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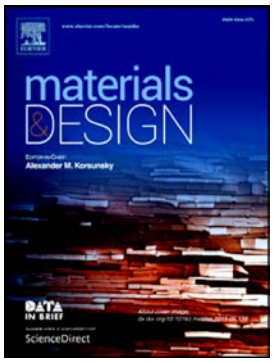


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# Cellular behaviour of bone marrow stromal cells on modified Ti-Nb surfaces

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## Abstract

The cellular behaviour of bone marrow stromal cells on titanium surfaces modified by niobium diffusion is presented in order to test their osteogenic differentiation response after culturing for 21 days. The surface modification of Ti substrates produced by powder metallurgy was performed through niobium diffusion treatments. Ti-Nb exhibited a β-Ti surface together with a microstructural (β / α+β / α) and compositional (Ti-Nb) gradient which enhances hardness, wear resistance and lowers the elastic modulus making it more similar to the human bone. Furthermore, the Ti-$Nb_{NH4Cl}$ by means of the activating agent achieved three times the hardness of Ti together with a porous surface.

The *in vitro* osteogenic differentiation response of bone marrow stromal cells on both Ti-Nb surfaces indicated the positive cell-material interaction. The osteogenic differentiation of cells was successful after 21 days, considering the positive response in terms of increased cell viability, lactate dehydrogenase-(LDH) activity, alkaline phosphatase-(ALP) activity expression (osteogenic marker) and bone-like nodules deposition by ST-2 cells as a bone mineralization cue. Therefore, the positive effect of a low elastic modulus Ti-Nb surface and a porous nitride Ti-$Nb_{NH4Cl}$ with suitable wettability and average roughness values on the osteogenic differentiation response of bone marrow stromal cells is demonstrated.

KEYWORDS: β-gradient Titanium, Surface modification, Powder technology, Osteogenic differentiation, Bone mineralization.

## 1. Introduction

*In vitro* cell behaviour is one of the main biocompatibility cues that a biomaterial has to satisfy to be employed as bone replacements. In this context, osteogenesis is one of the major reasons why there is a potential tendency toward enhancing this feature in Ti alloys, improving their integration with bone tissues [1]. Among the metal materials, β-Ti alloys exhibit superior mechanical performance-biocompatibility balance due to their

combination of lower Young's modulus with non-toxic elements. It is well known that the surface properties of the components in contact, i.e. bone and replacement material, are the main parameters that influence the interaction with the biological surroundings. In this context, chemical and physical properties together with the topography are the main aspects to alter in order to promote bone formation [2]. However, the final success of the complex osseointegration or osteogenesis processes depends on both, bulk and surface properties [3]. In this context, the surface modification of Ti allows specific surface properties to accelerate the osteogenic differentiation, preserving the advantage of the titanium lightness in the core [4].

Although the ideal surface properties for cell colonization and differentiation are not precisely specified because they are affected by the different cellular phenotypes, chemical composition, roughness, wettability and porosity have been considered as crucial keys. Regarding the chemical modifications of Ti-surfaces, the strategies followed to induce bioactivity are mainly focused on hydroxyapatite coating, calcium phosphate coating, deposition of Mg, P, Ca or other metallic ions like Sr to promote differentiation *in vitro* [1]. However, the introduction of other biocompatible metallic ions such as Nb, Zr or Mo allows the modification of the microstructure decreasing the elastic modulus and thus, improving the integration with surrounding bone and avoiding the death of bone cell because of the stress shielding [5]. Currently, numerous studies are focused on the development of β-Ti alloys in order to match the mechanical requirements and to better distribute the compression force during loading [6], [7], [8], [9], [10]. Recently, the Ti-24Nb-4Zr-8Sn alloy has been reported as a potential candidate in early osseointegration with osteogenic marker expression [11], [12]. Furthermore, Nb as alloying Ti element has increased the cell viability of different Ti-Nb compositions compared to the forged or melted Ti6Al4V alloy, being not related to the suppression of osteocalcin deposition and matrix mineralization as Al and V [3], [13]. Roughness is the other key factor to accelerate osteogenesis, especially moderate average roughness ($R_a$) values in the order of 1.2 - 1.5 μm have been proved as beneficial for ALP activity expression and cell differentiation *in vitro* [14]. Moderate rough Ti surfaces have been suggested as the most suitable, as average roughness ($R_a$) values higher than 4 μm can induce cell differentiation but suppress cell proliferation. Hydrophilicity seems to drive cell differentiation into the same direction; hydrophilic rough Ti surfaces promote cell differentiation but not cell proliferation *in vitro*. However there is opposite evidence in in vivo studies which can be affected by protein adsorption [3], [14]. Moreover the effect of porosity is essential for the cell-attachment and final osteogenesis. This can be introduced by means of different structures such as scaffolds, foams or surface porosity. The effect of porosity applied by different techniques has been reported in different studies i.e. the deposition of a micro-scale porous oxide layer on Ti by microarc oxidation, leading to positive cell proliferation and differentiation [11] or on the contrary, porous on-growth surfaces displaying an inverse rate of cell proliferation and ALP activity [15].

Based on the good bioactivity and cytotoxicity response offered by our modified Ti surfaces with hydroxyapatite formation after 21 days of immersion in SBF and non-cytotoxic response of osteoblast-like cells after 48 h of incubation [16], the goal of this study was to investigate their osteogenic differentiation potential. For this purpose, the cell-material response of mice bone marrow stromal cells to the designed Ti-Nb and Ti-$Nb_{NH4Cl}$ surfaces was evaluated after 21 days of cell culture through: i) cell viability and

proliferation, ii) cell adhesion and morphology, iii) cell differentiation and, iv) bone mineralization. Thus, this study is an attempt to understand the biological response of a new family of functionally gradient diffusion-based Ti surfaces to be further proposed as possible candidates for biomedical applications.

## 2. Experimental procedure

### 2.1 Modified Ti surface design and fabrication

Two different Ti surfaces (Ti-Nb and Ti-$Nb_{NH4Cl}$) designed by surface modification through Nb diffusion treatments have been produced [17], [18]. The surface modification has been performed on Ti substrates produced from Ti hydride powder (GfE Metalle und Materialien GmbH, Germany) with particle size below 63 μm using a conventional powder metallurgy route of uniaxial pressing at 600 MPa plus a high vacuum sintering at 1100 $^{O}$C for 60 min. Before the diffusion processes, an aqueous suspension of Nb or Nb + $NH_4Cl$ was sprayed on the Ti substrates. The particle size of the niobium powder used was between 1-5 μm (Alfa Aesar, Germany) and the activating agent $NH_4Cl$ was provided by D'Hemio Roy (Spain). The final surfaces were obtained after the diffusion process by means of a heat treatment at 1100 $^{O}$C for 3 h performed in high vacuum for Ti-Nb, and in an Ar atmosphere for Ti-$Nb_{NH4Cl}$ to allow the vapor creation with the activating agent. The heating and cooling rates were 5$^{O}$C/min. The surfaces were finished after a polishing step up to 1 μm for Ti, and a soft grinding step with 1200# SiC emery paper for Ti-Nb and Ti-$Nb_{NH4Cl}$, performed after the deposition process in order to ensure reproducible and homogenous surface characteristics. The different designing parameters followed for the material fabrication together with their final surface condition and nomenclature used further on are summarized in Table 1. The surface chemical composition and microstructure characterization are given elsewhere [16]. Finally, the samples with dimensions 15 mm in diameter and 3 mm in height were sterilized in an autoclave at 121 $^{O}$C for 90 min.

**Table 1.** Different designing parameters of the materials prepared with the nomenclature used further on in this study.

| ***Design materials*** | ***Design parameters*** | | | | | |
|---|---|---|---|---|---|---|
| | **Diffusion Element** | **Diffusion Process** | | **Final surface condition** | | |
| | *Nb* | *Diffusion*[(1)] | *TRD*[(2)] | *Surface finishing* | *Roughness (μm)* | *Wettability ($^{O}$)* |
| Ti | - | - | - | Polished (1μm) | 0.65±0.2 | 83.9±0.8 |
| Ti-Nb | x | x | | Ground (1200#) | 1.38±0.3 | 83.1±1.6 |
| Ti-$Nb_{NH4Cl}$ | x | | x | Ground (1200#) | 1.80±0.3 | 91.3±1.5 |

*(1) In high vacuum ($10^{-5}$ mbar)*

*(2) Thermo-reactive diffusion (in Ar atmosphere with activating agent ($NH_4Cl$))*

### 2.2 Characterization of modified Ti surfaces

The surfaces of the materials were exposed to X-ray diffraction measurements in grazing incidence condition (GIXRD) were carried out with a Bruker AXS D8 diffractometer equipped with an X-ray Co tube operating at 40kV and 30 mA and

Goebel mirror optics to obtain a parallel and monochromatic X-ray beam. 2θ scans over a range from 35 to 100º were performed at an angle of incidence of 5° with a step width of 0.02º and a counting time of 3 s/step. The microstructure and element distribution of the diffusion layers were analyzed by FE-SEM (FEI Teneo) equipped with EDAX device. In order to see the effect of niobium and porosity on the final material densities, they were measured by helium pycnometry. The results were expressed as an average of two samples (in total 10 measurements of each material).

2.3 Cell seeding and culture conditions

Samples used in this investigation, Ti, Ti-Nb and Ti-$Nb_{NH4Cl}$ were first sterilized in an autoclave at 121 $^{O}$C for 90 min, and then placed into an untreated 48-well polystyrene plate (Greiner, Germany; internal well diameter 15 mm). They were seeded with mice bone marrow stromal cells (ST-2) and suspended in a stimulating osteogenic differentiation medium: Dulbecco's modified Eagle's Medium (DMEM) containing 10 % fetal bovine serum, 1 vol % penicillin/streptomycin and doped with 50 µl/ml of ascorbic acid, 10 mmol of β-glycerolphosphate and 10 nM of dexamethasone. Finally, cells were cultured for 21 days in physiological conditions at 37 $^{O}$C in a 5 % $CO_2$ humidified air atmosphere. The medium was changed on the first and fourth day of each week. Five samples of each material were used for cell viability, three for cytotoxicity and cell differentiation, and two for bone mineralization and cell morphology studies.

2.4 Cell viability and proliferation

Cell viability was measured through Cell Counting Kit-8 (CCK-8) using WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt), which produces a water-soluble formazan dye upon bio-reduction in the presence of an electron carrier, 1-Methoxy PMS. CCK-8 solution is added directly to the cells and no pre-mixing of components is required. WST-8 is bio-reduced by cellular dehydrogenases to an orange formazan product soluble in tissue culture medium. The number of living cells is directly proportional to the amount of formazan produced. The measurement is based on a sensitive colorimetric assay to determine the number of viable cells in the proliferation and cytotoxicity assays. This test permits higher sensitivity detection than other tetrazolium salts such as MTT, XTT or MTS. After the cells were washed with PBS, 210 µL of solution containing 1 % WST-8 was added in each well and the plates were incubated for 1.5 h. Subsequently, the supernatant of all samples was transferred to 1 mL cuvettes and the absorbance was determined at 450 nm in a UV-VIS spectrometer.

Lactate dehydrogenase (LDH-) activity was assessed as an indirect measurement of the amount of attached cells on the samples. A commercially available LDH-activity quantification kit (TOX7, Sigma-Aldrich) was used to quantify cell number by the LDH enzyme activity in cell lysate. ST-2 cells cultured were washed with PBS and lysed with lysis buffer (1 mL/sample) for 30 min. 140 µL of each sample was added to each cuvette plus 60 µL of master-mix containing equal amounts of substrate solution, dye solution, and cofactor solution for LDH assay. The plates were left for 30 min in the dark and the reaction was stopped with HCl. A spectrophotometer (Specord 40) was used to measure the dye concentration at 490 nm.

2.5 *In vitro* differentiation and bone mineralization

Cell differentiation was assessed by determining the alkaline phosphatase (ALP) enzyme activity of the ST-2 cells after incubation during 21 days.
The cells were lysed with a cell lysis buffer containing 20 mM TRIS buffered solution (Merck) with 0.1 wt % Triton X-100 (Sigma, Germany), 1 mM $MgCl_2$ (Merck) and 0.1 mM $ZnCl_2$ (Merck). The cell lysate was incubated with a reacting solution containing 0.1 M Tris solution, 2 mM $MgCl_2$ and 9 mM p-Nitrophenylphosphate. 100 µL of ALP buffer solution was then added to 250 µL of the lysates obtained from each sample. After 87 min of incubation at 37 ºC, the color change to yellow was noticed and the reaction was stopped with 1 M NaOH solution. Then, absorption was measured at 405 nm using a spectrometer (Specord 40). The specific activity was calculated respect to the protein concentration of the cell lysates determined by a commercial kit based on Bradford assay (Sigma). It consisted of the release of p-nitrophenol from p-nitrophenolphosphate considering the amount of total protein. This reaction was incubated for 5 min and the change of color to dark blue appeared. Hence, the ALP activity (expressed as µmol of converted p-nitrophenol per min) was normalized by the amount of total protein, and finally expressed as nmol p-nitrophenol per min per mg protein.

Osteolmage™ Mineralization test (Lonza, Germany) was used for assessing the *in vitro* bone cell mineralization. The fluorescent assay can be used for the differentiation of osteogenic stem cells, based on specific binding of the fluorescent Osteolmage™ staining reagent to the hydroxyapatite portion of bone-like nodules deposited by cells. The *in vitro* mineralization was rapidly assessed qualitatively through laser confocal fluorescent microscope (DM6000 Leica CFS, Germany). The quantitative analysis was determined acquiring four images at 10x magnification of each sample (in total 8 images of each material) and analyzing the area of the bone-like nodules using ImageJ software.

2.6 4', 6-Diamidino-2-phenylindol staining

The nuclei of fixed cells were revealed by the fluorescence dye 4', 6-Diamidino-2-phenylindol (DAPI Roti$^R$-Mount FluorCare). Firstly, the staining of the samples was carried out by immersion in DAPI-solution (1 µL DAPI solution in 1 mL DAPI buffer) for 5 min in the dark. Finally, in order to eliminate the background, the samples were washed three times in PBS. The images were taken by fluorescence microscopy at 360 nm excitation and 460 nm emission; where the cell nuclei appeared as blue fluorescent spots. Four images at 10x magnification of each sample (in total 8 images of each material) were acquired and the number of cell nuclei was counted using ImageJ software.

2.7 Cell morphology and adhesion

The morphology of the ST-2 cells seeded on Ti, Ti-Nb and Ti-$Nb_{NH4Cl}$ was examined by FE-SEM after culture for 21 days. Before observation, samples were prepared by rinsing with PBS buffer, adding fixing solutions, dehydrating with isopropanol solutions

of increasing concentration, air drying and applying an extra thin gold coating using a sputter-coater.

### 2.8 Statistical analysis

Cell experiments were performed in duplicate using five samples for cell viability, three samples for LDH and ALP, and two samples for cell nuclei and bone mineralization (analyzing four pictures of each sample). All data are presented as mean values with standard deviations. Unmodified Ti samples (Ti) were the reference group. The results were evaluated by one-way analysis of variance (ANOVA, Tukey test) to determine statistical significant differences between the means of the different groups. The level of the statistical significance is given by *p*-values ($p < 0.05$ *significant*, $p > 0.05$ *no significant*) calculated by Origin software (version 8.5, OriginPro).

## 3. Results and discussion

### 3.1 Material characterization

Figure 1 shows the X-ray diffraction spectra for the titanium surface without treatment and the Ti-$Nb_{NH4Cl}$ and Ti-Nb surfaces obtained after diffusion and thermo-reactive diffusion treatments, respectively. It can be observed how Ti sample exhibits peaks corresponding to the α-phase whereas peaks related to the β-phase are intensely presented in Ti-Nb. In the case of the Ti-$Nb_{NH4Cl}$ sample, intense peaks corresponding to TiN as well as some less intense related to the β-phase are observed. This shows evidences of the phase transformation process of α-titanium into β-titanium due to Nb diffusion treatment in the Ti-Nb sample, which in the case of the addition of the activating agent during the thermo-reactive process also nitride the surface.

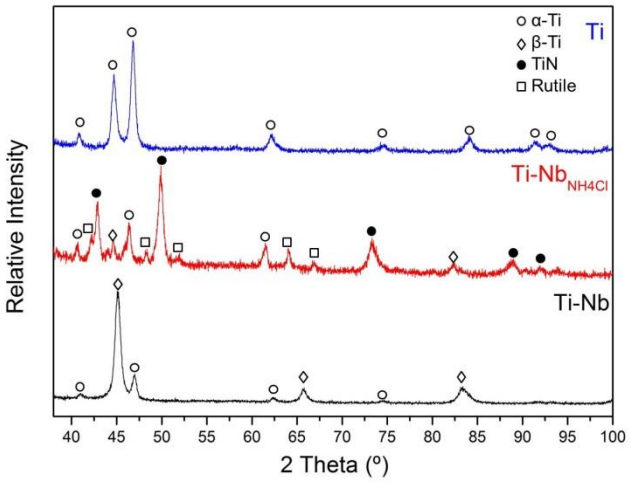
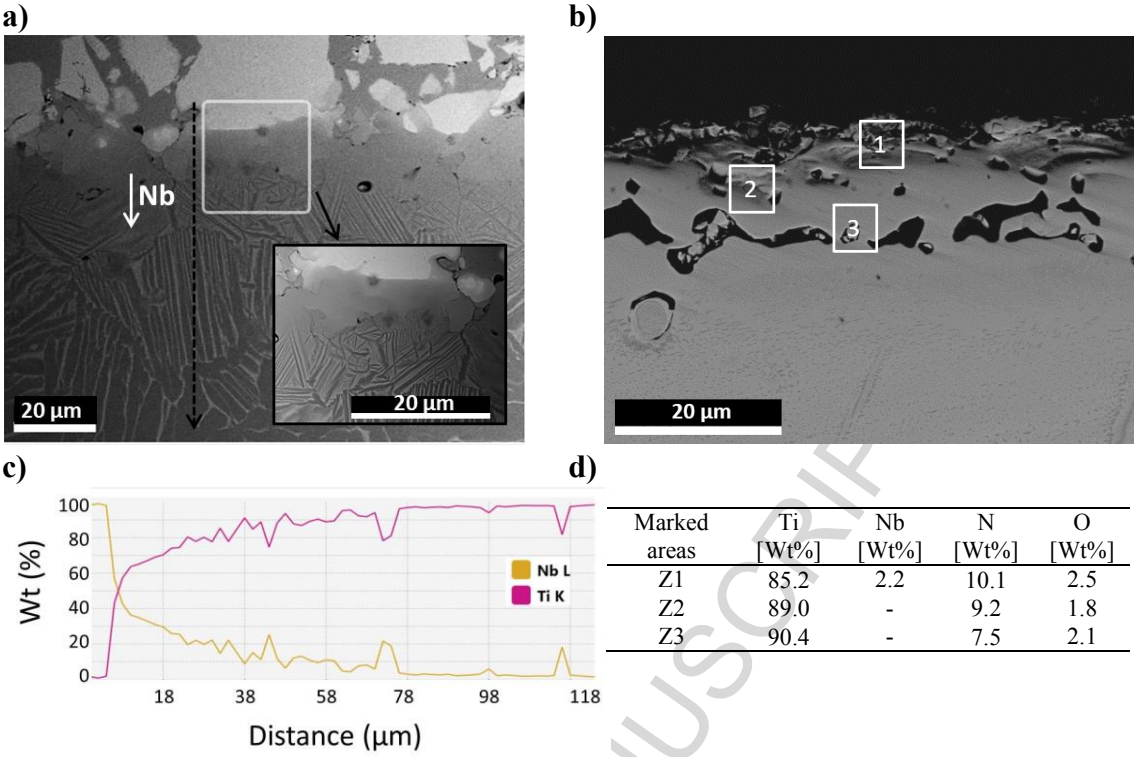



**Figure 1.** XRD spectra of the unmodified Ti surface and the modified Ti surfaces (Ti-$Nb_{NH4Cl}$ and Ti-Nb) obtained after diffusion processes.

The phase transformation process in Ti-Nb and Ti-$Nb_{NH4Cl}$ materials was further examined by SEM analysis and element distribution (Figure 2). Microstructural

examination showed that different diffusion layers were created depending on the diffusion process applied: diffusion (Figure 2a) or thermo-reactive diffusion (TRD) (Figure 2b). The niobium diffusion in high vacuum (Figure 2a) led to the phase-transformation of α into β, obtaining a microstructural gradient of β-surface and α-β moving inward. The lighter contrast regions coincided with higher amount of niobium while the dark areas correspond to the titanium substrate, where the Nb-rich region appears in the surface. The element distribution of Ti-Nb (Figure 2c) is shown through an EDS profile showing Nb diffusion along the diffusion layer. The fully β-region closer to surface ranged from 100 % to 30 % of Nb along the first 20 microns while getting deeper Nb becomes lower appearing a α+β-region until almost 120 microns of deepness. The thermo-reactive diffusion carried out in a controlled Ar atmosphere with $NH_4Cl$ as activating agent led to a nitride and porous Ti surface (Figure 2b). Figure 2d showed the element distribution for three different areas along the first 20 microns which allowed the observation of some nitrogen diffusion, remaining Nb and TiN on surface. One of the advantages of surface modification is to keep the lightness of titanium of the core. For this purpose, the effect of introducing Nb and porosity into the surface of Ti materials was studied by measuring the density of materials after diffusion treatments. Ti-Nb and Ti-$Nb_{NH4Cl}$ exhibited experimental density values of 4.5515 ± 0.0153 ($g/cm^3$) and 4.4954 ± 0.0120 ($g/cm^3$), respectively; whereas the value displayed for pure Ti was 4.4725 ± 0.0071 ($g/cm^3$). These values showed that the density of both modified materials was not dramatically increased. The slightly higher value of Ti-Nb was almost negligible compared to other full β-Ti alloys [7]. In the case of Ti-$Nb_{NH4Cl}$, the density value was also very similar to that of Ti, balancing the slightly increase caused by the nitride and niobium presence with the surface porosity as shown in Figure 2b.

In this context, the following surfaces were designed: i) a functionally gradient β-Ti surface with low elastic modulus (64 GPa), and ii) a hard nitride porous Ti surface (6 GPa); both with improved mechanical performance with respect to Ti [18].




d)

| Marked areas | Ti [Wt%] | Nb [Wt%] | N [Wt%] | O [Wt%] |
|---|---|---|---|---|
| Z1 | 85.2 | 2.2 | 10.1 | 2.5 |
| Z2 | 89.0 | - | 9.2 | 1.8 |
| Z3 | 90.4 | - | 7.5 | 2.1 |

**Figure 2.** Representative cross-sectional SEM micrographs with element distribution of: a) Ti-Nb with β-Ti surface and (β - α+β - α) microstructural gradient; and b) Ti-$Nb_{NH4Cl}$ with nitride porous surface. Element distribution analysis of: c) Ti-Nb and d) Ti-$Nb_{NH4Cl}$.

3.2 Cell viability and proliferation

Aside of cell differentiation and mineralization, assessing the cell viability and lactate dehydrogenase activity is important in order to understand the grade of cell proliferation and cytotoxicity of the materials [19]. The cellular viability and lactate dehydrogenase activity (LDH) of mice bone marrow stromal cells (ST-2) in contact with the different surface materials after 21 days of culture are presented in Figure 3. Figure 3a shows the cell viability of the materials respect to that of control reference material, Ti, set as 100 %. It can be observed that Ti-$Nb_{NH4Cl}$ showed cell viability higher than 75 % with respect to the Ti whereas Ti-Nb reached a value of 105 %. Although cell viability was lower for Ti-$Nb_{NH4Cl}$ and higher for Ti-Nb compared to Ti, no statistically significant differences were detected.

Additionally to cell viability, LDH activity is presented in Figure 3b. Similar LDH activity values (90-100 %) were obtained for three surfaces after 21 days of culture. There were no statistically difference between Ti-Nb and Ti-$Nb_{NH4Cl}$ compared to Ti. Thus, it can be suggesting similar amount of cells in both Ti-Nb and Ti surfaces. Similar LDH values were reported for different Ti-Mo-Nb alloys after only 3 and 5 days of culture [6]. Thus, even though the cell number indicated by LDH enzyme activity was similar on both Ti-Nb surfaces and Ti, the niobium and its low elastic modulus surface of Ti-Nb layer seemed to stimulate bone marrow stromal cells as indicated by increased cell viability as a sign of good biocompatibility and cell-material interaction. It could be stated that the average roughness ($R_a$) values of Ti-Nb and Ti-$Nb_{NH4Cl}$ surfaces, 1.38 µm and 1.80 µm, respectively were suitable for cell viability. Moreover, our findings are in

good agreement with recent literature that show results on different Ti-xNb [9] as well as in different surface states [20] where similar cell viability values ranging from 80 to 90 % after seven days of culture are reported.

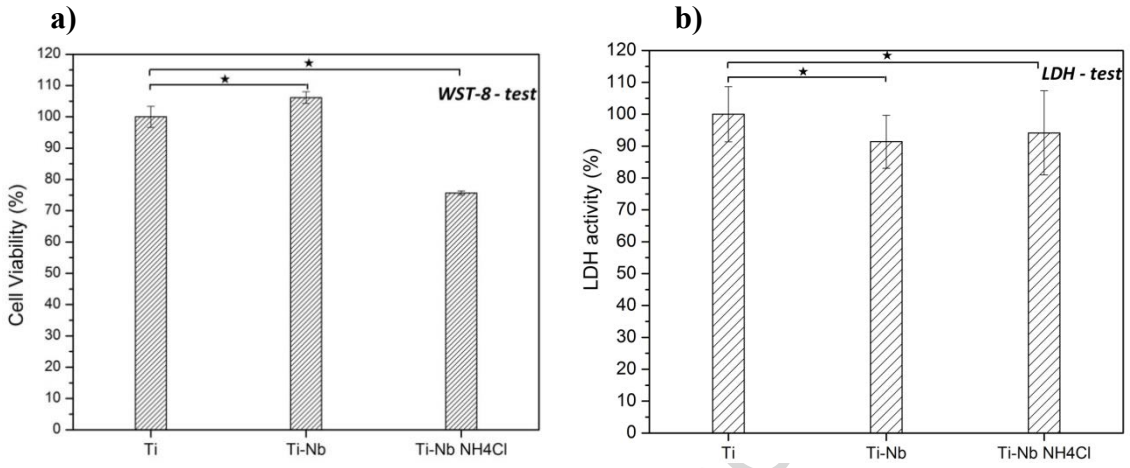



**Figure 3.** Viability of ST-2 cells grown on Ti, Ti-Nb and Ti-$Nb_{NH4Cl}$ after 21 days of incubation, as determined via WST-8 and LDH. a) WST-8 test, expressed as mean ± standard deviation of 5 samples (in total 10 measurements of each material). b) LDH test, expressed as mean ± standard deviation of 3 samples (in total 6 measurements of each material). * *$p>0.05$ is statistically insignificant compared to Ti.*

3.3 Cell differentiation and bone mineralization

The evaluation of ALP activity expression and bone-like nodules deposition were investigated according to cell differentiation and mineralization response [21]. Regarding the cell differentiation, alkaline phosphatase (ALP) activity is an essential indication of cells expression in their early differentiation stage and an important quantitative marker of osteogenesis [22]. Therefore, the osteogenic differentiation of the ST-2 cells in the Ti surfaces was monitored by the activity of alkaline phosphatase enzyme (ALP). The specific ALP activity is expressed in all samples after ST-2 seeding during 21 days (Figure 4). In particular, the ALP expression of the cells was significantly higher ($p<0.05$) in Ti-Nb than in Ti, whereas no statistical differences in ALP expression was detected between Ti-$Nb_{NH4Cl}$ and Ti. Hence, ALP was positively affected by the low elastic modulus of Ti-Nb as well as by the surface porosity of Ti-$Nb_{NH4Cl}$; promoting the ALP induction. This is in agreement with that reported about the positive effect of the Nb surfaces on osteogenesis [23]. These authors also investigated the ALP activity variation as function of incubation time (7, 14 and 21 days), obtaining the maximum ALP expression at 21 days reporting similar values to those obtained in our surfaces. Nb was found also beneficial on the ALP activity of the β-Ti alloy (Ti-Nb-Zr-Ta) as compared to the currently used Ti-6Al-4V alloy [24]. Moreover, in a similar study, slightly lower ALP values were obtained in dense/porous Ti/Ti-35Nb alloys where the material composition was reported as more relevant parameter on cell differentiation than porosity [25]. In this context, also surface treatments are relevant in ALP since studies about early osseointegration of Ti-24Nb-4Zr-8Sn treated by microarc oxidation reported higher ALP values for the treated surfaces compared to Ti [11].

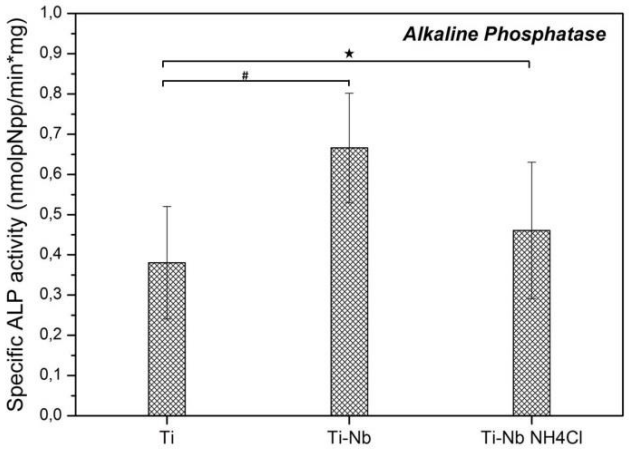



**Figure 4.** Alkaline phosphatase activity (ALP) of ST-2 cells after culturing on Ti, Ti-Nb and Ti-$Nb_{NH4Cl}$. Expressed as specific ALP activity considering the amount of total protein showing the mean value ± standard deviation of 3 samples (in total 6 measurements for each material). *$p$ *>0.05 is statistically insignificant and # $p$<0.05 statistically significant compared to Ti.*

The expression of calcium is considered as bone marker for osteogenic differentiation representing the mineralization process of newly formed bone matrix [26]. The calcium phosphate deposits were identified by fluorescence osteolmage staining after culture for 21 days (Figure 5). These green stained deposits revealed the main mineral content of bone which is mostly hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$]. Qualitatively, the amount of CaP nodules seemed to cover most of the area in the three surfaces (Figure 5 a-c). Although Ti-Nb sample exhibited slightly less green color, no significant difference were found in the detailed views. These results are also in agreement with the *in vitro* hydroxyapatite formation on these surfaces after 21 days of immersion in simulated body fluid [16]. In both cases hydroxyapatite is shown after 21 days, by immersion in SBF or due to the osteogenic differentiation of cells indicating the new bone matrix formation.

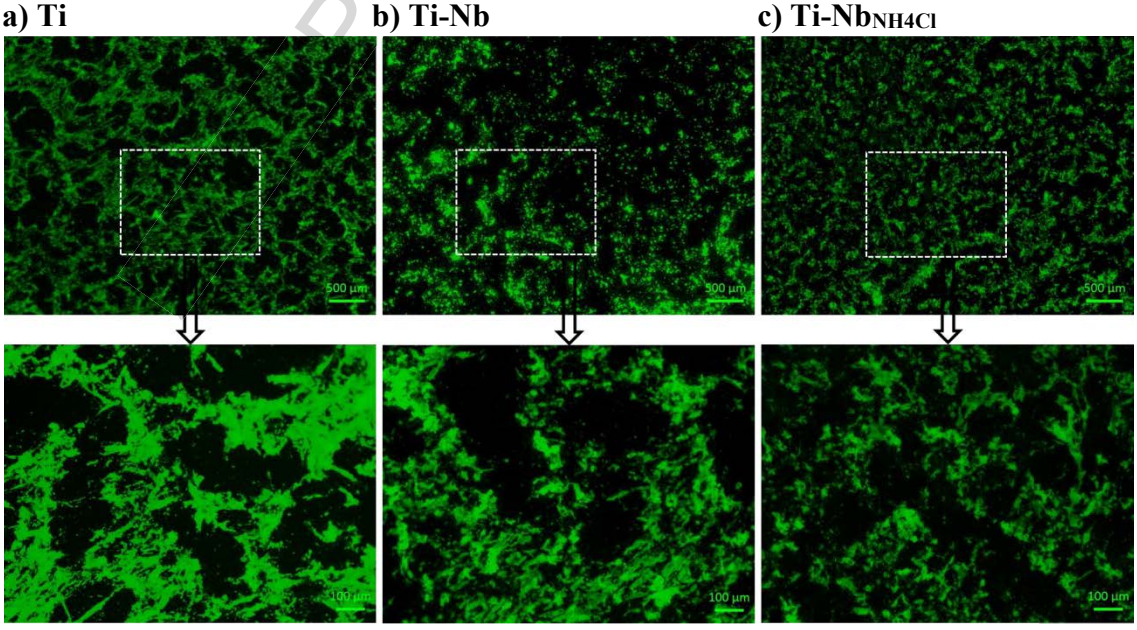


**Figure 5.** Fluorescence images from low to high magnification of the mineralized area formed on: a) unmodified Ti control, b) Ti-Nb and c) Ti-$Nb_{NH4Cl}$; showing the bone-like nodules deposited by ST-2 cells after incubation for 21 days and the *in vitro* bone mineralization.

Additionally, bone-like nodule formation together with the number of cells adhered onto the surfaces were analyzed from the recorded images using ImageJ and are quantitatively represented in Figure 6. The cells cultured onto Ti-Nb showed around 55 % of mineralization while Ti and Ti-$Nb_{NH4Cl}$ surfaces displayed the highest mineralization level, 65 % approximately. In contrast to mineralized area, the highest number of cells adhered was observed on Ti-Nb surface (5250) whereas approximately were observed 4500 on Ti and Ti-$Nb_{NH4Cl}$ surfaces. However, although no statistical significant differences were found between these values, the slightly higher mean value of mineralized area showed for Ti-$Nb_{NH4Cl}$ compared to Ti-Nb could be associated with the oxygen present in Ti-$Nb_{NH4Cl}$ reported as beneficial for osseointegration [27]. The double asterisk (**) means that the difference is statistically insignificant in cell number and also in mineralized area. These values of cell number (4500-5250) were in the order of those found in titanium-niobium-hafnium alloys where the influence of bio-functionalization with fibronectin recombinant fragments on bioactive coatings design was evaluated [28].

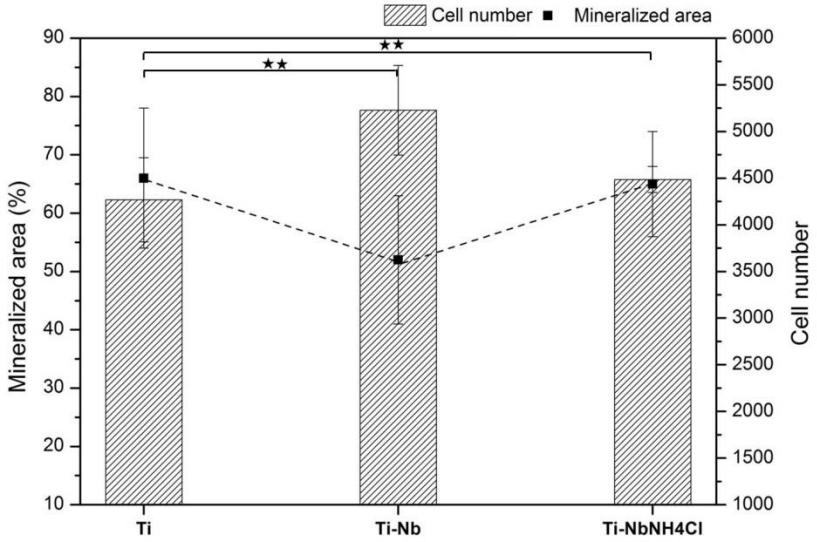



**Figure 6.** Percentage of mineralized area and number of ST-2 cells adhered to the materials after 21 days of culture. Data obtained by analyzing the fraction area of bone-like deposits and counting the cell nuclei and expressed as mean ± standard deviation of 2 samples (analyzing the whole surface by means of 4 pictures of each material). ** *$p>0.05$ is statistically insignificant compared to Ti in both parameters (cell number and mineralized area).*

3.4 Cell attachment and morphological features

The merged fluorescence micrographs representing the mineralized area (green) and distribution of DAPI-stained cell nuclei (blue) of mice bone marrow stromal cells are shown in Figure 7. The DAPI-stained confirmed the distribution of the cells on both, Ti and the modified Ti surfaces after 21 days of culture. The merged images (top row of

Figure 7) indicated a uniform cell distribution on the three surfaces, including mineralized part (green) and rest of the surface. The grey color images (bottom row of Figure 7) clearly showed the cell colonization; noting the high density of cell nuclei and thus, the good cell adhesion and cell-material interaction of the three materials. Similar bone matrix deposition together with cell nuclei results were presented in an osteogenic differentiation study of Ti13Nb13Zr compared to Ti6Al4V [29].

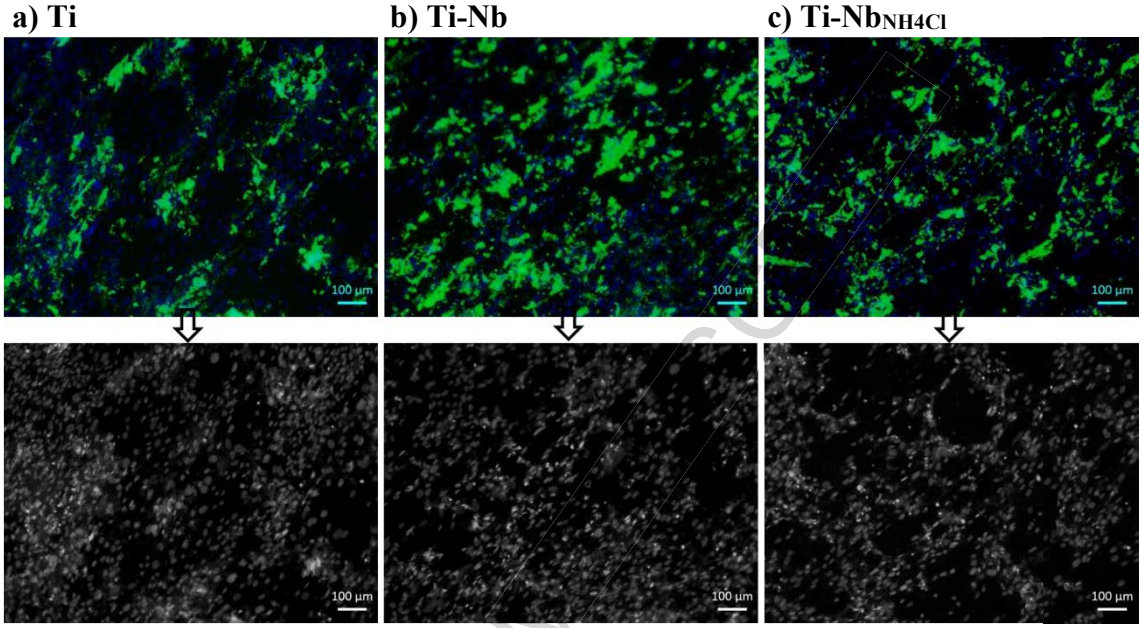



**Figure 7.** Fluorescence images of mineralized area and DAPI stained ST-2 cells cultured on: a) unmodified Ti control, b) Ti-Nb and c) Ti-$Nb_{NH4Cl}$ after incubation for 21 days. At the top: merged images of mineralized area (green) and nuclei (blue). At the bottom: grey color images focused only on nuclei distribution.

The cell morphology on the different Ti surfaces after 21 days of culture was further investigated with representative SEM images illustrated in Figure 8. The cells exhibited good adhesion on three surfaces (Figure 8 a-c). They displayed a fibroblastic phenotype shape on three materials although the cytoplasmic extensions appeared to be more numerous on Ti-Nb and Ti-$Nb_{NH4Cl}$ (Figure 8 b-c); promoting higher cell-cell communications. Furthermore, Ti-Nb (Figure 8b) surface showed a great number of extensions over the surface looking forward to link with other neighboring cells through the niobium particles and pores. The same cell morphology was observed on Ti-$Nb_{NH4Cl}$ where a large number of extensions were noticed at this stage, resulting in high proliferation due to communications with surrounding cells and a greater amount of extracellular matrix (ECM) deposited. These results about morphology agree with those presented for similar porous Ti surfaces and β-Ti alloys [11], [30].

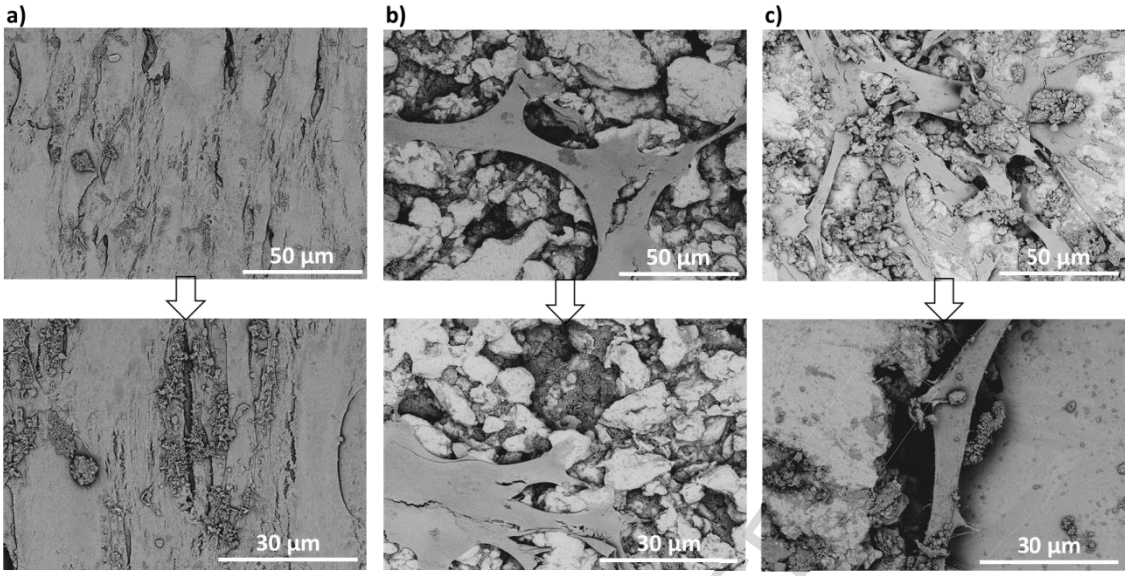



**Figure 8.** FE-SEM images from low to high magnification of mice bone marrow stromal cells (ST-2) cultured on: a) unmodified Ti control, b) Ti-Nb and c) Ti-$Nb_{NH4Cl}$ after 21 days of incubation.

## 4. Conclusions

In this *in vitro* study, the cellular behaviour of bone marrow stromal cells on two Ti surfaces designed by niobium diffusion processes has been presented. Ti-Nb and Ti-$Nb_{NH4Cl}$ have been studied and compared to titanium, evaluating the effect of a niobium rich β-Ti surface and a nitride porous Ti surface on cell differentiation and bone mineralization.

It has been demonstrated that Ti-Nb with low elastic modulus and Ti-$Nb_{NH4Cl}$ with surface porosity displayed high cell viability and proliferation after 21 days of culture. Moreover, cells displayed good adhesion and were seen to be well distributed over the whole surfaces. The lactate dehydrogenase activity was similar to that of Ti, indicating similar cell number but a slightly increase in cell viability of Ti-Nb. The bone-like nodules staining in the three tested surfaces resulted in existing bone mineralization, and the results of the ALP activity considerably showed higher cell differentiation for Ti-Nb followed by Ti-$Nb_{NH4Cl}$, meaning a more elevated grade of osteogenic differentiation for these materials in comparison to Ti. Therefore, overall the observations from this investigation of the biological response of bone marrow stromal cells (ST-2) to our designed Ti–Nb and Ti–$Nb_{NH4Cl}$ materials showed a similar or an improved cell-material interaction of these β-Ti surfaces promoting cell differentiation and bone mineralization. The enhanced osteogenic differentiation response considering cell viability, attachment, differentiation and bone mineralization was presented and thus, the good cellular behaviour of these Ti-Nb surfaces.

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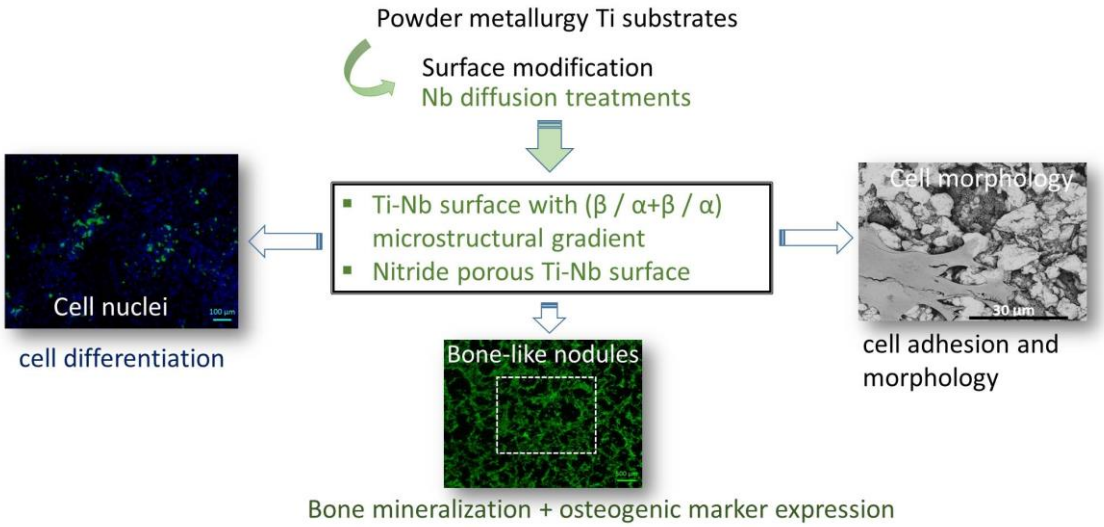


**Graphical abstract**

## Highlights

- The surface modification of titanium by niobium diffusion leads to cell viability values similar to bare titanium.
- The modified Ti-Nb surfaces increased the specific level of lactate dehydrogenase activity of titanium almost twice, pointing out cell differentiation.
- Bone-like nodules were deposited by stromal cells on the modified Ti-Nb surfaces indicating bone mineralization.