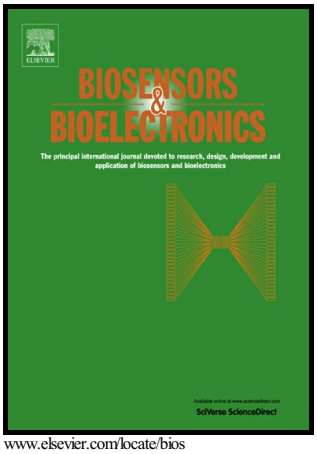

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Impedimetric antimicrobial peptide-based sensor for the early detection of periodontopathogenic bacteria

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Abstract

Peri-implantitis, an inflammation caused by biofilm formation, constitutes a major cause of implant failure in dentistry. Thus, the detection of bacteria at the early steps of biofilm growth represents a powerful strategy to prevent implant-related infections. In this regard, antimicrobial peptides (AMPs) can be used as effective biological recognition elements to selectively detect the presence of bacteria. Thus, the aim of the present study was to combine the use of miniaturized and integrated impedimetric transducers and AMPs to obtain biosensors with high sensitivity to monitor bacterial colonization. *Streptococcus sanguinis*, which is one of

the most prevalent strains in the onset of periodontal diseases, was used as a model of oral bacteria. To this end, a potent AMP derived from human lactoferrin was synthesized and covalently immobilized on interdigitated electrode arrays (IDEA). X-ray photoelectron spectroscopy (XPS) and electrochemical impedance spectroscopy (EIS) were employed to optimize and characterize the method of immobilization. Noteworthy, the interaction of *Streptococcus sanguinis* with AMP-coated sensors provoked significant changes in the impedance spectra, which were univocally associated with the presence of bacteria, proving the feasibility of our method. In this regard, the developed biosensor permits to detect the presence of bacteria at concentrations starting from 10^1 colony forming units (CFU)·mL^{-1} in KCl and from 10^2 CFU·mL^{-1} in artificial saliva. Moreover, the system was devoid of cytotoxicity for human fibroblasts. These results indicate that the proposed approach can be effective in the detection of initial stages of biofilm formation, and may be useful in the early prevention and treatment of peri-implantitis.

Keywords: Impedimetric biosensors, antimicrobial peptides, peri-implantitis, bacterial detection, biofunctionalization

1. Introduction

The restoration of missing or damaged teeth is an important aspect in modern dentistry. In this regard, dental implants have become the most frequently used materials for the management of tooth lost (Pye et al., 2009). However, despite significant progress in clinical success rates in recent years, the percentage of implant failure is still relevant (Algraffee et al., 2012; Karoussis et al., 2004).

Implant-associated infections are one of the most prevalent causes of implant failure in dentistry. Bacterial adhesion on the surface of the dental implants plays a crucial role in the onset of peri-implant diseases (Hall-Stoodley et al., 2012; Lee and Wang, 2010). Peri-implantitis is an inflammatory gum disease involving the destruction of the tissues surrounding the implant (loss of bone support), loss of attachment of the gums and the creation of a "pocket" between the teeth and the gums. Once gum disease has been diagnosed, immediate action is required in order to avoid biofilm progression. Biofilm establishment results from a specific sequence of events (Kolenbrander et al., 2010), starting with the attachment of early colonizers including *Streptococcus sanguinis* (*S. sanguinis*) and *Streptococcus gordonii* (*S. gordonii*), which subsequently facilitate the adhesion of late colonizers such as *Porphyromonas gingivalis* (Kuboniwa et al., 2006; Lee and Wang, 2010), considered one of the most important periodontopathogenic strains (Bosshardt and Lang, 2005; Leonhardt et al., 1999). Once fully structured, biofilms are highly stable and resistant to conventional antibiotic treatments (Brouwer et al., 2011). Hence, the rapid and early detection of primary colonizers would be of great value to effectively treat the initial development of peri-implantitis disease.

Many conventional laboratory-based methods of bacterial detection have been described, including cell culture techniques, microscopic analysis and biochemical assays. However, these methods typically have long

processing times, lack enough sensitivity and specificity, and are time-consuming. The use of real-time polymerase chain reaction (PCR) allows a rapid and sensitive analysis. Yet, its use entails some drawbacks, such as the complexity in primer design and the requirement of expensive equipment and reagents (Ahmed et al., 2014). In order to overcome these restrictions, new detection methods are required, and biosensors are considered very promising tools in this regard (Silva et al., 2014).

Biosensors are usually defined as an integrated system that includes a functional unit (receptor) and a transducer, which converts the detected signal into a measurable or detectable readout. The receptor element is based on a biomolecule responsible for recognition of the target substance through specific intermolecular binding. In this sense, antimicrobial peptides (AMPs) represent a highly interesting family of biomolecules because of their potent and broad antibacterial activity, ease of synthesis and intrinsic stability (Zasloff, 2002). Most AMPs are cationic (positively charged) peptides (Bahar and Ren, 2013), and as such they are electrostatically attracted to the negatively charged microbial cell membranes, but not to zwitterionic membranes of mammalian cells. This interaction is thus highly specific for bacteria. Upon binding to the membrane, AMPs can adopt an amphipathic structure which penetrates and disturbs the integrity of the lipid bilayer (Brogden, 2005; Yeaman and Yount, 2003). The capacity of AMPs to selectively interact with bacteria allows their employment as very potent detection systems. In this regard, Manoor et al. demonstrated the ability of an AMP (magainin I) as robust recognition moiety in biosensors (Mannoor et al., 2012, 2010). In these studies, an interdigitated capacitative biosensor was coated with magainin I and its capacity to detect *Escherichia coli* (*E. coli*) and *Salmonella typhimurium* evaluated by means of electrochemical impedance spectroscopy (EIS). Li et al. (Li et al., 2014) developed another impedimetric biosensor with magainin I conjugated to a structured film of ferrocene. This sensor was used to selectively detect *E. coli* O157:H7, and showed a limit of detection (LoD) of 10^3 colony forming units (CFU)·mL^{-1}. In another recent study, two synthetic AMPs with species-specific targeting (C16G2cys and G10KHc) were immobilized onto gold electrode surfaces to detect *Streptococcus mutans* (*S. mutans*) and *Pseudomonas aeruginosa* (Lillehoj et al., 2014), displaying LoDs of 10^5 CFU·mL^{-1} for both strains (Silva et al., 2014). Although those strategies have demonstrated good sensitivity for bacterial detection, some of them require extensive sample preparation and multiple assay steps prior to detection (Li et al., 2014). In other cases, the method used for peptide immobilization does not allow a covalent binding with the surface resulting in an unwanted release of the peptide over different conditions. Furthermore, none of those studies explored the detection of periodontopathogenic bacteria for application in periodontal diseases.

Among all different AMPs, short and linear cationic AMPs are particularly attractive for microbial cell sensing applications because of their small size, intrinsic stability, and easier synthesis (Dong and Zhao, 2015). The present work is focused on the N-terminal domain of the human antibacterial protein lactoferrin (LF), the hLF1-11 peptide, which contains the first eleven residues of the protein and retains its antimicrobial activity (González-Chávez et al., 2009). The effects and interactions of LF have been studied on

representative periodontopathogenic bacteria (Arslan et al., 2009; Dashper et al., 2012; Roseanu et al., 2010; Shi et al., 2000; Wakabayashi et al., 2010, 2009). Upon immobilization on a surface, the sequence hLF1-11 has shown remarkable antimicrobial activity by inhibiting the attachment and early biofilm formation of *S. sanguinis* and *Lactobacillus salivarius* while not affecting the viability and proliferation of human fibroblasts (Godoy-Gallardo et al., 2015a, 2015b, 2014). Moreover, the antimicrobial activity and minimum inhibitory concentration (MIC) of hLF1-11 have been defined for other strains involved in the development of biofilms such as *S. mutans* and *S. gordonii* (Huo et al., 2011). In another study, it was demonstrated that hLF1-11 very effectively kills different types of bacteria, including methicillin-resistant *Staphylococcus aureus* (MRSA), which is the most prevalent bacterial strain present in implant-associated infections (Dijkshoorn et al., 2004; Stallmann et al., 2005) but had no effect on erythrocytes and bone cells (Stallmann et al., 2005). This specificity is further proven by the fact that its use in vivo has not been associated with any type of toxic effects (Brouwer et al., 2011; Velden et al., 2009). Furthermore, another advantage of this peptide is that it is considerably shorter in size (11 amino acids, AA) than other AMPs that have been used for biosensing applications such as magainin I (23 AA), odorranin-HP (23 AA), leucocin A (37 AA), C16G2cys (39 AA), G10KHc (37 AA) or the sequence WK3(QL)6K2 (18 AA) (Etayash et al., 2014; Lillehoj et al., 2014; Liu et al., 2016; Mannoor et al., 2012, 2010). Thus, hLF1-11 is highly accessible by synthetic methods, and its production easier and cheaper compared to other AMPs.

Besides the biorecognition element, the transducer unit is another crucial element of a biosensor and has to be carefully designed. Amperometric and optical techniques have been the most commonly used over the last 30 years, however, nowadays the use of other methods, such as impedance and fiber optics, is rapidly expanding (Ahmed et al., 2014). Impedance-based biosensors have gained considerable interest due to their ability to perform label-free detection, low cost and ease of miniaturization.

Different kind of electrodes can be used as impedimetric transducers for bacterial detection. Of these, interdigitated electrode arrays (IDEA) present promising advantages compared to other impedimetric biosensors such as rapid detection kinetics, increase of signal-to-noise-ratio, fast establishment of a steady-state response or the absence of reference electrode (Varshney and Li, 2009). The use of functionalized IDEA for bacterial detection by the immobilization of diverse biorecognition elements on their surface like antibodies, peptides or lectins, has been recently reported (Brosel-Oliu et al., 2015). An IDEA transducer comprises two coplanar comb-like metal electrodes deposited on an insulating substrate. Conventional IDEA sensors are planar devices with a flat sensor surface. As it was previously reported (Bratov et al., 2012, 2008a) three-dimensional IDEA devices (3D-IDEA), with insulating barriers separating electrode digits, permit to enhance considerably the transducer sensitivity. This translates into a higher capacity to monitor changes in the surface charge when a target molecule binds to the sensor. On the basis of their small dimensions and high sensitivity, these devices may be regarded as promising tools for new biosensing platforms for bacterial detection.

In this work, we present a novel approach which combines hLF1-11, as a robust biorecognition moiety, and a miniaturized integrated IDEA transducer with the objective to obtain a peptide-based biosensor for direct label free bacteria detection. In this study, *S. sanguinis* was employed as a model of early colonizer (Kolenbrander et al., 2010) and promoter of biofilm formation on dental implants. The sensor modification strategy and measurement principle is schematically presented in Figure 1.

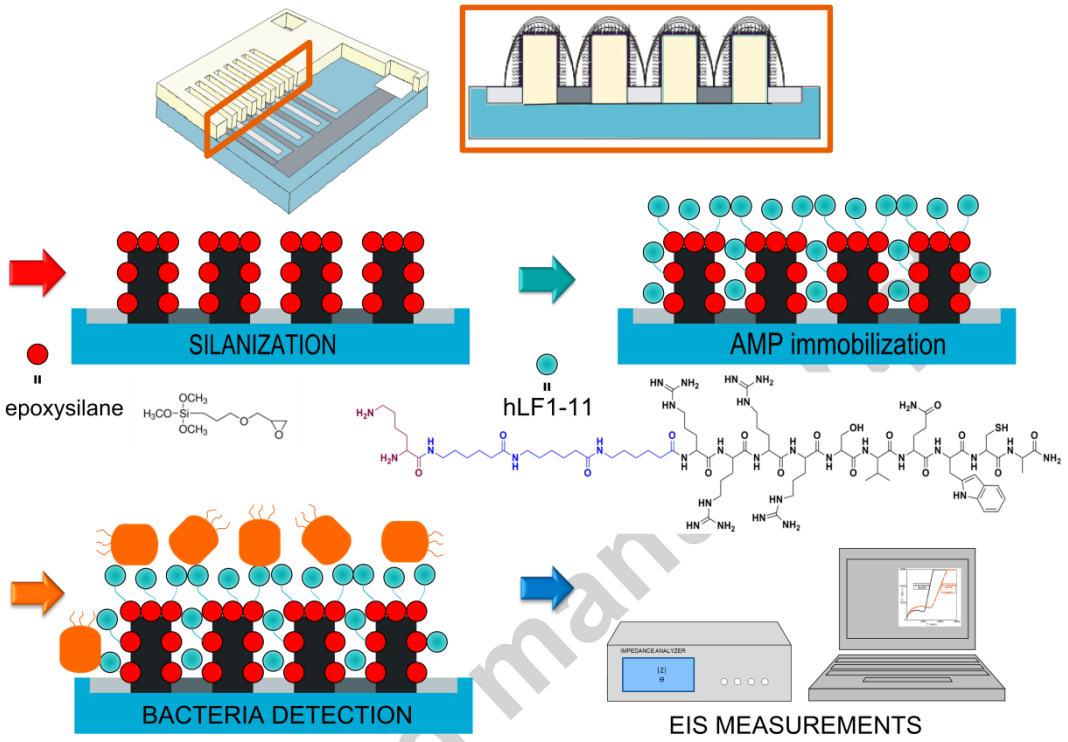

Figure 1. Sensor design and biofunctionalization method. Three-dimensional interdigitated electrode array (3D-IDEA) device in which the $TaSi_2$ electrode digits are separated by an insulating barrier of SiO_2. The electrodes were silanized in vapor phase with an epoxysilane. The hLF1-11 peptide was covalently immobilized on the surface. Bacterial detection assays were performed using *Streptococcus sanguinis* as model of oral bacteria.

2. Material and methods

2.1. Sensor Design

In this work two different types of interdigitated electrode array sensors with the same electrode geometry were used: flat (planar) and three-dimensional. The sensor chip and surface geometry of two devices are presented in Figure S1 of the Supplementary Information. Flat IDEA sensors (Figure S1B) had conducting electrodes formed directly on a silicon dioxide insulating substrate. In the case of 3D-IDEA sensors (Figure S1C, D) the electrode digits were separated by a 4µm high insulating barrier made of silicon dioxide

The design and fabrication process of both IDEA sensors is explained in more detail in Supplementary Information.

2.2. Synthesis of AMP

The peptide hLF1-11 (Figure 1) was manually synthesized in solid-phase following the Fmoc/tBu strategy and using Rink Amide- ChemMatrix® resin (0.4 g, loading of 0.48 mmol·g^{-1}) as a solid support. The synthesis and purification were adapted from a previous work (Godoy-Gallardo et al., 2014). Details on the structure (Figure S2) and characterization of this peptide are given in the Supplementary Information.

2.3. Biofunctionalization and characterization of IDEA sensors

2.3.1. Silanization of IDEA surface

Planar and 3D-IDEA sensor surfaces were first cleaned with isopropanol for 10 minutes, rinsed with distilled water and dried under nitrogen flow. Afterwards, both types of electrodes were treated with (3-glicidyloxypropyl) trimethoxysilane (Sigma-Aldrich, Spain) (epoxysilane) using two methods: vapour-phase (VP) and liquid-phase (LP) silanization. During VP silanization sensors were placed in a custom-designed cell and maintained at 50 ºC under a controlled atmosphere of epoxysilane vapor for 1 h. For LP silanization sensors were immersed into a solution of epoxysilane in absolute ethanol (1:10, v/v) and left for 1 h at 80 ºC. After this time, electrodes were rinsed with ethanol. These methods were adapted with some modifications from previous protocols (Wieringa, 2000). After silanization treatment, sensors were kept at room temperature.

2.3.2. Immobilization of AMP

A schematic representation of the silanization of the sensors and subsequent peptide immobilization is shown in Figure 1. Silanized sensors were biofunctionalized by immersion into a 100 µM solution (V = 350 µl) of peptide dissolved in PBS overnight at room temperature. The terminal epoxy group of the silane attached to the IDEA surface covalently reacts with the amino groups present at the N-terminus of the synthesized peptide. The same conditions were employed to physically absorb the AMP on non-silanized IDEA. AMP was dissolved in PBS buffer at neutral pH or at basic pH (8.5) to enhance the peptide anchoring. After peptide incubation, electrodes were gently washed with PBS to remove unbound peptide. Prior to impedance experiments, the sensors were maintained during 5 h in a KCl 10^{-5} M solution to remove possibly adsorbed PBS salts on the sensors surface and reduce their effect in the impedance measurements.

2.3.3. Chemical characterization

The immobilization method (physical adsorption or covalent grafting) of the peptide on the biosensor surface was characterized and optimized by X-ray photoelectron spectroscopy (XPS). This technique allowed the analysis of the chemical composition of the surface of the electrodes. XPS spectra of the samples were acquired with a non-monochromatic Mg anode X50 source, operating at 150 W and a Phoibos 150 MCD-9 detector (D8 advance, SPECS Surface Nano Analysis GmbH, Germany). Detector pass energy was fixed at 25 eV with 0.1 eV steps to record high resolution spectra at a pressure below $7.5 \cdot 10^{-9}$ mbar. Casa XPS software (Version 2.3.16, Casa Software Ltd., Teignmouth, UK) was used for fitting and peak integration of spectra. All binding energies were referenced to the C1s signal located at 284.8 eV. Three samples of differently modified sensors were studied.

2.3.4. Stability studies by XPS

To determine the stability of the antibacterial coatings, AMP-functionalized sensors were immersed either in KCl 10^{-5} M or in artificial saliva (0.4 $g \cdot L^{-1}$ NaCl, 0.4 $g \cdot L^{-1}$ KCl, 0.79 $g \cdot L^{-1}$ $CaCl_2 \cdot H_2O$, 0.69 $g \cdot L^{-1}$ $NaH_2PO_4 \cdot H_2O$, 0.005 $g \cdot L^{-1}$ $Na_2S \cdot 9H_2O$, pH=6.9, 1.0 $g \cdot L^{-1}$ urea) (Huang and Lee, 2005; Marino and Mascaro, 2004) for 2 h under stirring. Subsequently, the difference between conditioned samples and untreated electrodes was analyzed by XPS as described above.

2.4. Indirect cell cytotoxicity assay

2.4.1. Cell culture of human foreskin fibroblasts

Human foreskin fibroblasts (HFFs; Merck Millipore Corporation, Bedford, MA, USA) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (w/v) L-glutamine and 1% penicillin/streptomycin (50 $U \cdot mL^{-1}$ and 50 $\mu g \cdot mL^{-1}$), (all reagents from Invitrogen, Carlsbad, CA, USA) at 37 ºC in a humidified incubator and 5% (v/v) CO_2. The medium was renewed every 2 days. Cells at passage eight were used to carry out the experiments. Confluent HFFs were detached from the culture flask by incubation with TrypLE (Invitrogen) for 5 min. The HFFs solution was centrifuged at 300 G for 5 min and resuspended in new culture medium.

2.4.2. Cytotoxicity assay

Control and AMP-functionalized 3D-IDEA electrodes were used to perform an indirect cytotoxicity assay with HFFs. This assay was based on the measurement of the released lactate dehydrogenase (LDH) enzyme using the Cytotoxicity Detection Kit LDH (Roche Applied Science, Mannheim, Switzerland). A detailed description of this assay is found in the Supplementary Information (Figure S3).

2.5. Bacteria cultivation and detection assays

S. sanguinis was obtained from Colección Española de Cultivos Tipo (CECT) (CECT 480). *S. sanguinis* was grown overnight at 37 ºC in Todd-Hewitt (TH) broth (Scharlab SL, Spain) before each assay. Optical density at 600 nm (OD600) was measured and adjusted to around 0.2 ± 0.01, corresponding to a bacterial concentration of 10^8 CFU·mL^{-1}. Bacteria were then harvested by centrifugation at 9000 G for 10 minutes and resuspended in sterile 10^{-5} M KCl solution or artificial saliva at desired concentrations depending on the experiment.

Bacterial detection assays were optimized following the experimental procedure described in the Supplementary Information. In order to calculate the sensitivity of the sensors, assays in KCl and in artificial saliva were performed adjusting the concentration of *S. sanguinis* from 10^1 to 10^6 CFU· mL^{-1} under dynamic conditions to guarantee homogeneous distribution of bacteria within the sample volume. The electrodes were cultivated during 1 h in each concentration and subsequently the impedance measurements were registered.

Finally, to verify the exact concentration of *S. sanguinis* used on each experiment, bacterial solutions were also seeded on TH agar (Sharlau agar, Sharlau SL, Spain) plates, incubated for 36 h at 37 ºC and the resulting colonies were counted. All the experiments were done in sterile conditions to prevent contaminations.

2.6. Impedance Measurements

Characterization of the sensors was performed in a 100 Hz – 1000 kHz frequency range with 100 mV (amplitude) voltage excitation using Quadtech 7600 Plus LCRMeter. The measurements were performed in a non-Faradaic mode and no DC voltage bias was applied during the impedance measurements. Z-Plot/Z-View software package (Scribner Associates, Southern Pines, NC, USA) was used for impedance data treatment and for spectra fitting to an equivalent electrical circuit. All the measurements were performed in a KCl 10^{-5}M solution with a controlled conductivity ranging from 2.45 to 2.60 µS·cm^{-1}. The conductivity of the solutions was controlled with a commercial conductimeter EC-Meter GLP 31+ (Crison). All the assays were carried out in triplicates. For the simulation of mouth conditions artificial saliva was used. All the other chemicals and solvents were obtained from Sigma-Aldrich, and solutions were prepared with deionized water.

2.7. Scanning electron microscopy (SEM)

The adhesion of bacteria on IDEA sensor was verified by scanning electron microscope (SEM). IDEA chips exposed to different bacterial concentration during 4 h of incubation were fixed with 3% (v/v) glutaraldehyde in phosphate buffer 0.1 M for 24 h at 4 ºC. The chips were rinsed three times in H_2O, dehydrated in graded alcohol (ranging from 50% to 100%) and were subjected to critical-point drying with

CO_2 (Bal-Tec CPD030, Bal-Tec, California, US). Finally the chips were examined using a Merlin Fe-SEM (Zeiss, Germany) at an accelerating voltage of 10 kV (Zeiss).

2.8. Statistical Analysis

Significant differences between group means were analyzed by ANOVA test followed by post-hoc pairwise comparisons using Tukey's test, which corrects for experiment-wise error rate. Differences were also analyzed by Kruskal-Wallis non- parametric test obtaining very similar results. Confidence levels were set at 95%.

3. Results and Discussion

3.1. Characterization of the biosensor

As previously introduced EIS is an effective method to study surface modifications (Li et al., 2014). The impedance response of IDEA surfaces was studied in low conductivity KCl solutions in the absence of faradaic processes. To optimize the system, two types of sensor geometry were studied: flat and three-dimensional with barriers separating the electrode digits. In regards to the distribution of the electric field between adjacent electrodes of an IDEA sensor, it was shown that in 3D-IDEA sensors the main portion of the current goes not through the surrounding solution, as it occurs in planar IDEA sensors, but close to the surface of the barrier separating the electrodes (Bratov et al., 2008a). This permits to enhance the sensitivity of the device in detecting biochemical reactions of biomolecules attached to the surface.

The electrical equivalent circuit used for impedance spectra fitting is presented in Figure 2A and consists of the following components: R_C is the contact resistance introduced by wires and collector bars of the thin film electrodes; C_G is the geometrical (stray) capacitance between two electrodes; R_S is the resistance between two electrodes of the array; and CPE_{DL} is a constant phase element representing the capacitance of the electrical double layer at the electrode-water solution interface. Previously it has been reported that in low conducting solutions, surface conductivity plays an important role in this kind of sensors (Bratov and Abramova, 2013). Therefore, R_S is a parallel combination of bulk solution resistance (R_{BULK}) and the surface resistance (R_{SURF}) (Figure 2A). It is important to note that under experimental conditions used it is not possible to distinguish these two elements in the impedance spectra. However, it is possible to fix the bulk solution conductivity and attribute the changes in R_S to surface resistance (Bratov and Abramova, 2013; Brosel-Oliu et al., 2015).

Impedance spectra may be presented in different ways, though Nyquist plots (Z´ vs. Z´´) are the most common way to visualize the spectra and determine R_S. Nyquist plot of the experimental impedance spectra includes a semicircle at high frequencies corresponding to resistance R_S in parallel to the stray capacitance (Figure 2B). The intersection with the real axis on the left side gives the R_C values, while the intercept on the right side gives the value of R_S (which is the parallel combination of R_{BULK} and R_{SURF}). As all the

experiments were performed in KCl solutions with fixed conductivity, R_{BULK} values remained constant and determined, R_S changes were attributed to R_{SURF} alteration which is produced by the surface charge changes between the electrode digits generated by a biochemical reaction at the sensor surface (Brosel-Oliu et al., 2015).

Accordingly, IDEA surface modification by silanization, peptide immobilization and bacterial attachment, resulted in specific changes in R_S in subsequent experiments. In the case of bacterial attachment studies, the sensor response was presented as variations in R_S determined before (R_S^0) and after (R_S^1) bacteria interaction. This variation is calculated as: $\Delta Rs = Rs^1 - Rs^0$

As observed in the low frequency region of the Nyquist plots (Figure 2B), bacterial adhesion not only provokes changes in R_S but also modifies the interfacial capacitance (C_{DL}) and the constant phase element (CPE) alpha parameter. This is due to the formation of an additional layer over the electrodes, which results in an increase in C_{DL} and decrease in the alpha parameter as a result of the non-ideality of the double layer/interfacial capacitor. Subsequent experimental values (R_S, C_{DL}, alpha) obtained by fitting the spectra to the equivalent circuit in Figure 2A are presented in Table S1 of the Supplementary Information. Nevertheless, ΔRs was chosen as a principle sensor parameter due to its large scale changes and higher reproducibility.

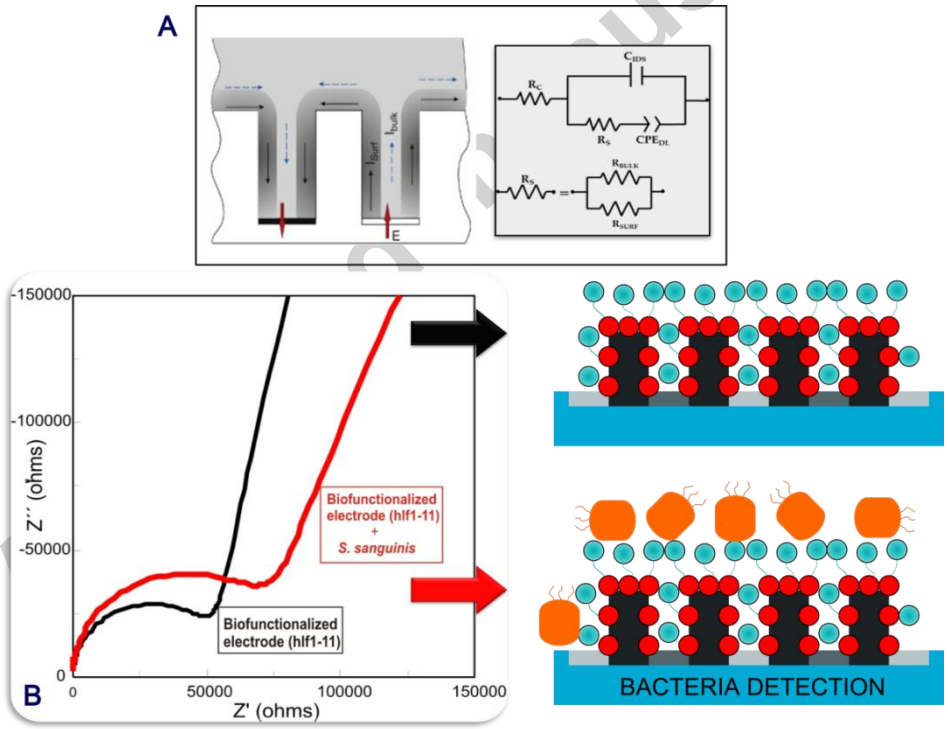

Figure 2. The electrical equivalent circuit used for impedance spectra fitting (A). Experimental impedance spectra are represented in Nyquist plots (B). Superficial conductivity was affected by *S. sanguinis* attachment, involving an increase in measured resistance (R_S). As the solution bulk resistance (R_{BULK}) remained constant these changes were attributed to surface resistance (R_{SURF}) increase.

3.2. Optimization of peptide immobilization by EIS

Firstly, the effect of varying the surface density of the immobilized AMPs on the detection of bacterial cells was investigated (see Figure S4 in the Supplementary Information). The impedimetric response to bacteria was found to increase with the concentration of immobilized peptide, reaching a maximum of detection at 100 μM. Thus, this concentration was chosen to conduct the experiments.

To obtain the highest density of AMPs on the IDEA surface, preliminary studies were carried out with planar and 3D-IDEA morphologies as well as using different immobilization strategies.

As follows from experimental data remarkable differences were observed on the basis of the techniques used to immobilize the AMP (Figure 3A). Impedance results indicate that VP silanization yields a higher increase in R_S values compared with LP silanization and physisorption methods. This suggests that VP silanization promotes the highest binding of AMP on IDEA surfaces. This result is in accordance with previous studies where VP silanization was also shown to be more effective than LP silanization in the adhesion of biomolecules (Zhu et al., 2012). Our data also indicate that the AMP can also be physically absorbed on the surfaces. Although physisorption is a simple and widely used method to immobilize molecules on a surface, it should be taken into consideration that the stability of the binding is significantly lower in comparison with silanization methods, which provides a covalent and stable binding (Chen et al., 2013).

Regarding the geometry of the sensor, as shown in Figure 3A, the impedimetric response in 3D-IDEA is much higher than in planar IDEA, which entails a considerable improvement in sensitivity. Owing to its specific design, when an AC potential current is applied between the electrodes of a 3D-IDEA, the main portion of the current flows not through the surrounding solution but close to the surface of the barriers that form a microcapillary over each electrode digit. Changes in the electrical charge distribution at the solid/liquid interface provoke respective changes in conductivity along the barrier surface which is registered by the device (Bratov and Abramova, 2013; Bratov et al., 2012). Therefore, the results were in agreement with previous reported studies (Bratov et al., 2008a, 2008b), and 3D-IDEA were chosen as the transducer element in the following experiments.

After the addition overnight of AMP 100 μM in PBS on silanized electrodes, the IDEA were maintained in a low conductivity KCl solution to reduce the unspecific effect of PBS in the EIS measurements. Figure 3A also indicated that after 3 h of this stabilization in KCl, the impedance value was maintained practically invariable in all conditions.

Therefore, from this preliminary assay it was concluded that the most optimal combination to perform bacterial detection studies was the use of 3D-IDEA biosensor functionalized with an AMP by VP silanization. Moreover, the importance to stabilize the electrodes during 3 h in KCl 10^{-5} M before the measurement was demonstrated. The sensor-to-sensor signal variability between biofunctionalized sensors was within 9%. Once the biosensor design was established, their effectiveness on detecting bacteria was

evaluated. To this end, four different conditions were tested: non-treated and VP-silanized electrodes, which were used as controls, and electrodes functionalized with the AMP, either via physisorption or VP silanization. The capacity of these sensors to detect the presence of bacteria was tested using *S. sanguinis* as a model of oral bacteria. As previously explained, the interaction of the bacteria with the AMP was expected to produce a measurable increase in the impedance spectra of the system.

As shown in Figure 3B, the presence of the AMP provoked an increase in the Rs response after 1 and 2 h of incubation, compared to control surfaces. This effect was more pronounced when the peptide was covalently attached to the sensors (Figure 3B). This effect is likely due to the presence of a spacer-anchor unit in the hLf1-11 peptide, which ensures a covalent binding of the peptide to the epoxysilane and an adequate availability and accessibility of the antimicrobial motif for interaction with bacteria (Godoy-Gallardo et al., 2014; Mas-Moruno et al., 2013) (see the Supporting Information for further details). In the absence of AMP on the surface, impedimetric changes were not statistically different. Therefore, this study suggests that the presence of the hLF1-11 peptide on the electrodes surface permits to register the binding of *S. sanguinis* within the first 2 hours of incubation. Thus, impedance-based sensors coated with AMPs can effectively detect the presence of bacteria on the surfaces.

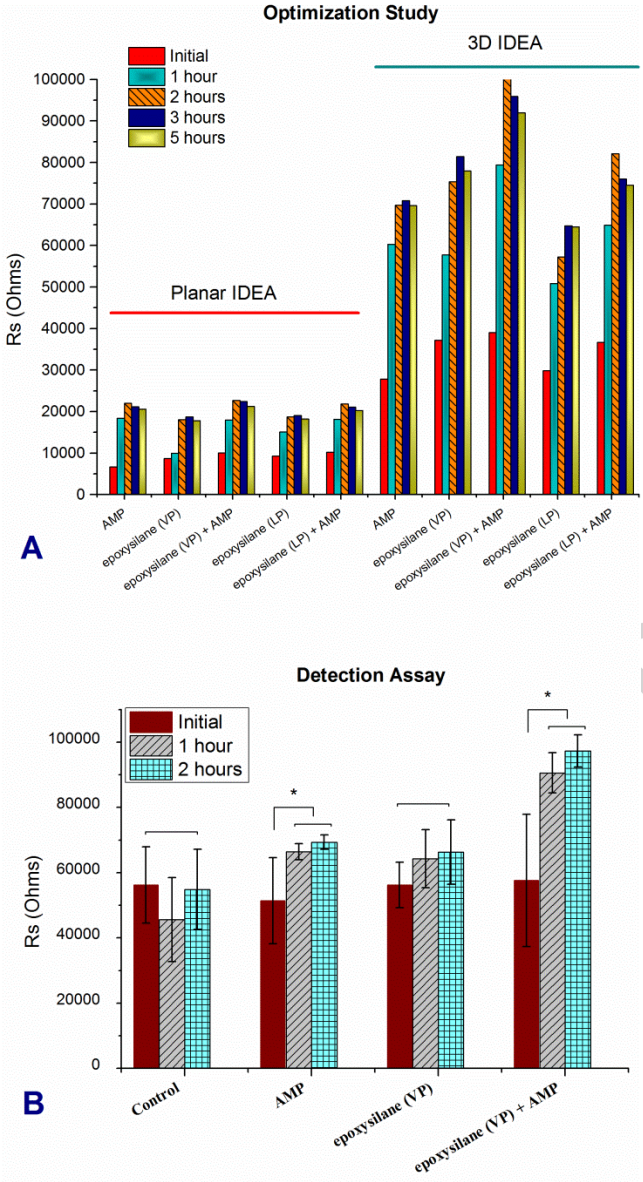

Figure 3. Optimization of peptide immobilization and bacterial detection by EIS in 10^{-5} M KCl solution. Optimization study between planar IDEA and 3D-IDEA and between different immobilization methods. AMP physisorbed (AMP), VP silanization (epoxysilane VP), VP silanization with AMP (epoxysilane VP + AMP), LP silanization (epoxysilane LP) and LP silanization with AMP (epoxysilane LP + AMP) (A). Bacterial detection assay in 3D-IDEA sensors in 10^{-5} M KCl solution with non-modified electrode (control), AMP physisorbed (AMP), VP silanization (epoxysilane VP), VP silanization with AMP (epoxysilane VP + AMP) (B). Statistically significant differences are indicated with an "*"($P < 0.05$).

In order to further confirm the presence of bacteria owing to AMP activity, the electrodes surface was visualized by SEM (Figure 4 and Figure S5 in the Supplementary Information). SEM images were in concordance with the impedance response measured. No bacterial attachment was observed on non-functionalized electrodes that were immersed in a bacterial solution of 10^5 CFU· mL^{-1}. In contrast, the presence of bacteria attached on the surface was clearly evident when the sensors were functionalized with

the AMP and treated under the same conditions. As expected, bacteria were neither observed in control sensors.

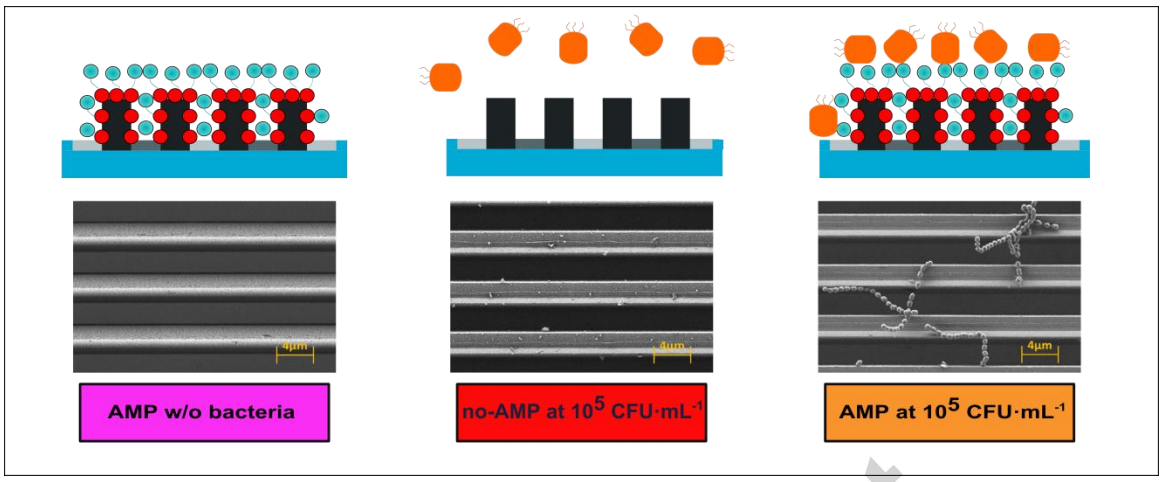

Figure 4. Visualization of bacterial attachment on the sensors by SEM. *AMP w/o bacteria*: biofunctionalized sensors in KCl solution; *no-AMP at* 10^5 *CFU·mL*$^{-1}$: non-functionalized sensors in bacterial culture; *AMP at* 10^5 *CFU·mL*$^{-1}$: biofunctionalized sensors in bacterial culture.

3.3. Chemical characterization and stability assay by XPS

The success in the functionalization strategy was further demonstrated by means of XPS studies. The results of the atomic composition of the surfaces (Table S2 in the Supplementary Information) show that coating the sensors with the AMP, either by physical adsorption or covalent immobilization, resulted in significant increases in the percentages of carbon (C 1s) and nitrogen (N 1s), together with reductions in the detectable amounts of tantalum (Ta 4f) . The tantalum signal comes from $TaSi_2$ layer forming the electrode digits. These results are in agreement with the deposition of the silane and/or peptide layers on the surfaces. In particular, the N 1s signal is an excellent indicator of peptide attachment, since this element is not present in non-functionalized surfaces. The C 1s signal cannot be used for such purpose, because it is also present in the control as a result of adsorbed organic contaminants from the environment. The largest percentage of nitrogen (N 1s) was found for VP silanization followed by peptide immobilization at pH=8.5 (i.e. a pH that facilitates the nucleophilic attack of the epoxide by the amino groups of the AMP), thus proving this method optimal for peptide attachment on the biosensors. Of note, these results are in accordance with impedance data (see Figure 3).

The stability of the coating was also verified by XPS. To this end, the electrodes were immersed during 2 h in KCl 10^{-5} M under stirring. The same treatment was done with artificial saliva to mimic conditions of salinity present in the mouth. XPS data illustrate (Table S3 in the Supplementary Information) that the percentage of nitrogen remained constant in both solutions after the stability treatment, hence demonstrating the coating is stable in KCl and in artificial saliva.

3.4. Cell cytotoxicity assay

The possibility to integrate the biosensing platform in a dental implant makes necessary to test that the electrodes are devoid of toxicity for human cells. Thus, the indirect cytotoxicity of the electrodes (both initial electrodes and AMP-coated) toward HFFs was studied for 1 day of incubation in different concentrations of solutions in contact with the material surface (Figure S6 in the Supplementary Information). These results were obtained using Cytotoxicity Detection Kit LDH (see Supporting Information for details). Noteworthy, all evaluated surfaces exhibited values of cell viability above 80% for all the dilutions studied. These results underscore that neither the sensors, nor the peptides, have cytotoxic effects for fibroblast cells.

3.5. Bacterial detection assays

3.5.1. Sensitivity of the system in KCl solution

To evaluate the sensitivity of the developed biosensor in bacterial detection, EIS analysis was performed using *S. sanguinis* at different concentrations. These studies were carried out in 10^{-5} M KCl solutions with a range of bacterial concentrations, from 10^1 to 10^6 CFU· mL^{-1}, after 1 h of incubation. This incubation time was established during optimization studies (see Supplementary Information and Figure S7 for details), and is substantially shorter than detection times described in previous studies (Li et al., 2014). Thus, after 1 h of incubation, the sensors were extracted from the electrochemical cells, rinsed gently with 10^{-5} M KCl and their spectra were measured in KCl solution with the same concentration. As previously indicated, KCl solutions with low conductivity were used to reduce the effect of the bulk solution electrolyte on the impedance response during bacterial incubation.

As illustrated in Figure 5A, a linear correlation between ΔRs and the logarithm of the cell concentration was observed in the range of 10^1–10^5 CFU· mL^{-1} with the correlation coefficient of R^2=0.924. The equation derived can be expressed using the linear model $y= bx +a$. This model is used to compute the sensitivity of the system, b, versus the logarithm of bacteria concentration, which was found to be 3.45 ± 0.5 kΩ per bacteria concentration decade. The LoD, can be determined as $LoD = 3\ S_a/\ b$ (Shrivastava and Gupta, 2011), where S_a is the standard deviation of the response and b is the slope of the calibration curve. The LoD was found to be as low as $3.5 \cdot 10^1$ CFU· mL^{-1}, which is comparable with or better than other detection limit values previously reported for direct label-free bacterial detection (Basu et al., 2014; Dweik et al., 2012; Escamilla-Gómez et al., 2009; Li et al., 2014; Silva et al., 2014; Varshney and Li, 2007).

3.5.2. Sensitivity of the system in artificial saliva

Artificial saliva (Huang and Lee, 2005; Marino and Mascaro, 2004) was used as an incubation medium for *S. sanguinis* to simulate the salinity and conductivity of the mouth environment and validate the use of the

developed biosensor for bacterial detection under physiological conditions. Moreover, this medium was used to prove whether the level of detection of the biosensor obtained in KCl is affected in more complex solutions.

Thus, bacterial detection assays were done as previously explained in the range of 10^1 CFU· mL^{-1} and 10^5 CFU· mL^{-1} using artificial saliva instead of KCl (Figure 5B). As observed in KCl measurements, the ΔRs response followed a linear correlation (R^2=0.872) with bacterial concentrations in the range of 10^1–10^4 CFU· mL^{-1}. In this case, the sensitivity was of 3.85 ± 1.3 kΩ per bacteria concentration decade and the LoD obtained was $8.6 \cdot 10^2$ CFU·mL^{-1}. This difference in regards to KCl data is probably due to the matrix effect induced by the saliva components that can mildly influence in biosensor response.

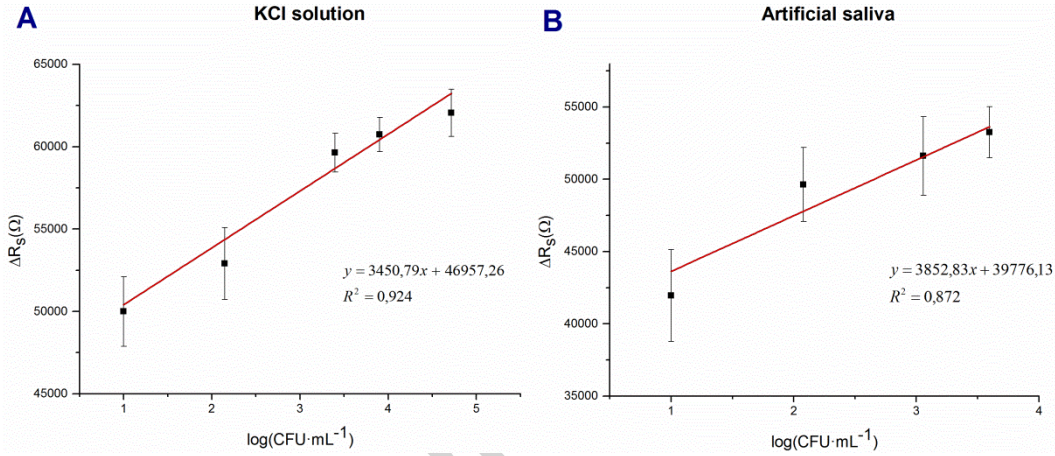

Figure 5. Linear regression fit of the measured impedance and the *S. sanguinis* concentration in KCl solution (A) and artificial saliva (B). The electrodes were in contact with each concentration during 1 hour.

4. Conclusions

In this study, an innovative impedimetric AMP-based biosensor has been introduced for the detection of the initial steps of biofilm formation, and consequent prevention of the development of peri-implantitis disease.

The type of biosensor and its biofunctionalization have been optimized by means of XPS and EIS techniques, concluding that 3D-IDEA sensors functionalized with the AMP hLF1-11 via VP silanization represent the most promising approach for detection of periodontopathogenic bacteria.

Impedance results clearly demonstrate that sensors biofunctionalized with this AMP sense the presence of pathogenic *S. sanguinis*, translating this interaction into measurable impedimetric signals. The biosensor developed in these studies has shown the possibility to detect oral bacteria at $3.5 \cdot 10^1$ CFU·mL^{-1} in a very short detection time (1 h) in 10^{-5} M KCl solution and at $8.6 \cdot 10^2$ CFU·mL^{-1} in a model of artificial saliva, thereby proving the feasibility and potential of this system. Although follow-up studies need to be done in more complex scenarios, e.g. in real saliva, to totally validate the adequateness and sensitivity of the sensor, it has to be noted that the detection assays of this impedimetric biosensor is comparable or better than those

described in the literature for similar sensors. Another important characteristic of our sensors is that the hLF1-11 is highly effective against different types of bacteria but has no effect on fibroblasts, erythrocytes and bone cells. Future experiments will investigate the selectivity of this sensor towards other types of cells and different strains of bacteria.

In summary, coupling of AMPs with 3D-IDEA biosensors has resulted in the implementation of a rapid, label-free, sensing platform with high sensitivity for the detection of the periodontopathogenic strain *S. sanguinis*, which was chosen as example of primary colonizer. This fully integrated AMP-based sensor array can be potentially used for the detection of a broad spectrum of bacteria and therefore could be a useful tool in the prevention of initial formation of biofilm and the consequent implant related-infections.

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Appendix A. Supplementary Material

Supplementary data associated with this article can be found, in the online version, at……..

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Highlights:

- A new impedimetric biosensor for bacterial detection with high sensitivity towards *Streptococcus sanguinis* is presented.
- The sensor combines an impedimetric transducer (3D-IDEA) and with an antimicrobial peptide (hLf1-11) (AMP) shorter and easier to synthetize than others AMPs.
- The antimicrobial peptide was covalently attached to the sensor via vapor phase silanization.
- The sensor effectively detected the presence of *Streptococcus sanguinis*, as a model of oral bacteria, within 1 hour of incubation with a limit of detection of 10^1 CFU·mL^{-1} in KCl and 10^2 CFU·mL^{-1} in artificial saliva.
- The limit of detection of the sensor was set at 10^1 colony forming units (CFU)·mL^{-1} in KCl solution and at 10^2 CFU·mL^{-1} in artificial saliva.
- This biosensor can be effective in the detection of implant-related infections initial stages of biofilm formation.