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Cell Behaviour of the Biomimetic Modifications on a New TiHfNb Alloy Developed for Orthopaedic Applications

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Abstract. The field of cellular behaviour and the biomimetic modifications on inorganic materials destined for biomedical applications shows a remarkable increase in scientific studies and, therefore, this is reflected in the investment priorities and the fundamental effort of the global research community in this area. The essential purpose of this study is to assess the cellular behaviour of a novel TiHfNb alloy with biomimetic modification. For the mimic surface, the samples were initially bioactive with oxygen plasma or with piranha, then the following samples were biofunctionalized with APTES + maleimide and finally different surface peptide sequences were immobilized (RGD, FHRIRIK, PHSRN or 50/50 mixtures of RGD / FHRIRIKA or RGD / PHSRN). To evaluate the biomimetic modification, adhesion and cell proliferation studies were carried out. Different techniques for chemical characterization were used, including: X-ray photoelectron spectroscopy (XPS), contact angle (CA), immunofluorescence, scanning electron microscope (SEM) and lactate dehydrogenase (LDH). With respect to the different peptide sequences used, the results indicated that the samples with RGD and the mixture exhibited more extended cells on the surface.

1. Introduction
Currently, there is an increase in the number of implants required by the population worldwide. In addition, patients who need these implants desire to maintain the same level of activity and quality of life. The implants have been manufactured with different materials, such as metals and their alloys, polymers, ceramics and composite materials. Then, a biomaterial design as an implant must provide mechanical stability, and durable bonding with the surrounding bone in a process called osseointegration. In addition, the biomaterial should avoid adverse tissue reactions and minimize the absence of infection. The most common biomaterials are metals based on Ti, Co and steel, because they have excellent mechanical properties. The polymers are the second group, the last group is composed of ceramic materials and less frequent compounds [1,2].

The success of an implant is conditioned to its acceptance by living tissues; as well as the formation of new bone around the implant. Several studies have reported that the bone-implant interface depends on the biological factors and factors associated with the patient. Therefore, it is important to know the design of the implant and its surface, the distribution of loads between the bone and the implant and the surgical procedure. Then, it is necessary to investigate the different strategies that modify the surface of the implant to determine if through these modifications it is possible to
obtain the best cellular response.[3] There are different methods to obtain modifications in the properties of the surface of the material; these promotes the cellular response, therefore, the validation of the biocompatibility of new surfaces could be based on experimental cellular studies on biomimetic surfaces.[3–5] Among the biomaterials, Ti and its alloys have shown a good combination between biocompatibility, corrosion resistance and lower density and lower modulus of elasticity.[2] For this reason, the aim of this study is to analyze the cellular response in a nobel Ti alloy (TiHfNb) with a low elastic modulus. Its surface was biomodified through the immobilization of different short sequences of peptides; it was carried out through the process of surface activation, silanization and immobilization of biomolecules. The variables that defined the behavior of the cells were number of adhered cells and their cell morphology, which are indicators of adaptation to the environment. Therefore, the biological behaviors on short peptide sequences were used to validate the biomimetic modification.

2. Materials and Methods.

2.1 Materials
Ti alloy studied (Ti-16Hf-24Nb) was fabricated into UPC Laboratory. The material was cutted into 2 mm of thickness. Each sample was polished using 600 and 1,200 grit silicon carbide (SiC), abrasive papers and finally, they were further polished with napped polishing pads with a colloidal alumina polishing abrasive (1 and 0.05 μm). Disks were degreased from macroscopic contamination by ultrasonication in: acetone, water and ethanol, and then dried with compressed air. Before the activation process, a chemical cleaning was made by ultrasonication in cyclohexane, isopropanol, ethanol, deionized water and acetone.

2.2 Biomimetic modification
The biomimetic modification involved three step: 1) Surface activation: through oxygen plasma (OP),[6–8] 2) Silanization: this stage consisted of incubation for 1h in a pentane basic solution containing the organosilane (3-aminopropyltriethoxysilane (APTES)). Then is modified with 3-(maleimido)-propionic acid N-hydroxysuccinimide ester for crosslinker,[8–10], and 3) Peptide immobilization: The samples were incubated overnight (ph = 7), room temperature in a phosphate buffer solution containing the desired peptide (CGGRGDS, CGGPHSRN, CGGFHRRIKA, and two mixtures (50/50) of CGGRGDS/CGGFHRRIKA and CGGRGDS/CGGPHSRN). GenScript provided the peptides used for the study. The strategy was to use biomolecules with cysteine (Cys) and glycine (Gly) as spacers. Because the Cys contains a sulfur atom in its side chain, which is highly reactive. Likewise, the peptides were acetylated (blocked by reacting the terminal amino group), thus, they only have the ability to react with silanes through the side chain of cysteine (-SH).[4,11–14] To the surface characterization, the techniques used were Contact Angle (CA), interferometry and X-ray Photoelectron Spectroscopy (XPS).

2.3 In vitro biological response:
All the samples were immersed for 30 min in a phosphate buffer solution (PBS / Invitrogen) and bovine serum albumin (BSA (1%)) in order to avoid unspecific protein absorption. The samples were sterilized in ethanol for 10 min [15], finally the surface were washed three times with sterile PBS.

2.3.1 Cell Adhesion: This process was evaluated to 6h at 37°C, The criteria used were the number of cell and their morphology. Were used these types of samples: 1) Two control: Sample, both samples without treatment (TisB), and one of them with PBS-BSA (1%), another sterilized with ethanol but without PBS-BSA (1%) (TiB), 2) Samples silanized with peptide sequences are showed at table 1. Samples were placed in each sample 6x10³ cells, and it was incubated in serum-free medium. The characterization techniques used were: Immunofluorescence, because the nuclei were stained with DAPI (4,6-diamino-2-phenol-dihydrochloride), the adhered cells were observed in a confocal
microscope, which allowed their quantification per unit area. Finally, the SEM images allowed to evaluate cell morphology and determine their area. The nomenclature used Control (Ti), Ti+OP (TiOP), TiO+APTES+maleimide (TiAM), TiAM+RGD (TiAMR), TiAM+ FHRRKA (TiAMF), TiAM+PHSRN (TiAMP) TiAM+RGD+FHRRKA (TiAMRF), TiAM+RGD+PHSRN (TiAMRP)

3. Results and Discussion.

3.1 Biomimetic Modification

Activation Process: The results indicated that Ti have a roughness 26.3 nm an TiOP 22.9nm; Thus, the samples can be considered smooth or minimally rough. [16] The hydrophilicity is associated with the reduction of the contact angle from 62.1° to 5.4°, these results indicated the introduction of hydrophilic groups (OH, O2, H2O) on the surface. Therefore, the treated samples seemed to provide excellent results in terms of cleaning and chemical modification of the surface.[6–8] However, the CA technique did not provide information on chemical composition; Then X-ray photoelectron (XPS) spectroscopy was used to evaluate the active functional groups (eg, OH−), since a higher concentration of OH− groups on the surface will benefit the next step of modification process (silanization).[8–10]

By XPS was possible to observe as activation method produced an increase of O 1s and also OH− groups; due Ti had 46% of oxygen while TiOP had 58%. The XPS high resolution of O 1s showed three contribution O2−, OH− and H2O[17,18], where the percentage of O2− (Ti = 28%, TiOP = 25%) and H2O (Ti = 8%, TiOP = 5%) were lower after the activation, however, the activation produced an increase of OH− group ranging from 10% up 28%. The percentage of O 1s together with the OH−O2− ratios demonstrated that OP was efficient, due to the OH−O2− ratio for TiOP was 1.12 while for the Ti was 0.36.

3.1.1 Silanization Process: After the silanization process, the samples increased the contact angle from 5.4° to 75.23°. These results confirm that the silanization process produced a superficial modification. By XPS it was possible to confirm the presence of silanes on the surface through the silanized samples showed the presence of Si 2p from 1% for TiOP up to 12% for TiAM. Also there was an intensification of the carbon content from 10% up 55%, due to the carbon chains of the silanes.

3.1.2 Peptide Immobilization: The aim of peptide immobilization on biomaterials is to induce a specific cell adhesion, thereby to improve the tissue-implant interface. The technique used to chemical characterization was XPS; The results are shown in table 1, these allowed confirming the presence of biomolecules because: in all the samples with peptides there was presence of S 2p, also the amount of Si 2p on the surface decreased due it was coated with biomolecules. Finally, there was an increase in the percentage of O 1s. The carboxyl groups (COOH) belong to the terminal chain of the peptide sequences or to the peptide bonds (-OC-NH-). [8,19]

| Element | TiAM | TiAMR | TiAMF | TiAMP | TiAMRF | TiAMRP |
|---------|------|-------|-------|-------|--------|--------|
| S 2p    | 0±0.0 | 1±0.1 | 1±0.1 | 1±0.2 | 1±0.0 | 1±0.2 |
| Si 2p   | 12±1.0 | 10±0.8 | 8±0.6 | 8±0.7 | 10±0.4 | 8±0.7 |
| O 1s    | 23±1.9 | 36±2.2 | 38±2.9 | 36±3.3 | 39±2.7 | 36±3.1 |

3.2 In vitro biological response

3.2.1 Cell Adhesion; the staining of the nuclei was carried out by immunofluorescence; through the quantification of the nuclei it was possible to determine the number of cells per cm² (figure 1), the best results are obtained in samples containing RGD, either alone or in mixtures.[11,13,20–24] The control samples contain a number of cells similar to that of biofunctionalized samples could be due the Ti alloy surface contains TiO2 and several studies have reported that this oxide favors the cell adhesion
process.[16] Also, when it is evaluated the cellular response, a relationship between the number of adhered cells and their morphology must be established; thereby, SEM images were obtained to visualize the shape of the cells and estimate their area. Figure 2.a shows that the immobilization of biomolecules influences the morphology of the cells. The control sample had a contracted cell and smaller extension area. Then, it is possible affirms that the RGD sequence promotes a more comfortable adhesion, because in surfaces with this sequence (RGDS alone or in mixtures) more widespread cells are observed, which have a larger area, and these exhibit the presence of filopodia. Corroborates what it is expressed by Petershans.et al., who expressed that the RGDS sequence, although it is the smallest motif in the extracellular matrix of fibronectin, acts as the receptor integrin of the cellular link, initiating the formation of local adhesion contacts. The quantification of the cells area i shown in figure 2.b, the best results are shown on surfaces with RGD and mixtures, although the standard deviation has a wide range, and then there are not statistically significant differences between them.

![Figure 1](image1)

**Figure 1.** Cell adhesion quantification using staining of the nuclei by DAPI
Figure 2. a) Cells Adhered on TiAM by SEM b) Area of the Adhered Cells

4. Conclusion
According to the results and in terms of the density of cells adhered, their morphology and the size, the samples with RGD (alone or in mixtures) showed the best results. When correlating the morphology and the area of the cell, we can affirm: 1) although in the untreated sample had a large number of cells, these were contracted, 2) the samples that show the best results are TIAMRF, because they have the greatest extension and marked presence of filopodia. Therefore, we can concluded that our nobel Ti alloy is biocompatible in terms of cellular adhesion, and also that the physical-chemical properties of the surfaces define the size, shape and distribution of the cells.

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