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THE INFLUENCE OF THE CRYSTALLINE PHASES ON CELL ADHESION AND PROLIFERATION ON TI-15MO SURFACES

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ABSTRACT

Surface texturing of titanium alloys implants have been improved by additive and subtractive modification methods. Recent progress on the highly controlled topographical and chemical properties has been guaranteed by laser ablation technology. The effects of Ti-15Mo textured surfaces (n= 12 surface textures; laser Yb:YAG irradiation) on the adhesion and growth of non-tumorigenic human keratinocytes cell line (HaCaT) were evaluated. It was evaluated the formation of the crystalline phases αTi, Ti_6O, Ti_3O and TiO_2 by the melting and fast cooling processes during irradiation. The resulting phases on the irradiated surface were correlated to the laser beam parameters. The aim of the present work was to control titanium oxides formations by using laser ablation technique. The cytotoxicity response of Ti-15Mo surfaces was evaluated in order to improve implants osseo integration. Thereby the use of laser ablation for post-processing should guarantee a clean, reproducible and controlled surface morphologies.

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INTRODUCTION

Metallic biomaterials have most attracted the attention of researchers for biomedical applications(Prasad *et al.*, 2017). For dental implants and orthopedic prostheses, Ti-6Al-4V alloy has been the clinical standard due to it combinates excelent biocompatibility, good mechanical strength and high corrosion resistance. However, the toxicity from released vanadium and aluminum ions has become in issue of concern (Lin *et al.*, 2005; Martins Júnior *et al.*, 2018; Okazaki and Gotoh, 2005). Research on biomaterials is being carried out focusing on β-type titanium alloys, aiming at obtaining desired mechanical properties, such as low modulus of elasticity, increased and improved tissue interactions response are obtained more easily when compared with Ti alloys of the β-type. Thus, Ti alloys of the β-type are being developed, presenting low modulus of elasticity and high resistance to corrosion and fatigue, besides being composed of non-toxic elements such as Nb, Ta, Zr, Mo, and Sn (Metikos-Huković *et al.*, 2003; Niinomi *et al.*, 2012). Thereby, the study of surface modifications on the Ti-15Mo alloy to the implant / prosthesis to better integrate with the host tissues has be cited in the field of orthopedics and dentistry(Liu *et al.*, 2017; Pennekamp *et al.*, 2007; Sasikumar *et al.*, 2011).Currently, advances in the metallic surface modification techniques have been applied to the dental implants and orthopedic prosthesis(Bagno and Di Bello, 2004; Qiu *et al.*, 2014). Several treatment and processing steps have been developed, including (1) the mechanical treatments with aim to increase surface roughness and (2) the chemical treatments that involve structural changes in the oxide layer obtained by ionization, ion deposition, vapor phase deposition, thermal projection coatings, diffusion and laser treatments, and others (Bandyopadhyay *et al.*, 2016;

Geetha *et al.*, 2009). One of the most promising surface modifications is by the laser beam irradiation(Cho and Jung, 2003; Götz *et al.*, 2004). Different types of lasers (CO_2, Yb: YAG and Nd: YAG lasers) have been employed in cutting, welding and surface modification application (Silfvast, 2004).The irradiation of the samples by the laser beam is a technique that consists in providing on the surface of the material a great irregularity in a "clean" way with the objective of creating a morphology with large specific area and physicochemical properties adequate to promote the interaction of the calcium phosphates. Simultaneouly, it provides the formation of a great diversity of oxides that aim to induce the osseointegration phenomenon more efficiently and in a satisfactory manner.

Surface characteristics such as macro and micro topography and chemical composition of surfaces affect short-term (migration and anchoring) and long-term (differentiation and matrix expression) cellular responses(Keller *et al.*, 1998; Silfvast, 2004).Therefore, the aim of the treatments or modifications of surfaces of a biomaterial is the establishment of a chemical and mechanical bond of the bone to the implant material, aiming at the creation of a physico-chemically favorable surfacewithout cell/tissue toxicity and with a reasonable cell adherence, so the phenomenon of osseointegration occurs.

MATERIAL AND METHODS

Laser-activated surface modification: Samples of Ti-15Mo alloy with dimension of 10x10x2.0 mm were manufactured. The surface modification was performed by Yb: YA Gmultipulse laser using a instrument model Laser *Omni Mark®* 20 F (Omnitek Tecnologia Ltda, São Paulo, Brazil) (λ = 1090 nm). All surfaces were modified under pressure and air atmospheric conditions. The influence of parameters (laser power, pulse frequency and scanning speed) with fluence (ablation) values of 0.019 to 0.055J/mm^2 (n= 12 conditions), Table 1. After irradiation, the samples (S0 to S12) were treated ultrasonically and separately in solutions of ethyl alcohol, acetone and distilled water, followed by oven-drying, and characterizated. Non-irradiated surfaces (S0) were used as control sample. The crystalline structures of samples were investigated by X ray powder diffraction (Seifert XRD 3000 TT diffract meter) and the quantitative phases analysis was obtained by Rietveld refinements (Rietveld and IUCr, 1969) using the program GSAS(Larson *et al.*, 2001). The phases considered in all refinements are listed in Table 2, according Inorganic Crystal Structure Database (ICSD)

Morphological and structural characterization: The samples were characterized by scanning electron microscopy (SEM), using a Zeiss EVO LS-15, Oxford Inca Energy 250. The X-ray diffraction analysis was performed in a Siemens D5000 X-ray diffract meter (Siemens, Munich, Germany), using a scan angle of 20 at 80º with a step size of 0.02 (2θ).
Each sample was subjected to a counting time of 10s/step in a Bragg-Brentano configuration, using Cu (kα1) radiation. Quantification by Rietveld refinement was performed in a Rigaku RINT-2000 X-ray diffract meter with rotating anode, operating under the experimental conditions at 42KV, 120mA, with divergence slits, scattering angle of 0.5º, 5 mm horizontal opening of the divergence slit, 0.3 mm receiving signal, 5°Soller, copper anode, and wavelengths of $K\alpha_1$ = 1.55056 Å and $k\alpha_2$ = 1.5444 Å, $I\alpha_2/I\alpha_1$ = 0.5.

Cell culture: Human keratinocyte cell line (HaCaT) mycoplasma-free were grown (9×10^5 cells/cm^2) in high glucose DMEM (Dulbecco's Modified Eagle's medium, Sigma-Aldrich, USA) pH 7.2, supplemented with 10% fetal bovine serum (Gibco, Invitrogen, USA), 100 U/mL penicillin and 100 µg/mL streptomycin, in a 5% CO_2 atmosphere at 37°C (Panasonic MCO-19AIC, Japan).

Citotoxicity assay: The samples of Ti-15Mowere autoclaved and positioned inside the well in sterile 24-well microplates (Greiner Bio One). Then, HaCaT cells (9×10^5 cells/cm^2) were platted (2.0 mL final volume) and incubated for 24 h for cell adhesion and growth. Experimental controls were conducted to exclude possible redox interference, where Ti-15Mo samples were incubated with MTT in the same experimental conditions without the cells and no MTT reduction was observed (data not shown). After the incubation time, the samples were removed and 0.1 mL of theMTT solution (5mg/mL) was added to each well and incubated for 4 h. The formazan crystals formed by the MTT reduction by cellular dehydrogenases were solubilized by the addition of 1.0 mL of 10% SDS and overnight incubation. After, an aliquot of 0.3 mL of each well was transferred to a96-well microplates and the absorbance of each well was read at 570 nm with background subtraction at 620 nm (Biochrom Asys Expert Plus Microplate Reader, Biochrom Ltd., UK). Cell viability was calculated in relation to the control (sample without laser irradiation, denominated sample 0), which was considered as 100% of viability.

Viability and Adhesion Assay: HaCat cells were incubated at the same experimental conditions described for the citotoxicity assay above. After 24 h incubation for cell adhesion, the supplemented medium was replaced by 1.0 mL of fluorescence buffer (1.5mM $CaCl_2$, 130mMNaCl, 5.6mMKCl, 0.8 mM $MgSO_4$, 1.0mM Na_2HPO_4 ,25 mM glucose, 2.5 mM $NaHCO_3$, 2.0 mMHEPES, pH 7.3), followed by staining with 2.5 mMHoechst 33342 (Cell Signaling Technology, EUA) and0.1 mg/mL propidium iodide (BD Biosciences, EUA). After 30 minutes, each well was washed twice and the fluorescence emission was analyzed by a widefield fluorescence microscopy (Leica AF6000, Leica Microsystems, Germany), using cube filters A4 (excitation 360/40 nm, dichromatic mirror 400 nm, emission BP 470/40 nm) and L5 (excitation 480/40 nm, dichromatic mirror 505 nm, emission BP 527/30 nm), an 20X Plan Apo objetive (NA 0.6), and a Leica DFC365 FX CCD digital monochrome camera.

Statisticalanalysis

Data were analyzed statistically usin gone-way analysis of variance (ANOVA) ($p < 0.05$) and Tukey's test for multiple comparisons among groups. Data were expressed as the mean± SD.

RESULTS AND DISCUSSION

Our research group has reported the effects of processing parameters on pulse repetition rate, scan speed, pulse energy and fluency must be take into accounting to the appropriate Tic.p (Braga *et al.*, 2007) and Ti-based alloy (dos Santos *et al.*, 2018)surface topographies. In this paper, we have evaluated the cytotoxic effects of different phases of titanium oxides obtained by laser irradiation process. In this perspective, the formation of the crystalline phases αTi, Ti_6O, Ti_3O and TiO_2 by the melting and fast cooling processes during irradiation

Table 1. Fluency obtained for the irradiation Laser beam. Samples (S) from 1 to 12

Parameters (Samples)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
Scanningspeed (mm/s)	125	100	125	125	100	75	125	100	75	100	75	75
Repetition rate (kHz)	20	20	25	30	25	20	35	30	25	35	30	35
Fluency (J/mm^2)	0,019	0,023	0,024	0.029	0.030	0.032	0.033	0.036	0.039	0.042	0.048	0.055

Table 2. Crystalline structures of identified phases in the irradiated structures

Phase	Crystalline system	Cell parameters (Å)	ICSD number
α-Ti	hexagonal	a = b = 2.95 c = 4.686	76144
Ti_3O	trigonal/rhombohedral	a = b = 5.1411 c = 9.5334	24082
Ti_6O	trigonal/rhombohedral	a = b = 2.95 c = 4.686	17009
TiO_2	tetragonal	a = b = 3.833 c = 9.436	202242

Table 3. Percentage of phases composed of Ti and O after refinement by Rietveld

	Fluency ($J.mm^{-2}$)											
	0.019	0.023	0.024	0.029	0.030	0.032	0.033	0.036	0.039	0.042	0.048	0.055
Phases(%)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
α - Ti	37,19	34,39	31,65	27,25	30,15	24,65	18,35	23,64	15,59	12,09	19,09	8,70
Ti_3O	26,42	23,00	23,04	27,01	25,19	30,68	29,57	30,52	38,36	41,06	32,26	49,55
Ti_6O	20,92	23,84	23,12	21,45	24,56	22,68	23,19	24,65	29,61	35,46	31,26	35,97
TiO_2	15,47	18,77	22,19	24,29	20,10	21,99	28,89	21,19	16,44	11,39	17,39	5,78

*SAMPLE: S

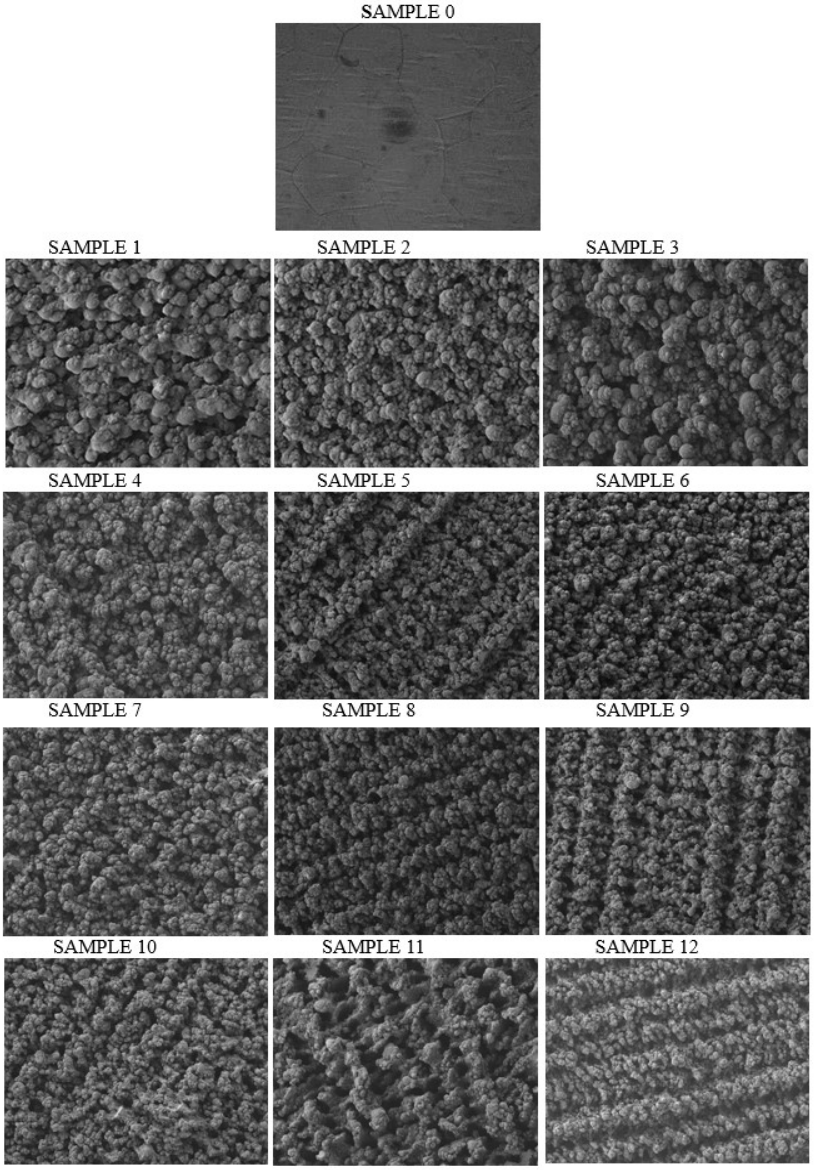

Figure 1. SEM of the Ti-15Mo alloy submitted to laser beam for Samples from 1 to 12. Amplitude: 100X.

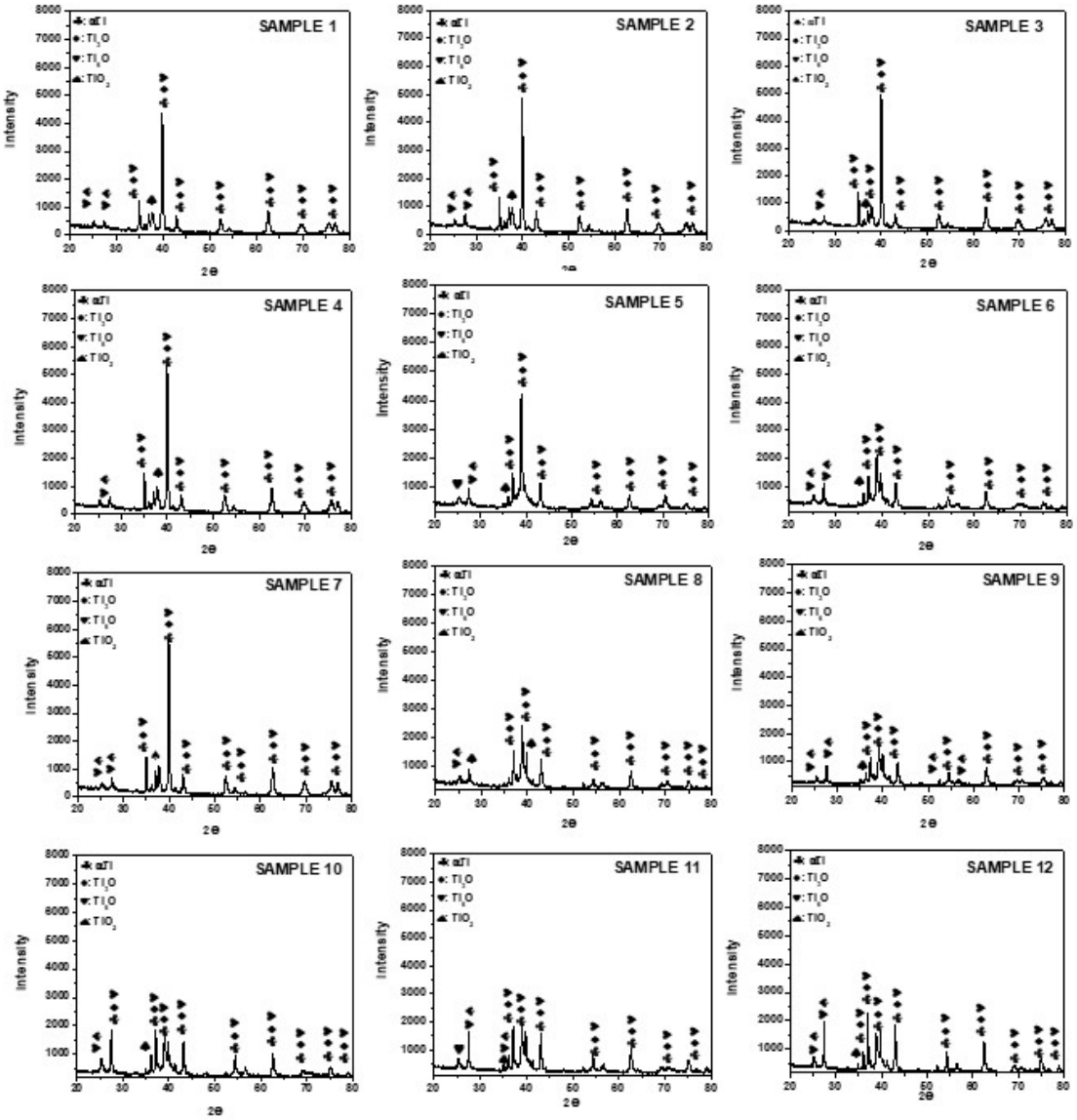

Figure 2. DRX of the Ti-15Mo alloy submitted to laser beam for samples from 1 to 12.

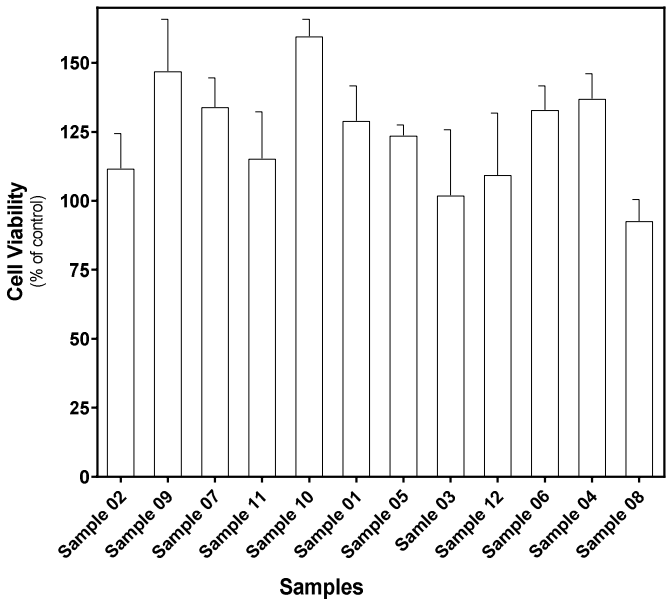

Figure 3. Effects of Ti-15Mo alloys on the HaCaT cell viability.

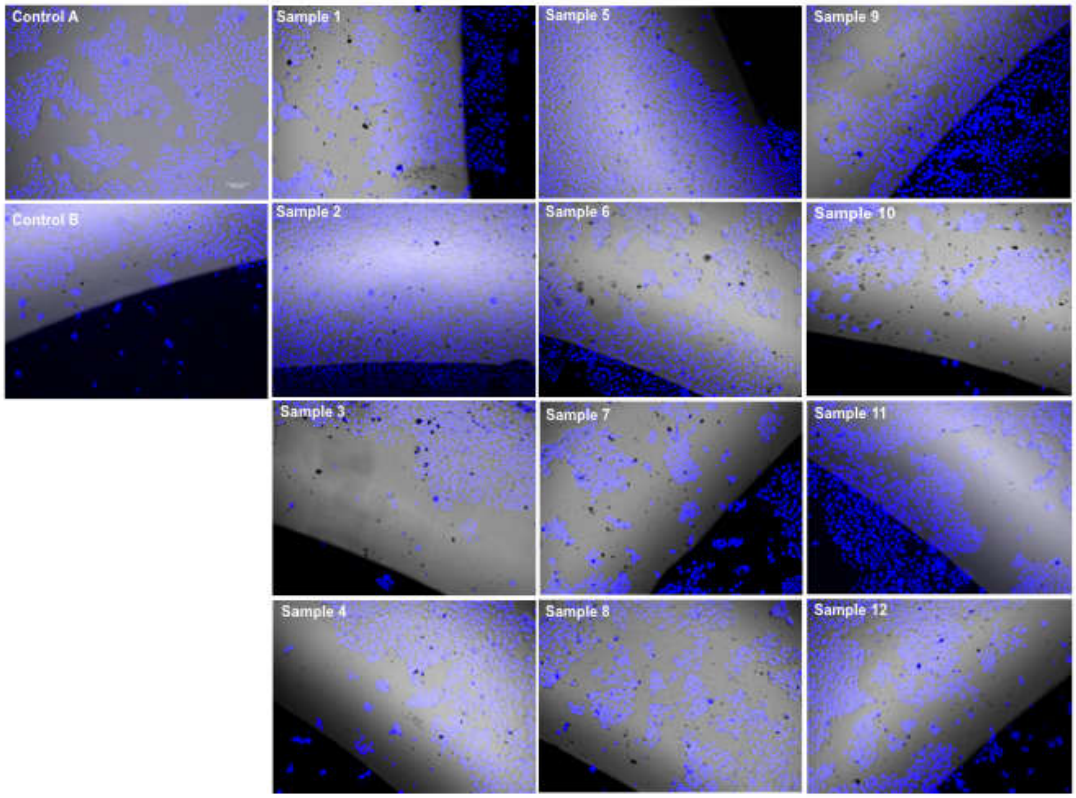

Figure 4. Growth and adherence of HaCaT cells on Ti-15Mo alloys

was thoroughly evaluated. Figure 1 shows the micrographs (SEM) of the non-irradiated surface (sample 0) and laser-irradiated Ti-15Mosurfacesfrom 0.019to 0.055J/mm^2 (samples 1 to 12, Table 1).It can be observed the increased fluency, due to longer exposure time of the laser beam to the alloy surface, produces a high roughness and typical morphologies with different surface energies. This can be explained through the formation of new structures (metal oxides) produced during the fast melt and solidification processes(Braga *et al.*, 2007; Lavisse *et al.*, 2002).The complete laser-metal interaction cycle is complex and non-linear. The phenomena involved occur with simultaneous and sequential effects. There are three fundamental components in the interaction of the beam with the surface of the material: laminar melting, evaporation and breaking of chemical bonds. The infrared-laser emission with a wavelength of 1090 nm (Yb:YAG), allows both phenomena such as plasma production and ablation, which consists on submitting the metal surface to rapid fusion and solidification process(Braga *et al.*, 2007; Lavisse *et al.*, 2002). During the thermal cycle could be presented a sequence of chemical reactions of non-equilibrium conditions: beam absorption, laminar melting, instability in surface tensions, change in roughness pattern, plasma explosions, heat loss through conduction, oxidation and migration of impurities. Laser-modified samples showed the formation of rougher surfaces than those of titanium alloys, which is attributed to the higher energy density generated on the surface, producing a larger zone of fused metal.

Figure 2 presents the graphics after Rietveld refinements for all samples; the Bragg peak positions of phases considered in the refinements were not presented to allow a better view. In all samples, peaks are slightly moved from their standard position due to the high residual stress developed during very fast heating and cooling process resulting from laser oxidation. However, although the uncertainty in the discrepancy factors of Rietveld refinements are in some cases high, it can be stated the phases percentages obtained are a good approximation and are in agreement with the literature (Lavisse *et al.*, 2002; Nanai *et al.*, 1998; Pérez del Pino *et al.*, 2002). As it can be observed in the samples XRD patterns (Figure 2), depending on the laser parameters set, different phases will be formed on metal surface. By modifying the scanning speed (mm/s), repetition rate (kHz) and pulse energy (mJ), the global energy delivered on the surface may be varied and the amount of diffused atoms in the treated surface will to depend on the repetition rate(Langlade *et al.*, 1998). The laser-titanium-molibdenium interaction only undergoes thermal effects, but during the laser pulse, the surface is heated and can be melted and vaporized creating plasma state on its surface. As a result, diffusion mechanism from species close to the irradiated area (especially oxygen and nitrogen) into the melted Ti-15Mo getter will take place. Thevery fast heating and cooling processes lead to non-equilibrium phenomena(Nanai *et al.*, 1998; Vajtai *et al.*, 1996), where the thermochemical reaction rate constants have an Arrhenius-type temperature dependence and also depend on other parameters such as a wavelength, power, polarization, time of irradiation, consistency of the environment gaseous atmosphere, focusing details, etc. Despite the high number of parameter influencing the control of phases and morphologies obtained at Ti-15Mo alloy surfaces, the accumulated laser fluency (Table 1) seems to be the more sophisticated. The accumulated energy per unit area (fluency) can be calculated as:

$$F = \frac{E_p f}{DV}$$

where E_p: pulse energy, f:repetition rate, D:spot diameter and V:scanning velocity. The presence of Ti_6O and Ti_3O is due to oxygen ordering in the hexagonal α-Ti and can be explained

by oxygen diffusion in the α-Ti initial lattice. The metallic titanium is heated between 400°C and 600°C under ambient pressure and air, it transforms into compounds with structures belonging to the TiO_{2-x} phases (György *et al.*, 2004; Sorensen, 1981). Some researchers have showed the higher the accumulated fluency, the higher the degree of oxidation (Lavisse *et al.*, 2002; Nanai *et al.*, 1998; Pérez del Pino *et al.*, 2002). These results suggest rapid fusion and solidification by laser beam irradiation in ambient air induces the formation of titanium oxides with different degrees of oxidation, indicating that the laser energy favors the diffusion of O atoms, as well as the rapid solidification (Lavisse *et al.*, 2002; Nanai *et al.*, 1998; Pérez del Pino *et al.*, 2002). Finally, samples were screened for their cytotoxicity in non-tumorigenic human keratinocytes cell line (HaCaT) by using the MTT reduction assay. Experimental controls were performed with the samples incubated at the same experimental conditions without cells and no redox interference of the modified Ti-15Mo surfaces (n=12 samples, according Table 1) with the MTT reduction was observed, showing this assay can be used for cytotoxicity evaluation with these samples. As observed in the Figure 3, none of the alloys decreased the cell viability in relation to control (non-treated surface, considered as 100 %), showing the absence of toxicity against these cells.

In fact, the absorbance in some wells was significantly higher than control, indicating a possible proliferative inducing effect exerted by samples 1, 2, 7, 9, 10, and 11, which will be further evaluated. *In vivo* experiments in hamsters revealed that these alloys induced an inflammatory response in skeletal muscle without high toxicity or dysfunction (Pennekamp *et al.*, 2007).In fact, it was showna beta type titanium alloy promoted an increase in the viability of murine embryonic fibroblasts 3T3 *in vitro* (Lee *et al.*, 2009), although the molecular mechanisms remain unclear. Considering the absence of cytotoxicity, we evaluated next the effects of surface treatment of Ti-15Mo alloys on adhesion properties of cultured keratinocytes. The nuclei of live HaCaT cells were stained with the cellular permeable fluorophore Hoescht 33342 and the fluorescence images were overlaped with the bright field images in order to allow the vizualization of the metal surface and cells simultaneously. As observed in the Figure 4, cells were able to proliferate in the presence of all samples.

However, it is noteworthy the surface modifications by laser irradiation affected the adhesion capacity of the cells on the material. The higher growth and adhesion capacity was observed in the same samples1, 2, 6, 7, 9, and 11. With the exception of sample 10, other samples were the same with higher viabilibity percentages in the Figure 3, and the number of cells in the field seems to be higher than found in the controls. All this mentioned samples were iradiated with low frequency (repetition rate, kHz), which indicates this is an important parameter to be considered regarding cellular growth and adherence. Electrolytic modification of Ti-15Mo alloys increased the adhesion of cultured cells on the surface (Kazek-Kęsik *et al.*, 2014)and also prevented the colonization of bacteria in their surface (Kazek-Kęsik *et al.*, 2016), pointing to the great potential of this type of materials in biomedical field.

Conclusion

The laser beam irradiation modifies the implant surface with significant characteristics which can help the osseointegation phenomenon, such asmorphology, surface roughness and compounds that facilitate the wetting and the physical chemistry properties,besides the absence of cytotoxicity, where the non-stoichiometric compounds can catalyze the bone reconstitution cells adhesion. Thereby, laser beam irradiation is a clean and reproducible process where the implant surface does not interact with any other materials for its modification.

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