







# Nanostructured Ti Grade 2 for Biomedical Applications: Evaluation of Cellular Interaction

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**Abstract.** Tissue engineering has as one of its objectives the development of biocompatible materials, capable of interacting with biological tissues, promoting regeneration or function repair. Titanium is widely used in dental and medical implants, due to its mechanical properties and biocompatibility. Alternatives of relatively low-cost titanium, with low elastic modulus, non-toxic and that maintain the mechanical properties are currently intended. Pure titanium grade 2 presents desired characteristics such as low density, excellent biocompatibility, and good corrosion resistance, although mechanical resistance may be improved. This work is focused on the biological evaluation of commercialized pure titanium grade 2 (Ti CP2), and its characteristics as a biocompatible material, viewing medical applications, such as osseointegration.

**Keywords:** Biocompatibility · Cellular adhesion · Cell culture techniques · Titanium · Nanostructure

## 1 Introduction

Tissue engineering has as one of its objectives the development of biocompatible materials, capable of interacting with biological tissues, and promoting regeneration or function repair. Simultaneously, the biomaterials mimetize the mechanical and biological properties of the specific tissue to be engineered [1].

Biomaterials also aim to be bioinert and bioactive, since it is necessary to minimize adverse effects on patients, generate an effective tissue interaction, be functional, and promote cell growth [2, 3]. In addition, metallic biomaterials have been idealized to be efficiently used in the development of medical devices, used as an alternative in lesions caused in bone tissues that need implants, in order to withstand high loads for fracture fixation, and also as substitutes for some joints. [3, 4]. Therefore, among the most used metallic biomaterials, stainless steels, cobalt-chromium alloys, and titanium alloys can be mentioned [4, 5].

Titanium is widely used in dental and medical implants, due to its mechanical properties and biocompatibility. However, some titanium related toxicity is known, especially in titanium alloys containing aluminum and vanadium, as Ti-6Al-4V [6, 7]. Alternatives of relatively low cost titanium, with low elastic modulus, non-toxic and that maintain

the mechanical properties are currently intended. Pure titanium grade 2 presents desired characteristics such as low density, excellent biocompatibility, and good corrosion resistance, although mechanical resistance may be improved. Nanostructured pure titanium can result in a more resistant material, without chemical changes that could result in associated toxicity.

This work is focused on the biological evaluation of commercialized pure titanium grade 2 (Ti CP2), and its characteristics as a biocompatible material. It is based on morphological analysis of initial cellular interaction with nanostructured pure titanium grade 2 samples, viewing medical applications, such as osseointegration.

## 2 Methodology

### 2.1 Titanium

To perform the cell interaction analysis, commercially pure titanium grade 2 (Ti CP2) was used, which has its chemical composition according to ASTM F67 requirements. The nanostructured Ti was obtained through severe plastic deformation routes involving ECAP (Equal Channel Angular Pressing), a combination of ECAP with lamination at different temperatures (cryogenic deformations) and HPT (High Pressure Torsion) [8–10].

The samples used were: 0X, control, without treatment; 2X, two ECAP process, at 300°C; 2X + CRLT, two ECAP process, at 300°C and lamination at  $-100^{\circ}\text{C}$ ; 2X + HPT, two ECAP process, at 300°C and HPT at room temperature. For more details on the ECAP and HPT processing please see reference 8. The preparation of samples was carried out from initial sterilization. Initially, the samples were cut into four pieces in a low-speed cutter (LabCut 1010) using a diamond blade. At sequence, using a sander and an ARAPOL-E universal polisher, the samples were sanded in water sandpaper with granulometry between 120 and 2000. The cleaning protocol of the samples pre-biological evaluation started with an ultrasonic bath with the presence of detergent lasting 20 min for each sample, from the ultrasound vat UltraCleaner 750 A. After these steps, the quadruplicates of each type were placed in beakers of 50 mL with the presence of distilled water and remain at rest for 24 h. At the end of this period, the samples were covered in packaging for autoclave sterilization.

### 2.2 Cell Culture

The *in vitro* cell interaction test, cells of the Vero lineage were used (Instituto Adolfo Lutz/SP, CCIAL 057) [11]. These cells originally are obtained from the renal epithelial tissue of an African green monkey (*Cercopithecus aethiops*), present a fibroblast-like type and they are anchorage-dependent, being widely used in biological evaluation, since it has a high rate of cell proliferation, do not have oncogenic factors and does not present risks to human health [12]. The cell cultures were maintained with HAM-F10 culture medium, supplemented with 10% Fetal Bovine Serum (FBS) and antibiotic, and were incubated at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$ .

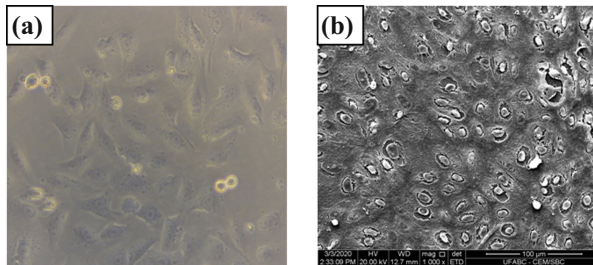
The Ti samples used were sterilized in an autoclave. The samples were positioned in 24-well culture plates. For the evaluation of the initial cellular interaction, the cells

were directly inoculated onto the Ti samples, and were kept in culture for two hours. After this period, it was possible to observe initial cellular interaction on Ti surface with Scanning Electron Microscope (SEM) (FEI, Quanta 250). For this, the samples were fixed in 2.5% glutaraldehyde and were washed with phosphate buffer following distilled water, dehydrated in critical point equipment (Leica EM CPD 300) and coated with gold (Leica ACE 200), so that they could be visualized with SEM.

Furthermore, for the morphological control of the experiment, light microscopy with phase contrast (AxioVert A1, Zeiss) was used.

### 3 Results and Discussion

Vero cells from the kidney of the African Green Monkey (*Cercopithecus aethiops*) present fibroblast-like morphology, and are widely used in biological evaluation assays [13]. In the ideal cell culture condition the characteristic morphology is polygonal, or elongated, with cytoplasmic spreading, forming a cell monolayer (Fig. 1) [11, 14].

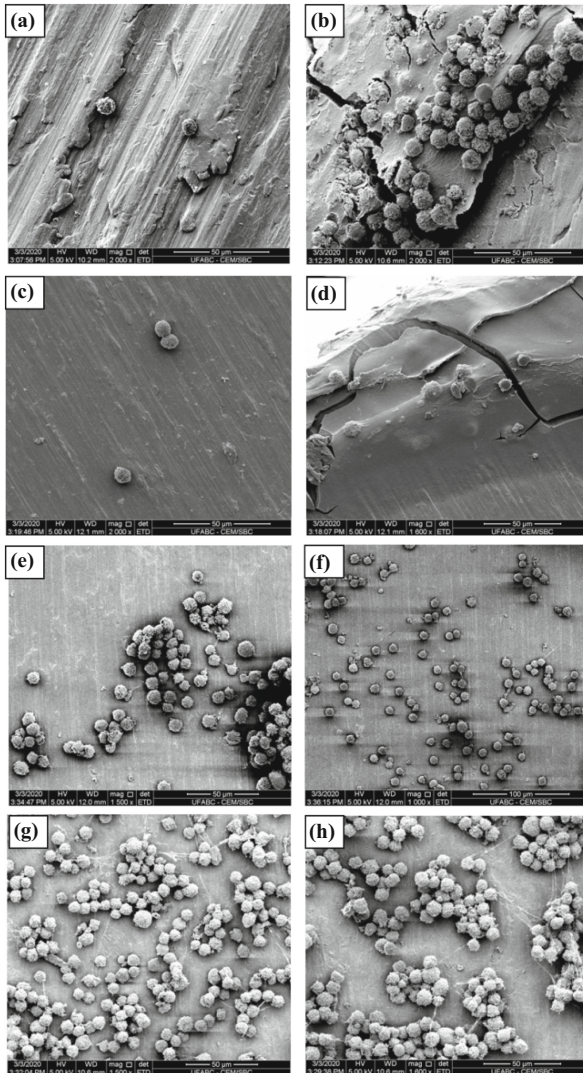


**Fig. 1.** Morphology of Vero cells after 7 days of culture, standard characteristic morphology for the lineage. **a** Phase-contrast light microscopy (200x); **b** Scanning electron microscopy (SEM).

The morphological analysis of the cells in the presence of the different Ti samples evidences morphological changes of cells in initial cellular interaction, characterizing adhesion assays. The cell attachment and spreading can occur within a 24 h period, depending on factors such as the solid substrate and cell line [15, 16]. Although it can characterize the initial interaction, the cell adhesion with the substrate can be observed in the first hours of culture for Vero cells [16–18].

In all samples, rounded cells were observed, with the beginning of adhesion onto the Ti substrate, and regions with less or less great cell density (Fig. 2).

In the Ti samples in which ECAP passes were performed (Fig. 2a, b) and HPT (Fig. 2c, d) the cells were preferentially deposited in regions of deformities or cutoffs of Ti, or close to the edges of the samples, with rounded morphology, and the little surface of contact between the cells and the metal. Few cells were observed in the process of spreading, mainly in samples with HPT (Fig. 2d). This morphology pattern was not observed in the other samples, being representative of a more stable interaction with the material. In samples with ECAP and cryogenic treatment (Fig. 2e, f) and in the control condition (Fig. 2g, h) the areas with the highest number of cells were observed in interaction with



**Fig. 2.** Scanning electron micrographs of Vero cell morphology after 2 h of interaction with Ti samples. (a, b) 2X; (c, d) 2X + HPT; (e, f) 2X ECAP + CRLT; (g, h) Control (0X).

Ti, in addition to the specific treatment (Fig. 2e, f), the geometry of these samples may also have favored the preferential interaction of cells, as they are surfaces more regular and flat. The samples submitted to ECAP and cryogenic lamination, represented by Fig. 2e, f, were the ones that showed the best adhesion to Vero cells, if not considering the control condition. Thus, these samples with better mechanical properties maintain the characteristics of biocompatibility.

As evidenced in the literature, nanostructured samples usually show greater adhesion than samples with natural microstructure and are related to surface roughness [15, 16].

However, in this mentioned situation, the titanium had been processed by four passes ECAP, and an even higher cell volume result was reported in the sample with eight passes. This may be the reason for this work to present a smaller number of cells in the sample with just two ECAP passes. In this way, more passes of both ECAP and HPT, could result in greater cellular adhesion. In a similar way, the cells were present in the regions that had grooves, according to what was evidenced by other authors [17, 18].

## 4 Conclusions

This work aimed to understand the interaction of nanostructured Ti CP2 with simulated biological conditions, simulated with in vitro experimentation using Vero cells, to consider the viability of cell growth for applications in Ti implants. With the biological tests performed, greater initial cell adhesion was observed in samples that underwent greater improvement in mechanical properties, indicating promising future osseointegration. Characterization of long term cellular interaction may be developed in the future.

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**Conflict of Interests** The authors declare that they have no conflict of interest.

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