Development of a versatile procedure for the biofunctionalization of Ti-6Al-4V implants

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Titanium (Ti) and titanium alloys are among the most-commonly used metallic materials for implantation in the human body for the purpose of replacing hard tissue. Although Ti and its alloys are widely used for such an aim, in implants of a long duration they exhibit some shortcomings due to the loosening of the very implant. This phenomenon is highly dependent on the interaction between the organic tissues and the surface of the implant. In this study, the authors introduce a surface treatment technique for functionalization of the surface of Ti-6Al-4V alloy with amino groups that could help to control this interaction. The functionalized layer was deposited by activated vapor silanization (AVS), which has been proven as a reliable and robust technique with other materials. The resulting biofunctional layers were characterized by atomic force microscopy and fluorescence microscopy, with the optimal conditions for the deposition of a homogeneous film with a high density of amino groups being determined. Additionally, the non-toxic nature and stability of the biofunctional layer were confirmed by cell culturing. The results show the formation of a homogeneous biofunctional amine layer on Ti-6Al-4V alloy that may be used as a platform for the subsequent covalent immobilization of proteins or other biomolecules.

1. Introduction

The ever-increasing need for implants to replace damaged parts of the human body calls for biomaterials with improved properties able to mimic the functions of failed organs. Among these biomaterials, metallic biomaterials are of significant interest for hard tissue replacement due to their advantageous mechanical properties. The biomaterials used for bone replacement are required to be chemically stable, be resistant to fracture and fatigue and, possibly, able possess an elastic modulus close to that of bone [1]. In this regard, titanium and titanium alloys offer a combination of properties that make them suitable as load-bearing implants for hard tissue replacement. Their combined properties of high tensile strength, low density, high corrosion resistance and low elastic modulus have made them the ultimate choice for load-bearing implants for such replacement [2]. In particular, their low elastic modulus compared with other metallic biomaterials (stainless steel and Co-Cr alloys) improves stress transfer to the adjacent bone. Consequently, titanium implants diminish bone resorption and reduce the risk of

a loosening of the implant [3], since its elastic modulus is closer to the 30 GPa of cortical bone [4]. Ti-6Al-4V is the most common titanium alloy used in biomedical applications, given that the biphasic structure of Ti-6Al-4V causes the alloy to have superior corrosion resistance and tensile strength compared with pure titanium [1].

However, and despite its extensive use as an implant for bone replacement, metals in general and titanium alloys in particular show a significant drawback as prostheses, since they are unable to integrate directly into the adjacent bone tissue. Thus, metallic prostheses constantly appear to be encapsulated by a layer of connective tissue [5]. Healthy functional tissue can grow on top of the connective tissue, though this non-specialized tissue remains as an arguably weak interface between the metal and the bone [6], and tend to cause problems in implants of long duration.

In order to improve the performance of metallic implants, different methods of chemical or physical surface modification have been employed. Application of these methods is based on the assumption that the reaction of the organism to the implant depends on the events that occur at the surface of the material. In particular, the proteins adsorbed on the surface and their recognition by distinct cell lineages are presumed to determine the response of the organism to the material [7,8]. Consequently, various surface modification procedures such as deposition of calcium phosphates [9], acid etching [10], plasma spraying [11]

and alkaline treatment [12] were proposed to monitor interaction between the material and the organism and to improve the osseointegration of Ti alloys. An increased interest has recently emerged in functionalization routes that allow the covalent immobilization of biomolecules on the surface of biomaterials in an attempt to trigger specific responses in the host tissue and thus improve cell adhesion and osseointegration [13].

Silanization is a commonly used technique for functionalization which results in an amine-terminated functional surface. Aminosilanes are used as precursors in this procedure because they are able to bond with hydroxyl groups which may be induced on the oxidized surface of metals [14,15] and, in particular, may be used to functionalize titanium and its alloys [16–19]. In turn, the resulting amino groups of the functionalized surface are capable of binding themselves to a wide range of biomolecules [20]. In general, this process proceeds by immersing the substrate in a solution of 3-aminopropyltriethoxysilane (APTS) with toluene or pentane [21] (immersion silanization, IS), although it has been found that this process does not necessarily lead to reproducible functionalized layers [22,23]. The difficulties associated with IS are firstly related with the need for surface activation to induce the appearance of –OH groups at the surface. Activation requires a pre-processing of the material by either immersion in acid [19,24] or with an oxygen plasma treatment [16]. Secondly, IS tends to result in a relatively low concentration of amine functional groups on the surface which, in addition, may be non-homogeneously distributed. These undesired features of IS seem to be highly dependent on the sensitivity of the process to the ambient conditions, since reactions are highly susceptible to hydrolyzation and oligomerization [22,23].

In order to address these obstacles, an alternative silanization technique referred to as activated vapor silanization (AVS) has been developed by the authors and is used in this work to functionalize Ti-6Al-4V substrates. This method has been previously employed to functionalize various materials, such as silicon, [25,26] successfully. In this procedure, the need for surface activation is eliminated, since the APTS molecules are activated in the vapor phase at high temperatures (700–800 °C) before impinging on the substrate. Additionally, as activation and deposition reactions take place under low vacuum ($P \approx 10^{-2}$ mbar), the reactions generally do not depend on ambient conditions, which then lead to higher reproducibility. As a result, AVS functionalized samples show a high concentration of amines which are homogeneously distributed. Lastly, the control exerted on the process through the deposition parameters allows a certain degree of variation in the properties of the functionalized layers.

This work demonstrates the ability of AVS for the effective bio-functionalization of Ti-6Al-4V alloy. Various biofunctional layers were prepared and characterized in terms of amine reactivity and surface topography. Optimal functionalization conditions that lead to the deposition of a homogeneous functional layer on Ti-6Al-4V were selected in terms of density of surface amine groups. Bio-compatibility of the biofunctionalized layer was assessed through *in-vitro* cell culture experiments. In particular, the proliferation of MC3T3-E1 cells on the functionalized Ti-6Al-4V samples was studied. Biofunctionalized layers were also shown to remain stable even after being used as substrates for cell culturing for a period of at least one week.

2. Materials and methods

2.1. Preparation of Ti-6Al-4V substrates

The substrates were cut from a block of commercial Ti-6Al-4V alloy into samples with dimensions of approximately $10 \times 10 \times 1$ mm. All the samples were subjected to a grinding process with SiC grinding papers grit no. 400. The samples that

Table 1

Parameters used in the AVS functionalization processes featured in this work. Deposition time was fixed at 20 min for all processes.

T_{Evap} (°C)	T_{Act} (°C)	P_{Ar} (mbar)
130	750	1
150	750	1
170	750	1
150	700	1
150	800	1
150	750	2
150	750	0.5
150	800	2

underwent this process only were labeled as 400 samples. The rest of the samples were subjected to a grinding process with SiC grinding paper grit no. 1200. Those that were not additionally polished were labeled 1200 samples. Lastly, the rest of the samples were polished with SiC grinding paper grit no. 4000 and labeled 4000 samples. After grinding, all the substrates were cleaned by sonication in acetone, isopropanol and distilled water.

2.2. Functionalization of substrates

The substrates were functionalized by activated vapor silanization (AVS) [25] by using APTS ($\text{NH}_2(\text{CH}_2)_3\text{Si}(\text{OC}_2\text{H}_5)_3$, (Fluka, Germany) as a precursor. Briefly, APTS is evaporated in an evaporation chamber and the vapor is then transported via an Argon flux (BIP, Purity $\geq 99,9997\%$) to the activation chamber in which the APTS molecules impinge on the substrate after being activated at 700–800 °C. The substrates were cleaned after deposition by sonication with acetone and isopropanol, rinsed with distilled water, dried by using the argon flux and stored in air.

The AVS process is defined by four parameters: evaporation temperature of APTS (T_{Evap}), activation temperature (T_{Act}), argon pressure (P_{Ar}) and deposition time (t). In order to determine the optimal conditions that lead to the deposition of a homogeneous amine layer on Ti-6Al-4V substrates, the first three parameters (T_{Evap} , T_{Act} , P_{Ar}) were varied while the deposition time (t) was kept constant, and the resulting amine layers characterized. The deposition parameters used in this study are presented in Table 1.

2.3. Characterization

2.3.1. Topography characterization

The topography of the bare Ti-6Al-4V and functionalized samples was studied by atomic force microscopy (Cervantes AFM, Nanotec S.L.). Profile data and roughness were analyzed by use of WSxM 5.0 [27] software. AFM measurements were performed in dynamic mode by using a pyramidal cantilever (Olympus OMCL RC800, semi-angle 39° , nominal resonance frequency 69 kHz). 10 substrates were scanned corresponding to each type of non-functionalized samples (*i.e.* 400, 1200 and 4000 samples) and two substrates for each functionalization condition.

In order to measure the thickness of the functionalized layers, 4000 samples were covered with a resin mask in some parts of the surface prior to functionalization. The resin mask was prepared by drop casting with photoresist (microchemical AZ5214E), and spun at 5000 rpm for 45 s in order to obtain a uniform thickness of 1.4 μm . The optical lithography was carried out with a Karl Süss MJB mask aligner. The resist development was made at $\text{AZ400K} + \text{H}_2\text{O}$ (1:5) for one minute and final rinsing in H_2O for 10 s. After the subsequent functionalization, the samples were sonicated in acetone to remove the resin. The step created between the regions previously covered by the resin and those covered by the functional layer was measured by AFM in contact mode.

2.3.2. Fluorescence labeling of amines

Fluorescein isothiocyanate (FITC) was used as a fluorescent marker to measure the density of amines on the surface. FITC is a fluorescent molecule which can react with the amino groups through the isothiocyanate group. After functionalization, relative amine concentrations were compared by examining the intensity of fluorescent light emitted from each sample. The measurement of the intensity of fluorescence under the same observation conditions represents a semi-quantitative procedure to determine the density of amine surface groups.

For this purpose, the samples were incubated in 0.5 mg/ml solution of FITC in phosphate buffer saline (PBS 10 mM, pH = 7.4) for 20 min. With the aim of removing any FITC non-covalently linked to the surface, the samples were cleaned in the same buffer for five minutes (three times) and subsequently rinsed with distilled water (three times). The presence of FITC was checked by fluorescence microscopy (Leica DMI 3000B) at an emission wavelength of 520 nm. The observation conditions were: exposure time: 1.3 ms, gain: 2.1X and gamma: 0.68.

2.3.3. Contact angle measurements

The contact angle of the bare Ti-6Al-4V 4000 samples (used as reference) and the F1 and F2 biofunctional layers (defined below) was measured according to the ASTM D7334-08 standard, by depositing a small volume of water ($V = 5 \mu\text{l}$) on each surface and taking a micrograph of the droplet using a precision camera (EO-1312C, Edmund Optics) equipped with a micro inspection lens system (Optem zoom 125). The measurement was performed on six samples of each condition and four times on each sample.

2.3.4. Reproducibility

In order to check the reproducibility of the F1 and F2 functionalization conditions, six samples of each condition were produced and characterized in terms of roughness (AFM) and amine concentration (FITC labeling).

2.3.5. Cell culture

The *in-vitro* cytocompatibility of bare Ti-6Al-4V and functionalized samples was compared by culturing osteoblast-like murine MC3T3-E1 cells on the materials. The cells were cultured in polystyrene Petri dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% of fetal bovine serum (FBS) and 1% penicillin/streptomycin in a humidified atmosphere at 37 °C and at 5% CO₂ until they reached confluence. The medium was changed twice a week. The cells were detached from the dishes by using Trypsin-EDTA (0.05% in HBSS, HyClone) when needed. Prior to cell culturing, the substrates were autoclaved and placed in 24-well plates. Cells cultured in blank wells under the same conditions were used as controls.

2.3.5.1. Cell viability. For cell viability experiments, MC3T3-E1 cells suspended in medium were seeded on the substrates in a concentration of 50,000 cells/ml and 0.5 ml of medium per well. After either four or 48 h of incubation at 37 °C in a humidified atmosphere of 5% CO₂, the cells were dyed with a combined solution of 1 μl /ml of calcein acetoxyethyl (Calcein AM, Life Technologies, 0.5 $\mu\text{g}/\mu\text{l}$ in DMSO) and 1 μl /ml propidium iodide (Sigma-Aldrich, 750 μM in PBS) in DMEM and incubated for another 30 min. Calcein AM is a non-fluorescent cell-permeable dye that inside the living cell is converted to green fluorescent calcein after acetoxyethyl ester hydrolysis by intracellular esterases, and is used to stain the viable cells [28]. Propidium iodide is a red-fluorescent nuclear marker impermeable to the cellular membrane. Therefore, the nuclei of cells with damaged membranes, *i.e.* dead cells, will be stained with this dye [29]. Lastly, the samples were observed by fluorescence microscopy at an emission wavelength of 520 nm and 620 nm for

calcein and propidium iodide, respectively, by using an inverted Leica DMIRB microscope equipped with a digital camera, Leica DC100 (Leica, Nussloch, Germany). Data were obtained from three independent experiments run in duplicate. At least three representative images were taken from the surface of each substrate. ImageJ software was used to determine the number of cells in each such image.

2.3.5.2. Cell proliferation. MC3T3-E1 cells suspended in medium (10,000 cells/ml) were seeded on the substrates (0.5 ml per well) and the substrates were incubated for either one or seven days at 37 °C in a humidified atmosphere of 5% CO₂. Cell proliferation was determined by using the Cell Proliferation Assay kit (AppliChem) following manufacturer instructions. This employs 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide salt (XTT). In living cells, mitochondria have the capability to reduce XTT to form an orange-colored, water-soluble dye [31]. Therefore, the concentration of this dye is proportional to the number of metabolically active cells. The absorbance of each well was measured spectrophotometrically at 450 nm by using an ELX808 microplate reader (BioTeK). For comparative reasons, the absorbance values were normalized according to the surface area of each sample. Data were obtained from experiments running in triplicate.

2.3.6. Determination of stability of the functional layer

In order to examine the stability of the functional layer exposed to cells and culture medium, after the cell culturing experiment was over (seven days of incubation) the samples were checked for the presence of the amine groups on the surface. The samples were removed from the medium, rinsed with distilled water and, subsequently, dyed with FITC following the protocol described above.

2.3.7. Statistical methods

Statistical analyses were performed with IBM SPSS Statistics 20 software by using one-way analysis of variance (ANOVA) followed by the Games-Howell post-hoc test [30]. $p < 0.05$ was considered significant. All data are presented as a mean value $\pm$ standard error.

3. Results and discussion

3.1. Characterization of non-functionalized samples

3.1.1. Roughness

Fig. 1 shows the root mean square (RMS) roughness for 400, 1200 and 4000 Ti-6Al-4V samples at three scan sizes (50 μm , 5 μm and 0.5 μm) measured by AFM. It can be seen that after each grinding step, the RMS roughness at 50 μm and 5 μm scan sizes decreases considerably. In contrast, roughness only shows a marginal decrease when the 0.5 μm scan size is considered. In all cases, all the samples are smooth at this scale with an average roughness of 16 ± 2 nm for the 4000 samples in 5 μm scan size.

3.1.2. Cell cultures on non-functionalized samples

In order to examine the possible effect of surface roughness of the substrates on the adhesion and viability of MC3T3-E1 cells, samples with distinct degrees of surface roughness were used as substrates for the cell culture. Cells seeded on polystyrene wells were used as controls. After seeding the cells on the substrates, the samples were incubated for four or 48 h. The number of viable cells attached to each substrate was then determined from the images of calcein AM/propidium iodide assay.

As can be seen in Fig. 2, after four hours of incubation, the cells adhered to the surface of the bare Ti-6Al-4V substrates, although they are not completely spread, and most have a circular shape. After 48 h of incubation, the cells are completely spread

Fig. 1. Comparison of RMS roughness values of Ti-6Al-4V substrates after each grinding step at different AFM scan sizes. Data are presented as a mean value $\pm$ standard error.

on the surface and exhibit a well-structured polygonal shape, typical for this cell line in its adhered state with many cytoplasmic protrusions extending along the surfaces. There is no obvious difference between the morphology of the cells that were seeded on polystyrene blank wells used as control, and of those on the substrates, although cell density seems to be higher in the former.

In order to compare the adhesion and viability rates of cells on each substrate after four hours of incubation, the number of cells present in each image taken from the surface of the samples was determined. The results showed (see the supplementary material) that after four hours of incubation time there is no sta-

Fig. 3. Absorbance values of XTT assay obtained from MC3T3-E1 cells cultured on Ti-6Al-4V substrates with different degrees of surface roughness for one and seven days. Data are presented as mean value $\pm$ standard error. * denotes statistically significant difference versus the corresponding control sample.

tistically significant difference between the number of viable cells on the substrates with distinct surface degrees of roughness (*i.e.* 400, 1200 and 4000). Moreover, the number of dead cells on each substrate was appreciably low and similar to that obtained when culturing the cells on polystyrene plates (control samples). In contrast, the number of viable attached cells on the control samples was significantly higher (Fig. 1 in the Supplementary material).

In order to examine the proliferation rate of cells on the substrates with different degrees of roughness at longer incubation times, an XTT assay was used. Fig. 3 shows the absorbance values after one and seven days of incubation time. It may be seen that as there is no significant difference between the absorbance values, the numbers of viable cells on all substrates are similar. In contrast, the number of cells is significantly higher on the controls at these

Fig. 2. Fluorescence microscopy images of MC3T3-E1 cells on substrates with different degrees of surface roughness after four and 48 h of incubation. Viable cells are dyed green; dead cells appear as red dots. Cells seeded on polystyrene wells are shown as control. Scale bar 100 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 4. Fluorescence microscopy images of Ti-6Al-4V substrates functionalized by using different deposition conditions after incubation with FITC. A non-functionalized 4000 sample incubated with FITC and observed under the same conditions is shown as control (c). Scale bar 1 mm.

longer incubation times. These results show that the roughness of the substrates in the range included in this study had no significant effect on the proliferation rate of cells. In all cases, Ti-6Al-4V samples were not as favorable for the proliferation of the cells as polystyrene controls.

Since no difference was observed between the substrates with a different surface roughness in terms of cell adhesion, viability and proliferation, those with the lowest surface roughness (4000 samples) were chosen as the substrates for functionalization. This choice was influenced by an interest in evaluating the possible increase in roughness due to the presence of the functional layer under the most demanding of conditions.

3.2. Characterization of functionalized samples

3.2.1. Roughness, contact angle and surface density of amino groups

Biofunctional layers deposited by AVS and by using APTS as a precursor are composed of Si, O, C, H and N, with an approximate empirical formula of $\text{SiO}_{2.5}\text{C}_{2.5}\text{H}_{2.6}\text{N}_{0.5}$, as determined in a previous paper by X-ray photoelectron spectroscopy (XPS) and Rutherford backscattering spectroscopy (RBS) [32]. This elemental composition is the result of the decomposition of the organometallic moiety, which results in a thin solid film stabilized by Si—O—Si bonds, while maintaining a relatively high proportion of the other elements, most importantly nitrogen. The analysis by FTIR of layers deposited with different AVS processing parameters also showed that,

although AVS always yields the same decomposition products, their quantitative proportion is highly sensitive to processing conditions. Quantitative variations are especially important with regard to the content of amino groups in the functionalized layer [33].

Various functionalized samples were prepared by varying the deposition conditions of the process: T_{Evap} , T_{Act} and P_{Ar} . The samples were characterized in order to establish at least one set of optimal functionalization conditions in terms of the high density of amino groups and low roughness.

Fluorescence microscope images of substrates functionalized under different conditions are shown in Fig. 4. By comparing these images with the non-functionalized 4000 samples (Fig. 4c) the presence of an amine layer is evident. By varying T_{Evap} from 130 to 170 °C (Figs. 4b, e and h), it can be seen that the amine layer produced at $T_{\text{Evap}} = 150$ °C is more homogeneous compared with the other two. Figs. 4d–f show samples that differ in T_{Act} and their comparison indicates that a $T_{\text{Act}} = 750$ °C leads to a higher concentration of amino groups present. Lastly, micrographs a, e and i show samples that differ in P_{Ar} . It can be seen that with increasing Ar pressure, the concentration of amines deposited on the substrates increases. Fig. 4g ($T_{\text{Act}} = 800$ °C, $P_{\text{Ar}} = 2$ mbar) exhibits the effect of increasing the activation temperature and argon pressure simultaneously. In this case, the fluorescence image shows a homogeneous functionalized surface with a high density of amino groups.

Fig. 5 shows the AFM topography images of functionalized samples under distinct functionalization conditions (scan size: $5 \mu\text{m} \times 5 \mu\text{m}$). From comparison of the surface morphology of a

Fig. 5. Atomic-force microscopy (AFM) micrographs of Ti-6Al-4V samples functionalized under different deposition conditions. The topography of a non-functionalized 4000 sample (c) is shown for comparison purposes.

Fig. 6. Comparison of (a) fluorescence intensity and (b) RMS roughness values of layers deposited under different functionalization conditions.

Fig. 7. Comparison of (a) fluorescence intensity and (b) RMS roughness values of biofunctional layers deposited under the F1 and F2 conditions. Six samples were deposited under each condition.

non-functionalized 4000 sample (c) with those of functionalized samples, it is apparent that the amine layer buries the characteristic scratches and grooves that result from grinding. In most of the functionalization conditions, the formation of a homogeneous thin film over the surface results in a reduction of roughness values as measured by AFM.

Fig. 6 summarizes the comparison of (a) surface density of amines (as measured by fluorescence) and (b) roughness (measured as RMS) of samples functionalized under different conditions. Two sets of optimal functionalization conditions were selected (F1): T_{Evap} : 150 °C, T_{Act} : 750 °C, P_{Ar} : 1 mbar, t : 20 min and (F2): T_{Evap} : 150 °C, T_{Act} : 750 °C, P_{Ar} : 2 mbar, t : 20 min. Both conditions result in homogeneous and smooth (low values of RMS from AFM micrographs) layers combined with a high density of amines (high values of fluorescence). Since the F1 and F2 sets of conditions were selected to continue the biological assessment, their characterization was extended to include the thickness of the film, the contact angle and

the reproducibility of the biofunctional layers deposited. The thickness of the functional layers was measured by using AFM and values in the range of 150–200 nm were obtained. The contact angle values obtained were F1: $66.2 \pm 0.5^\circ$ and F2: $58.9 \pm 0.7^\circ$, which can be compared with the value of $57.8 \pm 0.6^\circ$ measured for the bare Ti-6Al-4V 4000 samples. The reproducibility of the deposited layers was checked by characterizing six samples of each condition (either F1 or F2). Fig. 7 compares (a) the fluorescence intensity and (b) the RMS roughness values of the samples. The comparison of the error bars with the values of the corresponding feature is an indication of the excellent reproducibility obtained for the deposition of the biofunctional layers.

3.2.2. Cell cultures on functionalized samples

Long- and short-term cell culture experiments were performed to determine the response of cells to the functionalized layers in terms of their viability and proliferation rates. After either four or

Fig. 8. Fluorescence microscopy images of MC3T3-E1 cells on non-functionalized 4000 and two functionalized samples (conditions F1 and F2) after four and 48 h of incubation. Viable cells are dyed green while dead cells appear as red dots. Cells seeded on blank polystyrene wells are shown as control. Scale bar 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 9. Absorbance values of XTT assay obtained from MC3T3-E1 cells cultured on non-functionalized and functionalized substrates (samples F1 and F2) for one and seven days. Data are presented as mean value $\pm$ standard error. * denotes statistically significant difference versus the corresponding control sample.

48 h of incubation, cells were stained with calcein AM and propidium iodide and observed with a fluorescence microscope. Fig. 8 compares the morphology of cells adhered on functionalized and non-functionalized (4000) samples with polystyrene controls after four and 48 h of incubation. At four hours, the cells are adhered to the surface of all samples and possess a circular shape. This rounded cell shape is more pronounced in cells growing on the functionalized substrates. However, 48 h after seeding, the cells are

completely spread and the typical triangular shape of this cell line can be observed. The morphology of the cells appears to be similar in all substrates. This result indicates that cells can adhere to and spread over the amine layer, as well as they do on the non-functionalized Ti-6Al-4V samples.

The number of viable cells attached to the substrates four hours after seeding was determined from the micrographs taken from different samples and a statistical analysis performed (see the Supplementary material). The statistical analysis showed a significant increase in the number of cells attached to the functionalized compared with the non-functionalized substrates after four hours of incubation (Fig. 2 in the Supplementary material). Moreover, the number of dead cells (Propidium iodide positive cells) obtained for all substrates was somewhat low and close to that obtained with cells seeded on polystyrene controls, demonstrating a cytocompatibility for the functionalized substrates comparable to that observed on bare Ti-6Al-4V substrates.

In order to extend the comparison between functionalized and non-functionalized samples to longer incubation times, the proliferation rate of the cells on each sample after one day and seven days of incubation was compared by using the XTT assay (Fig. 9). According to the measured absorbance levels, the number of viable cells on the functionalized and non-functionalized samples at a given incubation time, does not show any statistically significant differences. In both types of substrates, however, the number of viable cells is considerably lower than that found in the controls.

From these results it can be seen that the functional amine layer does not inhibit cell proliferation in any way, and cells behave on the functionalized layer similarly as on the non-functionalized Ti-6Al-4V substrate.

Lastly, the influence of cell culturing on the biofunctional layer was assessed by removing the cells after seven days of culture and measuring the density of amino groups by using FITC and fluorescence microscopy (Fig. 10). These images show that although some degradation is observed, after cell culturing the amine layer keeps essentially its integrity and a high density of active amines at the surface is preserved after this incubation time.

Fig. 10. Fluorescence images of F1 and F2 samples before and after seven days of cell culture. Scale bar 1 mm.

4. Conclusions

In this study it is shown that the AVS technique is an effective, reliable and controllable method for depositing biofunctional amine layers on Ti-6Al-4V substrates. The amine layer was characterized by using fluorescence microscopy to determine the density of amines, and atomic force microscopy to measure the topography of the layer. Two sets of optimal functionalization conditions were defined that lead to the deposition of a homogeneous and smooth biofunctional layer on Ti-6Al-4V substrates.

MC3T3-E1 osteoblastic-like murine cells were used as a cell model for *in-vitro* experiments in order to assess the response of the cells to the biofunctional layer. It was shown that the amine thin film does not inhibit cell proliferation in any way and does not produce any toxic effect on the cells. Additionally, the stability of amine layer was confirmed after seven days of cell culturing.

The results confirm the formation of a stable, biofunctional and non-toxic amine layer which could create a platform that allows the subsequent covalent immobilization of biomolecules and proteins onto the surface of Ti-6Al-4V alloy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.apsusc.2016.06.139>.