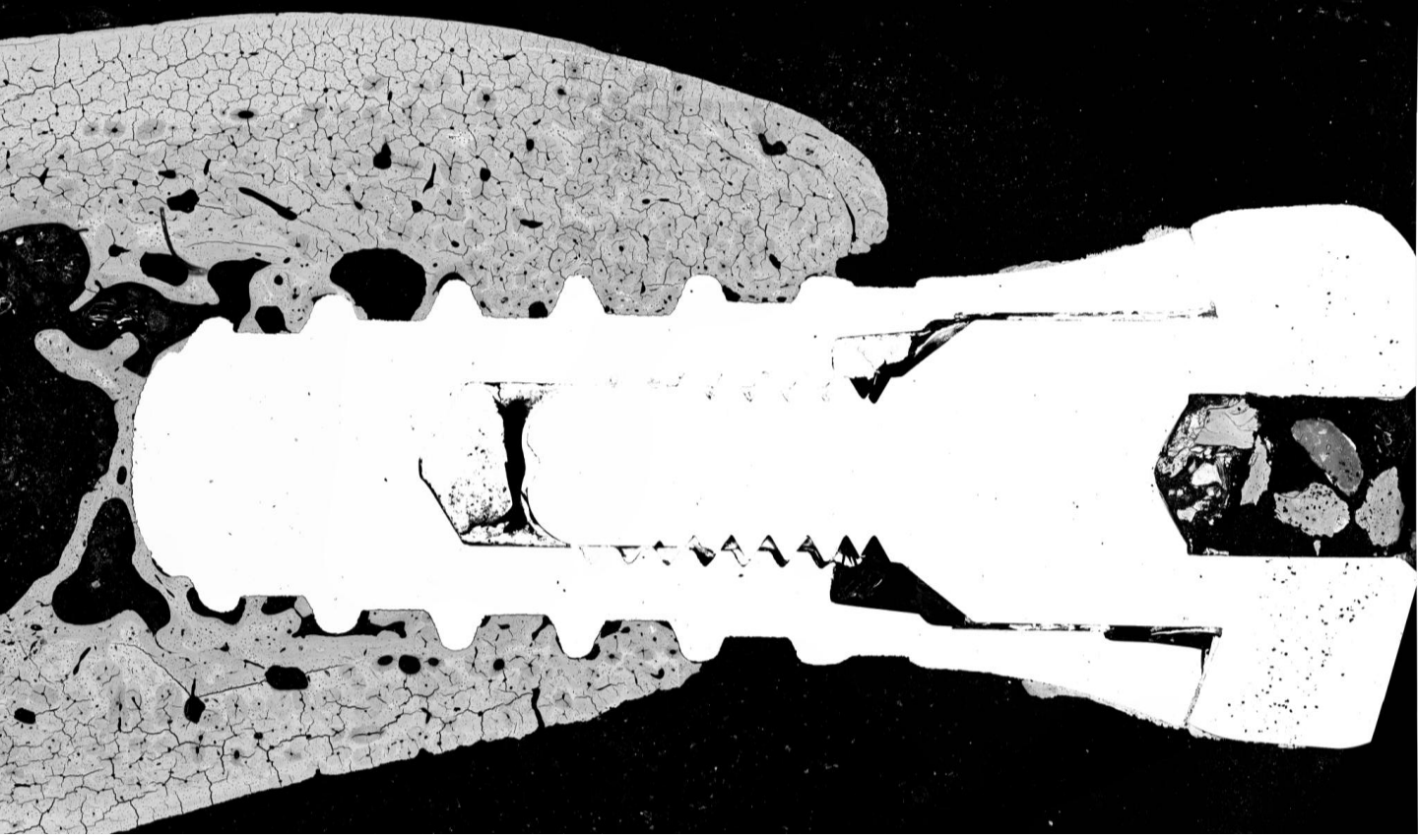
Development of Antibacterial Coatings for Titanium Dental Implants based on Different Strategies



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DOCTORAL THESIS IN
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List of Publications

This thesis is based on the following papers:

I. **Godoy-Gallardo M**, Mas-Moruno C, Yu K, Manero J.M, Gil F.J, Kizhakkedathu J.N, Rodriguez D. Antibacterial properties of hLf1-11 peptide onto titanium surfaces: a comparison study between silanization and surface initiated polymerization. Biomacromolecules. (DOI 10.1021/bm501528x; IF 5.788)

II. **Godoy-Gallardo M**, Rodríguez-Hernandez A.G, Delgado L.M, Manero J.M, Gil F.J, Rodriguez D. Silver deposition on titanium surfaces by electrochemical anodizing process reduces bacterial adhesion of Streptococcus sanguinis and Lactobacillus salivarius. Clin. Oral Impl. Res. 2014; 00:1-10. (DOI: 10.1111/clr.12422, IF 3.123)

III. **Godoy-Gallardo M**, Mas-Moruno C, Fernández-Calderón M.C, Pérez-Giraldo C, Manero J.M., Albericio F, Gil F.J, Rodriguez D. Covalen immobilization of hLf1-11 peptide on a titanium surface reduces baterial adhesion and biofilm formation. Acta Biomater. 2014; 10:3522-3534. (DOI doi:10.1016/j.actbio.2014.03.026, IF 6.191)

Article Presentation

The overall aim of this thesis was to develop new antibacterial coatings for dental implants. Implants designed for bone osseointegration have been subjected to modifications in order to improve the conditions for its osseointegration. While dental implant osseointegration is routinely achieved, development of antibacterial coatings, however, have been increasingly reported the last years because infection in dental implants is one of the main reasons to mid and long-term implant failure. Therefore, several strategies have been studied in this thesis to prevent biofilm formation on implant surfaces (**Paper I, II, III and Annex I**).

Oral biofilm mostly consist of a diverse microbial community, not randomly distributed and it is embedded in a protective extracellular polysaccharide (EPS) matrix which contributes to the spread of antibiotic resistance. After formation of the acquired pellicle, bacterial attachment with initial colonizers occurs on the implant surface. This process is followed by cell-to-cell adhesion with secondary colonizers. To initially determine the antibacterial effects of the antibacterial coatings developed through the project, two different oral strains have been used. They consist in *Streptococcus sanguinis*[1,2] which is considered as a early colonizer and *Lactobacillus salivarius* which interacts with other colonizers and their by-products are essential for the biofilm formation and maintenance[3]. As has been commented before, oral biofilm is the result of a combination of multiple bacteria, and its presence increases the risk of peri-implant diseases. Therefore, the antibacterial effect of all the optimized coatings was studied using a whole human oral plaque biofilm and the results compared to the more commonly used single species in vitro studies (**Annex**).

An in vivo study of selected antibacterial coatings on dental implants was performed in a dog animal model (**Annex III**) to evaluate their performance in real *in vivo* oral conditions.

Paper I: Silver deposition on titanium surface by electrochemical anodizing process reduces bacterial adhesion of Streptococcus sanguinis and Lactobacillus salivarius

Manuscript published; Clinical Oral Implant Research 2014;00:1-10. (DOI: 10.1111/clr.12422)

Author's contribution: Major part of planning and analysis. Performed all the experiments in the study and wrote the paper.

The manuscript titled "Silver deposition on titanium surface by electrochemical anodizing process reduces bacterial adhesion of *Streptococcus sanguinis* and *Lactobacillus salivarius"* is the result of a comprehensive original work aimed at the development and characterization of a new electrochemical surface treatment of titanium for a direct deposition of silver with antibacterial properties.

To our knowledge, this is the first time that a pulsed anodizing treatment has been developed for silver deposition on titanium surfaces. The antimicrobial properties of silver have been known for many years and many articles report the use of silver and silver-base compounds as antimicrobial agents. The simplicity of the technique allows envisaging the potential of this process as an antibacterial treatment for titanium dental implants.

The aim to this study was to optimize the silver electrodeposition onto titanium surfaces and determine his antibacterial properties on two oral bacterial strains (*Streptococcus sanguinis* and *Lactobacillus salivarius*). It was hypothesized that silver deposited onto titanium surfaces leads to reduce antibacterial adhesion and biofilm formation due to its wide range of activity.

Paper II: Covalent immobilization of hLf1-11 peptide onto titanium surface reduces bacterial adhesion and biofilm formation.

Manuscript published; Acta Biomaterialia 2014;10(8):3522-34. (DOI:10.1016/j.actbio.2014.03.02)

Author's contribution: Planned the study and performed all the experiments. Wrote together with other co-authors.

The manuscript entitled "Covalent immobilization of hlf1-11 peptide onto titanium surface reduces bacterial adhesion and biofilm formation" describes the development and characterization of new biofunctionalized titanium surfaces with antimicrobial properties against two oral bacteria strains responsible of peri-implantitis in dental implants.

The use of antimicrobial peptides as coating molecules has emerged in the biomaterials science as a powerful approach to overcome the limitations associated to the use of conventional antibiotics (i.e. development of bacterial resistance), and has potential to reduce the incidence of device-related infections. Implant failure due to bacterial infection is a growing cause of concern in the fields of dentistry and orthopedics.

This work has selected the potent antimicrobial peptide derived from human lactoferrin, hLf1-11, which has shown a broad spectrum of activity against bacteria and other parasites in previous reports. This peptide has been anchored to titanium following two distinct approaches and the resulting surfaces have been comprehensibly characterized in terms of physicochemical properties and antibacterial activity.

The purpose of the present study was to determine the antibacterial activity of the human hLf1-11 peptide attached onto titanium surfaces. It was hypothesized that lactoferrin peptide reduces the adhesion and early stages of biofilm formation of *Streptococcus sanguinis* and *Lactobacillus salivarius*.

The results presented in this study show an outstanding reduction in bacterial adhesion and early stages of biofilm formation for *Lactobacillus salivarius* and *Streptococcus sanguinis*.

Paper III: Antibacterial properties of hlf1-11 Peptide onto Titanium Surface: A Comparison Study between Silanization and Surface Initiated Polymerization

Manuscript published; Biomacromolecules (2014); **DOI:** 10.1021/bm501528x

Author's contribution: Planned the study together with the co-authors. Definition, development and analysis of many of the tests. Wrote together with one of the co-authors.

The manuscript entitled "Antibacterial properties of hLf1-11 Peptide onto Titanium Surface: A Comparison Study between Silanization and Surface Initiated Polymerization", details the comparison of two biofuncionalization methodologies in order to provide antibacterial properties to titanium surfaces.

The immobilization of antimicrobial peptides (AMPs) onto implant surfaces has emerged as a powerful approach to confer antibacterial properties to implant materials to reduce the risk of periodontal diseases, a major cause of implant failure in dentistry. Moreover, this strategy may be applied to other biomedical fields where bacterial infections represent a serious concern.

A key step in the functionalization of metallic materials is the grafting method used to covalently anchor the bioactive molecules to the implant surface. Among different immobilization strategies, silanization and grafting of polymer brushes by surface initiated polymerization represent well-established methodologies to coat surfaces with functional molecules. However, these two methods have never been compared in the same study, and their advantages and disadvantages seldom critically discussed.

In this work we selected the hLf1-11 peptide, previously reported by us as a potent AMP with the capacity to reduce bacterial adhesion and inhibit oral biofilm formation on titanium implants, and immobilized onto titanium surfaces by means of silanization or via polymer brushes. The two strategies were carefully characterized in terms of physicochemical properties, cellular toxicity and antibacterial activity.

Our study demonstrates that surfaces modified by polymer brush-based methods showed a greater decrease in bacterial adhesion and early stages of biofilm formation of *S. sanguinis* and *L. salivarius*, compared to direct silanization

Annex I: Anhydride-silane immobilized onto titanium surfaces induces osteoblast cell differentiation and reduces bacterial adhesion and biofilm formation

Manuscript submitted.

Author's contribution: Planned the study together with the co-authors. Major part of planning and analysis. Significant part of writing.

The manuscript entitled "Anhydride-silane immobilized onto titanium surfaces induces osteoblast cell differentiation and reduces bacterial adhesion and biofilm formation" reports a new silane which improves SaOS-2 differentiation and reduces bacterial adhesion and biofilm formation against one oral bacterial strain responsible of peri-implantitis in dental implants.

As far as we know, some materials are bioinerts (i.e. titanium), and in order to anchor covalently biomolecules and overcome its limitations associated to cell adhesion and antibacterial properties, silanes are use.

In this study, the aim was twofold. Firstly, it was determined the induction of SaOS-2 osteoblastic cell differentation by TESPSA immobilized onto titanium. Secondly, a prominent reduction in the adhesion and early stages of biofilm formation of *Streptococcus sanguinis* was observed. TESPSA immobilization could be an effective anchoring platform of biomolecules on titanium surfaces with improved osteoblastic differentiation and antibacterial properties.

Annex II: Antibacterial coatings on titanium surfaces: a comparison study between *in vitro* single-species and multispecies biofilm

Manuscript submitted.

Author's contribution: Planned the study together with the co-authors. Development of the major part of assays and analysis. Wrote together with the co-authors.

The manuscript, entitled "Antibacterial Coatings on Titanium Surfaces: A Comparison Study between in vitro Single-species and Multi-species Biofilm", expresses the comparison of five antibacterial coatings by two *in vitro* biofilm models.

Dental plaque is a biofilm that causes dental caries, gingivitis and periodontitis. Most of the studies in antibacterial coatings have been conducted by *in vitro* single-species biofilm formation but oral biofilm involves more than 500 different bacterial species that are able to interact. Therefore, new studies are focused on *in vitro* multispecies biofilm model that mimic *in vivo* biofilms.

Hence, the speculation in this study is based on the fact that antibacterial coatings developed during the thesis will have different efficiency when single or complete-biofilm are used. To address this issue, the antibacterial coatings have been studied in single-species biofilms with a dental plaque biofilm and compare the results obtained.

The physicochemical and biological properties of the surfaces have been characterized by scanning electron microscopy, contact angle, interferometry, XPS and cell adhesion studies. Moreover, antibacterial properties have been studied by viability and bacterial adhesion assays.

Our study demonstrates that multispecies biofilms method is a reasonable strategy to study antibacterial properties of novel coatings.

Annex III: Evaluation of antibacterial coatings on dental implants - An experimental study in dogs

Manuscript writing.

Author's contribution: Definition of the assay with one of the co-authors. sample's preparation, processing and histomorphometric analysis. Wrote together with one co-author.

The manuscript, entitled "Evaluation of antibacterial coatings on dental implants - An experimental study in dogs", refers to the study of two modified-dental implants in an *in vivo* dog model.

Silver electrodeposition and triethoxysilylpropyl succinic anhydride (TESPSA) silane molecule immobilization, result in an outstanding decrease in the oral plaque adhesion and biofilm formation in comparison with control titanium as has been studied in **Paper V**.

Therefore, we have assumed that the presence of silver and TESPSA onto surface reduces bacterial adhesion, bone reabsorption and peri-implantitis progress.

The goal in this work was to study the *in vivo* effect of both developed coatings in a dog model. Bone reabsorption and bone osseointegration have been characterized by clinical parameters, histomorphometry and histology assays.

The results obtained evidences that TESPSA coated dental implant increases bone osseointegration and a reduction in bone reabsorption was observed onto coated implants.

CLINICAL ORAL IMPLANTS RESEARCH

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Silver deposition on titanium surface by electrochemical anodizing process reduces bacterial adhesion of *Streptococcus sanguinis* and *Lactobacillus salivarius*

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Abstract

Objectives: The aim of this study was to determine the antibacterial properties of silver-doped titanium surfaces prepared with a novel electrochemical anodizing process.

Material and methods: Titanium samples were anodized with a pulsed process in a solution of silver nitrate and sodium thiosulphate at room temperature with stirring. Samples were processed with different electrolyte concentrations and treatment cycles to improve silver deposition. Physicochemical properties were determined by X-ray photoelectron spectroscopy, contact angle measurements, white-light interferometry, and scanning electron microscopy. Cellular cytotoxicity in human fibroblasts was studied with lactate dehydrogenase assays. The *in vitro* effect of treated surfaces on two oral bacteria strains (*Streptococcus sanguinis* and *Lactobacillus salivarius*) was studied with viable bacterial adhesion measurements and growth curve assays. Nonparametric statistical Kruskal–Wallis and Mann–Whitney U-tests were used for multiple and paired comparisons, respectively. *Post hoc* Spearman's correlation tests were calculated to check the dependence between bacteria adhesion and surface properties.

Results: X-ray photoelectron spectroscopy results confirmed the presence of silver on treated samples and showed that treatments with higher silver nitrate concentration and more cycles increased the silver deposition on titanium surface. No negative effects in fibroblast cell viability were detected and a significant reduction on bacterial adhesion *in vitro* was achieved in silver-treated samples compared with control titanium.

Conclusions: Silver deposition on titanium with a novel electrochemical anodizing process produced surfaces with significant antibacterial properties *in vitro* without negative effects on cell viability.

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Covalent immobilization of hLf1-11 peptide on a titanium surface reduces bacterial adhesion and biofilm formation

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abstract

Bacterial infection represents a major cause of implant failure in dentistry. A common approach to overcoming this issue and treating peri-implant infection consists in the use of antibiotics. However, the rise of multidrug-resistant bacteria poses serious concerns to this strategy. A promising alternative is the use of antimicrobial peptides due to their broad-spectrum activity against bacteria and reduced bacterial resistance responses. The aim of the present study was to determine the in vitro antibacterial activity of the human lactoferrin-derived peptide hLf1-11 anchored to titanium surfaces. To this end, titanium samples were functionalized with the hLf1-11 peptide either by silanization methods or physical adsorption. X-ray photoelectron spectroscopy analyses confirmed the successful covalent attachment of the hLf1-11 peptide onto titanium surfaces. Lactate dehydrogenase assay determined that hLf1-11 peptide did not affect fibroblast viability. An outstanding reduction in the adhesion and early stages of biofilm formation of *Streptococcus sanguinis* and *Lactobacillus salivarius* was observed on the biofunctionalized surfaces compared to control non-treated samples. Furthermore, samples coated with the hLf1-11 peptide inhibited the early stages of bacterial growth. Thus, this strategy holds great potential to develop antimicrobial biomaterials for dental applications.

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Antibacterial Properties of hLf1−11 Peptide onto Titanium Surfaces: A Comparison Study Between Silanization and Surface Initiated Polymerization

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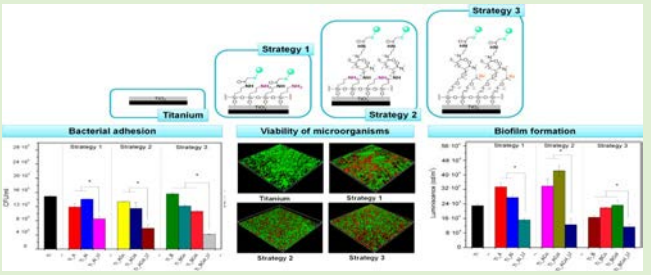
* *Supporting Information*

ABSTRACT: Dental implant failure can be associated with infections that develop into peri-implantitis. In order to reduce biofilm formation, several strategies focusing on the use of antimicrobial peptides (AMPs) have been studied. To covalently immobilize these molecules onto metallic substrates, several techniques have been developed, including silanization and polymer brush prepared by surface-initiated atom transfer radical polymerization (ATRP), with varied peptide binding yield and antibacterial performance. The aim of the present study was to compare the efficiency of these methods to immobilize the lactoferrin-derived hLf1−11 antibacterial peptide onto titanium, and evaluate their antibacterial activity in vitro. Smooth titanium samples were coated with hLf1−11 peptide under three different conditions: silanization with 3-aminopropyltriethoxysilane (APTES), and polymer brush based coatings with two different silanes. Peptide presence was determined by X-ray photoelectron spectroscopy, and the mechanical stability of the coatings was studied under ultrasonication. The LDH assays confirmed that HFFs viability and proliferation were no affected by the treatments. The in vitro antibacterial properties of the modified surfaces were tested with two oral strains (*Streptococcus sanguinis* and *Lactobacillus salivarius*) showing an outstanding reduction. A higher decrease in bacterial attachment was noticed when samples were modified by ATRP methods compared to silanization. This effect is likely due to the capacity to immobilize more peptide on the surfaces using polymer brushes and the nonfouling nature of polymer PDMA segment.



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Pages 33 to 46 of the thesis are available at the editor's web

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Results and Conclusions

Summary of the results and discussion

The work in this thesis has been aimed at developing potential antibacterial coatings for dental implants. This chapter highlights and discusses some of the main achievements of this study regarding the physicochemical characterization and biological properties of the antibacterial coatings developed. Notwithstanding the results and discussions in the papers, some of the results presented have not been published yet (as is shown in Annex I, II and III) or some of them are represented on the manuscripts and not are showed in this section. Therefore, the discussion based on the non-published results intends to complement the published work as well as, in an overall analysis, contribute to the field antibacterial coatings for dental applications.

Physic-Chemical Characterization

The physico-chemical studies of the treated surfaces demonstrated that all surfaces were successfully modified. Statistical analysis of the biological tests on the surfaces indicated that neither roughness nor wettability have a determinative influence in cell and/or bacterial adhesion. Therefore, the effects observed in the *in vitro* studies depend only on the modified chemical composition of the surfaces with the applied coatings.

Silver deposition onto titanium surfaces (Paper I)

One antibacterial coating developed consisted in silver deposition on titanium surface by means of an electrochemical anodizing process with complexated silver.Other silver deposition techniques present also antibacterial properties. An example is the use of RF magnetron sputtering[1,2] or the use of silver nanoparticle[3]. In both cases, however, the coatings show a initial rapid release of Ag ions. Moreover, silver nanoparticles show a higher surface area and will increase the Ag^+ release. This fast release of

silver ions could induce deleterious reactions in the body tissues, and it is therefore not desirable[4,5]. In comparison with the methodology optimized in the current thesis, silver particles are not released to the medium. Therefore, bacterial adhesion is reduced instead of affecting cells in surrounding tissues or planktonic bacteria, diminishing the possibility of development of silver resistance in bacteria. Furthermore, the concentration does not decrease onto the surface through the time.

The method developed in the present study has a low release of silver into the medium, with basal levels in animal model under detection limit for the ICP_MS technique (10 ng/g). Moreover, the antibacterial effect of silver-anodized surfaces does not require the application of external agents, such as UV irradiation[3].

SEM images showed a topographical effect of the anodization process on the treated substrates with the shape of rounded etching with deposits homogeneously dispersed on titanium surface. Besides, EDS analyses demonstrated that silver remained attached even after hard sonication.

To further study the topography of the treated samples, the roughness was also studied. White-light interferometry results showed that mean surface roughness changed after anodizing when compared to control titanium (Ti) with mean roughness values lower than 100 nm. It has been documented that surface textures below 150-200 nm did not increase bacteria adhesion because the size of the created irregularities is not big enough to indice measurable effects[6,7]. These results suggest that bacterial adhesion will not be influenced by the roughness increase of the treated surfaces.

In addition to roughness parameters, wettability was also analyzed by sessile drop method. Dexter *et al*[8] and Baier *et al*[9,10] proposed that an increase in contact angle rise the interaction between implant surface and biological environment which affects cell and bacterial adhesion[7,8,11]. Moreover, changes in wettability are good indicators of the presence of hydrophilic or hydrophobic chemical groups on a surface. The wettability results obtained in the present study do not present significant difference between control titanium and silver-treated surfaces. Hence, we can confirm that changes in bacteria adhesion can not be attributed to changes in surface wettability.

To determine the presence of silver, XPS analysis was performed. The deconvolution of the high resolution spectra revealed peaks at energy positions corresponding to silver oxide (AgO: 367eV, Ag_2O: 367.8eV) and metallic silver (Ag: 369eV)[12–16]. Moreover, the [Ag]/[Ti] ratio results suggest that silver deposition depends more on silver concentration in the electrolyte than on the number of cycles applied, in accordance with Liu *et al*[17] and Yin *et al*[18].

hLf1-11 immobilization by silanization (Paper II)

This study presents an study of the efficiency of lactoferrin antimicrobial peptide (hLf1-11) immobilization onto titanium surfaces by silanization by different processes.

Metallic samples were first activated by alkaline treated using a solution of NaOH. Consequently, a stable amorphous sodium titanate layer has been formed [19] with a micropore-structure (less than 1 μm in diameter). This layer produced a measurable increase in surface roughness of the surfaces, in contrast with plasma cleaning process (**Paper III**) or the electrochemical silver deposition process developed in **paper I.** As expected, the process of physisorption of hLf1-11 peptide, as well as the silanization process and subsequent covalent immobilization of hLf1-11, did not modify the surface roughness at any peptide coating concentration tested. Therefore, the roughness values are too low to significantly affect bacteria adhesion and aggregation.

Wettability was also studied in this study and control and modified samples were monitored by contact angle measurements. Alkaline etching activation drastically decreased contact angle values when compared to control titanium surfaces, which mostly indicates the removal of hydrophobic contaminants from the surface and the formation of free hydroxyl groups[20]. Despite the cationic nature of hLf1-11, the attachment of this peptide onto both types of silanized samples further increased water contact angle values, an effect attributed to the presence of hydrophobic residues within the peptide sequence (Ala, Trp and Val) and more relevantly the three 6-aminohexanoic acid (Ahx) units that constitute the spacer moiety. It should be mentioned that all functionalized samples showed a more hydrophilic character than control Ti with surface free energy (SFE) values ranging from 50 to 70 mJ/m^2. Since the biofunctionalized surfaces of this study present SFE values above 30 mJ/m^2, it is expected that the process of biofunctionalization applied to these materials will not compromise cell adhesion[21–23].

The success in the functionalization strategy was further demonstrated by means of XPS studies.The immobilization of hLf1-11 on titanium samples was accompanied with a clear increase in the high resolution signals of C 1s and N 1s, which is correlated with the presence of aliphatic carbons, amide and amino functionalities and other chemical groups characteristic of peptide molecules[24,25]. The presence of sulfur was another indicator of peptide attachment. High resolution XPS analyses of the S 2p peak confirmed the covalent attachment of hLf1-11 onto titanium samples. In this regard, the ratio S/Ti was calculated to compare the coating efficiency of the distinct functionalization approaches studied. For both silanes, the quantity of peptide attached increased with the concentration of peptide used in the coating solution. Interestingly, there were no significant differences in the amount of peptide bound to titanium comparing the use of APTES with CPTES silane, which is in agreement with a similar coating efficiency for the two silanes (as evidenced by Si/Ti ratios). On the contrary, the amount of peptide bound by physical adsorption was considerably lower.

Hence, these studies demonstrated that a) the methodology used successfully immobilized the hLf1-11 peptide on titanium, and b) silanization is a more effective method of biofunctionalization than physisorption to attach a higher amount of peptide on the treated surfaces.

Increase of hLf1-11 immobilized by atom transfer radical polymerization (Chapter 5)

This work has studied different methods to immobilize hLf1-11 onto titanium surfaces in order to increase peptide concentration, silanization as done in paper II, and silanization through the formation of polymer brushes on the titanium surface by two processes of atom transfer radical polymerization (ATRP) with DMA-APMA monomers.

First of all, wettability features were analyzed. Silanization of titanium surfaces significantly increased the water contact angle (Ti_A and Ti_B) together with a decrease in SFE due to the hydrophobic nature of the silane molecules. Moreover, copolymerization of DMA-APMA augmented the hydrophilic character due to the high number of amides and amine groups present in brushes and provoke a significant increase in the polar component of SFE. Covalent immobilization of hLf1-11 onto aminosilanized surfaces (TI_AI_Lf) reduced significantly CA values and on polymerized substrates increased. Comparing wettability of aminosilanized samples (Ti_AI_Lf), the CA of treated surfaces via brushes was similar for Ti_A_CoI_Lf or statiscally higher for Ti_BCoI_Lf.

Topographical properties were also determined. Overall, all three strategies displayed the same value of Ra (~30 nm), slightly higher than Ra of control samples (~25 nm) but such difference do not affect either HFFs or bacterial adhesion[26–32].

Additionally the thickness of coating layers after silanization and ATRP polymerization was studied. Comparing the different strategies, it is evident that polymerization increased the coating thickness more than mere silanization (~20 nm for copolymer brushes and ~3 nm for silanization), while, the addition of the crosslinker had an insignificant effect. Conversely, an increase of approximately 7-15 nm of thickness was observed after peptide immobilization. It can be presumed that an increase in the layer thickness suggests a larger number of hLf1-11 molecules on the surface. Furthermore, the 20 nm rise in thickness for both copolymer brush layers pinpoints to an interdifussion of the peptide into the coating and a corresponding increase of the peptide density on the surface.

The efficacy of the silanization process was characterized by the presence of silicon on the samples (10.2 % for Ti_A and 7.5 % for Ti_B). According to Si/Ti ratios, silanization was slightly more efficient for BPTCS than for APTES (Si/Ti ratio of 2.0 for Ti_A; and 2.9 for Ti_B), although these values were not statistically different. Moreover, it is important to verify the attachment of hLf1-11 peptide. The calculation of S/Ti ratios indicated that ATRP method allowed the conjugation of about 3-fold more peptide than standard silanization (S/Ti ratio of 0.4 for Ti_AI_Lf; 1.3 for Ti_ACoI_Lf; and 1.1 for Ti_BCoI_Lf). This result is consistent with the thickness measurements of the different coating processes.

The mass of the peptide (W_p) was calculated for all coating strategies with a result of 1.7 μg/cm² for Ti_ACoI_Lf, 1.3 μg/cm² for Ti_BCoI_Lf and 0.9 μg/cm² for Ti_AI_Lf. Although the peptide mass/area on Ti_ACoI_Lf samples presented higher values than that on Ti_BCoI_Lf samples, no statistically significant differences between both strategies were measured, which suggested that both are able to attach a similar amount of peptide onto titanium surface. As we expected, the amount of peptide immobilized onto the surface by ATRP is 2-fold higher in comparison with conventional silanization.

Finally, the stability of coating systems was analyzed by the immersion of representative samples in PBS and subjected to ultrasonication for 2 h. After this treatment, CA of silanized samples decreased, particularly Ti_B. On functionalized samples with hLf1-11, statistically significant differences were observed when using BPTCS (Ti_ BCoI_Lf) but not using APTES (Ti_AI_Lf and Ti_ACoI_Lf). Interestingly, this result insinuates that despite some extent of peptide detachment, the surface polymerized with APTES silane after ultrasonication may still present a layer of peptide similar to those found on the surfaces before ultrasonication.

TESPSA funcionalization (Annex I)

This study aimed to enhance the osseointegration and reduce bacterial adhesion and biofilm formation by incorporating a silane, TESPSA, on titanium dental devices.

As has been showed in **paper II**, a stable amorphous sodium titanate layer with nanoporous morphology was produced onto the surface after NaOH activation. Moreover, roughness was also influenced by the pre-treatment.

Additionally, wettability was also studied. The NaOH etching treatment induces a substantial decrease in contact angle value, which suggest the formation of hydroxyl groups[20]. Likewise, the contact angle values of TESPSA silanized sample revealed an increase in wettability in comparison with Ti_N due to its hydrophobic character. Besides, a cleaning effect on the surface was expected and it can affect the reduction achieved for both conditions (Ti_N and Ti_N_TSP). Some studies suggest that SFE values above 20-30 mJ/m² range present an optimal cell adhesion[21] and the results are higher than 30 mJ/m² in all surfaces. Therefore, we suggest that wettability of treated samples should show similar cell adhesion.

In order to determine the efficiency of TESPSA silanization, XPS was used. For Ti and Ti_N surfaces, no silicon was present which suggest that the presence of silicon obtained after TESPSA immobilization is attributed undoubtedly to the presence of the silane. Moreover, after several cleanings by sonication, silicon was still present and it demonstrates that silane molecules were chemically bound to titanium surface. Detailed peaks were also studied. Before NaOH activation there are three typical C 1s peaks (284.8, 285.7 and 288.1 eV) which come from hydrocarbon contamination (CHx, C-O and C=O bonds)[33–36]. After NaOH activation C1s spectra was fitted by two components

at 284.8 and 288.8 eV which can be attributed to C-H and C=O respectively[33–36]. When TESPSA is attached on titanium surface, an increase of C 1s peak at 284.6 eV (assigned to C-C)[33–37] is measured.

As expected, TESPSA was successful silanized onto titanium samples with a fully optimized process..

Biological characterization of the surfaces (Paper I, II, III, IV and Annex I)

Once all modified surfaces were physic-chemical characterized, to the focus of the study turned to the biological response to cell cytotoxicty and whether the treated surfaces improved the antibacterial properties.

In vitro cell viability

Cytotoxicity, viability and proliferation was analysed for all treated surfaces in different concentrations of cell culture extract for 1 day. Treated samples did not show significant differences in cell viability compared to control titanium (Ti) (**Paper I,II,III and Annex I and II**). Although some reduction in cell viability was observed for coated samples, such reduction was lower than 20% compared to control Ti. According to the International Organization for Standarization (ISO 10993-6:2007), reductions in cell viability below this value are not considered cytotoxic effects. Thus, all the treatment processes used in this study are noncytotoxicin vitro against HFFs[38–41].

Proliferation of HFFs on modified samples was also studied after 4 h, 1, 3 and 7 days of incubation. For 4 h and 1 day of culture, samples covered by ATRP strategy showed a lower number of viable cells than control titanium (**Paper III**). However, as evidenced by rates of proliferation, no statistically significant differences were observed between titanium and treated samples for up to 7 days of incubation.

Has been reported that the presence of TESPSA onto titanium surfaces can improve osteoblast differentiation [ES Patent P201 331756]. Thus, some studies with osteoblastic SaOS-2 line cells were developed to confirm this hypothesis (**Annex I**). The proliferation of SaOS-2 onto treated samples(Ti_N_TSP) was studied for up to 7 days of incubation. At day 1, etched surfaces (Ti_N and Ti_N_TSP) showed a decrease of the SaOS-2 cell number vs control surfaces. However, over time, these differences slightly decreased finally resulting in no statistically significant differences when compared to control Titanium.

In order to evaluate the osseointegration properties of TESPSA, osteoblast differentiation was analyzed through ALP activity and gene expression of osteoblast markers (**Annex I**). ALP results showed a peak of activity after 21 days of culture with osteogenic media. Both Ti_N and Ti_N_TSP exhibited higher values than control Ti but no significant differences between Ti_N and Ti_N_TSP were obtained. These results suggest that the pro-differentiation role can be influenced by Na-OH etching. However, the changes in the expression values of RUNX2, COL1A1 and BMP-2 points to another explanation. The presence of TESPSA on titanium surface enhanced RUNX2 gene expression

at 4h of culture, suggesting an increase in osteoblast activity, while it decreased during culture time. COL1A1 is an indicator of the transition between pre-osteoblast and immature osteoblast, suggesting that cells are probably in the mature state[42]**.** The results showed a slightly increase in COL1A1 expression after 24h of culture when TESPSA is immobilized, without significant differences compared with titanium. Finally, the expression of BMP-2 was also evaluated as an indicator of osteoblast activity. The combination of these results indicates a higher osteoblast activation in the presence of TESPSA.

In vitro single bacterial assay

After having established the non-cytotoxic character of the developed antibacterial coatings, the study focused on the antimicrobial properties for two common oral bacterial strains, *Streptococcus sanguinis* [43,44] , *Lactobacillus salivarius*[45] (**Paper I,II,III AND Annex I and II**). Both strains were chosen because they have siginificant roles in the oral biofilm. *S.sanguinis* is a primary colonizer of dental devices, whereas *L.salivarius* interacts with other secondary colonizers in the biofilm, and their by-products are important for the biofilm maintenance and maturation.

All the studied surface treatments significantly reduced *in vitro* the adhesion and biofilm formation of all bacteria suspensions, with the highest reduction measured for *S.sanguinis*.

Particularly, for titanium anodized with silver (**Paper I**) for 500 cycles(C1_500 and C2_500) presented lower bacteria count that surfaces treated with silver for 200 cycles (C1_200 and C2_200). Besides, the lowest amount of attached bacteria was measured for the sample C1_500 for both strains. These results confirm the effectiveness of silver anodizing against *in vitro* bacterial adhesion to treated surfaces. Moreover, a *post-hoc* statistical test was performed in order to evaluate possible relationships between surface properties and bacteria adherence onto surfaces. The Spearman correlation of bacteria adhesion with roughness and contact angle indicated a lack of correlation between the reduction in CFU/mm^2 and variations in both parameters. Furthermore, the relationship between the amount of adhered bacteria and the [Ag]/[Ti] ratio shows a statistically significant correlation between an increased deposition of silver on titanium surfaces and a decrease in bacterial adhesion to the silver-treated surfaces.

For the hLf1-11 immobilization process (**Paper II**), the study of the MIC values for the bacterial strain was performed. The kinetics showed that both strains are susceptible to the hLf1-11 peptide, even though *L.salivarius* has a lower susceptibility to the hLf1-11 peptide than the *S. sanguinis,* with MIC values of 1 and 8 $\mu g \cdot ml^{-1}$, respectively.

As expected, the presence of hLf1-11 peptide onto titanium surfaces resulted in a significant reduction in the adhesion of both strains. In the silanization process, this effect was more pronounced than when it was physically adsorbed. Probably because the peptide was covalently attached to the surface.

For *S.sanguinis* the most remarkably inhibitory effect were found when hLf1-11 was bound via CPTES and a concentration-dependent manner was also observed. The differences obtained in comparison with APTES-modified surfaces do not correlate with XPS data, which showed that similar amounts of peptide were attached to both surfaces. Hence, the variation observed may arise from other factors, like distinct chemical nature of the exposed functional groups of APTES ($-NH_2$) and CPTES (-Cl) that did not react with hLf1-11. Therefore, samples functionalized using CPTES (more hydrophobic) increased the contact angle values compared to the use of APTES. Interestingly, for *L.salivarius*, the inhibition bacteria adhesion seemed to be independent of both the peptide concentration and the method of peptide immobilization.

When the peptide was attached to polymer brushes (Ti_ACoI_Lf and Ti_BCoI_Lf) a higher antibacterial effect was observed in comparison with a standard silanization process, such as the one used in **papers II** . The best antibacterial adhesion results were achieved with the ATRP based method and they can be correlated to a combination of higher peptide density with the non-fouling properties of the PMA segment of the polymer brushes.

Finally, the effect of the antibacterial surfaces on biofilm formation was also evaluated. All functionalized samples with hLf1-11 attached to them showed a drastic reduction in the formation of the biofilm for both bacterial strains. ATRP-processed coatings showed a clearly higher inhibition in *S.sanguinis* biofilm formation, but for *L.salivarius* no significant reduction was observed between conventional silanization and the ATRP process.

Concurrently, the biological effect of the mechanical stability of the peptide coatings was analyzed (**Paper III**). After 2 h of sonication in PBS, the antibacterial properties of the functionalized samples decreased. These results may indicate a partial loss of hLf1-11 peptide presence on the surface, which might be due to an acceleration of hydrolysis process of the silane layer and/or polymer brushes due to the sonication process.

For TESPSA silanized samples (**Annex I**), LIVE/DEAD backlight bacterial viability assay was performed. A statistically significant decrease in the number of *S.sanguinis* was observed onto Ti_N_TSP in comparison with control samples. However, *L.salivarius* was presented with similar numbers onto all surfaces. In order to mimic better a clinical situation, a cell-bacteria co-culture study was carried out[46,47]. It was noteworthy that, on the total coverage surface of HFFs cells was different in the presence of bacteria among all samples, which is caused by a lower number of the adhering bacteria.

Taken together all the results obtained in section 6.1.2, the data suggest that titanium treated with any of the antibacterial coatings developed through the thesis (Ti_Ag, Ti_N_TSP, Ti_N_CM_Lf, Ti_ACoI_Lf and Ti_BCoI_Lf) could overcome some of the current limitations observed with conventional antibiotics which proved limited efficiency to remove oral biofilm[48–50].

In vitro multi-biofilm assay

Dental plaque consists in an oral biofilm which involves more than 500 different interaction bacterial species[51,52]. Most of the studies in antibacterial coatings have been conducted by *in vitro* single-species biofilm formation. Therefore, it is expected that the *in vitro* multispecies biofilm assay should achieve a closer similarity to *in vivo* biofilms.

Results of bacteria assay for *S.sanguinis*, *L.salivarius* and Oral plaque after 2 hours of incubation on treated samples and control titanium were obtained (**Annex II**). As expected, the results showed a reduction of bacteria presence on all modified coatings. Differences were also detected for the biofilms exposed to treated surfaces between mono-species biofilms and whole oral plaque biofilms. Samples with hLf1-11 peptide attached by ATRP polymerization (Ti_ACoI_Lf and Ti_BCoI_Lf) showed the highest reduction in all three bacteria biofilms. Interestingly, mono-specie biofilms had a higher inhibition in bacterial adhesion in comparison with multi-specie biofilm, indicating a synergistic protection effect when different bacteria strains are present in the biofilm.

These results are consistent with the fact that different strains have different sensitivities. Moreover, the increased resistance of whole bacteria biofilms to antimicrobial agents compared to mono-specie biofilms is possible due to the changes in bacterial metabolism and genetic expression associated with diverseoit communities[53].

Afterwards, the attention turned to the long-term antibacterial properties on biofilm formation of the antibacterial coatings. Results of mono-species bacteria and oral plaque cultures on modified surfaces and control titanium after 1, 2, 3 and 4 weeks of incubation showed a drastic reduction in the amount of viable bacteria onto all treated surfaces for both strains and the oral plaque. Control titanium surfaces showed no statistically significant differences on bacteria viability at different times for all three conditions. In particular, the highest antibacterial effect was observed when silver was electrodeposited onto titanium surfaces (Ti_Ag). Noteworthy, single-species biofilm (*S.sanguinis* and *L.salivarius*) displayed a higher decrease in comparison with oral biofilm (oral plaque) as has been showed in bacterial adhesion assay, possibly for the same reasons

In vivo study in dogs

Although *in vitro* tests give valuable information of the antibacterial activity of the new surfaces, *in vivo* studies have to be performed to assess the potential application of these coatings. Thus, two of the developed antimicrobial surface treatments were tested in dental implants inserted in dogs with a ligature-induced peri-implantitis model[54,55]. Based on the *in vitro* studies, the surfaces selected for the *in vivo* dog model consist of silver electrodeposition and TESPSA silanization.

No mortality was recorded in animals and the absence of complications confirmed *in vivo* the lack of cytotoxicity observed in the *in vitro* assays. The evolution of the clinical parameters confirmed the presence of peri-implantitis infection around the dental implant[56,55] but no statistically significant

differences among groups was detected. These results suggest that the infection provoked was not severe enough to detect differences between groups by means of the clinical evaluations within the statistical power of the protocol used.

The measurements of X-ray images displayed resorption in all the samples as early as 1 month after ligature placement with statistically significant differences between treated implants and the control group. Moreover, optical microscopy observations of histological samples showed a more continuous bone, implant contact and active remodeling in Ti_N_TSP. Then, it is reasonable to assume that this enhancement in bone regeneration is due to the TESPSA immobilization, which is able to induce cell differentiation[57–59]. A possible explanation for this finding is that coatings are able to decrease the resorption of the surrounding bone and tissues, and TESPSA is able to induce cell differentiation.

Additionally, the histomorphometric parameters were also evaluated. Both BIC and BAT values were higher for Ti_N_TSP implants with statistically significant differences in comparison with control surfaces.

Even though the in vitro cytotoxicity and the in vivo tissue cell response presented a good correlation, other results (i.e. the biological evaluation of the antibacterial and/or antifouling properties presented in this work) expose the limitations of assuming the results of in vitro studies in prima facie. The in vivo results displayed less intense responses than those expected a priori from the in vitro results, possibly because other factors not considered in vitro, such as the immune system, play an important role in the effects observed. Either better in vitro models are developed for testing anti-fouling and/or antibacterial properties, or the need of in vivo studies in a proper animal model will be maintained.

6.1 Conclusions

The present PhD dissertation has been devoted to: (i) the development and optimization of antibacterial surface treatments based on organic and inorganic agents. (ii) the study of the antibacterial activity of the different treated surfaces using *in vitro* mono-species and multi-species biofilm. (iii) the study of two antibacterial surfaces in an *in vivo* dog model.

From the results of the three published papers and one submitted, the following conclusions can be derived:

- Silver deposition by an electrochemical anodizing process has been developed as an antibacterial surface treatment for titanium. Roughness and wettability did not change enough to affect cell adhesion. Treated surfaces, however, presented a significant decrease on *in vitro* bacterial adhesion for the bacterial strains *Streptococcus sanguinis* and *Lactobacillus salivarius*. The results indicated a statistically significant correlation between an increase of silver concentrationon the modified surfaces and a decrease in bacterial adhesion. Moreover, all treated samples showed good *in vitro* biocompatibility.

- A chemical process to immobilize hLf1-11 onto titanium samples without affecting roughness and wettability has been developed. The antibacterial peptide hLf1-11 has been covalently immobilized onto the surface and the treated substrates showed a significant antimicrobial activity against *Streptococcus sanguinis* and *Lactobacillus salivarius* oral bacterial strains. Moreover, these antimicrobial surfaces were totally biocompatible with human fibroblasts.
- The hLf1-11 peptide concentration on the titanium surface has been successfully increased with the atom transfer radical polymerization (polymer brush) process compared to the conventional silanization process. No cytotoxic effects were observed against human fibroblasts. A decrease in *S. sanguinis* and *L. salivarius* adhesion to the treated surfaces was detected with a higher decrease measured for surfaces modified by polymer brush based methods compared to the conventional silanization process.

The fourth manuscript led to the following conclusion:

- A protocol to covalently attach TESPSA silane to titanium has been described. No cytotoxic effects were observed against human fibroblasts and sarcoma osteoblast cells. Additionally, TESPSA-modified substrates improved osteoblast cell differentiation and showed antibacterial activity against *Streptococcus sanguinis* and *Lactobacillus salivarius* strains. Besides, co-cultures of cells and bacteria on TESPSA-treated surfaces did not show any delay in HFFs cell adhesion compared to untreated titanium samples.

The Annex IIled to the following conclusion:

- All five surface treatments (Ti_Ag, Ti__N_CM_Lf, Ti_ACoI_Lf, Ti_BCoI_Lf and Ti_N_TSP) inhibit the bacteria adhesion and biofilm formation when a single-specie and multi-specie biofilm model was used. However, a higher decrease was measured when a single-species bacteria model was studied, especially for *S. sanguinis*.

The Annex III can be summarized in the following conclusion:

- Silver deposited and TESPSA silanized dental implant surfaces reduce soft tissue and bone resoption when exposed to a ligature-indeuced peri-implantits in dog model when compared to the untreated conventional dental implant surfaces. Moreover, TESPSA-treated surfaces enhance osseointegration compared to the untreated and silver-treated dental implants.

Future work

Even though the in vitro and invo results were highly satisfactory, some specific points of the developed titanium surface treatments should be improved. To begin with,, higher silver concentration onto titanium surfaces might be explored in order to further decrease bacterial and biofilm formation without compromising biocompatibility. A possible working line is to modify the temperature of the electrolyte while applying the treatment.

Regarding the hLF1-11 peptide, an interesting future work would be the further study the effect on stability of the studied immobilization processes, in order to verify if covalent attachment can sustain long-term thermo-mechanical stresses. Additionally, further work in TESPSA silanization should include the covalent attachment of specific peptides or protein fragments to the silane in order to improve cellular behavior and antibacterial properties. In this scenario, TESPSA biological effects may be synergized with the attached biomolecules to enhance these properties.

Finally, it is expected that the application of the coatings developed in this thesis is not limited only to dental implants, but that their use can be considered for metallic biomedical devices implanted in other parts of the human body.

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