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

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

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 and specificity for bacterial detection. 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. Moreover, the limit of detection of our sensor was set at 10^3 colony forming units (CFU)·mL^{-1}. These results indicate that our approach can be effective in the detection of early stages of biofilm formation, which could translate in the effective prevention and treatment of peri-implantitis.

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* (Lee and Wang, 2010)(Kuboniwa et al., 2006), 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. Several biological elements can be used for the manufacturing of biosensitive systems, such as enzymes, antibodies, antigens, nucleic acids, aptamers, lectins, phages and peptides (Arugula and Simonian, 2014; Caygill et al., 2010; Prodromidis, 2010; Silva et al., 2014; Šmuc et al., 2013).

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. 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) to serve as robust recognition moiety in biosensors (Mannoor et al., 2012, 2010). In these studies, an interdigitated capacitative biosensor for detection of *Escherichia coli* and *Salmonella typhimurium* was coated with magainin I and its capacity to detect these bacteria evaluated by means of electrochemical impedance spectroscopy (EIS). Li et al. (Li et al., 2014) developed another impedimetric biosensor also 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 antibacterial protein lactoferrin (LF), a rich source of cationic and hydrophobic AMPs. The antibacterial activity of LF has been widely documented both in vitro and in vivo for Gram-positive and Gram-negative bacteria, as well as for some acid-alcohol-resistant bacteria (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). Although many AMPs have been isolated from LF and characterized, only three of them have been studied in detail. These are LF1- 11, lactoferrampin, and lactoferricin (Sinha et al., 2013). LF1-11, as its name suggests, is the N-terminal peptide of LF, containing the first eleven residues of the protein. The potent antimicrobial effect of LF1-11 was attributed to the first two arginines at the N-terminus. When immobilized on a surface, the sequence hLF1-11 has shown remarkable antimicrobial activity by inhibiting the attachment and early biofilm formation of strain *S. sanguinis* and *Lactobacillus salivarius* (Godoy-Gallardo et al., 2015a, 2015b, 2014). Moreover, the antimicrobial activity and minimum inhibitory concentration (MIC) of LF1-11 have been defined for other strains such as *S. mutans* and *S. gordonii* (Huo et al., 2011).

Besides the biorecognition element, the transducer unit is another crucial element of the sensor and has to be carefully designed. Amperometric and optical techniques have been the most commonly used over the last 30 years, however, the use of more recent methods, such as impedance and fiber optics, is now 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). This type of transducer can be assembled into an IDEA with two coplanar metal electrodes separated by insulating barriers (3D-IDEA). Their ability to monitor changes in surface impedance when a target molecule binds on the sensor surface has been documented in previous reports (Bratov et al., 2012, 2008a).

In this work we describe a novel approach which combines the use of miniaturized and integrated IDEA and the ability of hLF1-11 as a robust biorecognition moiety to obtain a peptide-based biosensor with high sensitivity and low LoD to detect the presence of bacteria. The stepwise assembly of this sensor is schematically presented in Figure 1.

S. sanguinis was employed as a model of early colonizer (Kolenbrander et al., 2010) and promoter of biofilm formation on dental implants. The attachment and stability of the peptide on the sensor was characterized and optimized by means X-ray photoelectron spectroscopy (XPS) and EIS measurements. Bacterial adhesion and the LoD were analyzed by EIS and further confirmed by scanning electron microscopy (SEM). The cytotoxicity of the sensors was evaluated with human fibroblasts.

The proposed detection method is rapid, cost effective, sensitive, and may be regarded as a promising tool to detect the development of the initial biofilm formation and consequently prevent peri-implantitis disease.

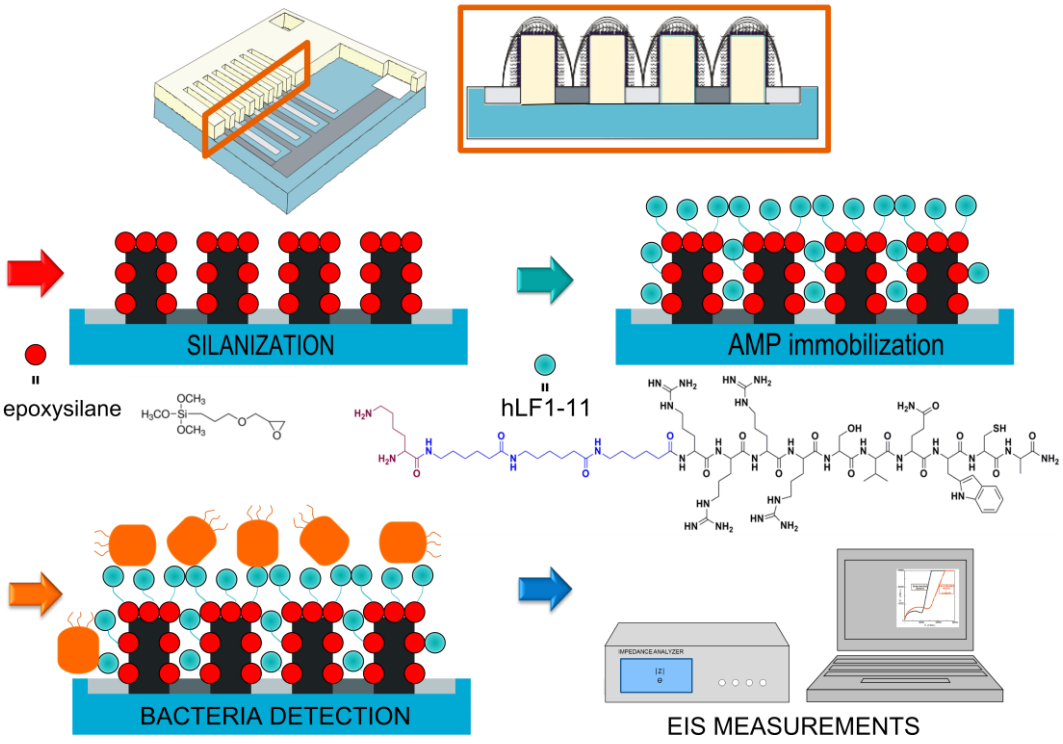

Figure 1. Sensor design and biofunctionalization method. Three-dimensional interdigitated electrode array (3D-IDEA) device in which the $TaSi_2$ electrode digits are separated by an insulating barrier of SiO_2. The electrodes were silanized in vapor phase with an epoxysilane. The hLF1-11 peptide was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

Material and methods

Sensor Design

The IDEA was formed on a silicon wafer covered with a 2.5 µm silicon dioxide layer by "wet" oxidation at 950 ºC. In the next steps, a 230 nm thick layer of highly conductive tantalum silicide ($TaSi_2$) was deposited using magnetron sputtering. The first photolithographic step defines collector bars and digits of two electrodes. The pattering is done by reactive ion etching technique, setting up interdigitated electrodes with 216 digits of 3µm width and 3µm gap between the adjacent electrodes. The aperture between the electrodes is 1.4 mm and the total length between the electrodes is 301 mm. To form contact pads 1 µm of aluminium was deposited and patterned using standard photolithographic and etching steps leaving metal solely at the extremes of the two collector bars.

The wafer of planar IDEA was covered with 700 nm thick silicon dioxide layer deposited by low pressure chemical vapour deposition (LPCVD) in which windows over electrode digits and aluminium contact pads were opened. In 3D-IDEA the final step is the barrier formation. The device is covered with 4 µm LPCVD silicon dioxide (SiO_2), and photolithography is used to define the openings in the oxide layer over the electrodes digits and contact pads. Complete technology of sensors is presented elsewhere (Bratov et al., 2008a).

Synthesis of AMP

The peptide hLF1-11, containing three units of 6-aminohexanoic acid (Ahx) as a spacer and 2 units of Lysine (Lys) as the anchoring moiety ($(Lys)_2$ -$(Ahx)_3$- GRRRRSVQWCA-NH_2) (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 [11] The purified peptide was characterized by analytical HPLC (10 to 35 % MeCN over 8 min, t_R = 4.888 min) and MALDI-TOF (m/z calcd. for $C_{80}H_{141}N_{31}O_{17}S$: 1840.08, found: 1841.82 $[M+H]^+$).

Biofunctionalization and characterization of IDEA sensors

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 the epoxysilane (3-glicidyloxypropyl)trimethoxysilane (Sigma-Aldrich, Spain) using two methods: vapour-phase (VP) and liquid-phase (LP) silanization. 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. 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 washed with ethanol. These methods were adapted with some modifications from previous protocols (Wieringa, 2000). After silanization treatment, planar and 3D-IDEA were kept at room temperature.

Immobilization of AMP

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 (see Figure 1). The same conditions were employed to physically absorb the AMP on non-silanized IDEA (physisorption). AMP was dissolved in PBS buffer at neutral pH or at basic pH (8.5) to enhance the anchor of the peptide. After peptide incubation, electrodes were gently washed with PBS to remove unbound peptide. Prior to impedance experiments, the sensors were maintained during for 5 h in a KCl 10^{-5} M solution to reduce the effect of PBS salts in the measurements. A schematic representation of the silanization of the sensors and subsequent peptide immobilization is shown in Figure 1.

Chemical characterization

The immobilization method (physical adsorption or silanization) 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 7.5×10^{-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.

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 $g \cdot L^{-1}$ $CaCl_2 \bullet H_2O$, 0.69 $g \cdot L^{-1}$ $NaH_2PO_4 \bullet H_2O$, 0.005 $g \cdot L^{-1}$ $Na_2S \bullet 9H_2O$, 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 analysed by XPS as described above.

Indirect cell cytotoxicity assay

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.

Cytotoxicity assay

Control and AMP-functionalized 3D-IDEA electrodes were used to perform an indirect cytotoxicity assay. To this end, three samples of each group were immersed in complete medium and incubated at 37 ºC for 72 h. Cell culture medium in contact with the electrodes will be referred as "electrodes-medium".

The viability of HFFs was quantified by measuring the released lactate dehydrogenase (LDH) enzyme using the Cytotoxicity Detection Kit LDH (Roche Applied Science, Mannheim, Switzerland). For that purpose, HFFs were seeded onto 96-well plates with a density of 10.000 cells per well/sensor and grown for 24 h. After this time, cell culture medium was aspired and replaced by "electrodes-medium" at different dilutions (ranging from 1:1 to 1:1000), and cells were subsequently incubated for 24 h. Cells were then lysed with 200 µl of M-PER® (Pierce, Rockford, IL, USA) per well and the release of LDH was measured spectrophotometrically at 492 nm using a multimode microplate reader (Infinite M200 PRO, Tecan Group Ltd., Männedorf, Switzerland). Cells cultured on tissue culture polystyrene (TCPS), but not treated with M-PER, were used as a negative control, and lysed cells cultured on TCPS were used as a positive control (minimum and maximum releasable LDH activity, respectively).

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 1×10^{-5} M KCl solution or artificial saliva at desired concentrations depending on the experiment.

Bacterial detection assays were carried out in different electrochemical cells of 150 mL immersing biofunctionalized IDEA sensors in 100 mL of bacteria resuspended in KCl solution. Bacterial detection assays in KCl solutions were performed adjusting the concentration of *S. sanguinis* at 10^2, 10^3, 10^4 and 10^5 CFU· mL^{-1} under dynamic conditions to guarantee homogeneous distribution of bacteria within the sample volume. Assays in artificial saliva were done only at 10^3 and 10^5 CFU· mL^{-1} bacterial concentrations. Two controls were included in the experiments: biofunctionalized IDEA sensors that were immersed in solutions without bacteria, and non-functionalized electrodes that were immersed in solutions containing the higher concentration of bacteria. All assays were performed in dynamic conditions to ensure a homogeneous distribution of bacterial cells.

Finally, to verify the 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 counted. All the experiments were done in sterile conditions to prevent contaminations.

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. 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 $\mu S \cdot cm^{-1}$. 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.

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).

Results and Discussion

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. The electrical equivalent circuit used for impedance spectra fitting is presented in Figure 2B 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 2). 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).

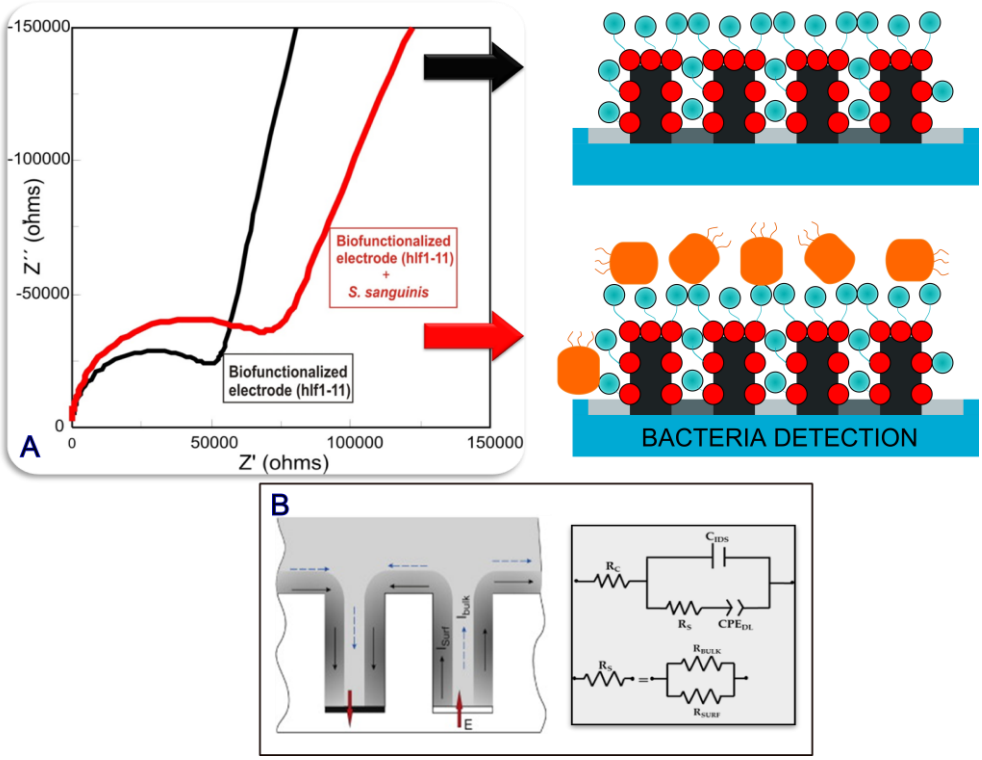

Figure 1. Experimental impedance spectra, represented in Nyquist plot (A), were analysed with an adjusted equivalent circuit (B). Superficial conductivity was affected by *S. sanguinis* attachment, involving an increase in measured resistance (R_S). As the solution bulk resistance (R_{BULK}) remained constant these changes were attributed to surface resistance (R_{SURF}) increase.

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 2A). 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: $\Delta Rs = Rs^1 - Rs^0$

Optimization of peptide immobilization by EIS

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 studies reported (Bratov et al., 2008a, 2008b), and 3D-IDEA were chosen to carry out the rest of the 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 2 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 2 h in KCl 10^{-5} M before the measurement was demonstrated.

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 of the system.

As shown in Figure 3B, the presence of the AMP provoked an increase in the impedance 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). In the absence of AMP on the surface, impedimetric changes were practically imperceptible. 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.

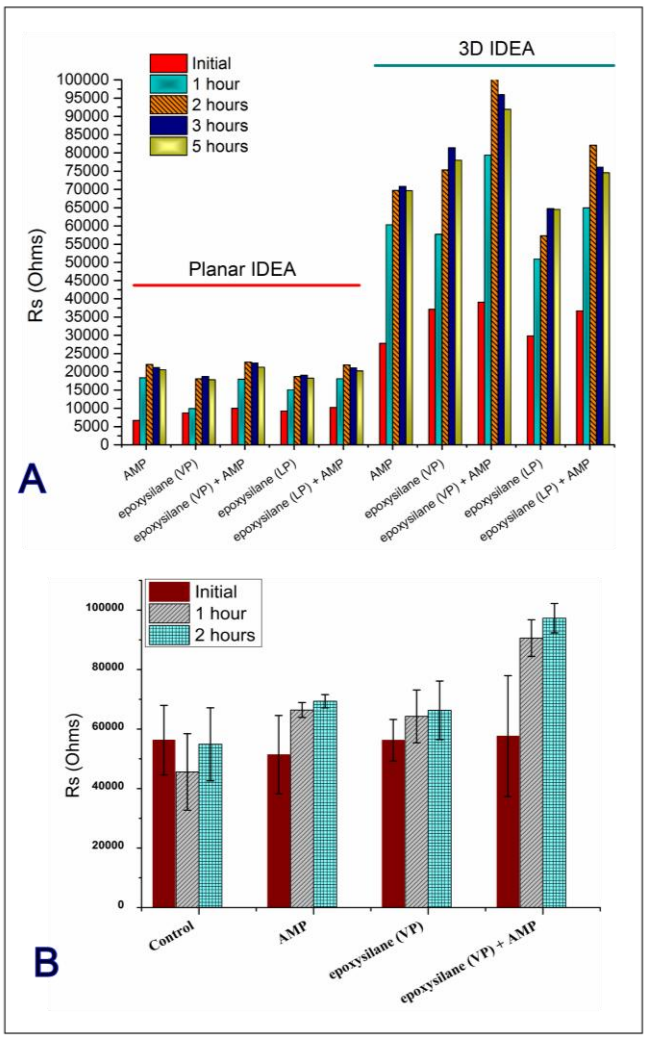

Figure 2. Optimization of peptide immobilization and bacterial detection by EIS. Optimization study between planar IDEA and 3D-IDEA and between different immobilization methods (A). Bacterial detection assay in 3D-IDEA sensors in KCl solution with AMP physisorbed (AMP), VP silanization (epoxysilane) and VP silanization with AMP (B).

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 were summarized in Table 1, 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) (Experiment a). 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 1, Experiment b) 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.

Table 1: Chemical composition (at %) of IDEA surfaces. (AS = Artificial Saliva).

	C1s	N1s	O1s	Si 2p	Ta 4f
Experiment a					
control	26.61±3.84	0.06±0.08	36.60±2.06	33.81±3.02	2.93±1.21
AMP	44.84±9.67	3.20±0.66	25.55±3.78	24.60±6.13	1.82±0.19
epoxysilane	37.43±8.65	0.09±0.10	28.81±7.65	27.45±6.64	2.31±1.07
epoxysilane+AMP	56.05±7.75	2.62±0.42	21.27±3.71	19.05±4.29	1.02±0.26
epoxysilane+AMP (pH=8.5)	51.19±8.63	3.71±0.26	21.62±3.73	21.87±4.30	1.62±0.43

Experiment b					
control	31.66±2.55	0.07±0.07	21.43±1.84	27.59±0.51	7.73±0.59
epoxysilane+AMP	51.46±4.07	3.06±0.32	23.09±1.00	18.64±3.61	3.75±0.76
epoxysilane+AMP (2h KCl)	45.03±4.80	3.20±0.54	25.62±2.15	22.84±2.59	3.31±0.51
epoxysilane+AMP (2h AS)	54.26±5.66	3.10±0.54	22.56±1.46	16.69±3.15	3.39±0.80

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 4). 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.

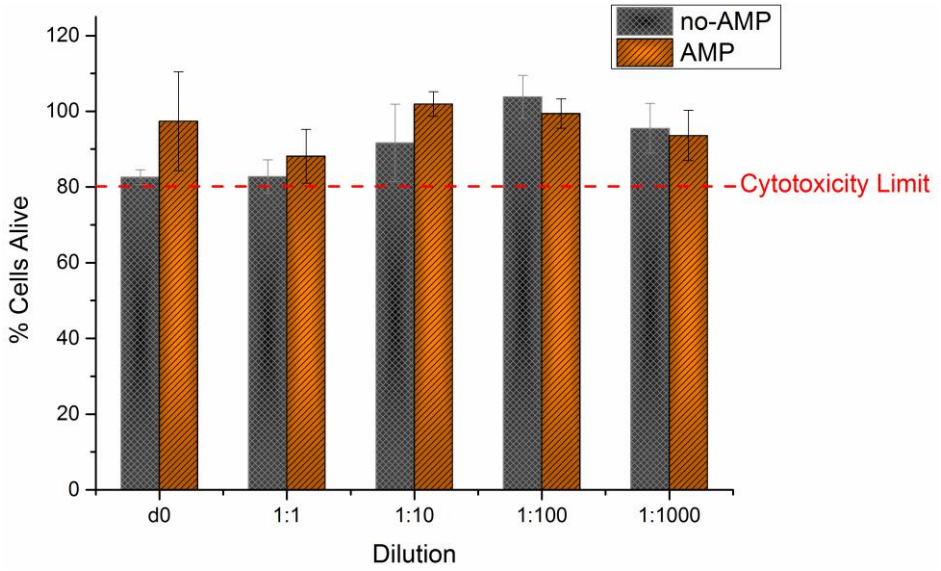

Figure 4. Cytotoxicity assay: cell viability (%) higher than 80% on all materials dismissed a potential cytotoxic effect.

Bacterial detection assays

Detection 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 performed in a 1×10^{-5} M KCl solutions

with a range of bacterial concentrations, from 3.60 × 10^2 to 1.52 × 10^5 CFU· mL^{-1}. Periodically, at 1, 2 and 4 h of incubation , the sensors were extracted from the sample, rinsed gently with 10^{-5} M KCl and their spectra were measures in KCl 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.

Figure 5A illustrates that impedimetric responses increase proportionally with the number of *S. sanguinis* present in the solution. Therefore, the higher bacterial concentration (i.e. 1.52 × 10^5 CFU· mL^{-1}) on the peptide-coated IDEA surface involved the highest impedance response. Interestingly, when non-functionalised 3D-IDEA were immersed in a solution with the same concentration of bacteria, no significant changes in impedance response were observed, thereby discarding the unspecific adhesion of bacteria on the sensors. Thus, the capacity of the biosensor surface to detect the presence of *S. sanguinis* was univocally attributed to the AMP biorecognition moiety, demonstrating the effectiveness and specificity of the developed biosensor.

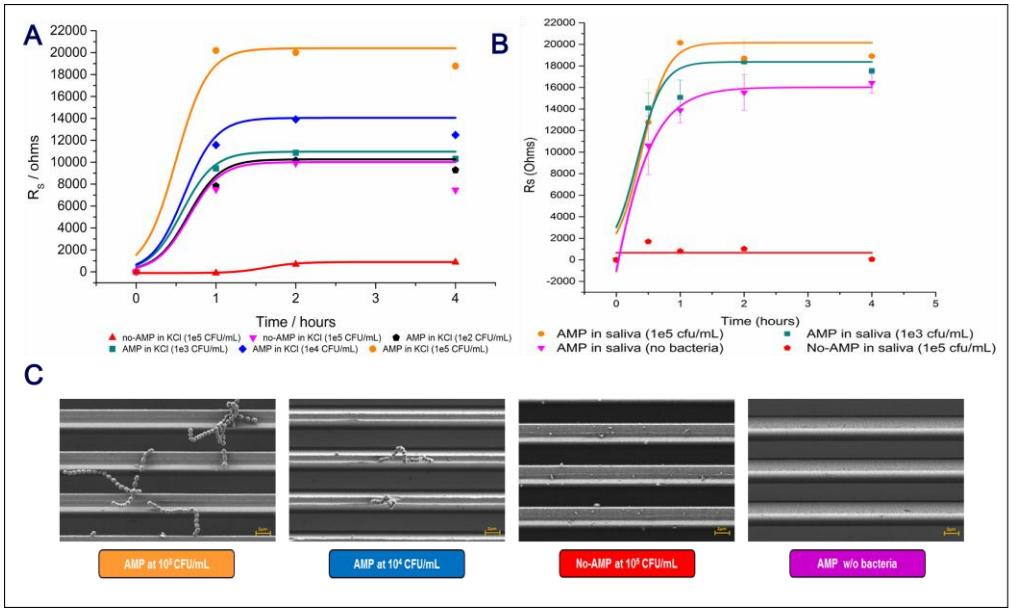

Figure 5. LoD assays in KCl and artificial saliva solutions. A: Assay with different concentrations of *S. sanguinis* showed that Rs values increase proportionally with number of bacteria. B: LoD assay in artificial saliva solution. The impedance data illustrates that LoD was not affected by artificial saliva. C: The presence of bacteria at different concentrations was visualized by SEM. The results showed that the presence of bacteria increases proportionally with their concentration in the incubation solution, in agreement with impedance data.

Noteworthy, changes in impedance were also correlated to the concentration of bacteria. Resistance increase was practically imperceptible at low concentration of *S. sanguinis* (3.60 × 10^2 CFU· mL^{-1}) compared with the control sensor. However, a clear increased in impedance was observed in the biosensors when higher bacterial concentrations were used. The minimum concentration of bacteria at which a significant variation in

impedance was observed in regards to the control was 4.18×10^3 CFU· mL^{-1}. Thus, this concentration was set as the LoD of the biosensor. Such LoD is comparable with other developed impedance sensors for direct label-free bacteria 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).

Another important parameter is the time of detection. As shown in Figure 5, variations in impedance were already observed during the first hour of incubation, reaching a saturation phase at longer incubation times. Thus, the detection time for this sensor was established at 1 h, which is substantially shorter than in previous studies (Li et al., 2014).

In order to further confirm the impedance results, the presence of bacteria in the electrodes was visualized by SEM (Figure 5C). The density of bacteria present on the surface was in concordance with the impedance response measured. No bacterial attachment was observed on non-functionalized electrodes that were immersed in a solution of 1.52×10^5 CFU· mL^{-1} of bacteria. 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. At lower concentrations, the presence of bacteria was proportionally reduced (shown for 10^3 CFU· mL^{-1} in Figure 5B). Below the LoD, no bacteria were observed (not shown). As expected, bacteria were neither observed in control electrodes.

Detection 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 LoD of the biosensor obtained in KCl is affected in more complex solutions.

Thus, bacterial detection assays were done as previously explained but using artificial saliva instead of KCl. In this case, only two bacterial concentrations were tested (2.25×10^5 and 4.10×10^3 CFU· mL^{-1}). As illustrated in Figure 5B no impedimetric response was obtained with non-functionalised IDEA at the highest concentration of bacteria. In contrast, the presence of the AMP provoked changes in impedance at the two concentrations tested, thereby demonstrating that artificial saliva does not alter the capacity of the sensors to detect bacteria. The sensors showed sensitivity to *S. sanguinis* at 10^3 CFU·mL^{-1}, which was comparable with the LoD obtained in the previous assays. Therefore, the kinetics of bacterial attachment was the same in saliva as in KCl, and the detection time was also established at 1 hour.

Conclusions

In this study, an 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 an AMP via VP silanization represent the most promising approach for bacterial detection.

In this regard, our work has proven that the AMP selected (i.e. the peptide hLF1-11) can be used as an effective molecular recognition element of bacteria in biosensing platforms. Impedance results clearly demonstrate that sensors biofunctionalized with this AMP specifically sense the presence of pathogenic *S. sanguinis* translating this into measurable impedimetric signals.

The biosensor developed in these studies has shown an excellent LoD (10^3 CFU· mL^{-1}) and very short detection times (1 h) in both 10^{-5} M KCl solution and in a model of artificial saliva, thereby proving the feasibility and potential of this system. Although more 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 LoD of this impedimetric biosensor is comparable or better than those LoD described in the literature for similar sensors.

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 a periodontopathogenic strain: *S. sanguinis*. 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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Figure 1

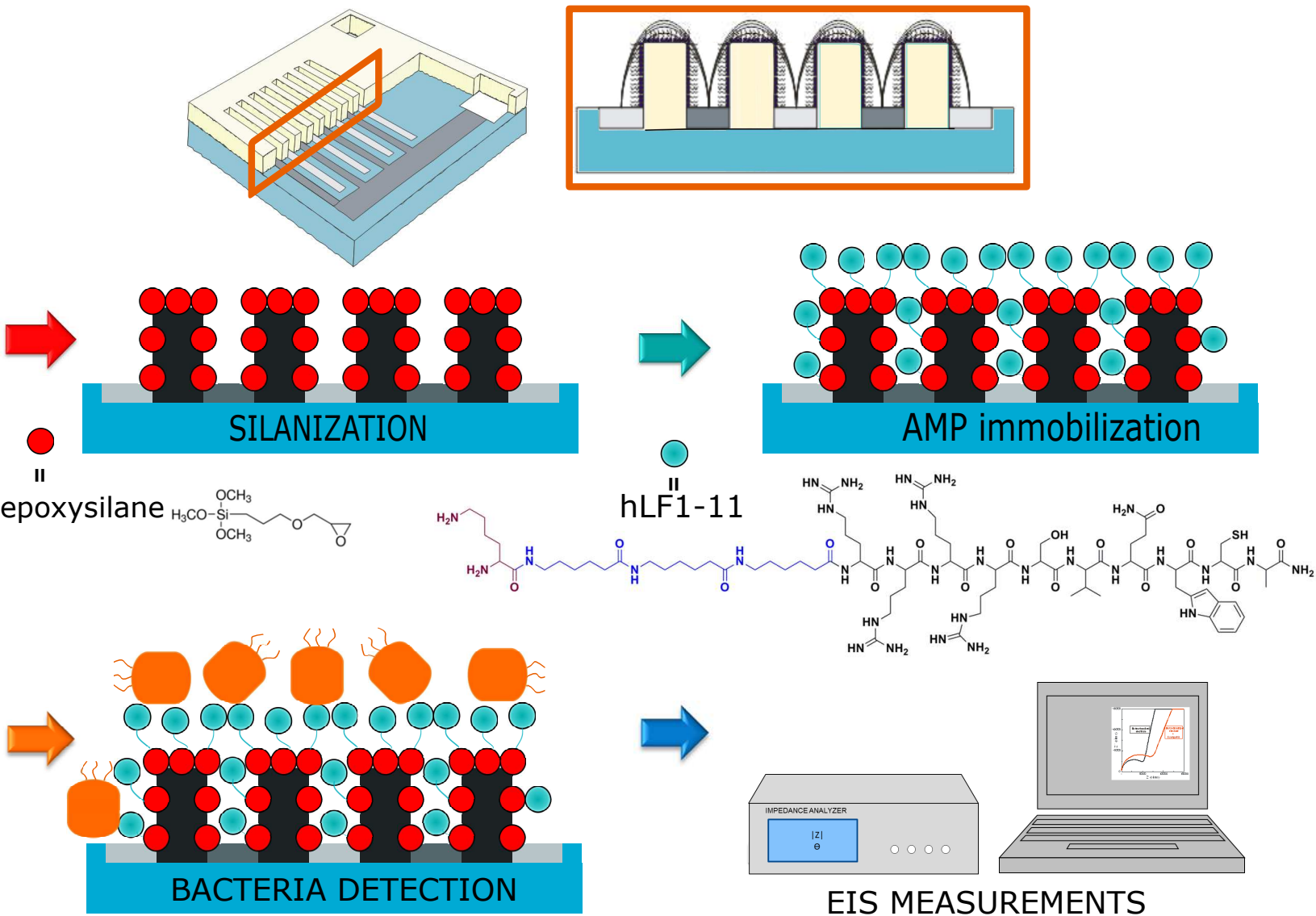
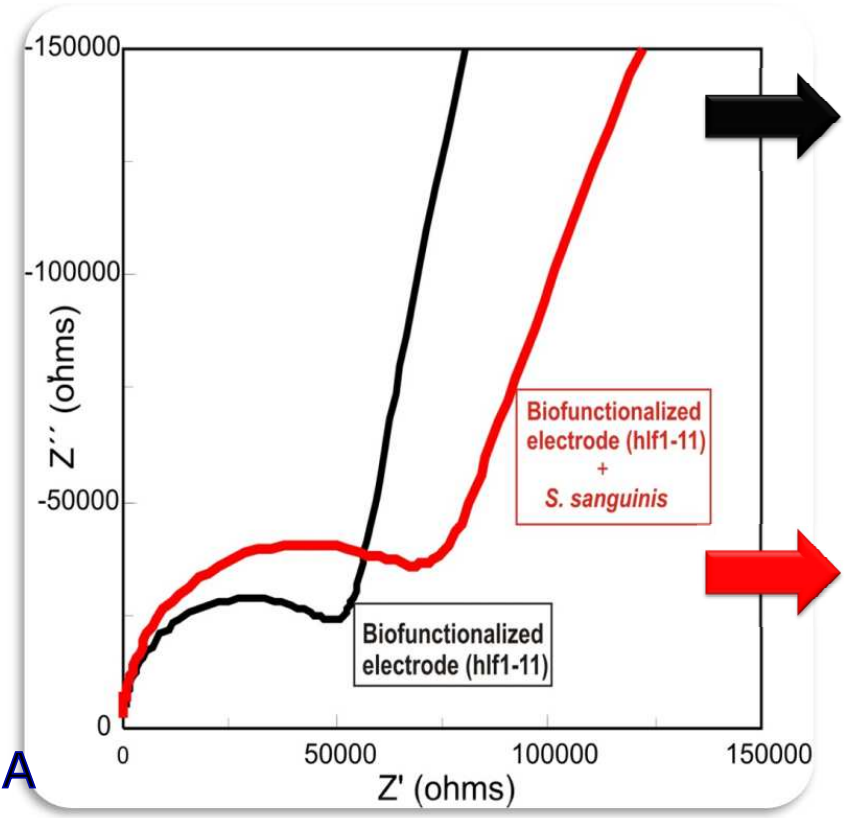
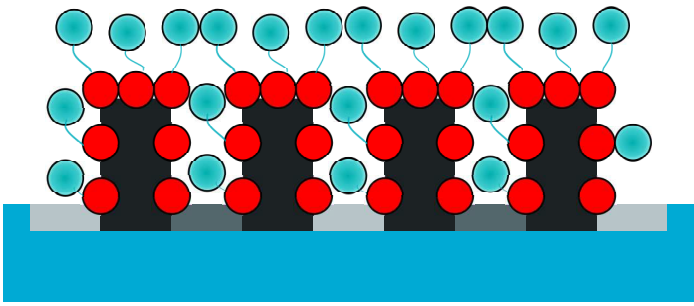
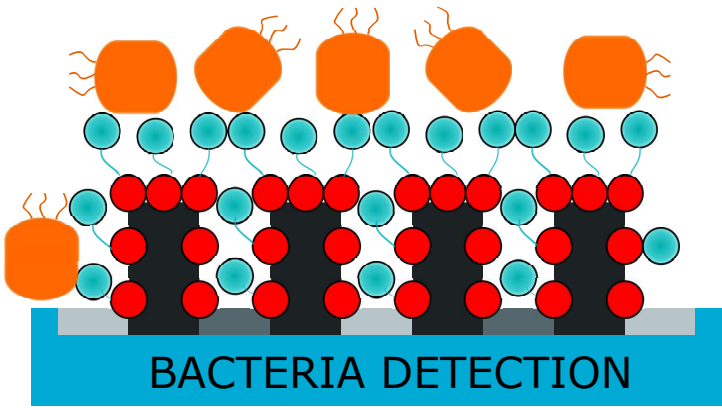
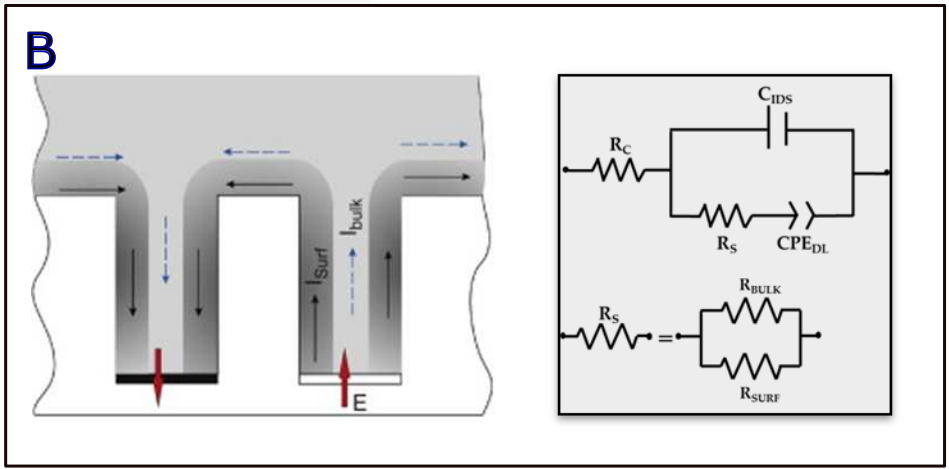

SILANIZATION
AMP immobilization
epoxysilane
hLF1-11
BACTERIA DETECTION
IMPEDANCE ANALYZER
EIS MEASUREMENTS

Figure 2


-150000
-100000
-50000
0
Z'' (ohms)
0
50000
100000
150000
Z' (ohms)
Biofunctionalized electrode (hlf1-11) + S. sanguinis
Biofunctionalized electrode (hlf1-11)
A




BACTERIA DETECTION


B
I_bulk
I_Surf
E
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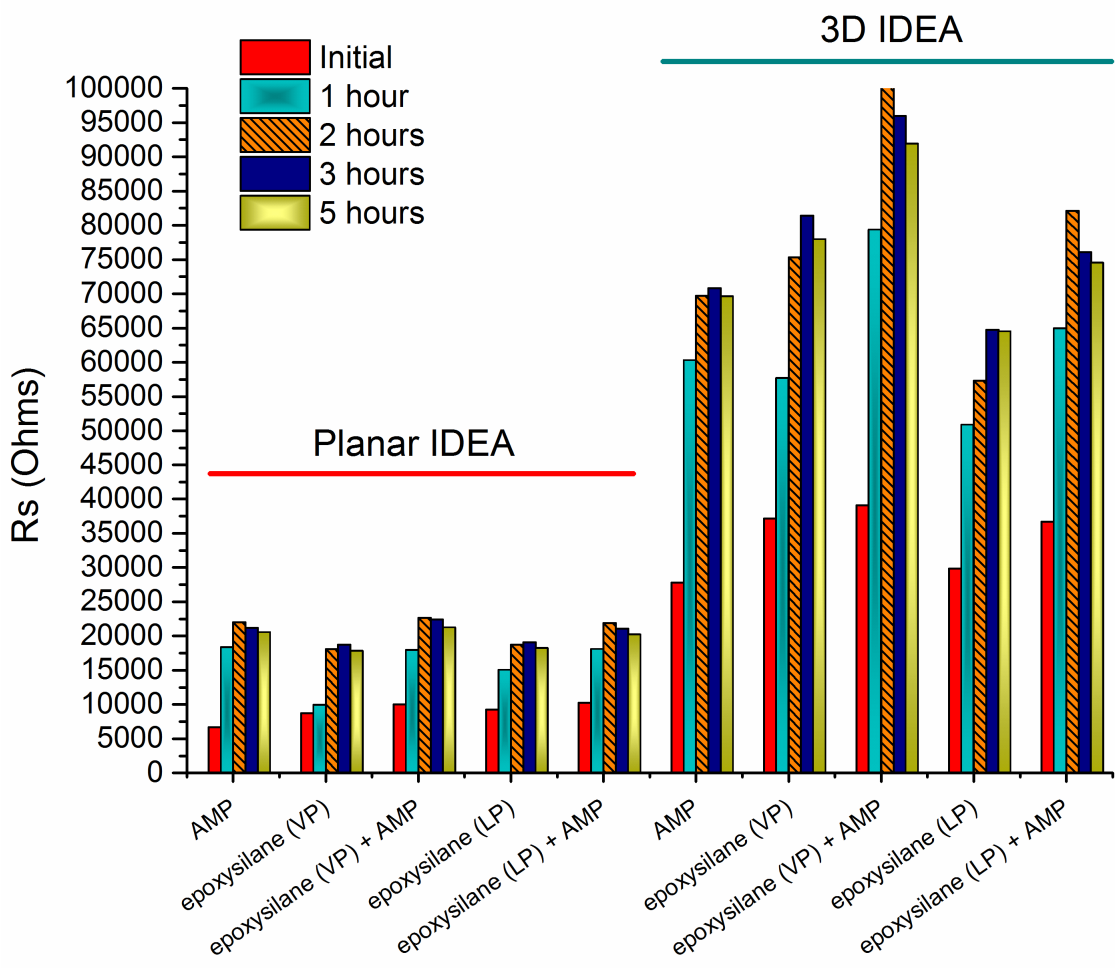
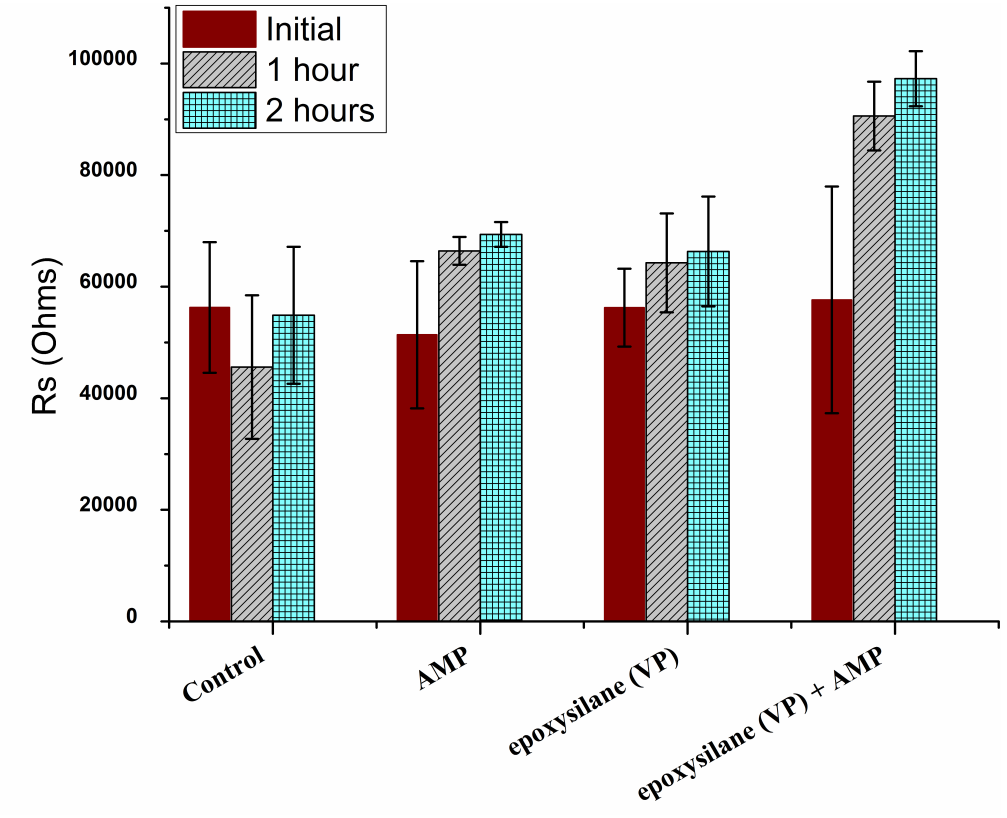
R_C
R_S
CPE_DL
R_BULK
R_S
R_SURF

Figure 3


3D IDEA
Initial
1 hour
2 hours
3 hours
5 hours
Planar IDEA
Rs (Ohms)
100000
95000
90000
85000
80000
75000
70000
65000
60000
55000
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
AMP
epoxysilane (VP)
epoxysilane (VP) + AMP
epoxysilane (LP)
epoxysilane (LP) + AMP
AMP
epoxysilane (VP)
epoxysilane (VP) + AMP
epoxysilane (LP)
epoxysilane (LP) + AMP

A


Initial
1 hour
2 hours
Rs (Ohms)
100000
80000
60000
40000
20000
0
Control
AMP
epoxysilane (VP)
epoxysilane (VP) + AMP

B

Figure 4

Click here to download high resolution image

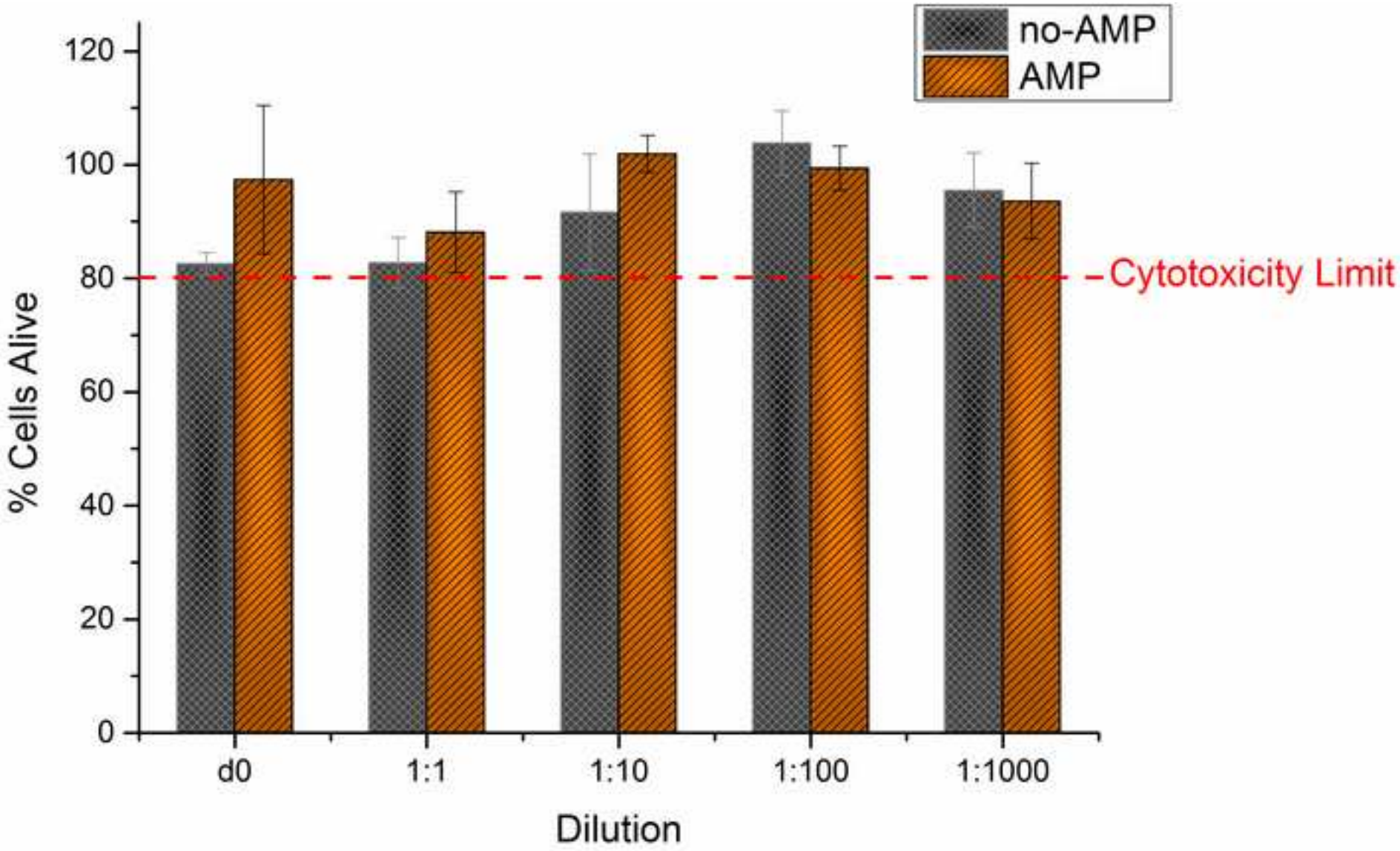
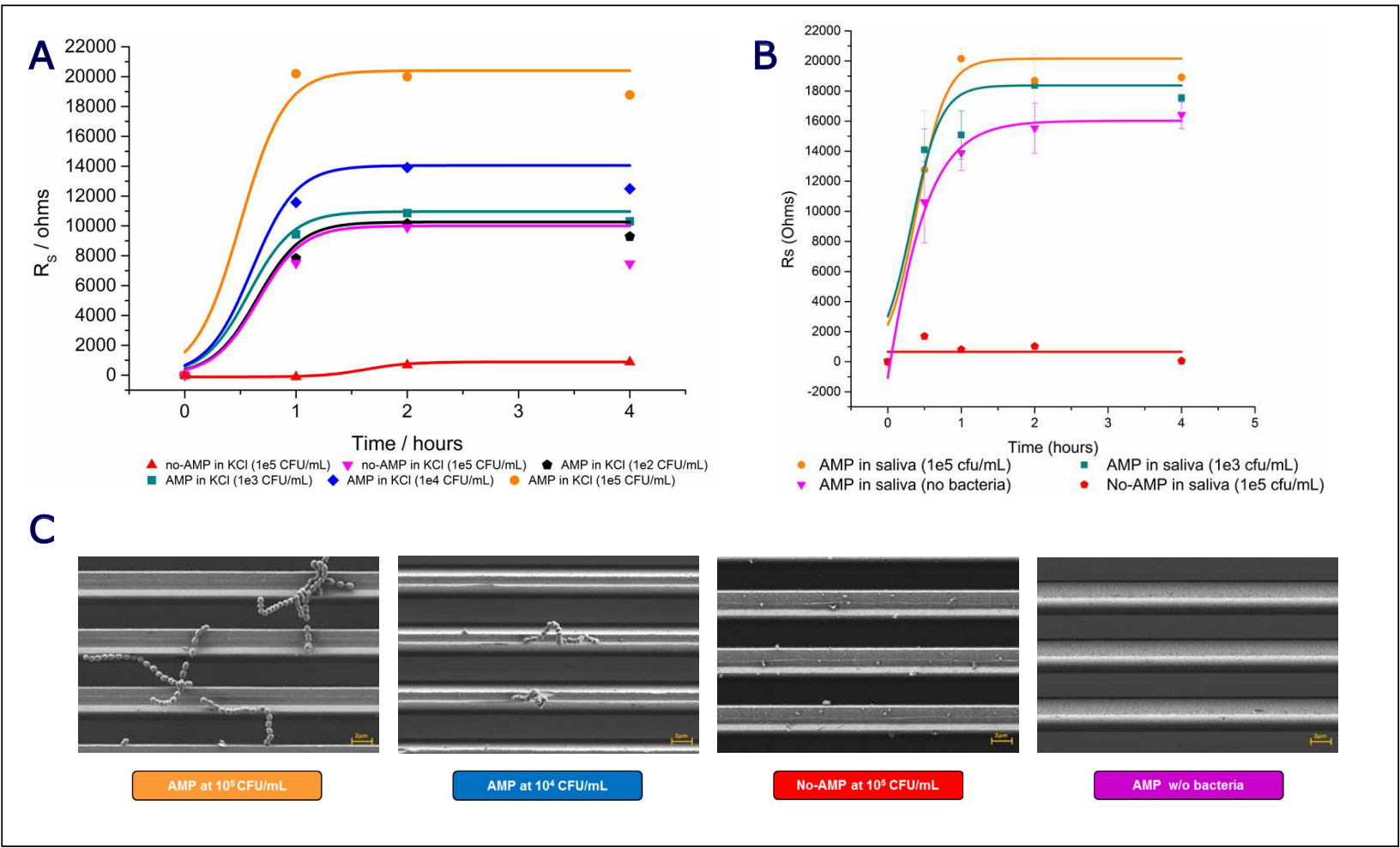


Figure 5


A
R_S / ohms
Time / hours
no-AMP in KCl (1e5 CFU/mL)
no-AMP in KCl (1e5 CFU/mL)
AMP in KCl (1e2 CFU/mL)
AMP in KCl (1e3 CFU/mL)
AMP in KCl (1e4 CFU/mL)
AMP in KCl (1e5 CFU/mL)
B
Rs (Ohms)
Time (hours)
AMP in saliva (1e5 cfu/mL)
AMP in saliva (1e3 cfu/mL)
AMP in saliva (no bacteria)
No-AMP in saliva (1e5 cfu/mL)
C
AMP at 10^5 CFU/mL
AMP at 10^4 CFU/mL
No-AMP at 10^5 CFU/mL
AMP w/o bacteria

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

Mireia Hoyos-Nogués, Sergi Brosel-Oliu, Natasha Abramova, Francesc-Xavier Muñoz, Andrey Bratov, Carlos Mas-Moruno, Francesc-Xavier Gil

Highlights:

- A new biosensor for bacterial detection is presented.
- The sensor combines an impedimetric transducer and an antimicrobial peptide.
- The antimicrobial peptide was covalently attached to the sensor via vapor phase silanization.
- The sensor effectively detected the presence of *S. Sanguinis*.
- The limit of detection of the sensor was set at 10^3 colony forming units (CFU)·mL^{-1}.
- This sensor can be effective in the detection of early stages of biofilm formation.