Universitat Autònoma de Barcelona

INTERDIGITATED ELECTRODE ARRAYS (IDEA) IMPEDIMETRIC TRANSDUCERS FOR BACTERIAL BIOSENSING APPLICATIONS

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– Tesi doctoral –

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Departament de Genètica i Microbiologia
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The thesis submitted by Sergi Brosel Oliu, graduate student in Biology and Master degree in Applied Microbiology, entitled **Interdigitated electrode arrays (IDEA) impedimetric transducers for bacterial biosensing applications** was carried out at Insititut de Microelectrònica de Barcelona (IMB-CNM, CSIC) under the supervision of Dr. Andrei Bratov Nikiforov, Senior Researcher, Dr. Natalia Abramova Pavlova, Researcher, and Dr. Naroa Uria Moltó, Post-Doc Researcher of IMB-CNM. The thesis satisfies the requirements for obtaining the title of PhD in Biotechnology.

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ABBREVIATIONS, SYMOBOLS AND UNITS

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List of abbreviations

3D-IDEA	Three-dimensional Interdigitated electrode arrays
AC	Alternating current
AMP	Antimicrobial peptide
Amp	Ampicillin
AST	Antimicrobial susceptibility tests
ATCC	American Type Culture Collection
AuNPs	Gold nanoparticles
BioMEMS	Biological microelectromechanical systems
BIS	*N,N´*-methylenebis(acrylamide) cross-linker
BOD	Biological oxygen demand
BSA	Bovine serum albumin
CBPs	Carbohydrate binding proteins
CD	Circular dichroism
CLSM	Confocal laser scanning microscopy
CMOS	Complementary metal-oxide-semiconductor
CNM	Centre Nacional de Microelectrònica
Con A	Concanavalin A
CPE	Constant phase element
CSIC	Consell Superior d'Investigacions Científiques
DC	Direct Current
DEP	Dielectrophoresis
DEPIM	Dielectrophoretic impedance measurements
DNA	Deoxyribonucleic acid
DRIE	Deep reactive ion etching
E. coli	*Escherichia coli*
EC	Equivalent circuit
EIS	Electrochemical Impedance Spectroscopy
Fig.	Figure
GFP	Green fluorescent protein
Gly	Glycogen
HMDS	Hexamethyldisilazane
HRP	Horseradish peroxidase
HUS	Hemolytic uremic syndrome
IDEA	Interdigitated electrode arrays
IMB	Institut de Microelectònica de Barcelona
ISE	Ion selective electrode
IUPAC	International Union of Pure and Applied Chemistry
LAL	Limulus amebocyte lysate method
LB	Luria-Bertani medium
LOD	Limit of detection
LPCVD	Low pressure chemical vapor deposition
LPS	Lipopolysaccharides

LTA	Lipoteichoic acid
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MIPs	Molecularly-imprinted polymers
MPTES	3-mercaptopropyl-trimethoxysilane
MRB	Methacryloxyethyl thiocarbamoyl rhodamine B
Mw	Molecular weight
OD	Optical density
p(NIPMAM)	Poly(N-isopropylmethacrylamide)
PBS	Phosphate buffer saline
PCB	Printed circuit board
PCR	Polymerase chain reaction
PDMS	Polydimethylsiloxane
PEI	Polyethyleneimine
PSS	Poly(sodium 4-styrenesulfonate)
RNA	Ribonucleic acid
SAM	Self-assembled monolayer
SDS	Sodium dodecyl sulfate
SELEX	Systematic evolution of ligands by exponential enrichment
SEM	Scanning Electron Microscopy
SI	Supplementary information
SPR	Surface plasmon resonance
TRIS	Tris(hidroxymetil)aminometane
UAB	Universitat Autònoma de Barcelona
UV	Ultraviolet
WBR	Western Blocking Reagent
WHO	World Health Organization

List of symbols

Δ	Increment
C	Capacitance
C_{CYT}	Cytoplasm capacitance
C_{DL}	Double layer capacitance
C_F	Biomolecules additional capacitance
C_G or C_{IDS}	Geometrical capacitance
C_{MEMEB}	Bacteria cytoplasm capacitance
C_S	Solution capacitance
I	Intensity or current
j	$\sqrt{-1}$
K_{CELL}	Cell constant
l	Length between electrode digits
R	Resistance
R_{BULK}	Bulk solution resistance
R_C	Contact resistance
R_{CT}	Charge/electron transfer resistance

R_{CYT}	Cytoplasm resistance
R_{MEMBR}	Bacteria Membrane resistance
R_S	Solution resistance/ resistance between the electrodes
R_{SURF}	Surface resistance
s	Spacing between electrode digits
SD	Standard deviation
T	Temperature
t	Time
V	Voltage or potential
w	Width between electrode digits
X	Reactance
Z	Impedance
$Z_{BACTERIA}$	Impedance of a whole bacteria
Z_C	Impedance of a capacitor
Z_{CPE}	Impedance of a Constant Phase Element
Z_{im} or Z''	Imaginary part of impedance
Z_R	Impedance of a resistor
Z_{re} or Z'	Real part of impedance
Z_S	Solution impedance
Z_W	Warburg impednace
α	Alfa
κ	Solution conductivity
ρ	Resistivity
ρ_S	Solution resistivity
ϕ	Phase
ω	Angular frequency

List of units

µS	Microsiemens
ºC	Celsius degrees
A	Ampere
CFU	Colony forming units
F; nF	Farads, nanofarads
G	Universal gravitational constant
g; mg; µg	Gram; milligram; microgram
h	Hour
Hz	Hertz
L; mL; µL	Liter; milliliter; microliter
M; mM; nM	Molar; milimolar; nanomolar
min	Minute
rpm	Rotation per minutes
s	Second
V	Volt
Ω	Ohm

SUMMARY/RESUM

SUMMARY

Biosensor technology, consisting of analytical devices that conjugate a bioreceptor with a transducer unit, has been applied in numerous research areas for the detection of different analytes of interest. Bacteria, especially pathogenic bacteria, are important targets to be sensed and identified in many fields, like clinical diagnosis, food industry or water safety, to prevent a great number of diseases in humans. However, bacteria can be employed in a wide range of beneficial applications, such as their use as biological indicators to determine the toxicity of various compounds.

In this thesis, the use of impedimetric transducers based on interdigitated electrode arrays (IDEA) has been proposed as a tool for the development of bacterial biosensing applications. Electrochemical Impedance Spectroscopy is a widely studied technique to characterize biosystems because it allows to monitor electrical events occurring on the surface of electrodes. This technique does not require additional markers for the transduction and can be used in a label-free operation mode and hence simplifying the biosensing assays. Among different types of impedimetric transducers interdigitated electrodes arrays are really advantageous in terms of easy-miniaturization, fast establishment of the steady-state signal response and increased signal-to-noise ratio. The utilization of IDEA devices as a base of a biosensor transducer permits reducing the time and cost per assay. In addition, the applicability of three-dimensional IDEA (3D-IDEA) is described and demonstrated, in which the electrode digits are separated by insulating barriers, to improve the sensitivity for the registration of superficial parameters compared with standard IDEA for bacteria sensing.

The main aim of this work is the elaboration and testing of robust and reproducible biosensing strategies using IDEA and 3D-IDEA impedance transducers with bacteria, as an analyte target or as a sensing element. In the first case, the detection of bacteria or bacterial endotoxins in liquid samples may be performed and, in the second one, novel microbial-based biosensors may be developed. To this end, IDEA devices have been (bio)functionalized using various grafting schemes for their use in four different applications.

First of all, the modification of planar IDEA with layer-by-layer method to assemble polyelectrolytes layers and concanavalin A lectin as a biorecognition element has been employed to study the immobilization and detection of *Escherichia coli* as a model bacterium. Here, the possibility to achieve low detection limits of bacteria concentration, along with a really short detection time, has been shown.

The follow-up work has been focused on the detection of endotoxins of Gram-negative bacteria, also defined as lipopolysaccharides, using 3D-IDEA devices functionalized by layer-by-layer technique to attach concanavalin A as the bioreceptor. The presented methodology is optimized by considering the prevention of unspecific bindings to enhance the selectivity. The results indicate that endotoxins can be detected in only 20 minutes with a remarkable low detection limit of 2 μg/mL.

The next part has been dedicated to the development of a highly selective biosensor for detection of *E. coli* O157:H7, a pathogenic bacterium that causes important infection diseases. A DNA aptamer that recognizes specifically outer membrane proteins of this bacterium has been selected as the biorecognition element on the 3D-IDEA transducers. The resulting aptasensor allows to determine *E. coli* O157:H7 with low detection limit (10^2 CFU/mL), high selectivity and short detection times. Moreover, an electrode regeneration methodology has been developed to use the same sensor several times.

Finally, a novel microbial biosensor approach using 3D-IDEA with *E. coli* specifically immobilized in the trenches between barriers has been employed to evaluate the bacterial response to ampicillin, a bacteriolytic antibiotic. The strategy employed is based on the immobilization of antifouling microgels on the top of barriers to concentrate *E. coli* within the trenches and improve the reproducibility and sensitivity for monitoring the bacterial response in presence of antibiotics by means of impedance variations. This work also opens new perspectives in bioassays for toxicity testing.

RESUM

La tecnologia dels biosensors, basada en dispositius analítics que combinen un bioreceptor amb una unitat de transducció, s'ha aplicat en nombroses àrees de recerca per a la detecció de diferents analits d'interès. Els bacteris, especialment els bacteris patògens, són agents biològics importants per ser detectats en diversos camps com el diagnòstic clínic, la indústria alimentària o la qualitat de l'aigua per prevenir malalties en els éssers humans. No obstant això, els bacteris també es poden utilitzar en un ampli ventall d'aplicacions; per exemple, com a indicadors biològics per determinar la toxicitat de diversos compostos.

En aquesta tesi es proposa l'ús de transductors impedimètrics basats en elèctrodes de tipus interdigitat (*interdigitated electrode arrays, IDEA* de les sigles en anglès) com a instrument per al desenvolupament d'aplicacions biosensores bacterianes. L'espectroscòpia electroquímica d'impedància és una tècnica àmpliament estudiada per caracteritzar biosistemes perquè permet monitoritzar els fenòmens que tenen lloc a la superfície dels elèctrodes. Aquesta tècnica no requereix marcadors en el procés de transducció i pot ser usada en un mode d'operació sense marcatge addicional; així se simplifiquen els assajos de biomonitoratge. Entre els diferents tipus de transductors impedimètrics, els elèctrodes de tipus interdigitat són realment avantatjosos en termes de miniaturització, d'obtenció d'una resposta ràpida i estable o d'increment en la relació senyal-soroll. La utilització dels dispositius IDEA com a base de transducció per a un biosensor permet reduir el temps i cost per assaig. A més a més, en aquest treball es detalla i demostra l'aplicabilitat dels IDEA tridimensionals (3D-IDEA), en els quals els dígits dels elèctrodes estan separats per barreres aïllants, que permeten millorar la sensibilitat en el registre de canvis superficials si els comparem amb els IDEA convencionals per a la detecció de bactèries.

Els objectius d'aquest treball són l'elaboració i la validació d'estratègies de biodetecció, estables i reproduïbles, utilitzant IDEA i 3D-IDEA per a la identificació de bacteris com a analit d'interès o bé com a element de sensat. En el primer cas, s'ha dut a terme la detecció de bacteris o endotoxines bacterianes en mostres líquides, mentre que en el segon s'ha desenvolupat un biosensor del tipus microbià. Per tal de dur-ho a terme, els dispositius IDEA s'han (bio)funcionalitzat mitjançant diverses metodologies per desenvolupar en quatre aplicacions.

En primer lloc, la modificació dels IDEA plans s'ha efectuat amb el mètode «capa a capa» per a la deposició de polielectròlits i la lectina concanavalina A, com a element de bioreconeixement, per estudiar la immobilització i la detecció d'*Escherichia coli* com a bacteri model. En aquest cas, es mostra la possibilitat d'obtenir límits baixos de detecció de concentració bacteriana en un temps molt curt.

La continuació del treball s'ha centrat en la detecció d'endotoxines de bactèries gramnegatives, també coneguts com a lipopolisacàrids, usant dispositius 3D-IDEA funcionalizats amb la tècnica capa a capa per adherir la concanavalina A com a bioreceptor. La metodologia presentada s'ha optimitzat tenint en compte la prevenció de les unions inespecífiques i la millora de la selectivitat. Els resultats indiquen que

les endotoxines es poden detectar en tan sols 20 minuts amb un límit de detecció considerablement baix, de 2 μg/mL.

La part següent està dedicada al desenvolupament d'un biosensor altament selectiu per a la identificació de l'*E. coli* O157:H7, un bacteri patogènic que causa malalties infeccioses greus. S'ha seleccionat un aptàmer d'ADN que reconeix específicament proteïnes de la membrana externa com a element de bioreconeixemnt, juntament amb els transductors 3D-IDEA. L'aptasensor obtingut permet determinar límits de detecció baixos (10^2 CFU/mL) per a l'*E. coli* O157:H7, amb una gran selectivitat i un temps de detecció curt. A més, també s'ha desenvolupat una metodologia de regeneració dels elèctrodes per tal d'utilitzar el mateix biosensor diverses vegades.

Finalment, s'ha confeccionat un nou biosensor microbià, utilitzant els 3D-IDEA i *E. coli* adherits a les trinxeres entre les barreres dels elèctrodes, per avaluar la resposta a l'ampicil·lina, un antibiòtic bacteriolític. L'estratègia emprada es basa en la immobilització de microgels antiadherents a sobre de les barreres per concentrar els bacteris a dins d'aquestes, de tal manera que millora la reproductibilitat i la sensibilitat per al monitoratge de la resposta bacteriana en presència d'antibiòtics a través dels canvis d'impedància. Aquest estudi obre noves perspectives en els bioassajos de toxicitat.

1. INTRODUCTION

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1.1. Biosensors in the field of microbiology

Bacteria are unicellular microorganisms with uncountable applications in food industry, biomedicine, pharmaceutics, chemical industry or environmental control. The use of bacteria in the field of biotechnology has been of high interest in global science in last decades, especially in large-scale industrial processes such as the production of ethanol, organic acids, enzymes, vitamins or pharmaceuticals or in the field of genetic engineering through the development of DNA technologies [1]. However, some bacteria are harmful pathogens that produce a wide range of diseases in humans and other organisms. According to the World Health Organization (WHO) [2] the number of deaths produced by infectious diseases is over 7 million people a year, mainly in the developing countries, and the majority are produced by bacteria. Hence, effective methods of analysis and detection of bacteria are highly demanded in various fields including medicine, environment, food safety or public health.

Conventional methods of bacteria detection in microbiology rely upon laboratory-based techniques such as cell culture and colony-counting, microscopic analysis, biochemical analysis or, more recently, molecular techniques. However, classical laboratory-based methods of bacterial detection and identification typically have long processing times, lack of sensitivity and specificity, and require trained-personnel, while molecular-based technologies need specialized equipment and expensive reagents, involving high costs and limited availability [3, 4]. For this reason, novel technologies involving the use of biosensors for determination of bacteria in different kind of samples and in different application fields, from environmental, food control or biomedical, are highly-required [5].

Compared with other bioanalytical methods biosensors offer rapid and cost-effective systems for bacterial detection and additional advantages like portability or multiple bacteria detection [6-8].

However, it also should be noted that bacteria are organisms that possess unique multifunctional properties, and not always have to be considered as potential pathogens. Bacterial cells are sensitive to different toxic reagents, so they can be also employed as biorecognition elements in the development of a specific type of biosensor, called microbial biosensor, as described later in the text.

1.2. Definition and origins of biosensor technology

According to the International Union of Pure and Applied Chemistry (IUPAC) nomenclature, a biosensor is a device that uses specific biochemical reactions mediated by isolated enzymes, immunosystems, tissues, organelles or whole cells to detect chemical compounds usually by electrical, thermal or optical signals [9]. More recent publications by authors of reference in this field like Anthony P. F. Turner define biosensors like analytical devices that incorporate a biological sensing element in conjugation with

physicochemical transducers to deliver complex bioanalytical measurements with simple and easy-to-use formats [10]. The recognition of the target is performed by a bioreceptor, which is a biomolecule that interacts specifically with an analyte, while the transducer transforms the response generated by the biorecognition event into a measurable signal [11]. Additionally, a signal processing system for the measurements is usually required [12].

The potential uses of biosensors can encompass every possible analytical application, ranging from medical diagnosis, food safety, process control and environmental monitoring. For this reason, it is not surprising that biosensors have mainly been employed in bacteria detection in the field of microbiology.

The development of chemical sensor and biosensor technology dates from the last century. The first sensor reported in 1922 by W.S. Hughes was the electrode for water solution pH (hydrogen ion activity/concentration) determination, but it was earlier, in 1909 that F. Haber and Z. Klemensienwicz reported their research on the glass pH sensitive electrode [13-15]. It is universally acknowledged that the first biosensor was developed by Leyland C. Clark and co-workers in 1962 as an amperometric enzyme electrode for detection of glucose [16]. Since the development of these first biosensors, this multidisciplinary field of research has exponentially increased in the last decades with thousands of publications each year, as reveal statistical data presented in Figure 1.

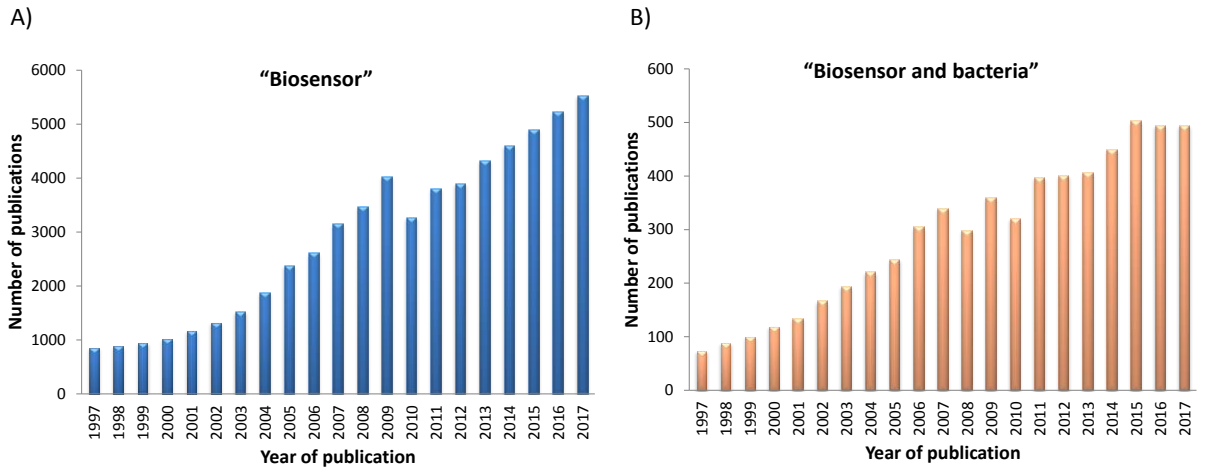


Figure 1. A) Number of publications on "biosensors" and B) "biosensors and bacteria" from 1997 to 2017 according to the Web Of Science.

1.3. Classification of biosensors

Biosensors are usually included as a sub-set of chemical sensors, though due to their importance they are treated as a singular category [17]. In general, biosensors are classified depending on two principles: the recognition element employed and the signal transduction mechanism. All sensors present various

advantages and disadvantages but, ideally, all sensors have to simplify the analytical process compared with classical methods and facilitate integration into a single system [18].

1.3.1. Transducer types

According to IUPAC chemical sensors may be classified depending on the operational principle of the transducer [9]. The following types of transducers are generally employed:

- Optical: Register changes in the system optical properties as a result of interaction of the analyte with the receptor part. This group can be subdivided depending on the type of optical properties: absorbance, reflectance, luminescence, fluorescence, refractive index, optothermal effect or light scattering. This group also includes surface plasmon resonance (SPR) effect-based sensors.

- Electrochemical: This transducer type converts the effect of the electrochemical interaction of the analyte and the electrode into a useful signal. Different sensors are included in this group, like amperometric and voltammetric, potentiometric or impedimetric sensors.

- Electrical: This type of sensors in some cases also includes electrochemical devices, although the signal arises from the changes of electrical properties caused by the interaction of the analyte. This group comprises electrolytic conductivity sensors or electric permittivity sensors.

- Mass sensitive: These devices transform the mass change at a specially modified surface into changes of the properties of the support material, produced by the accumulation of the analyte. Piezoelectric sensors are the most important group of this kind of transducers, including the quartz crystal microbalance-based sensors.

Other physical characteristics, like the paramagnetic properties of gases, can be monitored with magnetic devices. Also the heat effect of a specific chemical reaction or adsorption of an analyte can be measured with thermometric sensors.

1.3.2. Biorecognition elements

Biosensors are also classified depending on the type of biorecognition element employed. The recognition elements, also known as target receptors, play a crucial role in the performance of biosensors and are selected depending on the type of analyte to be detected. The main requirements for a bioreceptor are high binding ability and selectivity towards the target, as well as the stability during the biosensing process [19].

Last years are characterized by acquisition and utilization of new biorecognition elements for biosensing, from classic biomolecules obtained by means of living organisms, like enzymes, antibodies or whole-cells, to artificial recognition elements such as aptamers, molecularly-imprinted polymers (MIPs) or affibodies synthesized in the laboratory [20]. Moreover, the combination of different target receptors in the same platform and the advances in micro and nanotechnology have improved the analytical performance of

sensors and biosensors in terms of sensitivity, selectivity, limit of detection (LOD) and signal-to-noise ratio [21].

Below a brief description of some of the most widely utilized recognition elements employed in the development of biosensors is presented, paying special attention to some of those used in this thesis. Finally, main advantages and limitations of these bioreceptors are also summarized.

Antibodies

Among affinity-recognition elements antibodies are the most widely employed for the development of biosensors, named as immunosensors. The major advantage of antibodies is the specificity and sensitivity of biomolecular interactions between them and the target antigen. Antibodies may be produced with high specificity to a wide range of antigens, like protein-based molecules, toxins, viruses, bacteria, tissue cells or whole-cells [22, 23].

Antibodies can be divided into two types, polyclonal or monoclonal. Polyclonal are generated from a range of immune cells and bind the target analytes with different binding affinities and in different locations. On the other hand, monoclonal antibodies are produced by immune cells from an identical clone and have the same target region, the epitope (Figure 2). Thus, monoclonal antibodies are more selective and are usually employed in biosensors. However, in some particular cases, e.g. determination of pollutants in environmental analysis, it may also be interesting to use polyclonal antibodies showing sensitivity to a wider range of compounds [23, 24].

Despite the fact that antibodies are the most used bioreceptors in biosensor research, their high production and purification costs limit their practical application. Another problem is their stability that involves a decrease in binding efficiency. All these have provoked intensive search for new alternative biomolecules in the last years.

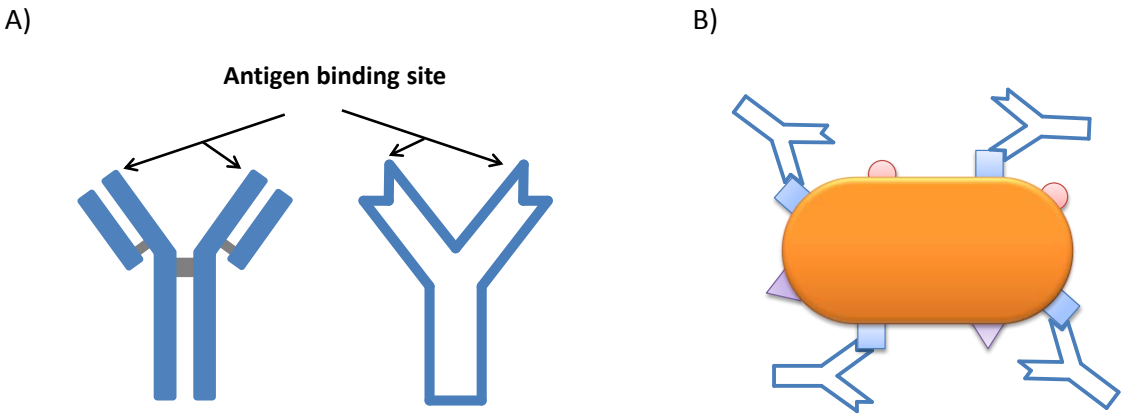


Figure 2. A) Representation of basic structure of monoclonal antibodies and B) selective interaction of antibodies with specific target molecules (epitope) on the bacterial membrane.

Enzymes

In enzymatic biosensors an enzyme is acting as the recognition element that selectively interacts and transforms substrate, the target analyte, catalyzing the specific biochemical reaction. Basically two kinds of measurements can be performed with enzyme-based biosensors. If the analyte is metabolized by the enzyme, the analyte concentration may be determined through measuring the catalytic transformation into the corresponding product. Otherwise, if the activity of the enzyme is inhibited by the analyte of interest, its concentration is associated with a decrease in the formation of the product. Therefore, these biosensors detect the products or the consumed species, respectively.

The most common transduction element associated with enzyme-based biosensors is electrochemical-based sensors, especially amperometric biosensors, using oxidase enzymes like laccases, tyrosine or horseradish peroxidase (HRP) [25]. However, one of the main disadvantages of these is the signal reduction due to fouling agents and interference from chemicals possibly present in the sample matrix [26].

Protein-based molecules: Lectins, antimicrobial peptides and affibodies

In this group different kinds of bioreceptor molecules may be included because proteins comprise a large variety of biomolecules with peptide nature formed by amino acid residues chains. Here we would like to focus on three different protein-related molecules: lectins, antimicrobial peptides and affibodies.

Carbohydrate binding proteins (CBPs), also known as lectins, are proteins of non-immune origin that specifically interact with carbohydrates without modifying those [27]. They can be derived from plants, microbial or animal sources. Most of lectins specifically recognize sugar units (mannose, galactose, fructose, glucose, *N*-acetylneuraminic acid, *N*-acetylglucosamine or *N*-acetylgalactosamine) [28]. Recently, due to their multifunctional biological properties, they have been employed in different optical and electrochemical biosensing devices.

Lectin biomolecules are more stable and smaller than antibodies, and they can neither be denatured easily nor lose their activity in time [29, 30]. Moreover, the small size of lectins allows to obtain higher densities of carbohydrate-sensing elemBents on a sensor surface, leading to higher sensitivity and lower non-specific adsorption [31].

Due to the binding ability to carbohydrate-residues the lectin-based biosensors have been employed in many fields, such as glucose sensors, detection of glycoproteins, microorganisms or cancer cells (Figure 3). Moreover, due to the binding ability of lectins to carbohydrates they are also employed for immobilization and assembling of other biomolecules like enzymes [32].

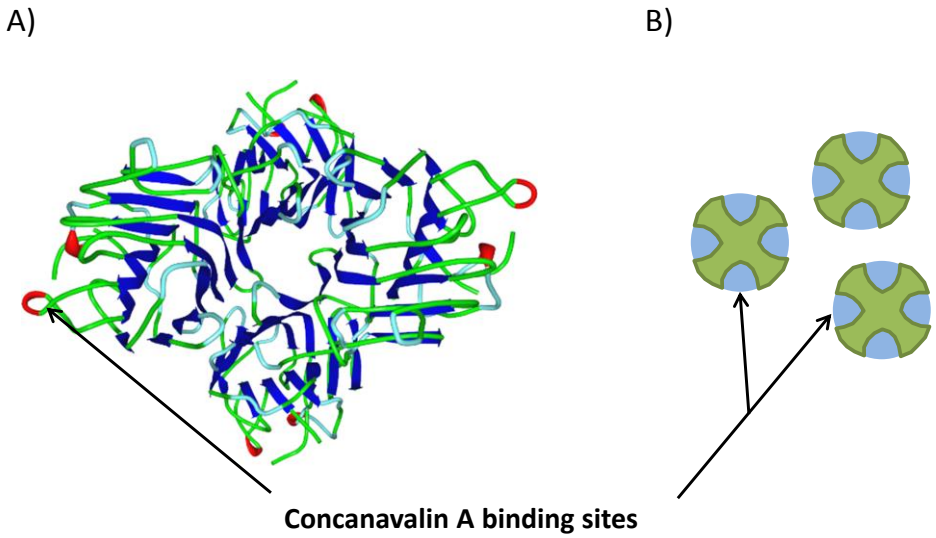


Figure 3. A) Molecular structure of concanavalin A (Con A), a typical carbohydrate binding protein (adapted from Matsumoto *et al*. 2015) and B) the corresponding schematic representation.

Another particular group of protein molecules that can be used as a biorecognition element for bacteria detection are antimicrobial peptides (AMP). AMPs are host defense molecules that are implicated in specific killing of bacteria through the binding to lipopolysaccharides (LPS) and lipoteichoic acids (LTA) components and disrupting the membrane [33]. These molecules are highly stable to adverse conditions and have the ability to bind to multiple target microbes [34]. They are categorized with consideration of their synthesis, composition and secondary structure. Typically AMPs consists on 10-40 amino acids and their hydrophobic residues provide amphipathic properties giving them the capability to interact with charged groups or lipids of the bacterial membrane [35]. For instance, short and linear cationic AMPs are particularly attractive for bacterial sensing applications because of their small size and easy synthesis.

Another promising group of bioreceptor elements are affibodies. Affibodies form a new class of engineered proteins, which have a considerable affinity and specificity to various specific targets, proteins or peptides, after their isolation. For the moment, they have been used in imaging diagnosis [36]. As well as antibodies, affibodies are affinity proteins that belong to the class of scaffold proteins but with improved properties, such as a robust spatially defined structure.

The isolation of affibodies is based on non-immunoglobulin scaffolds using synthetic combinatorial libraries and selection systems, mainly by phage display technology [36, 37].

Nucleic acids and aptamers

Nucleic acids (DNA or RNA sequences) are commonly used for the development of biosensors known as genosensors. Recognition of the target analyte is performed through hybridization of the DNA or RNA of different lengths to their complementary base pairs [38].

In last years a specific kind of genosensors appeared based on the use of synthetic short chain nucleic acids, the aptamers. Aptamers are short oligonucleotides of RNA or DNA single-strand sequences between 30 and 100 nucleobases produced *in vitro* and with high affinity to their targets. These biomolecules are more stable than antibodies. Aptamers are typically folded in a specific three-dimensional structure, which allows selective interaction with the corresponding target [20, 39] (Figure 4). However, the variations in shape and conformation may affect their binding affinity, involving a low or lack of interaction with the target. These interactions are mainly produced by complementarity, electrostatic and hydrogen-bonding forces. Biosensors with these molecules integrated in different sensor transducer platforms are called aptasensors.

Selectivity of an aptamer sequence to a specific analyte is obtained by means the systematic evolution of ligands by exponential enrichment, the SELEX method. This process, firstly described in 1990 by Ellington and Szostak [40], consists in screening of large random oligonucleotide libraries by repetitive cycles of *in vitro* selection of enzymatic amplification. The process starts with the incubation of nucleic acids sequence library with the specific target, followed by the separation and exponential amplification of the binding oligonucleotides. The process is repeated several rounds with more stringent conditions until the isolation of the desired sequence of the aptamer, that is finally cloned and sequenced [41].

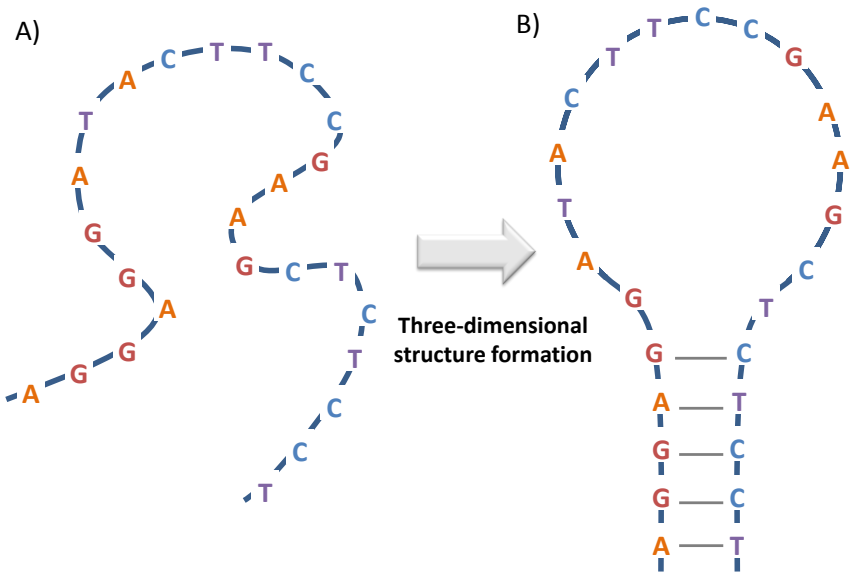


Figure 4. A) Schematic representation of a DNA aptamer sequence and B) typical "hairpin" format.

High interest in the use of aptamers as biorecognition elements of biosensors relies on the possibility to produce them for a wide variety of different targets, like small protein biomarkers, antibiotics, toxins, cell surface molecules, membranes fragments, virus or whole bacteria [41, 42]. Moreover, they present multiple advantages as biorecognition elements: binding affinity and specificity comparable to those of antibodies, reusable, low cost production, easy to synthetize (especially once the aptamer sequence is identified), stability at different temperatures and pH. Another advantage is that they can be modified with active sites for immobilization purposes or labeled with reporter molecules without affecting their

affinity. For this reason in the last years the number of publications on aptasensors research and development has significantly increased.

Whole cells

For the development of biosensors, various living organisms (bacteria, fungi, yeast, algae) and tissue-culture cells from animals or plants have been successfully used as recognition elements. In the case when microbes are utilized as a bioreceptor the resulting sensors are named microbial biosensors. These particular biorecognition elements offer an alternative to other biomolecules due to their massive and low-cost production by cell-culturing [43].

In whole-cell biosensors the metabolic activity of living organisms is monitored as the analytical signal. Thus, different parameters such as the inhibition of growth, cell viability, production of specific metabolic compounds, or the digestion of different substrates may be susceptible to be analyzed.

Among advantages of using cell-based bioreceptors are their tolerance to pH and temperature variations, longer lifetime, reduced purification requirements, low-cost and faster preparation compared with enzymes or antibodies. On the other hand, the main disadvantages are related to the metabolism of cells since it has to be maintained continuously in the majority of applications, the reproducibility of the immobilization of cells on a sensors surface, and the difficulty to ensure selectivity [31].

Bacteriophages

Bacteriophages (or phages) are viruses with either narrow or broad specificity, which infect certain host bacteria being ubiquitous in almost all environments [31, 44]. They have been used in biosensors for bacterial cell detection because they offer very high selectivity towards their host bacteria. Bacteriophages are inexpensive to produce and are resistant to harsh conditions like high temperatures or organic solvents. One of the main characteristics is that phages can discriminate between viable and non-viable bacterial cells because they only multiply in viable cells [45]. Moreover, due to their different functional groups phages can be integrated in different kind of transducers [46].

The replication cycle of phages consists in an initial step of binding to the membrane of the host bacteria and introduction of its genome into the host cell cytoplasm to utilize its ribosomes to manufacture structural proteins. Some phages use the lytic replication cycle in which the bacteria die releasing the new bacteriophage to infect another host cell, like the phage T4 that infects *Escherichia coli* bacteria presented in Figure 5. In the lysogenic cycle, phages integrate their genome into the host DNA, remaining dormant until stimulated for replication and propagation [47].

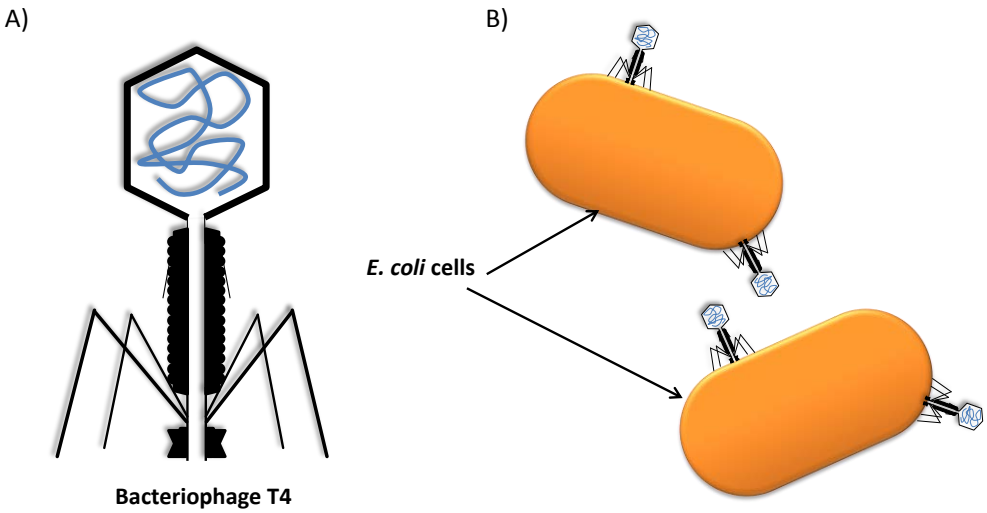


Figure 5. A) Schematic of T4 bacteriophage and B) representation of specific T4 infection of *E. coli* bacteria.

Other promising biorecognition elements: molecularly-imprinted polymers (MIPs)

Molecular imprinting is a type of template-assisted synthesis that results in selective formation of cavities in a 3D-polymeric network forming the synthetic matrix that can be used as a recognition element [48]. In some publications, MIPs are defined like a class of plastic antibodies employed in nanosensors [49].

MIPs are synthetized by polymerization of functional monomers in a mixture with cross-linkers and the presence of template molecules that are the target analytes. Afterwards the template molecules are removed and the recognition sites are formed in cavities of a MIP polymer matrix with optimal shape, size and functional groups to capture the desired molecules [49]. These sites formed around template molecules are identical in terms of the interaction sites, hydrophobicity and reactivity, with binding specificity similar to that of typical antibody-antigen interactions [22]. As a result the target analyte can be selectively bound in the molecular cavities of MIP. The functional monomer forming the network is responsible for providing the recognition sites in the imprinted cavity with the template molecule by covalent or non-covalent interactions. Thus, the stronger the interaction between the template and the monomer is, the higher will be the affinity between the target analyte and the recognition sites [20].

In summary, MIPs are synthetic cross-linked polymeric materials that have artificial recognition sites and possess the ability to mimic the biological activity of natural bioreceptors. However, their use in the performance of different biosensing platforms is still limited due to the difficulty to avoid unspecific interactions produced by the heterogeneity of the imprinted cavities.

In the Table 1 below the main advantages and disadvantages of the described biorecognition elements employed in biosensors development are summarized.

Biorecognition elements	Advantages	Disadvantages
Antibodies	- High affinity - Specificity	- Laborious and expensive production - Production requires use of animals - Lack of stability
Enzymes	- Specificity - Well-known mechanisms	- Purification is costly and time consuming - Poor stability - Efficient only at optimum pH
Lectins	- Stability	- Lack of selectivity
Affibodies	- Long shelf-life	
AMPs	- *In vitro* synthesis - Easy to modify - Stability - Low cost preparation - Thermally stable	- Lack of selectivity for different bacterial strains
Nucleic acids	- Stability - Low cost preparation	- Limited targets
Aptamers (DNA or RNA)	- *In vitro* synthesis - Easy to modify - Stability - Low cost preparation - Thermally stable - Possibility to reuse it by denaturalization and re-hybridization	- Active in limited conformations
Whole cells	- Low-cost preparation - Reduced purification steps	- Cells have to be alive in many applications
Bacteriophages	-Specificity - Sensitivity - Low cost preparation - pH stable	- Limited number of well-known phages for biosensing applications
Molecular Imprinted polymers (MIPs)	- Thermal, chemical and mechanical stability - Reusability - Low cost synthesis	- Complex fabrication methodology and time-consuming - Leakage of template molecules - Incompatibility with aqueous media

Table 1. Summary of advantages and disadvantages of main biorecognition elements (adapted from [20]).

1.4. Electrochemical biosensors

A great number of developed biosensors use electrochemical transducers for the detection of different analytes because of the low cost, easy-to-use, portability, high sensitivity, fast response, simplicity of construction and small sample volume [17]. Additionally, electrochemical detection compared with optical devices offers certain advantages in biosensors performance because they may be used in turbid fluids with optically absorbing and fluorescing compounds.

Electrochemical techniques are generally classified depending on the origin of the signal: current, potential or impedance.

- Amperometric sensors monitor the changes in the current resulting from the oxidation or reduction of electroactive species. Usually the measurements are performed by maintaining a constant potential at the working electrode, typically of gold, platinum or carbon, or an array of electrodes with respect to a reference electrode and the auxiliary electrode. The resulting current is directly correlated to the electroactive species concentration. One of the main problems of this kind of biosensors, where direct electron transfer between the electrode and the analyte or biomolecules is not possible, is that a redox mediator is required [50]. If the current is measured not at some fixed potential, but in a controlled potential range, this method is referred to as voltammetry.

- Potentiometric devices measure the electrical potential difference between an indicator electrode, in certain cases formed by a permselective membrane, and a reference electrode with a zero or no significant current that flows between them. The difference of potential between the two electrodes varies depending on chemical activity/concentration of ions in the sample. Thus, potentiometry provides information about ion activity in an electrochemical reaction. Different selective membranes can be employed to detect an ion of interest, in which the transducer is named as ion-selective electrode (ISEs) [18, 50-52].

- Impedimetric sensors working principle is based on the application of a potential of small amplitude and variable frequency to electrodes of a transducer, the resulting current is used to calculate the impedance of the system at each frequency [50, 51]. In the next parts of this thesis more detailed information on this technique is presented.

1.5. Electrochemical Impedance Spectroscopy (EIS) fundamentals

This section is focused on a global overview of Electrochemical Impedance Spectroscopy (EIS) technique as the main method employed in this thesis to characterize studied biosystems. The impedance method is used in a wide variety of applications, like material surface characterizations, corrosion process, electrode kinetics, membranes studies or fuel cell optimization [53]. During last years many efforts have been directed on the development of biosensors based on impedimetric transducers. This is due to the fact that EIS allows obtaining information on the interfacial phenomena occurring on the surface of the device or close to it, becoming a powerful tool for characterization of biosensors.

The impedance (Z) of a system is generally determined by applying a voltage perturbation (V, in V) of a sinusoidal wave of small amplitude and detecting the resulting current response (I, in A). In the case of electrochemical impedance measurements are performed in a conductive medium applying test voltage

between working and auxiliary electrodes. The exciting signal (V_t) with angular frequency ω is a sine wave of small amplitude that generates in the system under test a sinusoidal current (I_t) response of different amplitude and phase. The applied potential expressed as a function of time is defined as:

$$V_t = V_0 \sin\ (\omega\, t)\ , \tag{1.1}$$

where V_t is the potential at time t, V_0 is the amplitude of the signal, ω (expressed as radians/second) is the radial frequency and t is the time (in seconds). The radial frequency is associated with the frequency f (in Hz) as:

$$\omega = 2\,\pi\, f \tag{1.2}$$

The resulting AC current signal, I_t, is obtained in response to this applied potential, differs in phase (ϕ) and amplitude, I_0, (see Figure 6) and depends on frequency.

$$I_t = I_0 \sin\ (\omega\, t + \phi) \tag{1.3}$$

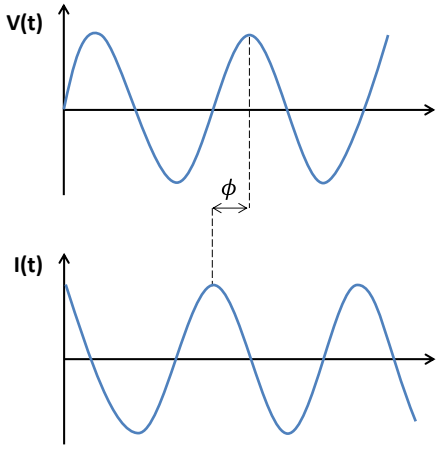


Figure 6. Diagram of alternating current (AC) potential applied and sinusoidal current response in terms of amplitude and phase variations.

The general Ohm's law allows determining the impedance with the following function:

$$Z_t = \frac{V_t}{I_t} = \frac{V_0 \sin\,(\omega\, t)}{I_0 \sin\,(\omega\, t + \phi)} = Z_0 \frac{\sin\,(\omega\, t)}{\sin\,(\omega\, t + \phi)} \tag{1.4}$$

Another common method to represent the impedance vector model is to use complex notation:

$$V_t = V_0\, e^{(j\,\omega\, t)}\ , \tag{1.5}$$

$$I_t = I_0\, e^{j(\omega\, t - \phi)}\ , \tag{1.6}$$

in which j is $\sqrt{-1}$ representing the imaginary term of the complex number.

According to the Euler's relationship:

$$e^{j\,\phi} = \cos\phi + j \sin\phi\ , \tag{1.7}$$

and the impedance may be expressed as:

$$Z\ (\omega) = \ Z_0\ e^{j\ \phi} = Z_0\ (cos\phi + j\ sin\phi) = Z_{re} + jZ_{im} \quad (1.8)$$

Graphical representation of impedance spectrum measured in a certain frequency range can be plotted as a real part of impedance (Z_{re} or Z') in the X-axis, and an imaginary part (Z_{im} or Z'') in the Y-axis into a diagram known as Nyquist plot. The imaginary component is also known as reactance (X), and the units are Ohms (Ω). In the Nyquist plot each point is the impedance at one frequency, representing a vector whose modulus coincides with the modulus of the impedance ($|Z|$) and the angle between the X-axis and the vector is equal to the ϕ, as shown in Figure 7.

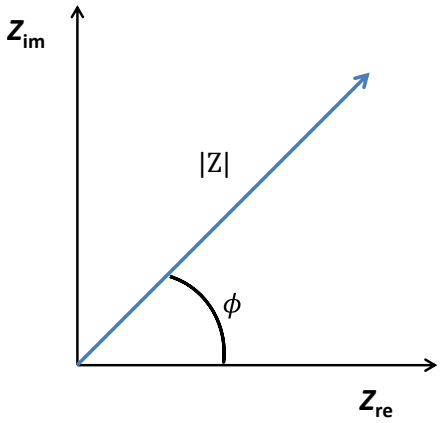


Figure 7. Nyquist plot representing the impedance vector in the complex plane.

Another common mode to present impedance spectrum data graphically is the Bode plot. In this case the impedance is plotted as values of $|Z|$ and ϕ on the Y-axis versus the logarithm of frequency (log f) on the X-axis.

Both diagrams, the Nyquist and Bode plots, can be employed to monitor the impedance changes occurring in an electrochemical cell and to evaluate the impedance response at different frequencies. Thus, the electrochemical impedance spectrum allows the characterization of a complex electrode system composed of surfaces, layers and membranes where electrical charge transfer and ion diffusion process may take place [54].

1.5.1. Interpretation of data and equivalent circuits

One of the most difficult parts of the EIS is correct interpretation of data and obtained spectra due to the complexity of physical, chemical or biological phenomena taking place in a system. Usually the spectra are analyzed using an equivalent circuit (EC), which is composed of different electrical components, combined in parallel or serially, representing the physiochemical properties of the studied system, in general modeled by resistances (R) and capacitances (C) [55].

Typically, more than one equivalent circuit model may fit the obtained experimental data equally well. This means that something should be known *a priori* about the electrochemical system under study in order to choose correct EC components that reflect the real physical-chemical phenomena occurring in

the system. Moreover, to guarantee the correctness of the chosen EC special experiments should be carried out in which some of the system parameters remain fixed and some others vary to see how it is reflected in the EC components values.

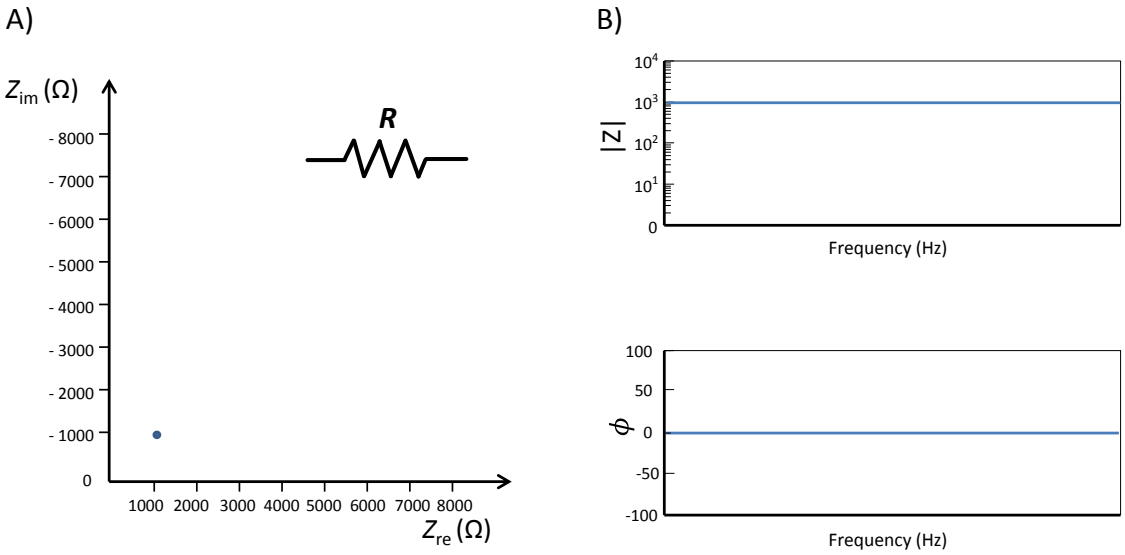
As noted before, many electrochemical system parameters may be represented by an EC composed of resistors and capacitors in different combinations. For pure resistances (*R*) the current through a resistor remains in phase with the voltage (ϕ=0). In this case the impedance of the resistor is independent of frequency and do not have the imaginary component, only the real one.

$$Z_R = R \tag{1.9}$$

On the other hand, the phase of the current through a pure capacitance (*C*) is shifted -90º with respect to the voltage (ϕ=-π/2) with only imaginary component of the impedance. The impedance of a capacitor decreases as the frequency increases.

$$Z_C = -\frac{1}{j\,\omega\,C} \tag{1.10}$$

Next graphs show the Nyquist and Bode plots for pure resistance and pure capacitance representations (Figure 8).



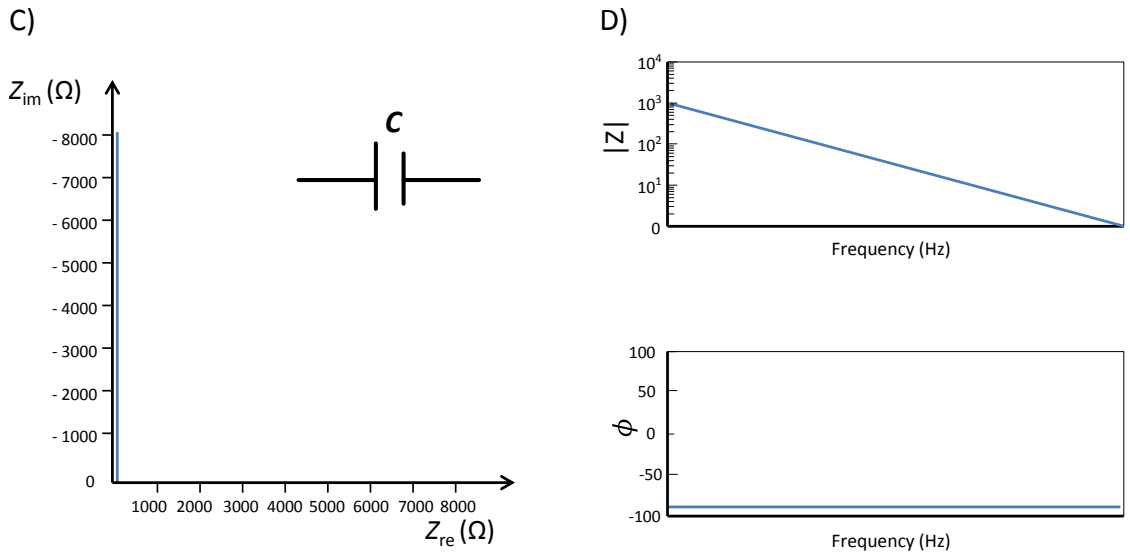


Figure 8. A) Nyquist and B) Bode diagrams for a pure resistor of 1000 Ω. C) Nyquist and D) Bode plots for a capacitor of 1 nF.

Each component of the EC gives its input into the total impedance depending on the applied frequency. Therefore, in complex systems the measurements are performed in a well-defined frequency range to simplify spectra interpretation. In some specific cases just one frequency may be selected to monitor an individual parameter of the EC. However, as it was noted above, real systems are quite complex and elaboration of a proper EC requires the profound knowledge of the system. Additionally, it has to be considered that in impedance measurements the whole electrochemical cell is studied and not only the working electrode. The impedance of the counter electrode, the surrounding solution, wires, stray capacitances, etc. should be taken into account and their effect should be minimized. In traditional EIS experiments commonly large size metal rods or plates are used as electrodes immersed in the medium to measure impedance, as shown in Figure 9 [56, 57], and the area of the counter/auxiliary electrode is chosen to be considerably larger than that of the working electrode to minimize the effect of its own impedance.

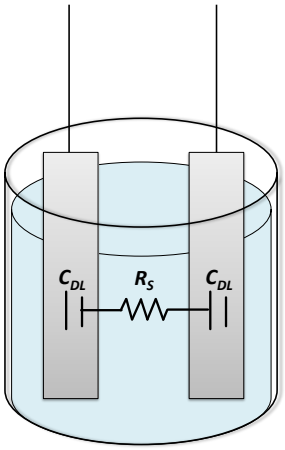


Figure 9. Representation of the electrical circuit elements of two metal electrodes in parallel immersed into an electrolyte solution (C_{DL}: double layer capacitance; R_S: solution resistance).

1.5.2. Faradaic and non-faradaic impedance

EIS measurements with metallic electrodes in an electrolyte solution can be achieved in two modes, in the presence of an additional redox probe used as a marker or directly in an analyte solution. In the presence of a redox pair in solution an electrochemical oxidation/reduction reaction takes place on the metal electrode surface, impedance measurement mode is usually referred to as faradaic. In non-faradaic conditions the redox probe is not required [58].

Impedance measurements in the presence of electroactive compounds are widely described in the general electrochemistry literature. The equivalent circuit employed is known as the Randles EC, presented in Figure 10A. This is a simple and well-known model used to present a faradic process and is widely employed to describe the behavior of impedance-based biosensors [59]. It is formed by different elements: the solution resistance (R_S), the double-layer capacitance (C_{DL}), the charge-transfer resistance (R_{CT}) and the Warburg impedance (Z_W). The bulk properties of the electrolyte and the diffusion of redox probe are represented by R_S and Z_W, while C_{DL} and R_{CT} depend on surface properties of the electrode/electrolyte solution interface. Depending on the frequency applied the impedance of the faradaic system may be limited either by kinetic or diffusion processes [60].

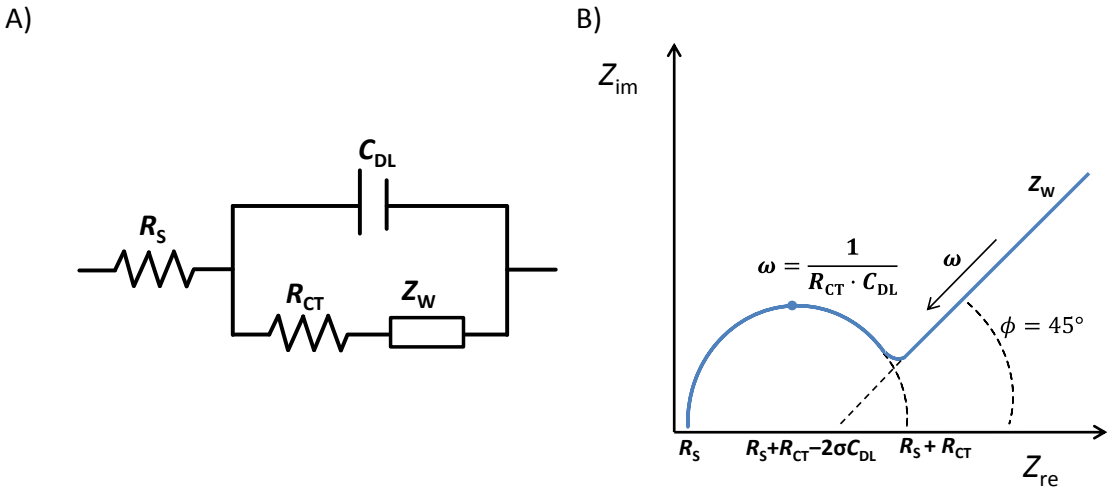


Figure 10. A) Randles equivalent circuit and B) corresponding Nyquist diagram.

The Nyquist plot (Figure 10B) is the best approach to study the Randles EC elements. The semicircle corresponds to the electron transfer limited process at high frequencies, while the linear part represents the diffusion limited process at low frequencies. The intercept of the semicircle with the real axis (Z_{re}) corresponds to R_S, and the intersection of the semicircle represents the sum of R_S and R_{CT}. The double layer capacitance C_{DL} can be calculated from the frequency at the maximum of the semicircle ($\omega = 2\,\pi\, f = 1/R_{CT}\, C_{DL}$) and the Z_W by extrapolating the 45º line observed in Figure 10B. In some analytical applications the effect of the Z_W is neglected by selecting a frequency range where no diffusive response is observed in the Nyquist plot and interfacial and bulk properties are predominant.

In the case of faradaic impedance, the main parameters to be monitored are the charge transfer resistance (R_{CT}) and the Warburg impedance (Z_W) (see Figure 10B). The R_{CT} is mainly affected by the kinetics of electrons transfer from the electrolyte to the metal and depends on the properties of the redox pair (concentration of species, temperature and applied potential) and the structure of the interface. The use electron mediators, such as $Fe(CN_6)^{3-/4-}$ (ferricyanide/ferrocyanide) or $Ru(NH_3)_6^{3+/2+}$ (hexaammineruthenium III/II ions), at sufficiently high concentration guarantee that impedance does not become limited by the process of charge transfer between the redox pair and the electrode surface [30]. Modification of the electrode surface provokes its blocking and affects the R_{CT}. This phenomenon is widely used in impedimetric biosensors to measure concentration dependencies.

The Warburg impedance (Z_W) results from the diffusion of the redox couple from the bulk of the electrolyte to the interface [54, 61]. At high frequencies the Warburg impedance is almost irrelevant since the diffusion path of reactive species is really short, while at low frequencies the reactants have to diffuse farther, increasing the Z_W [62].

In the case when a redox mediator is absent in the electrolyte solution the impedance is termed non-faradic [7]. Without a redox pair the electron charge transfer is not produced and R_{CT} becomes infinitely large. In the non-faradaic mode a transition current flows across the surface of the electrode and mainly depends on electrode interfacial capacitance and the impedance of the surrounding solution, Z_S. This mode is considered more amenable for direct biosensing and point-of-care applications [63].

The impedance of the solution, Z_S, is formed by two elements in parallel: the geometrical (or stray) capacitance, C_S, between the electrodes in the electrolyte solution, and the electrical resistance of the solution R_S, which depends of the conductivity of the medium. R_S is also affected by the temperature, and other geometrical features that will be explained in detail in the next sections. However, the effect of C_S at low frequencies is negligible and in the majority of systems the Z_S is simplified as R_S.

In the absence of a faradaic process the useful signal comes from the interfacial capacitance changes. This capacitance is mainly affected by the presence of absorbed species or the formation of layers on the electrode surface. Moreover, when an electrode is immersed in an electrolyte solution an electrical double layer is formed at the electrode/solution interface, defined as the double layer capacitance (C_{DL}) that basically depends on the ionic species concentration. The C_{DL} also contributes to the overall interfacial capacitance. Therefore, the interfacial capacitance modulated by the surface modifications may be used to monitor surface reactions on the electrode [30, 54, 57].

It should be noted that the impedance of solid electrode/electrolyte interface usually differs from purely capacitive behavior. Therefore, modelling the interface of the electrode/solution between the electrode and the solution can affect the correct interpretation of data. An ideal capacitor has a ϕ=90º, but the surface effects like the roughness of metal electrodes involves a small decrease in ϕ. The Constant Phase Element (CPE) is a capacitive element that is usually employed to model the behavior of the interface and is expressed as:

$$Z_{CPE} = \frac{1}{(j\,\omega)^{\alpha}\,C_{DL}} \tag{1.11}$$

In equation 1.11 j is the imaginary $j=\sqrt{-1}$, ω is the angular frequency (rad/s); C_{DL} (in F) the double layer capacitance that corresponds to the capacitance of the metal/solution interface, and α representing the behavior of the CPE. When the exponent α is equal to 1 the CPE behaves as a pure capacitor. If the value of α becomes 0 the CPE will behave as a resistor. In fact, the Warburg element is a specific type of CPE with a α= 0.5. Typical CPE_{DL} values of α for metal electrodes fluctuate between 0.7 and 0.98 [64] as shown in Figure 11.

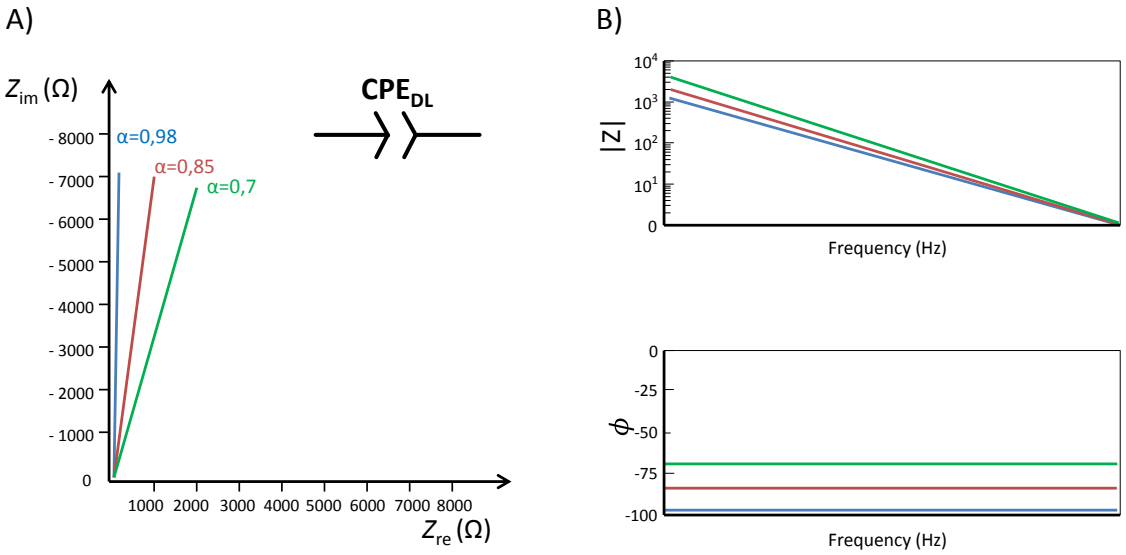


Figure 11. Representation of Constant Phase Element (CPE) with typical α values for metal electrodes.

To sum up, for an accurate interpretation of the impedance data it is important the correct choice of an equivalent circuit parameters of which reflect the real physical, chemical or biological phenomena at the electrode surface. In this thesis the impedance measurements have been performed in non-faradaic conditions, and the main parameters monitored to study the developed biosensing approaches are the changes in R_S and CPE_{DL}.

1.6. Interdigitated electrode arrays (IDEA) for impedance measurements: a tool for biosensing

Traditionally, macro-sized metal electrode systems are used to measure impedance [31], but application of modern technology and development of microfabrication techniques permitted to reduce significantly the sensor dimensions [65]. A well-stablished method is the formation of two electrodes microbands on a plane insulating substrate forming an interdigitated electrode array (IDEA). IDEAs have gained considerable interest in the last two decades as electrochemical transducers for chemical and biochemical sensing [66].

Among the advantages of IDEAs are: easy-miniaturization, absence of additional reference electrode, fast establishment of steady-state compared with other systems and increased signal-to-noise ratio [67-69]. An additional advantage of this type of sensor is the possibility to carry out label-free (direct) detection in biosensing [57]. Thus, IDEA sensors do not require additional markers for the detection of specific biomolecule analyte as its interaction with the sensor surface produces electrical changes that can be monitored directly by means of impedance measurements [64]. In the literature, IDEA sensors are usually classified as capacitive sensors because they allow to measure the changes in dielectric proprieties at the electrolyte-electrode interface [70]. However, as it will be explained later in this section, interaction of IDEA sensors with analyte can also produce surface conductivity changes that affect impedance of this type of transducers.

Common IDEA sensor design is shown in Figure 12. The device is composed by a pair of comb-like metal electrodes formed on an insulating substrate with their collector bars connected to the contact pads employed for wiring. The geometry of the sensor depends on number (*n*) and length (*l*) of the digits, as well as their width (*w*) and spacing (*s*) between electrode digits. The penetration of the electric field into the bulk solution under an applied potential is almost equal to the distance between the centers of the electrodes digits (*w*+*s*). Some studies reveal that the 80% of the current is distributed close to the electrode surface [71]. Thus, the miniaturization of the electrode width and the spacing between the electrodes allows the increase of the sensitivity related with reactions occurring on the sensor surface.

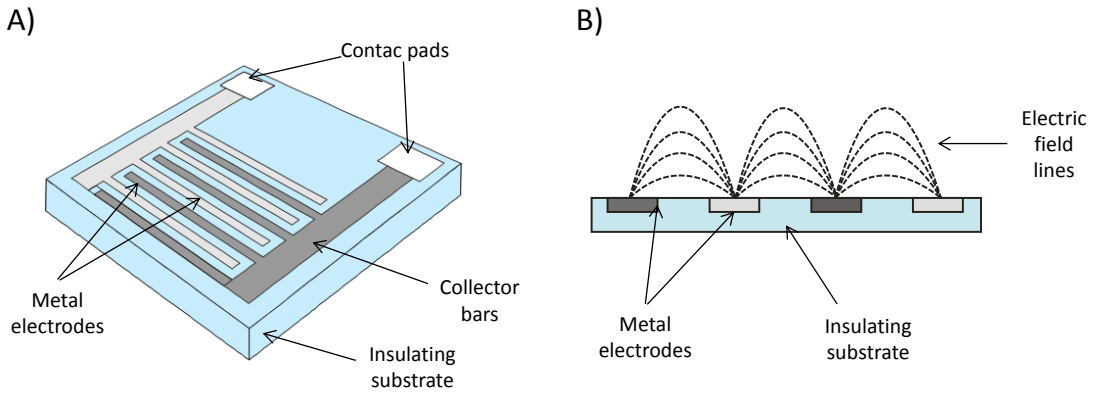


Figure 12. Planar interdigitated electrode arrays (IDEA) (A) and its cross-section with the electric field lines (B).

1.6.1. Equivalent circuit of IDEAs

Due to the fact that IDEA electrodes are typically symmetrical, of the same size and made of the same material the impedance measured between them is the sum of the impedance of each individual electrode that forms the digits. A typical Nyquist and Bode plots and the corresponding simplified equivalent circuit employed for the interpretation of the impedance spectra of an IDEA in non-faradaic mode is presented in Figure 13.

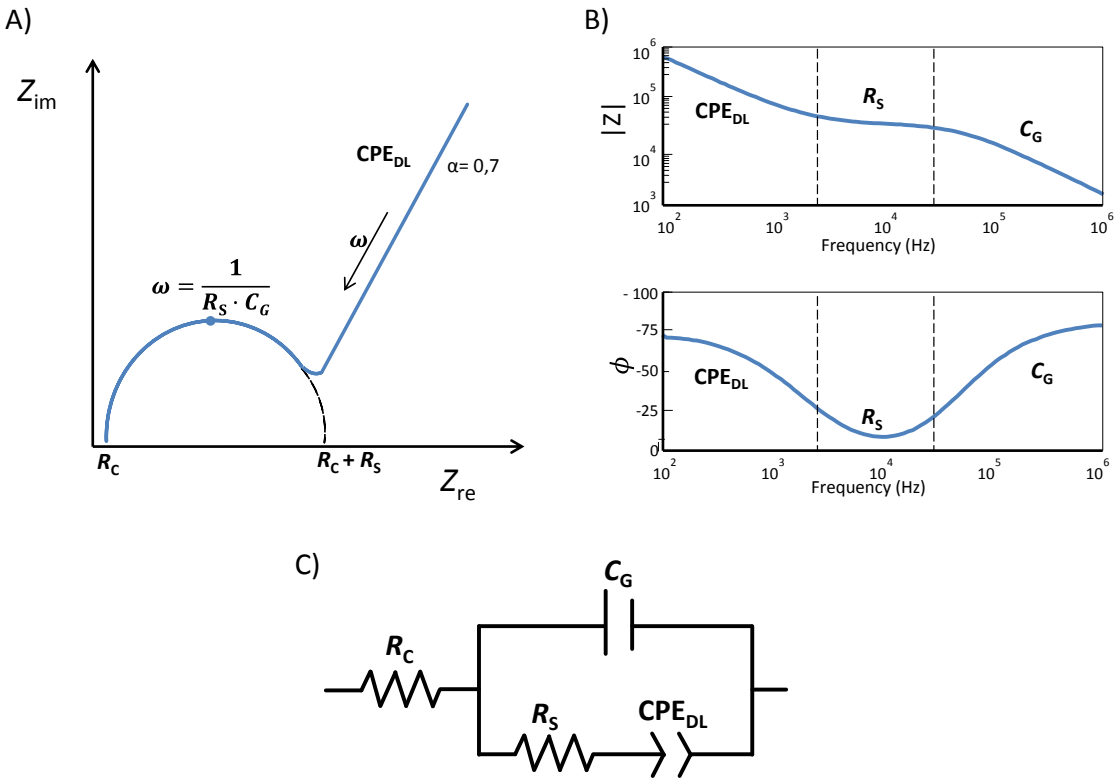


Figure 13. A) The Nyquist and B) Bode plots of the impedance of IDEA sensors measured in low conductivity KCl 10^{-5} M solution and C) the electrical equivalent circuit.

The corresponding elements of the EC are: R_C, the contact resistance of wires, contacts and collector bars; C_G, the geometrical capacitance between the two electrodes connected by the electrolyte solution; R_S, the electrical resistance between the electrodes introduced by the surrounding solution, and the C_{DL}, the double layer capacitance at the electrode/solution interface. Additionally, the introduction of a biomolecule layer on the IDEA surface may result in an additional capacitance (C_F) in series with the double layer capacitance. However, the combination of C_{DL} and C_F can be fitted by a single CPE_{DL}. As previously detailed, the roughness of the metal electrodes, but also the introduction of additional layers may affect the CPE parameters.

A semicircle observed in the Nyquist plot at high frequencies is associated with the parallel combination of C_G and R_S. The intercept with Z' axis on the left side gives the R_C value, and the intercept on the right side corresponds to the value of R_C + R_s. The linear response at low frequencies in the Nyquist plot is dominated by the CPE representing the electrical double layer capacitance.

The R_S of the equivalent circuit of an IDEA depends on the bulk solution resistivity, ρ_S, and a geometric factor, the cell constant K_{CELL}, as follows from:

$$R_S = \rho_S \cdot K_{Cell} \ , \tag{1.12}$$

where ρ_S (Ohm·cm) is the inverse of the solution conductivity κ (S/cm):

$$\rho_S = \frac{1}{\kappa}, \tag{1.13}$$

and the K_{CELL} depends on the geometry of the electrodes, basically on the number of digits, their width and the distance between them. Different approaches have been proposed for the calculation of K_{CELL} [72-74]. From eq. 1.12 it follows that R_S should be linear depending on ρ_S at all the solution concentrations till MilliQ water with a high resistivity (18 MOhm·cm). However, in previous studies of Olthius and co-workers [74, 75] it is noted that in low conductivity solutions, with a solution resistivity higher than 7-8 kOhm·cm, the sensor response in terms of R_S declines from linearity.

In other recent studies carried out in our group this behavior was also experimentally demonstrated and explained [76, 77]. As can be observed in Figure 14 the linearity between ρ_S and R_S is maintained in solutions with reasonable high conductivity, while in low conductivity solutions the IDEA presents a non-linear response. This effect was observed for IDEA devices with different electrode materials.

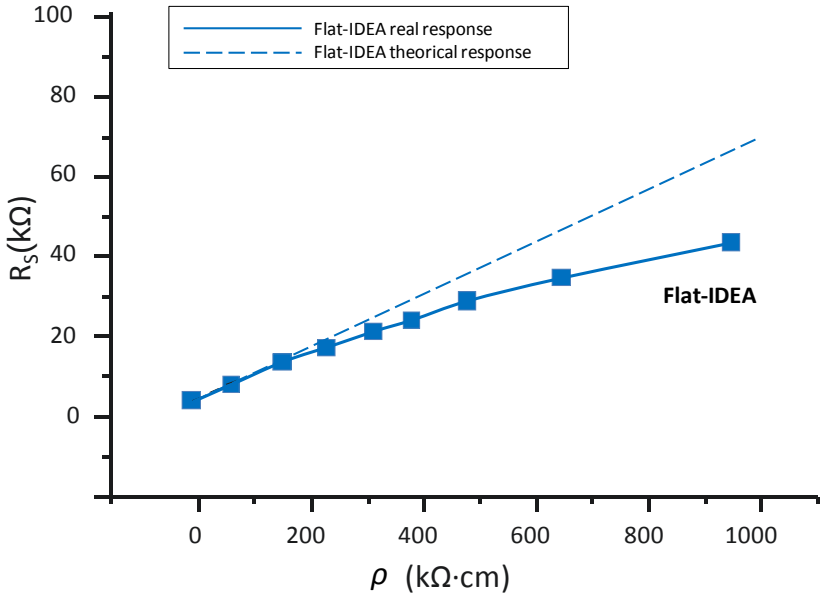


Figure 14. Solution resistances (R_S) obtained from equivalent circuit fitting as a function of the electrolyte resistivity for planar IDEA sensors. The dashed lines indicate the linear expected sensor behavior in low-conductivity solutions.

The decline of R_S in solutions of low conductivity is attributed to the effect of the surface conductivity within the interdigital spacing. Here, at neutral pH the SiO_2 or glass forming the isolating substrate is negatively charged due to the presence of ionized OH groups. These negative charges are compensated by cations from the bulk solution, accumulating positive counterions in the electrical double layer. Thus, when an electric field is applied between the electrode digits the higher concentration of ions within the electrical double layer over SiO_2 provokes that the electric current along the SiO_2 surface may be higher than through the bulk electrolyte solution [64] (Figure 15B).

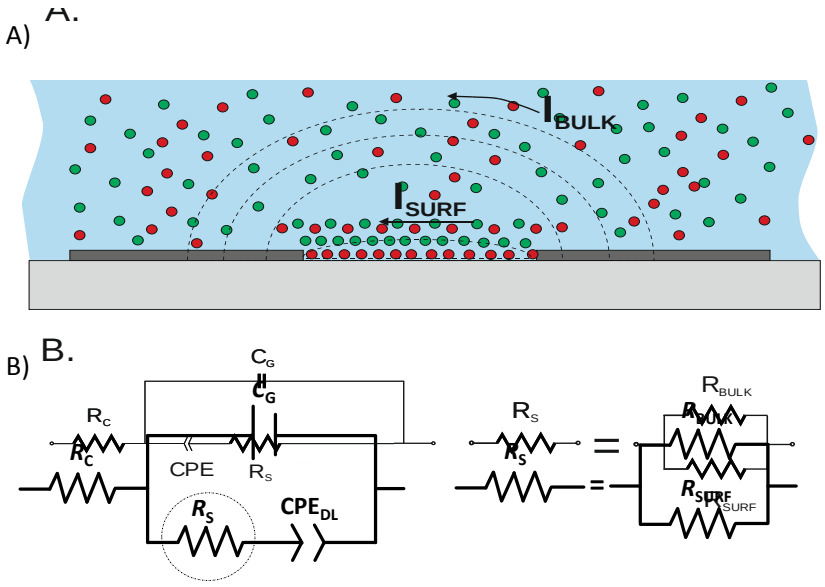


Figure 15. Physical model (A) and electrical equivalent circuit (B) of a planar IDEA device in low conducting solutions.

Consequently, R_S, which represents the resistance between the IDEA electrodes determined from impedance spectra, should be treated as a parallel combination of the bulk solution resistance (R_{BULK}) and the surface resistance (R_{SURF}) of the space between the electrode digits as shown in Figure 15B.

$$\frac{1}{R_S} = \frac{1}{R_{SURF}} + \frac{1}{R_{BULK}} \tag{1.14}$$

The effect of R_{SURF} on R_S will be higher the higher is the bulk solution resistance. In low conductivity solutions we may fix experimentally the solution bulk resistance, then any changes in R_S can be attributed to R_{SURF} variations. Therefore, the IDEA sensor response in controlled low conductivity solutions allows monitoring the changes in the surface charge due to the reaction with charged species. This property is fundamental to understand the changes that are occurring in the interface of the sensor surface.

1.6.2. Three dimensional interdigitated electrode arrays (3D-IDEA)

As previously mentioned, the 80% of the current flowing between the electrodes is located in a layer with a thickness equal to the separation between the adjacent electrode digits centers [71]. Thus, the reduction of the electrodes digits width and spacing under the micro scale may result in a higher sensitivity to surface parameters variations. However, it is demonstrated that the miniaturization of IDEA at the nano-scale the surface is exposed to carbon micro and nanoparticles contaminations outside the cleanroom facilities that alter the sensor electrical parameters, producing problems of short circuits between adjacent electrodes and consequently, reducing their reproducibility and stability.

To solve this limitation and enhance the sensitivity Bratov and co-workers [78, 79] proposed to separate the electrodes digits of an IDEA using SiO_2 insulating barriers to enhance the effect of surface conductivity

on the impedance measurements. In this case the penetration of the electric filed into the bulk solution is the same as for a planar/flat IDEA with equivalent geometry, but the current path along the SiO_2 surface is much longer, which permits to enhance one order of magnitude the sensitivity of the device to surface conductivity changes. Moreover, barriers reduce the effect of contaminations because the electrodes digits are electrically separated and conducting microparticles do not cause short-circuiting. This design with the insulating barriers is named as a three-dimensional interdigitated electrode array (3D-IDEA) and is shown in Figure 16).

Barriers of different height may be formed between the adjacent electrodes. In previous works the sensitivity of sensors was evaluated using various barrier heights and the same width of the barrier and the electrode digits (3 µm). The analysis of the sensors response demonstrated that devices with 4 µm barriers are more sensitive [79] (see Figure16 B). This type of 3D-IDEA sensors have been used in this work.

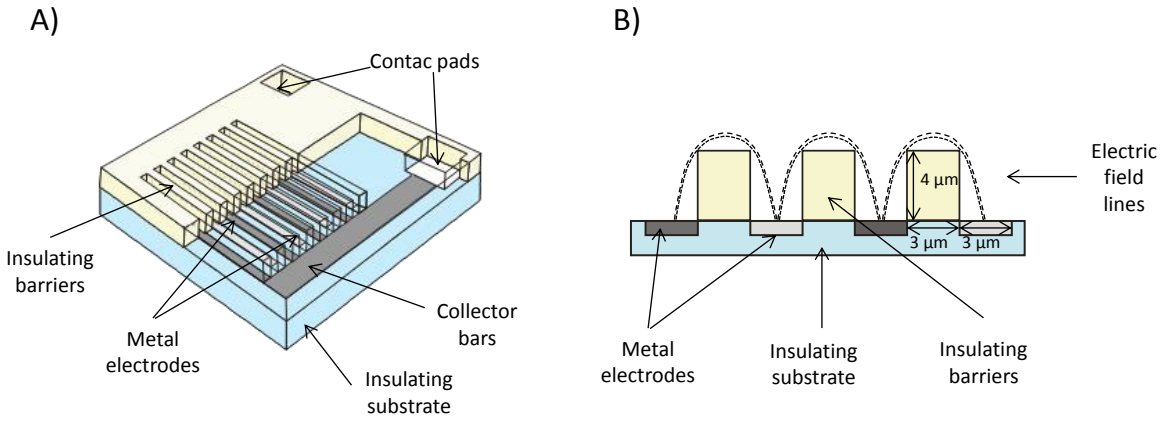


Figure 16. Three-dimensional interdigitated electrode arrays (3D-IDEA) design (A) and its cross-section with the electric field lines (B).

Differences between standard IDEA and 3D-IDEA with the same geometry can be easily examined measuring the impedance responses in the same low ionic-strength solution. As can be observed from Nyquist diagrams shown in Figure 17 the intercept of the semicircle with the X-axis, corresponding to R_S, becomes higher in the case of 3D-IDEA because the presence of barriers involves larger cell constants (K_{CELL}).

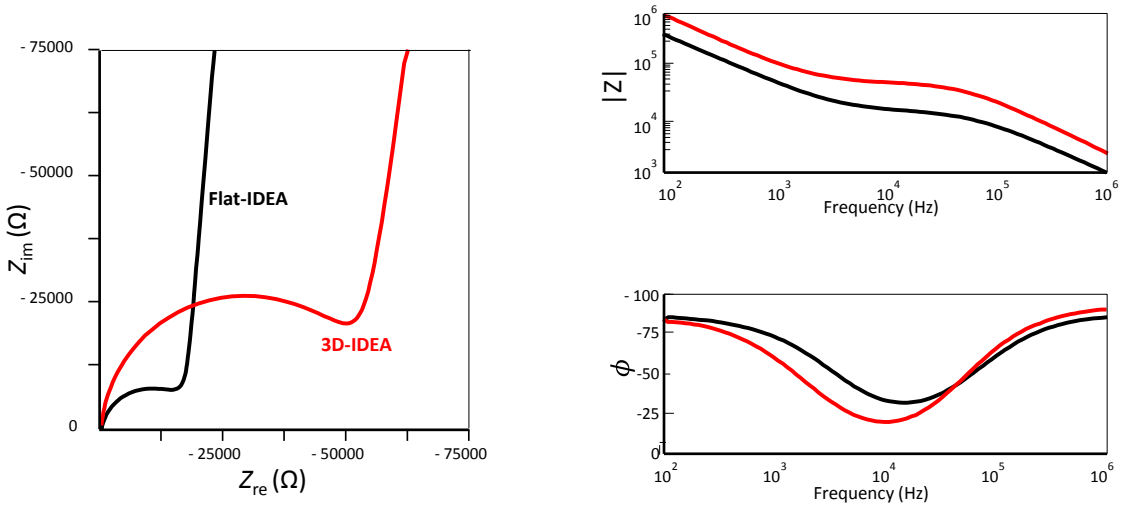


Figure 17. A) Nyquist and B) Bode diagrams of flat IDEA and 3D-IDEA of 3x3 µm (width and spacing) geometry, with same number and length of the digits, measured in the same conductivity solution (KCl 10^{-5} M).

Moreover, if the R_S values of flat and 3D-IDEA devices obtained in solutions with different electrolyte resistivity (ρ_S) are compared, one can see (Figure 18) that the difference between expected theoretical and measured R_S values is much larger in the case of 3D-IDEA. This shows that the presence of barriers enhances significantly the sensitivity to the surface charge.

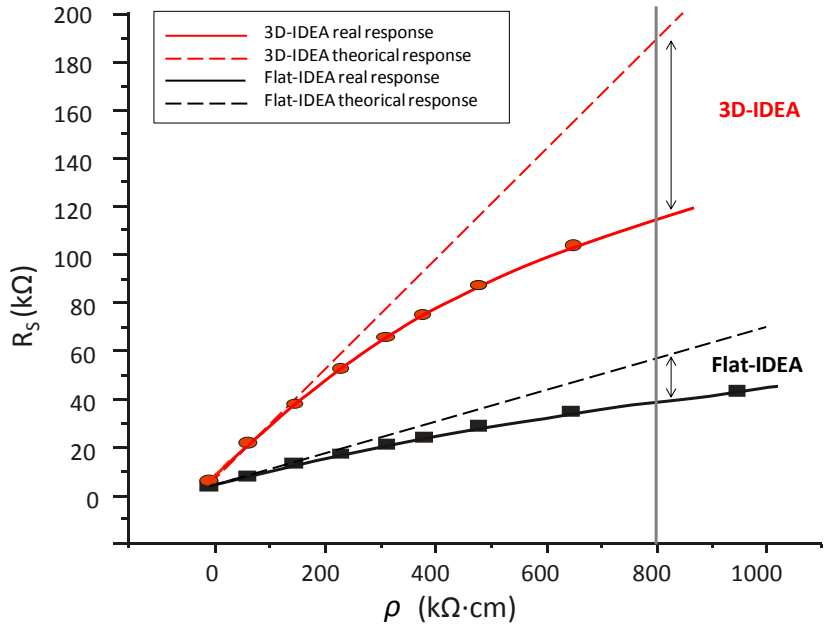


Figure 18. Solution resistances (R_S) obtained from equivalent circuit fitting as a function of the electrolyte resistivity for planar IDEA (black) and 3D-IDEA (red) sensors. The dashed lines indicate the linear expected sensor behavior in low-conductivity solutions.

To sum up, most of large molecules involved in biochemical reactions are electrically charged due to the presence of ionizable groups. IDEA-based sensors being sensitive to the surface charge variation within the interdigital spacing are a powerful tool for direct label-free detection of different analytes in solution. In the case of 3D-IDEA the higher sensitivity to the surface conductivity changes can be employed to easily

monitor the surface modifications. In this thesis, these sensors have been used to study the phenomena occurring on the IDEA interface for biosensing in a wide sense, from the study of the biofunctionalization strategies of the sensor surface, the bacterial adhesion by means of a biorecognition element or the changes produced by bacterial activity.

1.6.3. Fabrication and characterization of planar IDEA and 3D-IDEA sensors

Interdigitated electrodes are composed by parallel metal electrodes formed on an insulating substrate like glass, oxidized silicon surfaces or polymers. IDEA and 3D-IDEA sensors employed in this thesis were fabricated in the clean room facilities of the Microelectronics National Center of Barcelona (IMB-CNM, CSIC) on silicon wafers (of 4 inches diameter) using conventional microfabrication techniques and lithography process.

The first fabrication step is the formation of insulating silicon dioxide layer on a silicon wafer by wet oxidation of silicon at 950 ^{o}C to grow a SiO_2 coating layer of 2500 nm, isolating the silicon from the following deposition of metals. The second step is the magnetron sputtering deposition of a 230 nm thick layer of tantalum silicide ($TaSi_2$), which is a highly conductive material employed for the formation of the electrodes. The choice of $TaSi_2$ instead of other traditional metals like gold or platinum is conditioned by the contaminating nature of these metals in standard complementary metal-oxide-semiconductor (CMOS) processes employed for the fabrication of the devices.

The following phase is the photolithographic step to define the collector bars and digits of the two coplanar electrodes. The patterning is carried out by means of reactive ion etching technique. The sensors geometry and electrode dimensions are defined by design of a mask used in photolithographic processes. As previously mentioned, in this thesis a symmetrical interdigitated structure with 216 digits of 3 µm width separated by a 3 µm gap has been employed. The electrode digits are 1.5 mm long and the aperture (overlapping) between the electrodes is 1.4 mm. The total length between the electrodes is 301 mm.

The contact pads are formed by deposition and patterning of 1 µm of aluminum using photolithographic and etching steps, leaving the metal only at the extremes of the two collector bars. At this point there are some differences in the fabrication of flat IDEA and 3D-IDEA sensors.

In the case of the standard flat IDEA the final step is covering of the wafer with a 700 nm thick low pressure chemical vapor deposition (LPCVD) silicon dioxide passivation layer in which windows are opened in the area of electrode digits and contact pads. In the case of the 3D-IDEA an additional step of the barrier formation is required. To do this the wafer with the interdigitated electrodes is covered with a 4 µm thick LPVCD silicon dioxide, the layer that defines the height of barriers.

The last photolithographic step is deep reactive ion etching (DRIE) of 4 µm thick SiO_2 employed to open the $TaSi_2$ electrode digits and the aluminum contact pads. This DRIE process allows to obtain 4 µm high

3D-IDEA barriers with practically vertical walls. The spacing between the barriers is equal to electrode digit width (3 μm) and the barrier width is also 3 μm.

Finally, the wafers are cut and the individual sensors are glued on a printed circuit board (PCB) substrate and wire bonded for the electrical connections. The contact pads of aluminum and the wires of PCB are encapsulated using an epoxy resin (Figure 19). Optical microscopy and Scanning Electron Microscopy (SEM) images of flat and 3D-IDEA are presented in Figure 20.

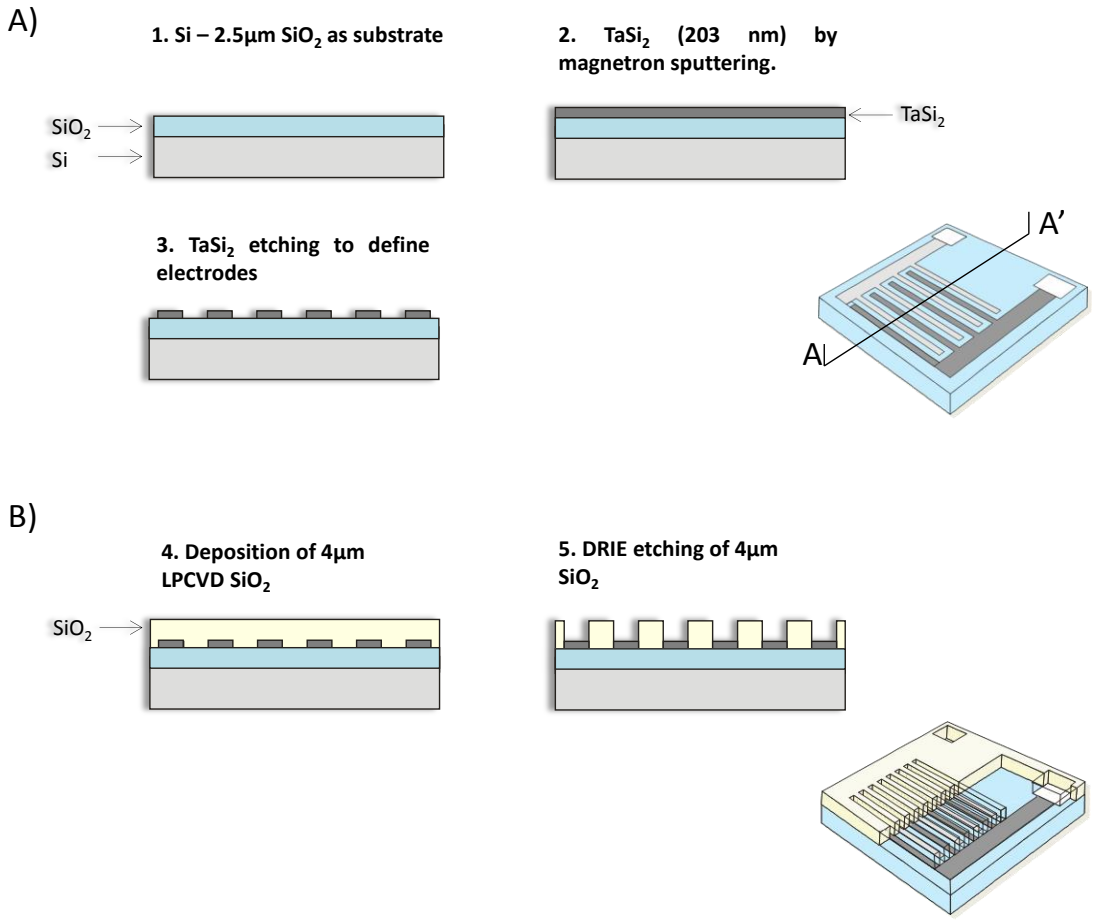


Figure 19. Basic microfabrication steps for standard A) planar IDEA and B) 3D-IDEA.

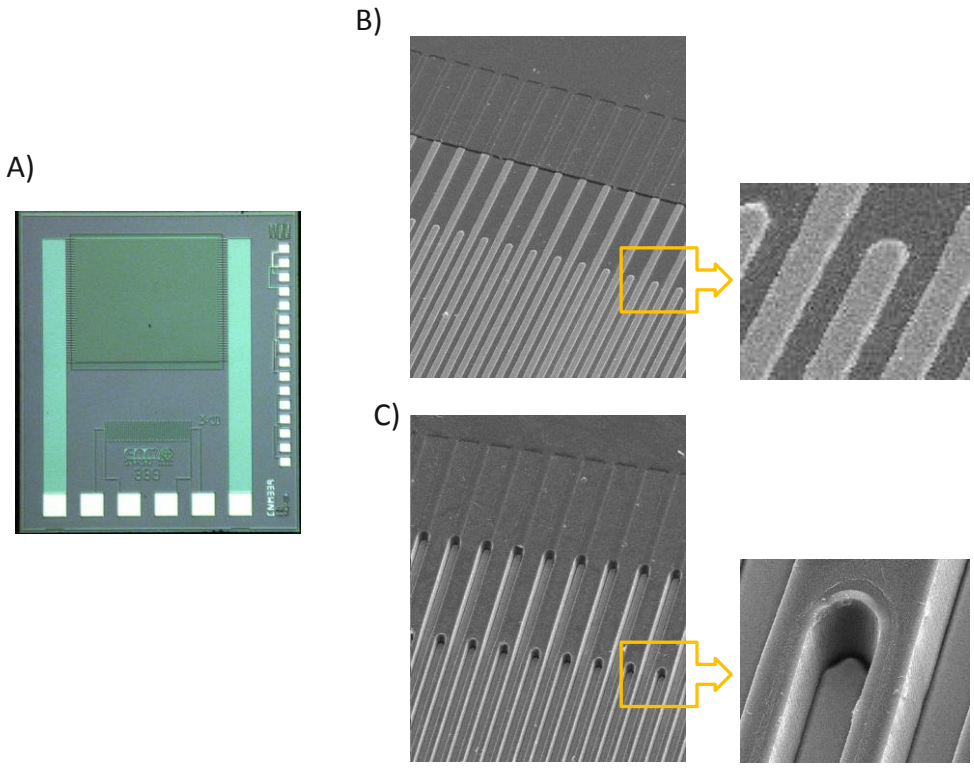


Figure 20. Optical image of IDEA surface (A) and SEM images of interdigitated electrode fingers of a planar IDEA (B) and of a 3D-IDEA with 4 mm barriers (C).

1.7. Impedimetric properties of bacterial cells

From a microbiological point of view, bacteria are usually classified depending on different parameters, like cellular structure (Gram-positive or Gram-negative), morphology (rod or bacilli-shaped), genetics (amount of DNA base pairs) or cellular metabolism (–autotrophic, heterotrophic or mixotrophic–; –litotrophic or organotrophic– and –chemotrophic or phototrophic–) [1].

All biological cells are formed by a complex spatial arrangement of biomaterials with very different electrical properties, for example, the cell membrane is highly insulating in contrast to the interior of the cell, the cytoplasm, which is highly conductive. Cell membrane consists of a lipid bilayer formed by different proteins, in which the lipid molecules are oriented with their polar groups facing outwards into the aqueous environment, and the hydrophobic hydrocarbons chains pointing inwards to form the membrane interior [60]. There are also differences in the bacteria cell wall structure. Gram-negative bacteria cell membrane is formed by two lipid bilayers separated by a thin layer of peptidoglycan, in which the external lipid membrane contains lipopolysaccharides and lipoproteins. Gram-positive bacteria comprise a thick layer of peptidoglycan forming the cell wall, which also contains teichoic acids and other compounds surrounding the lipid membrane [7] (see Figure 21). In both cases, the bacterial cell membrane contains various ionizable groups, such as carboxylic, phosphate or amino groups, with higher

concentration of anionic in front of cationic, and this result in a partial negative cell wall charge at neutral pH [80].

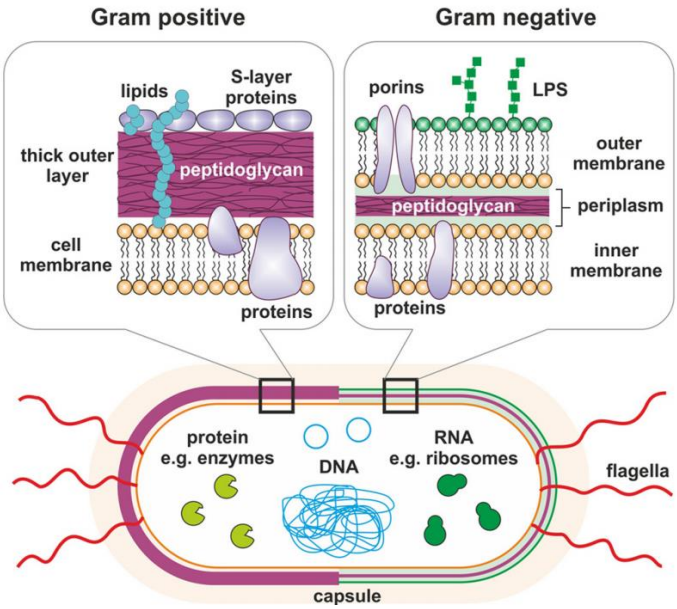


Figure 21. Bacterial architecture of Gram-negative and Gram-positive bacteria (adapted from [7]).

The intercellular bacteria structure forms their cytoplasm without defined organelles, like nucleus, chloroplast or mitochondria, and contains charged biomolecules like DNA, RNA, proteins as well as many dissolved charged ions, resulting in high conductivity [81].

In addition, a relevant aspect to take into consideration is impedimetric properties of bacterial cells. Under application of an external electric field the presence of bacteria affects the overall impedance of the system. Figure 22 shows a model of the basic electrical elements that defines bacterial cell impedance.

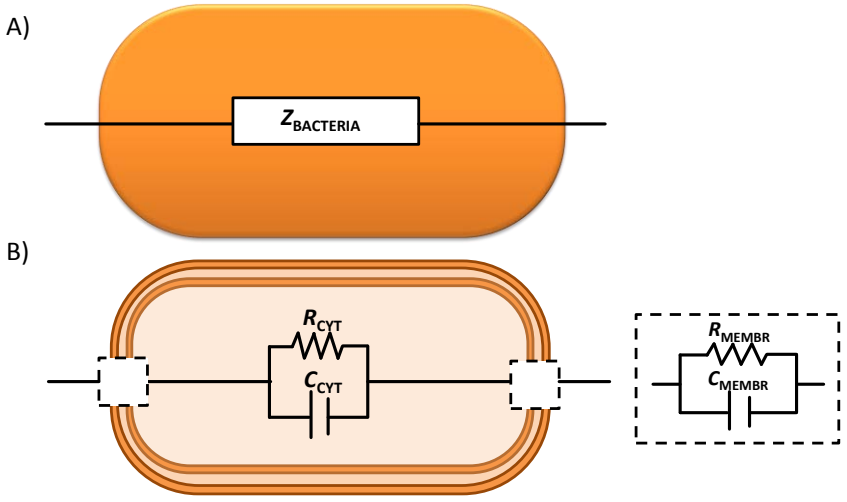


Figure 22. Impedimetric electric elements of a model rod-shaped bacterial cell, representing the total impedance ($Z_{BACTERIA}$), cytoplasm resistance and capacitance (R_{CYT}, C_{CYT}) and membrane resistance and capacitance (R_{MEMB} and C_{MEMBR}).

Therefore, considering the bacterial cells electrical properties, the attachment of bacteria on an electrode surface directly affects the superficial impedance. If bacterial cells remain intact after the immobilization onto an electrode, the active area of the electrode will be affected, with bacteria obstructing the pass of the current thus increasing the interfacial impedance [60]. The majority of impedance-based biosensors for detection of bacteria are based on this principle. In this sense, impedance sensors are also widely employed for monitoring the biofilm formation because of their insulating properties [82]. On the other hand, if the integrity of the cells is affected and the citoplasmatic material is released, changes in the interfacial properties (capacitive and resistive) will be produced by an increase in the conductivity at the electrode surface as consequence of the leakage of ions from the cytoplasm.

1.8. Impedimetric biosensing of bacteria: from impedance microbiology to impedimetric biosensors

Among different electrochemical techniques, impedance has been widely employed in different modes for bacteria detection and bacterial metabolism monitoring. Basically, sensing of bacteria using EIS may be performed in two ways: by the detection of metabolites produced by bacterial growth, that involves conductivity changes in the culture medium [83], or based on the impedance changes caused by interaction of target bacteria immobilized on the surface of an electrode [58, 84]. Additionally, impedance can also be employed for monitoring changes in the ionic concentration and conductivity of the medium caused by the activity of enzymes used as labels for the signal amplification [85]. It has to be taken into consideration that impedance allows to carry out label-free detection thus improving the performance of biosensors making the assays simpler and rapid. However, in some cases the labelling can increase the impedimetric response by using selective sandwich-based assays.

The first experiments that correlated the measured impedance with microorganisms growth was described in 1899 by Stewart and coworkers [86], but it was only starting from 1970s when much attention was put to this research [87-90]. The term *impedance microbiology* is referred to an impedance method for the detection of bacterial growth based on the changes produced in a culture medium resulting from the growth of bacteria [60]. This “classic method” based on the monitoring of bacteria metabolism also allows to distinguish between viable and non-viable cells. First studies employed a pair of metal electrodes immersed in a growth medium or reactant solution with bacteria and monitoring impedance changes in time. These changes are mainly associated with the release of ionic metabolites, like carbon dioxide and organic acids, produced by live bacterial cells after consumption of oxygen and sugars and resulting in increase of the conductivity of the culture medium. Other ionic species like K^+ and Na^+ are also released in the medium through ionic channels in the cell membrane to regulate the osmotic differences between the interior and exterior of bacterial cells. Therefore, to detect bacteria these impedance systems measure relative or absolute changes in conductance, capacitance and/or resistance at regular time intervals during the growth of bacteria at a specific temperature [60].

Several commercial analytical systems based on impedance microbiology has been developed for bacteria detection such as Bactometer (Bio Merieux, Nuertingen, Germany), Malthus systems (Malthus Instruments Ltd., Crawley, UK), rapid automated bacterial impedance technique (RABIT) (Don Whitley Scientific Ltd., Shipley, UK), and BacTrac (Sy-Lab, Purkersdorf, Austria [30, 60, 91, 92]. However, despite the fact that those systems have significantly reduced the detection times compared with traditional methods, like microbial colony counting, they are still time consuming (from 3.5 to 16 hours) [93] depending on bacterial type. In this sense, all techniques based on detection of the bacterial growth by metabolism products monitoring present certain limitations related with the detection time.

In the past few years the impedance technique has acquired more value for the study of bacterial detection by the development of microarrays of electrodes into a chip format applied to impedance microbiology methods [94]. For example, the use of interdigitated electrodes in impedance microbiology allows the reduction of the sample volume and shorter detection time. In the case of impedance microbiology, the main parameter that contributes to the total impedance is the medium or electrolyte impedance, which is progressively changing during the bacterial growth [95].

More recently the integration of impedance technique with biosensor technology has led to the development of impedance-based biosensors for direct bacteria detection [60, 96]. In this case, the electrode surface is functionalized with biorecognition elements to detect bacteria as the target analytes. Different surface modification techniques may be selected based on the electrode material and the addressable functional groups present on the biorecognition element and the surface of the electrodes [69].

1.8.1. Impedance biosensors for detection of bacteria

As previously mentioned the development of different types of impedance-based biosensors for detection of bacteria resulted in scientific publications that have grown exponentially during the last two decades. Most of the studied impedimetric biosensors employ immobilization of biorecognition elements on their surface that selectively reacts with a specific target analyte (bacteria) forming a complex that alter the electrical properties of the sensor surface.

Different kinds of electrode configurations exist for the development of impedimetric biosensors. One of the most common systems applied for impedance based biosensors is based on the three-electrode configuration. Here, a working, an auxiliary (also named counter) and a reference electrodes are employed to measure the impedance of the electrochemical cell. In this case, the working electrode is functionalized with the biorecognition element to interact with bacteria. The exciting signal and the current is measured between the working and auxiliary electrodes, and the reference electrode allows controlling the DC bias applied to the working electrode. This configuration is mainly employed in faradaic mode in systems that require an accurate control of redox process.

In the case of interdigitated electrodes, as previously described, a two-electrode configuration is employed combining parallel microband electrodes of identical size. The applied signal and the current response are measured between the pair of the electrodes without applying additional DC bias. As both electrodes are commonly of the same material it is assumed that the electric potential difference between them is close to zero. This eliminates the need for a reference electrode. In this section we will focus on previous published works dealing with interdigitated electrodes as the transducer for the development of impedimetric biosensors.

A crucial step in the performance of any kind of impedance based-biosensor is the immobilization of a bioreceptor on the sensor surface. Different strategies are used to promote the interaction: bioaffinity layers, Langmuir-Blodgett films, thiol containing self-assembled monolayers on gold (SAMs), chemical grafting by silanization strategies, layer-by-layer deposition of polyelectrolyte films or thin polymers [50, 54, 97]. Basically the appropriate immobilization technique depends on the biomolecules chemical structure, as well as the electrode material nature. In any case it should be taken into consideration that the biomolecules employed have to maintain their functionality after immobilization [98].

The following Table 2 summarizes the main works published in the last years related to the detection of whole-cell bacteria as well as some bacterial components considered as contaminants. The reported impedimetric biosensors summarized in the table are based on interdigitated electrodes of different geometries while the bioreceptors molecules promotes the selective interaction of bacteria on the transducer surface.

Target bacteria or bacterial components	Biorecognition element	IDEA material	Site of immobilization (M and/or O)	Application	Detection time	Limit of detection (range of application)	Supporting solution / medium	Reference
***E. coli* O157:H7**	Antibodies	Indium-tin oxide (ITO)	M+O	Detection of bacterial cells	–	10^6 CFU/mL	Redox probe $[Fe(CN)_6]^{3-/4-}$)	[58]
E. coli K12	Antibodies	Gold on silicon dioxide	O	Detection of bacterial cells	–	10^5 CFU/mL (10^5 to 10^7 CFU/mL)	0.1% peptone water	[84]
***E. coli* O157:H7**	Antibodies	Gold on silicon dioxide	O	Detection of bacterial cells in food samples (lettuce)	–	10^4 CFU/mL (10^4 to 10^7 CFU/mL)	0.1% peptone water	[96]
***E. coli* K12**	Antibodies	Chrome on glass	O	Concentration and detection of bacteria	–	10^4 CFU/mL	0.1M mannitol solution	[99]
Salomenlla enteritidis	Biotinylated rabbit anti-*Salmonella*	Gold on silicon dioxide	M	Detection of foodborne pathogens	3 min	10^4 CFU/mL in PBS and 10^5 CFU/mL in milk	PBS and milk	[100]
DNA of pathogenic bacteria	Oligonucleotide eco3 thiol-modified (39 base	Gold on glass substrate	M	DNA detection of pathogenic bacteria	30 min	–		[101]

	long)							
***E. coli* O157:H7**	Magnetic Nanoparticles with antibody conjugates	Gold on silicon dioxide	M	Detection of bacterial cells in food samples (ground beef)	35 min	7.4×10^4 CFU/mL (7.4×10^4 to 7.4×10^7)	Mannitol solution	[102]
***E. coli* O157:H7**	Magnetic Nanoparticles with antibody conjugates	Gold on silicon dioxide	M	Detection of bacterial cells in food samples (ground beef)	35 min	1.6×10^2 cells (4.2×10^4 CFU/mL	Mannitol solution	[103]
DNA sample from Salmonella choleraesuis	Oligonucleotide with amino group at 5'(38 base long)	Titanium and platinum on glass	O	Detection of pathogen DNA	30 min	50nM	PBS	[104]
E. coli* K12 and *Salmonella typhimurium	Antibodies	Gold on glass substrate	M+O	Detection of bacteria	40 min	10^4 CFU/mL (*E. coli*) and 10^5 CFU/mL (*Salmonella*)	PBS	[105]
E. coli, JM109	Antibodies	Conductive polysilicon	M+O	Selective Detection of Live/Dead pathogens	1 h	3×10^4 CFU/mL	Glycine buffer	[106]
E. coli ATCC 35218, E. coli O157:H7, S. typhimurium, and L. monocytogenes	Antimicrobial peptides (magainin I)	Gold on silicon dioxide	M	Detection of bacteria	–	10^3 CFU/mL	PBS	[107]
E. coli K12	Antibodies	Gold on quartz glass	M	Propose a quantitative estimation model of bacteria captured	–	10^3 to 10^6 CFU/ml	PBS	[108]
Staphylococcus aureus	Antibodies	Aluminium (covered with Al_2O_3)on silicon dioxide	M+O	Demonstrate the use of interdigitated array microelectrode for detection of bacteria	10 min	–	Distilled water	[109]
***E. coli* O157:H7**	Antibodies	Gold on glass	M+O	Detection of bacteria in food samples	–	$3x10^3$ CFU/mL	Grape and spinach samples	[110]
Legionella pneumophila	Antibodies	ITO on glass	M+O	Detection of Legionella in cooling towers	1 h	From 10^5 to 10^8 CFU/ml	PBS	[111]
Staphylococcus aureus* and *Helicobacter pylori	Antimicrobial peptides	Graphene with gold	M	Bacteria detection in saliva	–	(10^3–10^8 CFU/mL)	Distilled water	[112]
***E. coli* O157:H7**	Antibodies	Gold on glass	M	Detection of bacteria	45 min	$3x10^2$ CFU/mL	Distilled water	[113]
***E. coli* lipopolysaccharides/ endotoxins**	Polymixin B (antibiotic)	Silicon-based	M+O	Detection of bacterial endotoxins	–	0.1µg/mL (0.1-100µg/mL)	Water media	[114]
E. coli O157:H7	Antibodies on nanodiamonds	Gold on silicon dioxide	M+O	Detection of bacteria	≈ 1 h	–	Isotonic trehalose solution	[115]

E. coli O157:H7	Antibodies and lectins	Gold on ceramic substrate	M	Detection of bacteria	1 h	10^2 CFU/mL (10^2 – 10^7 CFU/mL)	Redox probe ($K_3Fe(CN)_6$/ $K_4Fe(CN)_6$/ in PBS)	[116]
E. coli O157:H7	Antibody-coated magnetic nanobeads	Gold on ceramic substrate	M	Detection of bacteria	≈ 1 h	$10^{4.45}$ CFU/mL (10^4 – 10^7 CFU/mL)	Redox probe $[Fe(CN)_6]^{3-/4-}$) in PBS	[117]
Salmonella enterica serovar (Salmonella typhi)	Antibody-coated gold nanoparticles	Platinum on glass	M	Detection of bacteria in clinics	–	10^2 CFU/mL	HEPES buffer	[118]
Streptococcus sanguinis	Antimicrobial peptides	$TaSi_2$ electrodes on silicon dioxide	M+O	Detection of bacterial in saliva	1 h	$3.5x10^1$ CFU/mL in KCl 10^{-5} M and $8.6x10^2$ CFU/mL in artificial saliva	KCl 10^{-5} M	[119]
***E. coli* DSMZ 17076**	Antibodies	Conductive polysilicon	M+O	Detection of bacteria	30 min	–	KCl	[120]
Enteropathogenic *Escherichia coli*	Antibodies	Silicon Carbon alloy	M+O	Develop a new testing IDEA	–	–	Luria-Bertani (LB) medium	[121]
E. coli O157:H7	Antibodies	Graphene with gold	M	Detection of foodborne pathogens	30 min	10-100 cells/mL	PBS	[122]
***E. coli* lipopolysaccharides/ endotoxins**	Concanavalin A	$TaSi_2$ electrodes on silicon dioxide	M+O	Detection of bacterial endotoxins and new blocking method development	20 min	2 µg/mL	KCl 10^{-5} M	[123]
Salmonella typhimurium	Antibodies	Gold on glass	M	Detection of foodborne pathogens	1 h	10^2 CFU ($76–7.6x10^6$) in 50µL	Redox probe $[Fe(CN)_6]^{3-/4-}$) in PBS	[124]
E. coli O157:H7 (E. coli DSMZ 17076)	Aptamer	$TaSi_2$ electrodes on silicon dioxide	M+O	Detection of bacteria in water samples	30 min	$2.9x10^2$ CFU/mL	KCl 10^{-5} M	[125]
***E. coli* DH5α**	Aptamer	Gold	M	Detection pof bacteria and LPS	30 s	267 cells/mL (10^2–10^6 cells/mL)	Distilled water	[126]

Table 2. Summary of more relevant works published last years in the development of impedimetric biosensors using interdigitated electrode arrays as the transducer for detection of different bacteria (M=metal, O=oxide).

In the last years to improve biosensors detection systems novel technologies like microfluidics and use of nanoparticles have been employed. Nanoparticles of various materials ranging from 1 to 100 nm possess unique physical and chemical properties due to their high surface area, small size and shape and are suitable for different applications. In this sense, they have been successfully employed for constructing different impedimetric biosensors for bacteria determination. For example, Yang and co-workers [127] developed a capacitive immunosensor for detection of *Salmonella* using gold nanoparticles (AuNP)

functionalized with antibodies, promoting the interaction of bacteria on the electrode surface through physical adsorption. Huang *et al*. [128] developed an impedimetric immunosensor for rapid detection of *Campylobacter jejuni* bacteria using thermally stables, chemically inert and non-toxic metal-oxide Fe_3O_4 nanoparticles to immobilize antibodies on its surface to interact with bacteria and to improve the response of the impedance-based biosensor.

Besides nanomaterials, microfluidics technique is a good strategy for improving the performance of impedimetric bacteria biosensors [31]. Microfluidics is a multidisciplinary field that involves different sciences like chemistry, biochemistry, engineering, physics and micro and nano-technologies, and allows to process low volumes of fluids improving the surface-to-volume ratio in chip formats [129]. The use of microfluidics allows continuous injection of bacteria samples in the impedance analysis system improving the throughput processing, enhancing the transport and the flow conditions, mixing of the sample with different reagents and the reduction of the analysis volume. Moreover, the possibility to utilize the same platform for both sample processing and detection may increase the sensitivity [130]. For example, Tan [131] presented a polydimethylsiloxane (PDMS) microfluidic sensor integrated with specific antibodies against *E. coli* O157:H7 and *Staphylococcus aureus* on a nonporous membrane for achieving a rapid detection of these pathogenic bacteria within 2 hours and a high sensitivity of 10^2 CFU/mL. Moreover, in other works the development of impedimetric detection in microfluidic biochip-format were also employed for rapid detection of bacterial metabolism as reported by Gomez and collaborators [94].

Another alternative technique employed for bacteria detection is the combination of impedimetric biosensors based on interdigitated electrodes and dielectrophoresis (DEP). Basically, DEP forces consist in the motion of particles due to its polarization in the presence of non-uniform electric fields [132]. Bacterial cells possess charged components, thus they can be polarized in the presence of an electric field. Since IDEA design is based on a periodic electrode structure they can generate DEP forces. The combination of impedance measurements with DEP is called dielectrophoretic impedance measurements (DEPIM) [99]. The dielectric properties of bacteria can be taken in advantage to move bacterial particles depending on the dielectrophoretic forces employed. For example, positive dielectrophoretic forces enable to trap bacteria on the edges of electrodes, while in the case of negative dielectrophoresis bacteria are pushed away from the electrode surface [69]. Suehiro and co-workers [99] proposed a method to immobilize antibody molecules on a chip surface in advance of *E. coli* bacteria trapping by positive DEP, demonstrating the selectivity of the proposed approach. In other works, Yang *et al*. [133] developed an IDEA integrated into a microfluidic system for the concentration and specific capture of *Listeria monocytogenes* by employing DEP and immobilizing anti-*Listeria* antibodies.

The main advantage of this separation technique is the possibility to concentrate bacteria in samples in which the concentration is really low, improving the attraction of bacteria on the electrode sensing surface. Additionally, the DEP based impedance detection requires almost no sample preparation step and is rapid and easy-to-use [69]. However, one of the main limitations is that the experimental

conditions of DEP forces depend on the conductivity and composition of the supporting solution, reducing the utility in *in situ* applications and complex samples processing.

All these complementary techniques and their combination should be considered for the development of new impedance-based biosensing systems for detection of bacteria and bacterial contaminants.

1.8.2. Impedimetric microbial biosensors

Microbial biosensors integrate microorganisms as the biorecognition elements with a transducer to generate a signal proportional to the concentration of an analyte [43]. The use of microbial-based biosensors for detection of different analytes has been developed for environmental, food and biomedical applications in the recent years. Although in the literature the concept of microbial biosensor is occasionally employed for the description of biosensors to detect bacteria, in this thesis the term "microbial biosensor" will be used to describe those sensors in which bacteria act as the biorecognition elements. In this section we will refer only to bacteria-related examples and no other type of microorganisms like yeast or microalgae.

In the development of microbial biosensors several important aspects should be considered. First of all, one of the most important problems is how to link the biological signal from the recognition element to the transducer. Here, the immobilization method plays an important role in the operation of microbial biosensors.

Many microorganisms have natural tendency to attach to surfaces and grow on them, so they can be passively immobilized. However, commonly used immobilization protocols include active immobilization like physical entrapment, covalent binding or gel entrapment [134]. Physical adsorption takes advantage of hydrophobic and adsorptive interactions to fix microorganisms on the transducer surface and have potential for high efficiency of immobilization of viable cells, but the main limitation is the lack of stability of the cells on the sensor surface [43]. Covalent binding uses different immobilization techniques, for example the use of antibodies or chemical reagents like glutaraldehyde, but in this case, bacteria are exposed to harmful conditions that may affect the cell membrane and their viability. The most frequently used microorganism immobilization technique is through entrapment in some gel-like matrix. Commonly utilized materials include natural polysaccharides (agars, carrageenans, chitosan or alginates), proteins (collagens and gelatins), synthetic polymers (polyacrylamide, polyvinyl alcohol, poly (ethylene glycol), polyurethanes, polyethyleneimine, etc.) or sol-gel-derived silicates [135-137]. However, these matrices also present certain variations in their properties depending on temperature, nutrient diffusion, degradation or lack of stability [134].

Another important aspect to consider in most of microbial biosensors applications is the viability of microorganisms. The majority of reported microbial biosensors rely on information related with the metabolic activity of bacteria. For example, the measurement of the respiratory activity can be correlated

with the biological oxygen demand (BOD) or metabolic activity related with consumed nutrients. In those cases, bacteria have to remain alive on a transducer surface to produce a measurable response. Other common applications of microbial biosensors are the monitoring of general toxicity (heavy metal ions, organophosphorus pesticides, phenol compounds and cyanides) in the environment, fermentation processes control, detection of antibiotics or toxins residues detection in food industry, or detection of hormones and glucose in clinical diagnosis [138].

Finally, the main inconvenience of microbial biosensors is that they typically suffer from poor selectivity to one target due to non-specific cellular response to substrates. However, with the development of biotechnology bacteria and other microorganisms can be genetically engineered with specific metabolic pathways (up-regulated or down-regulated), and their selectivity can be enhanced to specific targets [43].

A few works in the field of microbial biosensors using impedance-based transducers has been reported for different applications. García-Aljaro *et al*. [139] reported a method for detection of bacteriophages in aqueous media based on the response of *E. coli* biofilms on metal electrodes, in which the lysis of bacteria produces a decrease in impedance magnitude and capacitances. Hnaien and co-workers [140] developed a bacterial impedimetric biosensor for determination of trichloroethylene, an organic pollutant, using gold microelectrodes with carbon nanotubes functionalized with *Pseudomonas putida* using specific antibodies. In another work, a whole-cell based label-free capacitive biosensor using *E. coli* as bioreceptor was employed to evaluate the toxicity of Fe_3O_4 nanoparticles as models depending on their concentration and size [141]. Finally, a recent work of Ebrahimi *et al*. 2017 [142] studied the susceptibility of *E. coli* to ampicillin, a widely employed antibiotic, using non-faradaic impedance sensors to monitor changes in the electrical conductance depending on the antibiotic dosage and the treatment time.

Impedimetric transducers permit easy monitoring of changes in the resistivity of supporting solutions (that can be easily altered for the metabolic products of bacteria), as well as they give a possibility to study the variations in superficial resistance or capacitance on the transducer surface (directly resulting from the changes of cell membrane of immobilized bacteria on the surface). All this, along with the high sensitivity, makes impedance based sensors a promising tool for the development of novel microbial-based biosensors.

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2. OBJECTIVES

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The main goal of the present PhD thesis is the application of impedimetric transducers based on interdigitated electrode array (IDEA) for bacterial biosensing. Among different types of transducers applied for biosensor development, IDEAs are of great interest due to their high sensitivity for monitoring changes produced by physical, chemical and biological phenomena occurring on their surface. The aim of the work described in this dissertation is to take advantage of the outstanding properties of common planar IDEA sensors and three-dimensional interdigitated electrode arrays (3D-IDEA) to study various modification strategies for different applications such as bacteria or bacterial contaminants detection or the development of microbial biosensors. In order to carry out this goal, the following specific objectives have been addressed:

1. A study of different surface functionalization strategies for the development of simple, robust and sensitive methods for fast bacteria detection modifying the surface of planar IDEA impedance-based sensor.

The results of this work are presented in Section 3.1: ***Evaluation of sensitivity and response time of impedimetric transducers modified with polyethyleneimine for bacteria detection***.

2. Evaluation of different biofunctionalization strategies of concanavalin A immobilization on a 3D-IDEA transducers paying special attention to surface blocking procedures to prevent unspecific bindings and enhance the selectivity in determination of bacterial endotoxins.

The results of this study are presented in Section 3.2: ***Impedimetric sensor based on 3D interdigitated electrodes for label-free detection of bacterial endotoxins***.

3. Development of a reusable aptasensor for fast selective detection of pathogenic *E. coli* at low concentrations in water-based samples.

The results of this part are displayed in Section 3.3: ***Performance of a novel and reusable aptasensor for detection of Escherichia coli O157:H7***.

4. Development of a microbial biosensor, based on a 3D-IDEA with selective immobilization of pathogenic *E. coli* bacteria in the "trenches" of the sensor, for monitoring the activity of of antimicrobial drugs using ampicillin as a model.

The results of this work are presented in Section 3.4: ***3D impedimetric sensors as a tool for monitoring bacterial to antibiotics***.

3. METHODS AND RESULTS

3. METHODS AND RESULTS

3.1. Evaluation of sensitivity and response time of impedimetric transducers modified with polyethyleneimine for bacteria detection

This work has been published in the journal *Electroanalysis* (Annex I)

Abstract

An impedimetric transducer based on an interdigitated electrode array (IDEA) modified with polyethyleneimine was used to study interactions with bacteria present in a sample solution. Bacteria immobilization on the sensor surface affects the surface charge and produces changes in the superficial impedance. The sensor surface was chemically modified by layer-by-layer (LBL) method with oppositely charged polyelectrolyte layers by alternating polyethyleneimine (PEI) and poly(sodium 4-styrenesulfonate) (PSS). *Escherichia coli* Gram-negative bacteria were employed as a model for sensitivity and response time evaluation. Bacteria were immobilized on IDEA with PEI-PSS-PEI multilayer taking into account the ability of PEI to react chemically with outer membrane compounds of Gram-negative bacteria and thus improve purely electrostatic interactions. Detection limit of the sensor was found to be as low as 10^1 CFU/mL in *E. coli* cultures with response time around 20 minutes. In order to introduce a biorecognition element by bacterial detection, the lectin concanavalin A (Con A) was deposited on IDEA surface through PEI-Con A interaction, achieving a detection limit of 10^4 CFU/mL. The strategies developed show the possibility to achieve highly sensitive and rapid detection of bacteria using IDEA transducers.

1. Introduction

Rapid detection of low concentrations of microorganisms still remains a challenge in many fields such as water contamination, diagnosis, food safety and biological control. Conventional methods for bacteria detection as plating and culturing, biochemical tests, microscopy or flow cytometry are time-consuming while molecular techniques and immunosensors are non-cost-effective [1]. Therefore, highly sensitive, fast and low cost analytical methods are really required.

Electrochemical impedance spectroscopy (EIS) is a well-established experimental technique used to provide information on various physical and chemical properties of materials, as well as on interaction processes occurring in the bulk or at the surface of these materials [2]. Nowadays, this technique received much interest of biochemists due to its sensitivity to biomolecular recognition events occurring on the surface of metal or semiconductor electrodes modified with biomolecules like enzymes, antigens/antibodies or DNA molecules. A wide range of different impedimetric biosensors was reported and the number of publications grows from year to year [3].

This type of transducer for biosensor development may be easily miniaturized forming an interdigitated electrode array (IDEA) with two coplanar metal electrodes deposited on an insulating substrate and patterned to achieve the required interdigitated shape using standard microelectronic technology. In this case the impedance measurement is performed between parallel electrodes and no external counter or reference electrode is required, which allows to miniaturize the whole system. IDEA devices are produced using efficient microelectronic technology which permits to minimize the sensor dimensions, form multi-sensor arrays and integrate them with signal control electronics in one chip [4-7]

Different methods of impedimetric bacteria detection were studied that include bacteria binding by specific biomolecules like antibodies [8], lectins [9] or antimicrobial peptides [10]. To reduce detection limits concentration of bacteria using dielectrophoresis (DEP) was proposed [11] with posterior selective capture using antibody recognition with IDEA transducer, resulting in a high capture efficiency of bacterial cells.

Nevertheless, the majority of developed impedimetric biosensors for bacteria determination present some disadvantages. Biomolecules immobilized on electrodes surface suffer lack of stability in contact with harsh environments reducing lifetime of biofuncionalitzed systems. Another problem is the difficulty to establish a uniform and reproducible layer of bioreceptors on the surface [7]. In any case, the main problem in electrochemical bacteria sensing is the difficulty to detect low bacteria concentration in a short time [12].

Bacteria immobilization on an electrode surface allows increasing detection performance. The capture of bacteria on a solid by electrostatic interactions with positively charged surface was studied previously [13]. Polyethyleneimine (PEI) polyelectrolyte has been used to coat glass surface changing its potential to highly positive, improving bacteria immobilization by electrostatic interaction [14-15]. Along with this

antimicrobial chemical activity of PEI has been studied [16]. PEI has also been studied on IDEA transducers to detect target charged analytes in solution [17]. Basing on these results we may expect that PEI deposition on IDEAs may also result in improving immobilization and detection of bacteria present in liquid samples.

However, PEI interactions with bacteria are mainly of electrostatic nature. To make bacteria detection more selective the use of concanavalin (Con A) was proposed as a biosensor biorecognition element [18-20]. Con A is a glucose-binding lectin that reacts with specific terminal carbohydrates of bacterial pilis and outer membrane and may permit to enhance selectivity to this type of microorganisms.

The aim of this work is to develop a simple, robust and sensitive method to detect bacteria immobilization on IDEA surface. To perform the study *Escherichia coli* was used as a model Gram-negative bacteria. The main objective is to find if an impedimetric biosensor may achieve low detection limit and short time of bacteria detection.

2. Materials and methods

2.1. Electrode fabrication

The interdigitated electrode array was formed on a silicon wafer oxidized thermally by "wet" oxidation at 950 ^{o}C to give a good quality silicon dioxide layer of 2500 nm. As an electrode material a 230 nm 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. This resulted in an interdigitated electrodes array with 216 digits of 3 μm width and 3 μm gap between the adjacent electrodes. The aperture between the electrodes is 1.4 mm and the total length of two electrodes is 301 mm. To form contact pads 1 μm of aluminum was deposited and patterned using standard photolithographic and etching steps leaving metal only at extremes of the two collector bars. Finally the wafer was covered with a 700 nm thick LPCVD silicon dioxide passivation layer in which windows over electrode digits and aluminum contact pads were opened.

After being cut from the wafer the sensors are glued to a PCB substrate and are wire bonded for electrical connections. Contact pads and wires were encapsulated using epoxy resin [21].

2.2. Surface modification with polyelectrolytes (PEI-PSS-PEI) and Con A using a layer-by-layer method

Cationic polyethylenimine (PEI, branched, average molecular weight 25000, water-free from Sigma) and anionic poly(sodium 4-styrenesulfonate) (PSS, average molecular weight 70000, water-free from Sigma) were used for modification of IDEA sensor surface using layer-by-layer (LBL) self-assembling technique. Before the deposition IDEA sensors were cleaned in acetone and isopropanol and rinsed with distilled

water. To ensure cleanliness of their surface sensor were inspected by optical microscopy (Leica DM4000B-M).

To perform the deposition of polyions on sensors surface they were immersed into a PEI solution (1.5 mg/mL in distilled water) for 20 minutes, rinsed with water and dried with a nitrogen flow. To deposit the next layer sensors modified with PEI were immersed for 20 minutes into a solution of PSS (2 mg/mL in water), rinsed and dried. This procedure may be repeated several times until a desired number of layers are formed. Finally the devices with PEI-PSS-PEI layers were obtained.

Two methods of surface modification with concanavalin A (Con A, from Canavalia ensiformis, Sigma) were tested. In the first, Con A, acting as a polyanion at selected pH, was deposited over PEI by LBL method described previously by Lvov *et al.* [22]. Sensors with a PEI layer were put in contact with Con A (1 mg/mL in TRIS-HCl buffer, pH 7.4) solution for 90 minutes. This time was chosen from previous optimization experiments. In the second method [23-24] biotinylated Con A was attached via biotin-avidin reaction. Sensors surface was initially modified with PEI-PSS double layer on which avidin (from egg white, Sigma) was deposited by LBL method from solution (1 mg/mL in TRIS-HCl pH 7.4). Reaction with biotinylated Con A (from Canavalia ensiformis, Sigma) was carried out by immersing the electrodes in corresponding solution (1 mg/mL in TRIS-HCl pH 7.4) during 20 minutes.

After each deposition step impedance spectra of sensors were taken in fresh 10^{-5} M KCl solution to control the changes in surface properties of the sensors.

2.3. Bacteria cultures and sample preparation

The bacterial strain used was *Escherichia coli* (ATCC 10536) obtained from American Type Culture Collection. *E. coli* culture was grown at 37 ^{o}C overnight into Luria Bertani (LB) medium broth. The cells were then harvested by centrifugation at 9000 G for 5 minutes, washed with 10^{-5} M KCl solution and resuspended in the same solution. The cell concentration was determinate by colony counting after serial dilution followed by plating on LB-agar plates.

Samples with *E. coli* culture in 10^{-5} M KCl solution, used as medium during experiments, were prepared by adjusting concentrations by dilution as required in each case. All the experiments were done in sterile conditions to prevent contaminations. In all cases, colony counting was done also after each experiment.

2.4. Impedance measurements

All experiments were carried out by impedance measurements in a 10 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 applied for impedance data treatment and an equivalent circuit fitting.

The impedance measurements were always performed in 10^{-5} M KCl solutions with conductivity ranging from 2.4 to 2.6 µS/cm. The conductivity of solutions was controlled with a commercial conductimeter EC-Meter GLP 31+ (Crison).

All impedance tests were accomplished using the same methodology. Spectra measurements were taken at 0, 2, 5, 10, 20, 30, 40 and 60 minutes.

All experiments were performed in parallel at least on two electrodes. Corresponding results presented in this work are mean values obtained in each case.

3. Results and discussion

3.1. Equivalent circuit and sensors parameters

The impedance response of an IDEA device in low conductivity solutions in the absence of faradaic processes may be emulated by an electrical equivalent circuit [25] presented in Figure 1. It is formed by the following components: R_C is the contact resistance introduced by wires and collector bars of the electrodes; C_G is the geometrical capacitance between two interdigitated electrodes in a water solution; R_S is the resistance between two electrodes of the array; and CPE, which is a constant phase element [3] representing the capacitance of the electrode -water solution interface.

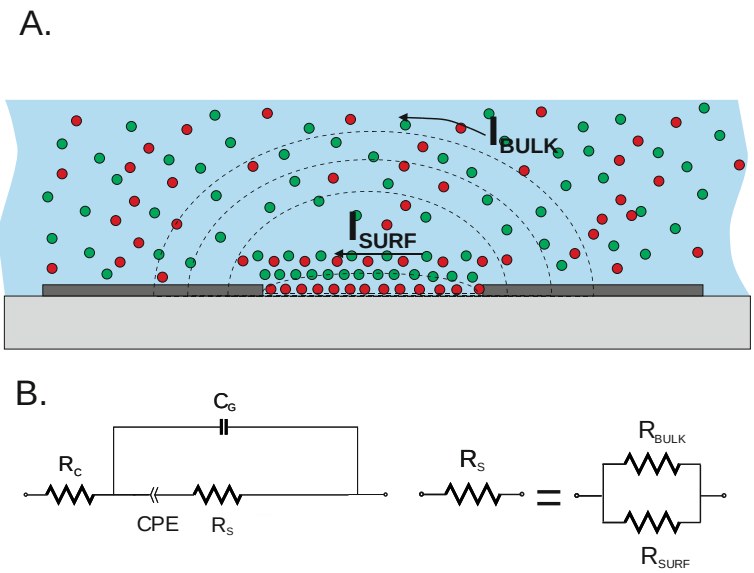

Figure 1. Physical model (A) and electrical equivalent circuit (B) of a planar IDEA device in low conducting solutions.

Earlier we have shown [26] that in poorly conducting solutions surface conductivity play an important role in the overall IDEA measured conductance/resistance. This implies that the resistance R_S (Fig.1B) is a parallel combination of the bulk solution resistance and the surface resistance. Unfortunately, we can not

resolve both of these parameters from an impedance spectra, but it is possible to fix the bulk solution conductivity performing experiments and attribute possible changes in R_S to surface resistance.

Figure 2 shows Nyquist plot of experimental impedance spectra. A semicircle at high frequencies appears due to resistance R_S in parallel to the stray capacitance. Its intercept with the Z' axes on the left side gives the R_C value. The intercept on the right side gives the value of R_S, which is the parallel combination of water solution bulk resistance and surface resistance. In all the experiments the solution resistivity was kept constant and observed R_S changes can be attributed to surface resistance variations associated with the surface charge in the spacing between the electrode digits. As reveals Figure 2, modification of a native SiO_2 sensor surface with highly charged polyelectrolytes gives rise to lower surface resistance due attraction of mobile ions from the water solution bulk to the charged surface (Fig. 1A). When this positive charge is compensated by adsorbed negatively charged bacteria cells the surface resistance significantly changes as it is shown in Figure 2.

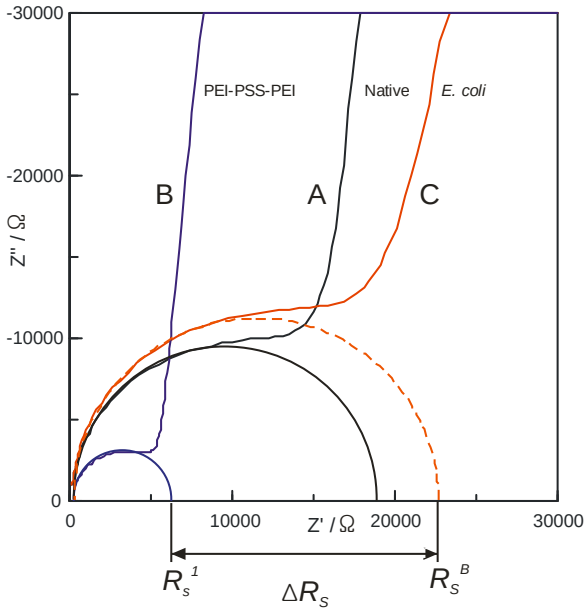

Figure 2. Impedance spectra of the IDEA surface after each modification step mesaured in 10^{-5} M KCl solution: (A) initial native SiO_2 surface, (B) after polyelectrolytes deposition and (C) after *E. coli* immobilization.

The sensors response was determined as changes in resistance R_S of a sensor before (R_S^1, Figure 2) and after (R_S^B) its contact with bacteria calculated as:

$$\Delta R_S = R_S^B - R_S^1 \quad (1)$$

3.2. *E. coli* adsorption on sensors modified with polyelectrolytes

The layer-by-layer self-assembling electrostatic deposition polyions has been demonstrated useful for different types of surface modification [17]. Here we applied this method for bacterial immobilization on IDEA sensors covered with positively charged polyelectrolytes. It may be also noted that PEI interactions

with Gram-negative bacteria may be not of purely electrostatic nature but include stronger amine/metal chelating interactions with bacteria outer membrane [16, 27]. Through these interactions PEI shows its antibacterial properties perforating bacterial outer membrane and making it permeable.

Response to *E. coli* bacteria presence was studied on three types of sensors: native SiO_2 surface without deposited polyions (used as a control); with one layer of PEI and with a multilayer structure PEI-PSS-PEI deposited on the sensor surface.

Experiment with polyions modified sensors (data not shown) in low conductivity solution (10^{-5} M KCl) confirmed that both polyelectrolyte systems are stable in time and no changes of the impedance signal were observed during their prolonged contact with the water solution.

Control and modified electrodes were immersed in *E. coli* culture at $6.4 \cdot 10^4$ CFU/mL to perform bacterial attachment. Figure 3 shows variations of ΔR_S (Ω) values determined by fitting the impedance spectra with the equivalent circuit model. Impedance changes were monitored during 1 hour at intervals indicated previously to determine kinetics of bacterial attachment.

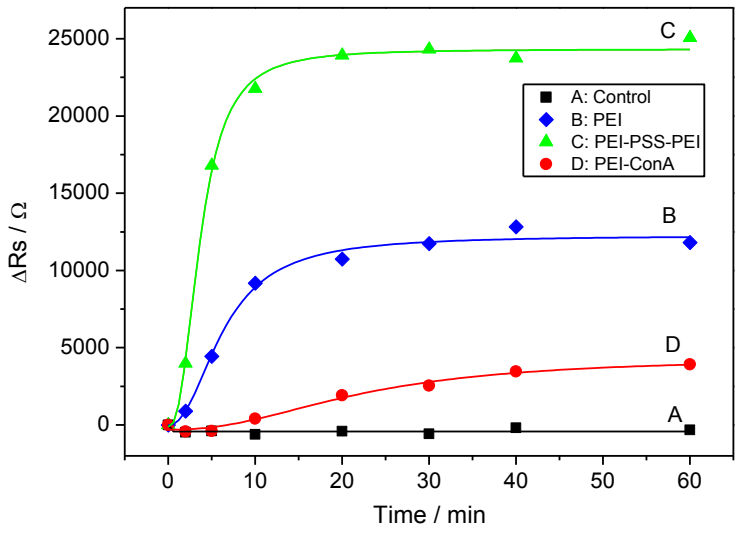

Figure 3. Sensors response measured in $6.4 \cdot 10^4$ CFU/mL *E. coli* in 10^{-5} M KCl: (A) Control (non-modified), (B) single PEI layer deposition, (C) PEI-PSS-PEI multi-layered and (D) PEI-ConA.

Obtained results demonstrate significant differences between three types of sensors. IDEA device with native silicon oxide surface layer used as a control remained stable in all measurements, indicating that no bacterial attachment was produced. Both sensors modified with PEI show increase of their impedance due to bacteria attachment to their surface but in the case of a multilayer structure (PEI-PSS-PEI) changes of the signal are larger. It was observed that impedance changes were produced quite rapidly reaching saturation in about 20 minutes. Differences between sensors with PEI and PEI-PSS-PEI layers most probably may be attributed to a more compact and dense layer in the case of multilayer IDEA surface coating [28].

In order to confirm the differences in bacterial attachment the same culture conditions and electrode modifications were reproduced on individual sensor chips to obtain Scanning Electronic Microscope (Fe-Merlin SEM, Zeiss) images of IDEA surface that are presented in Figure 4. Observed bacteria surface density is in concordance with impedance response of subsequent IDEA sensors. No bacterial attachment was observed on non-modified electrodes (Figure 4A). In the case of PEI (Figure 4B) and PEI-PSS-PEI (Figure 4C) modified electrodes bacterial surface density was substantial, but in accord with impedance results *E. coli* density was higher for PEI-PSS-PEI modified surface.

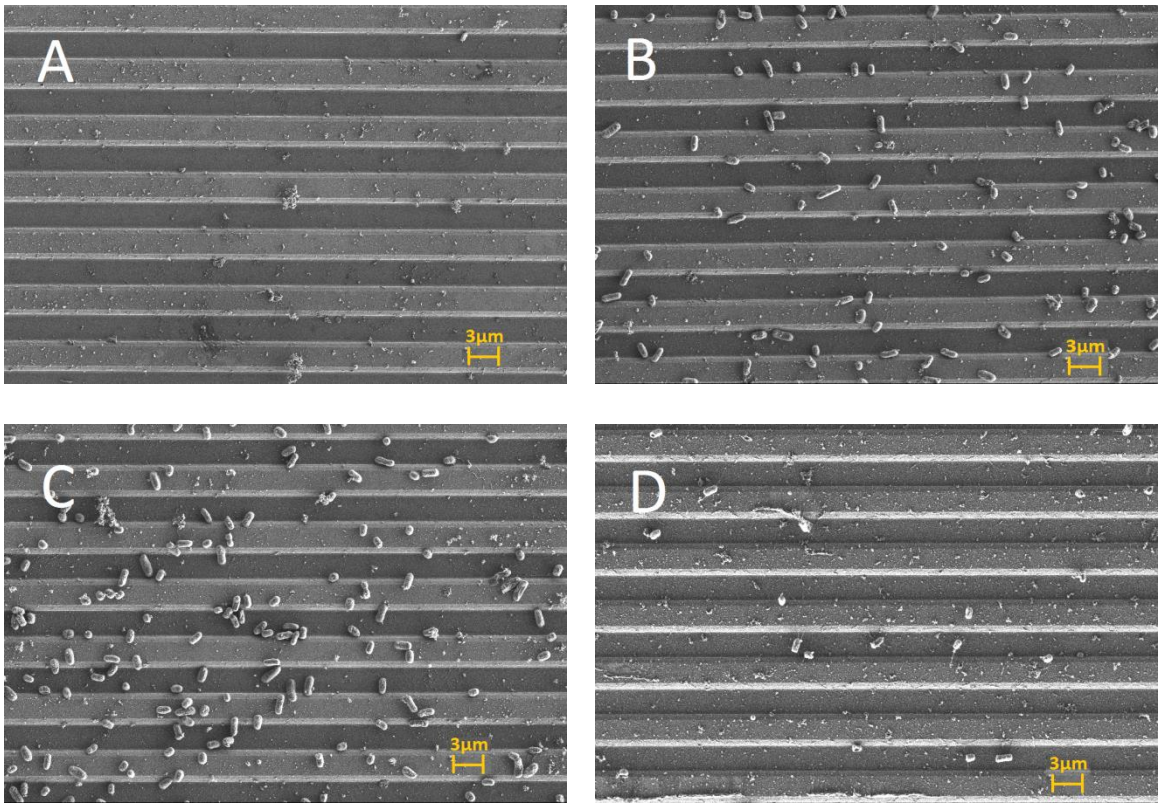

Figure 4. SEM images of IDEA sensors surface after 1 hour incubation in $6.4 \cdot 10^4$ CFU/mL *E. coli* culture. (A) non-modified electrode, (B) PEI, (C) PEI-PSS-PEI and (D) PEI-ConA modified electrodes.

3.3. *E. coli* adsorption on sensors modified with concanavalin A

In order to compare the efficiency of bacterial attachment with polyions a biorecognition system based on lectins was also tested. The major bacterial cell wall components in all Gram-negative bacteria are glycoconjugates called lipopolysaccharides. Lectins interact selectively with these carbohydrates and may be used for bacteria monitoring [20]. Concanavalin A (Con A) a mannose- and glucose-binding lectin can react specifically with terminal carbohydrates of bacterial outer membrane and has been used previously in various biosensors for bacteria detection [18-19].

As mentioned earlier, sensors modification with concanavalin A was performed in two different ways: by direct LBL deposition on the polyethyleneimine layer (PEI-Con A) [22] and by interaction of avidin at the

sensors surface with biotinylated Con A [23-24]. In the last case the sequence of the layers was PEI-PSS-avidin-Con A. To evaluate the activity of two modification pathways of Con A immobilization, impedimetric assays with a cellobiose polysaccharide (0.5-4.0 mg/mL) as a target molecule were performed. The objective was to simulate the interaction with polysaccharide present in *E. coli* bacteria membrane and to determine what kind of modification offers a faster kinetics of the surface reaction. Impedance results showed that the interaction with cellobiose was higher in the case of PEI-Con A (data not shown).

The *E. coli* response of sensors modified with PEI-Con A was studied with in parallel with other sensors in bacteria culture of $6.4 \cdot 10^4$ CFU/mL and is presented in Figure 3D. SEM image of electrode surface after 1 hour of contact with *E. coli* sample is shown in Figure 4D. Presented results confirm that *E. coli* bacteria interacts with Con A modified sensor surface but the signal magnitude and bacteria surface density are much less than in the case of PEI modified sensors.

3.4. Sensitivity of IDEA with PEI-PSS-PEI and PEI-Con A modifications

The sensitivity of PEI-PSS-PEI modified IDEA sensors to *E. coli* in water solutions was performed in a wide range of bacterial concentration from 10^1 to 10^6 CFU/mL. Sensors modified with PEI-PSS-PEI were placed in different *E. coli* cultures adjusted to desired concentrations and their impedance spectra was registered periodically. For each concentration a newly prepared sensor was used. In a control experiment modified IDEA sensors were immersed in a sterilized 10^{-5} M KCl solution without bacteria. The sensor response was defined as changes in R_S values, determined by fitting the impedance spectra to the equivalent circuit model. Results are presented in Figure 5.

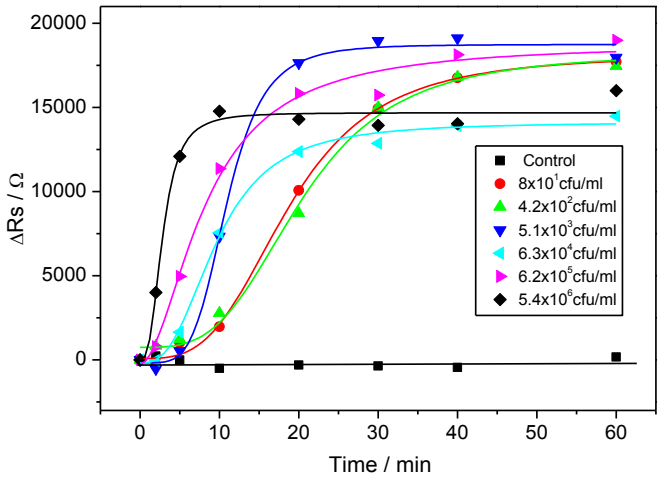

Figure 5. Variations of R_S values of sensors modified with PEI-PSS-PEI determined in 10^{-5} M KCl solution with *E. coli* bacteria concentrations from 10^1 to 10^6 CFU/mL.

Obtained results show that in all studied concentration ranges *E. coli* adheres rapidly to the sensors surface provoking significant increase in impedance. R_S values increase very rapidly in a short time (5-10 minutes) and this variation depends on bacteria concentration being faster when bacteria concentration is high ($5.4 \cdot 10^6$ CFU/mL). However, with increasing time and independently of the bacteria concentration all the sensors arrive to saturation with a very close R_S values. This shows that a maximum surface coverage may be reached at all studied bacteria concentrations and it is only the matter of time. Even at concentrations as low as 10^1-10^2 CFU/mL the saturation is reached within 30-40 minutes. Taking into consideration that in the control experiment (without *E. coli*) sensors signal remained unchanged, we may distinguish a remarkable sensitivity of the studied sensors and a very rapid detection time, around 20 minutes.

Experiments with PEI-Con A modified sensors were performed under similar conditions in *E. coli* concentration range from 10^2 to 10^5 CFU/mL in 10^{-5} M KCl solution. Sensors response is represented in Figure 6 and also show impedance variations due to bacterial attachment, though with less sensitivity than PEI modified sensors. Significant response was obtained only in solutions with 10^4-10^5 CFU/mL of *E. coli*, while in solutions with concentrations between 10^2 and 10^3 CFU/mL response is hardly observed.

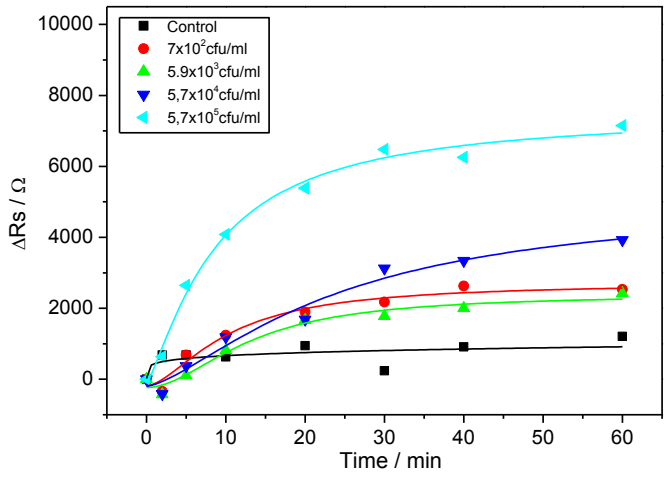

Figure 6. Variations on R_S values on sensors modified with PEI-Con A with increasing concentrations of *E. coli* bacteria from 10^2 to 10^5 CFU/mL.

It is known that presence of metal ions Ca^{2+}/Mn^{2+} is preferential for carbohydrate binding [29] because they promote the active conformation of Con A in water solution and the reaction between Con A and *E. coli* carbohydrates. It was also reported [19, 30] that to increase the sensitivity of sensors with immobilized Con A it is recommended to add free Con A into the sample solution. Unfortunately all our attempts to obtained stable results in solutions with 0.1-0.3 mM of metal ions and 100 nM of Con A were unsuccessful due to the drift of as the sensors in the control solution without bacteria, as well as the sensors in contact with *E. coli* culture.

Taking into consideration that the IDEA sensor responds to changes in surface charge associated with negatively charged *E. coli* interaction with its surface it may be concluded that this charge variation is much larger in case of PEI modified surface. Immobilization of Con A is a multiple stage procedure, so that the overall Con A surface density may not be very high and resulting surface charge changes produced by Con A- *E. coli* interactions are not very significant. Nevertheless, obtained in this case detection limits of 10^4-10^5 CFU/mL are nearly the same as reported for piezoelectric biosensor with immobilized Con A [19].

4. Conclusions

The behavior of interdigitated electrode arrays (IDEAs) modified with charged polyelectrolyte and lectin concanavalin A layers in the presence of *E. coli* bacteria in water solutions is studied. Results obtained from impedance measurements show that the IDEA sensor modified with PEI-PSS-PEI surface layer has very low detection limit of 10^1 CFU/mL and a fast response time of around 20 minutes. In the case of sensors with Con A upper layer the detection limit is of the order of 10^4 CFU/mL which coincides with one published in the literature [19] for piezoelectric biosensor with immobilized Con A. It must be noted that interaction of positively charged polyelectrolyte is not selective for different type of bacteria and also may react with other negatively charged large molecules or cells in a sample solution. However, presented results show that with impedimetric sensors it is possible to achieve very fast, cheap and robust method of analysis.

The experimental strategy presented in this work may be a promising approach for future development of a highly sensitive and rapid bacteria detection methods combining different type of selective biorecognition elements like enzymes, polypeptides, antibodies, modified nanoparticles, etc. and an impedimetric transducer.

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3.2. Impedimetric sensor based on 3D interdigitated electrodes for label-free detection of bacterial endotoxins

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Abstract

An impedimetric sensor based on a three dimensional electrode array modified with concanavalin A (Con A) was used for label-free detection of bacterial endotoxin: lipopolysaccharide (LPS) from *Escherichia coli.* The transducer permits the detection of the surface charge changes due to interaction of immobilized Con A biorecognition element and LPS of *E. coli* in test solution. The deposition of Con A on the surface was carried out using the layer-by-layer method with polyethyleneimine (PEI) polycation as an initial layer. The sensor surface characterization by means of electrochemical impedance spectroscopy technique allowed registering variations in superficial resistance provoked by surface charge changes and is demonstrated as an effective method to monitor sensor parameters at each modification step as well as to follow Con A – LPS reaction. In order to prevent non-specific adsorption of LPS on PEI covered surface different blocking strategies were tested to achieve the specific response between Con A and LPS. Results obtained in this work clearly show that blocking with bovine serum albumin (BSA) is not sufficient to prevent non-specific interactions of PEI and to ensure the selective biorecognition of LPS by Con A. To achieve more efficient PEI blocking a new method was proposed based on consecutive deposition of Con A-glycogen-Con A layers. Sensors modified with PEI-(Con A-Gly)$_2$-Con A multilayers are shown to be highly sensitive, selective and reproducible. Presented biosensor is able to detect bacterial LPS in a very short detection time (20 min) with 2 μg/mL limit of detection, which is much lower than reported for other biosensors with Con A.

1. Introduction

Endotoxins, also known as lipopolysaccharides (LPS), are ubiquitous markers of gram-negative bacteria considered as contaminants habitually found in food, environment and clinical products [1]. Lipopolysaccharides are the major structural component of external membrane of gram-negative bacteria composed of three distinct regions: O-antigen oligosaccharide that is specific to bacterial serotype, a hydrophilic core polysaccharide chain, and the lipid A - hydrophobic lipid section responsible for the toxic properties of the molecule [2]. Endotoxins can induce immune response on the internalization of mammalian cells, producing fever, multi organism failure or sepsis [3, 4].

Among food-borne pathogens responsible of many gastrointestinal diseases and the most common cause of urinary tract infections are *Escherichia coli,* well-studied gram-negative bacteria. Moreover, LPS of *E. coli* alone can cause an important number of diseases [5]. Taking into account an increasing concern in society for microbiological safety, detection of endotoxins is essential in controlling various biological and food products.

Although there are well established techniques for determination and quantification of endotoxins, like the rabbit pyrogen test and the standard limulus amoebocyte lysate (LAL) methods [6], they are relatively complex assays of multiple stages that require skilled operators [7]. Hence, the development of new detection strategies and techniques as well as rapid, compact, simple, highly sensitive, selective and high-throughput devices are required [8]. In this regard biosensors have been demonstrated as a promising alternative to classical techniques and the number of publications related to biosensors based on optical, piezoelectric, potentiometric or amperometric transducers for endotoxin detection increased considerably during last years [1, 8, 9].

In biosensors to achieve selectivity against endotoxins a specific biorecognition element should be immobilized on a sensor surface. The biological recognition elements that affect the selectivity of biosensors should possess a number of essential features: high specificity to the target, invariability under storage and detection conditions. More importantly, the reactions of recognition elements and targets should be accurate, rapid, reliable and reproducible. A large variety of different biorecognition elements were tested for LPS biosensor applications [1, 8, 9] including natural and synthetic proteins [10] and peptides, antibodies [11, 12] and aptamers [13].

Among them concanavalin A (Con A) lectin is a carbohydrate-binding protein that recognizes specifically α-D-glucose and α-D-mannose groups [14]. One of the main advantages is lectin's stability compared with other biomolecules like antibodies and their size allowing to obtain higher densities on a sensor surface [15]. Con A can be employed in biosensors development [16-22] due to its affinity for some bacteria [20, 21, 23], viruses [24], cells [25, 26] and including endotoxins [17]. Interactions of Con A with target analytes may be registered in a label-free mode using different experimental techniques, like surface plasmon resonance (SPR) [23, 27, 28], quartz crystal microbalance [16, 20-22, 29] or electrochemical impedance on

a Con A modified metal electrodes [11, 30-33]. Thus Con A is a widely reported molecule employed as biorecongition element.

In this regard, electrochemical impedance spectroscopy (EIS) is a powerful technique permitting to control changes at the solid/liquid interface of surface-modified electrodes produced by chemical, physical and biological interactions during the recognition events [34]. In many cases impedance measurements with lectin-based impedance sensors are performed in a Faradaic mode [18, 31, 32, 35] in the presence of the $K_4[Fe(CN)_6]/K_3[Fe(CN)_6]$ redox probe by registering changes in a charge transfer resistance associated with biorecognition processes at the modified metal electrode surface.

In the case when a redox pair is absent in the electrolyte solution, the impedance is termed non-Faradic and depends on the conductivity of the supporting electrolyte and impedimetric electrode interfacial properties (interfacial capacitance [36] or surface conductivity [37]). Among impedimetric sensors interdigitated electrode array (IDEA) transducers, in which a pair of comb-like metal electrodes are formed on a planar insulating substrate [38], present promising advantages, such as small size, rapid detection kinetics, increase of signal-to-noise-ratio and fast establishment of a steady state response [39]. These sensors may be used for monitoring both, the interfacial capacitance and the surface conductivity between the electrode digits.

In this work a three-dimensional (3D-IDEA) device [40], in which the electrode digits are separated by insulating barriers to enhance the sensitivity, was used as a biosensor transducer. In this case under applied potential the main portion of the current goes close to the surface of barrier and this permits to enhance the sensitivity compared with standard planar structures [41]. The principles of 3D-IDEA sensor operations are based on registration of the surface conductivity changes produced by variation in the surface charge and were discussed in detail earlier [37, 42]. This design has been demonstrated to be highly sensitive to changes in the electrical charge distribution at the solid/liquid interface produced by chemical and biochemical reactions [43]. Thus, it is a promising sensitive transducer for label-free biosensor development.

The functionalization of the IDEA surface to achieve selectivity against endotoxins is a crucial step in the sensor development and in this sense immobilization of Con A without decreasing its binding affinity is essential. Among different assembling techniques like absorption [24, 26, 30] or chemical grafting via self-assembled monolayers (SAM) [16, 22, 44], the layer-by-layer (LBL) technique has been demonstrated useful for immobilization of biomolecules like proteins, antibodies or lectins. This technique allows assembling oppositely charged polyions for the formation of multilayers [45]. As an initial anchoring layer a highly positively charged polycation, polyethyleneimine (PEI), is typically used for the formation of thin multilayer coatings with effective immobilization of biomolecules, negatively charged at neutral pH, like proteins layers [46, 47] , enzymes [43], lectins [48] and bacteria [49].

In the present work, in order to establish a robust methodology for endotoxin detection, the LBL method using PEI was used for immobilization of Con A. To demonstrate the effectiveness of the proposed system

surface characterization was carried out by impedance measurements on each surface modification step. Additionally, Con A lectin stability after the immobilization was evaluated to ensure that it maintains its specific recognition ability for carbohydrates, and consequently for bacterial LPS.

Another important aspect in the performance of biosensors based on surface reactions is the possible non-specific binding of the corresponding target or other molecules present in the sample to the sensor surface layer. Thus, to prevent non-specific interactions and enhance the selectivity in many cases it is necessary to use inert blocking reagents that cover the sensor surface without altering the specificity of a biorecognition element. As the PEI polycation was shown effective in binding endotoxins [50, 51], different blocking strategies to compensate this undesired effect in order to enhance the specificity and selectivity of Con A modified sensor to endotoxins have been studied. The sensor surface modification and blocking strategies used in this work are schematically presented in Figure 1.

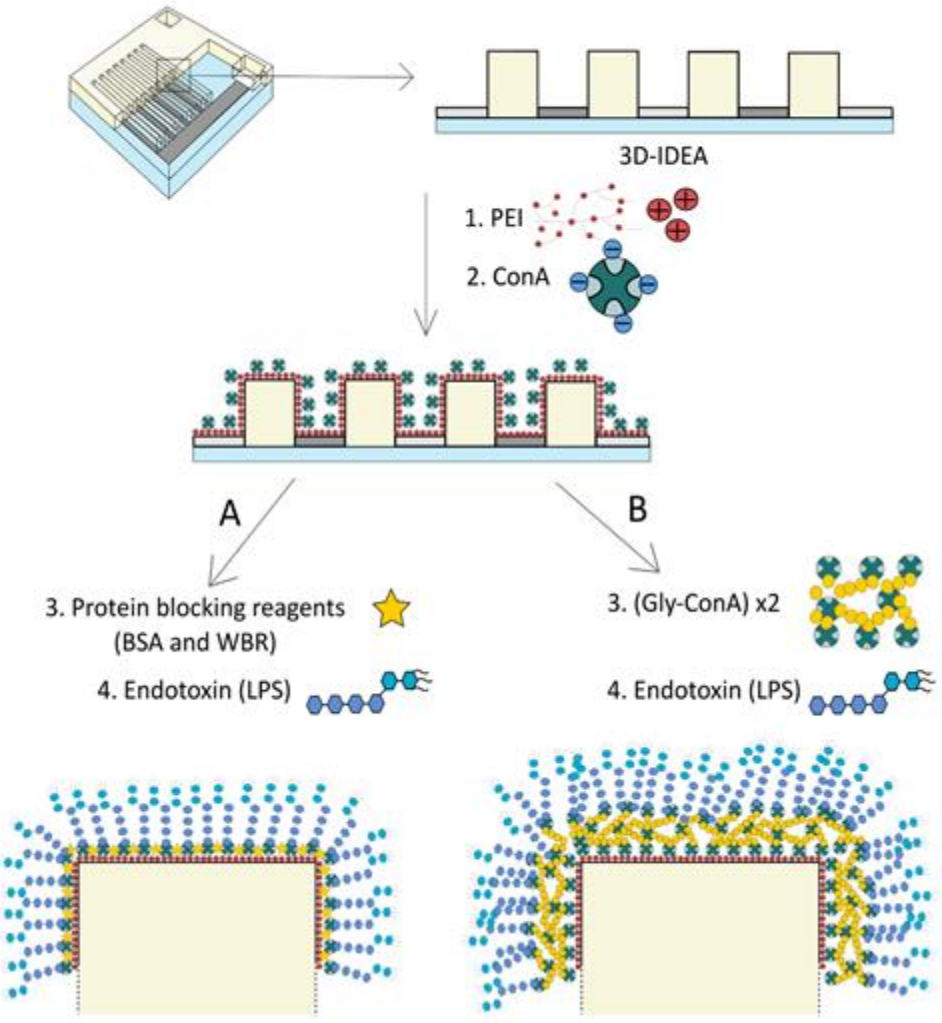

Figure 1. Sensor disign and schematic representation of the sequential steps of the sensor surface biofunctionalization.

2. Materials and methods

2.1. Electrode design and fabrication

The three-dimensional interdigitated electrode array (3D-IDEA) was fabricated using conventional microelectronic techniques. A silicon wafer oxidized by "wet" oxidation at 950 ^{o}C was employed as substrate to give a good quality of silicon dioxide layer of 2500 nm. As electrodes material a 230 nm layer of a highly conductive tantalum silicide ($TaSi_2$) was deposited using magnetron sputtering. The first photolithographic step defines collector bars and digits of the two electrodes. The pattering is done by reactive etching technique resulting in an interdigitated electrode array with 216 digits of 3 μm width and 3 μm gap between the adjacent electrodes. The aperture between the electrodes is 1.4 mm and the total length between them is 301 mm. To form contact pads 1 μm of aluminum is deposited and patterned by standard photolithographic and etching steps leaving metal only at extremes of the two collector bars.

The final step is the barrier formation. To do this the wafer is covered with a 4μm thick silicon oxide (SiO_2) layer deposited by a low pressure chemical vapor deposition (LPCVD). Photolithography is also used to define the openings in the oxide layer over the electrode digits and the contact pads. The aperture of these zones is carried out by deep reactive ion etching (DRIE) to obtain barriers with nearly vertical walls. The barrier separating the adjacent electrodes are 3 μm, 4 μm wide and 162 mm long and are opened at the top.

Finally, the electrodes cut from the wafer were glued to a PCB substrate and wire bonded for electrical connections. Contact pads and wires were encapsulated using epoxy resin. Complete technology is described elsewhere [40, 41, 43] and schematic design of device is also presented in Fig. 1.

2.2. Chemicals, solutions and reagents

Polyethyleneimine (PEI, branched, average Mw 25000, water-free) polycation and anionic poly(sodium 4-styrenbesulfonate) (PSS, average Mw 70000, water-free) were both dissolved in deionized water at 1.5 mg/mL and 2 mg/mL, respectively. These concentrations have been chosen in accord with previously published data [47]. Concanavalin A (Con A, from Canavalia ensiformis) lectin used as a biorecognition element was prepared at 25 μg/mL in 0.05 M Tris-HCl buffer (pH=7.4). Compounds employed as blocking reagents were: bovine serum albumin (BSA) (from Sigma-Aldrich)), Western Blocking Reagent (WBR) (from Roche Life Science) and glycogen (Gly), a polyglucose-type, all prepared in 0.05 M Tris-HCl buffer (pH= 7.4). The optimal concentration of Con A and the blocking reagents (BSA, WBR and Gly) were studied in additional assays. LPS from *Escherichia coli* 055:B5 were prepared using Tris-HCl buffer solution at the required concentrations. All chemicals were purchased from Sigma-Aldrich, Spain.

All the solutions were prepared with deionized MilliQ water (18 MOhm·cm) which was also used for the cleaning and rinsing processes. All chemicals were of analytical grade and were used as received without further purification.

2.3. Preparation and modification of electrodes for LPS detection

Prior to use, 3D-IDEA sensors were first cleaned with isopropanol for 10 minutes, rinsed with distilled water and dried under nitrogen flow.

2.3.1. Immobilization of Con A by LBL method

To perform the deposition of corresponding layers on the sensor surface, PEI polycation was employed as the initial assembling layer. First of all, the sensors were immersed into the PEI solution for 20 minutes to form a homogeneous self-assembled monolayer on SiO_2. The immobilization method of Con A as a biorecognition moiety on the 3D-IDEA surface was carried out over the initial layer of PEI. As at pH 7.4 Con A behaves as a polyanion, it was deposited by LBL method over PEI layer by immersing sensors in Con A solution during 60 minutes. After each modification step the sensors were thoroughly rinsed with water to remove non-bound molecules.

2.3.2 Immobilization of blocking reagents

To avoid unspecific binding of LPS to the sensor surface with PEI, different compounds were used to block the surface not occupied by Con A. Protein-based blocking reagents (BSA and WBR) and Gly multiple layers were employed to ensure the specific biorecognition interaction between Con A and LPS. For BSA treatment electrodes with PEI-Con A were immersed for 20 minutes into a BSA in Tris-HCl buffer solution with the BSA concentration in the range of 10 to 100 μg/mL. In the same way, the treatment with WBR was carried out in Tris-HCl buffer solutions with WBA concentrations in the range of 5 to 100 μg/mL and the treatment lasted up to 60 minutes.

In the experiments with glycogen as blocking-reagent, the immobilization was done taking advantage of the specific concanavalin-glucose interaction. Gly solutions were also prepared in Tris-HCl buffer at the concentration of 100 μg/mL according to procedure reported by Lvov *et al.* [47]. In this case Con A and Gly layers were alternatively deposited by immersing sensors into corresponding solutions during 60 minutes until the desired number of layers was obtained.

2.3.3 LPS detection assays

For LPS detection functionalized 3D-IDEA were incubated at room temperature in solutions of *E. coli* LPS with various concentrations ranging from 0 to 50 μg/mL. After this treatment electrodes were thoroughly rinsed with water to remove unreacted LPS and to reduce possible influence of adsorbed ions from Tris-HCl buffer with high salt concentration on the impedance measurements.

2.4. Impedance Measurements

For impedance measurements a QuadTech 7600 Plus highly precision LCR Meter analyzer was employed. Measurements were performed in a 10^2 Hz – 10^6 Hz frequency range with 100 mV (amplitude) voltage excitation. This voltage level is higher than typically used for EIS measurements with metal electrodes (10-

25 mV) and was chosen as the $TaSi_2$ electrodes have higher impedance and at low test signal level high noise appears. However, we have performed measurements with test voltages of 20, 50 and 100 mV to assure that the spectra parameters, except for the noise, are not affected by the test signal level.

Impedance data treatment and equivalent circuit fitting was performed using the Z-Plot/Z-View software package (Scribner Associates, Southern Pines, NC, USA). The fitting quality was evaluated using chi-squared values calculated as the square of the standard deviation between the original data and the calculated spectrum. In all fittings performed the χ^2 values were lower than 10^{-4} which reflects the correctness of the chosen equivalent circuit.

All experiments were done in duplicates at least on three electrodes under the same conditions.

All the impedance measurements were carried out at controlled room temperature in KCl 10^{-5} M solutions with the conductivity of 2.50 µS/cm. To guarantee the reproducibility of the bulk solution conductivity for each single measurement a fresh portion of solution was used and its conductivity was controlled with a commercial conductimeter (EC-Meter GLP 31+, Crison).

3. Results and discussion

3.1. Sensor characterization by impedance measurements

The impedance response of a 3D-IDEA in low conductivity solutions in the absence of faradaic processes can be emulated by an equivalent circuit [42] presented in Fig. 2A formed by the following components: R_C is the contact resistance introduced by wires and collector bars of thin film electrodes; C_G is the geometrical capacitance between two interdigitated electrodes in a aqueous solution; R_S is the resistance between two electrodes of the array; and CPE_{DL} is a constant phase element associated with the capacitance of the electrical double layer at the electrode – water solution interface that is attributed to the non-ideal behavior of the double layer capacitance [42, 43]. The impedance of the CPE_{DL} can be expressed as:

$$Z_{\mathrm{CPE}} = \frac{1}{(j\,\omega)^{\alpha}\,C_{DL}}, \tag{1}$$

where j= -1 (imaginary unit), ω is angular frequency (rad/s), C_{DL} (F) is the capacitance of the double layer and α is an empirical constant representing the behavior of the CPE. When the exponent α is equal to 1 the CPE behaves similarly to a capacitor. If the value of α becomes 0 the CPE will behave as a resistor. Typical values for 3D-IDEA with $TaSi_2$ electrodes of α parameter in low conductivity solutions are between 0.85 and 0.9 [42].

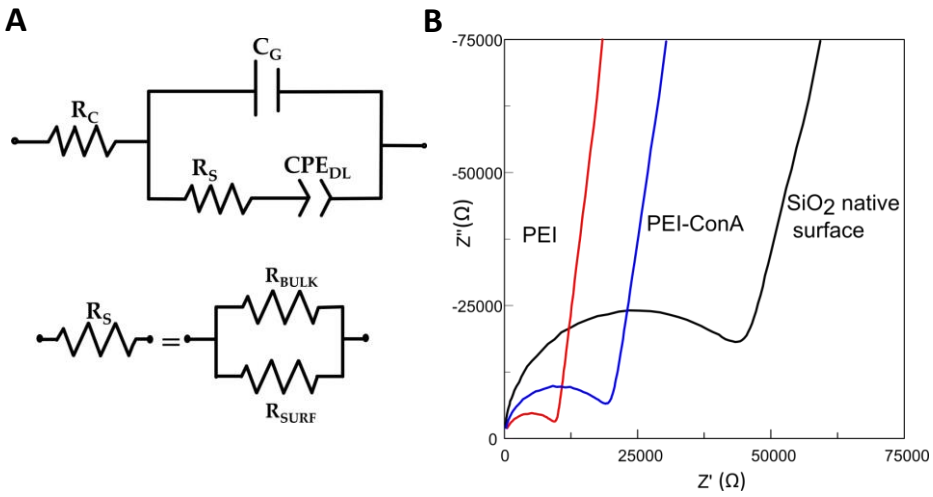

Figure 2. (A) Electrical equivalent circuit used for impedance spectra fitting. (B) The Nyquist plot of the 3D-IDEA measured in KCl 10^{-5} M solution with bare electrodes (SiO_2 native surface), after PEI deposition and with PEI-Con A layer.

Previously it has been reported [42] that at high electrolyte concentrations IDEA sensors respond mainly to bulk solution conductivity. However, in low conducting solutions surface conductivity, due to the presence of surface charge, plays an important role in IDEA sensors and this effect is much higher in 3D-IDEA devices. Therefore, R_S determined from the impedance spectra 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 individually these two elements in the impedance spectra. However, if we fix the bulk solution resistance, R_{BULK}, then all changes in R_S may be attributed to surface resistance, R_{SURF}, variations associated with the surface charge changes at the barriers surface due to surface (bio)chemical reactions [42, 49]. It is clear that the lower is the bulk solution conductivity, the higher is the sensitivity to surface conductivity changes. That is why the measurements are performed in a low conducting KCl solution with fixed and controlled conductivity of 2.50 µS/cm.

The impedance spectra presented in the Nyquist plot (Z' vs Z'') in Figure 2 allow to observe the formation of a semicircle at high frequencies corresponding to resistance R_S in parallel with the sensor geometrical capacitance. The intercept with the Z' axes at high frequencies on the left side of the plot gives the R_C value, while the intercept on the right side gives the value of $R_C + R_S$ (R_S being the parallel combination of R_{BULK} and R_{SURF}).

As all the experiments were performed in KCl solutions with controlled conductivity of 2.50 µS/cm, the solution resistivity was kept constant and the observed changes in R_S are attributed to surface resistance variations. The linear response at low frequencies in the Nyquist plot (Fig. 2B) is due to the CPE element of the interfacial capacitance.

As follows from the spectra in Figure 2B, modification of native SiO_2 of the barriers surface with highly positively charged PEI causes decrease in the surface resistance producing the attraction of mobile ions

from the water solution bulk to the charged surface. Accordingly, when Con A and other corresponding modification components are deposited on the surface the positive charge introduced by PEI is progressively compensated, producing significant increases in R_S.

To characterize the sensitivity of sensors to *E. coli* LPS their response was defined as changes in R_S of the modified sensor before (R_S^0) and after (R_S^{LPS}) reaction with LPS:

$$\Delta R_S = R_S^0 - R_S^{LPS} \quad (2)$$

It has to be taken in consideration that in the low frequency region of the Nyquist plots (Figure 2B), the surface modification not only provokes changes in R_S but also alters the interfacial capacitance and the α 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 α parameter as a result of the non-ideality of the interfacial capacitor. Subsequent experimental values (R_S, C_{DL}, α) obtained by fitting the spectra to the equivalent circuit in Figure 2 are presented in Table 1. All these parameters depend on the LPS concentration, however, CPE, attributed to the surface capacitance changes, is rather a complex parameter [52], that includes the double layer capacitance at solid/liquid interface and that of the deposited surface layer. On the other hand, ΔR_S is directly associated with the surface charge and due to its large scale changes and higher reproducibility ΔR_S was chosen as the main parameter. The quality of the equivalent circuit chosen for spectra fitting (Fig. 2A) was evaluated by chi-squared values, which were between 10^{-4} and 10^{-5} in the whole frequency range, demonstrating the goodness of fit and suitability of equivalent circuit employed.

	[LPS] (µg/mL)	R_S (kΩ)	C_{DL} (nF)	α
Native SiO_2	-	53.6±1.9	3.7±0.9	0.91±0.02
PEI	-	13.6±1.3	3. 8±0.9	0.94. ±0.01
PEI-ConA	-	20.4±2.9	3.9±1.1	0.94±0.01
PEI-ConA-BSA- LPS	-	31.3±4.2	4.2±1.1	0.93±0.01
PEI-ConA-BSA- LPS	1	31.5±1.1	3.8±1.5	0.92±0.03
PEI-ConA-BSA- LPS	2	33.3±0.9	4.9±0.9	0.92±0.01
PEI-ConA-BSA- LPS	5	50.7±4.8	5.3±0.2	0.90±0.01
PEI-ConA-BSA- LPS	10	59.8±3.3	5.9±1.2	0.90. ±0.01
PEI-ConA-BSA- LPS	20	77.8±0.4	5.7±0.9	0.89±0.01
PEI-$(ConA-Gly)_2$-ConA	-	64.6±0.4	5.1±0.3	0.89±0.01
PEI-$(ConA-Gly)_2$-ConA	1	66.2±0.2	5.5±0.1	0.89±0.01
PEI-$(ConA-Gly)_2$-ConA	2,5	68.1±0.6	5.4±0.1	0.89±0.01
PEI-$(ConA-Gly)_2$-ConA	5	74.1±0.3	5.3±03	0.89±0.01
PEI-$(ConA-Gly)_2$-ConA	10	77.1±1.7	5. 6±0.1	0.89±0.01
PEI-$(ConA-Gly)_2$-ConA	20	80.5±2.6	5.7±0.1	0.88±0.01
PEI-$(ConA-Gly)_2$-ConA	50	84.3±1.4	6.8±0.4	0.85±0.01

Table 1. Values of the equivalent circuit parameters obtained from the fitting of impedance spectra. Data are shown as means ±SD (n=3). C_{DL} and α are the parameters of the CPE impedance (see eq. 1).

3.2. Evaluation of the nonspecific binding on the electrode surface

The first step on the assembling process of different layers on the 3D-IDEA surface is immobilization of PEI, which is very fast and nearly irreversible. Colloid chemistry experiments show that PEI which bears

positive charge adsorbs strongly on silicon dioxide surface due to the presence of hydroxyl groups [53] and increases the surface conductivity. In our experiments performed in low conductivity KCl 10^{-5} M solutions this provokes the surface resistance, R_S, decrease [42] as shows Figure 2B. This effect is used to control changes in surface charging at liquid/solid interface due to adsorption process or surface chemical reactions. Employment of 3D-IDEA device allows to improve significantly the sensitivity to surface charge changes in comparison with traditional flat devices [37].

Multiple publications confirm that branched PEI used as the first layer over silicon dioxide in the LBL process acts as a uniform anchoring network for the formation of consecutive layers, resulting in homogeneous films [45]. PEI was also employed previously [47] to assemble multilayers of Con A and branched polyglucose by LBL method. In the present work the LBL method was used for immobilization on the sensor surface of Con A as a biorecognition element for the detection of bacterial endotoxins.

In addition, an important aspect to be considered is possible non-specific interactions of positively charged PEI on the IDEA surface with negatively charged molecules in test solution that may result in non-selective sensor response.

In order to study the effect of PEI in the nonspecific binding of LPS on 3D-IDEA sensors, a preliminary comparative experiment with electrodes modified with PEI and PEI-Con A was carried out. For this, both types of sensors were immersed in 25 µg/mL solutions of *E. coli* LPS and, subsequently, the impedance response was studied at different incubation times. Obtained results are presented as ΔR_S changes in Figure 3.

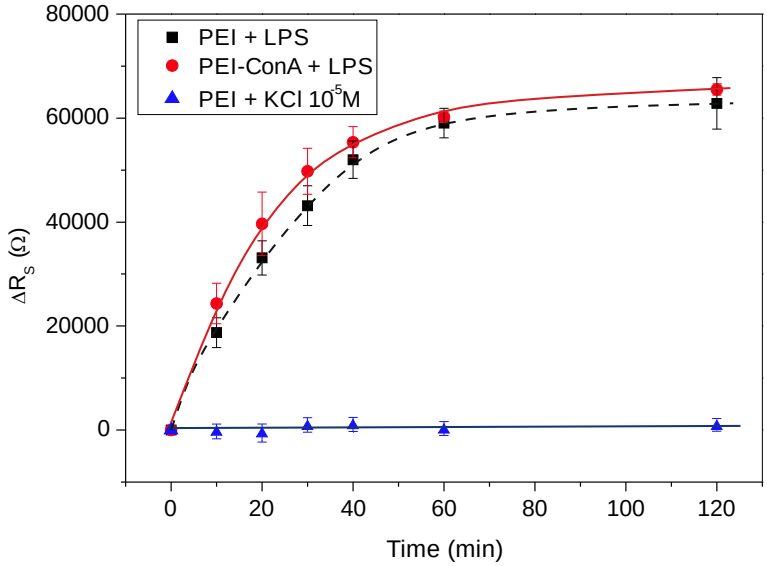

Figure 3. Resistance variations for sensors modified with PEI (squares) and PEI-Con A (circles) in the presence of *E. coli* LPS at 25 µg/mL. Response of a PEI modified sensor in the absence of LPS is also presented (triangles). Error bars show standard deviation (n=3).

It can be observed that the impedance response in the presence of the LPS changes rapidly in time in both cases, provoking significant increase in R_S parameter. Although in the case of PEI-Con A the response of modified sensors showed slightly higher sensitivity, the differences with PEI modified electrodes are practically imperceptible. The saturation of the signal for both types of sensors occurs within 2 hours. Thus, obtained results indicate that PEI and PEI-Con A modified 3D-IDEA have similar binding affinity for LPS. In contrast, in the control experiments without LPS in solution the signal of PEI modified sensor remained stable in time.

As previously described, the bacterial LPS are formed by carbohydrates with phosphorylated groups that contribute to the negative charge of this molecule. Hence, its interaction with polyethyleneimine is produced by electrostatic attraction. Therefore, obtained results are in accordance with other publications [50] confirming the ability of PEI to react strongly with endotoxins. On the other hand, the biorecognition process of Con A is based on the specific reaction with the glycosyl residues of LPS.

Obtained results clearly show the necessity of effective blocking of the sensor surface modified with PEI to prevent its non-specific interactions and to ensure the selective biorecognition of LPS by Con A. The following experiments based on the study of superficial properties were performed to test different blocking strategies aimed on reduction of the effect of the highly positive charges of PEI to ensure selective endotoxin detection by Con A.

3.3. Bacterial endotoxin detection

In order to establish a robust and reproducible methodology to avoid the direct reaction of PEI with LPS and, simultaneously, to guarantee the specific binding of LPS with Con A, two modification approaches using different surface blocking reagents were tested. Firstly, two protein-based blocking reagents, BSA and WBR (PEI-Con A-BSA and PEI-Con A-WBR) were utilized and, secondly, the application of glycogen as intermediate layer for Con A immobilization (PEI-(Con A-Gly)$_2$-Con A) was employed.

3.3.1. PEI-Con A and protein blocking reagents

Protein-based blocking reagents, especially bovine serum albumin (BSA), are widely used in different applications [54] and here BSA was employed due to its ability to interact with PEI [55]. It is well known that the isoelectric point of BSA lies between 4.7 and 5.6 [56], so in solutions close to neutral pH as in our case (pH=7.4), the net charge of BSA is negative. Thus, we suggest that BSA will strongly adsorb on PEI surface not covered by Con A lectin. To study the effectiveness of BSA as a blocking agent to isolate the electrostatic attraction of LPS produced by PEI, 3D-IDEA sensors modified by PEI and PEI-Con A were subjected to BSA blocking as presented in experimental section. The effect of the BSA blocking was studied by incubation of modified sensors in 10 µg/mL LPS solution during 1 hour. Subsequent results are presented in Figure 4.

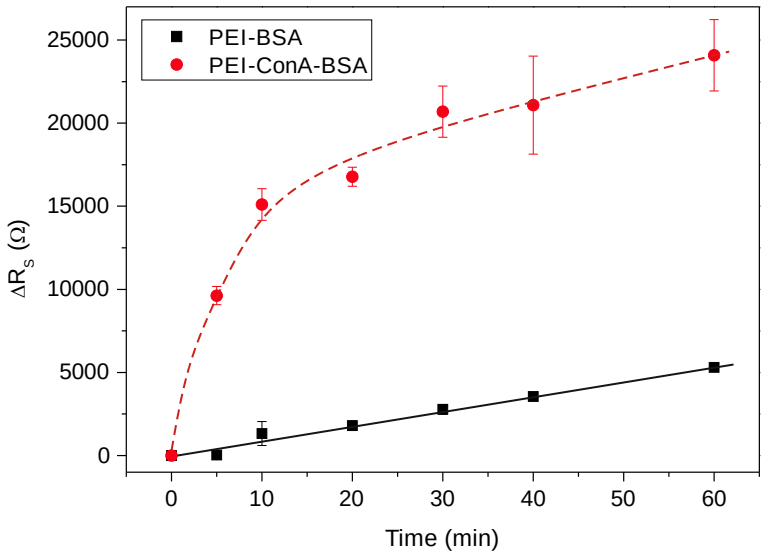

Figure 4. Variations in R_S of sensors functionalized with PEI and PEI-Con A after blocking with BSA. Response is measured in the presence of 10 µg/mL LPS in time. Error bars show standard deviation (n=3).

Figure 4 demonstrates that BSA acts as an effective blocking reagent in the case of PEI modified 3D-IDEA sensors. When the surface of PEI modified sensors is blocked with BSA the increase in R_S parameter in the presence of the LPS is rather small, especially in a short time, demonstrating low absorption of LPS. On the other hand, PEI-Con A sensors blocked with BSA show high sensitivity of the impedance response in presence of LPS which is stable after 1 hour of incubation.

Obtained results also demonstrate that Con A in PEI-Con A-BSA structures preserves its biological activity to recognize endotoxins. As previously reported [57] Con A can bind sugars, glycoproteins and glycolipids containing reduced terminal α-D-mannosyl and α-D-glucosyl groups and at pH above 6.9 exists in a tetrameric form, while below 5.9 as a dimer [58]. Thus, the solution at pH = 7.4 employed for Con A immobilization allowed to maintain the tetrameric structure with four binding sites for carbohydrates and, consequently, can stimulate the binding affinity to bacterial endotoxins.

Sensor response of PEI-Con A-BSA 3D-IDEA was measured in *E. coli* LPS solutions in 0 – 20 µg/mL concentration range during 1 hour. Subsequent results are presented in Figure 5. The R_S response increases proportionally with the LPS concentration with the sensitivity of 1.7 kOhm per µg/mL in the 5-20 µg/mL range. It may also be noted that the saturation phase is reached within 40 minutes at all concentrations. Significant differences may be noted between control tests without *E. coli* LPS in the solution and sensors response at low LPS concentration. These results confirm that BSA blocking method may be used as an effective strategy to ensure specific response of PEI-Con A layer in LPS biorecognition process.

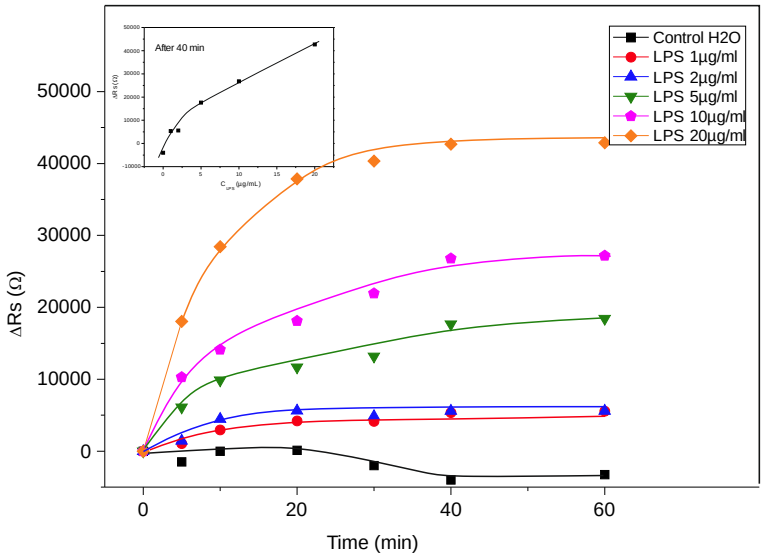

Figure 5. R_S variation in time of sensors modified with PEI-Con A-BSA at different LPS concentrations (0 –control–, 1, 2, 5, 10 and 20 µg/mL). In the inset the sensor response versus LPS concentration is shown.

Similar tests were carried out with WBR as a protein-based blocking reagent. 3D-IDEA sensors functionalized with PEI and PEI-Con A were subjected to blocking using solutions with different WBR concentrations (5, 10, 20, 40 and 100 µg/mL). Afterwards, sensors were immersed in a solution of 10 µg/mL of LPS for 1 hour to study the blocking effect of WBR. However, in the case of PEI-WBR modified sensors the blocking of PEI was not complete, especially at low concentrations of WBR (from 5 to 40 µg/mL). These test results are presented in Figure 6.

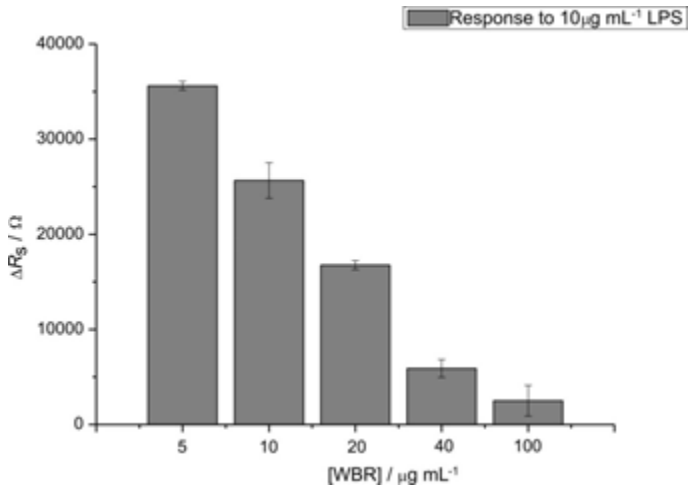

Figure 6 Response of PEI-WBR modified 3D-IDEA with different WBR concentration after 1-hour incubation in a 10 µg/mL *E. coli* LPS solution. Error bars show standard deviation (n=3).

In the case of WBR similar to BSA blocking effect was achieved only at WBR concentration of 100 mg/mL. However, when this concentration of blocking reagent was employed for PEI-Con A sensors, no response

to LPS was observed, demonstrating that WBA also blocks the activity of Con A making it impossible to detect bacterial endotoxins by means of Con A interaction.

3.3.2. Sensors with PEI-(Con A-Gly)$_2$-Con A multilayers

Though the BSA blocking methodology of PEI-Con A modified sensors described previously provides a good response and sensitivity to detect bacterial endotoxins, still a part of the R_S signal is produced by direct interaction of LPS with PEI. This may affect the selectivity in the presence of some other negatively charged molecules present in a test sample. Thus, an alternative approach of a robust methodology to immobilize Con A by means of PEI is required to prevent non-specific binding of bacterial LPS.

The second strategy to assemble Con A as a biorecognition element on the IDEA sensor surface is based on the use of glycogen to immobilize Con A and also to block undesirable activity of the underlying PEI layer. Glycogen (Gly) is a highly branched polysaccharide which consists of α-D-(1→4) linked D-glucose residues with α-D-(1→6) branch points and reacts easily with Con A at physiological pH [59]. The deposition of multiple layers of Con A and glycogen was performed by layer-by-layer method. As in the previous case the first two layers immobilized on the sensor surface were PEI and Con A. After this the sensor was treated alternately with glycogen and Con A to assemble the desired number of Con A-Gly layers on the device surface. Formation of Con A-Gly layers compensates the surface charges introduced by PEI and produces increase in the surface resistance as shown in Figure 7.

In order to test that thus constructed multilayer structure blocks efficiently the PEI layer positive charge, reaction with polystyrene sulfonate (PSS) polyanion was used. Being able to react with PEI by electrostatic interactions and to reverse the surface charge [45, 47], PSS is widely applied in the layer-by-layer method for deposition of polyelectrolyte multilayers. To demonstrate that the effect of PEI is completely blocked PEI-Con A-Gly-Con A and PEI-(Con A-Gly)$_2$-Con A structures were formed on the 3D-IDEA surface and were immersed in a solution of PSS for 20 minutes.

As follows from Figure 7, where R_S values determined after each modification step are presented, different response was observed in each of the cases. PEI-Con A-Gly-Con A modified electrodes showed a significant increase in R_S on addition of PSS (Fig. 7A) indicating that PEI positive charges were not totally compensated by Con A-Gly-Con A layers and maintain ability to react with LPS. On the other hand, for sensors modified with additional Con A-Gly double layer (PEI-(Con A-Gly)$_2$-Con A, fig. 7B) no changes in impedance were observed after the PSS addition which confirms the complete isolation of PEI in this case.

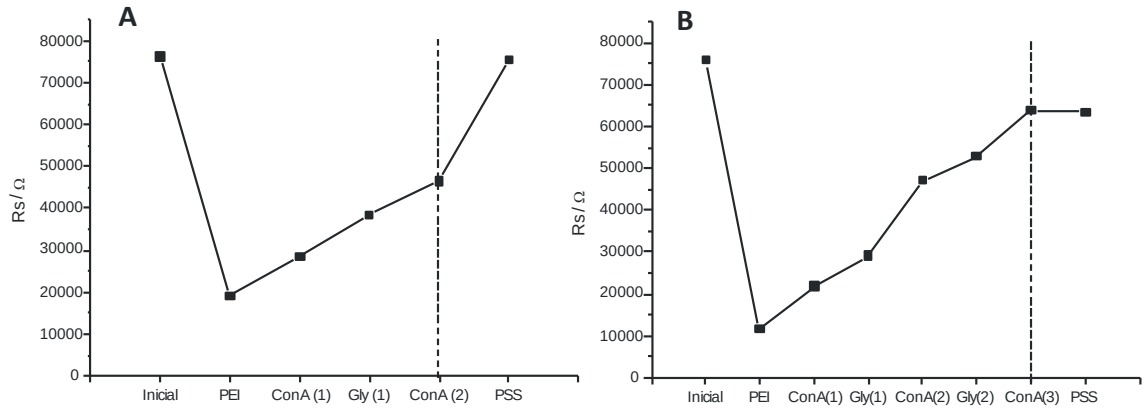

Figure 7. R_S values of the IDEA devices after the successive deposition of surface layers and final treatment with PSS. (A) PEI-ConA-Gly-Con A and (B) PEI-(Con A-Gly)$_2$-Con A.

Therefore, obtained results give the evidence that PEI-(Con A-Gly)$_2$-Con A multilayer structure completely blocks the PEI positive charges. This alternative approach of Con A immobilization may be regarded as a useful robust methodology for a selective bacterial LPS sensor development as presented below.

3D-IDEA sensors with PEI-(Con A-Gly)$_2$-Con A multilayered structure were used for detection of *E. coli* LPS. Figure 8 shows the sensors response at various concentrations of LPS (0-50 µg/mL) in time. It should be noted that kinetics of Con A – LPS interaction is quite fast and the saturation phase is reached in 20 minutes regardless of the LPS concentration. Modified sensors employed as control in the absence of LPS showed no changes in time, demonstrating the stability of the used multilayer structure.

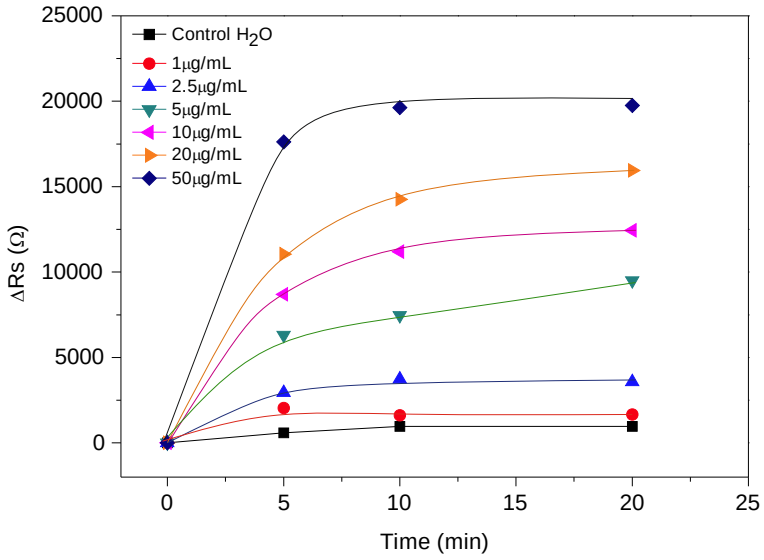

Figure 8. Variation of PEI-(Con A-Gly)$_2$-Con A 3D-IDEA sensors R_S values in time during incubation in solutions with different LPS concentrations (0 (control), 1, 2.5, 5, 10, 20 and 50 µg/mL).

Presented results confirm that the blocking is a crucial step in the development of functionalized surfaces for a biorecognition process in order to avoid non-specific interactions and enhance the selectivity and performance of biosensors based on label-free detection.

3.3. Sensor sensitivity and reproducibility

To evaluate the performance of developed devices 3D-IDEA sensors with PEI-(Con A-Gly)$_2$-Con A layers were used to test sensitivity, reproducibility and the limit of detection (LOD) of bacterial endotoxin from *E. coli*. The interaction between Con A and *E. coli* LPS were monitored during 20 minutes of incubation to reach the saturation phase at which the maximum response is obtained. The resulting response curve is presented in Fig. 9 showing significant sensitivity of the sensor to different LPS concentrations. The sensors response obeys the Langmuir adsorption isotherm typical for irreversible adsorption of analyte species on a solid surface that is usually presented as:

$$n = n_m \frac{bC}{1+bC}, \quad (3)$$

where *C* is the concentration of the adsorbate in the solution, *n* is the amount adsorbed species, n_m is the amount of *n* in saturation and *b* is a coefficient. However, Langmuir equation is based on simplified assumptions that do not take into account the possible interaction of the adsorbed molecules and the possible dependence of the chemical activity of the surface active sites with the number of adsorbed molecules. Often adsorption experimental results are fitted with an empirical Hill function [60] which is expressed as:

$$y = Rs_{max} \frac{x^n}{k^n + x^n}, \quad (4)$$

where:

x is the concentration of the adsorbate in the solution

n is the Hill coefficient that describes the cooperativity of the ligand binding. If it is more than 1 it means that once one ligand molecule is bound to the surface site, its affinity for other ligand molecules increases.

$R_{s\ max}$: The maximum R_S value at which saturation occurs and maximum surface concentration is achieved.

k: Ligand concentration producing half occupation.

As shows Fig. 9 the obtained experimental points can be perfectly fitted by the Hill function. The fitting of experimental data with the Hill's equation gives the following parameters: R_S max = 21.2±2 kOhm *n* = 1.2±0.2; *k* = 7.4±1.6 µg/mL. Langmuir type adsorption isotherms may be presented in a semilogarithmic plot thus giving a linear calibration regression of the sensor response. Results presented in the inset of Fig. 9 show that in the studied concentration range of 1 – 50 µg/L the sensors response is proportional to the logarithmic value of LPS concentration (µg/mL) with a correlation coefficient of 0.975 and with the sensitivity of 11.3 kOhm per LPS concentration decade.

The LOD may be defined as LOD = X_{b1}+ $3S_{b1}$ where X_{b1} is the output signal value measured in the blank and S_{b1} is the standard deviation of this signal [61]. The limit of detection of the studied sensors calculated in this way was as low as 2 µg/mL. The detection time required for the sensor was only 20 minutes, comparable or better than other published works in which ConA was employed. In that case, other detection system with an optical-based luminescence sensor for bacterial endotoxins allowed to detect from 0,2 to 20 µg/mL, but after an incubation time of 1 h [62] and in recent works da Silva and coworkers [17] performed an impedimetric sensor based on a Con A/polyaniline films with a detection starting from 50-100 µg/mL of bacterial LPS.

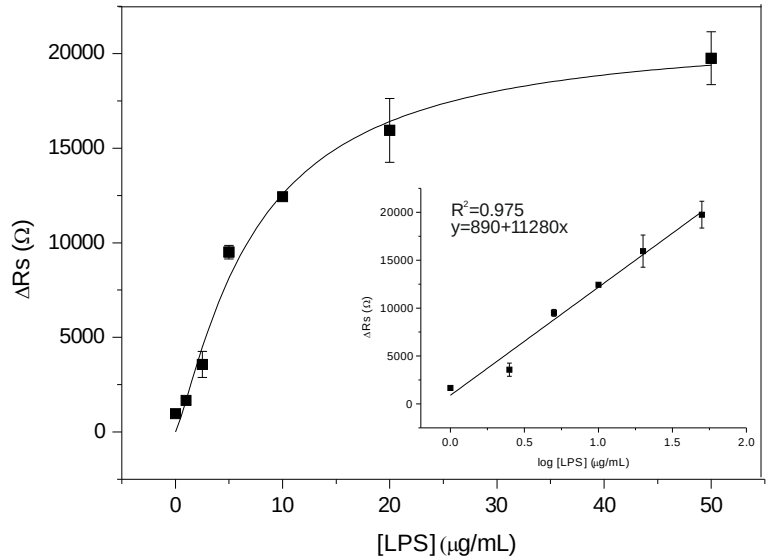

Figure 9. Response of sensors modified with PEI-(Con A-Gly)$_2$-Con A in solutions with different concentration of LPS (squares) and fitted by Hill's equation (line). In the inset - linear calibration regression of electrodes modified with PEI-(Con A-Gly)$_2$-Con A in LPS solutions. Error bars show standard deviation (n=3).

Finally, the reproducibility of the biofunctionalization strategy of the sensor with PEI-(Con A-Gly)$_2$-Con A was evaluated. In each experiment at least three sensors were used. Taking into consideration that the standard deviation of signals of different sensors was ± 3.3 kΩ we may speak about high reproducibility of the developed methodology of sensor preparation.

4. Conclusions

Presented study is focused on the development of new sensing strategies of endotoxins detection using impedimetric transducers with special consideration of the selective response between the biorecognition element and the target analyte.

In this work an impedimetric transducer based on three dimensional interdigitated electrode array was employed as a tool for the detection of the surface charge changes due to interaction of immobilized

concanavalin A lectin biorecognition element and bacterial endotoxins (lipopolysaccharides, LPS) of *E. coli* in test solution. The deposition of Con A on the surface was carried out using the layer-by-layer method with polyethyleneimine polycation as an initial layer. The sensor surface characterization by means of electrochemical impedance spectroscopy technique allowed registering variations in superficial resistance provoked by surface charge changes and is demonstrated as an effective method to monitor sensor parameters at each sensor modification step as well as to follow Con A – LPS reaction. In order to prevent non-specific adsorption of LPS on PEI covered surface different blocking strategies were tested to achieve the specific response between Con A and LPS. The typical blocking compound in many biochemical applications is BSA. However, its effectiveness is not always thoroughly studied. Results obtained in this work clearly show that blocking with BSA is not sufficient to prevent non-specific interactions of PEI and to ensure the selective biorecognition of LPS by Con A. To achieve more efficient PEI blocking a new method was proposed based on consecutive deposition of Con A-glycogen-Con A layers. Sensors modified with PEI-(Con A-Gly)$_2$-Con A multilayers are shown to be highly sensitive, selective and reproducible. The response of thus functionalized impedimetric sensors follows the Langmuir adsorption curve that is perfectly fitted by Hill's equation. The developed sensor permits to detect bacterial LPS in a very short detection time (20 min) with the limit of detection of 2 μg/mL.

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3.3. Performance of a novel and reusable aptasensor for detection of *Escherichia coli* O157:H7

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Abstract

Microbial safety of drinking water constitutes a major concern in countries at all levels of economic development. Thus, rapid, sensitive and cost effective methods of pathogenic bacteria detection, like common *Escherichia coli* O157:H7, which can cause important diseases, is highly required.

In this work an impedimetric transducer modified with *E. coli* specific aptamer is studied. To enhance the sensitivity a three-dimensional interdigitated electrode array (3D-IDEA) impedimetric transducer in which the electrodes separated by insulating barriers was used. In this sensor, chemical reactions at the surface of the barrier provoke electrical charge redistribution which causes changes in surface conductivity. A DNA aptamer that recognizes specifically the outer membrane proteins of the *E. coli* O157:H7 was selected as the biorecognition moiety.

Here we report a novel label-free impedance aptasensor for detection and quantification of pathogenic *E. coli* O157:H7 with a low detection limit, good selectivity and short detection times. The developed sensor shows a linear response (R^2=0.977), proportional to the logarithm of bacterial concentration present in the sample, with a limit of detection (LOD) of about 10^2 CFU/mL. No response was registered when the aptasensor was incubated with other bacterial strains, confirming the selectivity of suggested method. Additionally, the possibility of the sensor regeneration is shown, so that the detection may be performed several times with the same sensor. Moreover, suitability of the aptasensor for bacteria detection in real samples was demonstrated with a new approach involving bacteria pre-concentration.

1. Introduction

The quality of drinking or irrigation water is significant for health in both developing and developed countries worldwide. So, water plays a crucial role in economic development and social welfare, being a key factor in health, food production and poverty reduction [1].

Pathogenic bacteria in water are one of the main causes of human infection diseases and their detection with suitable, sensitive and fast methods is of great importance [2]. The presence of *Escherichia coli* bacteria in environmental samples, food or water usually indicates fecal contamination, lack of hygienic practices and storage conditions. So, the existence of this microorganism in water indicates that there is a high risk of the presence of fecal-borne bacteria and viruses, many of which are pathogenic [3].

Among the different strains, the enterohemorrhagic *E. coli* O157:H7 can produce from slight to life-threating diarrhea and is considered the major causative agent of hemorrhagic colitis (HC) and hemolytic uremic syndrome (HUS) due to the production of verotoxins [2, 4].Thus, the illness is associated with the consumption of contaminated food, including unpasteurized milk or fresh products like leaf lettuce and apples, as well as water that has not been properly disinfected [5]. Therefore, the presence of these bacteria is a major concern in food industry and water treatment. Additionally, the infection dose of entreohemorragic *E. coli* is around 100 colony forming units organisms, low compared with other virulence types [6], thus highly sensitive, rapid and selective detection methods of *E. coli* O157:H7 are on demand.

Conventional methods of bacteria detection, like microbiological culturing and isolation, show lack of sensitivity, involve enrichment steps with long processing times and require trained personnel [7]. Nowadays, the most commonly used methods are based on molecular techniques, like polymerase chain reaction (PCR), in which the analysis time is shorter compared to traditional methods, but it uses a complex set-up, expensive reagents and equipment [8, 9]. Therefore, new highly sensitive, fast and low cost analytical technologies are requested. In this sense biosensors, devices that integrate a biological recognition element and a physical transducer that generate a measurable signal proportional to the concentrations of analytes [10], are promising tools for bacteria detection.

Different biomolecules may be employed as a recognition element in biosensors for bacteria detection. These include enzymes, antibodies, nucleic acids or lectins [9]. Antibodies are the most widely used owing to their high selectivity and affinity with target molecules [11, 12]. Nevertheless, antibodies production is based on clone technology, involving a considerable cost. Additionally, their instability in a certain range of pH and temperature, short shelf life, easy degradation generate a problem for their application in robust biosensors development [12-15].

Aptamers are artificial oligonucleotide sequences of 30 to 100 nucleotides of DNA or RNA molecules that bind to specific ligands with high affinity and can be easily synthesized with high specificity to a certain target molecule. They are produced *in vitro* from pools of random nucleic acids sequences through the

selection evolution of ligands by exponential enrichment, SELEX process [16, 17]. Aptamers show binding affinity and specificity comparable to those of antibodies and they present important advantages, like better reproducibility in chemical production, stability under a wide range of pH conditions, resistance in harsh environments without losing their bioactivity, small size and low production cost [15]. Moreover, in the course of their chemical synthesis they can be easily modified introducing specific active terminal groups that help to immobilize effectively these molecules on different transducers, thus forming biosensors, commonly named as aptasensors [18]. In recent years a large number of publications have been focused on the development of aptamer-based biosensor for detection of pathogenic bacteria: *Salmonella* [19], *Staphylococcus aureus* [20], *Listeria* [21], and also *E. coli* [14].

More concretely, in the case of *E. coli* O157:H7 a great number of optical and electrochemical biosensors have been reported in the literature [22], however only few works are focused on the use of aptamers [23-25]. However, the majority of the reported aptasensors are based on indirect methods that complicate the detection process. Here we present a novel label-free impedance based biosensor to detect *E. coli* O157:H7 strain.

Electrochemical impedance spectroscopy (EIS) technique permits to study changes occurring on the solid/liquid interface on the surface of electrodes produced by physical, chemical or biological interactions [26]. The impedance measurements may be carried out in a faradaic or nonfaradaic mode. In nonfaradaic mode, a transient current flows across the interface that mainly depends on the interfacial capacitance. This mode of impedance measurements is considered as a more amenable method for direct biosensing applications. Different kind of electrodes can be used as impedimetric transducers for bacterial detection, but interdigitated electrode arrays (IDEA) present certain advantages, like small size, increased signal-noise-ratio and fast establishment of a steady state [27], compared to other electrodes systems.

Planar IDEA devices are formed by a pair of comb-like metal electrodes on a planar insulating substrate by conventional micro-fabrication techniques. In this case, impedance is measured between the two electrodes and depends on the solution conductivity and the interfacial properties of the electrodes: interfacial capacitance [28] and surface conductivity [29]. While traditional macro-electrodes with large surface area can be used to carry out measurements of interfacial capacitance, in micro-scale transducers the surface charge also plays an important role [29]. In this sense, to improve the sensitivity of standard IDEA sensors a three-dimensional interdigitated electrode arrays (3D-IDEA) impedance transducer, in which the electrodes are separated by insulating barriers, was proposed [30]. The 3D-IDEA devices are highly sensitive to changes in the surface charge at the solid/liquid interface produced by chemical and biochemical reactions, and previously have been demonstrated useful in different label-free detection processes [31, 32].

In this work, we report a novel label-free impedimetric aptasensor for the direct detection of *E. coli* O157:H7. The surface of the 3D-IDEA transducer was initially modified with a mercaptosilane to covalently

immobilize an *E. coli* specific 5' disulfide-modified DNA aptamer by means of a thiol/disulfide exchange reaction [33]. The biofunctionalization strategy is schematically presented in Figure 1. The impedimetric measurements allow registering the presence of target bacteria at different concentrations with low limit of detection in a short time. Moreover, the feasibility of the aptasensor was validated with real samples introducing a pre-concentration step with filtration system approach developed in our group. Finally, one of the main novelties of this study is the regeneration of the aptasensor after the detection of bacteria, confirming the great potential of its application compared with other label-free detection systems. The present study suggests a rapid and attractive method for bacterial detection especially in water quality analysis in environmental samples.

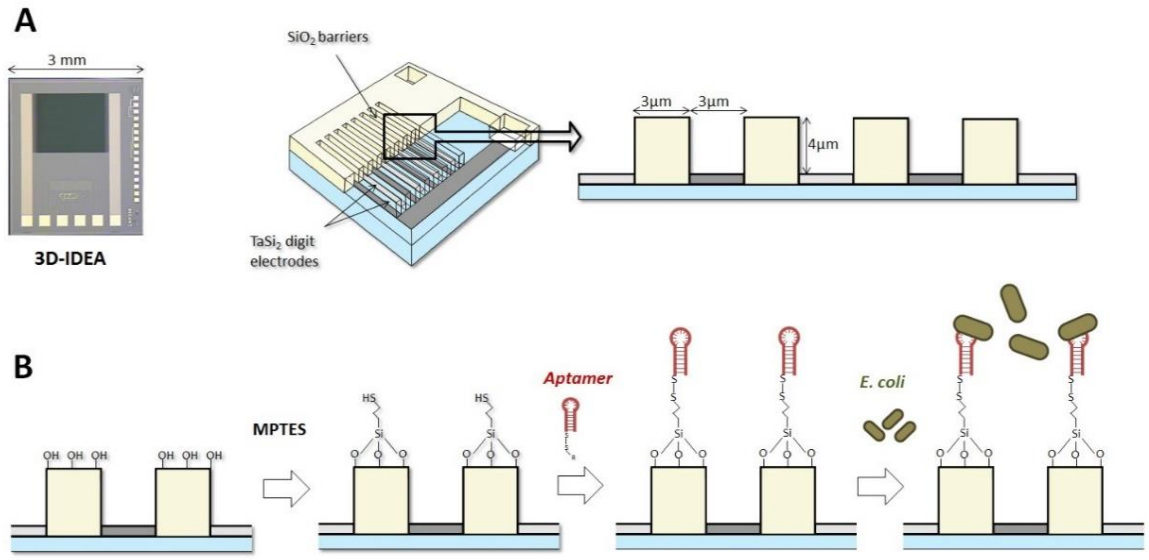

Figure 1. 3D-IDEA sensor surface image and its schematic representation (A) and different biofunctionalization steps (B).

2. Materials and methods

2.1. Electrodes fabrication

The interdigitated electrode array was formed on a silicon wafer covered with a 2.5 µm thick thermal silicon oxide layer. As an electrode material a highly conductive tantalum silicide ($TaSi_2$) was deposited using magnetron sputtering. This layer was patterned using conventional lithography giving as a result interdigitated electrodes with 216 digits of 3 µm width and 3 µm gap between the adjacent electrodes. The aperture between the electrodes is 1.4 mm and the total length between the electrodes is 301 mm. The wafer was covered by a 4 µm thick low pressure chemical vapor deposition (LPCVD) silicon dioxide in which electrode digits and contact pads of the transducers were opened by deep reactive ion etching (DRIE). Thus, 4 µm high barriers with nearly vertical walls separating the electrodes digits were formed.

Complete technology of sensor fabrication is presented elsewhere [30] and the resulting sensor is shown in Figure 1A .

2.2. Reagents, solutions and materials

DNA aptamer sequence 5'- S-S- ATCCGTCACACCTGCTCTGTCTGCGAGCGGGGC GCGGGCCCGGCGGGGGATGCGTGGTGTTGGCTCCCGTAT-3'used in this study against *E. coli* O157:H7 was selected from previous work [12, 23]. The aptamer was purchased in lyophilized form from Sigma-Aldrich (Spain). Prior to use the aptamer was purified by elution through Ilustra NAP 10 columns for a DNA gel filtration with Sephadex G-25 DNA grade (GE Healthcare Life Science, Spain) using deionized DNase free-water. DNA concentration was determined accurately by measuring the absorbance at 260 nm at room temperature by means of a spectrophotometer (ε= 650900 L/mol·cm) (DU 730 Life Science UV/Vis Spectrophotometer from Beckman Coulter). Stock solutions were divided in aliquots and stored at -20^{0}C until use at desired concentration.

Phosphate buffer solution (PBS) (0.01 M phosphate buffer, 0.0027 M KCl and 0.137 NaCl, pH=7.4) was used as the buffer solution to prepare the aptamer during the immobilization process and as a supporting solution to perform the bacterial detection assays. For the surface treatment 3-mercaptopropyl-trimethoxysilane (MPTES) was purchased from Sigma-Aldrich. All the solutions were prepared with deionized MilliQ water (18 MOhm cm) which was also used for cleaning and rinsing processes.

2.3. Preparation and characterization of aptasensor

Prior to use, 3D-IDEA sensors were cleaned with isopropanol for 10 minutes, rinsed with water and dried under nitrogen flow. Afterwards, the surface was treated with (3-mercaptopropyl) trimethoxysilane (MPTES) by means of vapor-phase silanization. During silanization sensors were placed in a custom-designed cell and maintained at 50 ^{o}C for 1 h under a controlled atmosphere of mercaptosilane. This method was adapted from previously reported protocol [34].

The covalent immobilization of 5' disulfide-modified DNA aptamer on the 3D-IDEA surface was performed via the mercaptosilane layer using a thiol/disulfide exchange reaction described elsewhere [33]. For this 3D-IDEA sensors were immersed into a 20 nM solution of aptamer dissolved in PBS (pH=7.4) overnight at room temperature with slight agitation. The complete biofunctionalization strategy is schematically presented in Figure 1.

In order to optimize the immobilization process of aptamer, different concentrations were tested (see Supplementary Information, Fig. S1) and 20 nM was selected to perform the detection experiments. Finally, the electrodes were rinsed with KCl 10^{-5} M solution to remove adsorbed PBS salts to reduce their possible effect on the impedance measurements.

2.4. Circular dichroisms (CD) and molecular absorbance measurements

The CD spectra were recorded on a JACSO spectropolarimeter J-815. The 2 μM aptamer samples were prepared in 10^{-5} M KCl and in standard PBS, and their spectra were registered at 25 ^{o}C in the range of 220-320 nm, with a scanning speed of 100 nm/min, a response time of 4 seconds, data pitch of 0.5 nm and a band-width of 1 nm [35].

2.5. Bacteria cultivation and detection experiments

E. coli DSMZ 17076 strain, a non-pathogenic but phenotypically similar to the entreohemorragic *E. coli* O157:H7, was employed to perform the detection assays. Bacteria were grown overnight at 37 ^{o}C in Luria-Bertani (LB) medium broth before each assay. The cells were harvested by centrifugation at 9000 g for 10 minutes, then washed three times in sterile PBS (pH=7.4) and re-suspended in a fresh PBS solution at desired concentrations to perform the detection assays. After each test bacterial concentration was determinate by colony counting in LB agar plates, incubated for 24 h at 37 ^{o}C.

E. coli detection assays were performed by incubation of aptasensors in solutions with bacterial concentration ranging from 10^{1} to 10^{5} CFU/mL. At least five different concentrations were used for the assay. 3D-IDEA sensors biofunctionalized with the aptamer were immersed in 100 mL of bacteria suspensions in PBS solution under stirring conditions to maintain a homogeneous distribution of bacteria. The aptasensors immersed into PBS solution without bacteria and non-functionalized 3D-IDEA in solutions with the highest concentration of *E. coli* were employed as controls. The experimental setup for bacterial detection assays was adapted from our previous work [32].

To test the selectivity of the detection method, cross reactions were studied using the bacterial strains *E. coli* K12, *Salmonella typhimurium* ATCC 14028 and *Staphylococcus aureus* ATCC 6538. In this case, the same procedure was used for bacteria samples preparation. All the different bacteria samples were adjusted to the same concentration of 10^{5} CFU/mL in PBS to compare the selectivity of the proposed biosensor. All the experiments were done in sterile conditions to prevent contaminations.

In the case of real drinking water samples, prior to be analyzed with the impedance-based aptasensor, they were subjected to a filtration pretreatment, as described in detail in Supplementary Information, to avoid possible interferences from unknown sample components.

2.6. Impedance measurements

A QuadTech 7600 Plus, a highly precision LCR Meter analyzer, was employed for all impedance measurements in a 10^{2}– 10^{6} Hz frequency range with 100 mV (amplitude) voltage excitation. The measurements were performed in a nonfaradaic mode and no DC voltage bias was applied during the impedance measurements. Impedance data treatment and equivalent circuit fitting was performed using the Z-Plot/Z-View software package (Scribner Associates, Southern Pines, NC, USA). All experiments were done at least on three biosensors under the same conditions.

Impedance measurements were carried out at controlled room temperature in 10^{-5} M KCl solutions. To guarantee the reproducibility of the bulk solution conductivity for each single measurement a fresh portion of solution was used and its conductivity of 2.50 µS/cm was controlled with a commercial conductimeter (EC-Meter GLP 31+, Crison).

3. Results and discussion

3.1. Characterization of the aptasensor by impedance measurements

The electrochemical impedance spectroscopy (EIS) technique used in this work is an effective technique to study electrochemical systems analyzing their complex electrical resistance, sensitive to surface phenomena and changes in bulk properties [36]. Therefore, it is a powerful tool to monitor electrode surface modifications in biosensing processes [37] and it was employed in this work also to characterize each step of the biosensor fabrication. Impedance changes were studied in a non-faradaic mode with IDEA in low conductivity KCl solutions in the absence of redox probes. Under this measurement conditions surface conductivity plays an important role due to surface charge presence. This effect is more pronounced in 3D-IDEA sensors, in which the major part of the electrical current between the electrodes goes close to the surface of the barrier separating the electrodes and not through the surrounding bulk solution [30]. This permits to improve the sensitivity of electrodes to biochemical reactions that occur on the sensor surface, thus monitoring the biofunctionalization steps and the detection performance.

The impedance response of the studied system may be presented by an equivalent circuit shown in Fig. 2A, formed by the following components: R_C is the contact resistance introduced by wires and collector bars of the thin film electrodes; C_G or C_{IDS} 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. As previously reported [38], R_S is a parallel combination of the bulk solution resistance (R_{BULK}) and the surface resistance (R_{SURF}) (Fig. 2A), but under the experimental conditions used it is not possible to distinguish these two elements in the impedance spectra. However, if the bulk solution conductivity remains fixed, as in our experiments, we may attribute the changes in R_S to the surface resistance changes produced by surface reactions and modifications [38].

Fig. 2B presents experimental impedance spectra as a Nyquist plot (Z' vs Z'') that allows to observe the formation of a semicircle at high frequencies, corresponding to the resistance R_S in parallel with the geometrical capacitance. The intercept with Z' axis on the left side gives the R_C values, while the intercept on the right side gives the value of $R_C + R_S$, where $R_S = (R_{BULK} + R_{SURF})$. The linear response at low frequencies in the Nyquist plot is produced by the CPE of the interfacial capacitance.

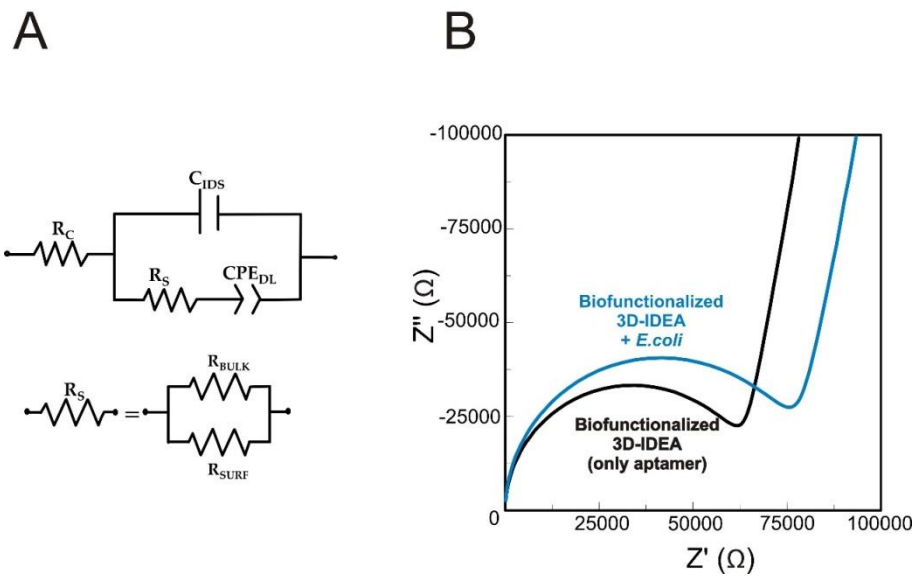

Figure 2. Electrical equivalent circuit used for impednace spectra fitting (A) and Nyquist plots of 3D-IDEA after biofunctionalization and after detection of *E. coli* O157:H7 measured in KCl 10^{-5} M solution (B).

3.2. Aptamer immobilization and optimization of incubation time for *E. coli* detection

In order to determine the optimal aptamer concentration for the sensor surface functionalization, 3D-IDEA was chemically modified via vapor-phase silanization with MPTES. The aptamer modified with a disulfide group at 5' terminal was selected to be covalently attached to the sensor surface, grafted with mercaptosilane, via thiol/disulfide exchange reaction between the disulfide terminal of oligonucleotide and the sulfhydryl group of mercaptosilane [33]. To ensure the completeness of the chemical reaction the electrodes were maintained overnight in the aptamer solution.

To confirm the importance of the aptamer concentration on the performance of impedance-based biosensor, aptasensors were modified in the aptamer solutions of different concentrations and then were incubated in the presence of 10^4 CFU/mL of *E. coli*. Figure S1 of Supplementary Information shows that in the presence of *E. coli* the impedimetric response increases with the concentration of immobilized aptamer with a maximum at 20 nM of aptamer solution employed. In the absence of aptamer practically no response to *E. coli* presence was observed, demonstrating that there is no nonspecific binding of bacteria on the sensor surface. Therefore, the aptamer concentration of 20 nM was selected as optimal for sensor functionalization and was used in the following experiments.

Aptamer immobilization on the surface and its subsequent interaction with *E. coli* produced significant changes in the impedimetric response, affecting R_S and C_{DL} values of the equivalent circuit. Bacterial presence on the functionalized interdigitated sensor surface modifies the double layer interfacial capacitance (C_{DL}) and the CPE α parameter due to the formation of additional layers over the electrode digits surface. However, the ΔR_S, associated with the surface conductivity due to the surface charge

presence, show higher reproducibility and a larger scale variation and was selected as the main parameter to monitor the aptasensor response.

The impedance sensor response within bacterial detection assays was expressed as variations in R_S:

$$\Delta R_S = R_S^{E.coli} - R_S^{aptamer} \quad (1)$$

where $R_S^{aptamer}$ is the resistance of 3D-IDEA biofunctionalized with the aptamer and $R_S^{E.coli}$ is the resistance values after the sensor interaction with *E. coli* cells .

Prior to the aptasensor sensitivity and selectivity characterization, the required incubation time was evaluated. One of the important parameters of biosensors is the response time, the time during which a sensor reaches the stationary signal level at a certain concentration of analyte in a sample. The optimal incubation time of aptasensors was evaluated in a 10^5 CFU/mL *E. coli* bacterial solution by periodic measurements of impedance spectra to register the evolution of R_S (Figure S2). It was observed that impedance response increases significantly reaching a stationary phase within 30 minutes. Thus, this time interval was chosen as the suitable incubation time to guarantee the completeness of bacteria reaction with the aptasensor, and, therefore, it was employed in the following experiments.

3.3. Study of the aptamer stability by CD and UV experiments

The stability of the aptamer as a biorecognition moiety is essential in the performance of the proposed impedance based biosensor. Aptamers, compared with other biorecognition elements, present certain advantages widely described in the literature [15], like the small size, cost effectiveness or resistance to harsh conditions. However, to preserve its bioactivity an aptamer should maintain intact its secondary structure. The aptamer employed in this study is composed by different loop-hairpin structures that allow to interact properly with the target molecules [39]. To ensure that under experimental conditions of this study the aptamer does not change its proper structure required for biorecognition, circular dichroism and molecular absorption measurements were done. CD is a spectroscopic method that allows to study the conformation of proteins and nucleic acids in solution, and is widely applied due to its sensitivity to structural changes in biomolecules [40]. Thus, this technique was used to analyze possible changes in the aptamer structure. These experiments were carried out by conditioning the aptamer in the physiological pH buffer (PBS), employed during the immobilization step and as the medium for *E. coli* detection, and in a low electrolyte concentration solution (KCl 10^{-5} M), used during impedimetric measurements. Results of CD and molecular absorption measurements are detailed in the Supplementary Information and are summarized in Figure S3, and the predicted structure of the employed aptamer is shown in Figure S4. As follows from the obtained data, in PBS solution the aptamer conformation remains nearly intact, however, slight changes in CD spectra were observed in KCl 10^{-5} M solution. Therefore, the aptasensor contact with KCl was reduced to minimum (1-2 min), only to perform impedance measurements and to ensure that the aptamer structure is not affected. All the rest of processes and bacteria assays were carried out in PBS.

3.4. *E. coli* detection with the aptasensor

The sensitivity and the limit of detection are the most critical parameters in the performance of a biosensor for its applicability [41]. To evaluate the sensitivity of the proposed aptasensor bacteria detection was performed after 30 minutes of sensors incubation in samples with different *E. coli* concentrations, ranging from 10^1 to 10^5 CFU/mL, as explained in the experimental section. After incubation the sensors were extracted from the bacterial solution, rinsed with 10^{-5} M KCl solution to eliminate PBS salts from sensor surface and, thus, to reduce their possible effect on impedance measurements. Finally, their impedance spectra were measured in 10^{-5} M KCl.

Moreover, two experiments were employed as controls. In the first case, to confirm the absence of non-specific bacteria adsorption on the initial, not functionalized sensor surface, electrodes were incubated in the solution with the highest (10^5 CFU/mL) bacterial concentration. On the other hand, sensors grafted with aptamer, in parallel with bacteria detection experiments, were immersed in a PBS solution without bacteria for the same period of time to guarantee the stability of the impedance response.

The results revealed that variation on the impedance response (ΔR_S) is directly proportional to the logarithm of bacterial cell concentration. Figure 3 shows the linear correlation, $\Delta R_S = S\ logC + R_o$, between ΔR_S and the logarithm of *E. coli* concentration in the range of $10^1 - 10^5$ CFU/mL with a good correlation coefficient of 0.977 and a sensitivity, S, of 2.6 ± 0.2 kΩ per bacteria concentration decade. In the case of controls, no impedance changes were observed, demonstrating the effectivity of proposed aptasensor and the absence of nonspecific interactions. The limit of detection (LOD) of the aptasensor was 2.9×10^2 CFU/mL. It was calculated using the method reported by Shrivastava *et. al* [42] as LOD = $X_{b1}+3S_{b1}$, in which X_{b1} is the mean signal value of the blank in the absence of bacteria, and S_{b1} is the standard deviation in the blank. The limit of detection is better or comparable with other previously reported biosensors for detection of *E. coli* O157:H7 [23, 24], which is of great importance taking into account the pathogenicity of this bacteria.

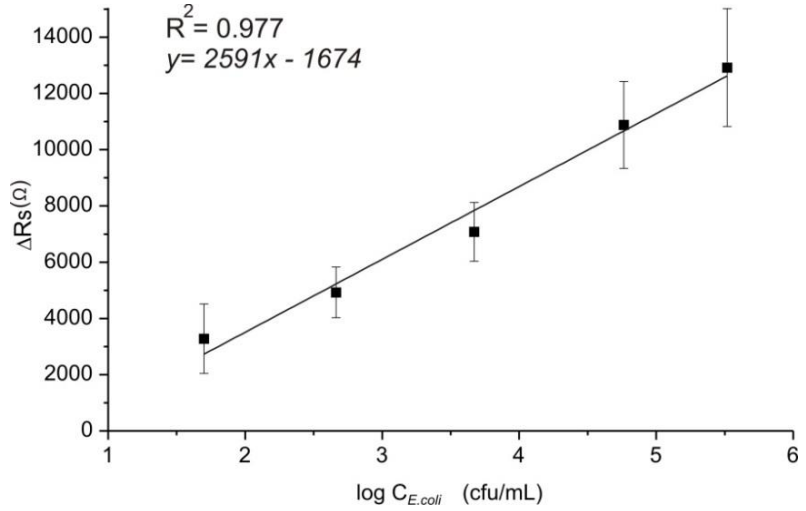

Figure 3. Calibration curve of impedance response, ΔR_S, expressed as a function of the logarithm of *E. coli* O157:H7 concentration from 10^1 to 10^5 CFU/mL.

3.5. Analysis in drinking water samples

The real sample analysis was performed in water directly collected from the tap and spiked with bacteria at 10^5 CFU/mL. As previously reported, the quality of drinking water for bacteriological safety is essential in many applications like human consumption, food preparation, washing or irrigation [1]. A previous filtration step was performed to retain bacteria in a specially designed filtration system. The system and methodology are presented in more detail in Supplementary Information. This filtration system may be employed for the treatment of different liquid samples in a short time (5 minutes for 100 mL of drinking water) and is of great interest to reduce the possible matrix effect of real samples on the sensor response. Furthermore, it has to be considered that this strategy is very useful to capture bacteria that are in low concentration in large volumes, which it is a recurrent problem in control of water-related infections, when the volume of samples to analyze may be considerable.

After the filtration, bacteria were recovered and transferred to PBS supporting solution to perform the detection assays. Transaction of the recovered bacteria into a fresh PBS and sensor incubation in this solution allowed to eliminate possible contamination and interference from “unknown” sample and to maintain the aptamer in the ideal conformation to detect bacteria and ensure a good response. Figure S5 shows the differential response between solutions in the absence of bacteria and in water samples inoculated with 10^5 CFU/mL. Applying earlier performed calibration (see Fig. 3) to thus determined ΔRs value of 10.70 ±1.96 kΩ we obtain between 5.53 and 4.02 log $C_{E.coli}$, that it is about 10^5 and 10^4 CFU/mL of the *E. coli* concentration. Taking into consideration the filtration system recovery rate of 92.3±1.2 %, determined concentration is practically the same to that of the sample.

3.6. Selectivity of the aptasensor

To ensure the selectivity of the proposed aptasensor in specific binding of target bacteria *E. coli O157:H7* experiments were performed in samples with different bacterial strains, two gram-negative, *E. coli* K12

and *Salmonella typhimurium,* and one gram-positive, *Staphylococcus aureus*, all adjusted at the same final concentration of 10^5 CFU/mL. Moreover, a blank without bacteria (only PBS) and the specific *E. coli* O157:H7 at 10^5 CFU/mL were analyzed in parallel. The test of specificity was carried out incubating the aptasensors in each of bacteria samples for 30 minutes, measuring their impedance response in each case.

As it can be observed in Figure 4, the sensor shows high selectivity to *E. coli* O157:H7, as in all other cases after the incubation of the aptasensor in different bacteria samples the observed impedance changes were insignificant. In the case of the blank without bacteria and the *E. coli* K12, the increase in impedance is almost imperceptible. However, in the presence of *Salmonella* and *S. aureus* the response is slightly higher. These results show that the selected aptamer immobilized on the sensor surface maintains its specificity in reaction with the *E. coli* O157:H7, giving significant ΔR_S increase, demonstrating the affinity for this bacterium. This may be considered as a validation of the selectivity of the proposed aptasensor.

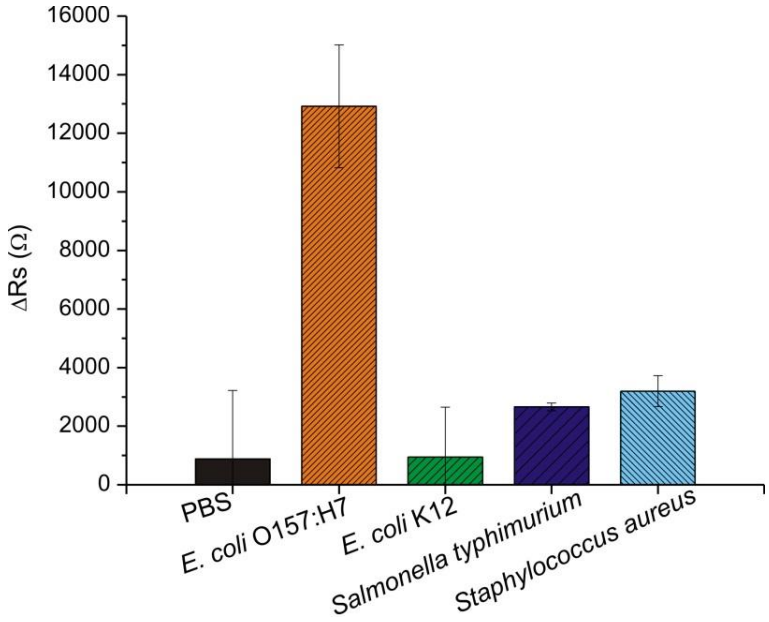

Figure 4. Evaluation of selectivity of the aptasensor for the detection of different bacteria strains (*E. coli* O157:H7, *E. coli* K12, *Salmonella typhimurium* and *Staphyolococcus aureus*) and control in PBS without bacteria.

3.7. Sensor regeneration

In the development of biosensors is essential to reduce costs per analysis and to do this regeneration strategies are particularly important [43]. In our case, experiments on regeneration of the aptasensor after its reaction with bacteria were performed. This can be done by provoking dissociation of the complex of aptamer and the *E. coli*, but it is important to guarantee that the aptamer recovers its structure necessary for the biorecognition after this treatment.

To break the interaction aptamer-*E. coli* the sensors were firstly immersed in distilled water at 80 ºC for 30 minutes to release the bacteria from the aptamer. Higher temperatures provoke the aptamer denaturation and the loss of its tertiary structure, resulting in the lack of affinity for bacteria. Afterwards the sensors were immersed in a PBS solution to restore the appropriate aptamer conformation. It should be taken into account that the interaction between the terminal DNA aptamer and the functionalized electrode surface is produced by a covalent interaction through a disulfide covalent bond. This bond is quite strong and the applied increase of temperature does not affect it, so the aptamer is retained immobilized on the surface.

To perform regeneration tests the sensors were incubated in 10^5 CFU/mL *E. coli* samples and then were subjected to regeneration process as presented above. In parallel, to check if the process affects the aptamer, the same experiments were made with sensors incubated in PBS in the absence of bacteria. Figure 5 shows the impedance changes observed after each incubation and regeneration step. As demonstrated previously, the changes in resistance R_S as a result of *E. coli* interaction with the aptasensor can be employed as the reference parameter to monitor the recovery of the sensor parameters. Firstly, the aptasensor was used to detect bacteria and, as previously observed, in the presence of *E. coli* an increase in R_S was registered. The control aptasensor stored in PBS showed no R_S changes. Secondly, after the first regeneration step, a decrease in R_S was produced with its final value coinciding with the control test without bacteria. This indicates that the bacteria are removed from the sensor surface and that the treatment does not affect the aptamer properties. The same process of bacteria detection was repeated showing subsequent recovery of the sensor response. Therefore, these results validate the efficiency of the regeneration methodology employed for the developed aptasensor.

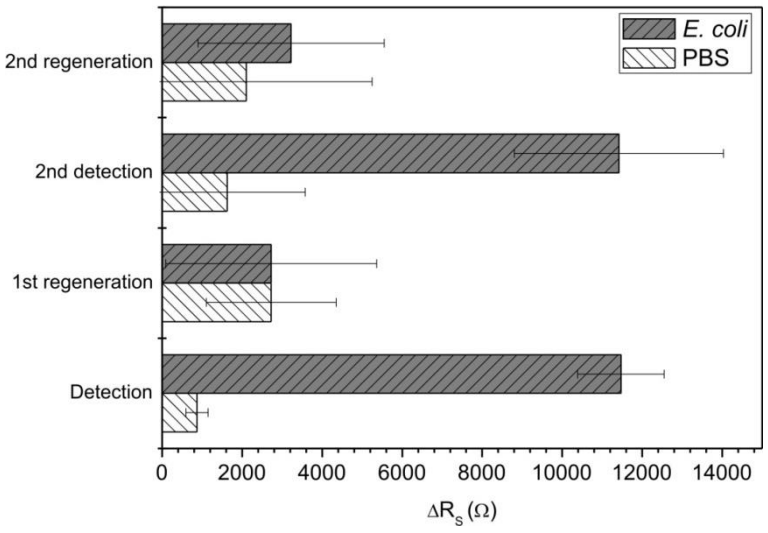

Figure 5. Impedance changes *(ΔR_S)* after the detection process of *E. coli* (10^5 CFU/mL) and the two consecutive regeneration steps with aptasensors (n=3).

4. Conclusions

In this study an innovative label-free aptasensor based on a 3D-IDEA impedimetric transducer has been introduced for the detection of the *E. coli* O157:H7, a well-known pathogenic fecal-borne bacteria.

The surface biofunctionalization with the aptamer and the detection of bacteria was characterized by EIS technique. The DNA aptamer was covalently immobilized on the 3D-IDEA surface grafted with mercaptosilane. Additionally, the effect of the assay conditions on the stability of the aptamer was investigated by means of circular dichroism technique, demonstrating that the aptamer in PBS remains stable, while in 10^{-5} M KCl some changes in its structure occur. This permitted to optimize the assay protocol to assure the aptamer stability.

The studied aptasensor showed a linear relationship (R^2=0.977) between the impedance changes and the logarithm of *E. coli* concentration in a broad range from 10^1 to 10^5 CFU/mL with a LOD about 2.9×10^2 CFU/mL. Moreover, the selectivity of proposed aptasensor has been validated with other bacterial strains, demonstrating high selectivity for the target *E. coli* O157:H7. The developed aptasensor allows to detect *E. coli* bacteria in PBS solutions in a very short time of only 30 minutes.

To study the applicability of the developed sensor to perform bacteria detection in real samples, tap water samples spiked with *E. coli* were analyzed. To reduce possible sample matrix effect on the sensor response a promising pre-concentration system for bacteria retention was employed that may be used for bacteria extraction from other liquid samples. Obtained results show satisfactory precision of the developed detection protocol.

Finally, it was shown that a biosensor regeneration methodology based on a simple temperature treatment in water allows to use the aptasensor multiple times, that is of great interest in biosensing applications.

In summary, coupling of aptamers with 3D-IDEA biosensors has resulted in the implementation of a rapid, label-free, sensing platform with high sensitivity and selectivity for the detection of the pathogenic strain *E. coli* O157:H7. The methodology proposed in the performance of this label-free biosensor can be potentially employed for the development of other rapid and selective aptasensors as promising tools for detection of other pathogenic bacteria in water samples.

Supplementary information

S1. Optimization of aptamer concentration

Different concentrations of aptamer were employed (0, 2, 20 and 200 nM) to optimize the sensor biofunctionalization protocol. The sensors were immersed overnight in the solutions of the selected concentration and then their impedance response was measured after 30 min incubation in 10^4 CFU/mL

E. coli bacteria solution in PBS. As follows from Figure S1, sensors prepared with 20 nM and 200 nM of aptamer showed similar response. Thus, 20 nM was selected as the optimal aptamer concentration to perform further experiments.

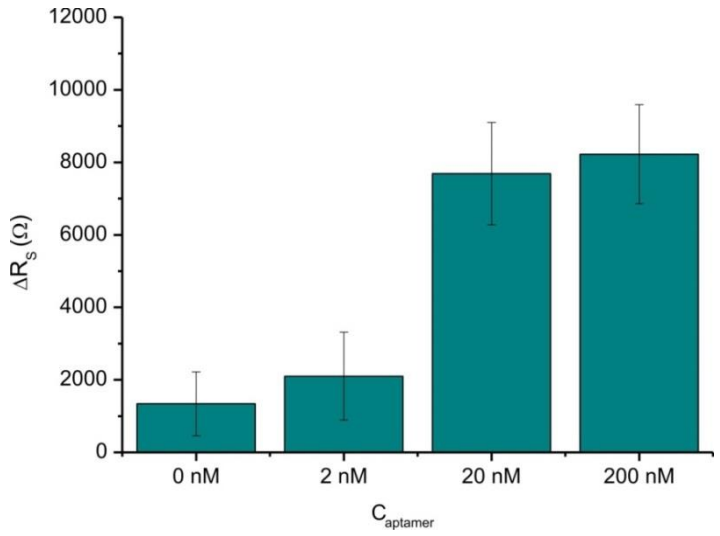

Figure S1. Impedance response at different concentrations of aptamer with *E. coli* bacteria at 10^4 CFU/mL.

S2. Incubation time

The incubation time of aptasensors was evaluated in a *E. coli* bacterial solution at a fixed concentration of 10^5 CFU/mL with periodic measurements of impedance response to register the evolution of R_S. Figure S2 shows that impedance response increases significantly reaching a stationary phase at 30 minutes. Thus, 30 minutes was chosen as the suitable incubation time.

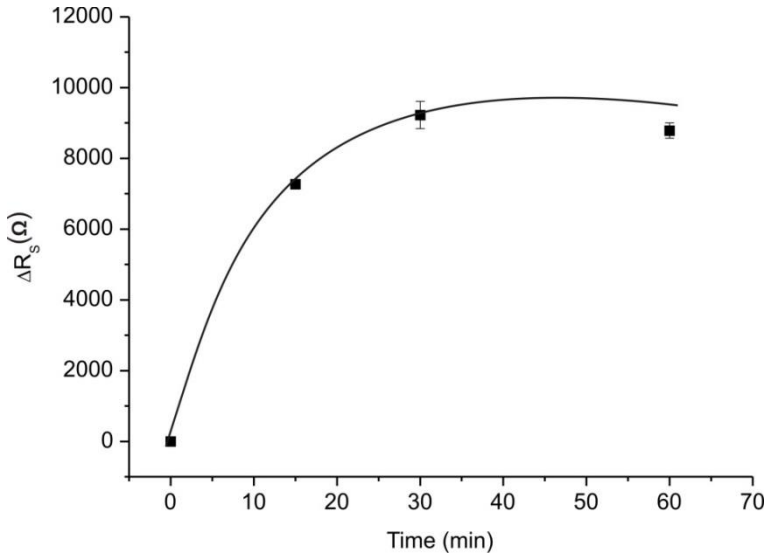

Figure S2. Changes in the impedance response (ΔR_S) of the aptasensor measured at bacterial concentration of 10^5 CFU/mL during 60 minutes.

S3. CD and molecular absorbance measurements

CD and molecular absorbance spectra of the DNA aptamer employed in this work are shown in the Figure below. The aptamer solutions were prepared by dissolving the aptamer in PBS buffer or KCl 10^{-5} M solutions at the concentration of 2 µM in both cases. Blank spectra of PBS and KCl solutions were also measured.

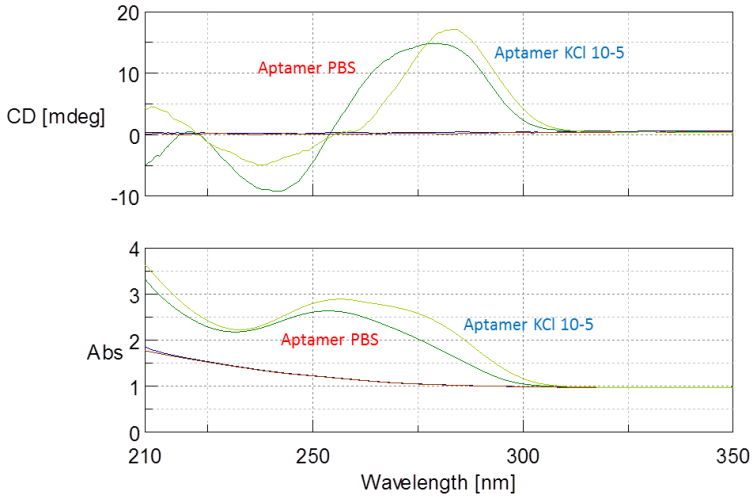

Figure S3. Circular dichroism and molecular absorption spectra recorded for the aptamer at 2 µM concentration in PBS (green line) and KCl 10^{-5} M solution (yellow line). Controls with PBS and KCl without aptamer are represented with black and red lines respectively.

CD spectroscopy is a technique that is sensitive to changes in the spatial structure of biomolecules, such as the aptamer studied here. In this sense, CD spectra presented in Figure S3 show clear differences between the spatial structure of the aptamer in PBS and KCl solutions. Also, molecular absorption spectra reflect changes in the stacking of bases in both solutions. The absorbance spectrum recorded in KCl shows hyperchromicity at all wavelengths, a fact that may be related to the unstacking of bases in a rather unfolded chain. In summary, CD and absorbance changes may be explained by differences in the aptamer spatial structure produced by the ionic strength of medium. In PBS solutions, in which the ionic strength is higher, the spatial structure is more stable due to the presence of salts and ions that stabilize the opposite charges of neighbor nucleotides [44] . However, in low conductivity solutions, like in the case of KCl 10^{-5} M used in our experiments, folded structures are less stable. Therefore, PBS was used in all the modification processes and for bacteria detection assays with the aptasensor, while KCl was only employed for impedance measurements to reduce the contact time of the aptamer with KCl.

S4. Aptamer folding prediction

The structure of the aptamer employed was obtained using a folding prediction based on *in silico* calculations with the web server located in: http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form. (M. Zuker, *Nucleic Acids Res.* 2003, 31, 3406). Calculations were done considering 25^{o}C and 150 mM Na^{+}.

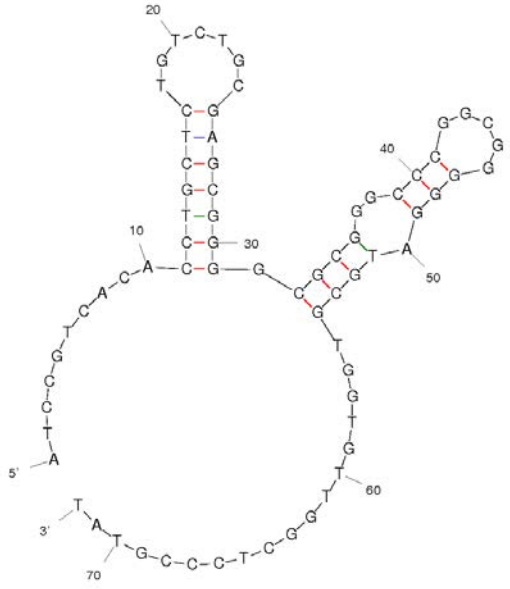

Figure S4. Folding prediction based on *in silico* calculations of the aptamer employed.

S5. Water samples treatment

S.5.1. Methodology

To analyze the drinking water samples, 100 mL of tap water was collected and inoculated with *E. coli* 0157:H7 bacteria was to have a final concentration of 10^5 CFU/mL. The precise concentration was determined by colony counting in LB agar plates. Afterwards, the 100 mL of water sample was pumped through a filtration system developed in our group to isolate bacteria. The system consists of a filter holder to anchor filters of polycarbonate (PC) (Whatman Nuclepore Track-Etched Polycarbonate, GE Healthcare Life science) with a pore diameter of 0.22 µm. The filter holder design and the polycarbonate membrane are presented in [45]. For the filtration of a 100 mL liquid sample the system requires around 5 minutes. After the filtration the filter was extracted from the holder, placed in a beaker with 10 mL of PBS and sonicated for 5 minutes to extract bacteria cells from the filter surface to the PBS solution. Finally, 90 mL of a fresh PBS was added to the recollected sample to obtain the same volume as the initial one. Moreover, the exact resulting concentration of *E. coli* in the 100 mL of PBS was also determined by colony counting.

The bacterial detection assay was performed in the 100 mL of PBS with *E. coli* recovered after the filtration step. The same protocol of impedimetric bacteria detection reported in the experimental section of this work was employed.

S.5.2. Results and discussion

To evaluate the efficiency of the proposed filtration system it is necessary to determine the system recovery rate, the difference between initial bacteria concentration and that obtained after the recovery step. Thus, the recovery rate of bacteria was expressed as (1):

$$\%\, recovery = \frac{\log C\ bacteria\ afetr\ filtration}{\log C\ bacteria\ before\ filtration} \times\ 100 \quad (1)$$

In this case, the resulting recovery obtained in PBS samples was 92.3±1.1 % of bacteria, demonstrating the high efficiency of the proposed system. The effectivity in the recovery of bacterial pathogens in liquid samples is essential to prevent infections, especially in case of *E. coli* O157:H7 in which the infection dose is really low.

After the recovery of bacteria in PBS, a detection assay was carried out with 3D-IDEA biofunctionalized with the aptamer to monitor changes in the impedance response as detailed previously. As shown in the Figure S5, significant changes of 10.70 ±1.96 KΩ were observed between the control without bacteria and the sample with the recovered bacteria in PBS after the filtration process.

Applying earlier performed calibration (see Fig. 3 of the work) to thus determined ΔR_S value we obtain between 5.53 and 4.02 log $C_{E.\ coli}$, that it is about 10^5 and 10^4 CFU/mL of the *E. coli* concentration. Bacterial concentration obtained by colony counting in LB agar plates, employed as the standard method to determine the number of bacteria, was 4.77±1.07 log C CFU/mL. Taking into consideration the filtration system recovery rate of 92.3±1.2 %, determined concentration is very close to that of the sample.

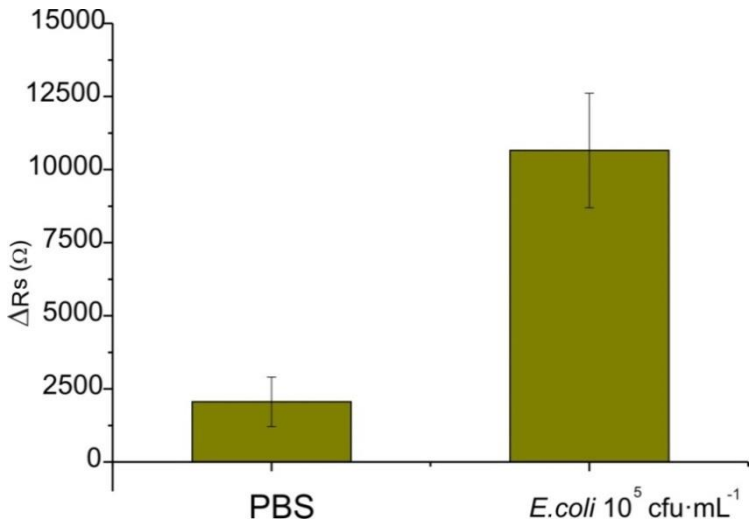

Figure S5. Variations in impedance response (ΔR_S) after the bacterial detection assay carried out after sample filtration and recovery steps in comparison with the blank (PBS) response.

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3.4. 3D impedimetric sensors as a tool for monitoring bacterial response to antibiotics

This work is currently under preparation for publication in *Lab on a Chip*

Abstract

The presence of antimicrobial contaminants like antibiotics in the environment is a major concern because they promote the emergence and the spread of multidrug resistant bacteria. Since the conventional systems for the determination of bacterial susceptibility to antibiotics rely on culturing methods that require long processing times, the implementation of novel strategies for these assays are highly required. Here the development and characterization of a label-free biosensing platform based on a microbial biosensor approach to perform antibiotic detection bioassays in diluted solution is presented. The microbial biosensor is based on a three-dimensional interdigitated electrode array (3D-IDEA) impedimetric transducer with immobilized *E. coli* bacteria. In 3D-IDEA to increase the sensitivity to superficial impedance changes the electrode digits are separated by insulating barriers. A novel strategy is employed to selectively immobilize bacteria in the spaces over the electrode digits between the barriers, referred here as trenches, in order to concentrate bacteria, improve the reproducibility in the *E. coli* immobilization and increase the sensitivity for monitoring bacterial response. For effective attachment of bacteria within the trenches an initial anchoring layer of a highly charged polyethyleneimine (PEI) polycation was used. To facilitate immobilization of bacteria within the trenches and prevent their deposition on the top of the barriers an important novelty is the use of poly(N-isopropylmethacrylamide) p(NIPMAM) microgels working as antifouling agents, deposited on top of barriers by microcontact printing.

The reported microbial biosensor approach allows to register the bacterial response to ampicillin, a bacteriolytic antibiotic, by means of impedance variations in a rapid and label-free operation that enables new possibilities in bioassays for toxicity testing.

1. Introduction

Infectious diseases and emerging bacterial pathogens are still one of the main public health problems worldwide [1]. To prevent bacterial infections antibiotics have been widely employed from the middle of the 20th century [2]. Antibiotics are antimicrobial drugs of natural or synthetic origin mainly used for the treatment and prevention of infectious diseases in humans and employed to promote growth of animals in the meat industry. Generally, antibiotics are classified by either their chemical structure or mechanism of action and can be divided in different subgroups, such as β-lactams, quinolones, tetracyclines, sulphonamides and others [3]. However, the extensive and abusive use of antibiotics in healthcare, medicinal veterinary and even in agriculture has led to the appearance of bacterial resistant genes, which are promoted because antibiotics also acts on commensal bacteria, creating a reservoir of resistant organisms [4, 5]. Food and water are important vectors for the spreading of these resistant organisms between humans and animals, resulting in an increasing pressure for ensuring safety in human consumption [6]. Hence, different methods are required to perform bacterial detection assays and identify possible bacterial resistances in common pathogens.

The standard methods in clinical microbiology to study the required dosage of antibiotics to different bacterial strains are based on the antimicrobial susceptibility tests (AST). Currently, AST are accomplished using classical methods or growth-dependent automated systems [7]. One of the earliest but still used methods is the broth dilution testing and the derived microdilution broth test based on the growth inhibition in liquid medium with antibiotics. The advantage of this method is the generation of a quantitative result, namely, the minimum inhibitory concentration (MIC), which is the lowest concentration of an antimicrobial reagent that prevents the microbial growth [8]. Another relevant parameter that can be evaluated is the minimum bactericidal concentration (MBC), the lowest concentration of antimicrobial agent need to kill bacteria, obtained after culturing the samples from the broth dilution test, and obtaining a negative microbial growth [9].

Other classic phenotypic methods are the agar dilution test, disk diffusion assays, the E-test or the use of chromogenic agars. In addition, automated instruments such as Dickinson Phoenix, the Siemens Micoscan WalkAway, or the bioMérieux Vitek 2, can standardize the susceptibility test results in a shorter time but all of them are based on the broth microdilution test and, consequently, the changes are produced by bacterial growth involving long processing times. Some added limitations are the requirements of a relatively large number of viable cells, analytical variability and high costs [7]. These methods typically take 1-2 days to get reliable information, involving a non-sufficiently fast diagnostic and limiting the point-of-care of these susceptibility tests [10]. Recently, some genotypic approaches have been developed for the determination of antimicrobial susceptibility by detecting resistant markers obtaining the genetic information by PCR. In this case it is necessary to know the mechanisms of resistance and is not possible to detect new variants of their antibiotics resistant mechanisms. Moreover, these methods are expensive and have not been adopted yet for resistance detection assays [11]. Hence, novel technologies for determination of antimicrobial drugs like antibiotics are in high demand.

A promising option is to monitor the response of bacterial cells to different treatments that affect their metabolic activity, the cell viability or structural changes. In this sense, microbial biosensors, consisting of a transducer with immobilized microbial cells are promising tools as they use the cellular response for the detection of biologically active agents [12]. A few works for detection of antibiotics using microbial-based biosensors have been reported. For example, a potentiometric microbial biosensor to detect the presence of β-lactams which inhibits the microbial growth [13], or an optical whole-cell biosensor using an engineered *P. putida* to detect structurally diverse antibiotics [14]. Despite the fact that different kinds of transducers may be employed to perform detection assays, limited numbers of works report the study of susceptibility of bacteria to antimicrobial compounds [15].

Among the electrochemical transducers, impedance based-sensors are really advantageous considering their ability to perform label-free detection [16], low cost production, easy miniaturization and integration with other technologies like microfluidics [17]. In the case of electrochemical impedance spectroscopy (EIS) the measurements may be performed in faradaic and non-faradic modes. In the first case a redox couple in the electrolyte solution is required to transfer a charge across an interface, while in non-faradaic measurements no additional reagent is necessary, and impedance mainly depends on the electrode interfacial capacitance [18-20].

To perform impedance measurements different electrode formats may be selected. It has been demonstrated that interdigitated electrode array (IDEA), consisting of a pair of comb-like metal electrodes deposited on an insulating substrate, are advantageous in terms of rapid detection kinetics, increased signal-to-noise ratio, fast establishment of steady-state and no-need of reference electrode [21]. The impedance of the IDEA sensor in a water solution is measured between a pair of electrodes and mainly depends on the solution conductivity and interfacial capacitance of the electrodes [22]. In a low conducting solution, surface conductivity in the spacing between electrode digits may play a significant role [20, 23]. To enhance the sensitivity of standard planar IDEA devices a new design based on three-dimensional interdigitated electrode array (3D-IDEA) sensor, in which an insulating barrier is introduced between the adjacent electrodes, is proposed [23]. Recently, application of 3D-IDEA biosensors for label-free detection of bacteria, have been reported [24, 25]. In these cases, the 3D-IDEA sensor surface was functionalized with different biorecognition molecules to selectively detect bacteria or bacterial contaminants

Accordingly, taking into account the high sensitivity of these 3D-IDEA sensors in comparison with conventional planar IDEA [23], we assume that these devices with immobilized bacteria may also be employed for monitoring the bacterial response to different antimicrobial compounds. Among different antibiotics it is well-known that ampicillin (Amp), of β-lactam group, inhibits bacteria cell wall synthesis, induce cellular stress and culminate in cell lysis [26, 27]. Thus, the bacterial membrane integrity is affected inducing a release of cytoplasmatic compounds.

In this work a new biosensing platform is developed using three-dimensional interdigitated electrode arrays (3D-IDEA) as impedimetric transducers and *E. coli* bacterial cells as the recognition element against ampicillin. One of the main challenges is the development of a reproducible method of bacteria immobilization on the transducer unit as the reproducibility of bacterial attachment is an important limitation in the performance of microbial-based biosensors. To achieve this, specifically synthesized poly(N-isopropylmethacrylamide) (p(NIPMAM)) microgels (μGel) have been used.

Microgels have found considerable interest due their ability to encapsulate, protect, and release bioactive components. Polymer microgels are small particles (typically between 100 nm to 1000 μm) whose interior consists of a three-dimensional cross-linked polymeric network that entraps a considerable amount of solvent [28]. One of remarkable features of microgel particles is that their properties respond to external stimuli like temperature, pH or electric field applied [29]. The use of microgels is reported in many fields, including biosensing applications, providing a favorable environment for enzymes and other biomolecules to preserve their activity and functional structure [30], and also in drug delivery systems [31]. In addition, due to the facility to tune their structure, physical and chemical properties microgels are also employed as antifouling agents in biointerfaces [21, 33] preventing cell adhesion.

In this study we present a novel strategy of spatially selective immobilization of *E. coli* bacteria cells in the spaces between the barriers of a 3D-IDEA, here referred to as “trenches”. In this case, one of the main objectives of the work is to prevent the adhesion of bacteria on top of barriers. To achieve this, poly(N-isopropylmethacrylamide) (p(NIPMAM)) microgels were used as antifouling agents to avoid the attachment of *E. coli* on top of barriers. A highly positively charged polycation, polyethyleneimine (PEI), typically used for the formation of thin multilayer coatings was employed as an initial anchoring layer. Finally, the changes produced in the medium in the presence of ampicillin and *E. coli* bacteria were monitored in a micro-incubation chamber with a small volume specifically designed to perform the impedimetric measurements in a simple and easy-to-use format (Figure 1).

The present study displays a promising biosensing approach that can be employed for determination of antimicrobial drugs, and can also be an attractive method for detection of other toxic contaminants that can affect the bacterial integrity.

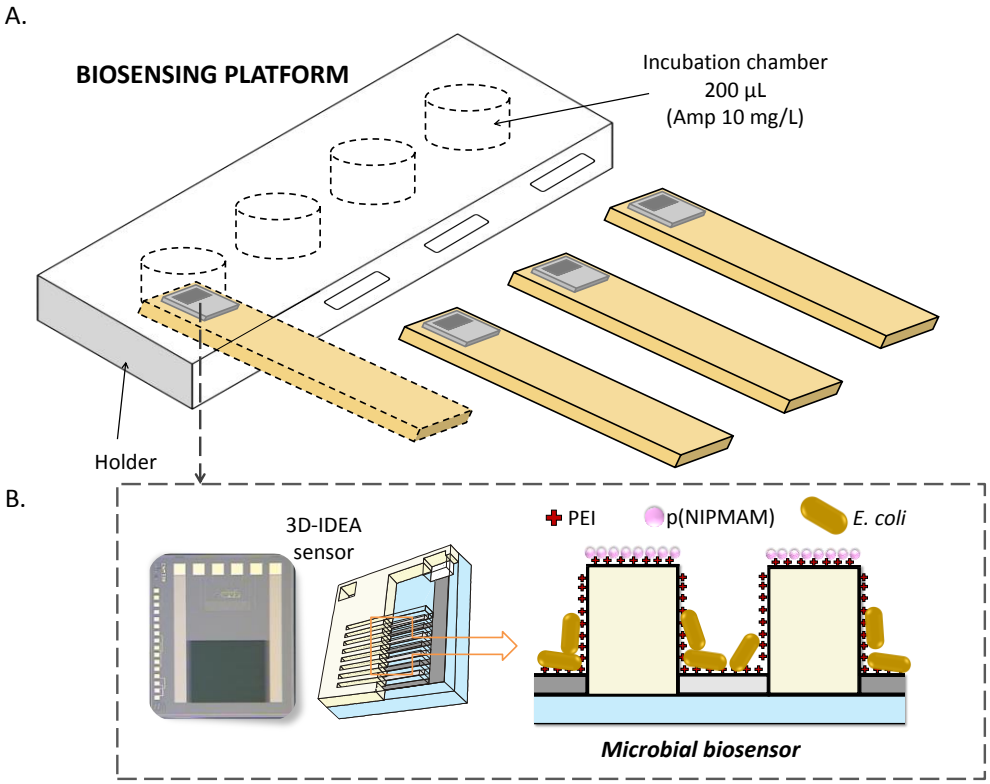


Figure 1. (A) Biosensing platform with the integrated (B) microbial biosensors to monitor the bacterial response to ampicillin.

2. Materials and methods

2.1. Reagents and materials

Polyethyleneimine (PEI, branched, average Mw 25000, water-free) polycation was dissolved in deionized water at 1.5 mg/mL in accord with previously published works [34]. N-Isopropylmethacrylamide (97%, NIPMAM), the cross-linker *N*,*N*´ methylenebis(acrylamide) (99%, BIS), the surfactant sodium dodecyl sulfate (SDS) and the initiator ammonium persulfate (98% APS) were purchased from Sigma-Aldrich. The dye methacryloxyethyl thiocarbamoyl rhodamine B (MRB) purchased from Polysciences, Inc. *N*-Isopropylmethacrylamide was recrystallized from hexane. Polydimethylsiloxane (PDMS) elastomer substrate was obtained by mixing 18 g of the prepolymer (Sylgard 184A) and cross-linker (Sylgard 184B, Dow Corning) vigorously stirred with a spatula at a 10:1 ratio. The viscous mixture was deposited on a 12 x 12 cm clean squared Petri dish under vacuum to eliminate the bubbles, and cured at 70 °C in an oven for overnight. Ampicillin sodium salt was dissolved in water at 10 mg/mL to obtain a stock solution that was diluted till the desired concentration.

All the solutions were prepared with deionized MilliQ water (18 MOhm · cm) which was also used for the cleaning and rinsing processes. Except for the *N*-Isopropylmethacrylamidell, all the chemicals were of analytical grade and were used as received without any further purification.

2.2. Fabrication of 3D-IDEA sensors

3D-IDEA sensors were fabricated using conventional microelectronic techniques. The interdigitated electrode array was formed on a silicon wafer covered with a 2.5 µm thick thermal silicon oxide layer. As an electrode material a highly conductive tantalum silicide ($TaSi_2$) was deposited using magnetron sputtering. This layer was patterned using conventional lithography giving as a result interdigitated electrodes with 216 digits of 3 µm width and 3 µm gap between the adjacent electrodes. The aperture between the electrodes is 1.4 mm and the total length between the electrodes is 301 mm. The wafer was covered by a 4 µm thick low pressure chemical vapor deposition (LPCVD) silicon dioxide in which electrode digits and contact pads of the transducers were opened by deep reactive ion etching (DRIE). Thus, 4 µm high insulating barrier made of SiO_2 with nearly vertical walls separating the electrodes digits were formed.

More detailed information and complete technology of sensor fabrication is presented in previously published works [22, 23].

2.3. Synthesis of p(NIPMAM) microgels

In a three-necked 250 mL flask equipped with a flat anchor-shaped mechanical stirrer, a reflux condenser and a nitrogen in- and outlet dissolving in 145 mL of water 1.812 mg (14.25 mmol, 95mol%) of NIPMAM, 116 mg (0.75 mmol, 5 mol%) of BIS, 5 mg (0.01 mmol, 0.05%) of MRB and 69 mg (1.6 mM) of SDS the reaction mixture was degassed with N_2 over 1 h. The solution was heated to 70 °C and the reaction was started by injecting the degassed initiator solution of 34 mg APS in 5 mL water to the reaction mixture (0.15 mmol, 1 mM). 10 min later, opalescence appeared and the reaction was continued for further 4 h at 70 °C and 300 rpm under nitrogen atmosphere. The reaction mixture was cooled down to room temperature and stirred overnight. The microgel dispersion was purified by ultracentrifugation (3 times at 40.000 rpm) and followed by re-dispersion of the sediment in water. The p(NIPMAM) microgel, was freeze-dried after purification for further use. The dried product is dissolved in water at 0.5 wt %.

2.4. Preparation of the biosensing platform

2.4.1.3D-IDEA modification with PEI

First of all, 3D-IDEA sensors were cleaned with isopropanol for 10 minutes, rinsed with distilled water and dried under nitrogen flow. To perform the deposition of PEI polycation coating on the surface the sensors were immersed into the PEI solution for 20 minutes to form a homogeneous monolayer. Afterwards the sensors were rinsed again with distilled water and dried.

2.4.2. Selective p(NIPMAM) microgels immobilization on the sensor barriers by microcontact imprinting

To attach the p(NIPMAM) microgels on top of barriers a two-step procedure was implemented. First, the microgels were immobilized on a flat PDMS to be used as a stamp and then transferred from PDMS surface to the barriers upper surface by microcontact printing.

To immobilize the p(NIPMAM) microgels small pieces of PDMS were initially cut with the same dimensions as 3D-IDEA chips, 3 x 3 mm. The PDMS slices were treated in an oxygen plasma system for 10 minutes at 100 mTorr and 0.4 L/min gas flow using a Diener electronic Femto plasma system (Diener Electronic GmbH, Germany) to turn the surface to hydrophilic. Immediately after microgels were sprayed 4-5 times on the treated PDMS pieces and were let drying at room temperature. Afterwards, to remove the excess and to obtain a monolayer of microgels on the surface the slices were washed 3 times during 24 hours by immersing the pieces in MilliQ water.

Once the PDMS stamp with the microgels was prepared, the microcontact printing was carried out on the 3D-IDEA sensors surface coated with PEI to transfer the microgels on top of barriers. To ensure the immobilization process the microcontact was performed overnight or during 24 hours.

2.4.2 Bacteria cultivation and immobilization

Standard bacteria of the Clinical and Laboratory Standard Institute (CLSI), *Escherichia coli* ATCC 25922, was employed as model bacteria to study the response to ampicillin antibiotic. *E. coli* was grown overnight at 37 ^{o}C in Luria-Bertani (LB) growth medium before each assay. Optical density at 600 nm (OD600) was measured and adjusted to around 0.2 ± 0.01 in LB, corresponding to bacterial concentration of 10^8 CFU/mL (CFU – colony forming units). Bacteria cells were harvested by 10 minutes centrifugation at 5000 G and washed three times in sterile KCl 10^{-5} M solution. Different concentrations from 10^5 to 10^7 CFU/mL in KCl 10^{-5} M were prepared by serial dilution. In the case of controls experiments with ampicillin-resistant *E.coli* (*E. coli* ATCC 25922 GFP) the same growth procedure was employed. Additionally, after each test the exact bacterial concentration was determined by colony counting in LB agar plates after incubation at 37 ^{o}C for 24 hours.

To immobilize bacteria within the 3D-IDEA trenches of sensors modified with PEI coating and microgels on top of barriers the following procedure was applied. A drop of 10 µL of KCl 10^{-5} M solution containing *E. coli* cells at $5 \cdot 10^7$ CFU/mL was pipetted into the interdigitated area and maintained for 20 minutes at 37 ^{o}C. A final washing step was required to remove the cells that are not strongly bound, as well as the ones remaining on top of the barriers. The whole process is schematically represented in Figure 2.

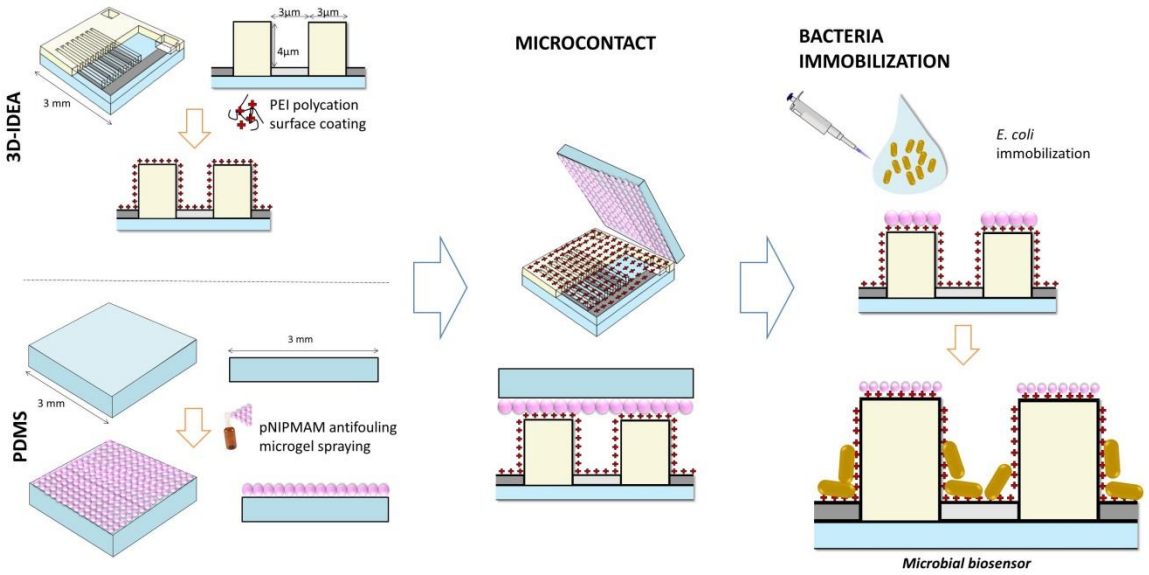


Figure 2. Scheme of the biofunctionalization process of 3D-IDEA impedimetric transducers with *E. coli* bacteria.

2.5. Impedance measurements

QuadTech 7600 Plus, a highly precision LCR Meter analyzer, and a VeraSTAT 3F potentiostat/galvanostat (Princeton Applied Research, USA) were employed for all impedance measurements in a 10^2 - 10^6 Hz frequency range with 100 mV (amplitude) voltage excitation. The measurements were performed in a non-faradaic mode and no DC voltage bias was applied during the impedance measurements. Impedance data treatment and equivalent circuit fitting was performed using the Z-Plot/Z-View software package (Scribner Associates, Southern Pines, NC, USA).

All experiments were done at least in triplicate using three sensors under the same conditions. Impedance measurements during the optimization and functionalization process were carried out in 10^{-5} M KCl solution with a controlled conductivity of 3 µS/cm. In the case of the microbial changes produced by antibiotics, impedance was registered in an incubation chamber containing 200 µL of ampicillin solution at 10 µg/mL with a conductivity of 3 µS/cm. The conductivity of the solutions employed in test experiments was controlled before and after the impedance measurements with a commercial conductimeter (EC-Meter GLP +, Crison). All the impedance measurements were carried out at controlled room temperature.

2.6. Microscopy imaging for characterization

Confocal laser scanning microscopy (CLSM) measurements were performed to verify the adhesion of microgels on top of barriers and the distribution of bacteria on the 3D-IDEA surface using a LEICA TCS SP2 CLSM equipped with 40X NA 0.80 and 63X NA 0.90 water immersion objectives. To be observed using CLSM the microgels were labelled with MRB fluorescent dye and bacteria were stained using a Live/Dead

Invitrogen Kit Bac Light (Invitrogen, Thermo Fisher Scientific) following the protocol detailed by the supplier.

Scanning electron microscope (SEM) experiments were employed to accurately determine the bacterial cell distribution on the IDEA sensor. After the impedance assay, the 3D-IDEA sensors were removed from the holder and bacteria were fixed with 3% (v/v) glutaraldehyde solution in water for 16 hours and maintained at 4 ^{o}C. Then, the 3D-IDEA samples were rinsed in H_2O, dehydrated in graded ethanol (ranging from 50% to absolute ethanol) and air-dried using hexamethyldisilazane (HMDS), following the methodology adapted from Murtey *et al.* [35]. Prior to SEM examination samples were coated with 20 nm gold layer with a Bio-Rad E500 sputter coater. Finally, the surface was examined using an Auriga-40 (Zeiss, Germany) SEM. PDMS stamps with microgels were dried at room temperature, coated with gold and also evaluated with SEM.

3. Results and discussion

3.1. 3D-IDEA biosensing characteristics

The sensitivity of 3D-IDEA sensors to monitor the surface changes produced by different chemical or biochemical reactions in different conditions was widely studied in previous works [23, 25, 36]. Electrochemical spectroscopy impedance is a powerful tool to investigate surface phenomena and changes in material bulk properties [37]. In the case of interdigitated-based sensors the impedance in high electrolyte concentrations in absence of a faradaic process mainly depends on the bulk solution conductivity. However, in low conductivity solutions the surface conductivity starts to play an essential role due to the presence of surface charges [23]. In the case of 3D-IDEA sensors this effect is more pronounced because the major part of the electrical current between the electrodes goes close to the surface of the barriers and not through the surrounding bulk solution. This permits to improve the sensitivity of these sensors for evaluation of processes occurring on the electrode surface. Here, 3D-IDEA sensors were employed to monitor the surface functionalization steps, the bacterial immobilization and, finally, the susceptibility of bacteria to antimicrobial reagents once integrated in the biosensing platform.

The equivalent circuit employed to study the impedance response is presented in Figure 3A, and is formed by the following elements: 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 [38] representing the capacitance of the electrical double layer at the electrode-water solution interface, attributed to the non-ideal behavior of the double layer capacitance. As previously reported [23], R_S is a parallel combination of the bulk solution resistance (R_{BULK}) and the surface resistance (R_{SURF}), but it is important to note that under employed experimental conditions is not possible to distinguish these two elements in the impedance spectra. However, if the bulk solution conductivity remains fixed, we may attribute the

changes in R_S to alterations in the surface resistance produced by superficial reactions and modifications [38]. In consequence, the lower is the bulk solution conductivity the higher is the sensitivity to surface conductivity changes [22].

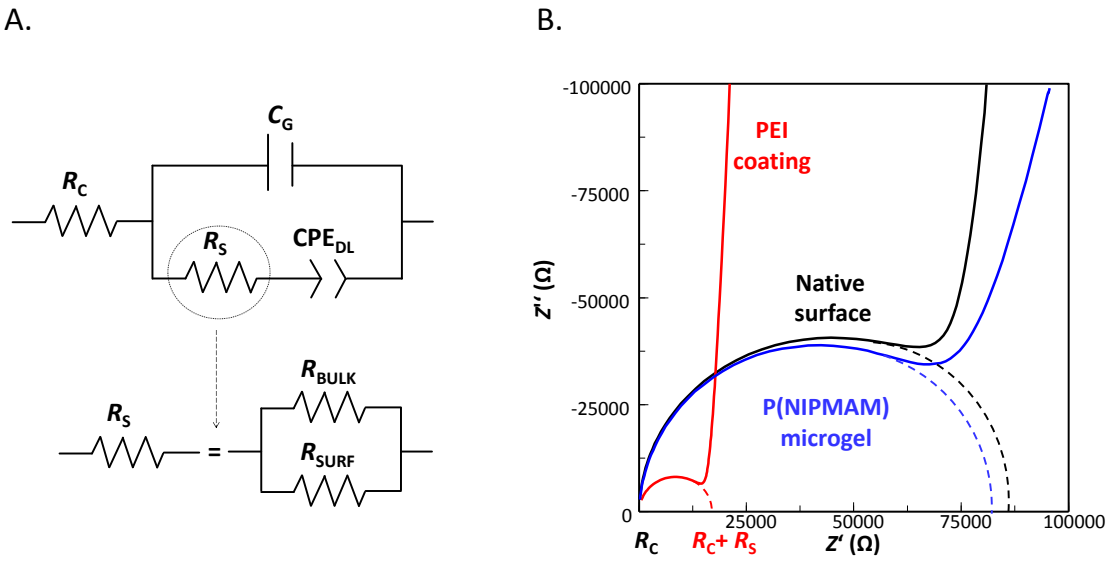


Figure 3. (A) Electrical equivalent circuit used for impedance spectra fitting. (B) The Nyquist plot of the 3D-IDEA measured in KCl 10^{-5} M solution with bare electrodes (SiO_2 native surface), after PEI deposition and with p(NIPMAM) microgel on top of barriers.

The experimental impedance spectra presented in the Nyquist plot (Z' vs Z'') (Figure 3B) allow to observe the formation of a semicircle at high frequencies corresponding to the resistance R_S in parallel with the geometrical capacitance C_G. The intercept with the Z' axis on the left side gives the R_C values, while the intercept on the right side gives the value of R_C + R_S, exemplified in Figure 3B with PEI-modified electrodes, where R_S is the parallel combination of R_{BULK} and R_{SURF}. The linear response at low frequencies in the Nyquist plot is produced by a CPE of the interfacial capacitance.

3.2. Immobilization and stability of the microgels

In order to immobilize the p(NIPMAM) antifouling microgels on top of the barriers, the 3D-IDEA surface was first modified with highly positively charged PEI acting as the anchoring layer. As observed on the Nyquist plot of Figure 3, the absorption of PEI on the surface produced a decrease in the resistance R_S. Previously it was shown that adsorption of branched PEI on the sensor surface of SiO_2 is very fast, homogeneous and nearly irreversible [23, 39]. Therefore, in this work PEI was used to immobilize the microgels on top of barriers and *E. coli* bacteria within the trenches.

P(NIPMAM) microgels synthetized via precipitation polymerization employed in this study are negatively charged in aqueous solution at neutral pH according to the measured zeta potential (ζ=-19 ± 3 mV) due to the use of negatively charged initiator for the polymerization. This gives the possibility to strongly adhere them on a positively charged PEI coating layer of the 3D-IDEA. Accordingly, interaction of p(NIPMAM) microgels with PEI by means of microcontact printing using PDMS stamps (Supplementary information,

Fig. S1) permitted to immobilize microgels on top of the 3D-IDEA barriers. As follows from the impedance spectra observed in the Nyquist plot of Figure 3, after p(NIPMAM) deposition by microcontact printing a significant increase in R_S was observed. The changes observed in the impedance spectra demonstrate that the interaction of PEI with the p(NIPMAM) microgels is taking place. We suggest that the positive charge introduced by PEI was partially compensated by negatively charged microgels producing the increase in the surface resistance. It has to be considered that the surface modification not only provokes changes in R_S but also slightly alters the interfacial capacitance (CPE) of the surface of the sensor and the electrolyte solution because of the formation of an additional layer over the barriers.

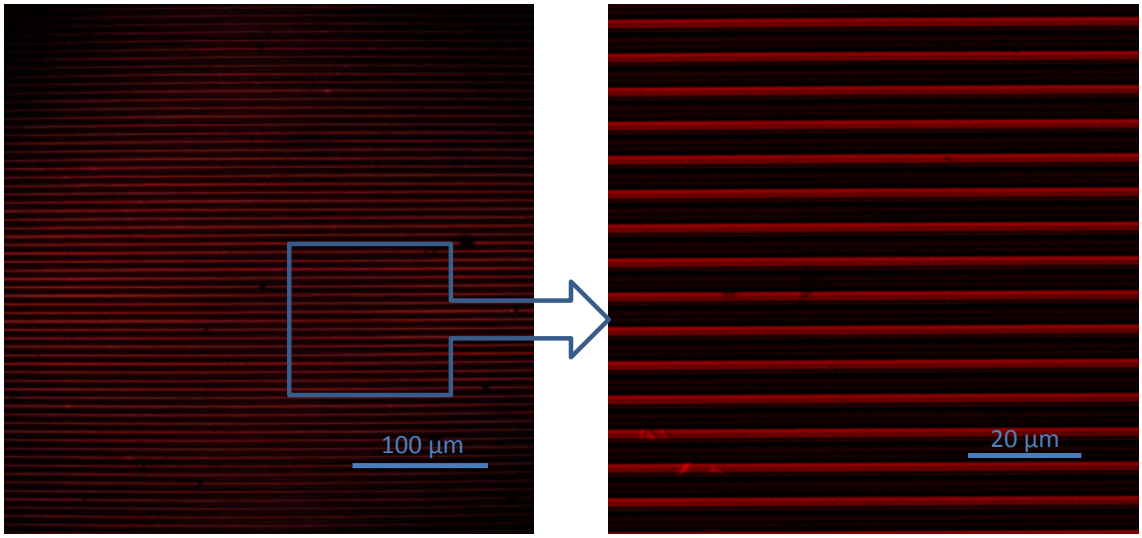


Figure 4. Confocal microscopy images overlay of the 3D-IDEA surface after the functionalization with rhodamine-labeled (red) p(NIPMAM) microgels on top of barriers.

In order to confirm the correct immobilization of microgels on the surface of 3D-IDEA and, consequently, to guarantee that the microgels are localized only on top of barriers, confocal microscopy images of the surface were obtained. In Figure 4 images correspond to the surface of a 3D-IDEA sensor immersed in the KCl 10^{-5} M solution after the microcontact printing process. The confocal imaging was carried out in KCl solution, the same as employed to perform the impedance measurements, in order not to alter the conditions of the microgels. As previously mentioned, the structure of microgels may be affected by environmental conditions, therefore, to maintain the hydrated swollen morphology p(NIPMAM) microgels remained in KCl solution [40]. Thus, to reduce the variations produced by the surrounding medium all the experiments were performed in KCl 10^{-5} M solution.

In Figure 4 an overlay of the sections imaged at different depths, from top to bottom of the electrodes, is presented. It can be clearly observed that microgels labelled with MRB (red color, corresponding to the emission spectra of rhodamine B) are completely aligned along the barriers patterns, demonstrating that immobilization on top of barriers by microcontact printing was successful. Additionally, the images illustrate that at the bottom of the electrode no fluorescence was detected, confirming the spatially

specific deposition of p(NIPMAM) microgels on the sensor surface. In the case of control electrodes modified only with PEI, without performing the microcontact process, no fluorescence was observed as expected (images not shown).

After the functionalization and analysis of the surface using CLSM the stability of microgels was also evaluated by studying the impedance response of modified electrodes over time. In this case, different electrodes functionalized with PEI + p(NIPMAM) microgels and only with PEI were maintained in KCl 10^{-5} M solution for two days to probe the robustness of the proposed methodology and guarantee that the microgels remain on the barriers. These results are shown in Figure 5, where changes in the determined resistance R_S are monitored for two days after the corresponding functionalization of the electrodes.

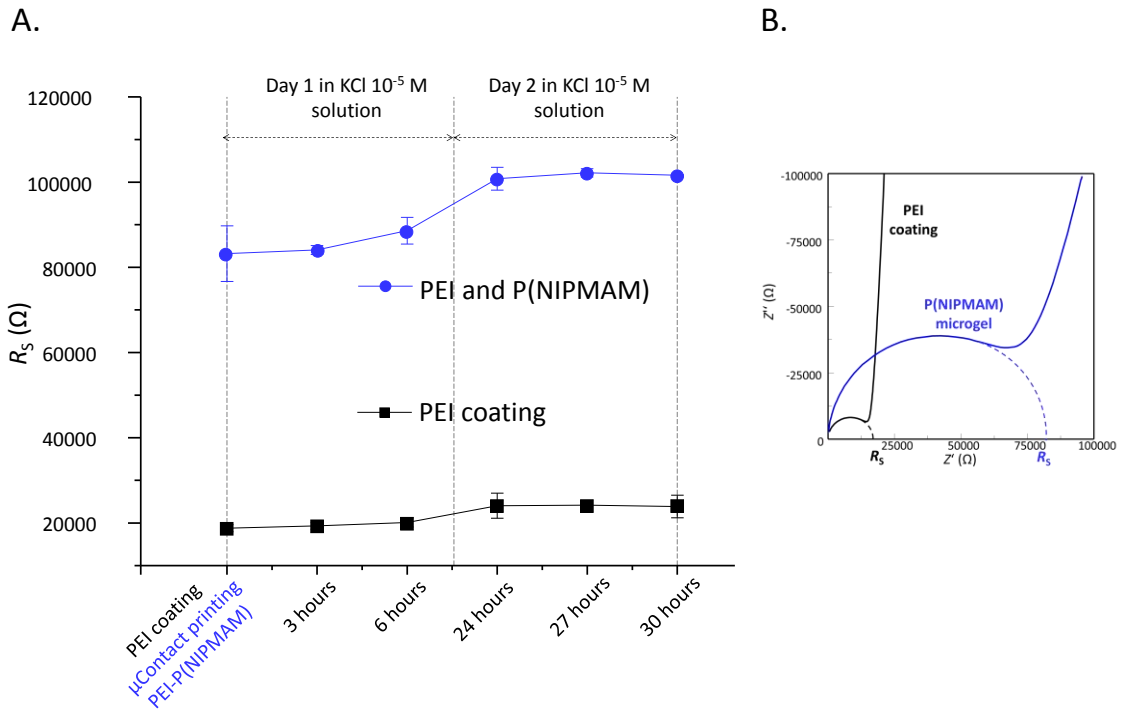


Figure 5. (A) R_S resistance variations in KCl 10^{-5} M solution for 3D-IDEA sensors modified with PEI (black squares) and PEI-p(NIPMAM) microgels (blue circles) in time, and (B) the Nyquist plot corresponding to the impedance spectra after each modification.

Here considerable differences between two types of modification can be observed. In the case of PEI modified electrodes the impedance response remained stable in time when the sensors were immersed in the KCl solution, confirming that no changes on the surface occur. For the sensors after the microcontact printing process presented higher initial values of R_S, corresponding to the reaction between PEI and p(NIPMAM) microgels on top of the barriers, but in this case a progressive increase was observed during the first 24 hours. We suggest that during the incubation in KCl solution, the microgels are reaching the optimal hydration, affecting the superficial resistance. Nevertheless, obtained results also confirm that after this period in the KCl solution the response remains completely stable.

Therefore, it was decided that before bacteria immobilization on the 3D-IDEA functionalized with PEI and microgels the sensors should remain at least 24 hours in KCl solution to achieve stable microgel properties on the surface, as well as to obtain a stable impedance response.

3.3. Site-specific bacterial immobilization

In order to establish the protocol of bacteria immobilization within the trenches of 3D-IDEA functionalized with p(NIPMAM) antifouling microgels on top of the barriers different conditions were tested. We studied the effect of bacterial concentration and incubation time required to obtain high density of bacteria within the trenches with high reproducibility and, at the same time, to diminish the number of bacteria on top of the barriers due to non-specific adsorption.

For this reason a drop of 10 µL of KCl solution containing *E. coli* concentrations from 10^5 to 10^7 CFU/mL was pipetted onto the sensor surface and incubated for either 20 or 60 minutes. At low bacteria concentrations (10^5 and 10^6 CFU/mL) the bacteria cells density on the surface was too low and, therefore, these concentrations were omitted considering that a reduced quantity of bacteria in the trenches would not produce significant response to ampicillin.

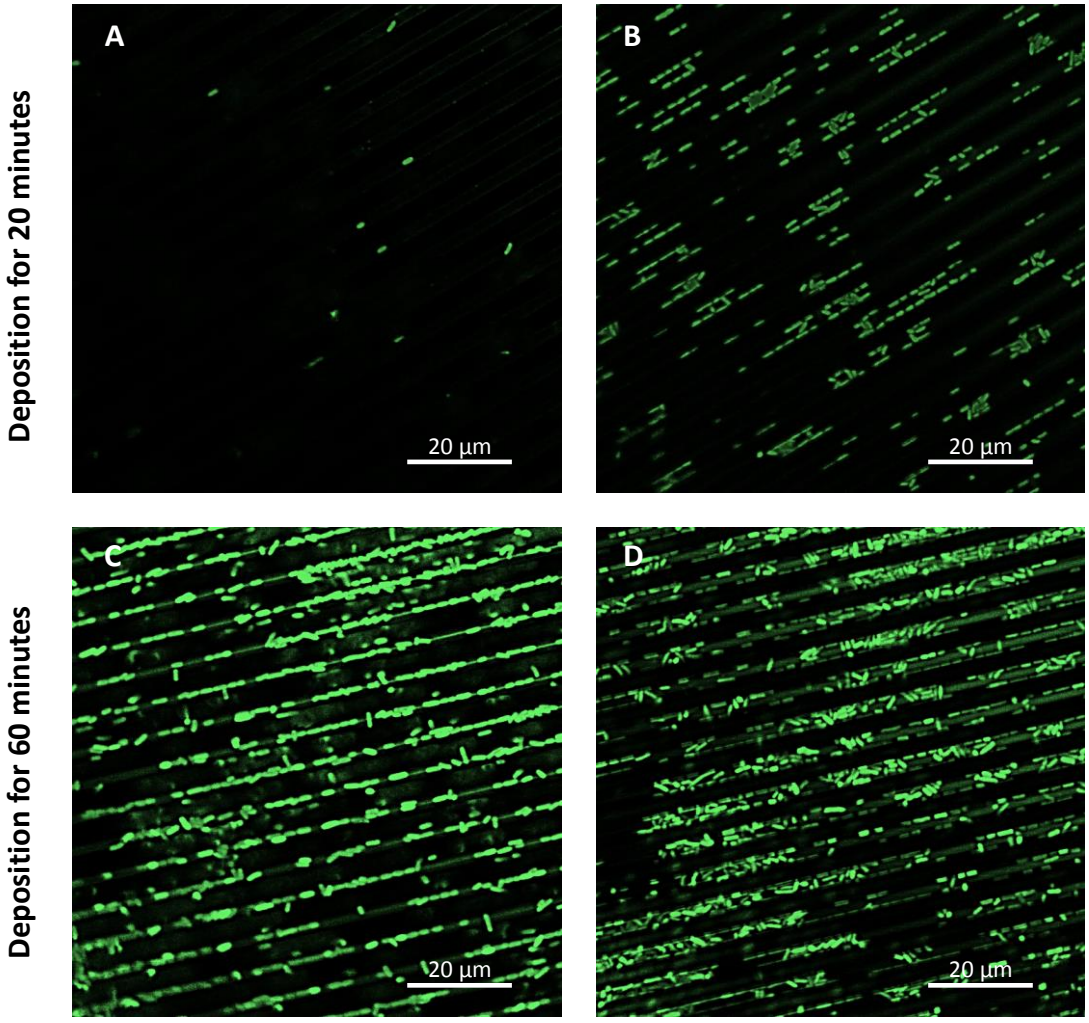


Figure 6. Confocal images of bacteria of the 3D-IDEA sensor surface corresponding to top of barriers (A, C) and the trenches (B, D) after the immobilization during 20 and 60 minutes of *E. coli* ATCC 25922 bacteria stained with Live/Dead reagent.

CLMS images of the electrode surfaces were acquired to compare the bacteria distribution of *E.coli* immobilized on the 3D-IDEA surface using concentration of 10^7 CFU/mL depending on the incubation time. Figure 6 shows the Live/Dead images corresponding to bacteria distribution after 20 and 60 minutes of incubation. The images demonstrate that bacteria were alive (green color) after the immobilization on the surface, while no dead bacteria were appreciated (red color). In both cases a final washing step was applied to remove loosely attached cells from the surface. Here it can be observed that after 20 minutes the majority of bacteria were at the bottom (trenches) of the electrode as desired, and just a few number bacteria remained on top of barriers. This proves the effectiveness of the p(NIPMAM) microgels as antifouling agent to prevent bacterial attachment, which was also demonstrated recently in more detail [41]. On the contrary, after 60 minutes of incubation a large amount of microorganisms were observed on top of barriers as well as in the trenches. Here, it is worth to note that after 60 minutes the drop of 10 µL of KCl solution containing *E. coli* bacteria was completely evaporated and may facilitate the fixation of bacteria on top of barriers even in the presence of antifouling microgels.

In summary, obtained results give the evidence that the optimal conditions for selective immobilization of *E. coli* within the 3D-IDEA trenches are 20 minutes of incubation using 10^7 CFU/mL bacteria solution. Under these conditions it was possible to establish a reproducible and controlled methodology to perform bacterial immobilization on cell-based biosensors, which is a crucial point in their application for different sample testing [42].

3.4. Integration of biosensors into the biosensing platform and monitoring of bacterial response to ampicillin

To validate the proposed microbial biosensor approach and demonstrate its effectiveness, experiments were conducted with sensors integrated into the biosensing platform, consisting of a specifically designed holder with 200 µL chamber over each individual sensor. Four 3D-IDEA sensors were introduced into the holder chamber to perform impedance measurements in 200 µL of solutions with controlled low conductivity (Figure 1). The chambers were sealed hermetically to prevent the solution evaporation. The use of solutions with reduced volumes at low conductivity allows to monitor easily the changes produced in the solution resistance as a result of metabolic processes or the release of ionic products by bacteria [43]. This integrated biosensing system was used to study the developed *E. coli* bacterial biosensors impedance response to ampicillin as antimicrobial agent.

E. coli ATCC 25922 is a reference strain with well-established minimum inhibitory (MIC) and minimal bactericidal concentration (MBC) values obtained by different antimicrobial susceptibility assays. The MIC values for ampicillin are about 2-8 mg/L and MBC mean values are around 8.8 mg/L [44, 45]. The objective of this work is to study the effect of ampicillin on the cell integrity of *E. coli* immobilized on the sensor surface by means of registering the impedance alterations. Ampicillin concentration of 10 mg/L was selected, that is slightly higher than the MBC values, to guarantee that ampicillin has a bactericidal effect. Additionally, the bactericidal effect of this ampicillin concentration was probed using standard protocols (Supplementary Information, Fig. S2).

In order to evaluate the effectivity of the proposed approach different sensor modifications were compared. 3D-IDEA sensors functionalized with microgels on top of the barriers and *E. coli* within the trenches were incubated in a solution of KCl 10^{-5} M supplemented with ampicillin at 10 mg/L and the variations in the impedance were monitored for 24 hours in the designed low volume biosensing system. It has to be noted that ampicillin at this concentration practically does not affect the conductivity of KCl solution, which is a relevant aspect to consider in the impedance measurements under these conditions.

Control measurements were performed using electrodes functionalized under different conditions. First, the same sensors with the microgels and *E. coli* but without ampicillin were used to test if the impedance response remained stable. Another control using 3D-IDEA with the microgels and without *E. coli* was also monitored in the presence of ampicillin. Finally, to demonstrate the higher sensitivity of our approach, in which the p(NIPMAM) antifouling microgels on top of barriers allow to improve the immobilization of bacteria in the trenches, the same conditions were employed but using 3D-IDEA functionalized exclusively

with PEI and *E. coli*. In this case, bacteria cells were distributed homogeneously on the sensor surface, within the trenches as well as on top of barriers (Supplementary information, Fig. S3). Corresponding results are presented in Figure 7.

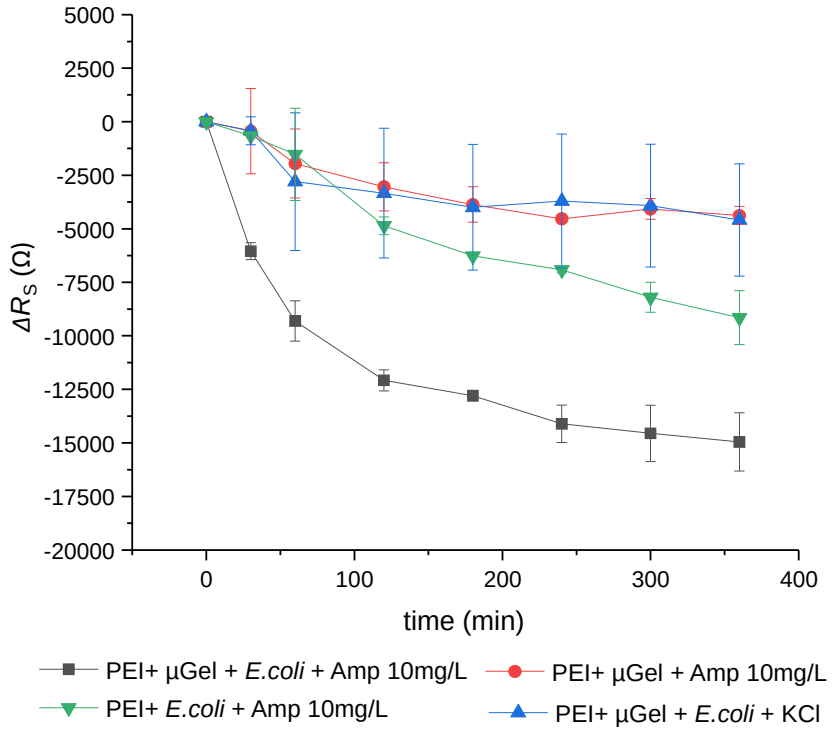


Figure 7. R_S variations in time of 3D-IDEA sensors modified with: PEI-p(NIPMAM) µGel and *E. coli* in KCl solution with Amp at 10 mg/L (black), PEI-p(NIPMAM) µGel with Amp at 10 mg/L (red), PEI-p(NIPMAM) µGel and *E. coli* in KCl solution (blue), and PEI modified electrodes with *E. coli* in KCl solution with Amp at 10 mg/L (green).

The impedance response, ΔR_S, presented as variation of R_S during the reaction process was calculated as:

$$\Delta R_S = R_S^{Amp} - R_S^0 , \quad (1)$$

where R_S^0 corresponds to resistance of sensors before applying ampicillin, and R_S^{Amp} corresponds to resistance after addition of ampicillin.

In the case of 3D-IDEA sensors with microgels and *E. coli* cells in the trenches a large decrease in R_S was observed in just 1-2 hours and the signal remained constant for around 5 hours. We assume that ampicillin was affecting the cell membrane of bacteria producing its disruption and the release of the cytoplasmic content into the surrounding solution. The cytoplasm of bacteria is formed by different dissolved charged macromolecules and ions [46], thus during bacterial lysis these components are washed out into the surrounding KCl solution increasing its conductivity and, consequently, decreasing the solution resistance. Moreover, as bacteria are located within the 3 µm wide trenches the diffusion of electrolyte from the trench to the outer solution is impeded and this possibly enhances the response.

At the same time, no significant changes in the impedance response were observed in control experiments. The functionalized sensors in KCl solution without ampicillin were stable over time, confirming that *E. coli* bacteria on the surface remained intact. The 3D-IDEA control with microgels on top of the barriers without bacteria also remained invariable; demonstrating that ampicillin does not interact with the sensor. In all cases, the response was stable for the duration of the measurements, 5-6 hours.

In addition, Figure 7 shows that sensors without microgels and only coated with PEI with immobilized *E. coli* cells also presented a decrease in the R_S values. This demonstrates that ampicillin is affecting the integrity of bacteria in a similar way as explained above. However, the sensitivity of this type of sensor is considerably lower. This ratifies the importance to concentrate bacteria within the trenches of 3D-iDEA impedance-based sensors in order to obtain better reproducibility and higher sensitivity, and it accentuates the role of antifouling microgels on top of the barriers to achieve it. The proposed approach allows detecting the microbial susceptibility to ampicillin in a short detection time as compared to other tests based on culturing methods that require between 18 and 48 h to provide results [8, 47].

In parallel, to validate that changes in impedance were caused by the cell damage provoked by ampicillin, an identical study with an ampicillin-resistant *E. coli* strain was carried out. The susceptibility of the two strains to ampicillin was previously tested showing sensitivity in *E. coli* ATCC 25922 and resistance in *E. coli* ATCC 25922GLP for the tested antibiotic (see Fig. S2).

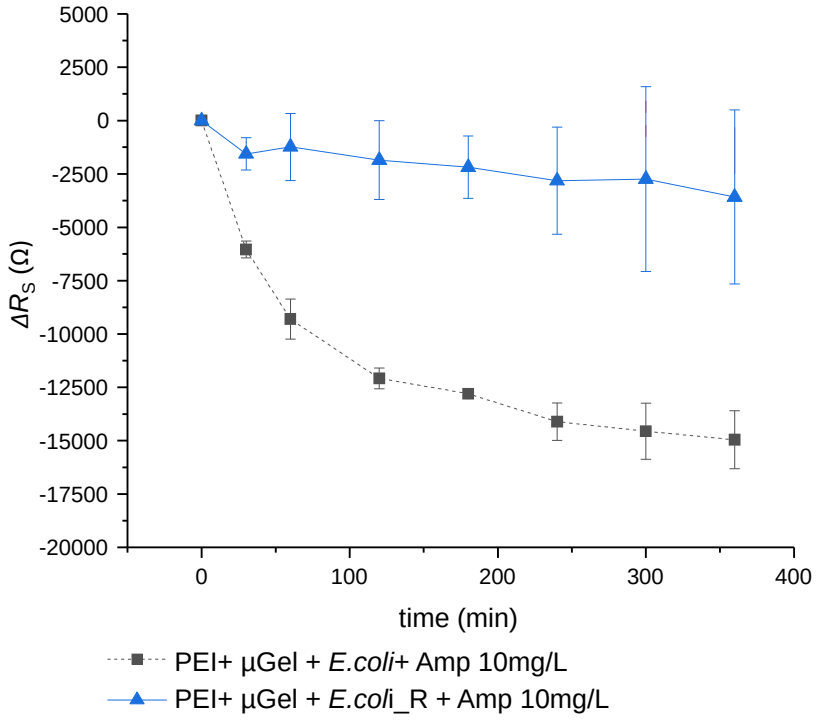


Figure 8. R_S variations in time measured in KCl solution with Amp at 10mg/L of 3D-IDEA sensors modified with: PEI-p(NIPMAM) µGel and *E. coli* ATCC 25922 (non-resistant to Amp) in black, and *E. coli* ATCC 25922GLP (resistant to Amp) in blue.

Figure 8 shows the impedimetric response of the ampicillin resistant and non-resistant *E. coli* in the presence of ampicillin. In the case of resistant *E. coli* there were no changes in R_S indicating that ampicillin does not affect the viability of bacteria as expected.

According to these results, the proposed novel method to functionalize the barriers surface of 3D-IDEA sensors with p(NIPMAM) microgels, the strategy to selectively immobilize bacteria within the trenches and the subsequent monitoring of impedance changes related with the bactericidal effect of ampicillin to *E. coli* cells have been validated. The developed approach is a versatile, rapid, compact and easy-to-use platform based on the microbial biosensor concept that can be useful in a wide spectrum of toxicity monitoring-related applications.

4. Conclusions

This work presents a microbial biosensor approach focused on the development of a novel and reproducible strategy based on the immobilization of *E. coli* bacteria on a three-dimensional interdigitated electrode array (3D-IDEA) impedimetric transducer. The performed microbial sensor is employed in a biosensing platform especially designed to monitor the bacterial response to ampicillin antibiotic. The surface functionalization, bacteria immobilization and the microbial response to ampicillin were characterized with EIS technique that allows registering variations in superficial resistance provoked on each modification step.

To improve the reproducibility in the immobilization and the sensitivity in the bacterial response *E. coli* cells were selectively immobilized within the 3D-IDEA trenches between the insulating barriers. One of the main novelties is the modification of the top of the barriers with antifouling poly(N-isopropylmethacrylamide) (p(NIPMAM)) microgels in order to prevent the deposition of bacteria. To achieve selective deposition, the microgels were first immobilized on a PDMS substrate, and afterwards transferred to the sensor surface via a microcontact printing procedure. Polyethyleneimine polycation (PEI) was employed as the assembling layer of *E. coli* cells in the trenches as well for the p(NIPMAM) microgels on the barriers. The suitability of the proposed immobilization method was demonstrated by confocal microscopy and scanning electron microscopy imaging. Both methods confirm the presence of microgels on the top of barriers, while *E. coli* bacteria are located within the trenches.

The applicability of the developed microbial biosensor was studied introducing the functionalized devices in the designed small volume biosensing system containing a bactericidal concentration of ampicillin (10 mg/L) in KCl 10^{-5} M solution. The impedance changes in terms of R_S shows a decrease attributed to release of cytoplasmatic components, promoted by the lysis of bacterial cell membrane, and demonstrating the response and sensibility to ampicillin. Accordingly, the same procedure was employed with an *E. coli* strain resistant to ampicillin, and no significant impedance changes were observed.

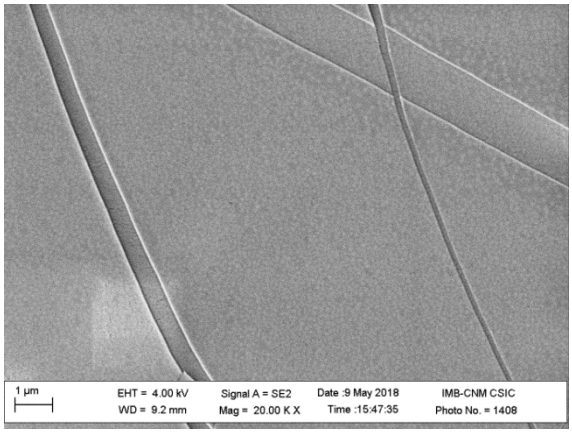
To sum up, the developed microbial biosensing approach may be very interesting in a broad spectrum of applications related with toxicity evaluation. In addition, the same procedure can be applied to study any other type of bacteria and its susceptibility to different antibacterial reagents.

Supplementary information

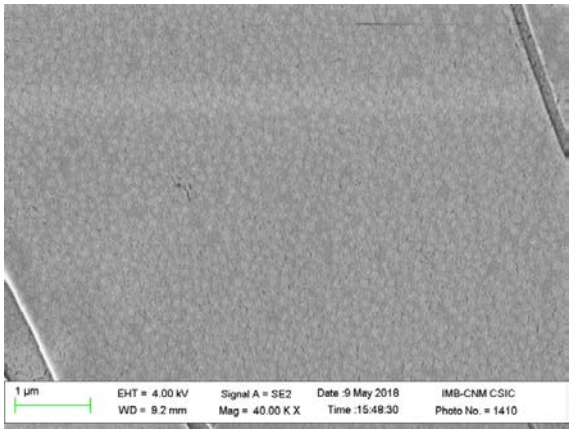
1. SEM images of PDMS stamps with p(NIPMAM) microgels

The diameter of employed p(NIPMAM) microgels is around 200 nm in swollen-state (in aqueous solution), but after the sample treatment for observation in SEM the size is considerable smaller.

Magnification: X 20000



Magnification: X 40000

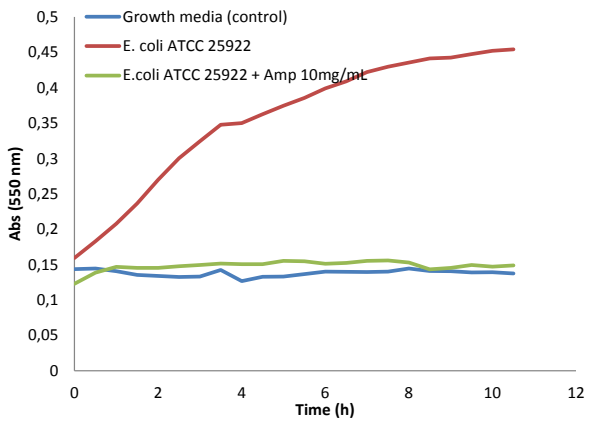


2. Growth curves of *E. coli* strains with ampicillin

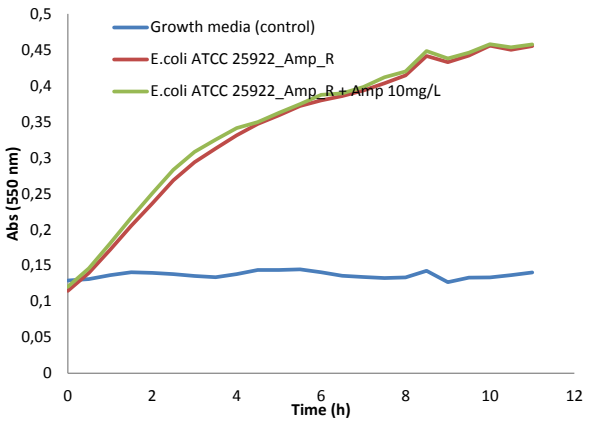
a) Bacterial growth of *E. coli* ATCC 25922 with and without ampicillin

b) Bacterial growth of *E. coli* ATCC 25922_GFP (resistant to ampicillin) with and without ampicillin

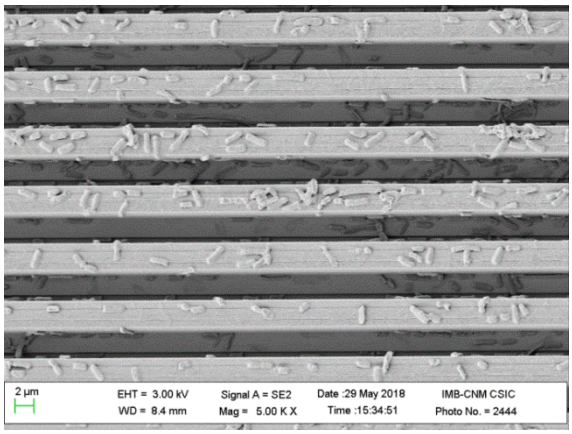
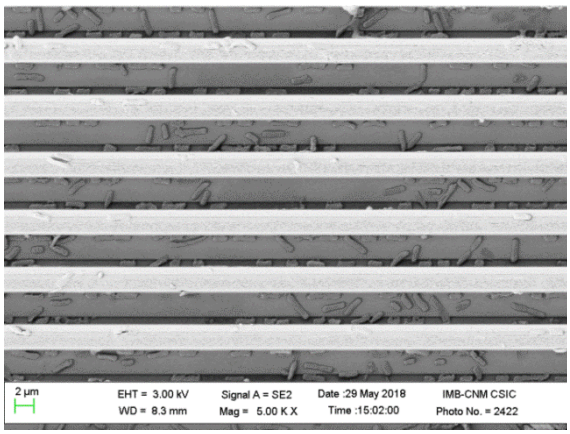
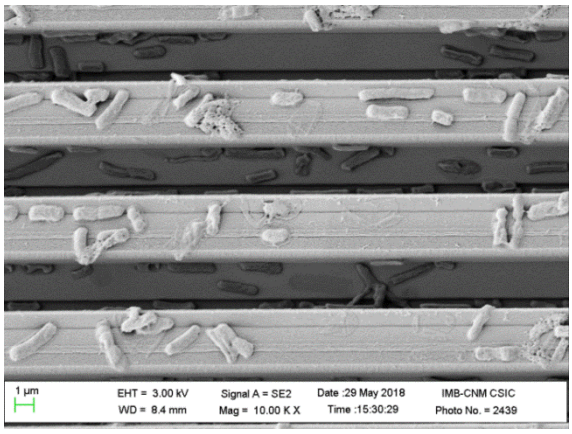
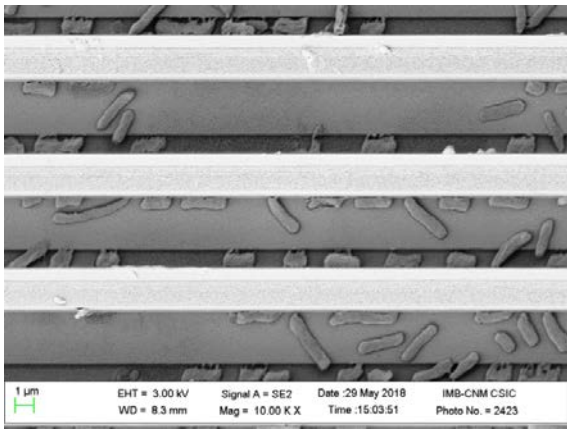
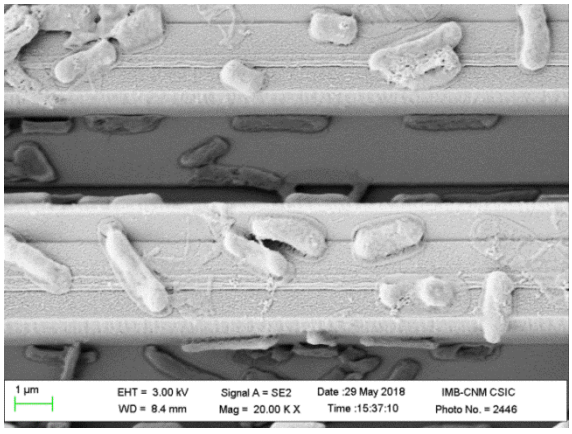
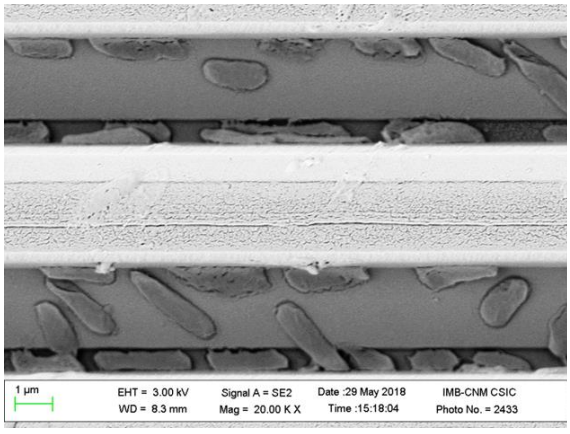
A)



B)



3. SEM images of 3D-IDEA after *E. coli* immobilization

	3D-IDEA with PEI (*E. coli* cells cover the entire surface)	3D-IDEA with PEI and OM33 microgel (*E. coli* cells in the trenches)
Magnification: X 5000		
Magnification: X 10000		
Magnification: X 20000		

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4. GENERAL DISCUSSION

4. GENERAL DISCUSSION

This thesis is focused on different bacterial biosensing applications of impedimetric transducers based on (bio)functionalizated interdigitated electrodes arrays (IDEA). Fast, cheap, sensitive and selective methods for bacteria detection, especially pathogenic bacteria, are highly required in various fields, like food safety or clinical diagnosis. In addition, the possibility to use bacterial as sensing elements may be applied to convert the cell response to analytical signals. Among different transducers that are commonly used for biosensors development, IDEA impedimetric devices are really advantageous in many aspects, such as easy-miniaturization, no need of reference electrode, fast establishment of steady-state, increased signal-to-noise ratio and label-free detection [1- 4]. There are many publications on IDEA-based biosensors; however, one of the main contributions of this work is the use of IDEA sensors in 3D configuration that, due to their specific geometry, offer higher sensitivity to surface parameters variations, mainly related to the surface resistance changes [5, 6].

One of the main problems of biosensors development is the elaboration of a robust and reproducible method of a sensor surface modification. That is why in the present thesis much attention is put on the study of biofunctionalization strategies. Taking into consideration that 3D-IDEA are very sensitive to the surface charge variation, they are used as a powerful tool to control and monitor each surface modification step. Sections 3.1, 3.2 and 3.3 of Methods and Results are focused on the functionalization of IDEA sensors with different polymers and biomolecules to achieve the immobilization and detection of bacteria and bacterial components, while section 3.4 revolves around the selective immobilization of bacteria cells for the development of a microbial biosensor. The described assay, aside from the study of different biosensing approaches, also intends to obtain a better understanding of the superficial and interfacial phenomena occurring on the IDEA and 3D-IDEA sensors during surface modifications, especially in the biosensing process. All the studies have been carried out using *Escherichia coli* as a model microorganism.

In ***Section 3.1,*** the assembling of polyethyleneimine (PEI) and poly(sodium 4-styrenesulfonate) (PSS) polyelectrolyte layers together with concanavalin A (Con A) as a bioreceptor on a planar IDEA sensor surface is used to immobilize and detect bacteria. Obtained results are of great relevance in the global achievement of this thesis but also as a starting point in the further work on the employment of surface modification protocols.

The adsorption of PEI polycation on the IDEA surface using the layer-by-layer (LBL) self-assembling method has been demonstrated to be really suitable for direct immobilization of bacteria, mainly by electrostatic interactions, and also for sensor functionalization with biomolecules like concanavalin A.

The use of PEI was widely employed in many sensor applications, like immobilization of enzymes [5, 7], oligonucleotides [8], or the formation of thin films with carbon nanotubes or nanoparticles [9, 10]. In our case, it has to be noted that the IDEA surface is mainly formed by SiO_2, which is negatively charged at

neutral pH due to the presence of hydroxyl groups. Negative charge of the surface facilitates the adsorption of cationic PEI on the sensor surface [11] and its use is really advantageous permitting to achieve a homogeneous layer of high stability on the device.

Under the experimental conditions tested the native surface of IDEA does not show the attachment of bacteria at different incubation times employed. However, the high stability of PEI on the employed impedance-based sensors permitted this positively charged surface to interact with negatively charged components of bacterial membrane, allowing reproducible immobilization of bacteria on the impedimetric transducer.

The deposition of PEI and, especially, PEI-PSS-PEI multilayers, results in a highly effective method to attach bacteria homogenously distributed on the surface, allowing to detect concentrations as low as 10^1 CFU/mL in about 20 minutes in water samples. Therefore, one of the main contributions of section 3.1 is the development of a really fast and robust methodology for the immobilization and detection of *E. coli* bacterium using planar IDEA sensors and PEI as the assembling layer.

However, in a strict sense, it cannot be considered that the modifications with only polyelectrolytes encompass the development of a biosensor approach due to the lack of a specificity and selectivity against bacteria of our system. To overcome this limitation, concanavalin A (Con A) lectin has been employed as a biorecognition element due to its ability to bind selectively the carbohydrates of LPS present on bacteria outer membrane. Con A immobilization have also been performed using the layer-by-layer method on PEI-coated electrodes but, in that case, the detection limits obtained are around 10^4 CFU/mL.

A controversial aspect in the utilization of PEI for the immobilization of bacterial cells is that in many works it is regarded as a permeabilizing reagent. This is because it can interact with anionic phosphates and carboxylic groups of LPS of bacterial cell membrane [12-14] compromising its viability. However, in other works the treatment of glass surfaces with PEI [15] or the functionalization of different types of fibers to immobilize *E. coli* cells for the development of whole-cell biosensors [16] was demonstrated to be really suitable to attach viable cells to a solid surface. Additionally, another important consideration is at which concentration PEI provokes a toxic effect on bacteria. In our case, to modify the IDEA surface a solution of PEI (branched, average Mw 25000) is prepared at 1.5 mg/mL in distilled water. The excess is removed from the surface by thorough rinsing with water. Therefore, we may assume that the concentration of PEI on the sensor surface is quite low, without affecting the attached bacteria viability. Finally, Live/Dead viability tests and bacterial growth experiments performed on IDEA sensors modified with PEI and immobilized *E. coli* have demonstrated that bacteria remain alive.

Section 3.2 explores the detection of bacterial endotoxins (lipopolysaccharides, LPS) with Con A as a biorecognition element immobilized on a 3D-IDEA to register the superficial changes. LPS, present on the external membrane of gram-negative bacteria, are considered as contaminants especially dangerous in clinical products and pharmaceutic industry [17,18].

Following the same principle as in 3.1, the work presented in section 3.2 uses the layer-by-layer methodology with PEI as the first anchoring layer to immobilize Con A molecules. This biosensing device has been used to determine the presence of LPS in water samples. Here, a 3D-IDEA transducer has been employed to monitor the changes in superficial resistance due to its higher sensitivity for the registration of surface charge changes compared with planar IDEA. However, taking in consideration that PEI may react with any negatively charged molecule in a sample solution, like anionic carboxylic groups of LPS [19-21], special attention is paid to the prevention of any unspecific binding between PEI and the analyte to ensure that the interaction is selectively occuring only between Con A and LPS.

To achieve an efficient methodology to prevent the adsorption of LPS on PEI covered surfaces a typical compound in biochemical assays like bovine serum albumin (BSA) [22] was first tested, but its blocking effect was found to be insufficient. Thus, an alternative approach based on the consecutive deposition of Con A-glycogen-Con A layers, in which glycogen polysaccharide allows to bind different Con A layers, have resulted very effective in blocking the PEI surface to guarantee the specific binding of Con A with LPS. 3D-IDEA devices modified with PEI-(Con A-Gly)$_2$-Con A multilayers are shown to be highly reproducible, sensitive and selective to determine bacterial LPS with a significantly low limit of detection (2 µg/mL) in a short detection time of 20 minutes.

Once the effect of PEI in the direct adsorption of LPS is appreciated, it is reasonable to reconsider the results presented in work 3.1. In that case, PEI-Con A functionalized planar IDEA has been employed for the immobilization of *E. coli* cells, but it has to be taken into consideration that the response may be partially produced by the direct interaction of PEI and LPS of the bacteria membranes. In any case, the use of Con A as biorecognition moiety has limitations in terms of selectivity for whole-bacteria detection since it reacts with carbohydrates of different types of bacteria strains. This reduces its applicability in some fields in which the determination of specific bacterial strains is crucial.

In ***Section 3.3***, the development and characterization of a highly selective and reusable 3D-IDEA biosensor with immobilized aptamers as the biorecognition element against a pathogenic *E. coli* strain, *E. coli* O157:H7, is presented. To achieve high selectivity a DNA aptamer has been employed due to its ability to bind membrane proteins only present in a specific bacteria strain.

Though LBL method may also be used for immobilization of DNA molecules [23], to overcome the limitations of selectivity mentioned earlier a different biofunctionalization strategy, consisting in the chemical modification of the surface via silanization, was employed to attach the aptamer to the 3D-IDEA transducer.

As noted previously, an important feature of biosensors for detection of bacteria in many applications is the demonstration of its selectivity [24]. In this work, a highly selective response of the aptasensor to *E. coli* O157:H7 was achieved and no impedance changes were observed in the presence of other Gram-negative bacteria, such as *E. coli* K12 and *Salmonella typhimurium* or Gram-positive like *Staphylococcus aureus*.

Moreover, the practical value of a biosensor for real applications is usually determined by the detection time and the limit of detection [25]. Here, the response of the developed aptasensor is really remarkable, detecting concentrations as low as 10^2 CFU/mL in 30 minutes. The immobilization and detection assays are performed in PBS solution since it is shown that the conformation of aptamer in the buffer solution is stable, but may be slightly affected in low conductivity solutions. Therefore, the impedance measurements are performed in KCl 10^{-5} M minimizing the exposition to ensure that the aptamer structure remains stable. In addition, the practical application of the aptasensor for bacteria detection in real samples has been demonstrated with a pre-concertation method based on the extraction of bacteria from liquid samples. This treatment allows to reduce the limitations attributed to the sample matrix effect [26].

Another relevant aspect of this work is the methodology developed to regenerate the biosensing surface after the interaction with bacteria, consisting in a temperature treatment to release the bacteria immobilized by the aptamer on the sensor surface and recover its binding-ability to employ the biosensor multiple times. Taking advantage of this, apart from their fast response, simplicity, selectivity and sensitivity, the performed biosensor is really promising for low-cost determination of bacteria. Following the same concept, the biosensing system may be modified with different aptamers for the detection of other bacteria of interest.

Finally, once validated the different strategies employing the IDEA impedimetric transducers to perform the detection of *E. coli* bacteria and bacterial endotoxins, the work was focused on the development of a novel microbial bioassay based on the monitoring of the response of bacteria immobilized in 3D-IDEA impedimetric transducers to antimicrobial reagents. These results are presented in ***Section 3.4***.

The particular geometric properties of 3D-IDEA [27] are taken into advantage to immobilize bacteria with spatial selectivity on the sensor surface to test the possibility to monitor and evaluate the effect of antibiotics on *E. coli* cells. In the presence of different toxic pollutants or antimicrobial drugs the bacterial growth may be inhibited producing changes in their metabolism or they even may be killed due to alteration of the cell membrane structure [28, 29]. These effects may provoke changes in the own impedance of bacterial cells as well as in the composition of the surrounding medium solution. These variations produced by the bacteria response are susceptible to detection with impedance-based sensors.

Therefore, the last section is mainly focused on the development, characterization and performance of a microbial biosensor exploiting the high sensitivity of 3D-IDEA to monitor changes in the superficial conductivity. In this case *E. coli* bacterium was employed as the biorecognition element and the previously acquired knowledge helped to successfully perform its immobilization. The proposed strategy is based on the selective deposition of *E. coli* cells in the interspace of 3 μm width and 4 μm high between the barriers of 3D-IDEA, denoted as trenches. We assume that high concentration of bacteria in the trenches may be really advantageous to establish a reproducible immobilization methodology, and also in terms of improving the sensitivity by the registration of impedance attributed to bacterial changes.

In this work, PEI has been selected again as the assembling layer considering its effectivity for the immobilization of *E. coli* bacteria as described earlier. To avoid the attachment of *E. coli* on top of barriers and guarantee the deposition of bacteria in the trenches, poly(N-isopropylmethacrylamide) (p(NIPMAM)) microgels were employed as antifouling agents fixed on top of barriers by microcontact printing. The PEI layer on the top of barriers also was found to be efficient for the anchoring of p(NIPMAM) microgels. Confocal and electron microscopy imaging confirmed the correctness of the chosen strategy – bacteria cells were located within the trenches and not on top of the sensor.

The impedance response of bacteria modified sensor was evaluated in a biosensing platform by exposing it to water samples containing antimicrobial drug ampicillin. This antibiotic, as well as other β-lactams, inhibits the synthesis of some wall components, induces cellular stress that affects the membrane integrity and can culminate in the cell lysis [30, 31]. Exposure of the microbial sensor to ampicillin resulted in the decrease of the sensor resistance, R_S, which can be related to a progressive release of cytoplasmatic compounds, charged molecules and ions from bacteria interior that alter the incubation solution conductivity. In parallel, the correctness of the strategy to use of microgels in order to deposit bacteria selectively within the trenches have been confirmed by using the same protocol of bacteria immobilization but without application of microgels on top of barriers. According to microscopy imaging bacteria cells are spread over the entire surface, over the barriers and within the trenches. In this case, the impedimetric response of bacteria to ampicillin resulted to be lower in comparison to the results obtained with bacteria concentrated in the trenches. This may be attributed to the fact that in this case the concentration of bacteria within the trenches is lower. In addition, the diffusion of electrolyte solution when bacteria are located in the trenches may be limited, enhancing the impedance response.

Thereby, obtained experimental results confirm that the proposed microbial biosensor fabrication approach is a promising way to construct biosensors for antimicrobial susceptibility tests, as well as real-time toxicity monitoring assays.

Summarizing, this PhD thesis implementing IDEA and 3D-IDEA impedimetric transducers tries to contribute to a better understanding of surface chemical/biochemical reactions paying special attention to practical applications. Different aspects are evaluated in this assay; the optimization and performance of novel methods for sensor surface functionalization, the strategies for bacteria immobilization, the study of blocking strategies to prevent unspecific adsorptions or the development of biosensors with biorecognition elements for detection of pathogenic bacteria with high selectivity. Finally, the performance of microbial-based sensors approach has been demonstrated as a valuable tool for label-free biosensing applications, achieving the objectives defined in this thesis. Results of the presented study may also be used as a guide for further biosensor development work using impedance transducers based on IDEA sensors.

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5. CONCLUSIONS

5. CONCLUSIONS

General conclusions:

The main conclusions presented in this PhD thesis are:

A. Interdigitated electrode arrays (IDEA) and three-dimensional interdigitated electrode arrays (3D-IDEA) sensors are shown to be a useful tool for different microbial biosensing applications including the detection of bacteria, the determination of bacterial endotoxins and the performance of microbial-based biosensors.

B. Application of Electrochemical Impedance Spectroscopy (EIS) technique for registration of impedance changes occurring in the solution and at the surface of conventional IDEA and 3D-IDEA sensors produced by redistribution of superficial charges has been proved as a highly sensitive and reproducible method for characterization of individual surface modification steps and the biosystem as a whole.

Specific conclusions:

In accordance with the initial objectives stated the conclusions obtained in this thesis assay are as follows:

1. Superficial modification of standard planar IDEA sensors with charged polyelectrolytes and concanavalin A lectin is demonstrated as a fast, reproducible and sensitive strategy for immobilization and detection of bacteria in water samples.

1.1. The modification of planar IDEA impedimetric transducers using the layer-by-layer technique to assemble polyelectrolyte alternating polyethyleneimine (PEI), poly(sodium 4-styrenesulfonate) (PSS) and concanavalin A (Con A) lectin are really effective to immobilize bacteria on the sensor surface. Changes in the superficial impedance can be effectively registered after each modification step helping to control and optimize the whole immobilization process.

1.2. PEI polycation reacts by electrostatic interaction and chemically with negatively charged compounds of the bacterial membrane, while Con A reacts specifically with the lipopolysaccharides (LPS) of gram-negative bacteria.

1.3. The functionalization of IDEA surface with PEI, PEI-PSS-PEI and PEI-Con A is a robust methodology that allows us to detect *E. coli* bacteria in water-based solutions with short detection times. In the case of modification with PEI-PSS-PEI, high sensitivity is achieved allowing to detect concentrations as low as 10^1 CFU/mL in 20 minutes. In order to obtain better selectivity IDEA were modified with PEI-Con A which resulted in lower sensitivity (10^4 CFU/mL) and longer detection times (40 minutes).

2. A novel functionalization method using the deposition of concanavalin A (Con A) multilayers on 3D-IDEA is developed for the detection of bacterial endotoxins.

2.1. Deposition of Con A as a bioreceptor carried out directly over PEI initial anchoring layer provokes unspecific adsorption of lipopolysaccharides (LPS) on PEI which required the study of different blocking methods. Among them consecutive deposition of Con A-glycogen-Con A layers was found to be really effective. 3D-IDEA transducers modified with PEI-(Con A-Gly)$_2$-Con A multilayers are shown to be highly sensitive, selective and reproducible for determination of LPS.

2.2. The impedance response of 3D-IDEA with PEI-(Con A-Gly)$_2$-Con A multilayers for detection of LPS follows the Langmuir absorption curve that can be fitted by Hill's equation. The resulting linear response between the impedance changes and the logarithm of LPS concentration allows to establish the detection limit of 2 μg/mL with a required detection time of 20 minutes.

3. A selective, reusable and sensitive apatsensor is developed for detection of *E. coli* 157:H7 detection in water samples.

3.1. The biofunctionalization of 3D-IDEA with a DNA aptamer allows to obtain an aptasensor for the detection of pathogenic fecal-borne bacteria *E. coli* O157:H7. The impedance transducer surface grafted via vapor-phase silanization with mercaptosilane (MPTES) is an effective method to perform the covalent binding of the aptamer. The immobilization is performed via thiol/disulfide exchange reaction between disulfide group at 5' terminal of the oligonucleotide and the sulfhydryl group of MPTES.

3.2. The developed aptasensor shows linear relationship between the impedance changes and the logarithm of *E. coli* concentration in a broad range from 10^1 to 10^5 CFU/mL (R^2= 0.977) with a LOD about $2.9 \cdot 10^2$ CFU/mL. The aptasensor allows to detect *E. coli* bacteria in PBS solutions in only 30 min.

3.3. Detection assays in samples with other bacterial strains (*Escherichia coli* K-12, *Salmonella typhimurium* and *Staphylococcus aureus*) validate the selectivity of the aptasensor for *E. coli* O157:H7.

3.4. The aptasensor regeneration process is developed which is based on a simple temperature treatment in water. This permits to use the aptasensor multiple times allowing to reduce costs per analysis.

3.5. The applicability of the aptasensor for bacteria detection in real samples is demonstrated using a promising pre-concentration system based on the filtration of water samples. The system permits the recovery of bacterial cells and eliminates the sample matrix effect on the sensor response.

4. Site-specific immobilization of *E. coli* bacteria within the trenches of 3D-IDEA sensors is validated as a promising approach to study the effectiveness of antimicrobial reagents on bacteria.

4.1. The performance of a microbial-based biosensor using the 3D-IDEA impedimetric transducer and *E. coli* as biorecognition moiety is demonstrated useful to monitor the response of bacteria to antimicrobial drugs. Impedance variations registered in solutions of low conductivity may be associated with the disruption of bacteria cell membrane producing the release of cytoplasmic compounds which affects the conductivity of the solution in the trenches.

4.2. *Escherichia coli* cells can be selectively entrapped within the trenches by modifying the top of the 3D-IDEA barriers with antifouling poly(N-isopropylmethacrylamide) (p(NIPMAM)) microgels. PEI polycation acts as the anchoring layer for bacteria on the trenches as well as for microgels that are deposited on top the of barriers by microcontact printing. This strategy of selective bacteria immobilization allows to improve the microbial biosensor reproducibility and sensitivity to monitor the bacterial response in comparison to the sensors with the bacteria immobilized on the entire surface.

4.3. The antimicrobial effect of ampicillin against *E. coli* bacterium with the proposed microbial biosensor can be detected within 1 - 2 hours which is a really a short detection time compared to other tests like culturing methods that require up to 2 days.

4.4. The developed microbial biosensor approach integrated in a specifically designed holder is a versatile, compact and easy-to-use biosensing platform that can be useful in a wide spectrum of bacterial bioassays for toxicity testing.

ANNEXES

ANNEXES

Published papers included in this thesis

- **Annex I:**

Paper 1. ***Sensitivity and response time of polyethyleneimine modified impedimetric transducer for bacteria detection***

Published in *Electroanalysis*, 2015, 27, 656-662 (doi: 10.1002/elan.201400575)

Sergi Brosel-Oliu, Natalia Abramova, Andrey Bratov, Núria Vigués, Jordi Mas, Francesc-Xavier Muñoz

- **Annex II:**

Paper 2. ***Impedimetric label-free sensor for specific bacteria endotoxin detection by surface charge registration***

Published in *Electrochimica Acta*, 2017, 243, 142-151 (doi: 10.1016/j.electacta.2017.05.060)

Sergi Brosel-Oliu, Dmitry Galyamin, Natalia Abramova, Francesc-Xavier Muñoz-Pascual, Andrey Bratov

- **Annex III:**

Paper 3. ***Novel impedimetric aptasensor for label-free detection of Escherichia coli O157:H7***

Published in *Sensors and Actuators B: Chemical*, 2018, 255, 2988-2995 (doi: 10.1016/j.snb.2017.09.121)

Sergi Brosel-Oliu, Rubén Ferreira, Naroa Uria, Natalia Abramova, Raimundo Gargallo, Francesc-Xavier Muñoz-Pascual, Andrey Bratov

Other publications related with this thesis

Book chapter I: ***Impedimetric sensors for bacteria detection***

Published in Biosensors-Micro and Nanoscale Applications, InTech, 2015, 257-288 (doi: 10.5772/60741)

Sergi Brosel-Oliu, Naroa Uria, Natalia Abramova, Andrey Bratov

Book chapter II: ***Label-free impedimetric biosensing using 3D interdigitated electrodes***

Published in Label-Free Biosensing: Advanced Materials, Devices and Applications, Springer International Publishing, 2018, 179-198 (doi: 10.1007/5346_2017_7)

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