Study on the use of 3-aminopropyltriethoxysilane (APTES) and 3-chloropropyltriethoxysilane (CPTES) to surface biochemical Modification of a Novel Low Elastic Modulus Ti-Nb-Hf Alloy

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ABSTRACT

The use of short bioadhesive peptides derived from the extracellular matrix has shown to efficiently enhance cell adhesion and improve biointegration in vitro and in vivo. One of the most commonly used method for binding biomolecules is by means of organosilanes. For pure titanium, there is a large diversity of literature suggesting organosilanes bearing different functional groups. Despite the wide variety of silane precursors available for surface modification, the majority of studies have employed aminosilanes, in particular 3-minopropyltriethoxysilane (APTES)**.** Recently, the 3-chloropropyltriethoxysilane (CPTES) is also proposed by other authors. Unlike APTES, CPTES does not require an activation step and offers the potential to directly bind the nucleophilic groups present on the biomolecule (e.g. amines, thiols etc.). The main objective of this work was to investigate and compare the efficiency of both organosilanes to chemically modify the surface of a new titanium alloy, Ti16Hf25Nb, with low elastic modulus. The percentage of silane covalent bonded to metal surface and its stability have been evaluated by XPS studies. Finally, in order to validate the experimental results obtained, a comparative study with mixtures of short peptides as RGD(Arg-Gly-Asp)/PHSRN(Pro-His-Ser-Arg-Asn) and RGD(Arg-Gly-Asp)/ FHRRIKA(Phe-His-Arg-Arg-Ile-Lys-Ala) have been carried out in terms of cell adhesion with rat mesenchymal cells. The effect of these mixtures of short peptides have already been studied but there are no comparative studies between them. This study provides insight to biomimetic modification of titanium alloys that can be applied for future fabrication of improved orthopedic implants.

1. INTRODUCTION

One of the most commonly used method to design biomimetic surfaces for binding biomolecules (short peptides or protein fragments) is using organosilanes capable, on one hand, to bind to the activated surface

materials and, on the other hand, to the biomolecule usually through a functional group that promotes a nucleophilic attack. Although, in the case of titanium, silanization is limited by the low surface hydroxyl group content of the titanium native oxide layer, it is frequently used. In fact, it has been demonstrated by X ray photoelectron spectroscopic (XPS) analysis that only about 15% of the surface oxygen is due to OH^- [1]. This problem can be overcome, at least in part, by adding a preliminary activation treatment. For pure titanium, there is a large diversity of examples in the literature suggesting different activation methods to provide reactive groups for covalent immobilization of biomolecules (mainly –OH groups) [2-11] and organosilanes bearing different functional groups [12-17]. Recently, a comparative study between two activation methods frequently used (Oxygen plasma and Piranha solution) was performed by V. Paredes [18]. In this work, it was demonstrated that the oxygen plasma technique was the best in terms of contamination removal and formation of hydroxyl groups. For this reason, in this study, all samples were pre-treated with this technique.

According to the literature, a large number of organosilanes has been employed. Nowadays, organosilane precursors bearing functional groups such as amino [12–22], thiol, carboxyl, phosphate, vinyl [23-25], cyanide [26], phenyl [24,27] or sulphhydryl groups are readily available. Despite the wide variety of silane precursors available for surface modification, the majority of studies have employed aminosilanes, in particular 3-aminopropyltriethoxysilane (APTES) [11–22]**.** Nevertheless, the 3-chloropropyltriethoxysilane (CPTES) is also proposed by other authors [2,4,10]. Unlike APTES, CPTES does not require an activation step and offers the potential to directly bind the nucleophilic groups present on the biomolecule (e.g. amines, thiols etc.). For example, a recently published paper proposed the co-immobilization of oligopeptides by means of a monolayer of 3-chloropropyltriethoxysilane (CPTES) as crosslinker [28].

In this paper, both organosilanes have been compared and characterized by means of a complete surface characterization using contact angle goniometry (CA), X-ray Photoelectron Spectroscopy (XPS) and Time-of-Flight Secondary Ion Mass Spectrometry (TOF SIMS). Finally, in order to validate the experimental results obtained, the strategy was to include the combination of well known mixed adhesive motifs (short peptides) including the RGD (Arg-Gly-Asp), a cell-binding domain derived, for example, from fibronectin, PHSRN (Pro-His-Ser-Arg-Asn) and FHRRIKA (Phe-His-Arg-Arg-Ile-Lys-Ala) putative heparin binding domains derived from fibronectin and bone sialoprotein, respectively. The heparin-binding domains has been reported to act as a cofactor in promoting cell adhesion and spreading [29-33], The effect of mixtures of short peptides as RGD / PHSRN [29-31] and RGD/ FHRRIKA [32-33] have already been studied by different authors in terms

of cell adhesion, differentiation and proliferation. Although, it´s not the main aim of this paper, a comparative study between them could be interesting because there is no comparative study in the literature.

In this research work, a biocompatible new titanium alloy Ti16Hf25Nb with low elastic modulus (45GPa) was selected and investigated for biochemical modification. This alloy has been widely studied and demonstrated to present a blend of attractive properties [34-37] ideal for fabricating orthopedic implants.

2. EXPERIMENTAL METHODS

2.1. Surface modifications methods

2.1.1. Silanization process

After the activation process, the disks were immersed for 1 h with 1 minute sonication every 20 minutes in a pentane solution containing 0.075 mL of diisopropylethylamine (DIEA) and 0.15 mL of the desired organosilane (CPTES or APTES). Silanization was carried out in an inert atmosphere of nitrogen and room temperature. Then, samples were washed with water, ethanol and acetone, respectively, dried and stored in a desiccator until the adhesion of biomolecules. For APTES, after silanization, the samples were placed in an inert atmosphere and were immersed in a solution of 3-(Maleimido)propionic acid N-hydroxysuccinimide ester (SMP) (2mg/mL) in N, N-dimethylformamide (DMF). They were left at room temperature with constant stirring for one hour. Then washed with DMF, water, ethanol and acetone, then dried and stored in a desiccator until the adhesion of biomolecules.

2.1.3. Peptide immobilization

The peptides used for the study were provided by GenScript USA Inc. The peptide amino acid sequences selected were: CGGRGDS, CGGPHSRN, CGGFHRRIKA and 2 mixtures (50/50) of CGGRGDS + CGGFHRRIKA and CGGRGDS + C GGPHSRN, respectively.

After the silanization process, TiNbHf alloy disks were washed in a series of ethanol, isopropanol, acetone and distilled water and dried with nitrogen. For CPTES silanized samples, disks were immersed in a water solution containing sodium carbonate (pH 11) and the desired peptide (500 µg/mol) and left reacting overnight. For APTES silanized samples, disks were immersed in a Phosphate-Buffered Saline at pH 7 for the same time. Finally, disks were washed in ethanol, isopropanol, distilled water and acetone and dried with nitrogen. To

ensure an appropriate distance between the active peptides and the implant material, a series of CGG (cysteine, gycine,glycine) will be used as spacer units.

2.2-Surface characterization

2.2.1. Contact Angle (CA)

Surface wettability was determined by the sessile-drop method using an OCA15 (*Dataphysics instrument Company, Germany*) equipment. Two different liquids (polar and non-polar) were employed; ultra pure MilliQR (*Millipore Corporation, USA*) water and diiodomethane (*Sigma- Aldrich*), a 1 µL droplet of each liquid was deposited at 1 mL/sec on the surfaces of the studied specimens. The drop image was captured by the video camera and analysed using the SCA20 software. Repetitive measurements (three drops of each liquid) were carried out on four samples of each alloy. The values of contact angle with diiodomethane were used for surface energy calculations. Surface free energy (SFE) of the samples was calculated following the Owens Wendt model (Eq.(1)) based on the contact angles determined previously [38].

$$\gamma_L(1+cos\theta) = 2\left[(\gamma_L^d\ \gamma_S^d)^{\frac{1}{2}} + (\gamma_L^p\ \gamma_S^p)^{\frac{1}{2}}\right] \qquad (1)$$

Where γ_L is the SFE of the liquid used in the measurement, θ is the contact angle, γ_S is the SFE of the test sample and the superscripts *d* and *p* denote the dispersive and polar components of SFE, respectively. The total SFE of the sample was calculated as the sum of its polar γ^p_S and dispersive γds components.

2.2.2. Interferometry (I)

Surface roughness was measured using a white light interferometer Wyko NT 9300 (Veeco) with a scanning speed of 25 µm/ s. The image anaparalysis was conducted on nine images with an area of 736 nm x 480nm using the software Vision 32. Roughness average (Ra) is the main height as calculated over the entire measured length, Rq is the root means square average between the height deviations and the mean line/surface, taken over the evaluation length/area and kurtosis (Rku) is a measure of the distribution of spikes above and below the mean line. For spiky surfaces, Rku > 3; for bumpy surfaces, Rku < 3; perfectly random surfaces have kurtosis 3.

2.2.3. X-ray Photoelectron Spectroscopy (XPS)

XPS measurements were obtained with an ESCA 5701 (Physical Electronics, PHI 10) instrument equipped with a Mg Kα X-ray source (E=1253.6 ev, 300.0 W). Survey spectra were collected with pass energy of 190 eV and high-resolution spectra were collected with pass energy of 25eV (O 1*s*, C 1*s*, N 1*s*, Si 2*p*, Cl 2*p*, Ti 2*p*,

Nb 3*d* and Hf 4*f* spectra). The binding energies were corrected by referencing the adventitious C 1s peak maximum at 284.8 eV for all the specimens used in this study. Multipak spectrum data analysis software was used to deconvolute the spectra and to calculate the elemental and component composition from the peak areas. Such analyses are believed to be accurate to ±10%. All binding energies reported have an error in the range of ± 0.1 eV.

2.2.4. Time-of-Flight Secondary Ion Mass Spectrometry (ToF SIMS)

A time-of-flight secondary ion mass spectrometer (ToF SIMS IV, Germany) was used to obtain the TOF SIMS depth profiles. Samples were bombarded with a pulsed Bismuth liquid metal ion source (Bi^{3+}), at energy of 25 keV. The gun was operated with a 20 ns pulse width, 0.3 pA pulsed ion current for a dosage lower than $5x10^{11}$ ions/cm^2, well below the threshold level of $1x10^{13}$ ions/cm^2 generally accepted for static SIMS conditions

2.3 Bioactivity of surfaces coated with oligopeptides

2.3.1 Cell culture

Mesenchymal cells of rat were used between passages 3 and 5, extracted from rat femur and tibia. Previous studies have reported that this cell type is suitable to examine the response of osteoblasts and to evaluate their morphology [39]. The cells were cultured on biofunctionalized samples for adhesion tests (6h). In order to block any surface nonspecific protein binding, samples were placed in phosphate buffer saline (PBS Invitrogen) and bovine serum albumin (BSA Sigma) 1% (PBS-BSA (1%)) for 30 min [40]. Sterilization was carried out by soaking 10 minutes with ethanol at room environment and finally samples were washed for 10 minutes with sterile PBS [41].

2.3.2. Cell adhesion

Mesenchymal cells of rat were seeded on tested surfaces at density of $6x10^3$ cells/well and cultured for 6h for staining immunofluorescence analysis. Cells were fixed on the surfaces with 4% paraformaldehyde in PBS for 20 min. After fixing, cells were lysed and permeabilized in PBS + 0.3% Triton-X-100 for 5 min, followed by blocking with 3% BSA to enhance the specific conjugation between antigen and primary antibody. Samples were immersed in antivinculin primary antibody (1:500 in 3% BSA) and incubated at 37 ◦C for 3 h. After washing with PBS + 0.1%Triton-X three times, samples were immersed in 3% BSA with Alexa Fluor 488 secondary antibody (1:500), DAPI (1:1500) and rhodamine phalloidin (1:5000), then incubated in dark at 37 ◦C for 1 h. Subsequently, the morphology and spreading of cells were observed by fluorescence microscopy. The

number of adhered cells was assessed by counting nuclei per image field using image analysis software (Image J). Three fields were selected for each sample and four samples were analyzed and aver-aged for each group.

3. EXPERIMENTAL RESULTS AND DISCUSSION

3.1. Silanization process

3.1.1. Contact Angle (CA)

Contact angle technique was employed for a qualitative physical characterization of the silanized surfaces. All silanized samples increased the contact angle from about 5° (control) up to values of above 66° thus demonstrating that a surface change was achieved (**Figure 1).** Similarly, we found that malemide derivatization also produced changes in the APTES silanized surface, as the angle decreased to 75°.

3.1.2. Time-of-Flight Secondary Ion Mass Spectrometry (ToF SIMS)

ToF SIMS spectra (not showed) of all silanized samples presented peaks in the positive region, due to Si+ (m / z ≈ 28) and Si-OH^+ (m / z ≈ 45), hence proving the presence of the silane on the surface. In addition, TiO^+ (m/z ≈ 57), TiO^{2+} (m/z ≈ 80) and Ti^+ (m/z ≈ 48), were observed in all samples, except for Ti+APTES, due to the presence of a multilayer silanised surface with a higher thickness [42-45]. The negative region showed the presence of O-Si-O^- (m/z ≈ 60), thus further confirming that all surfaces were silanized. In addition, other data demonstrated the presence of the organosilane, as for example, the presence of Cl^- (m/z ≈ 35 and 37) for CPTES and the CN^- and -CON (m/z ≈ 26 and 42) groups for APTES + maleimide [43,44,46].

3.1.3. X-ray Photoelectron Spectroscopy (XPS)

XPS results confirmed the covalent immobilization of the organosilanes on the activated surface. The atomic percentage compositions of samples treated with CPTES, APTES and untreated samples are provided in Table 1.

Si 2*p* spectra

In all silanized samples, a significant increase in silicon percentage content relative to the control sample (untreated) was observed being higher for samples treated with APTES (9 % of silicon compared to 5% for the CPTES). This increase is indicative of the presence of the organosilane on the surface metal and suggests that the silanization process performance was higher for APTES than for CPTES. It is important to underline that

for both silanized surface (APTES and CPTES) appeared a new component corresponding to siloxane bond O-Si-O at 103.3 eV [47,48], thus demonstrating that silane molecules were chemically bound to alloy surface via residual ethoxy groups on the metal substrate and not physiosorbed. The contribution at 102.3 eV can be assigned to some contamination due to polishing process by silica colloidal [57].

O 1*s* spectra

The high resolution spectrum of the core level O1*s* consist of at least three contributions from oxides species (TiO_2, HfO_2 or Nb_2O_5) at 530.2 eV, the bond (Ti-O-Si) at 531.3 eV and finally, hydrate and/or adsorbed water at 533.2 eV, respectively. It should be pointed out that the Ti-O-Si bond and the Ti-OH hydroxyl groups could be detected in the same range of energy binding. In both cases, the peak shift is so small that it is impossible to distinguish between them.

C 1*s* spectra

The high-resolution C1s XPS spectra were similar to those obtained by other authors [49-51] and they consisted of 3 components:

C1 (B.E. 284.8 eV): A carbon surface contamination is evidenced mainly due to aliphatic carbon [49], nevertheless after silanization, carbon concentration increased on both surfaces due to the presence of the aliphatic chain (C-H/C-C) of the organosilane from 6%, up to 22 % and 46 % for CPTES and APTES, respectively. Logically, APTES produced a greater increase because it had a longer aliphatic carbon chain

C2 (BE 286.6 eV): For APTES, this binding energy is assigned C-N groups confirming the covalent attachment of maleimide groups to silane (7%). However, for CPTES the C2 peak should not increase since there were no C-N or C-O groups. This increase could be related to partial hydrolysis of the silane producing C-OH groups instead of C-Cl.

C3 (BE 287.8 eV): From APTES modified to maleimide grafted surfaces, there was addition of a peak at higher binding energy of 287.8 eV which is due to the carbon atoms in imide groups [C(=O)-N-C(=O)]. This corroborated the covalent attachment of maleimide groups on the surface.

N 1*s* spectra

Finally, in the case of APTES, a significant increase of nitrogen content is observed (10% of nitrogen compared to 1% for not silanized alloy (TIOP). The N 1*s* peak decomposition of APTES functionalized

samples showed that it is mainly due to the imide group (O=C-N-C=O) of maleimide (contribution at 399.8 eV, table 1).

3.1.4 . Thermal and mechanical stability of the samples silanized

Upon thermal and mechanical stability tests, the amount of the surface adhered organosilane was assesed by means of high resolution Si 2*p* XPS curve. Samples silanized with APTES presented a greater stability than CPTES indicating that the silane-metal bond is fairly stable (Table 2). The silicon percentage is reduced from 12% to 7% for APTES, whereas for CPTES the reduction is from 5% to 2%. That is, for CPTES layer, the percentage of lost is around 60% whereas for APTES is 41.6%. This test performed in severe conditions provided information on the stability of the silane-metal bond and the general conclusion was that the APTES SAM´s seemed to perform better.

3.2. Biomolecule immobilization

3.2.1. X-ray Photoelectron Spectroscopy (XPS)

XPS results confirmed the covalent immobilization of the oligopeptides coatings on the silanized surfaces. The chemical composition of the XPS survey spectra showed a S 2*p* peak from cysteine (i.e. SGGRGD) hence confirming the presence of the oligopeptides at the surface (Table 1, *silane+peptide*). Furthermore, the deconvolution of the C 1s spectrum for biofunctionalized surfaces showed an increase in both peaks (C2 /C3) corresponding to the C-N, C-OH and C = O groups derived from peptides and, also, the appearance extra peak due to the peptide characteristic guanadine and O=C-N (binding energy at 288.9 eV) bonds (see table 2, silane+peptide) [46, 52-54].

3.3. Biological study

We assessed adhesion to the rat mesenchymal stem cells on TiNbHf surfaces coated with immobilized oligopeptides with RGD, PHSRN and FHRRIKA bioactive sequences. SEM pictures revealed the morphology of cells cultured on TNbHf alloy treated 6h after cell culture surfaces (Figure 3) whereas in Figures 2, the cell adhesion and spreading values are displayed. Experimental results demonstrated that cells cultured on Ti/APTES/peptides have a greater number of attached cells. These samples presented three times more attached cells than samples with CPTES as crosslinker. Moreover, although there is a large dispersion of data, cells cultured on Ti/APTES displayed larger areas than Ti/CPTES. Finally, although multi-component peptide

systems containing both RGD and PHSRN is capable of providing increased $\alpha_5\beta_1$-mediated adhesion and instructing the cells to adhere and spread more effectively than RGD alone [29-31], the best results are obtained for the mixtures RGD y FHRRIKA.

4.- DISCUSSION

4.1- Silanization process

For CPTES, the theoretical ratio between the atomic percentages of chlorine (organofunctional group) and silicon (silane) is one (Cl / Si = 1). Nevertheless, table 2 shows that this ratio ($Cl/Si_{(103.3\ eV)}$ has a value of 0.69 indicating that there is a loss of chloride ions during the surface modification process (efficiency of 61%). A possible explanation is that, although the experiments were performed in an anhydrous environment, some traces of water could be still be present either on the metal surface or in the chemical reagents, causing some hydrolisis. Furthermore, this explanation is corroborated by the unexpected increase of C2 peak *(BE 286.6 eV)*. As previously explained, for CPTES it should not increase since there were no C-N or C-O groups. This increase is related to partial hydrolysis of the silane producing C-OH groups instead of C-Cl.

For APTES + SMP, the theoretical ratio between the atomic percentages of nitrogen and silicon (silane) is 2 and the experimental ratio between the atomic percentage of nitrogen peaks (9.6% at 399.8 eV) versus silicon (5.6% at 103.3 eV) is 1.76 (efficiency of 85%). Probably the croslinker (SMP) grafting occurs without a systematic bonding between SMP and APTES molecules due to the over-size of crosslinker molecules. This may result in unreacted free amine groups (N1*s* peak at 399.8 ev). The fact that the ratio N/Si (APTES) was obtained away from the theoretical value shows that unexpected chemical reactions have been produced. The excess of silicon detected on APTES samples is indicating the formation of multilayer or unreacted free amine group.

In summary, the best results obtained in terms of thermal / mechanical stability (Table 2), percentage of silicon detected (9%), as well as, the experimental ratio N/Si (1.76) for APTES, seem to suggest that this organosilane produces a better quality of self-assembled monolayers (SAM). All these factors may account for a higher amount of immobilised peptide, a more homogeneous dsitribution, higher stability and consequently for an enhanced cell adhesion response. Nevertheles, these results must be confirmed by further test, as for example, its biological behaviour.

4.2 Biomolecule immobilization. Biological study

Cell adhesion assays showed that when the peptide immobilization is carried out by APTES silanised samples, an improved biological behavior in terms of number of cells attached and spreaded is observed. It seems that the better quality of the APTES SAM surface, discussed before, was corraborated by cell adhesion assays.

Nevertheless, independently of the effect of biomolecules, it is proposed that unexpected secondary chemical reactions could modify the substrate's physicochemical characteristics and the future peptide immoblization. The superficial electrostatic charge is a parameter to be considered in designing biomimetic surfaces due to that the most mammalian cells are coated with a hydrated, negatively-charged carbohydrate layer known as the glycocalyx. For example, Mark H. Lee et al [55-56] studied the adhesion strength of K100 erythroleukema cells to the functional substrates and they found that the NH_2 SAM exibited greater adhesion strengh than the epoxide, COOH or CH_3 SAMs due to its electrostatic charge.

In our study (biological media; pH 7.2-7.4), one part of CPTES SAMs is essentially arrays of Cl^- (unreacted silane) or OH groups (hydrolyzed silanes) which can interact electrostatically with the cell glycocalyx increasing the electrostatic repulsive force. By contrast the hydrolized or free silane SAMs remaining on the substrate after the biomolecule immobilization could produce a density of positively-charged NH3+ species which could contribute to improve the cell adhesion. This proposal is consistent with a study conducted by P.Sevilla [57] where it is suggested that the electrostatic interacctions might play a big role in the adsorption and retention of peptides on treated surfaces when the difference of electrical charge between peptide and surface is very high. In his study, the most electronegative sample is the CPTES silanized surface compared with APTES or APTES-malonic acid surfaces.

Finally, although the multi-component peptide systems containing both RGD and PHSRN is capable of providing increased $\alpha 5\beta 1$-mediated adhesion and instructing the cells to adhere [29-31], the best results are obtained for the mixtures RGD and FHRRIKA (Figures 4-5). In the case of RGD and FHRRIKA mixture, it is proposed that two different mechanisms of cell adhesion may occur. Cell adhesion to RGD peptide takes place via transmembrane receptors, type integrins, while adhering to FHRIKKA peptide is given through transmembrane proteoglycans. Therefore, the co-immobilización of oligopeptides as RGD and FHRIKKA could produce both types of cell adhesion mechanisms whereas in the case of RGD and PHSRN only one. This fact could explain that the best cell adhesion results have been obtained for RGD y FHRRIKA. Nevertheless this is not the aim of this work and it will be desirable further studies, as for example, cell proliferation and diferentation studies.

5. CONCLUSIONS

- Even though the 3-chloropropyltriethoxysilane (CPTES) has been proposed in the literature, APTES with (3-(Maleimido) propionic acid N-hydroxysuccinimide ester) produces a better quality of self-assembled monolayers than CPTES SAMs in terms of thermal/mechanical stability, unexpected chemical reactions (ratios N/Si or Cl/Si) and preliminary biological studies.

- It is proposed that the unexpected chemical reactions (hydrolisis or unreacted free functional gropus) that could modify the surface´s physicochemical characteristics is a parameter to be considered in designing biomimetic surfaces by organosilanes.

- Although systems containing both RGD and PHSRN is capable of providing increased $\alpha 5\beta 1$-mediated adhesion and instructing the cells to adhere, the best results in terms of cell adhesion are obtained for the mixtures RGD and FHRRIKA.

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Figure captions

Figure 1 The static contact angle measurements for silanized samples (TiOP: oxyegn plasma, TiC: CPTES, TiAM: Aptes + maleimide)

Figura 2 a) Number of living cells per unit of surface area attached to the different substrates studied and the TiNbHf as control material at 8 hours of incubation. 2b) Spreading cell values

Figure 3. SEM micrographs showing rat m esenchymal cells cultured for 8h: a) Alloy + Oxygen plasma + Aptes. b) Alloy + Oxygen plasma + CPTES. Nomenclature: Ti: TiNbHf; A: APTES; C: CPTES; M: maleimide, F: CGGFHRRIKA; R: CGGPHSRN; R: CGGRGD, RF: CGGRGD +CGGFHRRIKA (50/50) and RP: CGGRGD+ CGGPHSRN (50/50)

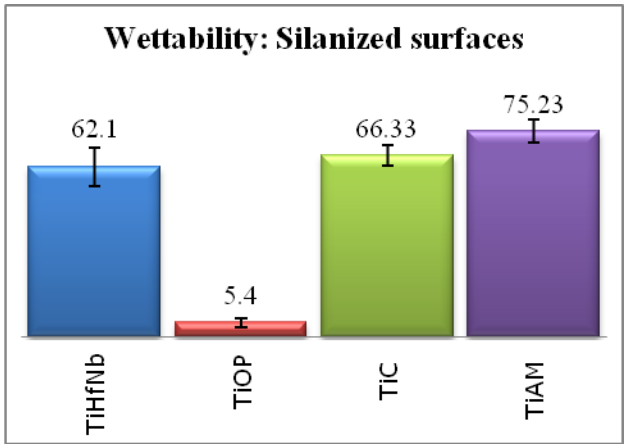
Wettability: Silanized surfaces
62.1
5.4
66.33
75.23
TiHfNb
TiOP
TiC
TiAM

Figure 1

Figure 2

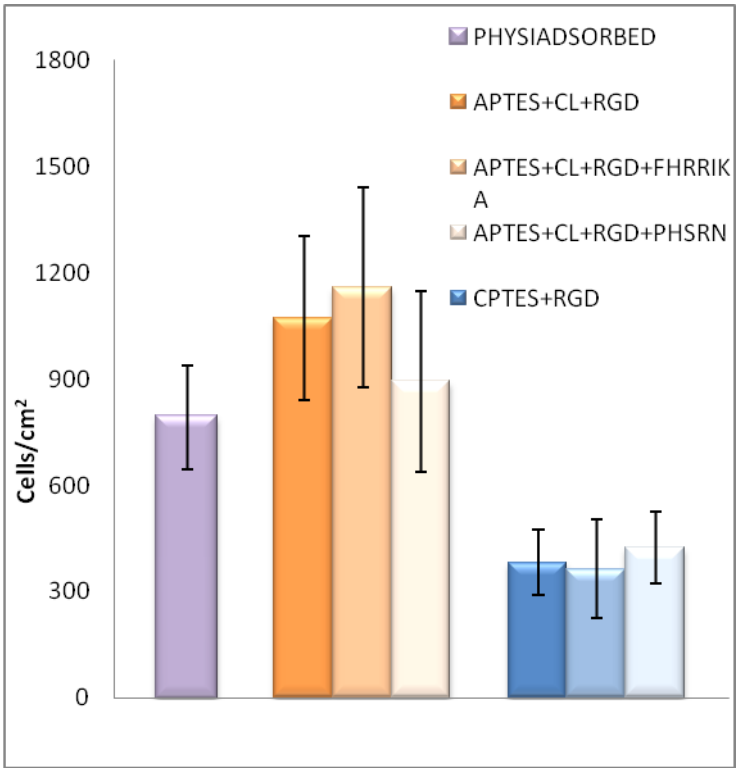

Figure 2a

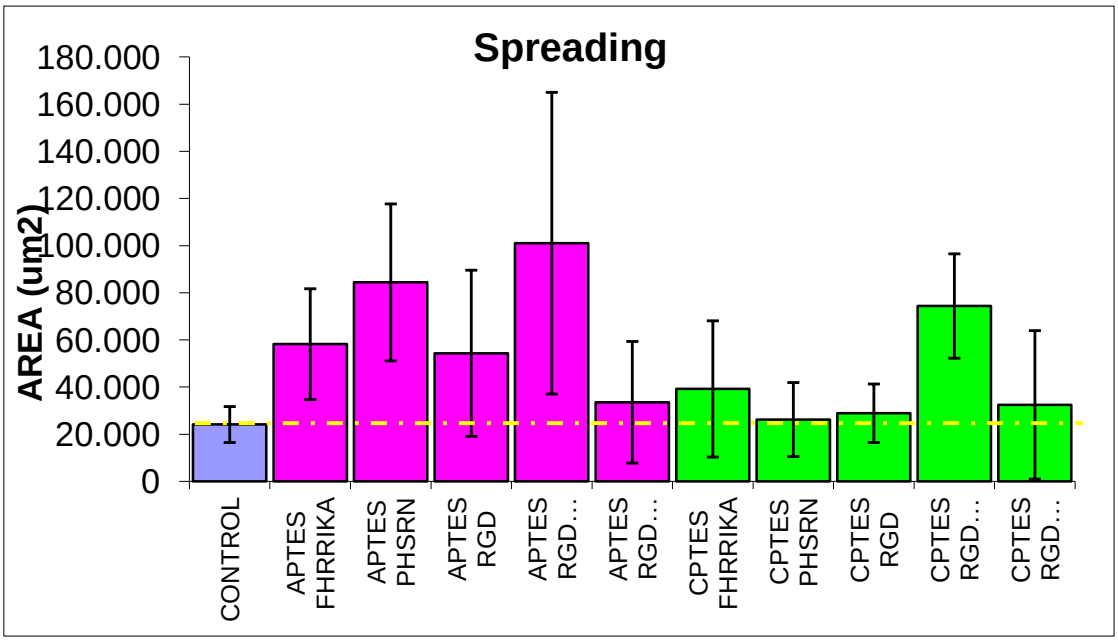

Figura 2b

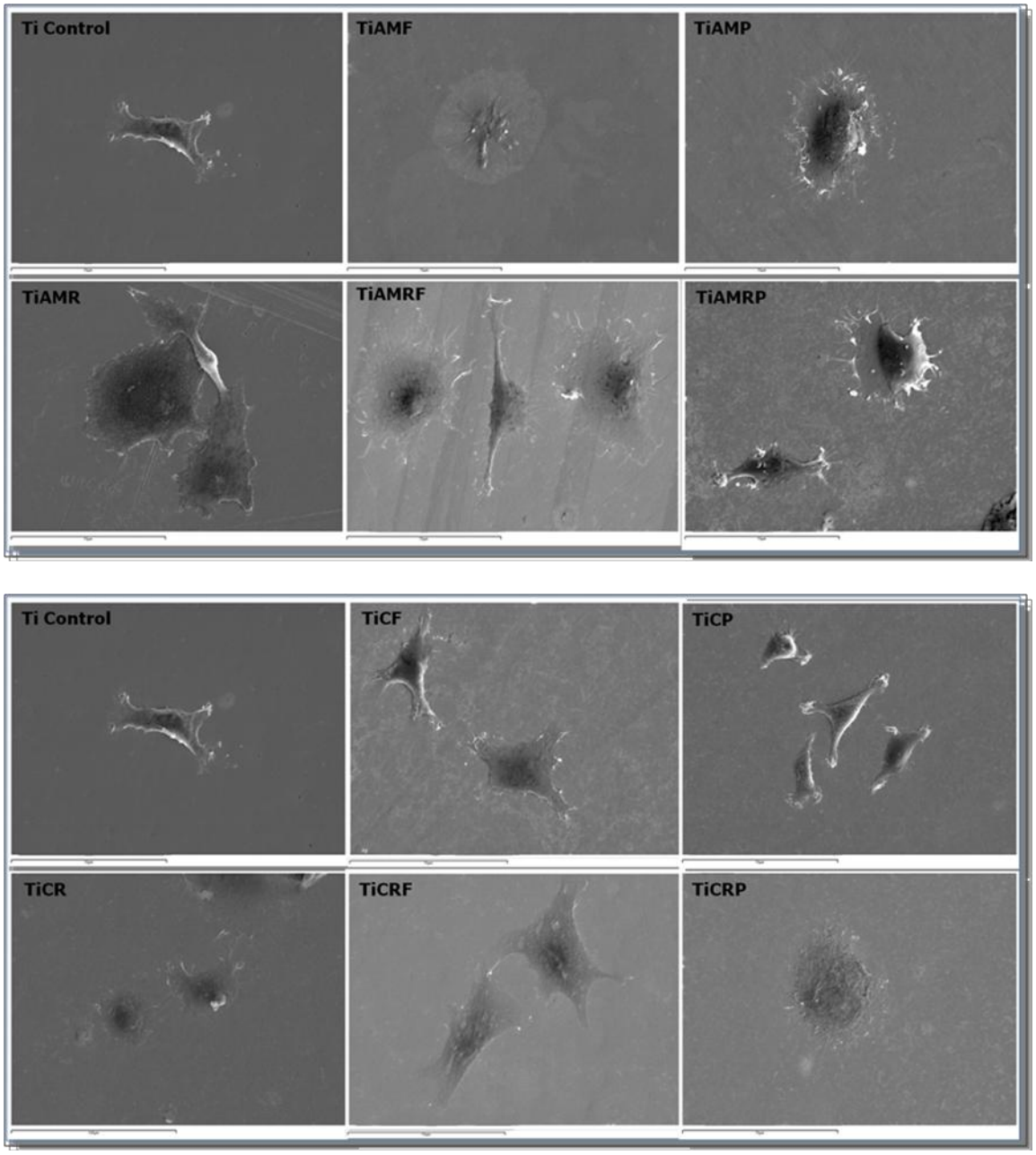
Ti Control
TiAMF
TiAMP
TiAMR
TiAMRF
TiAMRP
Ti Control
TiCF
TiCP
TiCR
TiCRF
TiCRP

Figure 3