Biocompatible, Smooth, Plasma-Treated Nickel–Titanium Surface – An Adequate Platform for Cell Growth

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ABSTRACT: High nickel content is believed to reduce the number of biomedical applications of nickel–titanium alloy due to the reported toxicity of nickel. The reduction in nickel release and minimized exposure of the cell to nickel can optimize the biocompatibility of the alloy and increase its use in the application where its shape memory effects and pseudoelasticity are particularly useful, e.g., spinal implants. Many treatments have been tried to improve the biocompatibility of Ni–Ti, and results suggest that a native, smooth surface could provide sufficient tolerance, biologically. We hypothesized that the native surface of nickel–titanium supports cell differentiation and insures good biocompatibility.

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Figures 1–5 and 7 appear in color online: http://jba.sagepub.com

0885-3282/12/06 0707–25 $10.00/0 DOI: 10.1177/0885328211416023

Three types of surface modifications were investigated: thermal oxidation, alkali treatment, and plasma sputtering, and compared with smooth, ground surface. Thermal oxidation caused a drop in surface nickel content, while negligible chemistry changes were observed for plasma-modified samples when compared with control ground samples. In contrast, alkali treatment caused significant increase in surface nickel concentration and accelerated nickel release. Nickel release was also accelerated in thermally oxidized samples at 600°C, while in other samples it remained at low level. Both thermal oxidation and alkali treatment increased the roughness of the surface, but mean roughness R_a was significantly greater for the alkali-treated ones. Ground and plasma-modified samples had 'smooth' surfaces with $R_a = 4$ nm. Deformability tests showed that the adhesion of the surface layers on samples oxidized at 600°C and alkali treatment samples was not sufficient; the layer delaminated upon deformation. It was observed that the cell cytoskeletons on the samples with a high nickel content or release were less developed, suggesting some negative effects of nickel on cell growth. These effects were observed primarily during initial cell contact with the surface. The most favorable cell responses were observed for ground and plasma-sputtered surfaces. These studies indicated that smooth, plasma-modified surfaces provide sufficient properties for cells to grow.

Keywords: nickel–titanium, bioactivity, plasma modification, surface engineering, cell modulation, thermal oxidation

INTRODUCTION

In the past decade, nickel-titanium alloys (also NiTi, Nitinol^{TM}) have been tried for dierent types of implants.[1–3] The most successful and well-established applications of the alloy are: cardiovascular stents, orthodontic wires, and endodontic instruments (e.g. files). Both shape memory eects and pseudoelasticity are used in these applications to obtain the desired functionality. For example, cardiovascular stents employ one-directional shape memory eect to treat coronary artery constrictions,[4] while in orthodontics the pseudoelasticity of the material is used to apply controlled and constant forces on teeth to correct their position.[5] Also, highly deformable and wear-resistant endodontic instruments made of NiTi alloy are used to obturate the root canals of teeth.[6] These mechanical properties create opportunities for the use of the nickel–titanium alloys in orthopedic implants. Some reports have already pointed to the possibility of using the NiTi for intramedullary nails, and spinal implants and discs.[1–3,7–9] The wider use of the alloy is constrained by high, nominally 54.5–57% (ASTM F2063-00) nickel content, which is considered to limit the biocompatibility of the material. To allow the alloy to be widely applied for orthopedic implants, biological properties *in vivo* need to be improved. The mechanical properties of NiTi can transform and enhance many treatments that typically use

implants made of other metals (Ti6Al4V, stainless steel and cobalt alloy) if high biocompatibility is insured. For example, using NiTi pseudoelastic foams, it could be possible to create spinal discs that pose highly desirable 'shock absorber' characteristics. Similarly, in the case of orthodontic implants, the pseudoelastic behavior of NiTi can enable us to apply controlled forces to spinal segments, and thus correct their positions more eectively. This kind of implant can significantly improve the results of scoliosis treatment, especially in growing spines[10–12] but they require sucient biocompatibility and osteoconductivity.

The ability of a material to support and influence the production of new bone *in vivo* is one of the major challenges for the materials used for orthopedic implants; materials that have an ability to support bone formation show a significantly lower rate of adverse reaction.[13–15] On the other hand, there is a large group of temporary implants, such as wrist plates, wires, and intramedullary nails, which require good biocompatibility but low osseointegration, minimal disturbance to the biomechanics, and ease in the removal of the implant.[16–20] The manipulation of only surface characteristics, such as local stiness, topography, surface charge, and structure, makes it possible to trigger or inhibit biological action, e.g., cell dierentiation and mineralization.[21–25] Good biocompatibility and/or osseointegration remain a challenge for nickel–titanium. The improvement of the biocompatibility is the primary aim of this article.

Some reports showed evidence of the toxic eects of nickel released from the Ni–Ti surface, and thus a variety of treatments have been developed to limit nickel release.[26–31] In contrary, other studies demonstrated that despite a high nickel surface content and high nickel release rate, cell response was not drastically aected.[1,9,21,32,33] To modulate cell response, simple surface treatments, such as thermal oxidation or polishing, were shown to be as eective as more expensive and complex treatments such as diamond-like coating (DLC).[1,2,14,27,34–39] Considerations of cell–surface interactions *in vivo* must *always* be related to the specific application, which dictates the desired properties. The deployment of NiTi almost always involve significant material deformation (e.g., pre-operation shaping) and most of the surface coatings and layers lack good mechanical integrity with the substrate and can crack and delaminate, which can lead to extensive corrosion. For these reasons, the use of nickel–titanium for biomedical application raises the question whether coatings and layers are of benefit, or whether the native nickel–titanium surface can provide sucient interface in bodily environment.

Taking together, the primary aim of our study was to investigate whether smooth and native NiTi surface insures sucient

biocompatibility and support cell growth. In our studies, we focus on the spinal applications of NiTi, which are divided into two groups: (1) applications that require high osteoconductivity – spinal discs, screws; (2) and those that require high biotolerance but limited cell attachment – distractors, bars, and pins. Our hypothesis is that: *plasma sputtered, native surface of nickel–titanium insures a good biocompatibility of the alloy*.

MATERIAL AND METHODS

In this study, pseudoelastic nickel–titanium alloy (Johnson Matthey, Inc. UK) was used. The concentrations of nickel and titanium in the alloy were 54.8% and 45.2% (producer certificate). A 1 mm thick nickel–titanium sheet was cut into 12 mm discs and the samples were pre-treated: grinding to a mirror finish (SiC 400–2000) and cleaning (5 min ultrasonication in isopropanol followed by washing with deionized water [dH_2O], soaking in 60% HNO_3 for 10 min, and finally ultrasonicated in dH_2O for 5 min). The pre-treatment was designed to insure the same initial conditions for all the samples prior to further modification.

Three types of treatments were applied to modify the surface properties:

a. alkali treatment – first, the samples were immersed in 10 M NaOH for 24 h at 80°C with continuous stirring. Second, the samples were heat treated at 600°C for 1 h (samples were placed in the furnace [Carbolite] and the temperature was ramped at 1°C/min; at 600°C, temperature was held for 1 h and then samples were cooled down at the rate of 1°C/min to room temperature) – sample coded **BNT**;
b. thermal oxidation at 400°C, and 600°C (temperature ramp, hold, and cooling rate for sample BNT) – sample code 400, 600; and
c. inductively coupled plasma sputtering: samples were placed in the vacuum chamber (PDC-002, Harrick Plasma) and sputtered with the plasma generated at 29.6 W (40 mA DC) applied to RF coil for 30 min – sample coded PC.

Control ground and pre-treated samples (coded as NT) and Thermanox® (coded as TX), for cell culture experiments, were used.

Chemical compositions of the surfaces were characterized using X-ray Photoelectron Spectroscopy, (XPS PHI 5700 Physical Electronics) utilizing an Al-Kα monochromated X-ray source. Survey scan for each sample was obtained at 100 eV pass energy between 0 and 1400 eV.

Five scans for each sample were collected. High-resolution spectra for the separate elements were collected at 20–50 eV pass energy. The elemental composition (atomic concentration) was calculated from high-resolution scans using CasaXPS (Casa software Ltd) and Multipak (Physical Electronics) after subtracting Shirley background. Evaluation was done in triplicate.

Surface wetting ability (water contact angle) was assessed utilizing the sessile drop method (KSV, CAM 100, Finland). Droplets of approximately 5 μL of ultra pure water (polar liquid, 18 MΩcm) were placed on the sample surface using a manual syringe. The drop images were recorded at 1 s intervals for 1 min. Three drops on each sample were measured and the measurements carried out in triplicate. For highly hydrophilic samples, only one drop per sample could be measured, because the drops spread on the entire sample surface.

Roughness (R_a) of the samples was measured using atomic force microscope. In this study, $40 \times 40\ \mu m^2$ scans were obtained using non-contact mode Atomic Force Microscopy (AFM) (Park System, XE-70). To calculate the average roughness, six line profiles on each image were analyzed. Measurements were conducted on three sample of each type.

Nickel Ni^{2+} ion release was measured using inductively coupled plasma atomic emission spectroscopy (Agilant inductively coupled plasma optical emission spectrometry (ICP-OES) 710). The instrument was calibrated using single element standard (Sigma–Aldrich TraceCERT®) in the range 0.001–5 ppm. To measure the cumulative release of nickel, a $2\ cm^2$ sample (PC, NT, 400, 600, and BNT) was placed on a V-base vial and 10 mL of cell culture media (α-MEM containing 10% fetal bovine serum (FBS) and 2% antibiotics – PAA Australia) was added to each vial; three samples of each type were incubated in separate vials at 5% CO_2/95% air and 37°C for 1, 3, 7, 14, and 21 days. After each time point, samples were gently washed with the media and placed in another vial with 10 mL of fresh media. The concentration (ppm) of nickel was measured at each time point and presented as a cumulative release. Average release for single sample was obtained from five measurement repeats.

The deformability of the surface layers (layer flexibility) was analyzed using SEM (Philips XL 30 CP). The samples were placed in a device that was capable of applying forces from three directions on the samples and uniformly deforming the samples (Figure 1). The device allowed the deformation to be maintained while samples were mounted in the microscope. This enabled the evaluation of deformed samples which was otherwise not possible due to the pseudoelastic properties of the material. The samples were deformed at an angle of 175° and tests conducted at room temperature.

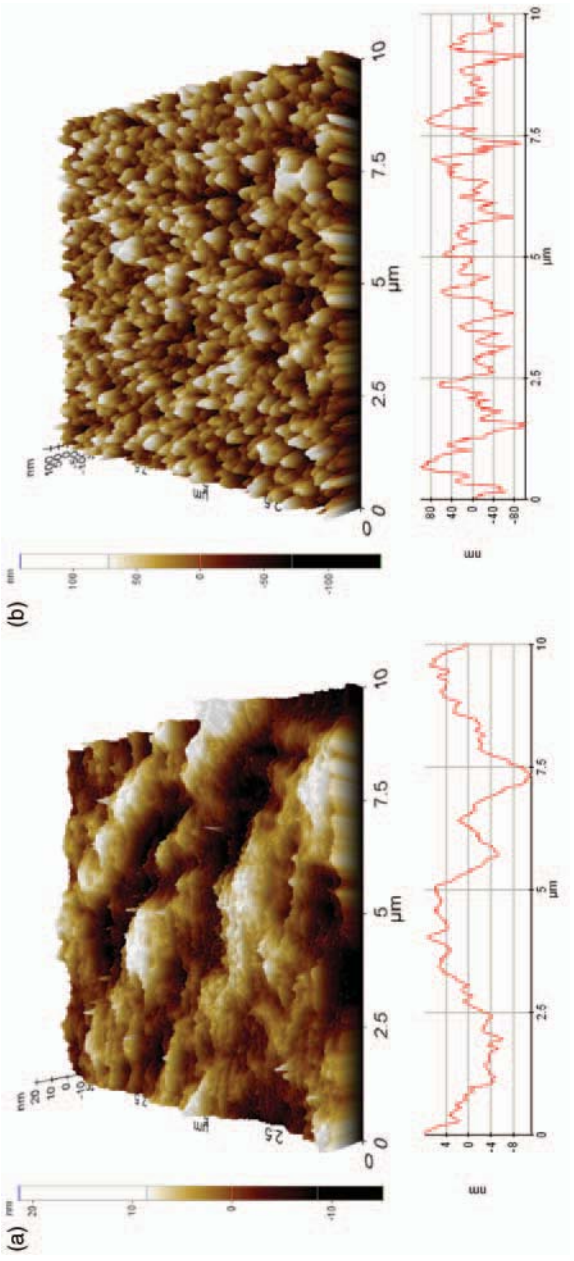

Figure 1. Atomic force microscope images and line profiles for the NT (a) and 600 (b) samples.

Cytoskeleton: Human primary osteoblast (HOB) cells obtained from PromoCell® (Heidelberg, Germany) were used. Cells were derived from human hipbone biopsies. Before trypsinization and seeding onto the samples, cells were cultured in α-MEM containing 10% FBS and 2% antibiotics for 10 days. HOBs, taken as representatives of a cell type that orthopedic materials would need to influence *in vivo* in order to produce new bone, were seeded on the samples with density 1×10^3 cells per sample and the culture was maintained in basal medium (α-MEM containing 10% FBS and 2% antibiotics). To assess the formation and organization of the cytoskeleton, cells were cultured for 3 days and stained (early time point to allow viewing of individual cells). The cells were fixed in 4% formaldehyde/PBS, with 1% sucrose at 37°C for 15 min. Fixed cells were washed in PBS and incubated in a permeabilizing buer (10.3 g sucrose, 0.292 g NaCl, 0.06 g $MgCl_2$, 0.476 g Hepes buer, 0.5 mL Triton X, in 100 mL distilled water, pH 7.2) at 4°C for 5 min, and then incubated in 1% BSA/PBS at 37°C for 5 min. As primary antibodies, anti-β-tubulin or anti-vinculin was used (1:100 in 1% BSA/PBS; tub 2.1 or Hvin1 monoclonal anti-human raised in mouse, (IgG1) Sigma, UK) for 1 h at 37°C. Rhodamine phalloidin was added for the duration of this incubation (1:100 in 1% BSA/PBS, Molecular Probes, OR, USA). Subsequently, the samples were washed in 0.5% Tween 20/PBS (5 min× 3), and secondary antibody was added (1:50 in 1% BSA/PBS monoclonal horse anti-mouse (IgG), Vector Laboratories, UK) for 1 h at 37°C. Finally, the samples were washed as described previously, and Fluorescein isothiocyanate (FITC)-conjugated streptavidin was added (1:50 in 1% BSA/PBS, Vector Laboratories, UK) for 30 min at 4°C. Samples were mounted in Vectashield fluorescent mountant and nuclei counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and viewed using fluorescence microscopy (Zeiss Axiovert 200 M).

In addition to cytoskeleton evaluation, the spreading of the cells was assessed on the basis of total surface area of cells after 3 days in culture. The cells were viewed using fluorescent microscopy (Zeiss Axiovert 200 M) with a magnification of 4; FITC filter (actin) was used. For each sample, 10 images in dierent locations were recorded. An external cell contour (threshold) was created on each image using ImageJ software (downloaded from the National Institute of Health, Bethesda, MD, USA; free download available at http://rsb.info.nih.gov/ij/) and the contours were subtracted from the background; subtracted area indicated the total area of cells for each image For each sample type, the cell surface area was calculated as an average obtained from all images. This analysis did not consider the number of cells but cells area was assessed in relation to surface area for 10 expositions. The results were

presented as a percentage of the area of cells on test sample versus area of cells cultured on Thermanox. Therefore, increase or decrease of cell area in relation to cell area on TX was presented.

Dierentiation of the cells was analyzed on the second group of the samples, which were cultured for 21 days. At this time, the cells will be preparing themselves for mineralization (if osteoblast dierentiation is supported). The cells do this by secreting bone-specific extracellular matrix proteins (osteocalcin (OC) and osteopontin (OPN); immunofluorescence). Both are produced by osteoblasts just prior to bone formation. Hence, they provide an indication as to whether cells would be able to mineralize on the material surfaces.

The culture media was replaced every 3 days. After 21 days (late time point to allow maturation), the cells were fixed with glutaraldehyde and stained for OC and OPN with a protocol similar to the one described above. The primary antibodies used were OC4-30 and AKm2A1 from Autogen Bioclear, UK. Samples were then viewed by fluorescence microscope (Zeiss Axiovert 200 M). Tests were conducted in triplicate.

RESULTS

Surface chemistry

The analysis of the surface chemistry showed that surface nickel concentration was around 8% for control ground (NT) samples and dropped to about 6% and 2% after thermal oxidation at 400°C and 600°C. Nickel concentration was not altered by plasma cleaning. However, its concentration in the samples treated with NaOH and thermally oxidized was twice as high as observed for the control samples.

Nickel for both thermally oxidized sample groups was recognized mainly as Ni_2O_3 (binging energy BE = 855.5 eV) with small contribution of NiO (BE = 854.1 eV). In alkali NT and BNT samples, nickel was predominantly present in the form of NiO, while in PC samples it was present as Ni_2O_3 with a small amount of metallic nickel (BE = 852.7 eV). Similar changes were observed for titanium; insignificant increase in titanium concentration was observed after thermal oxidation, and about 2% decrease in Ti was observed in plasma-cleaned samples. The most significant decrease in titanium concentration was recorded for alkali-treated samples (Table 1). Titanium for all the samples was recognized as TiO_2 (BE = 456.5 eV); however, a small amount of metallic titanium was observed in both NT and PC samples. The level of carbon contaminations was the highest in alkali-treated samples, followed by plasma sputtered and thermally oxidized ones at 600°C.

Table 1. The elemental chemical compositions of the samples surface evidenced by XPS, wetting ability, and roughness.

	C 1s (at.%)	Ca 2p (at.%)	N 1s (at%)	Na 1s (at%)	Ni 2p (at%)	O 1s (at%)	P 2p (at%)	Ti 2p (at%)	S 1s (at.%)	Contact angle (°)	Roughness, R_a (nm)
NT	10.9 (1.3)	–	0.3 (0.1)	5.1 (0.4)	8.0 (1.2)	57.5 (0.8)	–	18.2 (0.4)	–	51 (3)	4.1 (1.1)
400	7.3 (0.6)	0.2 (0.1)	–	5.7 (3.1)	6.1 (3.0)	60.6 (0.2)	–	20.1 (0.3)	–	55 (8)	7.6 (1.1)
600	13.6 (6.9)	–	–	4.1 (1.9)	1.9 (0.6)	59.2 (4.4)	–	21.2 (1.3)	–	45 (3)	22.1 (2.7)
BNT	27.4 (1.6)	1.6 (0.4)	0.4 (0.1)	1.3 (0.2)	20.2 (0.5)	44.3 (1.7)	–	1.4 (0.1)	–	14 (5)	102.2 (3.3)
PC	17.7 (1.1)	0.4 (0.1)	1.4 (0.1)	–	7.7 (0.4)	55.3 (0.8)	0.4 (0.1)	16.9 (0.6)	0.5 (0.1)	6 (6)	3.2 (1.4)

Note: Standard deviation values are given within parentheses.

The surface of the plasma-cleaned samples contained a small amount of other laboratory contaminations such as Ca, P, and S. All the other samples also contained some amount of Na contamination, which is another typical laboratory contamination built into the surface during the surface preparations.

Surface Wetting Ability

The analysis of the water contact angle showed that thermal oxidation at 400°C did not have a major influence on the wettability of the surface (Table 1). A drop of 10% was observed in the contact angle after the oxidation took place at 600°C. Significant changes in the wettability were observed after alkali treatment and plasma treatment. The contact angle values dropped to 14° and 6°, respectively, which indicated that the surfaces were highly hydrophilic (Table 1). The overall plasma treatment aected the surface wettability the most.

Roughness

Results of the roughness measurements were compiled in Table 1. R_a for the ground nickel–titanium was around 4 nm and it was not aected by plasma sputtering. Some increase in roughness was observed after oxidation at 400°C (Table 1). For both the samples, thermally oxidized at 600°C and alkali treated, the roughness parameters were significantly greater. For thermally oxidized, R_a was around 22 nm. AFM images evidenced that the surface had a nodular morphology; nodules were dense and uniform, but randomly distributed on the surface (Figure 1). For alkali-treated samples, R_a was around 102 nm.

Nickel ion release

Nickel ion release showed significant dierences in the concentration of nickel in the culture media when samples were immersed up to 21 days. The greatest amount of nickel was released from BNT and 600 samples and reached almost 2.5 and 0.1 ppm in 10 mL of media after 21 days. Nickel release increased steadily for these samples during the experiment. Elevated nickel concentration at day 1 was observed for samples BNT and PC. The release from sample PC changed insignificantly in the next time points – the released amount was negligible. In the BNT sample, a large amount of nickel was released. In general, nickel release

for NT, PC, and 400 samples remained at the a low level and did not exceed 0.04 ppm after 21 days (Figure 2).

Deformability

The deformation of the samples that had relatively thick oxide layers (600, BNT) resulted in delamination and layer cracking (Figures 3 and 4). Severe delamination was observed for samples 600 and BNT (Figure 4a). However, this eect was observed not in the area of the largest deformation, but close to the sample edges where the compressive forces were applied (Figure 4). In the area of largest deformation for samples 600, small spots of removed layer (bright spots – Figure 4(a)) were evidenced (Figure 4(b), right). In contrast, the layer heavily delaminated and large debris were produced (Figure 4(b), center) on the areas near the edge. In addition, in Figure 4(b), right, small dark spots were observed and these were suggested to be the areas of greater height or delaminating areas. Neither delamination nor cracking was observed for samples NT, PC, and 400.

In summary, deformability examination showed that samples treated at 400°C, control NT ones, and plasma-cleaned ones had the highest deformability with minimal surface disruption.

Cytoskeleton After 3 days

The cytoskeletons and focal adhesions (actin, tubulin, and vinculin) for all tested samples were well organized with defined actin stress fibers. Particularly well-organized cytoskeleton (actin and tubulin) was observed for samples 400, NT, and PC (Figure 5(a), (b), and (e)). Vinculin staining of focal adhesions showed that the cells formed large contacts with all the tested surfaces. This was particularly evident for PC samples (Figure 5(e)). Cytoskeleton organization on samples NT, PC, and 400 (Figure 5(a), (b), and (e)) was found to be more developed than that observed on control samples (TX; Figure 5(f)).

In the cells cultured on samples BNT and 600, the cytoskeletons seemed to be less developed in comparison with cells on the other samples (Figure 5(c) and (d)). The cells typically displayed good expression of stress fibers with slightly less developed tubulin networks. Cells were seen to be more rounded and the cytoskeleton was organized around nuclei. Some focal contacts with the surface were also formed but were less evident than those found on samples NT, PC, and 400.

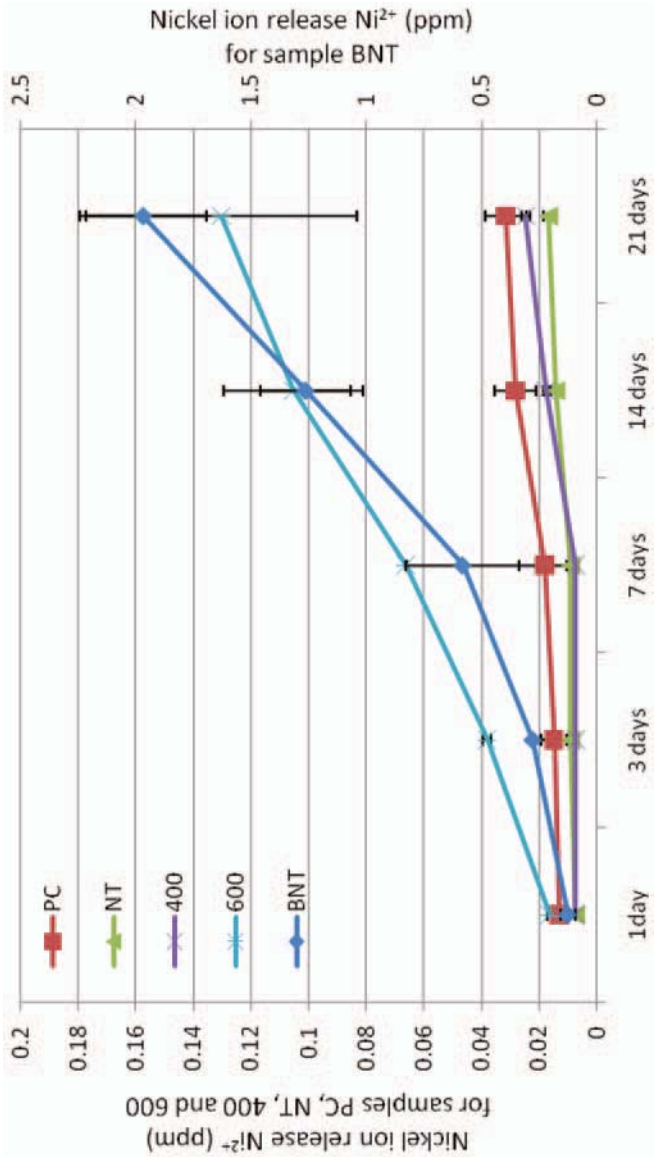

Figure 2. Nickel ion Ni^{2+} release in 10 mL of culture media up to 21 days evidenced by ICP-OES.

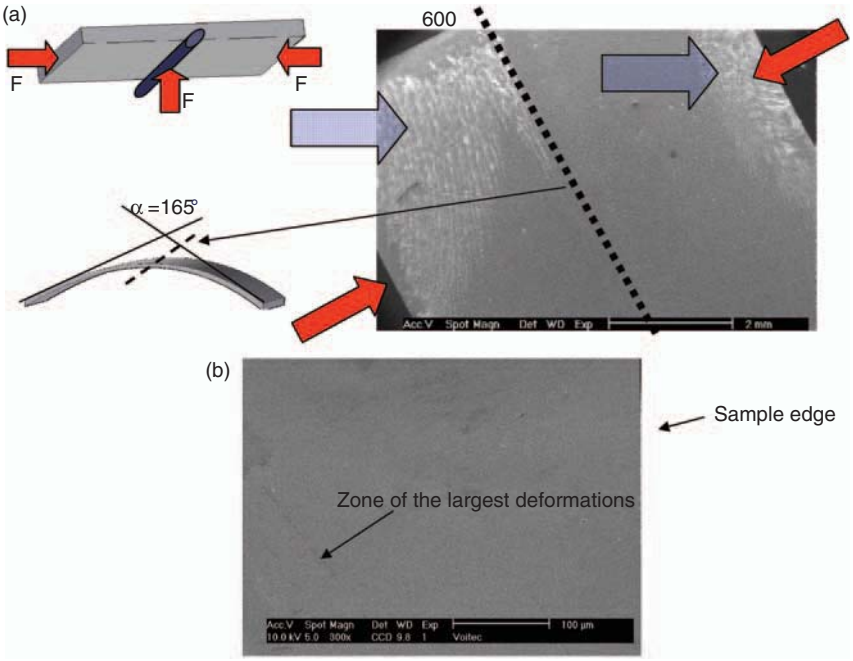

Figure 3. (a) Schematic representation of the deformability test; SEM image of the sample 600 after deformation. Visible impairment of the layer was evidenced in the areas near the sample edges (blue arrows). Red arrows – applied force direction and (b) SEM image of sample 400 after deformation showing no impairment to the layer.

Surface Area

Cell spreading analysis, which was done on the basis of the total cell surface area on the samples after 3 days in culture, showed that the area covered by cells significantly increased for the samples NT, PC, and 400 in relation to control TX (Figure 6). Total surface area covered by cells was the greatest for sample PC. In both samples 600 and BNT, a drop of the total cell surface area was observed. The drops were 30% and 51%, respectively, and statistically significant for both types of samples.

OC and OPN Immunofluorescence

Osteoblast cells after 21 days in culture showed positive staining for OC for all tested samples with the exception of sample 600. In samples 400, NT, and PC, the expression of the OC was particularly high. OPN

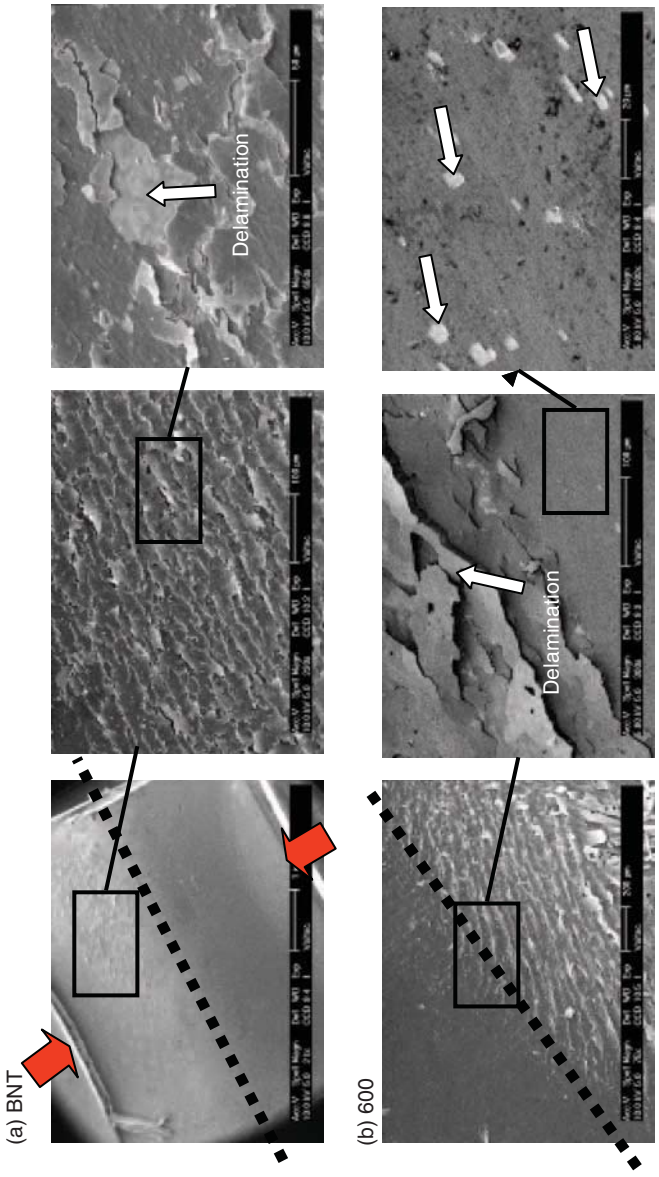

Figure 4. SEM images of the deformed samples: (a) sample BNT and (b) sample 600. Dotted line represents area of the largest deformations. On both samples, severe delamination of the layers is seen.

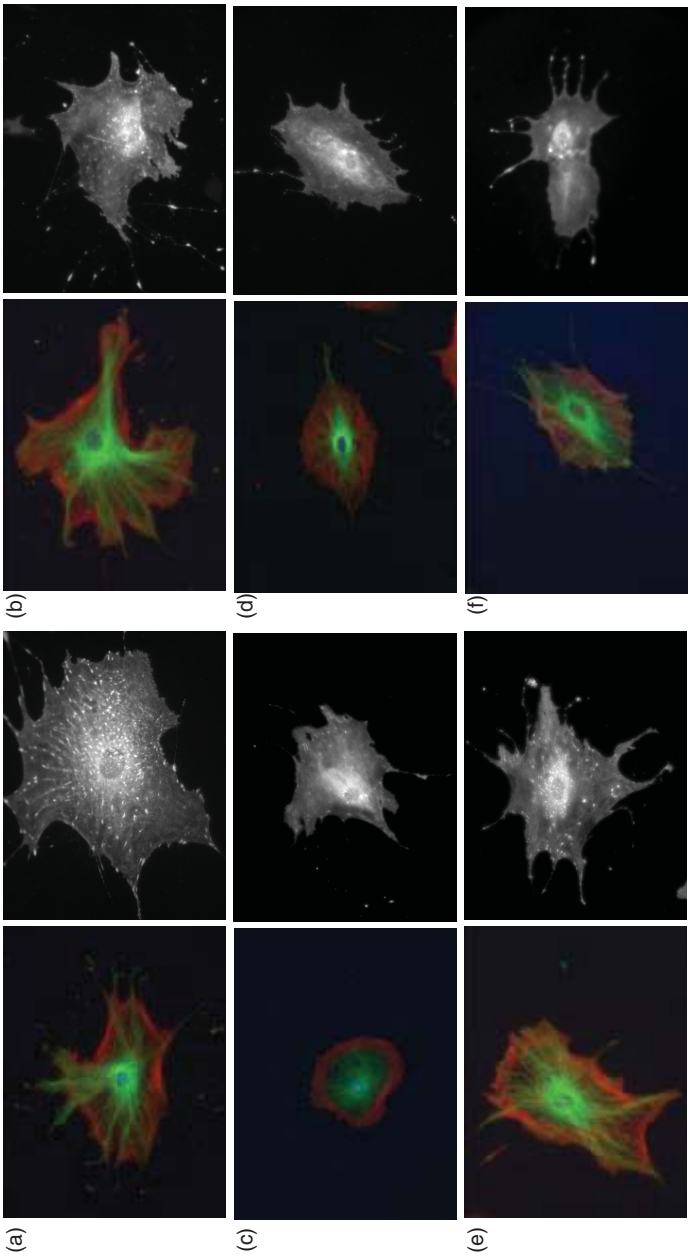

Figure 5. Cytoskeleton and focal adhesion staining in HOBs cultured on samples: (a) NT, (b) 400, (c) 600, (d) BNT, (e) PC, and (f) TX. Red – actin filaments; green tubulin microtubules, gray – vinculin focal contacts; blue – cell nucleus. Cells cultured on NT, 400, and PC samples display a well-defined cytoskeleton and large focal adhesion sites, whereas on the 600 and BNT cells have a less organized cytoskeleton and fewer, smaller focal adhesions.

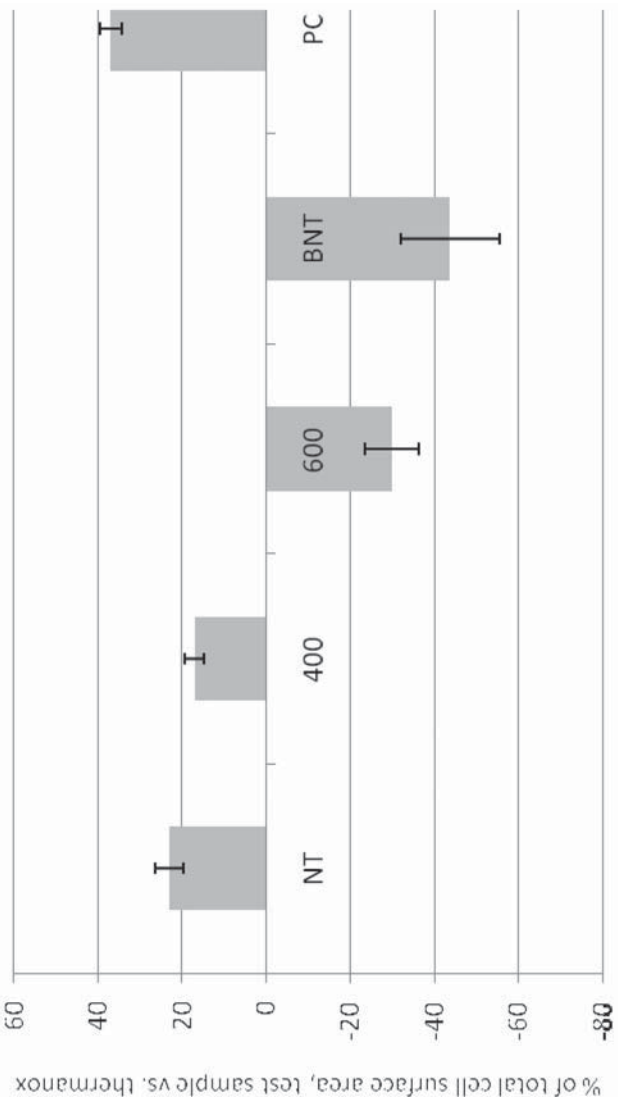

Figure 6. Percentage of increase or decrease of total surface area covered by cells for test samples (NT, 400, 600, BNT, and PC) in relation to control Thermanox®.

after 21 days of culture was evident for all tested samples at the level similar to that observed for control samples (Figure 7).

DISCUSSION

Nickel–titanium alloys have been a subject of significant controversy since they were introduced for medical applications.[9,27,29,40–42] The major disadvantage is the high nickel content. Nickel, despite being a vital trace element in the human body, can be toxic in high doses.[43] However, some reports suggested that nickel release from nickel–titanium was at a lower level than that observed for stainless steel, which has four times less nickel.[26,28,36,42,44–45] Despite the high nickel content, the toxicity of the alloy is debatable and this could be related to the oxidation state of the nickel, and typically its lower concentration on the surface as the surface is primarily occupied by titanium dioxide. This thin TiO_2 coat may be critical for good biocompatibility.[27,28] The exceptional mechanical properties of the alloy encourage investigation into the use of the alloy in dierent medical devices; nowadays, stents and orthodontic brackets are primarily produced from NiTi alloys. Also, new, advanced, and smart implants and coatings are made of porous nickel titanium foams.[3] However, to improve the biological performance of the alloy, which mostly relates to the suppression of the nickel release, many types of treatments have been investigated.[1,14,15,21,27,30–37,39,46,47] A few types of these treatments dominate the literature: electropolishing, thermal, anodic-oxidation, and coating formation using Chemical Vapour Deposition (CVD) or Physical Vapour Deposition (PVD) methods focused on DLC coating. The large benefits of electropolishing, should be viewed in the context of the high risk related to the volatile mixtures used (sulfuric acid + methanol)[48] and highly corrosive (i.e., HF) electrolytes; the use of such media is questionable when alternatives are available. While mechanical polishing may oer similar benefits, it has already been shown that residual stresses observed as a result of the process may aect surface properties and introduce inconsistencies.[27] Thermal and anodic oxidations can be easily applied to complex geometries, which is an advantage and these treatments are eective methods to improve biocompatibility.[49] The close control of the process parameters allows us to produce surface layers and coatings with dierent chemical compositions and topographies, which can be used to manipulate cell or bacterial activity.

Some reports already pointed to the likelihood that a ground, smooth surface of nickel–titanium may be sucient for biomedical applications.[28,50] In our studies, we concentrated on the potential application

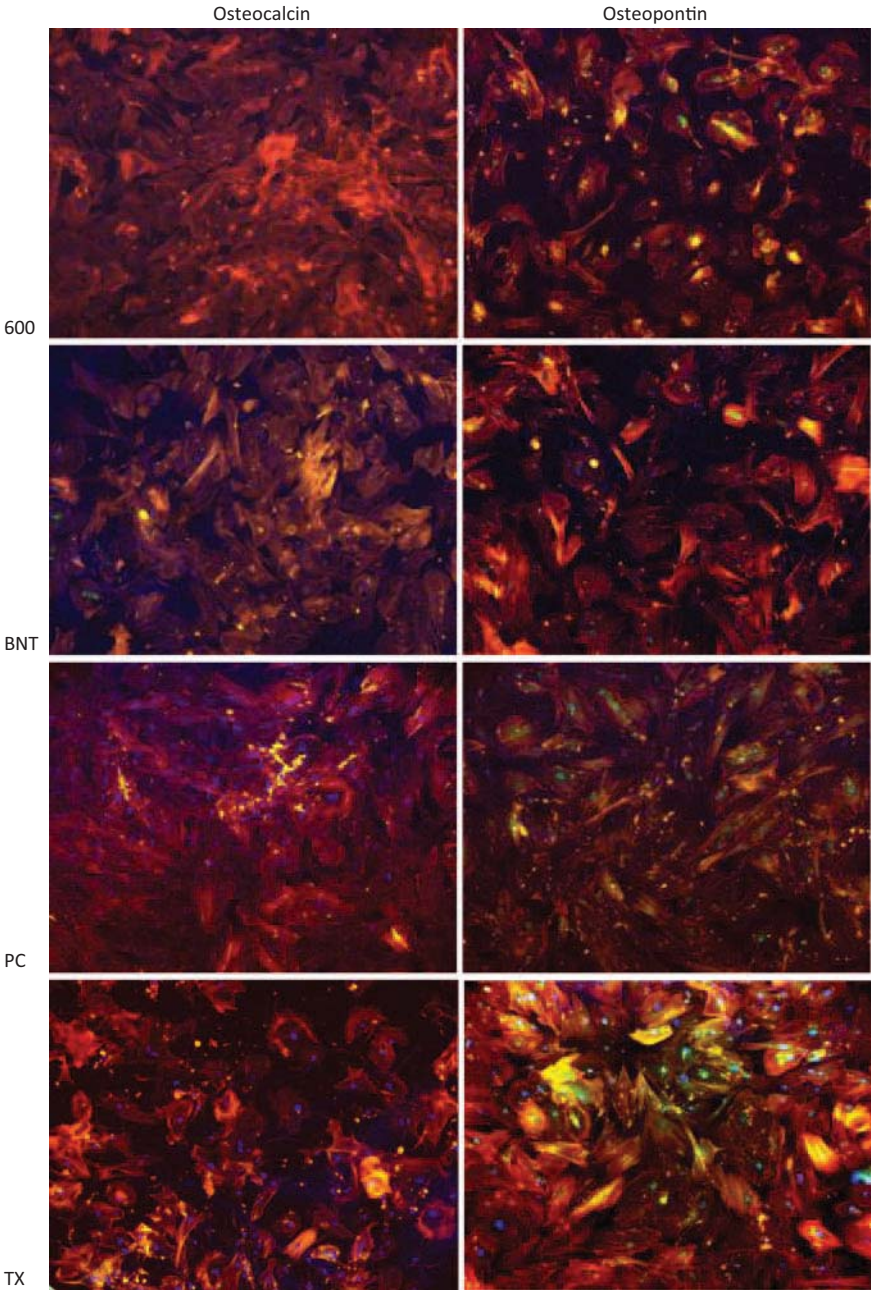

Figure 7. Fluorescence images after 21 days in culture for 600, BNT, PC, and Thermanox® samples; OPN and OC staining (red – actin; green – OC or OPN; blue – nuclei), magnification 20×.

of nickel–titanium for orthopedic implants. It is already well known that the vast majority of metal-based orthopedic implants remain in the body temporarily and to ease their removal, their integration with the body tissue should be minimal. Therefore, a smooth finish is preferred for such implants.[19,20] It has already been demonstrated that smooth titanium alloy implants have a very good biocompatibility and is particularly relevant for many orthopedic applications.[16,18,19] Taken together, these findings raise the tantalizing possibility that the native and smooth surface of nickel–titanium can be sucient for medical application and thus, expensive and complex treatments may not be required.

In our study, we prepared some dierent sample groups: all the samples were pre-treated – ground to a mirror finish and cleaned using well-established methodology published before;[33] specimens prepared in that way were used as a control. Other three groups were: (1) thermally oxidized at two dierent temperatures, (2) chemically treated in NaOH, and (3) plasma sputtered. Chemical analysis showed that chemical treatment increased surface nickel content; the nickel concentration of the alkali-treated samples was twice as high as that observed in ground samples. In addition, nickel release was considerably higher from alkali-treated samples than from any other tested samples. It was suggested that alkali treatment etched titanium from the top surface, thus forming a nickel-rich layer. Because cells contacted the surface almost directly, nickel became more 'visible' to them and aected their behaviors – less developed cell cytoskeletons were observed for these samples after 3 days in the culture. The reduction of nickel surface concentration was observed after thermal oxidation when compared with ground samples. Nickel release from the samples oxidized at 600°C was elevated when compared with samples treated at 400°C, ground ones and plasma–sputtered ones, but it was considerably lower than that from BNT samples. In general, release of nickel was the highest from sample BNT followed by sample 600. The release level was negligible for samples 400, NT, and PC and the cell response was the most favorable for these samples.

In particular, cell culture experiments investigated whether cell response was aected by the surface chemistry, ion release, and wettability and importantly whether a smooth, native, and cleaned surface on NiTi is biologically relevant. Three major cell structures were assessed (cytoskeleton organization[51]): *actin* is a major structural protein and gives information on the general condition of the cells, indicating adhesion and spreading capability of the cells. *Tubulin* is an important structural protein in cell metabolism processes, where vesicles are moved into and out of the cell (endocytosis and exocytosis) along the tubulin microtubules. *Vinculin* is a protein involved in cell adhesion; staining for

vinculin shows where the cells contact the materials. These adhesion points are important in cell signaling, proliferation, and dierentiation. The cell-spreading capability was assessed on the basis of the total cell surface area on the sample surface. The analyses of the cell cytoskeleton showed very well-developed tubulin, actin networks for samples that were ground, thermally oxidized at 400°C, and plasma sputtered. These samples had smooth surfaces ($R_a = 4$ nm) and moderate wetting ability. The organization of the cytoskeleton was better on these samples than that was observed on control TX samples. Clear and large focal adhesions were observed in both ground and plasma-sputtered samples, while in samples that were chemically treated and oxidized at 600°C, cells were smaller and their cytoskeletons less organized. Only few, small, focal adhesions were observed for these samples. Because samples 600 and BNT had very low and very high surface nickel contents, respectively, it was concluded that the surface nickel concentration was not a dominating factor that impacts cell development. But both samples released greater amount of nickel when immersed in culture media, which can be the major reason for the limitation of the cell development. Sample 600 showed hydrophobic character and had rough (random nanoscale ''noodle-like' protrusions) surface, which together with elevated nickel release caused less favorable cell responses. It has been previously reported that samples oxidized at 600°C show limited bioactivity when tested in simulated body fluids.[21,33,52] It must be highlighted that despite very high nickel content on the BNT samples that aected cell development, the cells still conserve their phenotype and remained vital. Cell behaviors might have been related not directly to the concentration of the elements but also their form and stability. Nickel on thermally oxidized samples and plasma-cleaned samples was present as Ni_2O_3, while in alkali-treated and ground samples, it was present as stable and non-soluble NiO_2. However, there is no clear evidence in literature that one oxidation state of nickel or the other is more favorable for cells, but NiO is much better characterized. Titanium was in the form of stable TiO_2 in all the samples, but some amount of metallic titanium was found in ground and plasma-sputtered samples.

The analysis of the total surface area of cells that covered the surface showed the least cell surface area for samples 600 and BNT. This result aligned with previous findings and confirmed impaired cell responses to these surfaces (BNT and 600), while the total area of cell for the three groups of samples (400, NT, and PC) was significantly greater than observed for TX. The most significant increase was observed for the plasma-sputtered samples. Finally, the analysis of protein expression (OC and OPN) from the cells after 21 days in culture showed positive

results for both OC and OPN for all the samples with the exception of the samples 600.

Presented results suggest that the smooth, native, and cleaned surface of nickel–titanium can support cell growth and insure good biocompatibility. It was observed that high surface nickel content impaired the development of the cells on alkali-treated samples. However, after this initial impairment of the cell cytoskeleton development (at early time points), the cells grew in density and conserved their phenotype-expressing osteogenic proteins. The least favorable cell response was noted on thermally oxidized samples at 600°C; we concluded that it was related to the specific random nano-topography, low wettability of the surface, slightly elevated nickel release, and the previously reported limited bioactivity for these samples. These results indicated that the high nickel content can impact cell development but cells are capable of recovery and in the long run they maintain their phenotype and function even in the presence of a relatively high nickel content. This could also suggest that nickel may not be particularly cytotoxic and the form of nickel as well as other surface parameters such as topography and wetting ability contribute to the cell response.

In summary, these studies demonstrated that the topography and wetting ability of the surface were major factors that influenced cell response; chemistry (mainly nickel), on the other hand, had some impact on cell response but it was not a key factor. Cell response to smooth and cleaned nickel–titanium surface was very positive; therefore, simply grinding and/or plasma sputtering was an eective surface preparation method to insure good biocompatibility. The results demonstrated that plasma treatment is a promising method for achieving surface modifications of NiTi, enabling the possibility to improve biocompatibility.

Taken together, these finding suggest that *the native surface of nickel–titanium supports cell dierentiation and insures biocompatibility of the alloy* – hypothesis of this study. It was also found that *thermal oxidation at 600°C can suppress cell dierentiation but the oxide layers were poorly adhered to the substrate and such a treatment is not appropriate for load-bearing implants*.

CONCLUSIONS

(1) Smooth and native surfaces of nickel–titanium insures good cellular response.
(2) Release of nickel from the samples impaired cell development at early time points.

(3) Relatively thick oxide layer was formed on NiTi substrate by thermal oxidation at 600°C and as a result of chemical treatment, it lacks mechanical bonding with the substrate, which results in cracking and delamination.
(4) Plasma sputtering is a promising, easy, and cost-eective technology used to modify the surface of nickel–titanium for biomedical applications.

On the basis of the results, it can be concluded that by cleaning the surface using plasma sputtering, grinding, or thermal oxidation at 400°C, it is possible to improve the cell response to NiTi alloy. At the same time, the deformation of the material will not cause damage (cracking, delamination) to the surface layers.

ACKNOWLEDGMENT

This work was supported in part by WCU Program through the National Research Foundation of Korea(NRF) funded by the Ministry of Education, Science and Technology (No. R31-10069). MJD is supported by grants from EPSRC and BBSRC and the Chief Scientists Oce (Scottish Health Executive). The authors also acknowledge the facilities as well as scientific and technical assistance from sta in the AMMRF (Australian Microscopy & Microanalysis Research Facility) at the Australian Centre for Microscopy & Microanalysis, The University of Sydney.

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