cell–materials interactions under FESEM.

Cells morphology was assessed by field emission scanning electron microscope (SEM) observation. Duplicate samples were prepared for FESEM analysis. Cultured samples for SEM observation were rinsed with 0.1 M phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde/2% glutaraldehyde in 0.1 M cacodylate buffer overnight at 4°C. Following this, post fixation for each sample was made with 2% Osmium tetroxide (OsO₄) for 2 h at room temperature. Fixed samples were then dehydrated in an ethanol series 30%, 50%, 70%, 95% and 100% three times, followed by a hexamethyldisilane (HMDS) drying procedure. Dried samples were then mounted on aluminum stubs, gold coated and observed under a field emission scanning electron microscope (FESEM).

A colorimetric assay (MTT assay), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide was used to evaluate cell proliferation on anodized samples. The culture medium was changed every 3 days for the duration of the experiment. Samples for testing were removed from culture after 3, 7 and 11 days incubation. A 5 mg/ml MTT (Sigma, St. Louis, MO) solution was prepared by dissolving MTT in phosphate-buffered saline and filtering using a 0.2 µl pore size filter paper. An aliquot of 500 µl of diluted MTT solution (50 µl in 450 µl of DMEM/F12) was then added to each sample in 24-well plates. After 2 h incubation 500 µl of solubilization solution made up of 10% Triton X-100, 0.1 N HCl and isopropanol was added to dissolve the formazan crystals. An aliquot of 100 µl of the resulting supernatant was transferred into a 96-well plate, and read by a plate reader at 570 nm. Statistical analysis was performed using Student's t-test, with $p < 0.05$ being considered statistically significant. Triplicate samples were used in MTT assay experiments to ensure reproducibility.

3. Results

3.1. Phase Analysis of As-processed NiTi Alloy

Fig. 2a shows the X-ray diffraction (XRD) results of laser-processed NiTi alloy. The XRD results showed that as-processed samples contain a mixture of austenite (B2) and martensite (B19'). Further, no additional peaks representing undesirable intermediate precipitates/compounds such as Ti₂Ni, Ni₄Ti₃, and Ni₃Ti were found in the laser-processed samples. To compliment the XRD results and to confirm the absence of intermediate phases in the laser processed NiTi alloy DSC analysis was performed. The results of DSC tests on LENS-processed NiTi alloy samples are shown in Fig. 2b. The transformation sequence for laser-processed samples was one-stage B19' ↔ B2. The endothermic peak during heating is associated with B19' → B2 transformation and exothermic peak during cooling is due to B2 → B19' transformation. The XRD analysis of LENS-processed NiTi alloy confirms the absence of undesirable intermediate phases and the present samples contain a mixture of austenite and martensite with high amount of austenite. The one-stage transformation sequence of laser-processed samples further substantiate the absence of secondary precipitates/compounds, which are known to induce intermediate R-phase formation during heating/cooling [31].

3.2. Anodized Surface Morphology

Initially all the samples were examined for their surface topography as anodization invariably change the surface characteristics of base metal. Fig. 3 shows different surfaces that were obtained by anodizing NiTi alloy for varying durations at different electrolyte

pHs'. As-polished surfaces before anodization appeared to be smooth without any artifacts or surface asperities (Fig. 3a). However, anodization of NiTi alloy resulted in micro/nano textured surfaces (Figs. 3b–e) with varying asperities. Close inspection of anodized surface features showed that both the electrolyte pH and anodization time strongly influence the surface morphologies. Anodization produced porous surfaces on the order of micron and nanometer sized pores. Comparison of surface morphology of NiTi alloy anodized in an electrolyte with a pH of 4.5 for 15 min to that of surface anodized for 30 min, shown in Figs. 3b and c, respectively, indicate that surface asperities such as pits/pores not only grow in number but also in size covering the entire surface of the sample. Decreasing the pH of the electrolyte appeared to increase the reaction rate between the electrolyte and the NiTi alloy surface as shown in Fig. 3d. Samples anodized at lower electrolyte pHs found to exhibit extremely fine pores and large pits with nano scale asperities on the surface (Figs. 3d and 3e). In addition, as shown in Fig. 4, decreasing the electrolyte pH completely eliminated the equiaxed/spherical pores or asperities and entirely new angular features projecting out of the matrix were observed at pH 1.5. Table 1 shows the average r.m.s. roughness values for different NiTi alloy surfaces. There is almost an order of magnitude difference in roughness for the as-processed NiTi alloy and anodized surfaces. As-processed and polished NiTi alloy samples show a smooth surface with an r.m.s roughness value of 0.008 μm , which increased to a maximum value of 0.075 μm for the surface anodized for 60 min. in an electrolyte with 1.5 pH. In general, increasing the anodization duration and/or acidity of electrolyte (decreasing pH) increased the surface roughness.

3.3. Contact Angles and Surface Energy

To evaluate the influence of micro/nano textured surfaces created via anodization on the of wettability of NiTi alloy, contact angles between the alloy surface and two different liquids were measured, and the results are shown in Fig. 5. As expected NiTi alloy surface before anodization showed highest average contact angles of $33 \pm 2.3^\circ$ and $29 \pm 1.8^\circ$ with water and cell media (DMEM), respectively. The contact angles decreased on anodized samples. By varying the anodization parameters, it was possible to improve the wettability of Nitinol surface via further decrease in the contact angles. Lowest contact angles of $\sim 5^\circ$ and $\sim 8^\circ$ with cell media and water, respectively, were achieved by anodizing at electrolyte pH of 1.5 for 60 minutes. In general, the contact angles decreased with increasing anodization time and also with decreasing electrolyte pH. These observations corroborate the measured surface roughness of respective surfaces. The lower contact angles of anodized surfaces compared to bare NiTi alloy indicate that the surface become highly hydrophilic due to anodization.

A lower contact angle of anodized surfaces suggests their relatively high surface energy than un-anodized surface. Fig. 6 shows the surface energies NiTi alloy surfaces calculated using contact angles measured with two different well characterized liquids, before and after anodization. For comparison the surface energy of commercially pure (CP) Ti was also measured. Before anodization Nitinol surface exhibited considerably lower surface energy of $49 \pm 0.98 \text{ mN/m}$ than CP Ti with an average surface energy of $53 \pm 1.3 \text{ mN/m}$. However, experimental results show that after anodization Nitinol can achieve significantly higher surface energy than un-anodized CP Ti. Highest surface energy of $63 \pm 1.3 \text{ mN/m}$ was observed for the surface anodized at electrolyte pH of 1.5 for 60 minutes, which also exhibited lowest contact angles (Fig. 5). Therefore, the anodization parameters that decreased the contact angles resulted in high surface energies. A maximum of 29% increase in the surface energy of NiTi alloy was observed after anodization compared to un-anodized surface. The surface energy results corroborate the observations on contact angles on these sample surfaces.

3.4. In vitro Cell-materials Interactions

The hFOB cell attachment, growth and spreading on Nitinol sample surfaces with and without anodization was evaluated using FESEM. All the samples showed well spread and flattened cell morphologies after 7 days of culture period as shown in Fig. 7. However, anodized samples clearly produced cells adhering to each other with cellular micro extensions (e.g. filopodia) and were connected to the substrate in addition to the neighboring cells (Figs. 7b and 7c). Further, the anodized Nitinol surfaces with the lowest contact angle (5°), with associated high surface energy, showed significantly higher number of cells that flattened and spread covering the entire surface of the sample even at 7 days of culture period suggesting significantly better cell attachment and spreading. This sample also showed that cells grew in a monolayer and had a flat, well-spread-out morphology on the textured surface. Cells were also found to have covered the micropores and were observed to bridge the micropores on a macro scale. In contrast, on Nitinol surface without anodization (Fig. 7a), cell growth was less pronounced, where limited establishment of cell spreading can be noticed after 7 days of culture. Fig. 8 shows cell morphology after 11 days of culture period on NiTi alloy samples before and after anodization. After 11 days of culture, cells appeared to be more elongated and fully confluent. On anodized samples the cell numbers increased and the entire sample's surface was covered with cells having numerous filopodia extensions grabbing the surface irregularities as shown in Fig. 8b and 8c. Distinctive cell-to-cell attachment was noticed, with small calcified nodules as an early sign of cell differentiation after 11 days in both the samples with anodized surfaces. After 11 days of culture, cells on anodized Nitinol surfaces with lowest contact angle (5°) became elongated forming a dense and confluent cellular layer (Fig. 8c). For this sample, extracellular matrix (ECM) mineralization can also be seen on the cell surface. However, on Nitinol surfaces without anodization, relatively fewer cells were observed, although they increased in number from day 7 culture period.

The MTT assay was used to quantitatively determine the proliferation of viable hFOB cells on NiTi surface with and without anodization, and cpTi surface was used as a control. MTT assay is a measure of mitochondrial activity that reduces MTT to formazan dye giving purple color. The optical density of the solution is a measure to quantify the cell viability/living cell count on the surface using MTT assay. A higher optical density represents a higher concentration of living cells. In case of human osteoblast cells, a higher number of living cells relates to better biocompatibility of the material. Fig. 9 shows a comparison of viable cell densities for different samples after 3, 7 and 11 days of culture. For all culture durations, cells proliferated in greater numbers on the anodized NiTi samples compared to cpTi and un-anodized NiTi samples. The differences in cell densities were significant after 11 days culture duration between cpTi and NiTi, cell numbers were almost 50% higher on the cpTi samples compared to un-anodized NiTi surfaces. Also, statistical analysis revealed that a significant difference ($p < 0.05$) in cell density was always present between the NiTi samples before and after anodization for all culture days. It was observed that, during the initial culture days, cells proliferated most rapidly on cpTi and anodized NiTi samples with 10 and 5° contact angles. However, after 11 days of culture there was no statistically significant difference in cell densities between the cpTi and anodized NiTi samples with contact angle 20°.

3.5. Ni Release

Anodization of NiTi alloy can increase the corrosion resistance by forming oxide layer that acts as a barrier layer to prevent Ni^{2+} ions from leaching out of the bulk material. Fig. 10a shows the cumulative Ni released (ppb) from different surfaces as a function of immersion time in SBF. It can be seen from the results that anodization significantly reduced the Ni release from NiTi alloy surface. However, all the Nitinol surfaces showed more or less

similar and gradual Ni release, and none of the surfaces exhibited saturation or plateau in the Ni release. This observation suggests that anodization does not alter the Ni release rate of NiTi alloy, although it can significantly decrease the amount of Ni release from the alloy surface. Comparison of Ni release between anodized surfaces showed that the release can be altered by changing the anodization parameters. Among the anodized samples, the surfaces with the lowest contact angles (or the highest surface energy) showed smaller amount of Ni release. Fig. 10b compares the total Ni concentration in SBF after 8 weeks of immersion for various samples. Nitinol sample without anodization showed the highest Ni concentration of 268 ± 11 ppb, which decreased to 190 ± 13 ppb and to 161 ± 9 ppb and finally to a minimum of 136 ± 15 ppb as the contact angle of surface decreased from $20^\circ \pm 1.5$ to $10^\circ \pm 1.1$ and to $5^\circ \pm 0.7$, respectively as a result of anodization. From the present Ni release data it can be said that the anodization parameters that decreased the contact angles and enhanced the in vitro cell-materials interactions were also effective in minimizing the Ni ion release.

4. Discussion

Biomaterials interact with the body through their surfaces. Consequently, the anodized outermost layers of a material are critically important in determining both biological responses to implants and material responses to the physiological environment. Our experimental results show that anodization of NiTi alloy changes the smooth as-polished surface to micro/nano textured surfaces with varying asperities depending on anodization duration and electrolyte pH. The NaF in the anodization electrolyte provide fluorine source, which forms HF and catalyzes etching of the substrate. Similarly, the citric acid serves as buffer and stabilized the electrolyte. During anodization in H_2SO_4 electrolyte, the thickness of NiTi alloy samples reduced from 2 mm to 1.82 mm (180 μm reduction) suggesting that both Ni and Ti are consumed during the anodization process. Therefore, it is hypothesized that the development of varying surface morphologies on NiTi alloy arise from the competitive interplay of field-assisted oxide formation at the metal-oxide interface and oxide dissolution at the oxide-electrolyte interface [32]. As the anodization process begins the initial oxide layer [33], formed due to interaction of the surface Ti^{4+} ions with oxygen ions (O^{2-}) in the electrolyte, while the Ni dissolves in the electrolyte. However, in sulfuric acid electrolytes, the formation of TiO_{2-x} layer seems to be in competition with the field-assisted dissolution reaction at local regions yielding localized pits or pores. As the anodization process proceeds, the pits grew both in number and size. The oxide layer again forms on the newly exposed surfaces and the process of dissolution continues as the anodization duration increases. As the anodization duration increased to 60 min, large pits with nano scale asperities were formed on the pits' surface due to increase in the amount of dissolution. The elimination of equiaxed/spherical pores or asperities and the formation of new angular features projecting out of the matrix in the samples anodized at pH 1.5 for 60 min are attributed to high dissolution rate of the surface. Longer anodization times and the increase in acidity at low pH resulted in more pronounced surface irregularities/surface roughness. It has been shown that highly-ordered TiO_2 nanotube arrays could be generated on the surface of titanium [34] due to the simultaneous occurrence of field assisted oxidation, field assisted dissolution, and localized chemical dissolution. However, the formation of an irregular surface layer rather than nanotube arrays on the NiTi alloy surface in similar electrolyte might be due to domination of field-assisted dissolution reaction at local regions yielding localized pits or pores. Pores tend to develop in the locally thinner regions of the initial oxide layer where the current is concentrated and field-assisted film dissolution increases. Even slight changes in the specific characteristics of the metallic surface seem to exert appreciable effect on the development and structural features of the present anodized samples.

Several investigators reported enhancement of surface wettability in terms of low contact angles on anodized or other surface treated Nitinol [35] and Ti [34]. However, it is not clear from those studies that what actually contributed to the observed increase in the surface wettability of modified surfaces of these metals/alloys. In line with above earlier works, present anodized Nitinol samples also showed significant decrease in the contact angles indicating their enhanced wettability. It is hypothesized that the enhancement in surface wettability of present Nitinol samples is due to formation of oxide layers as a result of anodization. The ceramic oxide layers, particularly TiO_2 , formed on the surface of Nitinol samples have high ionic character, therefore exhibit higher wettability [36] than the passive oxide films on Nitinol surface without anodization. The electronegativities of $\text{Ti} = 1.54$ and $\text{O} = 3.5$ result in predicted ionic character of TiO_2 at about 63%. It has been reported that the compounds in water and water based liquid readily chemisorbs to ceramics surface than to metals, thereby increasing their wettability [36]. Further, such adsorption activities were found to increase with ionic character of ceramics [36]. Therefore, the oxide layers, formed during anodization, on Nitinol surface can provide a favorable surface with higher wettability. Another important characteristic that can affect the wettability is the surface topography or roughness. Wenzel [37] and Onda et al. [38] showed that materials' hydrophilicity or hydrophobicity enhances with increase in the surface roughness, when the initial contact angle is $< 90^\circ$ or $> 90^\circ$, respectively. Since the present NiTi alloy surface before anodization showed contact angles lower than 90° , the surfaces became relatively more hydrophilic (low contact angles) after anodization. This suggests that the changes in surface topography due to anodization, is also responsible for the enhanced wettability of Nitinol surface after anodization. The observed increase in the surface roughness of NiTi alloy samples after anodization is consistent with above discussion. From the current results it can be said that improved wettability and surface energy of anodized surfaces are combined effects of surface oxide layer, reduction in surface Ni concentration [39] and increased surface roughness after anodization (Table 1). Similar improvement in wettability, polar component and total surface energy has been reported for oxidized NiTi alloy [39].

It is known that the cell attachment and growth are primarily associated with the material's surface characteristics such as chemistry, topography, wettability and surface energy. In general, contact angles lower than 20° indicates hydrophilic nature of the surface. Since the cell media are water based, the cellular attachment will be better in any hydrophilic surface. Reports show that the highest level of cell attachment is on moderately hydrophilic surfaces [40]. A high contact angle with a hydrophobic nature tends to show poor cell attachment on NiTi surface compared to cpTi and anodized NiTi surface. Also, a low contact angle of anodized surfaces suggests its high surface energy, which is another factor that can contribute to improving the cell attachment and proliferation on anodized surfaces. Therefore, NiTi surface with lower surface energy of 49 mN/m showed inferior cell attachment and proliferation when compared cpTi with a surface of energy of 53 mN/m . After anodization the surface energy of NiTi alloy increased to a maximum value of 63 mN/m with contact angle of approximately 5° . Anodized NiTi surfaces, with contact angle of $\sim 5^\circ$, show better cell-material interactions over un-anodized NiTi and cpTi samples due to both its high wettability and high surface energy. Osteoblast cell attachment to the sample surface and growth took place via filopodia and lamelliopodia. More filopodia on the anodized NiTi surface with contact angle of 5° indicates favorable surface characteristics for cell attachment, especially in the early stages of cell culture. The MTT results also demonstrate that cell attachment and proliferation depend on sample surface topography/roughness in addition to material chemistry. The results show that cells proliferate rapidly on the samples with the highest surface wettability, energy and roughness, i.e. anodized NiTi with contact angle of 5° . Other studies also showed that a better cell attachment takes place on surfaces with higher surface energy [22,34,41–43].

As with total surface energy, the surface roughness can significantly alter cell-materials interactions. It has been reported that nano and micron level surface roughness influences the binding and differentiation of cells by providing the anchorage points [44–46]. Therefore, current superior cell adherence and proliferation of anodized surfaces could also be due to presence of micro/nano textured surface asperities and resultant high surface roughness. In addition, enhancing the polar component of the surface energy via compositional or surface modifications is also shown to enhance the cellular adhesion and proliferation [41,47]. Total surface energy calculated from measured contact angles include a dispersive component associated with general forces acting between any atoms and molecules, and a polar component that translates into strong chemical interactions between the liquid and the substrate. The polar and dispersive components of total surface energy of different surfaces are summarized in Table 2. The experimental results clearly show that the polar component (γ^p) of NiTi alloy surface gradually increased from 15.43 mN/m, before anodization, to about 23.91 mN/m after anodization for 60 min in an electrolyte with 1.5 pH. The enhancement of polar component as a result of anodization suggests stronger chemical interactions of the liquid with the surface that could be related to both surface chemistry and nano/micro level surface roughness. Similarly, the increase in dispersive component (γ^d) can promote wettability of the surfaces. Therefore, the improved wettability and high polar component of anodized surfaces resulted in enhanced cellular adhesion and proliferation on Nitinol surfaces comparable and/or better than that of cp Ti.

Despite the fact that Ni is an essential element in our body, the maximum daily intake should not exceed between 300 and 600 μg [48], and a threshold concentration of 30 ppm is required to trigger any cytotoxic response during in vitro experiments [49]. Our experimental results clearly demonstrate that the amount of Ni released due to passive dissolution can be significantly decreased via anodization of NiTi surface. Further, the amount of Ni released during 8 week period is calculated to be 2.43 ppb/day for anodized samples, which is significantly less than un-anodized samples (4.79 ppb/day). The observed Ni levels for anodized samples are negligible compared to daily intake of Ni in the diet [48] and in vitro cytotoxicity limit [49]. The total amount of Ni ions released during the first week of immersion is high for all the samples, which indicates that it is possible to decrease the Ni ion release even more by simply soaking the implant in a simulated body fluid for the first week during which a high concentration of Ni from the surface can be removed before the implant is placed in the human body. It is intuitive to expect the highest amount of Ni release from un-anodized NiTi samples as the natural passive oxide layer on these samples can not act as protective barrier for Ni release for long durations in SBF. The lowest total amount of Ni ions released during first 4–7 days between 104 to 18 ppb (ng/mL) for anodized samples are in line with earlier investigations [6–9]. At the end of 8 weeks in SBF, the amounts of Ni leached from the anodized NiTi samples are ~ 2 times lower than those leached from the un-anodized samples. Thus, the oxide films developed during anodization constitutes an effective barrier layer that mitigates the outward diffusion of Ni from the substrate. Among the anodized samples, Ni release decreased with either increasing anodization duration or decreasing the electrolyte pH. This release phenomenon could be due to a decrease in the effective Ni concentration on the NiTi alloy surface [50] as a result of increased dissolution/etching of Ni from the surface due to increase in the anodization duration or decrease in the electrolyte pH. This is clearly evident from the time required to achieve ~ 50 ppb of Ni in SBF for the sample ($\theta = \sim 5^\circ$) anodized at pH 1.5 for 60 min, which took around 2 weeks compared to 3–4 days for other anodized samples. Further, the present Ni concentrations were well within the toxic limit (150 to 300 $\mu\text{g/day}$ maximum) [48] indicating that the present anodized NiTi alloy samples have considerable application potential. Further, it is important to note that present anodized samples have higher surface area due to pits and surface asperities, but still exhibit lower Ni ion release rate than un-

anodized samples. The actual Ni ion released per unit area will be considerably lower than the un-anodized and polished samples as mentioned above.

5. Conclusions

Anodization treatment was successfully applied to laser processed NiTi alloy to improve cell-materials interactions and also to reduce Ni ion release and associated allergic and toxic reactions. Experimental results showed that electrolyte pH and anodization duration strongly influence the surface morphology/chemistry leading to improvement in wettability of NiTi surface in terms of low contact angles in the range of 5 and 30°. Surface energy of various surfaces showed that the hydrophilic character of NiTi surfaces improved due to increase in the polar component of surface energy as a result of anodization. The differences of surface energy between untreated and anodized surfaces found to have a direct influence on osteoblast cell adhesion, growth and proliferation. The anodization significantly improved the cell-material interactions on NiTi surfaces, which are comparable to that of cp Ti. Finally, a significant drop in Ni ion release from 268 to 136 ppb has been observed for NiTi surfaces after anodization. Overall present results show that anodization of NiTi alloy not only improve *in vitro* bone cell-materials interactions but also mitigate toxic Ni ion release.

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Fig. 1.
Schematic diagram of LENS process.

Fig. 2.

X-ray diffraction pattern (a) and DSC results (b) of LENS-processed NiTi alloy. B2: austenite (A), JCPDS No. 18-899, B19': martensite (M), JCPDS No. 35-1281.

Fig. 3. FESEM micrographs showing the surface morphology of NiTi alloy (a) before anodization, $\theta = \sim 33^\circ$, (b-e) anodized surfaces, (b) pH 4.5, 15 min, $\theta = \sim 27^\circ$, (c) pH 4.5, 30 min, $\theta = \sim 20^\circ$, (d) pH 2.0, 30 min, $\theta = \sim 10^\circ$, (e) pH 1.5, 60 min, $\theta = \sim 5^\circ$. Contact angles (θ) were measured using cell media (DMEM).

Fig. 4. High magnification FESEM images showing variations in surface asperities formed on NiTi alloy surface as a result of anodization (a) pH 2.0, 30 min, $\theta = \sim 10^\circ$, (b) pH 1.5, 60 min, $\theta = \sim 5^\circ$.

Fig. 5.
Influence of anodization time and electrolyte pH on the contact angles of NiTi surfaces with distilled water and cell media (DMEM).

Fig. 6.
Surface energies of cpTi and NiTi surfaces before and after anodization.

Fig. 7.

FESEM micrographs illustrating hFOB cell morphology after 7 days of culture on Nitinol surfaces (a) before anodization, (b) anodized at pH 4.5 for 30 min, $\theta = \sim 20^\circ$, (c) anodized at pH 1.5 for 60 min, $\theta = \sim 5^\circ$.

Fig. 8. FESEM micrographs illustrating hFOB cell morphology after 11 days of culture on Nitinol surfaces (a) before anodization, (b) anodized at pH 4.5 for 30 min, $\theta = \sim 20^\circ$, (c) anodized at pH 1.5 for 60 min, $\theta = \sim 5^\circ$.

Fig. 9.

MTT assay of cells measured after 3, 7 and 11 days of culture. There were statistically significant differences in optical density between anodized samples at 11 day culture period.

Fig. 10.

(a) Ni ion release of NiTi alloys with and without anodization as a function immersion duration in SBF, (b) Average concentration of Ni released from different NiTi surfaces after 8 weeks of immersion in SBF.

Table 1

Surface roughness of NiTi surface with and without anodization.

Surface	Average r.m.s value (μm)
NiTi As-processed	0.008 ± 0.001
NiTi Anodized (20°) pH – 4.5, Time – 30 min.	0.021 ± 0.005
NiTi Anodized (10°) pH – 2.0, Time – 30 min.	0.032 ± 0.007
NiTi Anodized (5°) pH – 1.5, Time – 60 min.	0.075 ± 0.004

Table 2

Polar and dispersive components of total surface energy (mN/m) of CP Ti and NiTi surface with and without anodization.

Surface	Dispersive Component (γ^d)	Polar Component (γ^p)	Total Surface Energy (γ^{tot})
CP Ti	27.99 ± 0.53	24.85 ± 0.38	52.84 ± 0.63
NiTi As-processed	33.71 ± 0.76	15.43 ± 0.69	49.14 ± 0.76
NiTi Anodized (20°) pH – 4.5, Time – 30 min.	34.83 ± 0.45	18.56 ± 0.55	53.39 ± 0.85
NiTi Anodized (10°) pH – 2.0, Time – 30 min.	38.07 ± 0.87	20.03 ± 0.91	58.10 ± 1.08
NiTi Anodized (5°) pH – 1.5, Time – 60 min.	39.61 ± 0.64	23.91 ± 0.70	63.52 ± 1.01