Enhancement of plasma protein adsorption and osteogenesis of hMSCs by functionalized siloxane coatings for titanium implants

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ABSTRACT

A series of sol-gel derived silicon based coatings were developed to improve the osseointegration of commercial titanium dental implants. The osseointegration starts with a positive interaction between the implant surface and surrounding tissues, which is facilitated by the adsorption of plasma proteins onto the biomaterial surface immediately after implantation. It is likely that the enhancement of protein adsorption to titanium implants leads to a better implant/tissue integration. In addition, silica based biomaterials have been shown to promote osteoblast differentiation. To improve the protein adsorption and the osteogenesis, methyltrimethoxysilane (MTMOS), tetraethoxysilane (TEOS), 3-glycidoxypropyltrimethoxysilane (GPTMS) and gelatin were selected to coat titanium surfaces. Compared with non-coated titanium, the functionalized coatings enhanced the adsorption of adhesive proteins such as fibronectin and collagen. The Si release was successfully modulated by the control of the chemical composition of the coating, showing a higher dissolution rate with the gelatin and GPTMS incorporation. While the roughness of commercial implants seemed to promote the adhesion of mesenchymal stem cells (MSC), the osteogenic differentiation was greater on surfaces with Si-coatings. In this study, an improved osteogenic surface has been achieved by using the siloxane-gelatin coatings and such coatings can be used in dental implants to promote osseointegration.

Keywords: siloxane-gelatin coatings, silicon, protein adsorption, osteogenesis, GPTMS

INTRODUCTION

The number of dental implant procedures used worldwide has been steadily increasing over the past few decades. According to the American Dental Association, dental implants are placed in two million people each year in the USA.[1] Titanium is the most widely used metal to manufacture dental implants due to its many advantages over other materials such as superior mechanical properties and biocompatibility.[2] However, titanium is relatively inert due to the spontaneously formed oxide layer on its surface, and hence it cannot directly bind to bone. Therefore, osseointegration via this oxide layer can be slow.[3] The reported healing time range from 3-4 month in lower jaw to 5-6 months in upper jaw,[4,5] what might be a greater limitation for patients with poor bone regeneration ability. To overcome this problem, most of the recent investigations on titanium implants are focused on optimizing surface composition and topography to increase the bone-to-implant contact (BIC) ratio and thus enhance cell-implant interaction and consequently promote the osseointegration.[6-8]

New titanium surfaces with controlled characteristics are being developed to enhance and accelerate the osseointegration process by the control of the processes that occur at the implant tissue interface. Sol-gel technique is a promising route for the fabrication of versatile materials for very different applications. It provides high purity homogeneous coatings in substrates with large and complex shapes, and the strong covalent bonds between the coating and the metallic surface prevents delamination.[9-11]

Silicon alkoxide precursors have been extensively used to prepare inorganic-organic hybrid coatings for corrosion protection of metallic substrates[12,13] and for biomedical applications.[14-16] These coatings are resorbable in aqueous media, which is beneficial considering the widely recognized role of Si stimulating the activity of bone-forming cells.[17] Even though the mechanism by which Si promotes bone formation is not clear, soluble orthosilicic acid is known

to stimulate collagen type I (Col I) synthesis in human osteoblastic-like cells and promote osteoblastic differentiation.[18-20]

Li et al. showed the ability of pure silica gels obtained by the sol-gel process of tetraethoxysilane (TEOS), to induce the formation of bone-like apatite on its surface when soaked in simulated body fluid.[21] However, the use of organically modified silicon precursors has gained more interest due to the improvement of some characteristic of the resulting material, such as the reduction of the brittleness of the film and the possibility of introducing functionalized groups into the siloxane network. Juan-Díaz et al.[22] found an improvement of the corrosion protection ability for coatings synthesized combining TEOS with methyltrimethoxysilane (MTMOS). Beganskiene et al.[23] assessed cell proliferation of this type of methyl modified silica coatings, obtaining better results for the organically modified material than for the pure SiO_2. One of the most interesting organoalkoxysilane precursor is 3-glycidoxypropyl trimethoxysilane (GPTMS) due its epoxide ring, which provides corrosion protection of metals[24,25] and is useful in the synthesis of scaffolds for biomedical applications.[26-27]

In this context, a variety of sol-gel coatings were designed starting from different MTMOS, TEOS and GPTMS molar ratios. This relatively inexpensive inorganic polymerization process allows the use of low temperature during almost all stages of the reaction making possible the incorporation of different biomolecules. Hence, gelatin was also introduced into the siloxane network due to its well-known biocompatibility and capacity to facilitate cell adhesion and proliferation.[28,29]

Since the first event taken place at the tissue-implant interface after implantation is the formation of a layer with blood proteins, the first aim of this work was to assess the preference of different plasma proteins to be adsorbed on developed surfaces. There are many proteins in human blood plasma and among them the most prominent, well-known and extensively studied ones are albumin

(Alb), fibrinogen (Fbg), fibronectin (Fn) and Col I. Alb is the major protein component in blood, which acts as a multifunctional transporter of different nutrients and metabolites, and it is also known to eliminate cell attachment and block non-specific binding.[30] Fbg is a large plasma protein that plays a predominant role in mediating platelet adhesion.[31] Fn is a large non-collagenous adhesive protein presented in extracellular matrix (ECM). It plays important roles in organizing the ECM and possesses arginine-glycine-aspartic acid (RGD) domains which are of particular importance since are recognized by the respective cell-surface receptors enabling cells to attach to it.[32,33] Col I, the most abundant fibrous ECM protein, also presents this RGD sequence. It has structural functions and interacts with cells via integrin receptors and with other ECM proteins, e.g. Fn.[34] The adsorption behavior of these proteins were correlated with surface properties such as wettability, topography and functionality that are known to affect biological response.[35-38]

The second aim was to test the effectiveness of the released Si from the sol-gel coatings promoting osteogenic differentiation of hMSCs on the surface of the materials and to verify if their performance is better than the one of a commonly used commercial dental implant treatment. For this purpose, real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was used to determine the expression of various differentiation markers of the osteogenic pathway.

MATERIALS AND METHODS

Materials

The selected silicon precursors for the synthesis of the organic-inorganic hybrid coatings were trimethoxymethylsilane (MTMOS), 3-glycidoxypropyltrimethoxysilane (GPTMS) and tetraethyl orthosilicate (TEOS), all of them purchased from Sigma-Aldrich and employed without further purification. Gelatin from porcine skin, Type A was also purchased from Sigma-Aldrich.

Sample preparation

Hybrid coating, identified as MT, was made by mixing MTMOS and TEOS precursors in a molar ratio of 70:30. The coating identified as MTg was prepared in the same manner as MT but with the incorporation of 0.9 wt% gelatin. A third hybrid coating, MGTg, is a ternary hybrid of MTMOS:GPTMS:TEOS (35:35:30 molar ratio), and a 0.9 wt% of gelatin (Table 1).

In the case of the MT coatings 2-propanol (Sigma-Aldrich) was used as solvent, while for hybrids with gelatin an isopropanol:water 1:1 (v:v) mixture was used as solvent which was found to fulfill all the requirements to ensure the miscibility of the system and obtaining a homogeneous and transparent sol. The volume ratio among precursors and solvent was 1:1 in all the cases. A stoichiometric amount of hydrochloric acid (HCl, 0.1 N) was added drop-wise as the catalyst of the reaction. For the synthesis of the MTg sol, gelatin was added dissolved in the HCl, and for MGTg hybrid a pre-hydrolysis step was incorporated for the chemical anchorage of gelatin with the epoxy ring of GPTMS. After the addition of the catalyst, solutions were kept under stirring for 2 h. Once the sol was obtained different substrates were coated depending on the assay for which they were intended.

For the protein adsorption study gold-sensors (fundamental frequency of 5 MHz) purchased from Q-sense (Sweden) were spin coated with the three different types of sols and, subsequently, a heat treatment of 80 ^{o}C for 2 h was applied allowing obtaining condensed and solid films of about 100 μm thickness. Titanium sensors (QSX 310) were also purchased from Q-sense (Sweden) and used

as received. These sensors consisted of gold-coated quartz crystals covered using physical vapor deposition with a 120 nm thick Ti layer.

Grade 4 titanium discs measuring 10 mm in diameter and 1.2 mm in thickness provided by Illerimplant S.L. (Spain) were used for the silicon release test, the study of the roughness and the in vitro evaluation. They presented a heterogeneous rough surface produced by a blasting with corundum (Al2O3) particles and an acid-etching with nitric acid and sulfuric acid solution (ADS® Surface Treatment), the same treatment as the one applied to commercial titanium dental implants. Discs were dip-coated with the different sols and the same curing treatment as mentioned before was applied.

Surface characterization

The chemical composition of the surface was assessed with an X-ray photoelectron spectrometer (K-alpha XPS Instrument, Thermo Scientific). Survey spectra were recorded with a pass energy of 200 eV, a binding energy range from -10 to 1350 eV, and a scan size of 400 µm. Resulting spectra were analyzed (Advantage Data Spectrum Processing Software) and peaks areas were used to calculate the elemental composition.

The wettability was determined by the measurement of the contact angle of deionised water on the different surfaces using an automatic goniometer (DataPhysics OCA 20). Test was carried out at room temperature and a sessile drop of 10 µL of deionised water was placed on the coated surfaces. The given value is the mean of at least 11 measurements.

Surface topography was studied by atomic force microscopy (AFM). The nanoscale surface roughness of the coating was characterized by the non-contact tapping mode AFM (Nanoscope III Multimode AFM, Bruker). For the measurements etched silicon TESP-V2 tips with a force constant around 42 N/m and a resonance frequency of 320 Hz were used. Images were collected

with a frequency of 1 Hz and an angle of 0°. Five randomly distributed areas were analyzed per surface using NanoScope Analysis 1.5 Software. Roughness was reported as the mean roughness (R_a).

Qualitative changes in the topography of the titanium discs after coating them by the sol-gel process were investigated by the scanning electron microscopy (SEM; Amray 1830I; acceleration voltage of 20kV). Cell morphology was also analyzed at different periods of time of the assay. For this, cells were fixed in 4 % paraformaldehyde phosphate buffer solution (PFA, pH 7.4) at room temperature, cleaned with DPBS and dehydrated stepwise in a series of ethanol solutions of 50 %, 65 %, 75 %, 85 % and 100 % (v/v %). Finally, the samples were critical point dried (CPD 0202) and sputter-coated with gold/palladium (SCD 004, 30 milliAmps for 120 s). Images were recorded at 100X, 250X, 500X and 1000X.

Protein Adsorption

Protein solutions. Bovine serum albumin (BSA; Sigma-Aldrich), human Fbg (Merck Millipore), human Fn (Sigma-Aldrich) and Col I (Sigma-Aldrich) were used at different concentrations established to match human blood plasma ratios. BSA was used at 5 mg/mL in a phosphate-buffered saline solution (PBS; Sigma), Fbg at 500 µg/mL in PBS, Fn at 50 µg/mL in PBS and Col I at 10 µg/mL in acetate buffer (0.1 M, pH 5.6).

Adsorption from monoprotein solutions. A QCM-D system (Q-sense Model E4, Biolin Scientific, Sweden) was used to monitor protein adsorption study by measuring the frequency and dissipation values during the process. Experiments were conducted at 37 °C and with a constant flow rate of 25 µL/min. After a stable baseline was reached with PBS, each of the protein solution was individually introduced into the QCM-D module, and the flow was maintained until reaching saturation. Then, PBS was again introduced into the chamber to remove the reversibly adsorbed

proteins on the surface and to obtain a new plateau. QCM-D data were analyzed using the Q-Tools Software using the Voight model.[39,40]

Silicon release test

Si released during the degradation of the coating was monitored using inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700). Test was performed by sinking coated titanium discs into 50 mL of phosphate buffer saline (PBS, Sigma-Aldrich) and keeping them at 37 °C for 8 weeks. Aliquots of 500 µL were taken at 1, 2, 4 and 8 weeks after immersion. Each data point is an average of three individual measurements.

In vitro test with hMSCs

Cell culture. Human mesenchymal stem cells (hMSC) (passage 2-4) were purchased from (Texas A&M University) and cultured in α-Minimal Essential Medium (αMEM; Gibco, Life Technologies) supplemented with 10 % (v/v) fetal bovine serum (FBS) and gentamicin (25 µg/mL, GIBCO, Life Technologies) at 37 °C in a 5 % CO2/ 95 % air atmosphere with 100% humidity.

For the differentiation studies, cell culture medium was changed to an osteogenic medium, consisting of complete α-MEM medium supplemented with 20mM β-glycerophosphate, 50 µg/mL ascorbic acid and 100 nM dexamethasone (all chemicals were from Sigma-Aldrich).

Cell attachment and proliferation. Cells (passage 2-4) were seeded onto coated and uncoated grade 4 titanium disks at a density of 104 cells per sample in 0.50 mL of medium. Uncoated titanium discs with a commercial surface treatment were used as control. Cell attachment was assessed after 24 h of incubation and proliferation was studied up to 14 days. Discs were cleaned with DPBS (Gibco, Life Technologies) and transferred to a new 48 well plate at each time point, to avoid interferences of non-adherent cells. Subsequently 200 µL of 1X Lysis Buffer (Cell Signaling Technology) were added to each well and cells were incubated at RT on the orbital

shaker for 30 min. The total DNA amount was quantified using the fluorometric Quant-iT™ PicoGreen® dsDNA Assay Kit (Invitrogen). After adding the PicoGreen stain, samples were incubated in dark for 5 min and then the fluorescence was measured at 480 nm excitation and 520 nm emission using a microplate fluorescence reader (Tecan).

qRT-PCR analysis for osteogenic differentiation. Cells were seeded onto coated and uncoated titanium discs at a density of 104 cells per sample and incubated for 24 h, after which they were rinsed with DPBS, transferred to a new 48 well plate and incubated up to 14 days in 0.50 mL of osteogenic medium.

To assess the ability of each material to promote the differentiation, gene expression of runt related transcription factor 2 (RUNX2), alkaline phosphatase (ALP) and bone gamma-carboxyglumate protein (BGLAP) was determined using a real time quantitative reverse transcription polymerase chain reaction (qPCR) assay. The qPCR assay was performed as described elsewhere.[41] Briefly, total RNA was isolated from the samples using a SV 96 Total RNA Isolation Kit (Promega) and RNA concentration was measured with a NanoDrop spectrophotometer (Thermo Scientific). RNA was transcribed to cDNA using the Reverse Transcription reagents (Promega) and a T-Gradient Thermocycler (Biometra). Real time PCR was performed using the SYBER Green PCR kit (Roche) and LightCycler 480 (Roche). The primers used for the detection of the osteogenic differentiation were purchased from QIAGEN: RunX2 [QT00020517], ALP [QT00211582] and BGLAP [QT00232771]. GAPDH [QT00079247] was used as internal control. Relative gene expression was normalized to the expression in the experimental control.

Statistical Analysis

All the experiments were performed at least in triplicate. Results are expressed as the mean ± standard deviation (SD). One-way analysis of variance, followed by a Tukey's post hoc test was employed as appropriate to determine the statistical significance observed. Statistical significance was defined as having $p \leq 0.05$.

RESULTS

Surface characterization

Prior to the study of protein adsorption and cell differentiation, the surfaces under study were characterized. The study of the chemical composition of the Ti-crystal surface by XPS analysis revealed the presence of Ti, O, C and N; obtained values are consistent with those in literature (Table 2).[42] Oxide film, mainly the stable oxide TiO_2, is known to grow spontaneously on the surface of titanium metal in contact with air, thus resulting in a O/Ti ratio ~ 2. The O/Ti ratio in our samples was ~ 2.6. This difference and the presence of C and N can be attributed to a contamination layer formed by the rapid adsorption of molecules,[43] most likely from solvents used in the cleaning protocol or air. As shown in Table 2, synthesized sol-gel coatings are characterized by a predominant amount of Si and O due to the formation of the siloxane network. The C signal at the surface of MT and MTg coatings corresponds to the presence of the methyl group of the MTMOS precursor. The increase of the atomic percentage of C for the MGTg coating can be explained by the insertion of an epoxide group along with the addition of the GPTMS silicon precursor.

The wettability of developed surfaces was studied by means of contact angle measurements. Results are shown in Table 3. It is observed that the more hydrophilic surface is Ti, obtaining a contact angle value of 15.8°, while the less hydrophilic one is MT coating, 70.0°. The addition of

both gelatin and GPTMS precursor into the formulation decreases the contact angle, being more significant for the MTg material.

As shown in Figure 1 and summarized in Table 3, the topography of all the studied material was in the nanolength scale. On the basis of the AFM data (Ra values) the substrates were categorized by decreasing the roughness in the order Ti > MT > MTg > MGTg, where the main differences are between the Ti-crystal and the sol-gel coatings.

Protein adsorption.

The adsorption behavior of four key serum blood proteins on Ti, MT, MTg and MGTg substrates was studied. The data summarized in Table 4 shows that with Fbg, Fn and Col I, for all the studied surfaces, the changes in dissipation upon protein adsorption (ΔD) is greater than a 5 % of the corresponding changes in frequency (Δf), $\Delta D/\Delta f \geq 5$ %. In addition, there were significant differences between overtones (data not shown). This behavior indicates that the adsorbed protein forms a viscoelastic layer.[40] Therefore, Voigt model, with data from the 3rd to the 9th harmonics, was used to calculate the adsorbed mass density and the results are reported as mass per area (g/cm2). For BSA, in contrast, ΔD was close to zero, between 1.2 and 0.8 (1E-6) (Table 4), denoting the formation of a rigid layer and allowing the use of the Sauerbrey Equation.[39]

Examples of the adsorption kinetics for the four studied proteins, plotted as frequency and dissipation curves, are shown in Figure 2 for titanium surface. The behavior was similar in all the cases regardless the substrate type; there were differences in the amount of adsorbed protein mass but not in the adsorption kinetics; in all cases, there is a rapid decrease in frequency after the introduction of each protein in all surfaces. However, the time to achieve saturation is different for each protein because of its size and complex shape and structure. For instance, Col I takes more

time to saturate the surface because of its fibrous structure whereas albumin, a globular protein, saturated much faster.

Figure 3 shows the adsorbed protein mass density for each different substrate. BSA was the least adsorbed protein on all the surfaces, reaching values three folds smaller than the ones for Fbg, Fn and Col I. MGTg showed the highest Fbg adsorbed mass, where no significant differences were found between Ti, MT and MTg. A similar behavior was obtained for the study of Fn and Col I. Nevertheless, in these cases, the three developed Si-based coatings gave higher protein mass density compared with Ti control. This performance was even better in the case of MGTg due to the functionalization of the surface by the epoxide groups.

Silicon release

The polysiloxane network degrades in an aqueous media by hydrolysis releasing Si compounds. As shown in Figure 4, there was a progressive increase in Si release over time for all the materials up to 8 weeks of assay. In the same way, gelatin makes the coating more soluble, as can be seen by the detection of more Si in the media with MTg coating than with MT coating. Nevertheless, the highest Si values were obtained with coatings prepared using GPTMS precursor, MGTg hybrid.

Cell attachment and proliferation

In order to determine the osteogenic activity of the developed siloxane hybrids coatings, hMSCs were cultured and differentiated on MT, MTg and MGTg-coated discs in comparison with uncoated Ti discs with a commercial surface treatment.

Firstly, the attachment of cells was evaluated. As shown in Figure 5A, there was a significant decrease in hMSC adhesion for all the coated Ti discs when compared to the uncoated control. This observation was unexpected because an enhanced protein adsorption was detected on Si-

based coatings (Figure 3). However, the coating of MT, MTg or MGTg changed the topography of the Ti discs by smoothing the roughness of uncoated Ti discs decreasing the affinity of cells toward these coated Ti discs, where they present a more extended shape and more filopodia bound onto uncoated-Ti discs compared with the coated ones (Figure 5B and C). On the other hand, among the coated surfaces, hMSC cells adhered better to MGTg coated Ti discs compared with MT or MTg coated ones. Despite the initial lower cell attachment, cells showed higher proliferation on coated surfaces than on uncoated titanium (Figure 6).

Osteogenic differentiation

For the evaluation of the osteogenic differentiation qRT-PCR technique was used. The expression of three different genes corresponding to the osteogenic pathway was quantified. Gene expression of the transcription factor RunX2 was evaluated 7 and 14 days after the addition of the osteoinductive medium. Results plotted in Figure 7A shows an increase of the expression level of this gene over time for all the materials. The highest values for the both studied periods of time were obtained with the MGTg surface, being four folds higher in some cases.

The expression of ALP in hMSCs also increased with time on all the studied surfaces (Figure 7B). At short cell culture times (Day 7), siloxane-gelatin coatings did not have an effect on ALP expression and no statistically significant differences were found between the different surfaces. In contrast, after 14 days of culture, cells on treated Ti discs with MT, MTg and MGTg coatings, exhibited significantly higher levels of ALP gene expression than cells on uncoated Ti.

Finally, the expression of BGLAP gene was quantified. As shown in Figure 7C, lower expression values were obtained for this gene compared with RunX2 and ALP since is a marker of the last stages of the differentiation. Comparing the values obtained for the four studied surfaces, the expression was significantly higher when cells were cultured on MGTg coating.

DISCUSSION

The surface chemical composition, wettability and roughness of biomaterials determine the biological response at the biomaterial/tissue interface. Therefore, prior to the protein adsorption evaluation, XPS analysis, contact-angle measurements and AFM study were carried out on the four materials under study: Ti-sensor, and spin-coated MT, MTg and MGTg.

According to the XPS study, sol-gel coatings with homogeneous surface composition were obtained. Moreover, this study allowed confirming the effective incorporation of gelatin into the hybrid network according to the presence of a small atomic percentage of N in MTg and MGTg coatings (see Table 2).

The surface wettability is known to be influenced by the surface chemistry and the topography.[44,45] Based on obtained contact angle and roughness data (Table 3), a rougher and more wettable surface is obtained by the vapor deposition of titanium in contrast with the spin-coated sol-gel materials where surfaces are smoother and with higher contact angle values. Silica based materials are usually distinguished by a smooth nanostructured surfaces.[46] These parameters will determine the interaction of developed materials with biological fluids. It is widely accepted that surfaces with contact angle values below 90° can closely interact with biological fluids allowing the interaction with serum proteins and cells;[35,36] studied materials fulfill this requirement and can be categorized as hydrophilic materials. Moreover, sol-gel hybrids with an intermediate hydrophilicity of around 70° have been reported to promote cell growth.[47] The incorporation of gelatin to the formulation of the coatings is translated into a decrease of the contact angle value, an increase of the wettability, from 70.0° in the case of MT surface, to 58.2° for MTg, behavior consistent with those in literature.[29,48]

Protein adsorption study with QCM-D showed an adsorption profile dependant with the protein type and the surface chemistry. In terms of mass density, BSA was the least adsorbed protein on all the studied surfaces, while Fbg, Fn and Col I are uniformly higher (Figure 3). BSA's lowest adsorbed mass density, in spite of it being the most abundant protein, is due to its low molecular weight (66 kDa) and rigid packed globular shape that undergoes little reorganization once adsorbed onto the surface.[49] In contrast, Fbg is a rod-like protein with a higher molecular weight (340 kDa), that may undergo a multistage adsorption process where the protein is initially adsorbed with its long axis parallel to the surface and then undergoes a rearrangement resulting in the long-axis perpendicular to the substrate that increases the adsorbed mass.[49] Similarly, the compact conformation of Fn, with a molecular weight (440 kDa) six-fold higher than that of BSA, may be disturbed when it is adsorbed onto a surface, perhaps leading to the formation of a more hydrated increasingly viscoelastic layer. In both instances, higher $\Delta D/\Delta f$ results are obtained (Table 4). Col I, despite its concentration being the lowest one, gave the highest adsorbed mass values. This is most likely because Col I self-assembles to form collagen fibrils in overlapping rows.[50] Such an arrangement traps a significant amount of solvent, that manifests in high dissipation, with spread of overtones and a slower adsorption kinetic; Col I took much longer to reach saturation, ~ 250 min, compared with the other proteins (Figure 2). Additionally, at low concentrations, proteins like collagen Fbg and Fn are able to spread and reach a conformation that minimizes the energy after adsorption.

Among the four substrates that were studied here, the highest surface mass adsorbed for almost all the proteins was observed for MGTg coating (Figure 3). For BSA the adsorbed mass density decreased as Ti > MT ≈ MTg > MGTg. This trend follows the roughness (Table 3). This may suggest that this protein has not specificity to any given surface and it adsorbs equally onto any

substrate forming a compact monolayer where the only decisive parameter is the available surface area for the adsorption. While BSA, because of its non-specific adsorption, displays lower affinity towards coated surfaces, Fbg and Fn do not.[45] Opposite effect was observed for the adsorption profile of Fbg, Fn, and Col I, where MGTg in spite of being slightly smoother than the other surfaces, adsorbed higher amounts of protein. More interestingly, the proteins involved in the subsequent cell attachment process due to their cell binding domains, such as Fn and Col I, adsorbed preferably onto developed siloxane coatings compared to Ti control. This behavior was enhanced in the case of MGTg substrate due to the presence of epoxide groups on the surface which may mediate the binding of these proteins. These findings show that the functional groups can selectively increase the adsorption of proteins, and corroborates the previously reported results.[45,49] Moreover, studies with QCM-D have revealed that an increase in the viscoelastic properties of the adsorbed Fn layer correlates to the unfolding of the molecule once is adsorbed on the surface, increasing the exposure of the RGD cell binding sites and in consequence enhancing the bioactivity of the protein.[51,52] In this case an increase of D and $\Delta D/\Delta f$ values (Table 4), from 4.0 to 5.5 and from 6.8 to 9.4 respectively, was observed in the cases where protein was bound to silicon-based coatings compared to Ti; this indicates the formation of a more viscous layer, what may suggest an open conformation of the Fn molecule.

No correlation was found between water contact angle values and protein adsorption levels. Furthermore, there was no noticeable effect of gelatin content as can be seen from the similar mass density values obtained for the MT and MTg coatings (Figure 3). The most significant difference is found in the functionalization of MGTg coatings through the epoxide groups, suggesting the specific effect of surface chemistry. However, this increase of adhesive protein adsorption on sol-gel coatings compared with Ti was not translated into an improvement of hMSCs' adhesion. As

shown in Figure 5, more cells were attached to uncoated Ti discs. Since both the protein and cell attachment are complex processes where different variables converge: surface chemistry, wettability, topography or competitive adsorption of proteins and their conformation;[36,56] the protein adsorption alone may not be the predictor of cell adhesion. However, when studying the proliferation rate (Figure 6) cells were able to proliferate without detriment onto developed coatings, and apparently (not statistically significant differences) with a higher rate.

As previously mentioned, there is evidence that Si affects the bone density by increasing bone matrix synthesis, ALP activity, osteoblastic differentiation and mineralization.[18,53,54] Despite exact biological role of Si is still not clear, a number of possible mechanism have been suggested including the synthesis of collagen and/or its stabilization, and matrix mineralization.[19,55] For that reason, it was important to study the dissolution kinetic of developed sol-gel siloxane coatings to test their ability to release this element; where it was found that MGTg was the one that released the highest amount of Si to the media and also the one that induced earlier the osteogenesis of hMSCs.

As previously stated, one of the main goals of this work was to assess if the developed Si-coatings promote the osseointegration by inducing an earlier osteoblastic differentiation of hMSCs. To that end, the expression of three genes involved in the osteogenic differentiation of hMSC was evaluated. According to obtained values, showed in Figure 7A, there was a significant increase in RunX2 expression for cells cultured in the presence of MGTg coating, the same material that released the highest amounts of Si at shorter periods of time (see Figure 4). Thus, since RunX2 is essential for the initiation of the osteogenic differentiation and bone formation, the obtained data suggest that the MGTg surface promotes the earlier osteogenic differentiation of hMSCs compared with Ti control.

In the case of ALP gene expression there are not statistically significant differences among the three sol-gel coatings (Figure 7B). At short cell culture times (Day 7), siloxane-gelatin coatings did not have an effect on ALP expression and differences were not statistically significant between the different surfaces. In contrast, after 14 days of culture, cells on treated Ti discs with MT, MTg and MGTg coatings, exhibited significantly higher levels of ALP gene expression than cells on uncoated Ti. Not statistically significant differences were obtained between the siloxane coatings. ALP is an enzyme produced early during osteogenic differentiation and important for the mineralization step, therefore the greater expression of ALP gene on MT, MTg and MGTg surfaces suggested better osteogenic activities of these coated surfaces. Despite being widely accepted that rough surfaces lead to an increase on ALP activity,[57] the better osteogenic activities observed on the smooth coated surfaces may be attributed to the enhanced adsorption of adhesive proteins such a Co I and Fn.

BGLAP gene encodes osteocalcin protein formation, which is an osteoblast-specific protein, and is a marker of late stage of osteogenic differentiation and maturation.[58] Maybe for that reason the study done by qRT-PCR showed low expression of BGLAP gene compared with that of RunX2 and ALP (Figure 7), because a small number of cells has reached the last stage of the differentiation during the studied period of time. At Day 14, cells on MGTg surfaces expressed higher BGLAP mRNA than all the other surfaces (Figure 7C). This observation is correlated with the highest Si released from the MGTg coating.

Although the beneficial effect of Si-based materials promoting bone formation is accepted, the exact role by which Si enhances cell differentiation and the specific needed dose is still unclear. Many conflicting results in literature can be found. Hench[59] demonstrated 20-40 ppm Si level to stimulate osteogenesis, while other authors obtained similar performance with lower values

around 4-8 ppm.[3,53] Moreover, it has been reported the decrease of cells' proliferation or increase of cell apoptosis with a Si concentration higher than 200 ppm.[26,54,59] In our study, we developed siloxane based coatings to release the amount of Si not only nontoxic but promote the osteogenic differentiation of hMSCs.

CONCLUSIONS

Our study demonstrated that MT, MTg or MGTg coating enhanced the adsorption of serum proteins onto Ti surfaces. While the hybrid coatings seemed to inhibit the initial cell adhesion to coated discs, they equalized the proliferation rate of cells on uncoated Ti and in some cases improved osteogenic differentiation of hMSCs. Furthermore, we showed a positive correlation between the osteogenic activity and the Si released from the coated surfaces.

MGTg coating stood out giving the highest Fn and Col I adsorption values, faster dissolution kinetic and promoting earlier the osteogenesis with significantly higher values than Ti control according to the RunX2, ALP and BGLAP genes expression. This suggests that functionalized sol-gel Si-based coatings are good candidates to be used as surface treatment to produce titanium implants with enhanced osseointegration ability.

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FIGURE LEGENDS

Figure 1. Height images (5 x 5 μm) of (A) Ti, (B) MT, (C) MTg and (D) MGTg surfaces obtained by AFM.

Figure 2. Example of the frequency and dissipation shifts, of the fifth harmonic, over adsorption time of BSA, Fbg, Fn and Col I onto Ti sensor.

Figure 3. Adsorbed mass density of BSA, Fbg, Fn and Col I onto Ti, MT, MTg and MGTg after saturation at 37 ^{o}C. Statistically significant differences between surfaces are indicated by asterisks (*, $p \leq 0.05$).

Figure 4. Cumulative Si released from MT, MTg and MGTg coatings in PBS at 37 ^{o}C up to 8 weeks.

Figure 5. (A) Number of hMSCs attached on Ti control and MT, MTg and MGTg coatings after 24 h in culture (expressed as μg DNA/disc). Statistically significant differences are indicated by asterisks (*, $p \leq 0.05$). SEM image of hMSC cultured onto rough Ti disc (B) and Ti coated disc with MT material (C). hMSCs present a more extended shape with more filopodia bound to the substrate onto uncoated-Ti compared with MT coated Ti disc.

Figure 7. (A) Runx2, (B) ALP and (C) BGLAP genes expression of hMSCs on Ti, MT, MTg and MGTg surfaces measured by qRT-PCR after 7 and 14 days of cell culture. Values are presented as ratios of each gene relative to the housekeeping gene (GAPDH) and normalized relative to the uncoated titanium. Statistically significant differences are indicated by asterisks (*, $p \leq 0.05$).