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

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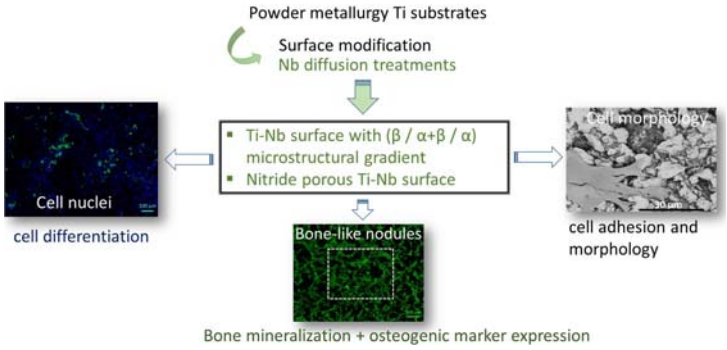
HIGHLIGHTS

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

GRAPHICAL ABSTRACT



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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.

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

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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 °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 and 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 °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 °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 °C for 90 min.

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 40 kV and 30 mA and Goebel mirror optics to obtain a parallel and monochromatic X-ray beam. 2θ scans over a range from 35 to 100° were performed at an angle of incidence of 5° with a step width of 0.02° 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).

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 (°)*
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