Biofunctional polyethylene glycol coatings on titanium: an *in vitro*-based comparison of functionalization methods

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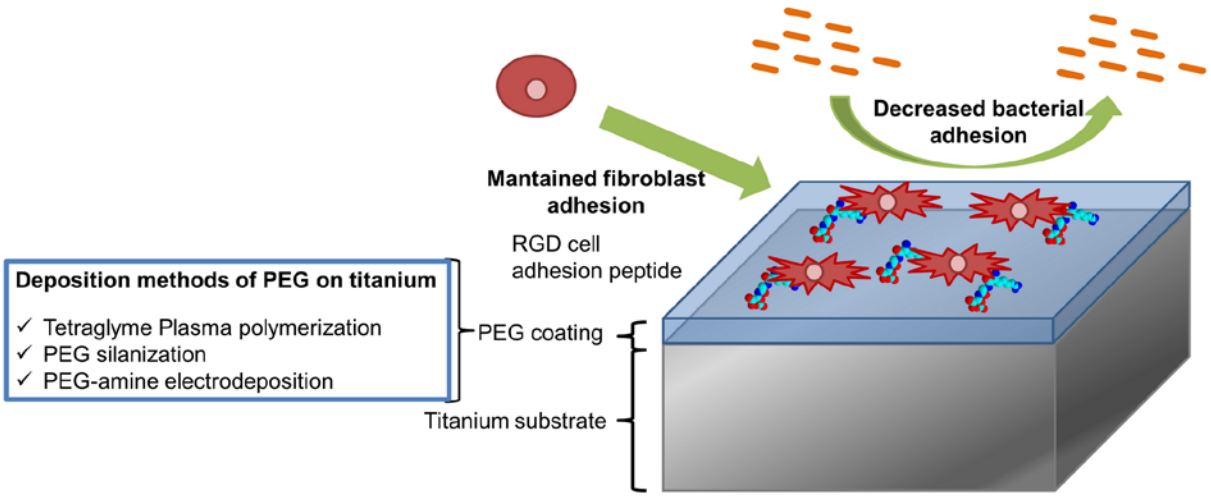
ABSTRACT. Three methods for the production of Polyethylene glycol (PEG) coatings on titanium are compared, i.e. plasma polymerization, electrodeposition and silanization. The compared deposition methods presented similar wettability (hydrophilic coatings), chemical composition assessed by XPS and thickness around 1nm. The coatings lowered albumin adsorption and presented a decreased fibroblast, *Streptococcus sanguinis* and *Lactobacillus salivarius* adhesion. Immobilization of a cell adhesion peptide (RGD) presented a higher fibroblast adhesion and no alteration of the bacterial adhesion, giving three methods for the biofunctionalization of titanium for dental implants. The feasibility of each methodology is compared in terms of the process parameters in order to provide a guide for the election of the methodology.

Graphical abstract



INTRODUCTION

Polyethylene glycol (PEG) is a well-known polymer employed to develop antifouling surfaces. When immobilized on a substrate it renders hydrophilic character to the surface, which has been related to the reduction of protein adsorption[1–3], mammalian cell adhesion[4,5] and bacterial adhesion[6–8]. These coatings also reduce platelet adhesion, leading to a lower risk of thrombus formation, tissue damage and other cytotoxic effects[9]. Thus, PEG coatings have been used to obtain anti-infective medical implants[10–13], biosensors with improved signal-to-noise ratios[14,15] and low-fouling membranes[16–18], among other applications.

Titanium is widely used for the production of implantable devices, such as dental and orthopedic implants[19–21]. The use of titanium in contact with body fluids and tissues may lead to unspecific protein adsorption, cell adhesion and bacterial adhesion, which can induce adverse pathogenic problems in clinical practice, such as thrombosis, lack of osseointegration and biomaterial related infections[22,23].

Infections related to titanium implants are difficult to treat since bacterial adhesion often leads to the formation of a biofilm, which is a multi-species community embedded in a polysaccharide extracellular matrix produced by the bacteria. The biofilm protects the bacteria community against the immune response and provides them with resistance to antibiotic treatments[24–26]. Bacterial ecology found in a biofilm is complex and formed by several species. For example, in the case of dental implants, a number of early colonizers including *Streptococcus sanguinis*[27–29] initially adhere to the implant surface, and subsequently guide the adhesion of later colonizers, such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Aggreggabactiter*

actinomycetemonitans[30,31]. Other species have a role on maintaining the stability of the biofilm through interactions with other bacterial strains, like *Lactobacillus salivarius*[32,33].

Different methods have been studied in order to produce PEG coatings. Their preparation can be done via physical adsorption[34], self-assembled monolayers[35], or covalent bonding[14]. Several studies dealt with the influence of the PEG properties on the antifouling abilities of the coating[35–37]. However, there is no data on the differences between the deposition methods in terms of their antifouling properties.

Among the existing methods, a promising strategy is the use of plasma polymerization. Plasma treatments are a suitable technique for the production of polymeric layers on different substrates, such as polymers[38–40] or stainless steel[41]. In this regard, we have previously reported the production of nanometric PEG-like layers on titanium by plasma polymerization of tetra(ethylene glycol) dimethyl ether[42]. Plasma-based methods are solvent-free, allow the treatment of several samples at the same time and are suitable for the treatment of complex geometries. The properties of plasma coatings can be controlled by modifying the parameters of the plasma treatment, such as the pressure, power of the plasma and the type of precursor used, among others[39].

Alternatively, electrochemical treatments are able to produce a great variety of coatings, such as hydroxyapatite[43], phospholipid-based polymers[44] and silver-doped surfaces[45]. Electrodeposition of polymers can be performed via electropolymerization[46,47] or by electrostatic attraction of the polymer to the sample, which acts as one of the electrodes of the system[48]. Production of PEG-coatings by means of electrodeposition was developed by Tanaka et al.[49]. In their work, an aqueous solution of PEG-amine terminated containing sodium chloride was used as the

electrolyte for the electrodeposition. This coating showed a reduced biofilm formation of *Streptococcus gordonii* and *Streptococcus mutans* on titanium surfaces[50]. The use of PEG electrodeposition implies the use of water as a solvent and represents a simple and fast procedure.

Finally, PEG silanization is a technique that has been widely used for the preparation of PEG-coatings on silicon wafers. It was initially developed by Zhang et al.[51], by using PEG and silicon tetrachloride ($SiCl_4$), which in turn reacted with the silanols of the silicon surface. This method was also used by Kocijan et al.[52] to coat stainless steel. A significant reduction of protein and bacterial adhesion was observed for the immobilization of the silanized PEG on both silicon and stainless steel. Methods for the silanization of silica surfaces can also be applied to titanium, due to the presence of a titanium oxide layer[53]. In the present work, this approach is applied to the titanium surface in order to achieve a similar coating.

Integrin-binding peptide sequences can be immobilized on the titanium surface to improve the bioactivity of the implant[54–56] The best well-known cell adhesive motif is the arginine-glycine-aspartic acid (RGD), a sequence found in proteins of the extracellular matrix such as fibronectin, which is able to bind cells via specific interactions with integrins. The RGD sequence is thus capable of promoting the adhesion of a large number of cells[57–59]. In this regard, the use of RGD peptides in combination with PEG-based coating has been investigated to enhance tissue integration in the presence of the antifouling polymer [60–62]; yet a comparison of the effect of the cell adhesive peptide depending on the PEG coating is missing.

The main aim of this work is to compare the *in vitro* biological performance of three different methods for coating titanium with PEG layers, namely, plasma polymerization, electrodeposition

and silanization. Plasma polymerization renders different properties on the polymer, since the polymerization process is different from the traditional one (i.e. radical polymerization). The comparison of the *in vitro* response of the three techniques is of special interest taking into account the differences between a plasma process and the wet chemical processes. Surface characterization was performed by means of water contact angle and XPS. In a second stage, a cell adhesion peptide was added to the surfaces in order to improve the response of the mammalian cells to the PEG coatings while maintaining the antifouling effect for bacteria. Following chemical characterization of the layers, the response of mammalian cells and bacteria was tested in order to check the biocompatibility of the coatings.

MATERIALS AND METHODS

Materials

Commercially pure grade 2 titanium was purchased as 2m rods of 10mm in diameter, which were cut into disks of 2mm disks (VDM metals, Germany). Silicon carbide grinding paper of different particle size (P400, P600, P800, P1200 and P2500) and colloidal silica suspension (Eposil M11, particle size 0.06µm) were purchased from Neurtek (Spain). Isopropanol (>99.7%), ethanol (96%) and acetone (99%) were obtained from Panreac (Germany) and used without further purification. Toluene (99.9%), tetraethylene glycol dimethyl ether (tetraglyme, 99%), PEG (M_w=1000g/mol), bis(3-aminopropyl)-poly(ethylene glycol) (PEG-amine), trimethylamine, silicon tetrachloride were purchased from Sigma Aldrich (USA) and used without further purification. Phosphate buffer saline (PBS, pH=7.4, Gibco, US) is purchased as 5g tablets which are dissolved in 500mL of distilled water and autoclaved at 121ºC for 1h before using.

Titanium samples preparation

Titanium disks were grinded with silicon carbide paper and polished with colloidal silica. Afterwards, samples were solvent cleaned by sonication in a series of solvents: toluene, isopropanol, water, ethanol and acetone. Samples polished and cleaned as described were used as control (Ti). Before coating with PEG with any of the three methods evaluated, samples were plasma activated in low pressure plasma. Details of the plasma treatment can be found elsewhere[42]. Briefly, a 5 min argon plasma treatment at 100W and at 0.40mbar was performed with a radio frequency (13.56MHz) low pressure plasma system (Femto Plasma System, Diener, Germany).

Coating procedures

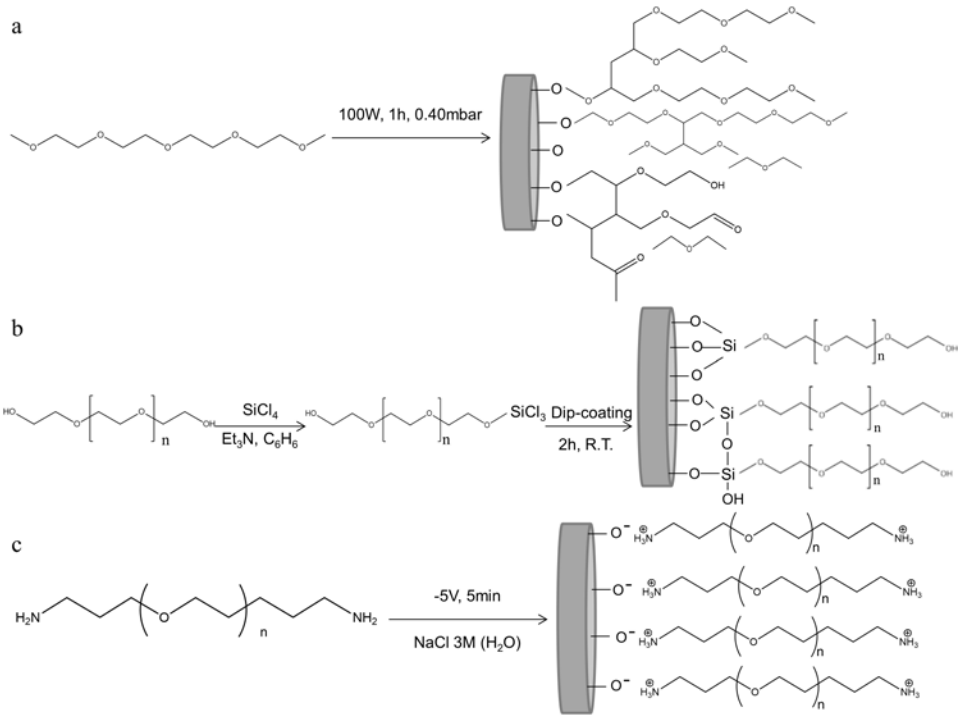
Plasma polymerization was performed right after plasma activation in the same reactor without breaking the vacuum. The polymerization precursor was tetra(ethylene glycol) dimethyl ether introduced by bubbling argon in the reactor, and the parameters used were 100W, 0.40mbar, and 1h, according to a previous work[42]. The process was performed in pulsed mode with a t_{on}=20µs and t_{off}=20ms (Scheme 1a).

For the preparation of silanized PEG, a solution of 0.98% of PEG (M_w=1000g/mol) in anhydrous toluene (99.8%) was mixed with 136µL of trimethylamine during 1h in an inert atmosphere. After this time, 20µL of silicon tetrachloride was added and left to react for 5min. This solution was filtered and the plasma activated samples were immersed in this solution for 2h (Scheme 1b).

Electrodeposition was done in an electrolytic cell with the plasma activated sample as the cathode, a saturated calomel reference electrode and a platinum contraelectrode, connected to a

potentiostat (Princeton Applied Research, Parstat 2273). The procedure was an adaptation of Tanaka et al[49]. The electrolyte was an aqueous solution containing 2% in weight of bis(3-aminopropyl)-poly(ethylene glycol) (PEG-amine) and 0.3M of NaCl. A potential of 5V was applied to the system during 5min (Scheme 1c).

Scheme 1. Scheme reaction for the production of plasma polymerized tetraglyme (a), PEG silanization (b) and electrodeposition of PEG-amine (c)



Peptide immobilization

The RGD peptide was prepared by solid-phase peptide synthesis and characterized as explained in previous studies[54]. For the physisorption of RGD, Ti and PEG-coated samples were immersed overnight at room temperature in a solution of 100µM of the peptide in Phosphate Buffer Saline. After the immersion, samples were cleaned twice with PBS.

A summary of the sample codes used in this study is presented in table 1.

Table 1. Sample codes used in this study. Polishing, solvent cleaning and plasma activation was done for all samples

Sample treatment	**Sample code**
Control	Ti
Plasma activated	Ti-PA
Plasma polymerized PEG	Ti-PEG-PP
Silanized PEG	Ti-PEG-S
Electrodeposited PEG-amine	Ti-PEG-E
Control+RGD	Ti-RGD
Plasma polymerized PEG+RGD	Ti-PEG-PP-RGD
Silanized PEG+RGD	Ti-PEG-S-RGD
Electrodeposited PEG-amine+RGD	Ti-PEG-E-RGD

Surface characterization

Static water contact angle was measured by the sessile method with ultrapure water (MilliQ, Millipore Corporation), with OCA15 goniometer (Dataphysics instrument Company, Germany). A 2µL droplet was placed at 1µL/s. The shape of the drop was analyzed using the software SCA20 (Dataphysics instrument Company, Germany). Three areas of each sample were measured in triplicate for each condition.

Chemical analysis was performed by means of X-ray photoelectron spectroscopy (XPS). Spectra were acquired in ultra-high vacuum ($5.0 \cdot 10^{-9}$ mbar) with an XR50 Mg anode source operating at 150 W and a Phoibos 150 MCD-9 detector (D8 advance, SPECS Surface Nano Analysis GmbH, Germany). Spectra were recorded at pass energy of 25 eV with a step size of 1.0 eV for survey spectra and 0.1 eV for high resolution spectra of C1s, O1s, Si2p, Cl2p, N1s, and Ti2p. C1s peak was used as a reference. CasaXPS software (Casa Software Ldt, UK) was used for the

determination of atomic elemental composition applying the manufacturer set of relative sensitivity factors. Two samples per condition were analyzed.

Film thickness was estimated by the attenuation of the XPS titanium signal according to the equation 1, where I_{Ti} is the intensity of Ti2p from the clean surface (Ti-PA), I_{Ti}^{0} is the intensity of the Ti2p from PEG coated substrate, L_{Ti} is the electron attenuation length for Ti peaks and θ is the take-off angle for XPS measurements.

$$I_{Ti} = I_{Ti}^{0}\exp\left(-\frac{t}{L_{Ti}sin\theta}\right) \qquad \textbf{Equation 1}$$

Electron attenuation length was assumed as 2.8Å according to Ruiz-Taylor et al.[63] and the take-off angle was 90º for all the measurements.

Biological evaluation

Protein adsorption on the titanium surfaces was tested by immersing the samples in bovine serum albumin (BSA, Sigma Aldrich). BSA was stained with fluorescein isothiocyanate (FITC, Thermo Scientific, USA) with the Kit Pierce Antibody Labeling Kit (Thermo Scientific). The staining was performed by dissolving BSA in a phosphate-borate buffer, mixed with a FITC solution and purified in a resin to remove the non-reacted FITC. Samples were then immersed in 150 µL of FITC-BSA at a concentration of 100µg/mL during 1h in the darkness. Next, the protein was fixed with paraformaldehyde (Sigma Aldrich) at 2.5% v/v in PBS. After each step samples were washed with PBS. Coverslips were mounted on the samples in Mowiol (Merck Millipore Corporation, Bedford, MA, USA) mounting medium. Samples were photographed with a Nikon E-600 fluorescence microscope, and an Olympus DP72 camera (Nikon Corporation Instruments

Company, USA). To assess protein adsorption, four images were taken for each sample and the pixel intensity was calculated by the software Image-J (NIH, MD, USA).

Potential cytotoxic effects of the coatings were evaluated according to ISO 10993-5 standard on human foreskin fibroblasts (hFFs, Merck Millipore Corporation) using three samples for each condition. All specimens were sterilized by immersion in ethanol 70% during 30min. Extracts of the samples at concentrations of 1:1, 1:10, 1:100 and 1:1000 were prepared by immersing the samples in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, Carlsbad, CA, USA) for 72h at 37ºC. 5000 cells/well were seeded on a 96-well tissue culture polystyrene (TCPS) plate and incubated with media for 24 h. Afterwards, culture media was replaced by the extract dilutions. After 24 h, cells were lysed with mammalian protein extraction reagent (mPER, Thermo Scientific, USA) and quantified by the activity of lactate dehydrogenase enzyme (LDH) with the LDH Cytotoxicity detection kit (Roche, USA).

Cell adhesion tests were performed with hFFs. Samples were sterilized with ethanol and cleaned twice with PBS. $2 \cdot 10^4$ cells per samples were seeded on the samples and incubated for 6h at 37ºC in DMEM. After the incubation time, samples were washed twice with PBS and the cells remaining attached to the sample were lysed with mPER. The number of adherent cells was assessed by quantification of the activity of LDH. For observation of the cells with scanning electron microscopy (SEM, Focused Ion Beam Zeiss Neon40), samples with the adhered cells were fixed in a 2.5% paraformaldehyde solution in PBS during 1h at 4ºC. Dehydration of the samples was done by immersion in a sequence of ethanol solutions at 50, 70, 90, 96 and 100% (v/v) for 15min each solution. After dehydration, samples were carbon coated for the SEM

observation. SEM images were taken at 5.0kV. Triplicates for each condition were used for the quantification, and one extra sample was prepared for SEM observation.

Bacterial adhesion tests were performed with two bacterial strains: *Streptococcus sanguinis* (*S. sanguinis*, CCUG 17826, Culture Collection University of Göteborg (CUG), Göteborg, Sweden) and *Lactobacillus salivarius* (*L. salivarius*, CECT 4063, Colección Española de Cultivos Tipo (CECT), Valencia, Spain). Todd-Hewitt broth (TH) (Sharlab SL, Spain) was used as culture media for *S. sanguinis* and Man, Rogosa and Sharpe broth (MRS) (Sharlab SL) as culture medium for *L. salivarius*. Cultures were grown overnight prior every assay, and the optical density at 600nm was adjusted at 0.2 ± 0.01, giving approximately $1 \cdot 10^8$ colony forming units per mL (CFU/mL). Samples were immersed in 1mL of the bacterial suspension during 2h at 37ºC. In order to detach bacteria from the samples, 5min of vortexing were applied in 1mL of PBS. Serial dilutions of the PBS were seeded on medium-agar plates and incubated for 24-48h at 37ºC to count the CFU adhered to the samples.

Statistical analysis

Results are presented as the average ± standard deviation (SD), for at least three independent samples. Statistical analysis was performed with Minitab 17™ software (Minitab Inc, State College, PA, USA). Data were analyzed by Student's t-test and one-way ANOVA tables with Tukey's multiple comparison tests in order to evaluate outliers and statistically significant differences between sample groups, respectively. The differences were considered as statistically significant when $p<0.05$.

RESULTS AND DISCUSSION

The application of PEG coatings on certain medical devices is intended to decrease bacterial adhesion in order to reduce the rate of infections related to the implantation. In the case of dental and orthopedic applications, cell adhesion should be maintained in order to achieve a proper integration and stability of the implant. In the present study, three different methods for the deposition of PEG on the titanium surface have been characterized and compared in terms of their biological and microbiological *in vitro* performance.

The first step for the coating of the titanium samples was an activation of the surface. Plasma activation is a well-known procedure for the cleaning and activation of metals, polymers and ceramics[64]. In this study, the titanium surface was activated in order to remove organic contamination of the surface and increase the functionality of the titanium oxide by grafting reactive hydroxyl groups on the titanium surface. According to the water contact angle (Figure 1), this treatment renders high hydrophilicity. The enhanced wettability is due to the combined effect of the removal of contaminants and the formation of hydroxyl species[65,66], as confirmed by the reduced amount of carbon found by XPS (table 2).

Control samples showed the most hydrophobic behavior. PEG coatings render a hydrophilic surface, as can be observed by comparing the water contact angle of the PEG-coated surfaces with that of the control. All PEG-coated samples are significantly more hydrophilic, with the plasma process (Ti-PEG-PP) leading to the highest wettability ($\theta \approx 10^{o}$). Silanized PEG (Ti-PEG-S) and electrodeposited PEG (Ti-PEG-E) presented approximately the same hydrophilicity (around 50^{o}). Lower values for the contact angle of the plasma polymerized sample can be explained by the formation of oxygen functionalities in the polymer due to the plasma process [67],

and the different structure of the PEG-like layer obtained, possibly more cross-linked and with shorter chains than the PEG obtained by the other processes, as shown in Scheme 1. The water contact angles for PEG-coatings have been reported to be in the range of 15-60º depending on the substrate, the deposition method and the chemical properties of the immobilized molecule [1,68,69], thus, the wettability measured for the PEG-coated titanium is indicative of the presence of the polymer.

The adsorption of the RGD peptide on the surfaces resulted in a decrease in the water contact angle for all samples with the exception of Ti-PEG-PP. Nonetheless, Ti-PEG-PP-RGD presented a lower contact angle compared with the rest of samples containing RGD.

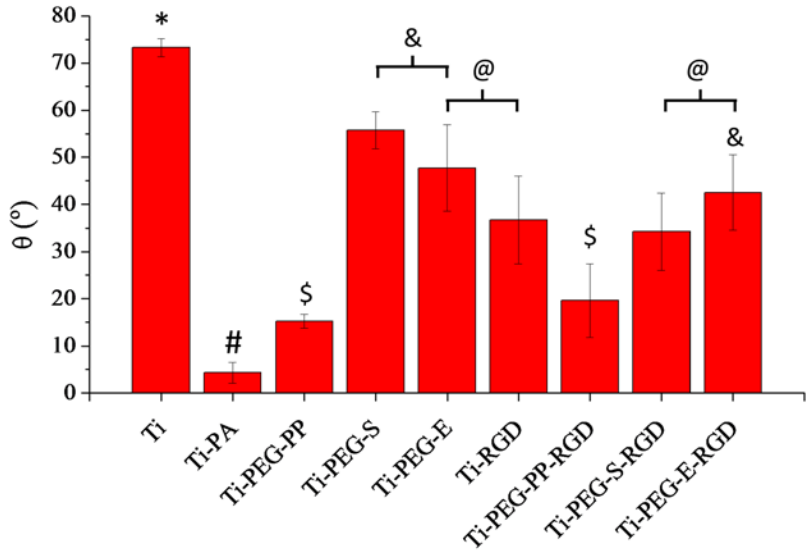


Figure 1. Water contact angle of the PEG-coated samples by the three different methods and the PEG-RGD samples. Samples with the same symbol have no statistically significant differences ($p<0.05$).

The presence of a certain percentage of carbon as measured by XPS (table 2) indicates the presence of an organic contamination layer on the titanium surface[70,71]. The amount of carbon on the surface decreases when applying the activation plasma treatment (Ti-PA) due to the removal

of organic contaminants. In contrast, an increase in the C1s signal can be observed in all PEG-coated samples as compared to Ti-PA, indicating the presence of the polymer layer. After the RGD immobilization, this percentage further increases as a result of the deposition of the peptide. Changes in the Ti2p signal followed an opposite trend: the presence of the PEG and RGD coatings reduced the amount of detectable Ti by XPS. The electrodeposition of amino-PEG on the surfaces (Ti-PEG-E) was also accompanied by a significant increase in the N1s signal. Alternatively, the incorporation of PEG by silanization (Ti-PEG-S) resulted in significant percentages of Si2p and Cl2p present in silicon tetrachloride. Adsorption of RGD on the titanium surface rendered an increase on the %C 1s and %N 1s (Table 1), showing the presence of the peptide.

Table 2. % atomic composition of the Titanium with the different PEGylation treatments and RGD adsorption.

	C1s	**O1s**	**N1s**	**Si2p**	**Cl2p**	**Ti2p**
Ti	23.5 ± 0.8	59.6 ± 0.8	0.6 ± 0.1	0.2 ± 0.0	0.1 ± 0.1	16.0 ± 0.1
Ti-PA	15.0 ± 0.7	62.1 ± 0.7	0.3 ± 0.1	0.3 ± 0.0	0.0 ± 0.1	22.3 ± 0.1
Ti-PEG-PP	21.8 ± 1.0	64.0 ± 0.6	0.2 ± 0.1	0.3 ± 0.0	0.1 ± 0.1	12.0 ± 0.6
Ti-PEG-S	17.7 ± 0.5	57.7 ± 0.8	0.3 ± 0.0	10.9 ± 1.2	1.8 ± 0.0	13.4 ± 0.9
Ti-PEG-E	27.1 ± 1.2	55.0 ± 0.7	1.3 ± 0.2	2.7 ± 1.1	0.4 ± 0.3	13.5 ± 0.1
Ti-PEG-PP-RGD	37.5 ± 2.1	51.5 ± 1.4	0.6 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	10.1 ± 0.7
Ti-PEG-S-RGD	25.8 ± 1.8	58.9 ± 1.0	0.4 ± 0.1	5.2 ± 0.6	1.7 ± 0.3	8.0 ± 0.5
Ti-PEG-E-RGD	30.1 ± 1.6	54.4 ± 1.0	1.0 ± 0.0	2.4 ± 0.5	2.0 ± 0.2	10.2 ± 0.8

The decomposition of the C1s peak of the PEG-coated samples shows a peak at 286.5eV, attributed to the presence of C-O bonds (ether) characteristic of PEG (Table 3) confirming the formation of a PEG coating in all the PEG-coated samples. The other peaks found at 285.0eV and 287.0eV can be attributed to the presence of C-C (hydrocarbons) and C=O bonds (carbonyl group), respectively. Ti-PEG-PP presented an additional peak at 288eV corresponding with the presence of O-C=O (carboxyl groups), probably due to the dissociation and formation of new species in the plasma reactor or etching of the deposited polymer by argon employed as carrier gas and further reaction with air after treatment [72,73], as indicated in Scheme 1. Presence of Ti-PEG-E was also supported by the detection of a higher amount of nitrogen on the treated surfaces, due to the presence of the amino groups at the end terminals of the amino terminated PEG. For Ti-PEG-S, higher silicon and chlorine amount are caused by the use of silicon tetrachloride employed for the silanization of PEG (Scheme 1).

Regarding the oxygen signal (Table 3), two different peaks were found. The peak at 532.0eV corresponds to a combination of the titanium oxide surface and to the presence of hydroxyl groups, while the peak at 533.4eV can be attributed to ether bonds from the PEG. For the Ti-PEG-S, this peak can be also attributed to the presence of silanol and siloxane groups.

Table 3. Contribution of the peaks on the XPS spectra of C1s and O1s

	C1s				O1s	
Binding Energy (eV)	285.0	286.5	288.0	288.8	532.0	533.4
Ti-PEG-PP	15.9 ± 0.5	54.4 ± 1.2	17.6 ± 0.7	11.9 ±1.1	49.6 ± 0.9	50.4 ± 0.8
Ti-PEG-S	44.5 ± 0.9	46.9 ± 0.7	8.5 ± 0.8	-	45.1 ± 0.7	54.9 ± 0.4
Ti-PEG-E	38.8 ± 1.1	39.9 ± 0.9	22.1 ± 0.5	-	40.4 ± 0.8	59.6 ± 0.5

The coating was assumed to be homogeneous according to SEM images (not shown). Thickness of the coatings estimated by XPS revealed the presence of an ultra-thin coating, with values of 1.4nm for Ti-PEG-PP and Ti-PEG-E, and a value of 1.8nm for Ti-PEG-S. Thus, very thin layers are obtained by any of the techniques. These results are in agreement with the thickness obtained by Papra et al [74], and Tanaka et al [49]. Both authors gave thicknesses values between 1-2nm for either silanized PEG and electrodeposited PEG respectively.

Notwithstanding the low thickness of the layers, albumin adsorption on the PEG-coated samples is lower compared with the bare titanium, showing the antifouling character of the coatings (Figure 2). No differences on the fluorescence intensity of the FITC-BSA were observed between the samples. Measurement of the fluorescence intensity of FITC labeled proteins is an established method for the determination of protein adsorption on different substrates, as the fluorescence intensity can be considered proportional to the presence of protein[75,76]. Therefore, the three methods are adequate to confer similar antifouling properties to the titanium surface.

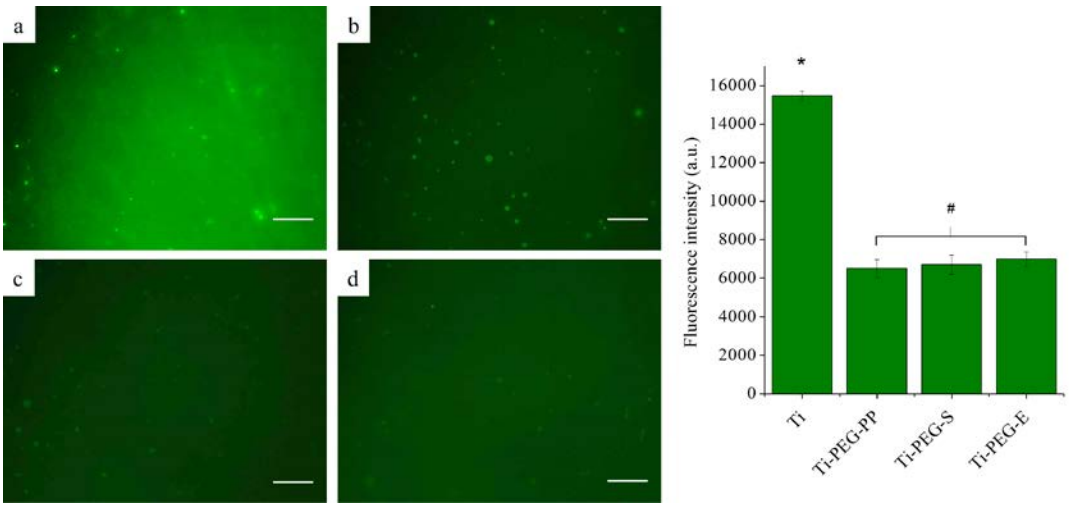


Figure 2. Fluorescence microscopy images (left) of the samples with adsorbed BSA-FITC a) Ti, b) Ti-PEG-PP, c) Ti-PEG-S, d) Ti-PEG-E. Scale bar represents 50µm. Quantification by image analysis (right). Samples with the same symbol have no statistically significant differences.

Indirect cytotoxicity assays revealed no toxic effect for the eluents of both PEG-coated and RGD-coated samples, as the survival of cells in contact with these eluents was above 80% for all the dilutions tested (data not shown).

Cell adhesion of human fibroblasts was impaired by the presence of the PEG-coating in the case of Ti-PEG-PP and Ti-PEG-S. However, while cell adhesion of Ti-PEG-E tends to decrease, the difference is not statistically different from the control (Figure 3). Fibroblast adhesion is enhanced with the presence of the RGD with no statistically differences found within the methods of deposition, enhancing the biocompatibility of the coatings. Cell morphology

observed by SEM was not changed in presence of both PEG and PEG with RGD, with the cells spread on the surface.

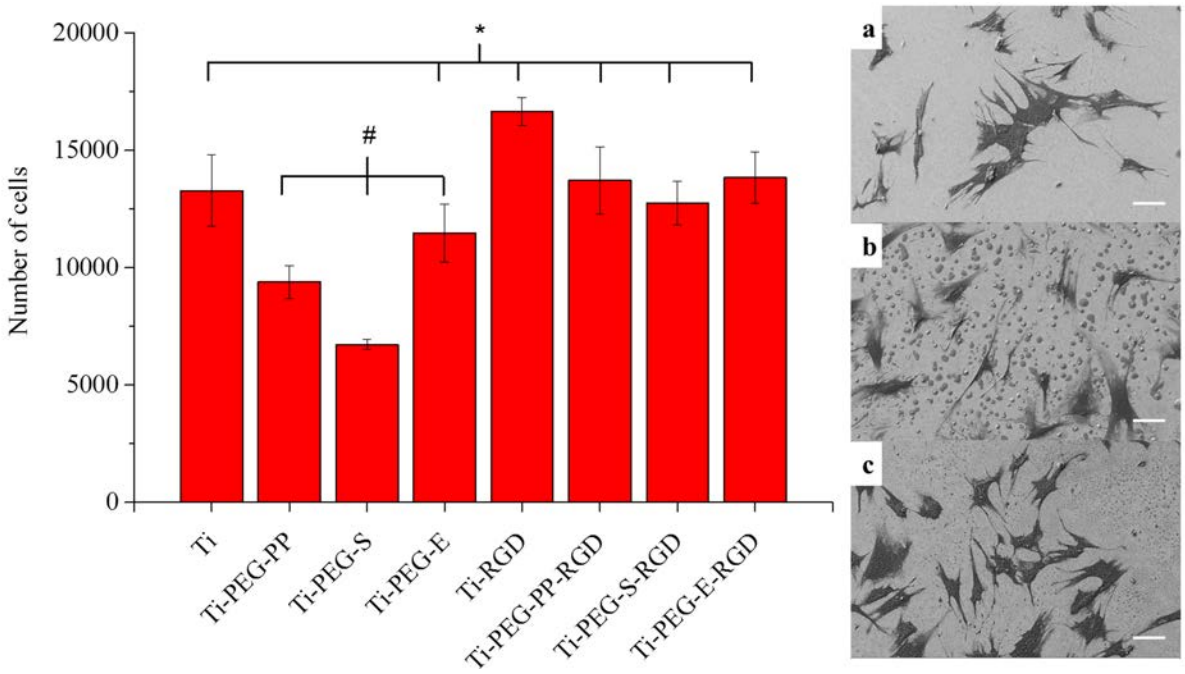


Figure 3. Number of cells present on the samples (right). Representative SEM images of the adhered cells. a) Ti, b) Ti-PEG-S, c) Ti-PEG-S-RGD. Scale bar represents 50µm. Samples with the same symbol have no statistically significant differences ($p<0.05$).

In views of dental applications, two oral bacterial strains were tested. Bacterial adhesion assays with *S. sanguinis* (Figure 4a) and *L. salivarius* (Figure 4b) presented a significant decrease ($p<0.05$) on the number of bacteria attached on all PEG-coated samples with respect to control titanium surfaces. Bacterial adhesion of *S. sanguinis* and *L. salivarius* (Figure 4) also presented a decrease compared to bare titanium, as observed in other works with an antifouling coating[50,77,78]. The different methodologies employed to obtain the PEG coatings do not show statistically significant differences, so all the techniques are suitable to prevent bacterial adhesion. However, Ti-PEG-S tends to have a lower bacterial adhesion for the two tested strains.

Bacterial adhesion of both strains was not affected by the presence of RGD (Figure 4), as it was observed in previous studies for other bacterial strains [62].

Dental implants are placed in a naturally contaminated environment, where bacteria are in competence with the host cells such as fibroblasts to colonize the surface. This situation was described by Gristina[79], and it is commonly known as "the race for the surface". This means that the proper integration of the implant is reliant on the ability of the surface of minimizing bacterial adhesion and/or maximizing cell adhesion. To test *in vitro* this situation, Zhao et al.[80] developed a co-culture experiment in which a bacterial strain is allowed to colonize the surface, and after a certain period of time, fibroblasts are seeded on the samples. This assay intends to more closely reproduce the clinical situation. Since the three compared methods rendered comparable *in vitro* results for the mono-culture experiments, a co-culture study was only done for Ti-PEG-E (see Figure S1 and S2 in the Supplementary Material), which is a fast and eco-friendly method, carried out in an aqueous solution. The adhesion and spreading of hFFs in the presence of bacteria was higher for the samples coated with PEG (Ti-PEG-E) than for the control samples (Ti), showing that the PEG coatings are able to withstand bacterial adhesion while allowing cell colonization. This result is in agreement with the mono-culture experiments.

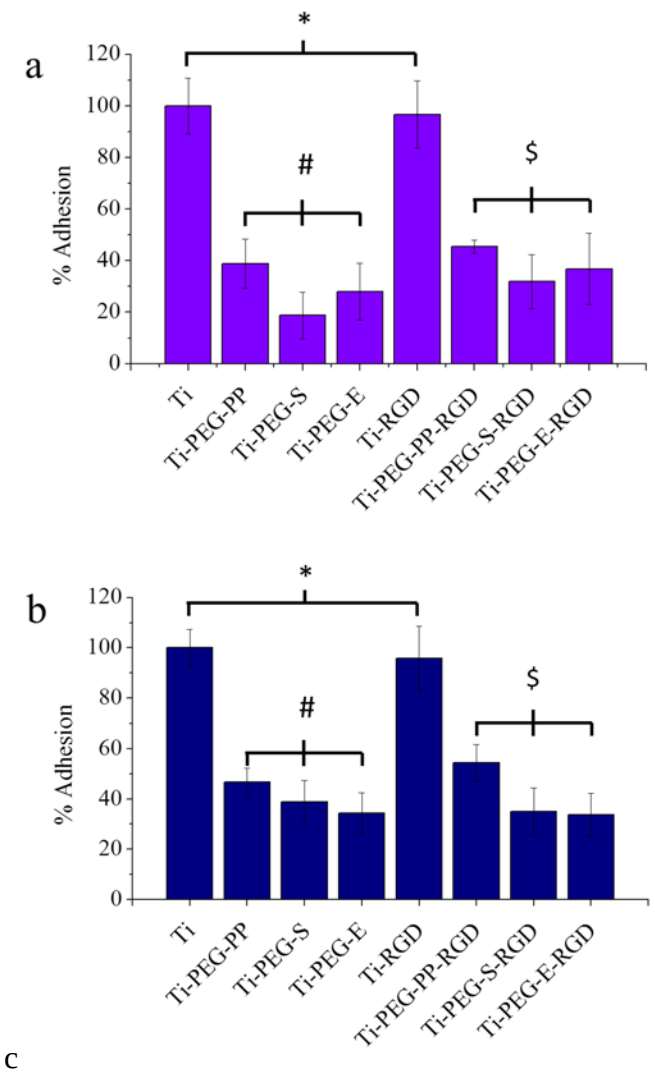


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Figure 4. Bacterial adhesion on the samples. a) *S. sanguinis*, b) *L. salivarius*. Samples with the same symbol have no statistically significant differences ($p<0.05$).

The different techniques studied have advantages and disadvantages which need to be taken into account in the selection of the most suitable one for a particular application. For instance, silanization tends to reduce more the bacterial adhesion, reaching values of 70% of reduction compared to control. Indeed, it is possible to treat more than one sample at a time since the concentration of silanized PEG is high. However, to silanize, organic solvents (toluene) are employed and vacuum is required, which makes the process more expensive and less eco-

friendly. Electrodeposition is a quick process which takes place using water as a solvent, at atmospheric pressure and room temperature. It renders a surface which reduces bacterial adhesion up to 65% compared to control, while cell adhesion is almost not affected. Lastly, plasma polymerization is a solvent free technique. The treatment of several samples at the same time is possible. It is a low pressure technique, which enhances the purity of the PEG-like coating. Plasma polymerized samples showed a reduction of 60% on bacterial adhesion, and the cell adhesion is also impaired to a similar extent. Taking this versatility into account, the selection of one method or another should be based on more practical parameters, such as the final application or the details of the manufacturing process.

CONCLUSIONS

Three different PEG coatings have been obtained on the titanium surface by different methods: plasma polymerization, electrodeposition and silanization. Chemical composition of the coatings and improved wettability correspond to the presence of the PEG polymer on the surface. The coatings have antifouling properties against BSA and significantly decrease the bacterial adhesion of *S. sanguinis* and *L. salivarius*. The impaired cell adhesion due to the antifouling character of the PEG coatings was improved by the immobilization of a cell adhesive motive RGD, while maintaining the effect on the bacterial adhesion. The three deposition methods in the studied conditions do not present major differences in terms of biological performance, demonstrating that these methods are a good option for the preparation of PEG coatings on titanium.

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ABBREVIATIONS

Polyethylene glycol, PEG; Arginine-Glycine-Aspartic Acid, RGD; Phosphate Buffer Saline (PBS); Fluoresceine Isothiocyanate, FITC; Bovine Serum Albumin, BSA; human Foreskin Fibroblasts, hFFs; Tissue Culture Polystyrene, TCPS; mammalian Protein Extraction Reagent, mPER; Lactate Dehydrogenase, LDH; Dulbecco’s Modified Eagle Medium, DMEM; Colony Forming Units, CFU; Todd Hewitt broth, TH; Man, Rogosa and Sharpe broth, MRS.

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Supplementary material: Evaluation of the material in co-cultures of bacteria and cells

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As expected, the co-culture experiment showed a decrease in cell adhesion in the presence of bacteria (Figure I). For the plain Ti surface, the surface coverage is highly reduced, to values of around 25% compared to the Ti surface without bacteria. Moreover, the cell spreading is much lower (Figure II a, b, c), showing that the cells are not able to colonize the surface when the bacteria are present. This result was also found in a previous study using *S. sanguinis* and *L. salivarius*[3]. In contrast, for the Ti-PEG-E surface, the surface coverage is twice the one observed for the Ti, due to the antifouling character of the PEG coating. The cell spreading is higher compared to the found for the control sample (Figure II d, e, f). This is indicative of the better performance of the Ti-PEG-E to favor the cell adhesion while discouraging the bacteria adhesion.

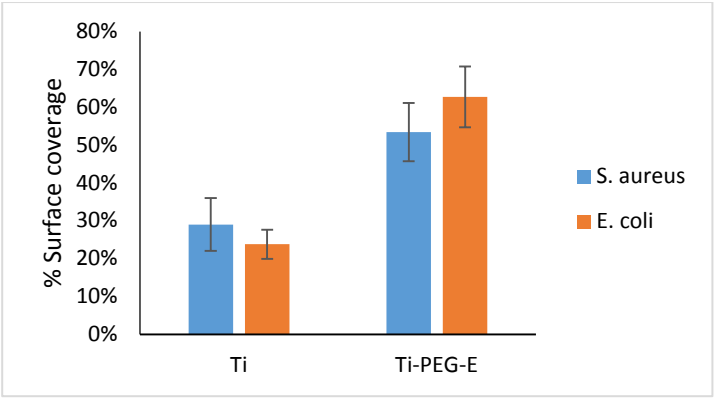

Figure S1. Quantification of the area covered by hFFS on the surfaces coated with bacteria compared to the same condition without bacteria (which is employed as control with 100% surface coverage)

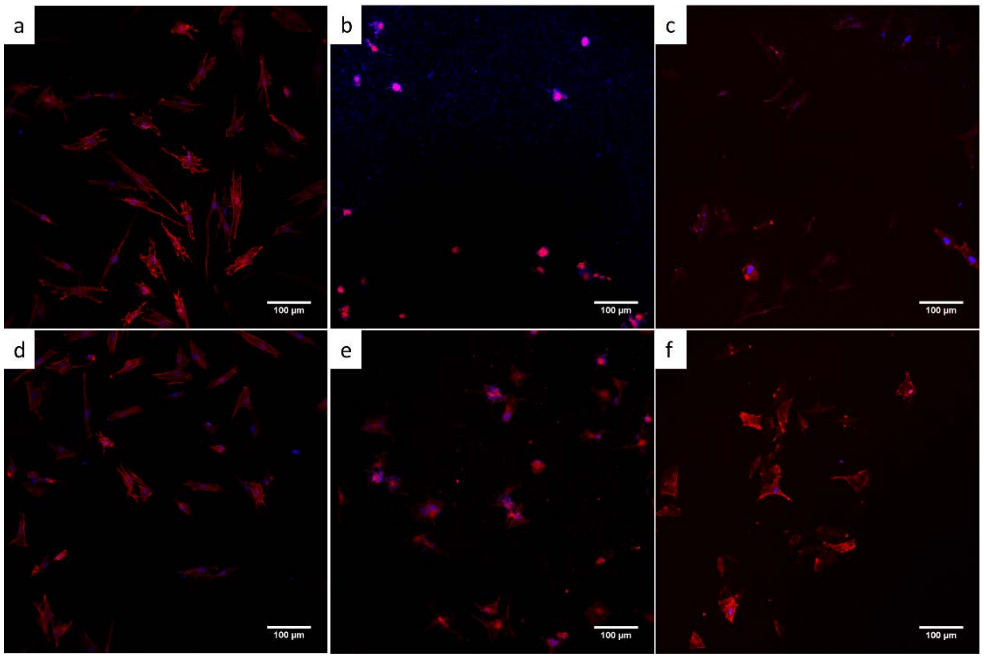

Figure S2. Representative images of the surfaces tested in the co-culture experiments. (a) Ti + hFFs, (b) Ti + *S. aureus* + hFFs, (c) Ti + *E.coli* + hFFs, (d) Ti-PEG-E + hFFs, (e) Ti-PEG-E + *S. aureus* + hFFs, (f) Ti-PEG-E + *E. coli* + hFFs

Experimental

Cell-bacteria co-culture experiment was an adaptation from [2,3]. *Staphylococcus aureus* CCUG 15915 (Culture Collection University of Göteborg (CCUG), Göteborg, Sweden), and *Escherichia coli* CECT 101 (Colección Española de Cultivos Tipo, Valencia, Spain) were used as the bacterial model. For co-culture experiments, a suspension of either *S. aureus* or *E. coli* was adjusted to an optical density of 0.2 in BHI, giving approximately $1 \cdot 10^8$cells/ml. 5µl ($5 \cdot 10^5$cells/sample) of the bacterial suspension were seeded on each sample. After 2h at 37ºC, samples were washed three times in order to eliminate the non-attached bacteria, and hFFs in modified DMEM (DMEM with 2% BHI) at $2 \cdot 10^4$cells/sample were seeded and incubated for 24h. After the incubation time, all the samples were washed twice with PBS, fixed and stained with Phalloidin-Rhodamin (Invitrogen) and DAPI (Invitrogen) for observation. Images were taken with a Leica TCS SPE confocal microscope and analyzed with Image J software. Five images were taken for each sample for the quantification, using duplicates for each condition.

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