Universitat Autònoma de Barcelona

HIGH-DENSITY BACTERIAL IMMOBILIZATION STRATEGIES FOR THE DEVELOPMENT OF MICROBIAL BIOSENSORS

Núria Vigués Frantzen

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HIGH-DENSITY BACTERIAL IMMOBILIZATION STRATEGIES FOR THE DEVELOPMENT OF MICROBIAL BIOSENSORS

Memòria presentada per

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A thesis submitted by Núria Vigués Frantzen entitled **High-density bacterial immobilization strategies for the development of microbial biosensors** was carried out at Genetics and Microbiology Department in UAB under the supervision of Dr. Jordi Mas and Dr. Xavier Muñoz Berbel.

Aproval of the directors,

Dr. Jordi Mas Gordi

Dr. Xavier Muñoz Berbel

Bellaterra, June 2020.

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Al Jordi, a la Jana,

al Martí i als meus pares.

Life is like riding a bicycle.

To keep your balance

You must keep moving.

-Albert Einstein-

INDEX

List of abbreviations and units

BOD	Biochemical Oxygen Demand
CNT	Carbon Nanotubes
CV	Coefficient of Variation
DAPI	4′,6-diamidino-2-phenylindole
DCP	Dichlorophenol
DGDE	Diethylene glycol diglycidyl ether
DMSO	Dimethyl sulfoxide
DMF	N,N-dimethylformamide
DNA	Deoxyribonucleic acid
DTP	10-(4H-dithiyeno[3,2-b:2',3'-d]pyroll-4-il)decan-1-amine
E.coli	*Escherichia coli*
ECH	Epichlorohydrin
EDC·HCl	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride
Ig	Immunoglobulins
IUPAC	International Union of Pure and Applied Chemistry
Fig	Figure
FET	Field Effect Transistor
LB	Luria Bertani medium
MFC	Microbial Fuel Cells
MIP	Molecular Imprinted Polymer
MTES	Methyltriethoxysilane
MWCNT	Multiwalled nanotubes
NA biosensors	Nucleic acid biosensors
NHE	Normal Hydrogen Electrode
NHS	N-hydroxysuccinimide
NIPS	Non-solvent Induced Phase Separation
NPG	Nano Porous Gold
PDDA	Poly(diallyldimethylammonium chloride)
PEDOT	Poly (3,4-ethylenedioxythiophene) polystyrene
PEI-ECH	Polyethylenimine- epichlorohydrin
PEI-DGDE	Polyethylenimine- diethylene glycol diglycidyl ether
PES	Polyethersulfone
PVA	Polyvinyl alcohol

RNA	Ribonucleic acid
SCE	Satured Calomel Electrode
SEM	Scanning Electronic Microscopy
SHE	Standard Hydrogen Electrode
SPE	Screen-printed electrodes
SPR	Surface Plasmon Resonance
TEOS	Tetraethyl orthosilicate
UV	Ultraviolet

List of units

A	Ampers
CFU	Colony Forming Units
EC50	Half-maximal effective concentration
I%	Inhibition percentage
mC	MiliCoulombs
min	Minutes
mM	Milimolar
nm	Nanometers
µm	Micrometers
mL, µL	Mililiters, microliters
Pow	Partition coefficient of the solvent in an equimolar mixture of n-octanol and water
s	Second
V	Voltage
w/v	Weight/volume

Summary

Microbial biosensors are analytical devices that use microorganisms as recognition elements. Microorganisms are immobilized on the surface of a transducer in such a way that the microorganism-analyte interaction generates a signal (electrochemical, optical, among others) that can be quantified. These microbial biosensors can be applied in the fields of clinical, industrial or environmental diagnosis with the advantage of being portable, simple and inexpensive alternatives to many laboratory-based methods. Unfortunately, development of microbial biosensors has been hindered by important technical limitation related to: (i) poor reproducibility, due to non-reproducible cell immobilization protocols, (ii) low sensitivity, by the difficulty of immobilizing high bacterial concentrations, and (iii) short life-time, due to cell death during immobilization or storage.

This thesis describes the development of two immobilization strategies that allow reproducible confinement of microorganisms at the electrode surface, with high densities and in a reproducible manner, while providing a physiological environment that allows adequate diffusion of nutrients, ensuring the functionality and viability of the trapped microorganisms.

In one of the strategies, (1) microbial cells have been trapped in an **alginate-graphite** polymeric matrix electrodeposited at the electrode surface using very soft and biocompatible conditions (i.e. room temperature, aqueous medium, neutral pH, etc.). Conductive alginate-coated electrodes are obtained after potentiostatic electrodeposition of graphite-doped alginate samples (up to 4% graphite). The presence of graphite reduces electrode passivation and improves the electrochemical response of alginate-coated sensors. Bacterial entrapment in the conductive matrix is highly efficient ($4.4x10^7$ cells per electrode), reproducible (CV < 0.5%) and does not compromise bacterial integrity or activity.

In the second strategy, (2) microorganisms are trapped in **polyethersulfone** when the polymer, initially dissolved in organic solvents, precipitates in aqueous medium through a process of phase inversion. Polyethersulfone, as other synthetic polymers (e.g. polyvinyl alcohol) presents improved stability at the expense of complex protocols involving chemical/physical treatments that decrease their biological compatibility. In an attempt to explore new solutions to this problem, we have developed a procedure for the immobilization of bacterial cells in polyethersulfone (PES) using phase separation. In this process, the polymer initially dissolved in an organic solvent is precipitated by the use of a non-solvent, a liquid in which the polymer is insoluble. Any component present in the non-solvent solution during the formation of the membrane may be retained inside its porous matrix. We have shown that microorganisms can be incorporated during membrane formation and remain viable. With this method, 300 µm PES membranes were reproducibly

obtained containing up to $2.3x10^6$ cells per electrode, with an entrapment efficiency of 8.2%, while maintaining acceptable levels of cell integrity or viability.

Both systems have been applied to immobilize *E. coli* at the surface of screen-printed electrodes to develop biosensors in which microorganisms act as recognition elements. Biosensing has been performed electrochemically through ferricyanide respirometry, with metabolically-active entrapped bacteria reducing ferricyanide in the presence of glucose. The analytical performance of the two amperometric microbial biosensors has been assessed carrying out a toxicity assay using 3,5-dichlorophenol (DCP) as a model toxic compound. In both cases, biosensors provided a concentration-dependent response to DCP with half-maximal effective concentration (EC_{50}) of 3.5 ppm (alginate) and 9.2 ppm (polyethersulfone), well in agreement with reported values. This entrapment methodology is susceptible of mass production and allows easy and repetitive production of robust and sensitive microbial biosensors.

Resumen

Los biosensores microbianos son dispositivos analíticos que utilizan microorganismos como elementos de reconocimiento. Los microorganismos están inmovilizados en la superficie del transductor de manera que la interacción microorganismo-analito genera una señal (electroquímica, óptica, entre otras) que puede ser cuantificada. Los biosensores microbianos se pueden aplicar en diferentes campos como el diagnóstico clínico, la industria alimentaria o la monitorización medioambiental, con la ventaja de ser portables, simples, baratos, así como una buena alternativa a los métodos de laboratorio. Desafortunadamente, la implementación de los biosensores microbianos se ha visto obstaculizada por: (i) su pobre reproducibilidad, debido a la poca reproducibilidad de los protocolos de inmovilización de las células, (ii) su poca sensibilidad, por la dificultad de inmovilizar elevadas concentraciones de microorganismos, y (iii) su corta vida útil, debido a la muerte celular durante el proceso de inmovilización o almacenamiento.

Esta tesis describe el desarrollo de dos estrategias de inmovilización que permiten el confinamiento reproducible de microorganismos en la superficie del electrodo, con altas densidades y de manera reproducible, al tiempo que proporcionan un entorno fisiológico que permite una adecuada difusión de nutrientes, asegurando la funcionalidad y viabilidad de los microorganismos atrapados.

En uno de los sistemas, las células microbianas se atrapan en una matriz polimérica de alginato-grafito electrodepositada en la superficie del electrodo utilizando condiciones muy suaves y fisiológicas (temperatura ambiente, medio acuoso, pH neutro…). Los electrodos recubiertos de alginato conductor se obtienen después de la electrodeposición potenciostática de muestras de alginato dopadas con grafito (hasta el 4% de grafito). La presencia de grafito reduce la pasivación del electrodo y mejora la respuesta electroquímica de los sensores recubiertos de alginato. El atrapamiento de microorganismos es altamente eficiente ($4.4x10^7$ células por electrodo) y reproducible (CV <0.5%) sin comprometer la integridad o actividad microbiana.

En la segunda estrategia, los microorganismos quedan atrapados en una matriz de polietersulfona, un material soluble en solventes orgánicos y que sólo precipita en contacto con medio acuoso en un proceso llamado de inversión de fase. La polietersulfona, como otros polímeros sintéticos (por ejemplo, el alcohol de polivinilo) presenta una elevada estabilidad a expensas de requerir protocolos complejos de polimerización que involucran tratamientos físicos o químicos que disminuyen su biocompatibilidad. En un intento por explorar nuevas soluciones a este problema, hemos desarrollado un procedimiento para la inmovilización de células microbianas en membranas de polietersulfona (PES) utilizando la inversión de fase. En este proceso, el polímero inicialmente disuelto en un solvente orgánico precipita mediante el uso de

un “no solvente”, un líquido en el cual el polímero es insoluble. Cualquier componente presente en la solución no soluble es retenido dentro de la matriz porosa del material durante la formación de la membrana. Hemos demostrado que los microorganismos pueden incorporarse durante la formación de la membrana manteniendo cierta viabilidad. Con este método, se obtuvieron de manera reproducible membranas de 300 µm, con $2.6x10^6$ células en su interior, que mantienen niveles aceptables de integridad y viabilidad celular.

Ambos sistemas se han utilizado para inmovilizar *E.coli* en la superficie de electrodos serigrafiados para desarrollar biosensores en los que los microorganismos actúan como elementos de reconocimiento. La biodetección se ha realizado electroquímicamente mediante respirometría de ferricianuro, de manera que los microorganismos atrapados dentro de la matriz pueden reducir el ferricianuro en presencia de glucosa y convertirlo en ferrocianuro. Se ha evaluado el rendimiento analítico de los dos biosensores microbianos amperométricos llevando a cabo un ensayo de toxicidad utilizando 3,5-diclorofenol (DCP) como compuesto tóxico modelo. En ambos casos, los biosensores proporcionaron una respuesta dependiente de la concentración de DCP con una dosis efectiva (EC_{50}) de 3.5 ppm (alginato) y 9.2 ppm (polietersulfona), de acuerdo con los valores reportados. Esta metodología de atrapamiento es susceptible a la producción en masa porque permite una producción fácil y repetitiva de biosensores microbianos robustos con buena sensibilidad.

Resum

Els biosensors microbians són dispositius analítics que utilitzen microorganismes com elements de reconeixement. Els microorganismes s'immobilitzen a la superfície del transductor de manera que la interacció microorganisme-analit genera una senyal (electroquímica, òptica, entre altres) que pot ser quantificada. Els biosensors microbians es poden fer servir en diferents àmbits d'aplicació com a el diagnòstic clínic, la indústria alimentària o la monitorització mediambiental amb l'avantatge de ser portables, simples, barats, i per tant bones alternatives als mètodes de laboratori. Desafortunadament, el desenvolupament de biosensors microbians es veu obstaculitzat per: (i) la baixa reproductibilitat que presenten els protocols d'immobilització de les cèl·lules, (ii) la seva poca sensibilitat, deguda a la dificultat d'immobilitzar elevades concentracions de microorganismes, i (iii) la curta vida útil dels mateixos, deguda a la mort cel·lular durant el procés d'immobilització o emmagatzematge.

Aquesta tesi descriu el desenvolupament de dues estratègies d'immobilització que permeten el confinament reproduïble de microorganismes a la superfície d'un elèctrode, amb elevades densitats i de manera reproduïble, i al mateix temps proporciona un entorn fisiològic que permet una adequada difusió dels nutrients, garantint la funcionalitat i la viabilitat dels microorganismes atrapats.

En la primera estratègia, les cèl·lules microbianes romanen atrapades en una matriu polimèrica d'alginat-grafit electrodepositada a la superfície de l'elèctrode mitjançant condicions molt suaus i fisiològiques (temperatura ambient, medi aquós, pH neutre…) de polimerització. Els elèctrodes recoberts d'alginat conductor s'obtenen després de l'electrodeposició potenciostàtica de mostres d'alginat dopades amb grafit (fins al 4% de grafit). La presència de grafit redueix la passivació de l'elèctrode i millora la resposta electroquímica dels sensors recoberts d'alginat. L'atrapament de microorganismes és molt eficient ($4.4x10^7$ cèl·lules per elèctrode) i reproduïble (CV <0.5%), sense comprometre la integritat ni l'activitat microbiana.

En la segona estratègia, els microorganismes romanen atrapats en la matriu de polietersulfona, un material soluble en solvents orgànics que precipita en contacte amb medis aquosos en un procés anomenat inversió de fase. La polietersulfona, com altres polímers sintètics (per exemple, l'alcohol de polivinil) presenta una elevada estabilitat a costa de protocols de polimerització complexes que involucren tractaments físics o químics que disminueixen la seva biocompatibilitat. En un intent d'explorar noves solucions a aquest problema, hem desenvolupat un procediment per la immobilització de les cèl·lules microbianes en la matriu de la polietersulfona (PES) fent servir la inversió de fase. En aquest procés, el polímer inicialment dissolt en un solvent orgànic precipita al entrar en contacte amb un "no solvent", un líquid en el qual el polímer és insoluble. Qualsevol component present en la solució no soluble es retingut

dins la matriu porosa durant la formació de la membrana. Hem demostrat que els microorganismes poden incorporar-se durant la formació de la membrana mantenint certa viabilitat. Amb aquest mètode, s'obtenen de manera reproduïble membranes de 300 μm amb $2.6x10^6$ cèl·lules en el seu interior, que mantenen nivells acceptables d'integritat i viabilitat cel·lular.

Tots dos sistemes s'han utilitzat per immobilitzar *E. coli* a la superfície d'elèctrodes serigrafiats per desenvolupar biosensors, en els quals, els microorganismes actuen com a elements de reconeixement. La biodetecció s'ha realitzat electroquímicament mitjançant la respirometria de ferricianur, de manera que els microorganismes atrapats dins de la matriu poden reduir el ferricianur en presència de glucosa i convertir-lo en ferrocianur. S'ha avaluat el rendiment analític dels dos biosensors microbians amperomètrics portant a terme un assaig de toxicitat utilitzant 3,5-diclorofenol (DCP) com a compost tòxic model. En tots dos casos, els biosensors van proporcionar una resposta depenent de la concentració de DCP, amb una dosi efectiva (EC_{50}) de 3.5 ppm (alginat) i 9.2 ppm (polietersulfona), d'acord amb els valors reportats. Aquestes metodologies d'atrapament són susceptibles de producció en massa perquè permeten una producció fàcil i repetitiva de biosensors microbians robusts amb una bona sensibilitat.

CHAPTER 1

INTRODUCTION

1. INTRODUCTION

1.1. Biosensors: a history of sensing with biological elements

In 1997, the International Union of Pure and Applied Chemistry (IUPAC) defined for the very first time a biosensor as “an analytical device that uses specific biochemical reactions mediated by enzymes, tissues, organelles, immune-systems or whole cells to detect chemical compounds usually by electrical, thermal or optical signals”. According to this, biosensors combine biological material with sensitive transduction mechanisms to detect biological or chemical species directly from the samples. Thanks to this combination, they can transform specific chemical or biological information into a measurable quantitative or semi-quantitative response.

Although not defined until the late nineties, the history of biosensors began much earlier with Leland C. Clark who is, in fact, considered the father of biosensors. In 1962, Clark and Lyons describe the first amperometric biosensor. The device combined a Clark’s oxygen electrode with the activity of the enzyme glucose oxidase to determine glucose concentration in blood through oxygen consumption (Clark and Lyons 1962). The concept was further refined by Updike and Hicks in 1967 in a seminal article published in Nature in which they describe the immobilization of glucose oxidase in the surface of an oxygen electrode. The authors used a polyacrylamide gel as the immobilization matrix, and they referred to their amperometric device as an “enzyme electrode”. After that, progress was fast and successful and in 1975, the first biosensor reached the market when Yellow Spring Instruments (YSI) produced and sold the first glucose amperometric analyzer for diabetic patients (Palchetti et al. 2010).

In parallel, other types of biosensor were being developed. In 1969, Guibault and Montalvo described the first potentiometric enzyme biosensor for urea detection, based on the activity of urease immobilized on a selective ammonium electrode. Somewhat later, in 1975, Lubbers and Opitz used the term "optode" for the first time to describe an optical biosensor based on an optical fiber with immobilized alcohol hydrogenase, for oxygen measurement (Setford et al. 2005, Voelkl et al.1980). The same year, Diviès described the first biosensor that used microorganisms (*Acetobacter zylinum*) as recognition element for the measurement of alcohol (Diviès et al. 1975). Biosensors incorporating antibodies as recognition elements (also known as immunosensors), were developed in the 70’s by immobilization of antibodies in piezoelectric or electrochemical transducers (Liedberg et al. 1983). Artificial redox mediators were introduced in 1979 by J. Kulys and in 1984, Cass introduced the first ferrocene-mediated amperometric glucose biosensor which was commercialized by MediSense Inc. in 1987 for home blood-glucose monitoring (Kulys et al. 1979, Cass et al. 1984).

In the early 1980s, other important discoveries related to biosensors took place: Arnold and Rechnitz described the first tissue-based and organelle-based biosensors (Rechnitz 1978), the

first DNA-based biosensors appeared (Zhai et al. 1997), and magnetic biosensors capable to detect magnetic particles in microfluidic channels were developed (Chon 2008, Giorundi et al. 2017, Nabaei et al. 2018). More recently, concepts such as nanobiosensors, implanted biosensors, aptameric biosensors or DNAzyme biosensors, among other, have been developed (Bhalla et al. 2016). These different milestones have been represented in figure 1.

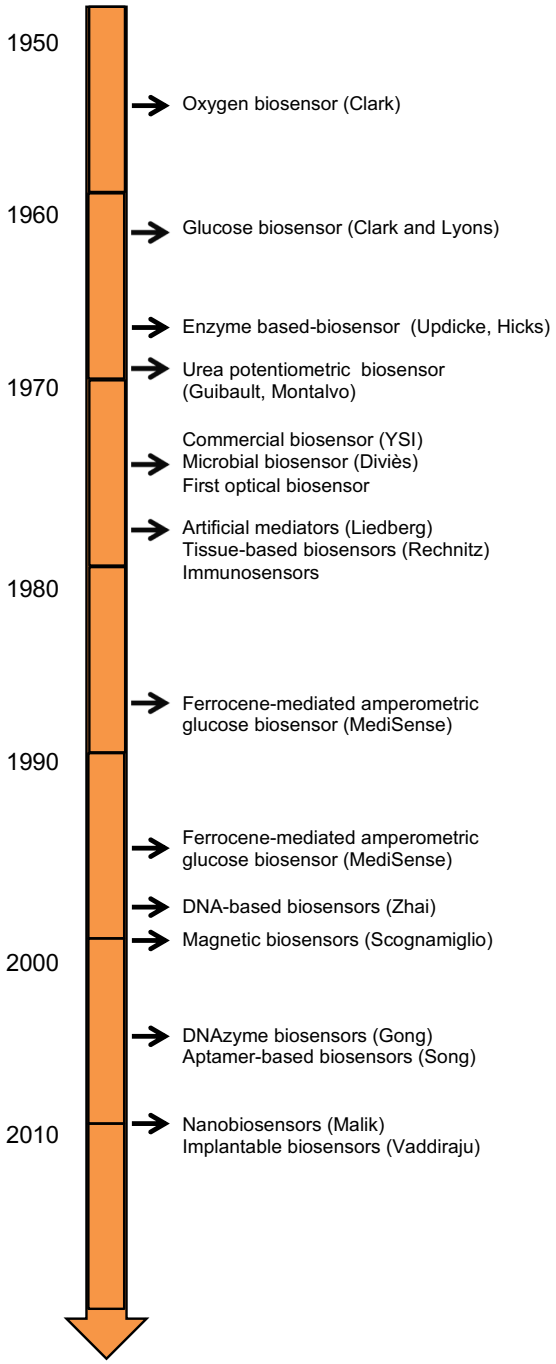

Figure 1. Timeline showing the most relevant milestones in the field of biosensors.

Although development in this field, as indicated by the number of publications, has been intense over the last 40 years (figure 2), the number of biosensors reaching the market has been particularly low. Similarly, the number of publications involving microbial biosensors has risen in recent years (figure 3), but with an even more limited number of final products ready for the costumers. In spite of this, the overall biosensors market has reached a considerable volume now estimated at USD 21.2 billion, and is expected to grow at a compound annual growth rate of 8.3% during the next 5 years achieving a volume of USD 31.5 billion by 2024 (Biosensors Market by Type (Sensor patch and embedded device), Product (Wearable and non-wearable), Technology (Electrochemical and optical), Application (POC, Home Diagnostics, Research Lab, Food & Beverages), and Geography - Global Forecast to 2024. Markets and Markets. www.marketsandmarkets.com, last checked November 2019).

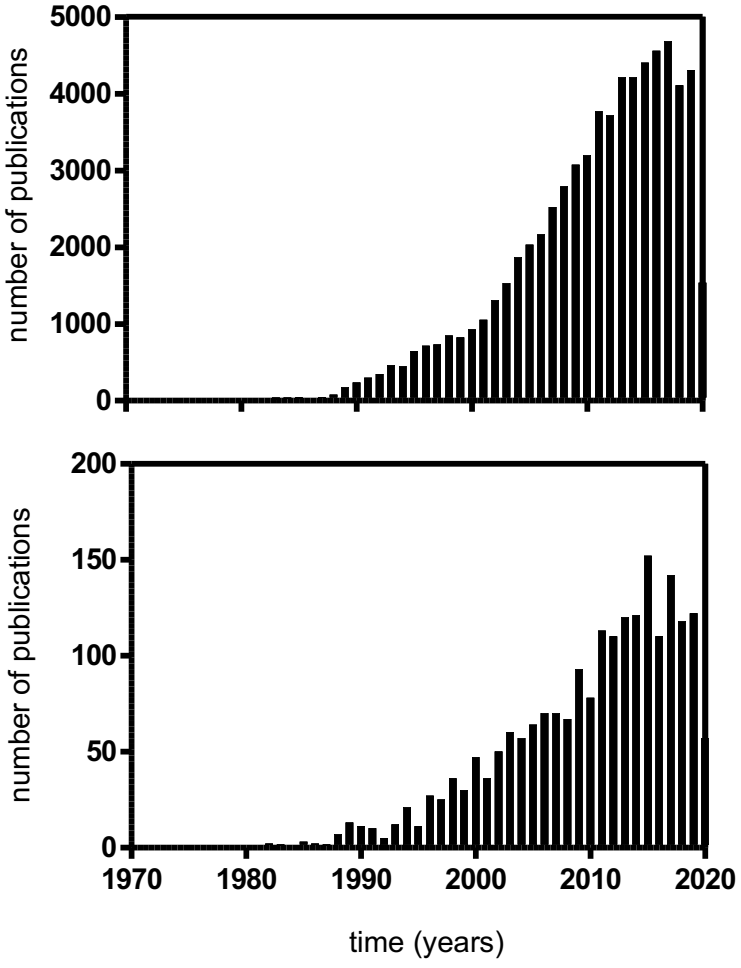

Figure 2. Number of biosensor (A) and microbial biosensor (B) publications during the last 50 years (Scopus).

The early commercial success of Yellow Spring Instruments in 1975 and MediSense in 1987 has been further capitalized by companies like Abbott Point of Care Inc. (US), ACON Laboratories, Inc. (US), Biacore (Sweden), Bio-Rad laboratories Inc. (US), Biosensors International Pte. Ltd. (US), DuPont (US), F. Hoffman-La Roche Ltd. (Switzerland), LifeScan Inc. (US), LifeSensors Inc. (US), Medtronic Inc. (US), Nova Biomedical Corp. (US), Pharmaco-Kinesis Corporation (PKC) (US), Sysmex Corporation (Japan) and Universal Biosensors Inc. (Australia) that currently constitute the main players in this market. Therefore, biosensor in general and microbial biosensors represent an active and evolving field that requires continued progress and further efforts in order to meet the evolving requirements of the industrial sector.

1.2. Biosensor components and classification

Biosensors are composed of three main elements: (i) the biological recognition element (or biochemical receptor), (ii) the transducer and (iii) the electronic components for signal amplification and processing (Mehrotra et al. 2016, Thévenot et al. 2001) (figure 3).

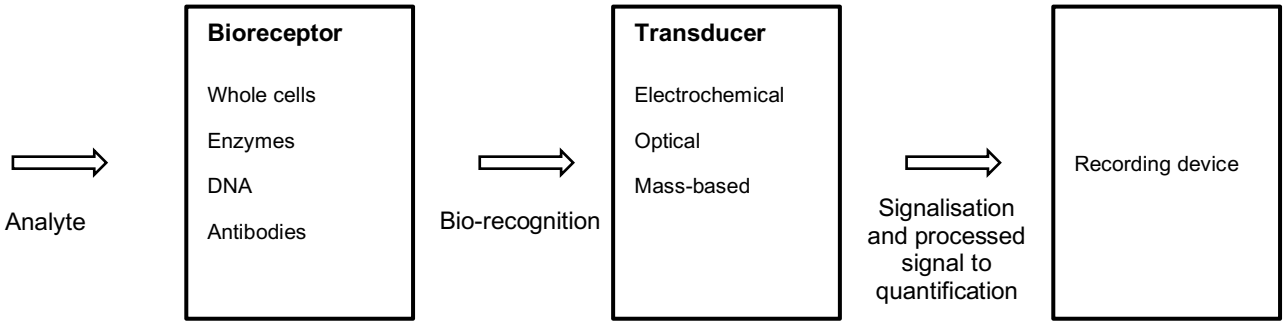

Figure 3. Outline of the main elements of a biosensor.

The biological recognition element provides the sensor with selectivity for a specific analyte, i.e. the substance to be detected. The interaction of the bioreceptor with the analyte generates a response through the bio-recognition reaction. Different types of biological elements have been used, including enzymes, antibodies, DNA, aptamers, organelles or whole cells, among others (Chambers et al. 2008).

The transducer is the element responsible for transforming the changes produced by the interaction between the analyte and the bioreceptor into a measurable signal. Depending on the nature of the signal, it can be detected electrochemically, optically, acoustically, mechanically, calorimetrically or electronically (Malhrota et al. 2003). The signal produced by the transducer is proportional to the amount of analyte-bioreceptor interaction and may allow quantification.

Finally, the electronic part is necessary to amplify and to convert the transduced signal into a digital signal. The received signals are stored and displayed for easy interpretation of the data by the user in a display.

Biosensors can be classified according to either (i) **the principle of the operation of the transducer** or (ii) **the nature of the bioreceptor element.**

1.2.1 Biosensors classification according to the transduction mechanism

There is no universally accepted methodology to classify biosensors according to their principle of operation and different authors have considered different approaches. In this thesis, the one published by IUPAC has been adopted (figure 4).

The IUPAC classifies biosensors according to their transduction mechanism as electrochemical, optical, or mass sensitive (Malhrota et al. 2003, Grieshaber et al. 2008).

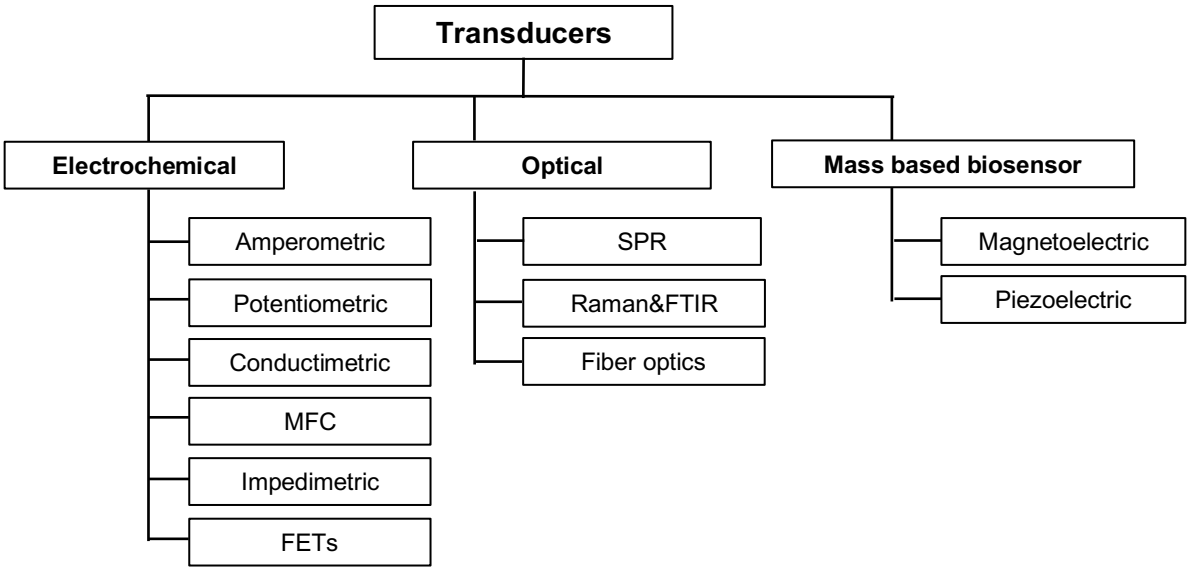

Figure 4. Classification of biosensors according to IUPAC.

1.2.1.1 Electrochemical:

Electrochemistry is a branch of chemistry that studies the relationship between electrical and chemical effects. Electrochemical methods are employed as tools in the study of chemical process that occurs in electrode-solution interface when an electric potential is applied and current passes through (Bard et al. 2001).

The IUPAC defines an electrochemical biosensor as “an integrated autonomous device, which is able to provide specific quantitative or semi-quantitative analytical information using a biological recognition element (biochemical receptor) that remains in direct and spatial contact with the transduction element”. Biosensors based on electrochemical transducers have the

advantage of being economical and presenting a rapid response; the possibility of automation allows their application to a large number of samples (Grieshaber et al. 2008).

Electrochemical approaches are widely used in the development biosensors due to their simplicity, miniaturization, low-cost and portability. Depending on the detection principle, electrochemical biosensors are divided into **amperometric**, **potentiometric**, **conductimetric**, **microbial fuel cells** (MFCs), **impedimetric** and **field effect transistors** (FETs) (Hammond et al. 2016).

This thesis is based on amperometric biosensors and thus this mechanism will be described in more detail.

Amperometric biosensors

Amperometric biosensors are based on bio-recognition reactions that involve, either directly or indirectly, electron transfer processes. At a given potential, there is an electron flow between the working electrode and redox molecules, these molecules being either the substrate, or the product of the bio-recognition reaction. Redox molecules are oxidized or reduced in the electrode surface, producing a current flow proportional to the concentration of analyte (Mehrvar et al. 2004). In general terms, amperometric biosensors are faster, more sensitive and more precise than other electrochemical transducers (e.g. potentiometric) (Ghindilis et al. 1998).

The most common electrochemical cell employs three electrode configuration consisting of a working, a reference and a counter electrode. In terms of operation, a voltage difference is applied between the reference and the working electrodes, while the current flows between the working and the counter electrode (Pohanka et al. 2018). A more detailed description of the role, geometry and composition of each electrode is included below.

The working electrode is the electrode where the redox reaction of interest is taking place. Depending on whether the reaction at the electrode is a reduction or an oxidation, the working electrode acts as a cathode or an anode, respectively (Bard et al. 2001). These electrodes should present high conductivity, fast electron exchange kinetics and long-term stability. Most common materials for the working electrode are metals (Pt, Au, Al, Ag, among others), carbon-based material (graphite, glassy carbon, diamond, carbon-nanotubes, etc.), liquid metals or even semiconductors (Si). The effective area of the electrode is an important parameter to be considered in the electrochemical measurements.

The reference electrode is designed to provide a stable and known potential over time independently on the experimental conditions and environmental factors. This electrode fixes with precision the potentials applied to the working electrode during the reduction or oxidation process under study. The current flow between the working and the reference electrode is

minimal or null to ensure the stability and durability of the reference electrode. Most common and internationally accepted reference electrodes are: the standard hydrogen electrode (SHE) or normal hydrogen electrode (NHE), the saturated calomel electrode (SCE) and the silver-silver chloride electrode. Potentials are often measured respect to the NHE. SCE potential is 0.242 V vs. NHE and the silver-silver chloride electrode has a potential of 0.197 V vs NHE (Bard et al. 2001).

To guarantee the stability of the potential, these electrodes are immersed in a solution of defined and well-known composition and protected with a glass barrier to ensure electrical contact with the sample under study. Also, pseudo-reference electrodes based on metals (Au, Ag, Pt, etc.) or even Ag/AgCl have been used and described in the literature (Ilzet et al. 2013). These are normally in direct physical contact with the sample under study and may not be as stable as the conventional ones.

The counter electrode, also called auxiliary, is the electrode used to close the electrical circuit. It is connected to the working and collects (or produces) the electrons that flow from one electrode to the other, generating the electrical current. In the design of the electrochemical cell, the counter electrode has a much larger area than the working electrode to avoid electron flow limitations. Auxiliary electrodes are often fabricated in platinum due to its high stability and fast kinetics, although other metals such as gold or carbon have been also used.

To complete the electrical circuit, electrodes are submerged in an electrolyte solution, where anions and cations circulate. In this phase, charge is carried by the ions moving in a low resistance aqueous solvent to ensure the best electron exchange between the electrodes.

From the large number of amperometric transducers currently available, we chose screen-printed electrodes (SPEs) to carry out the amperometric tests described in this thesis. Screen-printing is an additive technology consisting of the deposition layer by layer of inks on a solid substrate. The geometry of the sensor is defined with a screen or a mesh with the patterns corresponding to the design of the sensor. The fabrication process includes the selection of the screen, the selection and preparation of the inks, the selection of the solid substrate and the printing. This final step includes the deposition through the mask at specific regions in the support material, the drying and the curing stages (Alonso-Lomillo et al. 2010).

The emergence of screen-printed electrodes (figure 5) has contributed to reduce the costs of conventional electrodes. SPEs are simple to fabricate and allow mass production, are compact, relatively inexpensive with design flexibility and a wide selection of materials in a reproducible system that avoids long cleaning processes of the electrodes. This technology allows the immobilization of biomolecules on the surface, such as, immunoglobulins, DNA, enzymes and

microorganisms for different fields of application (Alonso-Lomillo et al. 2010, Turodache et al. 2007).

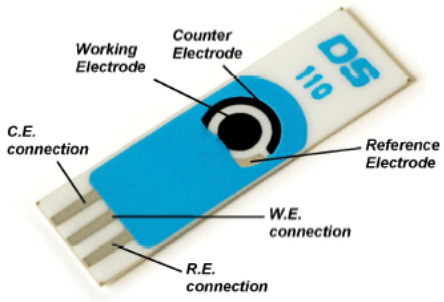

Figure 5. Screen-printed electrode used in this study, Dropsens (image from webpage).

Potentiometric biosensors

Potentiometric biosensors are defined as an analytical device comprising a biological sensing element connected to an electrochemical potential transducer. In this case, the transducer converts the biological response into an electrical potential. The potentiometric measurement consists of the determination of the potential difference between two electrodes, namely the reference electrode and the working electrode. The first provides a constant potential while the second is sensitive to the changes of the analyte of interest. The difference of both potentials is related by the Nerst equation (Yunus et al. 2013). The potentiometric electrode is normally modified with a membrane selective to the analyte of interest, either a substrate or a product of the bio-recognition reaction. The presence of the analyte changes the interface potential, which can be measured by comparison with the reference electrode, providing a constant potential over time. Potentiometric devices can measure changes in pH and ion concentration with a high selectivity and good sensibility (Malhrota et al. 2003).

Conductimetric biosensors

Conductimetric biosensors detect changes in the electrical conductivity of the solution as a function of the concentration of analyte. The changes in conductivity of the medium are a consequence of the consumption or production of ions (Dzyadevynch and Jaffrezic-Renault 2014, Thévenot et al. 2001). With this technique, there are no electrochemical reactions on the electrode surface, but only conductivity variations in the boundary layer of the electrode surface. In this case, the properties of the electrolyte are very important, because variations in these properties are related to the detection of the analyte. Conductimetric biosensors have high sensitivity but low selectivity, consequence of the number of processes that can modify conductivity (Mikkelsen and Rechnitz 1989, Lim et al. 2015).

Microbial fuel cells

Microbial fuel cells (MFC) use the bio-catalytic capabilities of viable microorganisms to oxidize organic or inorganic matter to generate an electrical current (Logan 2006). Microbial fuel cell has two compartments: the anode and the cathode, which are separated by a membrane that only allows the passage of protons. Microorganisms remain in the anode compartment generating electrons and protons with their metabolism and the electron acceptor remains in the cathode. Electrogenic microorganisms can transfer electrons to the anode either directly through components of their electron transport chain located in the outer membrane or, when these components are not present, through self-synthesized soluble redox molecules that act as redox shuttles. When microorganisms are not electrogenic, it is necessary to add external redox mediators that enable electron flow between the microbial electron transport chain and the surface of the anode. MFCs operated using mixed cultures currently achieve substantially larger power densities than those with pure cultures (Rabaey et al. 2004).

Electrical impedance biosensors

Electrical impedance biosensors measure the opposition that some components of the electric circuit (representing the electrochemical cell) present to the electronic/ionic flow. The analyst imposes an electrical perturbation to the electrochemical cell, either an alternate current or potential, and observes how the dependent variable (potential or current) responds to these electrical changes. Impedance is usually measured by a bridge circuit and a reference module is often included to measure and correct nonspecific changes, such as temperature (Daniels and Pourmand 2007). Electrical impedance biosensors are very sensitive and versatile, allowing the measurement of both ionic and electron exchange modifications, and sensitive. However, they present important selectivity problems that limit they application in biosensing (Borghol et al. 2010).

Field effect transistor-based biosensors

Field effect transistor-based biosensors are designed to detect changes in the electric field. A transistor is a semiconductor electronic device used to deliver an output signal in response to an input signal. It fulfils functions of amplifier, oscillator, switch or rectifier. The term "transistor" is the English contraction of transfer resistor ("transfer resistor"). Field effect transistors (FET) are devices whose principle of operation is based on the control of the electric current flowing in a semiconductor placed between two electrodes (source and drain). A dielectric layer is inserted between the semiconductor and an electrode (gate) to be able to modulate this current following the effect known as field effect. That is, when the metal potential is charged, the electric field induces changes in the semiconductor channel, so that charges are generated in the channel carriers, which causes current-voltage changes. Hence, building a FET requires combining at

least three different types of materials: conductors (metals or conductive oxides), semiconductors (inorganic material such as silicon or metal oxides) and the dielectric (aluminum oxide or silicon oxide). Semiconductor conductivity can be modulated by a field effect, which allows the device to be on or off. Dielectric allows capacitive control of the charge in the channel (Fortunato et al. 2016).

The biosensor based on FET has been proposed and has become an emerging field due to the rapid development of the technologies associated with them. As most biomolecules carry electrostatic charges, and changes in their concentration involve changes in electrical potential, the FET-based biosensor becomes a good candidate in applications that require great sensitivity and good time response (Syu et al. 2018).

1.2.1.2 Optical

Optical detection is performed by exploiting the interaction of the optical field with a biorecognition element. Optical detection can be done in two modes: label-free and label-based. In the first mode, the detected signal is generated directly by the interaction of the analyte with the transducer (Peltomaa et al. 2018). In the second mode, a label attached to the biomolecule and the optical signal is generated by a colorimetric, fluorescent or luminescent method (Damborsky et al. 2016).

Colorimetric methods have great potential for economic applications of daily life because they are easy to use, low cost and do not require expensive instruments for operation. In many occasions, the color change of the substrates can be observed with the naked eye. Fluorescence would be an example of the label-based optical mode. In this case, the biomolecule covalently binds to a fluorescent probe that allows highly sensitive detection of the analyte of interest.

Surface Plasmon Resonance (SPR) is another optical technique. In this case, the changes of index of refraction produced by the union of the analyte to the recognition elements are measured. The phenomenon occurs on the surface of a conductive material, i.e. gold, when it is illuminated with polarized light at a specific angle. This generates surface plasmons and, consequently, a reduction in the intensity of the light reflected at a specific angle known as the resonance angle. The SPR phenomenon can detect changes in the refractive index of the sensor surface, which are proportional to the analyte concentration (Pilliarik et al. 2009, Damborsky et al. 2016).

1.2.1.3 Mass sensitive

Piezoelectric or acoustic biosensors are based on the vibration of crystals at specific frequencies after application of a potential. The adsorption of certain molecules to the surface of the crystal alters this resonance frequency that can be measured with an electronic device as a potential

change (Pohanka et al. 2018). Piezoelectric transducers (e.g. quartz crystal microbalances, surface acoustic wave) are most commonly used in immunosensor configurations. In these devices, an antigen or antibody is immobilized on the surface of the crystal. The selective interaction of these elements with the analyte produces a change in the resonant frequency due to a modification of the mass in the crystal. The frequency change is proportional to the concentration of analyte.

1.2.2 Biosensors classification based on the biological recognition element

The most commonly recognition elements are: enzymes, nucleic acids, antibodies, whole cells and biomimetic receptors (figure 6). In any case, it is important to achieve a specific interaction through bio-recognition and, therefore, it is important to avoid any non-specific interaction that may interfere in the detection (Justino et al. 2015). The activity of the biological component for a substrate can be monitored through oxygen consumption, hydrogen peroxide formation, change in NADH concentration, fluorescence, absorption, pH change, conductivity, temperature or mass.

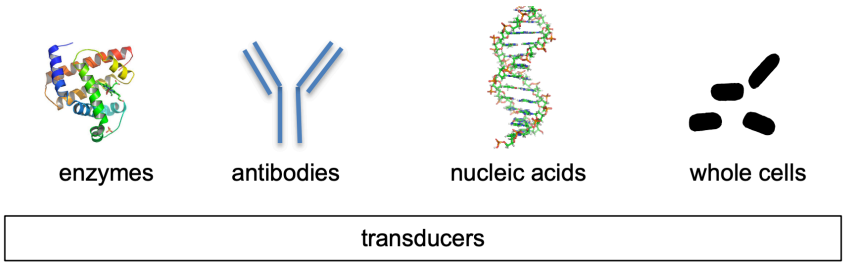

Figure 6. Scheme of the most used recognition elements in the bibliography.

1.2.2.1 Enzymes

Enzymes are the most widely employed bioreceptors. Enzymes are biomolecules involved in the cellular metabolism of living organisms. They selectively catalyze reactions to accelerate biological processes and often require the presence of cofactors. As bio-catalyzers, enzymes are not consumed by the chemical reaction, so they are available for new reactions. They can catalyze a large number of reactions with efficiency and selectivity, in a way that they have suitable characteristics to become biosensors (Mulchandani 1998).

The enzymes used in biosensors production are divided into two large groups, namely: oxidases and dehydrogenases, and both require coenzymes during catalysis. Examples of oxidases-based biosensors include: glucose oxidase (Hossain et al. 2016), glutamate oxidase (McMahon et al. 2006), alcohol oxidase (Chinnadayyala et al. 2015), lactate oxidase (Rocchita et al. 2016), ascorbate oxidase (Wen et al. 2012), cholesterol oxidase (Lata et al. 2016), laccasse (Gonzalez-Rivera et al. 2015) and tyrosinase (Campanhã et al. 2016), among others, mostly combined with amperometric sensors. Regarding dehydrogenases, biosensors using: alcohol dehydrogenase (Gómez-Anquela et al. 2015), glutamate dehydrogenase (Liang et al. 2015), glucose

dehydrogenase (Guo et al. 2016) and lactate dehydrogenase (Lin et al. 2014), have been already published, also mainly in amperometric biosensors. The main limitation of enzymes is their stability, because these proteins lose their biocatalytic activity if their three-dimensional architecture changes, and therefore have important storage and transport limitations, in addition to the high cost of purifying them.

1.2.2.2 Nucleic acids

Nucleic acids are the molecules that carry genetic information and are made up of phosphoric acid, sugars and a mixture of organic bases (purines and pyrimidines). The two main classes of nucleic acids are deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

The study of biosensors based on nucleic acid (NA biosensors) is gaining importance due to their efficiency, specificity and long-time stability. Most of these biosensors are composed of a single-strand (ss) DNA that could hybridize with the complementary strand. The well-known specific hybridization property between NA chains is the fundamental principle of detection of DNA sensors. Within this type of sensors, there are Aptamer Based Biosensors (Aptasensors) (Zhou et al. 2014). Aptamers are single nucleic acid chain (between 70 and 100 nucleotides) isolated from oligonucleotide libraries using the SELEX method. These simple chains are able to recognize different types of target specifically and with high affinity by three-dimensional folding of the chain. Since 2004, it is possible to find biosensors with this recognition element. They are ideal for the recognition of small molecules such as thrombin or many toxins such as aflatoxin, ochratoxin, etc., but present important limitation in the identification of bigger compounds. More recently, DNAzymes have been discovered and used in the development of biosensors (Gong et al. 2015). They are DNA sequences with bio-catalytic activity resembling that of enzymes (Silverman 2016).

1.2.2.3 Antibodies

Antibodies or immunoglobulins are proteins produced by the immune system in response to the presence of foreign substances (antigen). Each antibody consists of four polypeptides joined to form an "Y" molecule: two heavy chains and two light chains. In both types of chains, we find constant zones and variable zones. It is in the latter where the specificity to bind to the antigen is found. Antibodies are divided into 5 classes (IgM, IgG, IgA, IgD and IgE) depending on the constant zone and its function in the immune system. The antibodies could be of two main types, some belong to the innate response (these defend immediately but not specifically) and within the second group we find the acquired response antibodies (in this case the antigen is recognized and attacked in a specific manner). In the case of biosensors, the latter are used: antibodies that specifically recognize antigens providing sensors with high specificity and sensitivity. Antibodies for a specific molecule can be monoclonal (recognize one antigen epitope) or polyclonal

(recognize more than one antigen epitope). In the development of biosensors, when possible, the first option is preferred since confers the system a higher selectivity. As in the case of enzymes, antibodies are very sensitive to environmental factors and they lose their recognition capacity when changing their structure. Another important limitation of immunosensors is the cost of the antibodies, always very expensive due to the tedious purification processes required to obtain them. Recent modifications employed in the development of biosensors are nanobodies (fragments of antibodies maintaining the selectivity of the others) (Keller et al. 2019).

1.2.2.4 Biomimetic receptors

The bio-recognition property of enzymes, antibodies, cells and tissues can be mimicked by the molecular imprinting technique. Organic polymers are often used in the printing process thus producing the so-called molecular imprinted polymers (MIPs). Using a template, the different monomers are polymerized by temperature, UV radiation or using an initiator. Biosensors with biomimetic receptors (MIMs-Based Bio-Mimetic Sensors) are more stable than biological molecules (enzymes, cells...) and easy to integrate into a device. However, their selectivity is normally lower than that obtained with molecules from biological origin (Vasapollo et al. 2011).

1.2.2.5 Whole cells

In the development of biosensors, living organisms (bacteria, yeasts, fungi) have been used as recognition elements. In this case, the whole cell, rather than a single molecule, is immobilized in the transducer surface. In whole-cell biosensors, for example, the metabolic activity of living immobilized cells is monitored as the analytical signal. When microorganisms are immobilized in a transducer, we refer to such system as a microbial sensor (Su et al. 2011).

Microorganisms offer a good alternative to enzymatic biosensors since, for example, they contain full enzymatic cascades in a protecting envelop (cell membranes and walls, depending on the organisms) that confers some protection to external factors. Additionally, they can be mass produced and do not require complex purification steps (i.e. they can be purified with a simple centrifugation step), which reduces the production costs and the price of the final biosensor. However, the use of whole cells present important problems, for instance: i) the metabolic activity of the cells does not remain continuous over time; ii) it is difficult to achieve selectivity; iii) it is not easy to reproduce because the number of cells can vary between biosensors; and iv) not all immobilization methods maintain cells viability (Wang et al. 2012). Thus, one of the most limiting factors in the development of whole cells biosensors is the stable, bio-compatible and reproducible immobilization of the cells on the transducer.

This thesis is based on microbial biosensors, and particularly, on the optimization of the immobilization protocols to achieve reproducible microbial biosensors for future

implementation in the biosensors market. For this reason, microbial biosensors and immobilization methods will be widely discussed in the following sections.

1.3. State of the art of microbial biosensors

As previously stated, microbial biosensors combine microorganisms, as bio-recognition element, and a transducer to generate a measurable signal proportional to the concentration of the analyte (Lei et al. 2006). These biosensors take benefit from the enzymatic cascade reaction present in the microorganism, normally as part of its own metabolism, to determine the presence of the molecule of interest, either directly as a measurable product/substrate (Sanahuja et al. 2015) or indirectly by the effect on bacterial metabolism (Pujol-Vila et al. 2015, 2016, 2018).

It is important to remark that, in some cases, the same enzymatic cascade reactions present in the microorganisms may be replicated with isolated enzymes, once extracted from the microorganisms and purified (Hold et al. 2016). Thus, microbial and enzymatic biosensors may compete in some applications.

Currently, enzymatic biosensors are preferred by the high selectivity and sensitivity, short time-to-result and simplicity. However, some authors started pointing the advantages of microbial biosensors. D'Souza, for instance, claimed that 90% of enzymes are found within cells and the use of whole cells in the construction of biosensors is a good alternative when the process under scrutiny requires the sequential participation of several enzymes. Apart from that, microbial biosensors present other advantages, e.g. the cost. In this regard, enzymes require purification, which is costly and time-consuming. In contrast, microorganisms are ubiquitous, easy to grow and to produce in large quantities, as well as easy to purify with simple centrifugation steps. Additionally, microorganisms are able to metabolize a large number of chemical compounds (Lei et al. 2006). Finally, it is important to consider that enzymes are unstable in complex biological matrices and environmental conditions (Rocchita et al. 2016).

Therefore, microbial biosensors are positioned as a promising technology in clinical diagnostics, food analysis, bioprocessing and environmental monitoring (Xu et al. 2011, Fracchiolla et al. 2013). The importance of microbial biosensors results from their high specificity and sensitivity, which allows the detection of a broad spectrum of analytes in complex sample matrices (blood, water or food), with minimum sample pre-treatment. The use of viable microorganisms in environmental applications, like determination of the biochemical oxygen demand (BOD), or general toxicity assessment by monitoring the inhibition of microbial respiration by contaminants, are two examples of potential applications.

Microorganisms have been integrated in a variety of transducers that turn a number of biological responses into detectable signals. Table 1 summarizes works published during the last ten years related to the field of microbial biosensing, emphasizing the transduction mechanism, the microorganism used as bio-recognition element, the immobilization strategy, the material employed, the life-time of the sensor and the application.

Table 1. Summary of the main contributions published during the last ten years related biosensors that use microorganisms immobilized using different strategies.

Technique	Microorganism	Immobilization	Material	Viability	Application	Reference
Electrochemical						
amperometry	*G. oxidans,* *P. fluorescens*	adsorption	poly(1-(4-nitrophenyl)-2,5-di(2-thienyl)-1 *H*-pyrrole (SNS(NO2))		respiratory activity	Tuncagil et al. 2009
amperometry	*P. syringae*	adsorption (filtration)	micro-cellular polymer (MCP)		organic compound degradation	Kara et al. 2009
amperometry	*G. oxydans*	entrapment	chitosan		glucose consumption	Odaci et al. 2009
amperometry	*Lactobacillus sp*	entrapment and crosslinking	gelatin	10 days at 4ºC	phenolic compounds detection	Sagiroglu et al. 2011
amperometry	*E. coli*	adsorption	polyaniline	15 days at 4ºC	lindane determination	Anu Prathap et al. 2012
amperometry	*S. cerevisiae*	entrapment	PVA, alginate	4ºC	pesticide biotoxicity	Qian et al. 2014
amperometry	*T. duodecadis*	adsorption (filtration)	cellulose filter		vitamin B12 determination	Ovalle et al. 2015
amperometry	*P. aeruginosa*	entrapment	polypirrole		BOD	Hu et al. 2015
amperometry	*C. violaceum*	entrapment	alginate		BOD	Hooi et al. 2015
amperometry	*P. aeruginosa,* *A. calcoaceticus,* *S. marcescens,* *E. coli,* *S. oneidensis*	entrapment	polypirrole		bacterial activity	Le et al. 2015
amperometry	*G. oxydans*	entrapment	alginate, cellulose sulphate and poly(methylene-co-guanidine)	60 days at 4ºC	2-phenylethanol biooxidation	Schenkmayerová et al. 2015
amperometry	*P. angusta,* *C. curvatus*	entrapment	silane precursors (TEOS, MTES)	8-26 days	effect of heavy metal ions and UV exposure	Ponamoreva et al. 2015
amperometry	*S. cerevisiae,* *E. coli*	adsorption	collagen fibers	several months at 4ºC	BOD	Zhao et al. 2017
amperometry	*G. polyisoprenivorans*	adsorption	glass fiber paper	300 days at 4ºC	BOD	Emelyanova et al. 2017
amperometry	*P.aeruginosa*	entrapment	graphene-polypirrol	60 days	BOD	Hu et al. 2015

amperometry	*S. cerevisiae*	entrapment	chitosan with boron-doped nanocrystalline diamond (BND) particles		biotoxicity assessment	Gao et al. 2017
amperometry	*G. oxydans*	crosslinking	DTP	15 days at 4ºC	glucose detection	Cevik et al. 2018
amperometry	*E. coli*	entrapment	alginate		toxicity determination	Vigués et al. 2018
amperometry	*G. sulfurreducens*	entrapment	silica (sol-gel technique)		artificial bioelectrodes	Estevez-Canales et al. 2018
amperometry	*E. coli*	entrapment	agar, gelatin, agar/gelatin, chitosan, PVA		toxicity determination	Liu et al. 2018
amperometry	*E. coli*	entrapment	polyethersulfone	30 days at -80ºC	toxicity determination	Vigués et al. 2020
amperometry	*E. coli*	adsorption	nanoporus gold (NPG)		cathecol detection	Liu et al. 2019
conductimetry	*B. ammoniagenes*	entrapment	polystyrene sulphonate-polyaniline (PSS-PANI)	7 days at 4ºC	urea detection	Jha et al. 2009
impedance	*E. coli*	adsorption	cysteamine, sulfo-NHS-LC-biotin, and avidin		glucose detection	Borghol et al. 2010
potentiometry	*S. cerevisiae*	entrapment	polyvinyl alcohol (PVA)		organic water pollution	Chiappiani et al. 2010
MFC	*S. algae*	entrapment	alginate/polyaniline/TiO2/graphite	7 days	miniaturize the bio-anode	Szöllösi et al. 2017
potentiometry	*T. thioparus*	entrapment	gelatin		hydrogen sulfide monitoring	Ebrahimi et al. 2014
			Optical			
luminescence	*E. coli*	adsorption	cotton, polyester, viscose rayon, and silk		study of the immobilization capacity and effectiveness	Chu et al. 2009
absorbance	*Sphingomonas sp.*	crosslinking	polystyrene		methyl parathion detection	Kumar et al. 2010
luminescence	*P. leiognathi*	entrapment	agar, carrageenan and alginate	30 days at 4ºC	heavy metals and pesticide detection	Ranjan et al. 2012
absorbance	*E. coli*	entrapment	agarose	28 days at 4ºC	phenolic compounds detection	Shi et al. 2012
SPR	*E. coli*	crosslinking	MPA self-assembled monolayer (SAM)/ EDC/NHS		T4 detection	Xiao et al. 2012
absorbance	*A. fischeri*	entrapment	alginate, cellulose membrane	48 days	heavy metal detection	Futra et al. 2014
luminescence	*P. phosphoreum*	entrapment	PVA	-80ºC	ecotoxicants monitoring	Efremenko et al. 2014
absorbance	*E. coli*	entrapment	alginate		catechol determination	Sanahuja et al. 2015
fluorescence	*E. coli*	entrapment	bacterial cellulose, silk composite matrix		UV light stress study	Drachuk et al. 2017

reflectance	*R. planticola*	adsorption	acrylic microspheres	3 days	nitrite ion quantitation	SNSM Zuki et al. 2018
fluorescence	*H. pylori*	adsorption	polymethylmethacrylate		response to acidic stress	Belova et al. 2018
absorbance	*M. lysodeikticus*	adsorption	(PDDA)-coated-glass		lysozyme detection	Dinca et al. 2018
absorbance	*B. cepacia*	adsorption and crosslinking	polystyrene and glutaraldehyde		organophosphate insecticide detection	Pootawee et al. 2018
fluorescence	*C. vulgaris*	crosslinking	PEI-ECH and PEI-DGDE		cross-linking effect	Vasilieva et al. 2018
SPR	*B. subtilis*	adsorption	gold sensor hydrophobic surface		EPS changes in contact with Cd^{2+} and Pb^{2+}	Zhang et al. 2017

As a rule, most of the microbial sensors gathered in Table 1 have good performances (detection limits and specificity). However, for many of them, fundamental information about repeatability, reproducibility, stability during use and storage and durability, is missing. Careful analysis of the information available allows us to draw some interesting conclusions about the state of the art:

1. Transduction mechanism. Most electrochemical microbial sensors use amperometry (81%) as the transduction mechanism although there are also some examples involving potentiometry and conductimetry. In the case of optical microbial sensors, the transduction method is more evenly distributed between absorbance (40%), fluorescence (20%) and luminescence (20%), with some examples of reflectance and surface plasmon resonance (SPR).

2. Immobilization method. The most common method of immobilization is entrapment (67%), followed by adsorption (25%) and cross-linking (8%). Entrapment is carried out in polymeric and biocompatible matrices. Different materials such as hydrogels, chitosan, polyvinyl alcohol (PVA), silica, agar, agarose, polypyrrole, gelatin, or mixtures of the above are used for the matrices.

3. Microorganisms used. The microorganism that appears with higher frequency in the examples presented in the Table 1 is *E. coli*. Sensors using this microorganism as the recognition element represent 36% of the works found. In second and third place come microbial sensors that use different species of the genus *Pseudomonas* (16%) or *S. cerevisiae* (8%). Most of the biosensors described immobilize a single type of microorganism but in 11% of the cases two or more species are immobilized.

4. Application. Most of the sensors described in Table 1 are used in environmental applications. Among them, detection of environmental pollutants such as heavy metals, phenolic compounds, hydrocarbons or pesticides stand out constituting the main application for 42% of the publications reviewed followed close behind by microbial sensors for the determination of the biochemical oxygen demands (BOD) that comes up in 20% of the contributions, using different microorganisms, methods and immobilization material. A very popular application, as BOD is essential for the management of wastewater treatment processes and conventional methods (BOD_5) require several days to deliver results.

As it is claimed in many of the papers and reviews devoted to microbial sensors, one of the most critical points is the **immobilization (or bio-functionalization) of living microorganism on the transducer**. For suitable performance, microbial biosensors require a close contact between microorganisms and transducer, so that the changes produced by them take place near the transducer. In addition, the bio-recognition elements should be immobilized **i)** in high concentrations (to provide low detection limits and high sensitivities), **ii)** permanently (to guarantee stability), **iii)** in a reproducible manner (to ensure reproducibility and repeatability of the measurement) and **iv)** not compromising their structure or functionality (particularly important

because the sensors require living microorganisms to operate). Microorganisms can be easily inactivated by factors such as high temperature, harsh reagents, UV irradiation, etc., which are often used in the curing process of most of polymeric precursors.

This critical point, the immobilization of living organisms on the transducer, is the main objective of the current thesis and **will be discussed at length in the following section.**

1.3.1 Transducer bio-functionalization in microbial biosensors

Immobilization of microorganisms in a stable matrix in close contact with the transducer has several advantages when compared to the use of cells in suspension (Junter et al. 2004):

1. Viable immobilized cells can proliferate within the support matrix, increasing volumetric reaction rates.
2. Immobilization provides stability and minimizes the risk of cell release.
3. Depending on the matrix and the storage conditions, immobilization can provide a better conservation of the cells.
4. The polymeric matrix protects microbial cells conferring them high resistance against toxic compounds (pollutants, xenobiotics, antibiotics...) and reducing nutrient limitations or environmental stress. This resistance has been observed in alginate entrapped bacteria exposed to sanitizers (Trauth et al. 2001).

Despite the existence of clear advantages in the use of immobilized organisms, the immobilization process itself has been one of the most critical and limiting steps in the development of microbial biosensors.

Between 1950s and 1960s, when the first biosensors were developed based on the use of enzymes, different methods for covalent immobilization of this bio-recognition element were proposed. Unfortunately, most of them were not very efficient and required extensive optimization and validation.

Cell immobilization emerged at the end of 1970s with the first microbial biosensors (Cheetham et al. 1979). Immobilization protocols took as a starting point the covalent bonding protocols developed for enzyme immobilization. From that moment on, bio-functionalization protocols for cells have widely evolved regarding two main factors: the type of immobilization support and the experimental conditions of the immobilization method (Mohamad et al. 2015).

First, the properties of the support material should be adapted to the application and the characteristics of the bio-recognition element in terms of mechanical and chemical stability, durability, biocompatibility or biodegradability, toxicity, porosity, weight, size, swelling or compression, among others (Leenen et al. 1996, Zacheus et al. 2000).

On the other hand, the physicochemical conditions experienced by the microorganisms during the immobilization process are critical to guarantee their structural integrity and activity. As mentioned previously for enzyme sensors, conditions should allow the enzymes to maintain their structural stability and catalytic activity (Datta et al. 2013), but this is even more critical in the development of microbial biosensors where microorganisms should remain alive.

A number of strategies have been used in cell immobilization based on ***adsorption*** (Kalmokoff et al, 2001), ***cross-linking***, ***covalent bonding*** (Schriver-Lake et al. 2002), ***encapsulation/entrapment*** (Salalha et al. 2006, Trelles et al. 2013) (figure 7). These strategies are described below.

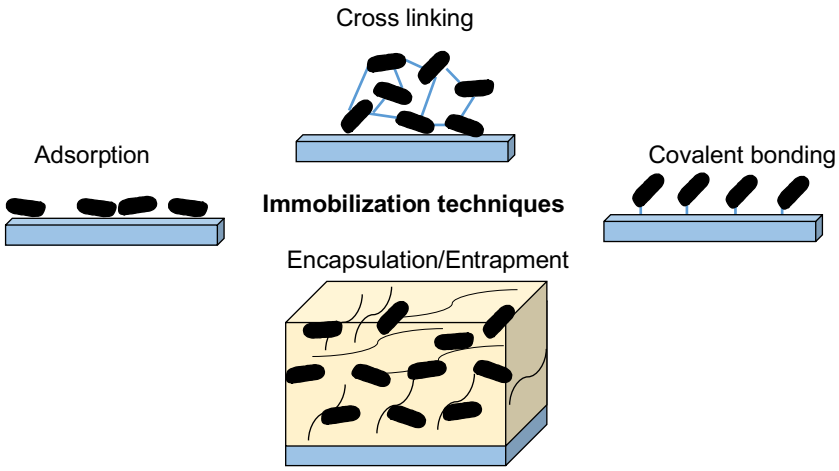

Figure 7. Representation of the different immobilization techniques.

1.3.1.1 Adsorption

Adsorption mechanisms are classified according to the type of interactions as more as physical (Van der Waal forces) or chemical (electrochemical/ionic interactions).

From an experimental point of view, physical adsorption is a simple method of immobilization in which the bio-recognition element is deposited on the support material and, after incubation, the non-absorbed fraction is removed by rinsing with aqueous solutions. Physical absorption can be carried out under static (without agitation) or dynamic conditions (with mechanical agitation).

In chemical adsorption, electrostatic charges attract and immobilize the bio-receptor on the support material. Although any support material can be used to immobilize the bio-receptor, not all of the bio-receptors will immobilize successfully in all supports (Jesionowski et al. 2014). In some cases, it is possible to modify the surface of the sensors with molecules, called carriers, that improve bio-receptor/support affinity and thus the efficiency of immobilization. These carriers can be soluble or insoluble (organic or inorganic compounds according to their origin) (Jesionowski et al. 2014). Common carriers in microbial sensors development are the positively-charged poly-L-lysine, polyethyleneimine (PEI), chitosan or even silanes (Brosel-Oliu et al. 2015). The positive charge of these molecules attracts negatively charged bacteria to the sensor surface for immobilization.

Since absorption is based on weak interactions, immobilization of the biocatalyst on the support material is reversible (long or short depending on the affinity between bio-receptor and support

material), difficult to control and not reproducible due to the difficulty to control the density of microbial cells that are immobilized (Homaei et al. 2013) (figure 8).

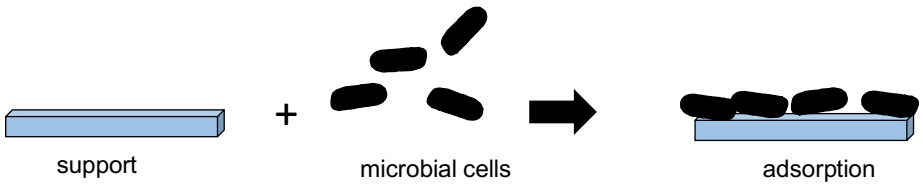

Figure 8. Scheme of the adsorption immobilization technique.

1.3.1.2 Cross-linking

Cross-linking is an irreversible immobilization protocol that depends on the creation of intermolecular bonds between the biocatalyst and the cross-linker, a molecule with high reactivity that can interact with at least two more elements to create an aggregate (figure 9). One of the most common cross-linkers is glutaraldehyde that can react with two amine groups from the bio-receptor creating an aggregated matrix of bio-recognition material.

The polymerization reaction differs widely depending on the nature and mechanism of the cross-linker. Some cross-linkers are photo-activated and require exposure to selective wavelengths, e.g. ultraviolet (UV) light, while others are sensitive to temperature (Mohammad et al. 2015).

In the case of microbial biosensors, cross-linking methods create strong bonds between microorganisms and are commonly used for obtaining immobilized non-viable cell preparations containing active intracellular enzymes (Abelyan 2000).

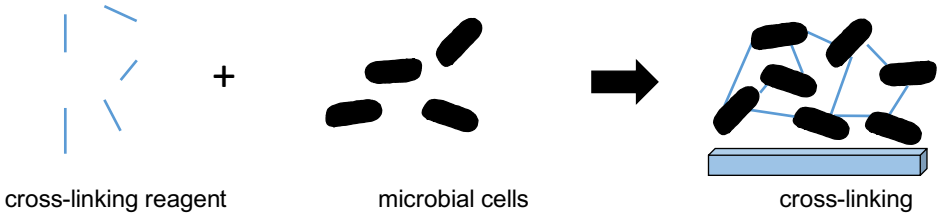

Figure 9. Scheme of the cross-linking immobilization technique.

1.3.1.3 Covalent bonding

Covalent bonding methods involve the formation of covalent bonds between the support surface and external functional groups of the bio-catalyst (figure 10). The functional groups more commonly present in proteins and biological material are amino, carboxyl, phenol, indole or imidazole groups (Datta et al. 2013). These groups are preferably attached to polymeric or silicon-based substrates, such as polysaccharides, polyvinyl alcohol, mesoporous silica and porous glasses to quote the most usual (Ispas et al. 2009).

The irreversible and stable attachment of the bio-receptors to the support increases the average lifetime and thermal stability of the microbial cells. As a disadvantage, structure and function of the bio-receptors may be altered during covalent bonding, leading to an important loss of activity. For this reason, covalent bonding is rarely used for the immobilization of living microorganisms (Valuev et al. 1994).

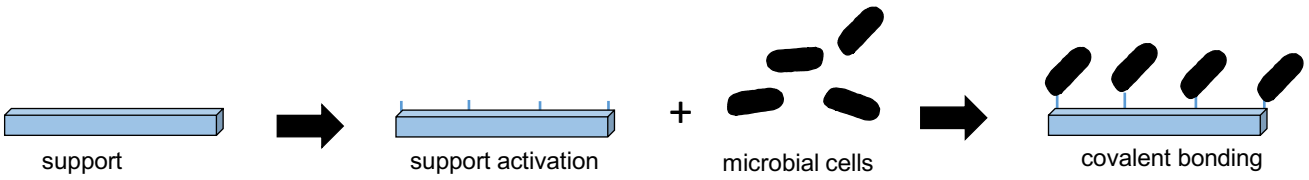

Figure 10. Scheme of the covalent bonding immobilization technique.

1.3.1.4 Entrapment

Entrapment is usually an irreversible immobilization method based on physical trapping of the sensing element inside a small-size porous matrix or a hollow fiber (figure 11). With this strategy, the matrix creates a protective barrier around the immobilized bioreceptors that prevents them from escaping, while allowing the free circulation of analytes or metabolites by diffusion. In general, the entrapment matrix provides the microbial cells with a physiological microenvironment (Mohamad et al. 2015). In opposition to covalent bonding where the bioreceptor may loss activity due to changes in the structure/function or a non-suitable orientation of the recognition regions, in entrapment methods, the biomolecules are immobilized in a matrix cavity that does not compromise their structure or activity. Additionally, entrapment processes are normally fast, cheap and use mild conditions, which make them very attractive for the immobilization of sensitive bioreceptors, particularly microbial cells.

For all these reasons, and as previously stated, entrapment is the most widely reported method in the development of microbial biosensors (Costnier et al. 1999, Gupta et al. 2007, Trelles et al. 2013). In the development of entrapment microbial sensors, the biocompatibility of both the matrix and the polymerization procedure are additional factors to be considered since the sensor requires living cells to be functional. Thus, the selection of a suitable entrapment matrix is essential to guarantee the functionality of the microbial biosensor (D'Souza 2001, Gupta 2007). Two types of polymers, namely natural and synthetics polymers, have been successfully applied to cell immobilization (Nwankwegu AS 2017). From the variety of natural polymers, hydrogels are the most frequently used in bacterial entrapment. Agar, alginate, agarose or chitosan are examples of this kind of materials. Hydrogels are water-swollen and cross-linked polymer networks produced by the polymerization of simple monomers (Ullah et al. 2015). The formation of hydrogels occurs under soft conditions and they retain a significant fraction of water within

their structure, which makes them suitable to preserve biological functions. Their main limitation, also expanded to other natural polymers, is their low mechanical strength, durability and stability.

Alternatively, synthetic biopolymers present mechanical strength, durability, high retention of water, have the capacity to produce well-defined structures and are able to stand sharp and strong fluctuations of temperature (Webb and Dervakos 1996, Ahmed et al. 2015). However, these materials require polymerization protocols that may compromise microbial activity. This is the case of PVA, one of the most reported synthetic polymers in microbial biosensors development. PVA is an excellent biodegradable and water-soluble biopolymer with excellent mechanical properties and durability. However, chemical and/or physical crosslinking methods are needed to stabilize PVA membranes, including chemical crosslinking with glutaraldehyde, formaldehyde, sulfur-succinic acid or boric acid as well as physical modification by UV irradiation or cyclic freezing–thawing. Other examples of synthetic polymers used in microbial biosensors development are poly(carbamoyl)sulphonate (PCS), polyhydroxyethylmethacrylate (polyHEMA), polyacrylamide, polyurethane or poly(ethylene glycol) prepolymer), among others.

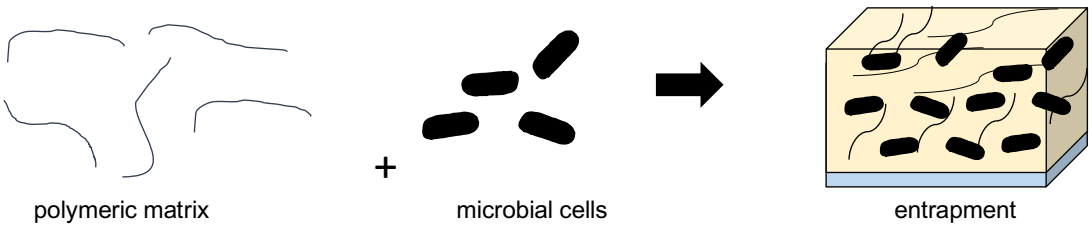

Figure 11. Scheme of the entrapment immobilization technique.

In order to analyze how these immobilization principles have been carried out in practice, we have collected a number of relevant works published during the last 10 years describing immobilization of microorganisms using different strategies for a host of diverse applications ranging from biosensing to ethanol production. These publications, summarized in Table 2, have been grouped by immobilization method.

Table 2. Summary of different publications describing the immobilization of microorganisms using different methods and materials.

Microorganism	Material	Viability	Application	Authors
Entrapment				
E. coli	agar, gelatin, agar/gelatin, chitosan, PVA		toxicity determination	Liu et al. 2018
E. coli	agarose	*28 days at 4ºC*	phenolic compounds detection	Shi et al. 2012
C. violaceum	alginate		BOD	Hooi et al. 2015
E. coli	alginate		water toxicity monitoring	Wasito et al. 2019
E.coli	alginate		toxicity determination	Vigués et al. 2018
E.coli	alginate		catechol determination	Sanahuja et al. 2015
A. fisheri	alginate, cellulose	45 days	heavy metal detection	Futra et al. 2014
S.algae	alginate/polyaniline/TiO_2/graphite	7 days	bio-anode	Szöllösi et al. 2017
G.oxidans	alginate, cellulose, sulphate and poly(methylene-co-guadine)		2-phenylethanol biooxidation monitoring	Schenkmayerová et al. 2015
S. cerevisiae	alginate, methacrylic alginate		bioethanol production	Cha et al. 2012
Sphingomonas sp	alginate, PVA	90 days at 4ºC	phenol degradation	Ruan et al. 2018
S. cerevisiae	alginate, PVA	4ºC	pesticide toxicity assay	Qian et al. 2014
Nitrifying bacteria	alginate, PVA		ammonia-oxidizing activity	Bae H et al. 2014
E. coli	alginate, Fe_3O_4		growth and viability analysis	Kiprono S.J. et al. 2018
S. cerevisiae	chitosan with diamond particles		biotoxicity assessment	Gao et al. 2017
Lactobacillus sp	gelatin	10 days at 4ºC	phenolic compounds detection	Sagiroglu et al. 2011
T. thioparus	gelatin		hydrogen sulphide monitoring	Ebrahimi et al. 2014
Thermosipho sp. *T. kodakarensis*	gellan and xanthan gums		growth capacity, mechanical stability	Landreau M. et al. 2016
E. coli	polyethersulfone	1 month at -80ºC	toxicity determination	Vigués et al. 2020
P. aeruginosa *A. calcoaceticus* *S. marcescens* *B. subtilis* *E. coli*	polypyrrole		novel technique for viable immobilization	Tokonami et al. 2012
Pseudomonas sp.	silica	1 month	PHA degradation	Sakkos et al. 2016

Synechococcus *Cyanothece*	silica	2-12 weeks	viability and activity	Rooke et al. 2018
Synechocystis sp	silica		hydrogen production	Dickson et al. 2009
E. coli	silica	60 days at 4ºC	effects of sol−gel entrapment on the metabolic pathways	Eleftheriou et al. 2013
E. coli *L. elongisporus*	silica		continuous-flow cascade reactions	Nagy-Györ et al. 2018
Sphingomonas sp	silica	28 days at 4ºC	phenol degradation	Li et al. 2016
Chlamydomonas reinhardtii	silica, chitosan		biocompatibilty and growth	Homburg et al. 2019
Synechocystis sp	silica (sol-gel technique)		review biotechnology applications	Dickson and Ely 2013
G. sulforeducens	silica (sol-gel technique)		artificial bioelectrodes	Estevez-Canales et al. 2018
A. tertiaricarbonis	ceramics (sol-gel technique)	1 day at 4ºC	MTBE degradation	Pannier et al. 2010
P. angusta *C. curvatus*	silane precursors (TEOS, MTES)	8-26 days	effect of heavy metal ions and UV exposure	Ponamoreva et al. 2015
Rhodococcus sp. *A. tumefaciens*	PVA-PAC (powdered activated carbon)		C8-HSL removal	Zeng Z. et al. 2018
P.aeruginosa	graphene-polypirrol	60 days	BOD	Hu et al. 2015
S. cerevisiae	Poly(glycerol) silicate	60% of viability on 10 days	viability	Harper J.C. et al. 2011
P. aeruginosa *A. calcoaceticus* *S. marcescens* *E.coli* *S. oneidensis*	Polypirrole		bacterial activity	Le et al. 2015
Adsorption				
R. planticula	acrylic microspheres	3 days	nitrite ion quantification	SNSM Zuki et al. 2018
T. duodecadis	cellulose filter		vitamin B12 determination	Ovalle et al. 2015
S. cerevisiae *E.coli*	collagen fibers	several months at 4ºC	BOD	Zhao et al. 2017
G. polyisoprenivorans	glass fiber paper	300 days at 4ºC	BOD	Emelyanova et al. 2017
B. subtilis	gold		EPS changes for Cd^{2+} and Pb^{2+}	Zhang et al. 2017
E.coli	nanoporus gold		cathecol detection	Liu et al. 2019

E. coli	polyaniline films	15 days at 4ºC	lindane determination	Prathap et al. 2012
H pylori	polymethilmethacrylate		response to acidic stress	Belova et al. 2018
M. lysodeikticus	PDDA-coated-glass		lysozyme detection	Dinca et al. 2018
Crosslinking				
G.oxydans	*DTP*	15 days at 4ºC	glucose detection	Cevik et al. 2018
E.coli	MPA self-assembled monolayer/EDC/NHS		T4 detection	Xiao et al. 2012
C. vulgaris	*PEI-ECH and PEI-DGDE*		crosslinking effect	Vasilieva et al. 2018

Of the different publications appearing in Table 2, 60% immobilize microorganisms to develop biosensors, either electrochemical or optical. The remaining 40% immobilize microorganisms in different supports to exploit their activity and improve the efficiency of certain applied processes. As an example, in one case, the authors immobilize *A. tertiaricarbonis* in ceramic biofilters to increase the capacity of the organism to degrade recalcitrant environmental pollutants during the treatment of contaminated water (Pannier et al. 2010). In these applications, the ability to achieve high concentrations of microorganisms improves degradation rates or increases production of a substance of interest, as happens during production of hydrogen by *Synechocystis* immobilized in sol-gel silica (Dickson et al. in 2009).

If we consider the type of immobilization used, cross-linking appears is 6% of the cited publications. In these cases, the authors use three different cross-linkers: glutaraldehyde (Cevik et al. 2018), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl) and N-hydroxysuccinimide (NHS) (Xiao et al. 2012) or epichlorohydrin (ECH) - diethylene glycol diglycidyl ether (DGDE) (Vasielieva et al. 2018). In all cases, cross-linkers reduce the viability of trapped microorganisms due to their high toxicity. Probably due to this reason, the use of this type of approach is not extended. Adsorption represents 20% of the publications cited. In this type of immobilization, microorganisms are reversibly adhered to different surfaces without using chemical substances that create covalent bonds as happens with cross-linkers. In many cases filtration is used to increase the efficiency of immobilization as for example, in the work of Ovalle et al. published in 2015. Finally, the most widely used immobilization procedure, representing 74% of the cases, is entrapment. A variety of matrices are used for this purpose with materials as diverse as agar, agarose, alginate, chitosan, gelatin, polypyrrole or silica, among others. The two materials used more extensively according to the examples listed in Table 2 are alginate and silica.

Alginate is a hydrogel that is often used to encapsulate microorganisms within beads or microspheres for different applications, for example, to determine toxicity (Wasito et al. 2019, Futra et al. 2014, Cha et al. 2012, Ruan et al. 2018 and Bae et al. 2014). In other instances, alginate is deposited as thin films on various supports or within confined structures (Sanahuja et al. 2015). Chitosan and gelatin are also used for immobilization, but to a lesser extent. The remaining matrices of the table are synthetic polymers with less water retention, but which have adequate porosity to allow mass transfer. Silica has good mechanical properties, is biocompatible, has suitable characteristics of porosity in its structure and displays good durability and storability of the matrix. Thus 25% of the papers cited use this material as matrix entrapment.

Polypyrrole is a conductive polymer that polymerizes under controlled conditions, allows controlling the thickness of the thin sheet it generates and the amount of entrapped bacteria. This

material resists a wide range of pH and allows immobilizing a large number of viable cells, metabolically active for multiple applications.

Apart from a variety of matrices, these works also presented a variety of immobilized microorganisms, although the immobilization of *E.coli* stands out with 25%, followed by the use of different species of *Pseudomonas* and *Saccharomyces cerevisiae*.

In terms of survival of the immobilized organisms during storage, less than half of the publications provide any information about the topic. The remaining half provides information that indicates that survival of the immobilized organisms after immobilization varies greatly, from no more than one day to more than 300 days. Storage conditions are not always specified, but when they are, temperature is always 4ºC or below.

All the data shown above, indicate clearly the existence of a host of contributions during the last ten years that aim at immobilizing active, functional bacteria for a number of different applications, most prominently, biosensing. Careful analysis of these contributions indicates the existence of a number of issues that plague immobilization technologies and that hamper the development of reliable and robust microbial based biosensors. The main issues these immobilization techniques have been dealing with, with varying degrees of success are: reproducible entrapment of high numbers of microorganisms, high matrix porosity to allow for nutrient and analyte diffusion, survival during entrapment and durability during storage. The information available until now indicates that the problem of achieving a robust microbial sensor fulfilling these characteristics is far from being solved. **The aim of this thesis is precisely to contribute to progress in this field by addressing the topic of microbial immobilization in biosensor development by exploring novel and advanced ideas that lead to testable prototypes.**

The work described in this memory has progressed along two main lines of research. In one of them it has been explored the entrapment of bacteria by electrodeposition of alginate hydrogels at the electrode surface in conditions that allowed precise control of both, the thickness of the hydrogel and the amount of microorganisms immobilized. To improve the results obtained in this first approach, the alginate matrix was doped with different concentrations of graphite to make it conductive and increase the response signal. The second line has been focused on the use of polyethersulfone, a synthetic organic polymer, to entrap the microbial cells. The process of immobilization is unprecedented and uses non-solvent induced phase separation in the presence of bacterial cells to fabricate a microbial polyethersulfone membrane. Through this process, microorganisms are trapped and strongly retained within the matrix while maintaining their viability.

In both chapters, the matrices are characterized taking into account the reproducibility of the method, survival during entrapment and possible storage. In addition, both matrices are tested

in the development of amperometric microbial biosensors for toxicity detection. Biosensing is performed electrochemically through ferricyanide respirometry, with metabolically-active entrapped bacteria reducing ferricyanide in the presence of glucose. The analytical performance of the sensors is assessed using 3,5-dichlorophenol (DCP) as a model toxic compound.

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CHAPTER 2

Electro-addressable conductive alginate hydrogel for bacterial trapping and general toxicity determination

2. Electro-addressable conductive alginate hydrogel for bacterial trapping and general toxicity determination

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Abstract

In biosensors development, alginate hydrogels are a first choice for enabling stable biomolecules entrapment in biocompatible membranes obtained under soft physiological conditions. Although widely exploited, most alginate membranes are isolating and poorly repetitive, which limit their application in biosensing. Significant steps forward on improving repeatability and conductivity have been performed, but to date there is no single protocol for controlled deposition of live cells in replicable conductive alginate layers. Here, cell electrotrapping in conductive alginate hydrogels is examined in order to overcome these limitations. Conductive alginate-coated electrodes are obtained after potentiostatic electrodeposition of graphite-doped alginate samples (up to 4% graphite). The presence of graphite reduces electrode passivation and improves the electrochemical response of the sensor, although still significantly lower than that recorded with the naked electrode. Bacterial electrotrapping in the conductive matrix is highly efficient ($4.4x10^7$ cells per gel) and repetitive (CV < 0.5%), and does not compromise bacterial integrity or activity (cell viability = 56%). Biosensing based on ferricyanide respirometry yielded four times increase in biosensor response with respect to non-conductive alginate membrane, providing toxicity values completely comparable to those reported. Cell electrotrapping in conductive hydrogels represents a step forward towards high-sensitive cell-based biosensors development with important influence in environmental analysis, food and beverage industry as well as clinical diagnosis.

2.1 Introduction

Biosensors development requires stable immobilization of biomolecules, including enzymes, antibodies, aptamers and cells, among others, on the transducer surface. A good immobilization method may allow to control and maintain the amount of biomolecules without compromising the integrity and activity of the biomolecule or the biosensor performance. Cell immobilization is particular because, apart from previous stability and reproducibility requirements, the immobilization methods should ensure cell viability and metabolic activity (Bickerstaff 1996). The methods for cell immobilization include adsorption (Klein and Ziehr 1990, Kalmokoff et al. 2001, Fleming and Wingender 2010), covalent binding (Shriver-Lake et al. 2002), encapsulation

(Sultana et al. 2000, Salalha et al. 2006) and entrapment. Although this variety, cell entrapment in polymeric matrices is advantageous for preserving cell integrity and viability, at the time that ensures stability in a physiological environment (Cosnier 1999, Gupta et al. 2007).

A number of polymeric matrices have been used for entrapping cells, such as gellan/xanthan matrix (Landreau et al. 2016), SiO_2 sol-gel (Inama et al. 1993), PVA/alginate (Bae et al. 2014), alginate (Sanahuja et al. 2015) and polypyrrole (Tokonami et al. 2012, Le et al. 2015), among others. From all of them, alginate is one of the preferred for enabling gel formation at very soft experimental conditions, i.e. room temperature, aqueous medium and pH 7, through a cation-mediated cross-linking process (Lee et al. 2012, Sanahuja et al. 2015). Additional advantages are in the low toxicity, low antigenicity and permeability of these membranes, allowing the diffusion of small molecules (Lee et al. 2012, Varaprasad et al. 2017). Main limitations of alginate hydrogels for biosensors are the lack of standardization and repeatability of the gelling protocols, and the electrical insulation of alginate membranes, particularly relevant in the development of electrochemical biosensors. It is worth mentioning that electrochemical transduction, for simplicity, cost-efficiency and miniaturization/integration capacities is still the first choice in biosensors development.

In recent years, significant efforts to improve hydrogels repeatability and conductivity have been performed in separate. Regarding repeatability and homogeneity of the hydrogels, electrodeposition techniques are preferred for providing strict control of the deposition region and the architecture of the hydrogel, i.e. size, shape, thickness and porosity (Liu et al. 2009, Cheng et al. 2011, Márquez-Maqueda et al. 2016, Márquez et al. 2017), properties that can be modulated by adjusting the potentiostatic conditions (Márquez et al. 2017). Mammalian and bacterial cells have been successfully immobilized by alginate electrodeposition (Shi et al. 2009, Cheng et al. 2012, Betz et al. 2013, Ozawa et al. 2016). On the other hand, a growing trend to improve hydrogel conductivity is the inclusion of conductive nanomaterials within the polymer matrix (Le et al. 2017). Conductive hydrogels based on graphite (Qu et al. 2014), graphene (Xu et al. 2010, Zhang et al. 2011) and carbon nanotubes (MacDonald et al. 2008), have attracted a great interest (Guiseppi-Elie 2010). These conductive matrices combine advantages of hydrogels and conductive composites in a hybrid material, the properties of which can be modulated by shifting the ratio between the alginate and the doping conductive nano-material (Le et al. 2017). These conductive hydrogels have been used to immobilize bacteria and to demonstrate their electroactivity for energy production (Mottet et al. 2018). However, to the best of our knowledge, both improvements have been never combined in a single electrodepositable and conductive alginate hydrogel for cell trapping.

In this work, bacterial electrotrapping in conductive alginate hydrogel-coated electrodes is examined and applied to biosensing. Graphite microparticles are used as conductive doping

material. The analytical performance of the conductive hydrogel-coated electrodes is characterized and hydrogel thickness is measured by profilometry. The biocompatibility and reliability of the electrotrapping protocol is assessed by confocal microscopy and plate counting. Biosensing is performed based on ferricyanide respirometry (Catterall et al. 2010, Pujol-Vila et al. 2015, 2016, 2018) and applied to toxicity assessment.

2.2 Materials and methods

2.2.1 Chemicals

Alginic acid, calcium carbonate, calcium chloride, potassium ferricyanide, potassium ferrocyanide, glucose, graphite, 3,5-dichlorophenol, potassium di-hydrogen phosphate and di-potassium hydrogen phosphate 3-hydrate were purchased from Panreac (Spain) and were of analytical grade and all the solutions were prepared with distilled water.

2.2.2 Alginate electrodeposition and electrochemical analysis

Gold screen printed electrodes (SPE; Dropsens 220BT) integrating a round gold working-electrode (4mm of diameter), a gold counter-electrode and an Ag pseudo-reference electrode were used in this work. Alginate electrodeposition and electrochemical measurements were performed with a Dropsens μSTAT8000 potentiostat controlled by Dropview 8400 software.

Briefly, precursor solution containing 1% (w/v) sodium alginate, 0.125% (w/v) $CaCO_3$, graphite (between 0 and 4% w/v) and a bacterial concentration up to $1x10^9$ colony forming units (cfu) per mL were prepared in distilled water. Graphite was pre-treated and introduced in the solution following the protocol described elsewhere (Yong et al. 2013).

A volume of 100μL of the precursor solution was dropped on the SPE surface and electrodeposited at 1.5V (vs. Ag pseudo-reference) for 90 seconds. Thick and stable conductive alginate hydrogels were obtained.

2.2.3 Microorganisms

Escherichia coli ATCC 10536 was grown aerobically in Luria-Bertani (LB) broth for 18 h at 37ºC in a shaker bath (110 rpm). Grown cultures were centrifuged at 10100g for 10 minutes and re-suspended in distilled water. For cell quantification, optical density of re-suspended bacteria was

measured at 550 nm in a SmartspecTM Plus spectrophotometer (BioRad). Agar plate counting was carried out for viable cell determination.

2.2.4 Confocal microscopy

Three-dimensional reconstructions of bacteria-containing conductive alginate hydrogels images were used to evaluate the biocompatibility of the electrodeposition protocol (through the number of live and dead bacteria) and the distribution of bacteria in the gel.

Electrotrapped bacteria were stained with the Live/Dead Invitrogen Kit BacLight (Invitrogen) following the protocol detailed by the supplier. The conductive alginate hydrogel was incubated in 100µL of Live/Dead staining solution for 15 minutes, washed with water (three times) and imaged by confocal microscopy (Leica TCS SP5) at excitation wavelength of 470 nm. Z-stacks were acquired every 1 µm for a total thickness of 300 µm. Reconstruction of individual stacks was performed with the IMARIS software. In the three-dimensional reconstruction, live bacteria, stained with SYTO9, appeared in green (emission wavelength = 630 nm), while dead bacteria, stained with propidium iodide, emitted in the red region of the visible spectra (emission wavelength = 530 nm).

2.2.5 Profilometer

Hydrogel thickness was measured with an optical profilometer PLµ2300 from Sensofar controlled with PLµ Confocal Imaging Profiler.

2.3. Results and discussion

2.3.1 Conductive alginate hydrogel

Conductive alginate hydrogels were produced by electrodeposition of precursor solutions doped with 1, 2 and 4% (w/v) graphite and compared with non-conductive hydrogels (Figure 12a). Above 4% hydrogels presented poor colloidal stability and were discarded. The presence of graphite doping particles in the precursor solution increased the thickness (Figure 12b), the electrodeposition current and the electrodeposition charge, the latter calculated as the variation of electrodeposition current over time (Figure 12c). Concretely, the charge associated to the electrodeposition process and the hydrogel thickness increased linearly with the graphite concentration in the precursor solution (Figure 12d). This result suggested that the presence of conductive graphite particles improve charge transfer during electrodeposition producing thicker, more stable and more repetitive (n=12, CV < 0.3%) hydrogels.

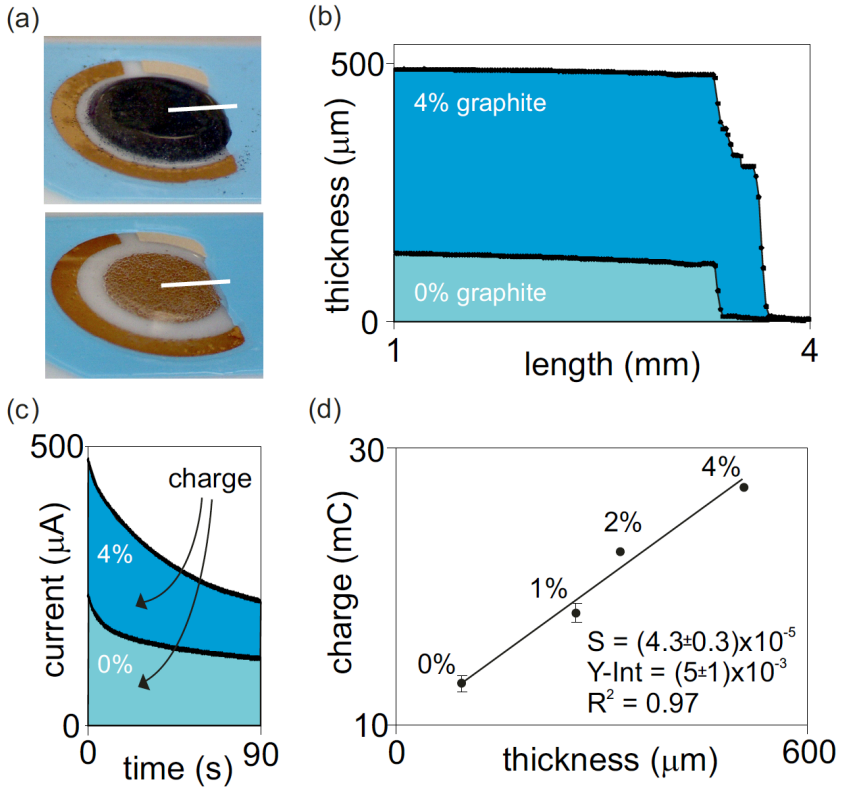

Figure 12. a) Image of conductive (dark) and non-conductive hydrogel (transparent) electrodeposited on working electrode. Inset, a white line indicating the region analysed by profilometry. b) Profiles of the conductive (dark blue) and non-conductive (clear blue) hydrogels obtained by profilometry. c) Electrodeposition current curves for the two hydrogels, with the associated electrodeposition charge. d) Representation of the variation of the charge (mC) and thickness of hydrogels with 0%, 1%, 2% and 4% graphite concentrations.

The electrochemical behaviour of the hydrogels was examined by cyclic voltammetry using equimolar mixtures of ferricyanide and ferrocyanide (10mM). Cyclic voltammetry measurements of the hydrogels were carried out 5 minutes after sample inoculation to ensure diffusional equilibrium and compared with naked electrodes. Results are illustrated in Figure 13. The presence of non-conductive alginate membranes reduced a 73% the anodic current of the electrode. A progressive increase was observed when increasing the graphite concentration in the hydrogel. Best performance was obtained by alginate hydrogels containing 4% graphite, which presented a reduction of only 36% with respect to the naked electrode. Graphite-doping, therefore, enhanced the electrochemical response of alginate-coated electrodes thus overcoming some of the traditional limitations of electrically passivating polymers. Hydrogels containing more than 4% graphite were expected to present better electrochemical behaviours, but those were not possible to obtain with the current electrodeposition protocol.

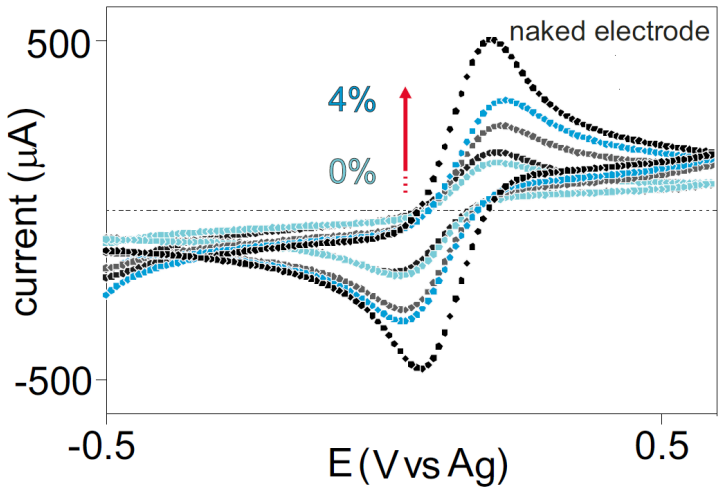

Figure 13. Cyclic voltammograms corresponding to the measurement of equimolar mixtures ferricyanide/ferrocyanide (10mM) with alginate hydrogels containing 0%, 1%, 2% and 4% graphite concentration. The cyclic voltammetry corresponding to the naked electrode (black dots) is included by comparison. Measurements are performed at a scan rate of 50mV·s^{-1}.

2.3.2 Bacterial electrotrapping in conductive alginate hydrogel

Bacterial suspensions of *Escherichia coli* (*E. coli*) from 10^6 to 10^9 cfu·mL^{-1} were mixed with the precursor solution and electrodeposited in conductive (4% graphite) and non-conductive alginate membranes under optimal conditions. Hydrogel formation was achieved in bacterial suspensions containing up to (1.52±0.02)x10^8 cfu·mL^{-1}. Higher bacterial concentrations were too isolating and impeded electrodeposition, being discarded for future experiments. Unless otherwise stated, the maximum bacterial concentration enabling electrodeposition was used in subsequent experiment for providing maximum sensitivity in the development of general toxicity biosensors based on microbial metabolism.

To evaluate the effect of cells on electrodeposition of conductive and non-conductive hydrogels, cell viability and the thickness of bacterial hydrogels was determined and compared with that corresponding to hydrogels without bacteria. Results are presented in Figure 14.

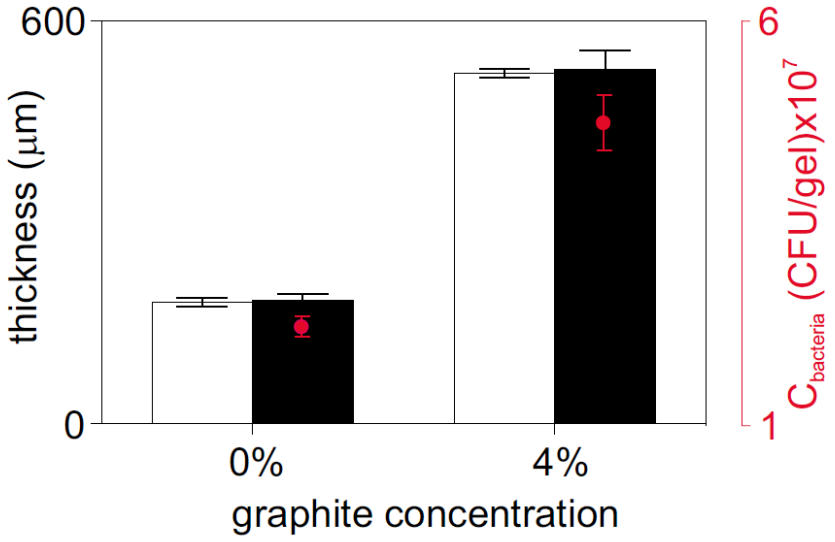

Figure 14. Variation of the thickness of non-conductive (0%) and conductive (4%) alginate hydrogels in absence (white) or presence (black) of *E. coli* bacteria. Red dots in the plot correspond to bacterial concentration entrapped in the hydrogels determined by agar plating.

As shown, the presence of bacteria had an almost negligible contribution to hydrogel thickness in any case. At the same time, plate counting of hydrogels dissolved with 0.1M phosphate buffer demonstrate high cell viability in the hydrogels, i.e. 1.4 x 10^7 cfu·gel^{-1} for non-conductive and 4.4 x 10^7 cfu·gel^{-1} for conductive hydrogels (n = 10, CV < 0.1%), which demonstrated that electrodeposition did not compromise cell viability. The difference in the number of living bacteria in each case was consistent with the higher thickness of conductive alginate hydrogels, which retained more bacteria. In fact, it was a linear correlation between graphite concentration, hydrogel thickness and bacterial concentration (supplementary information) suggesting that electrotrapped cell quantity was proportional to hydrogel thickness (and thus to graphite content). Additionally, electrodeposited cell-containing hydrogels were stained with Live/Dead fluorophores and imaged by confocal laser microscopy. Due to the high opacity of graphite-doped hydrogels, only transparent non-conductive hydrogels were used in this case. The 3D reconstruction of the confocal images and three selected planes are illustrated in Figure 15.

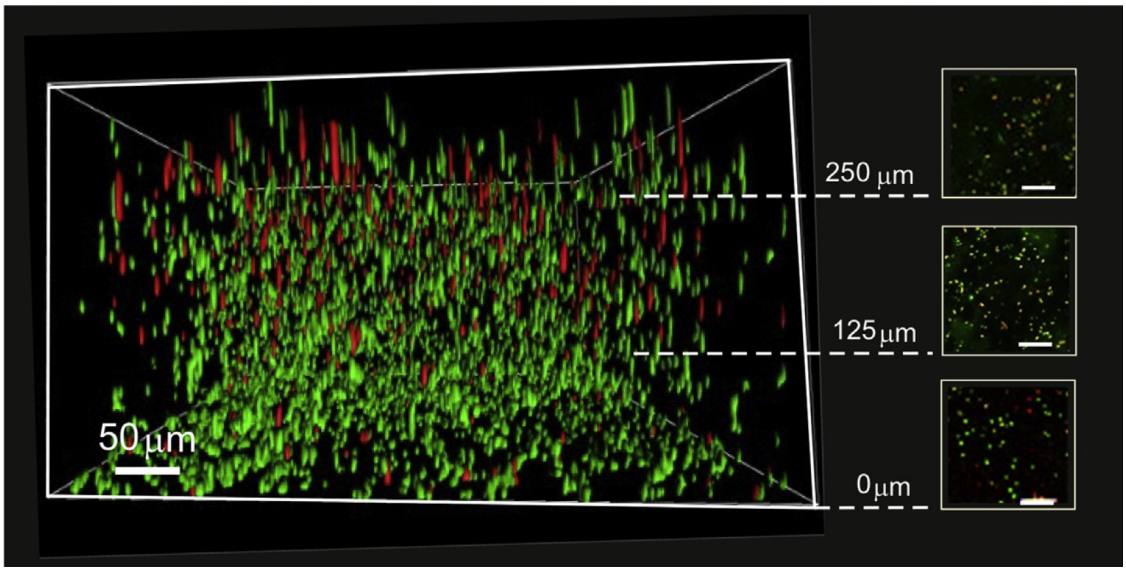

Figure 15. Confocal 3-D image reconstruction image corresponding to a non-conductive alginate hydrogel containing bacteria. Due to the staining, live cells appear in green and dead cells in red. Three individual stacks corresponding to a height of 0, 125 and 250 μm versus the electrode surface are also included to demonstrate the homogeneous distribution of cells in the gel. Scale bar in the stacks corresponds to 25 μm.

The reconstruction showed a homogeneous vertical distribution of living cells (green) and dead cells (red) along the gel, which coincided with the analysis of individual stacks at different depths (0, 125 and 250 μm). The elongated morphology of the cells in the upper part of the image appeared due to depth of field limitations of the microscope. Quantitative image analysis revealed that 55±9% of total electrotrapped bacteria were alive, which was in agreement with the percentage observed in *E. coli* overnight cultures (66±15%).

Considering these results, the electrodeposition conditions may be considered harmless for cell entrapment and validate electrotrapping in the generation of cell-based biosensors with viable bacteria.

2.3.3 Cell-based conductive alginate biosensor

Electrodeposited conductive alginate hydrogels containing *E. coli* were employed in the production of biosensors for general toxicity assessment. Bacterial metabolism of entrapped bacteria is used to determine the toxicity of samples through ferricyanide respirometry (Catterall et al. 2010, Pujol-Vila et al. 2015, 2016, 2018). Briefly, ferricyanide acts as a final electron acceptor for bacterial metabolism. That is, living and metabolically active bacteria are able to reduce ferricyanide to ferrocyanide in their electron transport chains, whereas dead or

metabolically inactive cells are unable to reduce this redox compound. Therefore, ferrocyanide production over time is directly proportional to bacterial metabolic activity and thus, to the number of live bacteria.

For toxicity assessment, a 100 µL 1 mM ferricyanide solution supplemented with 0.2% glucose was inoculated to the hydrogel-containing electrode. Chronoamperometry recordings were then acquired at 0.4 V (vs Ag pseudoreference), corresponding to the ferrocyanide oxidation potential, for a maximum of 90 minutes, to evaluate the accumulation of metabolically-produced ferrocyanide. Current values of conductive (4% graphite) and non-conductive alginate hydrogels were acquired at 0, 30, 60 and 90 minutes of assay (Figure 16a). Both conductive and non-conductive hydrogels showed a progressive increase in current magnitude over time resulting from the metabolic production of ferrocyanide due to bacterial activity. The main difference between them was in the current magnitude. Conductive hydrogels showed larger current values due to (i) the presence of graphite particles, which improve charge transfer between redox molecules and the electrode, and (ii) the number of bacteria, higher in the thicker conductive membrane as discussed before. It is worth mentioning that the hydrogels lost their consistency after 90 minutes of accumulation and the optimal time assays was set at 60 minutes.

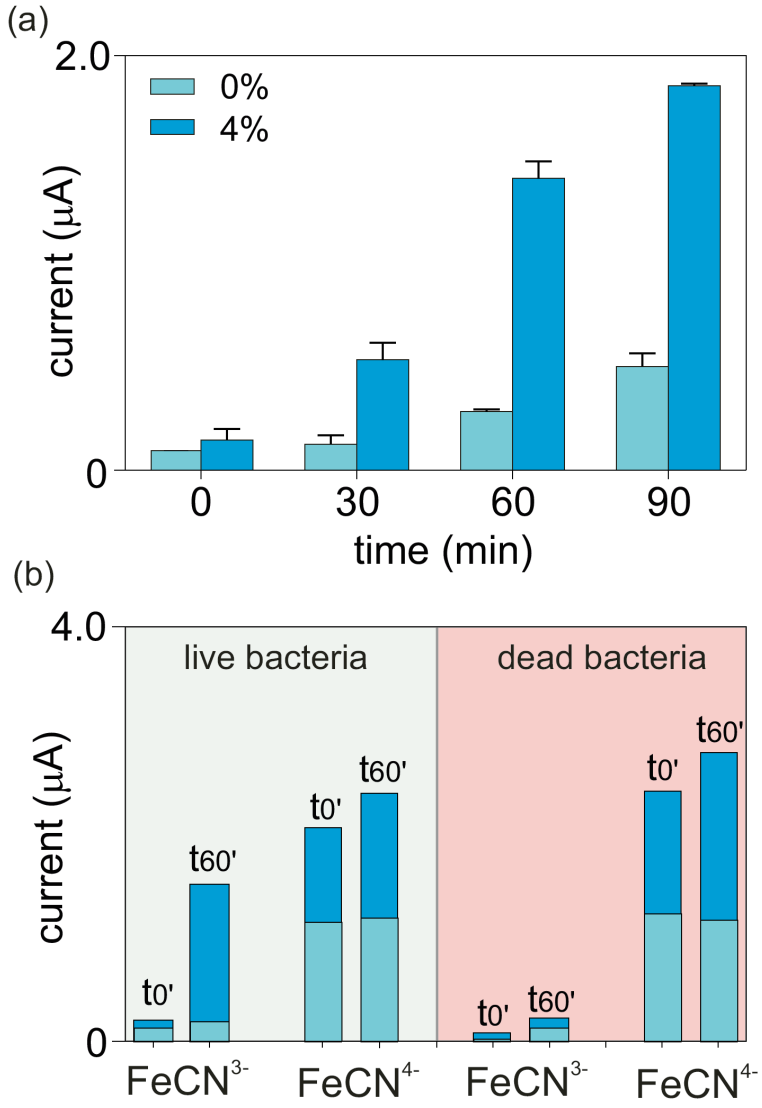

Figure 16. a) Variation of the current values at 0.4 V with the accumulation time for conductive (dark blue) and non-conductive (clear blue) alginate hydrogels containing $1.5x10^8$ cfu·mL^{-1} bacteria. The accumulation time is defined as the time that entrapped bacteria are in contact with 1mM ferricyanide (supplemented with 0.2% glucose) to metabolically produce ferrocyanide, which is finally measured at 0.4 V. b) Current values of live and dead bacteria at the beginning of the experiment and after 60 minutes of incubation with ferricyanide or ferrocyanide solutions supplemented with 0.2% glucose.

To ensure the metabolic origin of ferrocyanide, the response of conductive and non-conductive hydrogels containing live and dead bacteria (by incubation with 4% glutaraldehyde) was compared. In both cases, measurements were performed in solutions containing either 1 mM ferricyanide or 1 mM ferrocyanide, the latter used as control. Results after 0 and 60 minutes of incubation are presented in Figure 16b. Important differences between hydrogels containing live and dead bacteria were observed when comparing the response to ferricyanide. That is, hydrogels with live bacteria always presented higher current values associated to the production

of ferrocyanide, which may confirm that this production was metabolic. However, dead bacteria hydrogels still presented a current that increased over time, probably due to the presence of some live bacteria in the gel. Control measurements with ferrocyanide provided similar results for live and dead bacteria hydrogels, which remained almost stable over time.

These results, therefore, confirmed the metabolic origin of the ferrocyanide in the sample as a consequence of live bacteria activity, as well as the potentiality of these simple cell-based biosensors for toxicity assessment based on ferricyanide respirometry.

2.3.4 General toxicity assessment

The cell-based conductive alginate hydrogel biosensor was finally applied to general toxicity assessment using the well-known toxic agent 3,5-dichlorophenol (DCP). Toxicity assessment involved in situ generation of the conductive alginate hydrogel membrane with bacteria and 15 minutes of incubation with the toxic solution. DCP concentrations from 0.1 to 100 ppm were evaluated. Then, initial solution was replaced by the sensing solution containing 1 mM ferricyanide and 0.2% glucose. After 60 minutes of incubation, the accumulated ferrocyanide was measured by chronoamperometry at 0.4V. DCP toxicity results are presented in Figure 17 as inhibition (in percentage) by comparison with the response of the biosensor to samples without toxic.

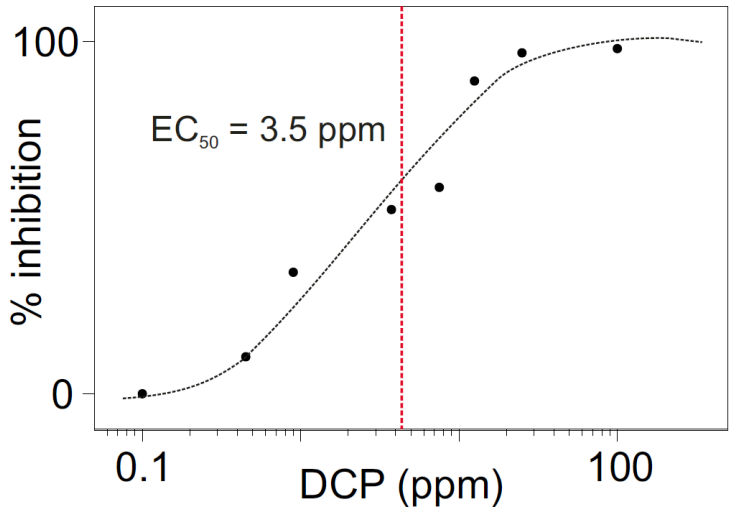

Figure 17. Dose-response curves obtained by plotting the inhibition percentage against DCP concentration. Inset, the EC_{50} magnitude, corresponding to the half-maximal effective concentration of a toxic, is also included.

As shown, there was a clear concentration-dependence in the response of the biosensor. At low toxic concentration the inhibition of the metabolic production of ferrocyanide was low, but it increased with the toxic concentration up to 10 ppm DCP where it reached a 100% of inhibition. The half-maximal effective concentration (EC_{50}) magnitude for DCP with the current biosensor was of 3.5 ppm, which was in agreement with the already reported values (Tizzard et al. 2004, Qian et al. 2014).

Therefore, the conductive alginate hydrogel presented in this work was suitable for stably and reproducible immobilization of viable bacteria which may be used for fast and simple determination of general toxicity through their metabolic activity and using ferricyanide as final electron acceptor.

2.4. Conclusions

Cell electrotrapping in conductive alginate hydrogels, containing up to 4% graphite microparticles, is here examined and applied to the development of general toxicity biosensors. Electrotrapping of bacteria (up to $1.5x10^{8}$ $cfu \cdot mL^{-1}$) in the conductive hydrogels provides repeatability and simplicity of the entrapment process, and ensures cell stability in a biocompatible matrix that not compromises its integrity and activity. Hydrogels conductivity, moreover, improves charge transfer during electrodeposition, producing thicker hydrogels with more bacteria than non-conducting counterparts. Regarding the latter, the determination of the metabolic reduction of ferricyanide to ferrocyanide, used in the development of the general toxicity biosensor, is enhanced when using conductive hydrogels due to the increased bacterial number and the improved charge transfer between electrode and redox molecule. When applied to general toxicity, the biosensor presents a dose-response curve with a EC_{50} magnitude of 3.5ppm for DCP, completely comparable to reported values. The presented entrapment approach represents a step forward towards reliable, controlled and repetitive cell immobilization and the production of simple and fast-response biosensors with enhanced sensitivity.

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CHAPTER 3

Fast fabrication of reusable polyethersulfone microbial biosensors through biocompatible phase separation

3. Fast fabrication of reusable polyethersulfone microbial biosensors through biocompatible phase separation

This work has been published in the journal Talanta (Annex)

Abstract

In biosensors fabrication, entrapment in polymeric matrices allows efficient immobilization of the biorecognition elements without compromising their structure and activity. When considering living cells, the biocompatibility of both the matrix and the polymerization procedure are additional critical factors. Bio-polymeric gels (e.g. alginate) are biocompatible and polymerize under mild conditions, but they have poor stability. Most synthetic polymers (e.g. PVA), on the other hand, present improved stability at the expense of complex protocols involving chemical/physical treatments that decrease their biological compatibility. In an attempt to explore new solutions to this problem we have developed a procedure for the immobilization of bacterial cells in polyethersulfone (PES) using phase separation. The technology has been tested successfully in the construction of a bacterial biosensor for toxicity assessment.

Biosensors were coated with a 300 μm bacteria-containing PES membrane, using non-solvent induced phase separation (membrane thickness ≈ 300 μm). With this method, up to $2.3x10^6$ cells were immobilized in the electrode surface with an entrapment efficiency of 8.2%, without compromising cell integrity or viability. Biosensing was performed electrochemically through ferricyanide respirometry, with metabolically-active entrapped bacteria reducing ferricyanide in the presence of glucose. PES biosensors showed good stability and reusability during dry frozen storage for up to 1 month. The analytical performance of the sensors was assessed carrying out a toxicity assay in which 3,5-dichlorophenol (DCP) was used as a model toxic compound. The biosensor provided a concentration-dependent response to DCP with half-maximal effective concentration (EC_{50}) of 9.2 ppm, well in agreement with reported values. This entrapment methodology is susceptible of mass production and allows easy and repetitive production of robust and sensitive bacterial biosensors.

3.1. Introduction

In biosensors, the recognition element (i.e. cells, enzymes, antibodies, etc.) is immobilized in close physical contact with the transducer in order to ensure compactness, sensitivity and short response times (D'Souza 2001). Ideal bio-functionalization protocols allow stable and controlled immobilization of the recognition element, without compromising its integrity or activity (Bhalla et al. 2016). Regarding biosensors based on living bacteria, the biocompatibility of the immobilization protocol represents an additional and critical factor to be considered (Lei et al. 2006, Michelini et al. 2012). Until now, the most popular fabrication procedure has been the entrapment in biocompatible polymeric matrices (Akylmaz et al. 2006, Gupta et al. 2007, Jha et al. 2009, Xu et al. 2011), such as polymeric hydrogels of natural origin and synthetic polymers. Bio-polymeric hydrogels (e.g. alginate, agar, agarose and chitosan, among others) are usually preferred since they require soft reaction conditions (room temperature, neutral pH) and use harmless chemical reagents (Lee et al. 2012, Sharma et al. 2018), which do not compromise the stability and activity of the biological elements. However, their application is compromised by the lack of reversibility of these materials to dehydration (they do not recover the initial polymeric structure after being dehydrated) and their poor chemical and biological stability (Lee et al. 2012). On the other hand, synthetic polymers such as polyvinyl alcohol (PVA) (Lozinsky et al. 1998, Efremenko et al. 2014, Ruan et al. 2018), polyacrylamide (Simpson et al. 1998) and polyurethane (König et al. 1996) improve the chemical and structural stability as well as mechanical strength (Zhang et al. 2006) of the matrix, but they require the use of toxic reagents (Ahmad et al. 2012), chemical/UV-light cross-linking and/or thermal treatment (Bolto et al. 2009), which may compromise the function of the immobilized molecules.

In this work, we explore the possibility of using polyethersulfone deposited by phase separation, for the entrapment of active and viable bacterial cells. Phase separation is a simple and quick procedure for the preparation of polymeric porous membranes that combines biocompatibility (extraction may be performed with a water-based solution) and stability of the final membrane. The procedure consists of the removal of solvent from the polymer to induce the formation of a porous solid membrane. Solvent removal from the polymeric solution can be achieved with temperature, evaporation or addition of a non-solvent solution (usually water) in which the polymer is insoluble (Tadros 2013). This last approach, also known as non-solvent induced phase separation (NIPS), has been already used in the development of biosensors by immobilization of enzymes and antibodies in PES porous membranes (Prieto-Simón et al. 2006, 2007; Ordoñez et al. 2007, González-Bellavista et al. 2009), but so far has not been reported for the entrapment of whole microorganisms.

In this paper we explore for the first time the immobilization of *E. coli* by entrapment in polyethersulfone membranes through NIPS. Immobilization is carried out on the surface of a

screen-printed electrode in order to obtain a working microbial activity biosensor. The integrity, viability and biological activity of the cells after the entrapment process is evaluated. Bacterial activity in the PES membrane is tested using electrochemical measurements and the applicability of the resulting biosensor is evaluated in an electrochemical respirometry assay for general toxicity assessment (Morris et al. 2001, Catterall et al. 2010, Pujol-Vila et al. 2015, 2016; Vigués et al. 2018). Biosensor reusability, reproducibility and stability under different storage conditions are also evaluated.

3.2. Methods

3.2.1 Chemicals and materials

Commercial polyethersulfone (PES) was obtained from BASF (BASF Ultrasons S3010, Frankfurt, Germany). Screen-printed carbon electrodes (DRP-110) with carbon working and counter electrodes and Ag pseudo-reference were purchased from Dropsens (Spain).

Potassium ferricyanide, 3,5-dichlorophenol, glucose were purchased from Panreac (Spain) and dimethyl sulfoxide (DMSO), γ-butyrolactone, ethyl-lactate, methyl-lactate and N,N-dimethylformamide (DMF) from Sigma-Aldrich (US). All chemicals were of analytical grade and all solutions were prepared using Milli-Q water.

3.2.2 Microorganisms

Escherichia coli ATCC 10536 was grown aerobically in 100 mL of LB broth for 18 hours at 37 ºC. Grown cultures were centrifuged at 10100 x g for 10 min and re-suspended in 2 mL of Milli-Q water, providing a final bacterial concentration of $5.6x10^9$ cells·mL^{-1}. Viable numbers were determined using LB agar plates after suitable dilutions. Total cell numbers were determined by epifluorescence microscopy of samples filtered through 0.2 µm polycarbonate filters, stained with 4′,6-diamidino-2-phenylindole (DAPI) fluorescent stain and observed with a Zeiss AXIO Imager A1 fluorescence microscope.

3.2.3 Survival of microorganisms in selected organic solvents

Successful immobilization of live microorganisms in matrices of organic polymers requires that the cells survive the contact with the solvent used to prepare the polymer solution. To study the effect of different solvents in bacterial viability, bacterial pellets from overnight cultures of *Escherichia coli* were re-suspended in 500 µL of solvent, i.e. DMSO, DMF, ethyl-lactate, methyl-lactate, γ-butyrolactone and phosphate buffer used as control, and incubated for 5 min, 30 min

and 24 h. After incubation, bacterial suspensions were centrifuged (5 minutes at 10100xg) to remove the solvent. The pellet was resuspended in phosphate buffer and centrifuged again. The procedure was repeated three times to ensure that any remaining solvent had been removed from the sample. Total and viable bacterial numbers were then determined as detailed above.

3.2.4. Biosensor fabrication

Non-solvent induced phase separation (NIPS) methods involve three components, namely the polymer, the solvent and the non-solvent. Membrane preparation started with the dissolution of the polymer, i.e. PES, in DMSO. Specifically, PES was dissolved (12% w/v) in DMSO at room temperature and stirred until obtaining a homogenous solution. Five microliters of the resulting solution were deposited on the working element of a screen-printed electrode (Dropsens, DRP-110). Then, 100 µl of non-solvent were deposited on top of the PES/DMSO solution to induce phase separation and the formation of the porous membrane on the working electrode. For the electrodes used for the physical and electrochemical characterization of the membranes, the non-solvent was MilliQ water. In the case of the electrodes used in the toxicity assays, the repeatability and reproducibility assays, and the storage stability assays, the non-solvent was MilliQ water containing $5.6x10^9$ cells·mL^{-1}. Finally, for the electrodes used to assess the effect of cell concentration on electrode output, the non-solvent was MilliQ water containing variable concentrations of microorganisms (0, $5.6x10^8$, $2.8x10^9$ and $5.6x10^9$ cells·mL^{-1}).

3.2.5. Biosensor characterization

Profilometry and SEM imaging. A Leica DCM 3D dual core optical profilometer was used to study the thickness and surface roughness of the deposited membranes.

Images of the inner structure of the PES membrane were acquired by Field Emission Scanning Electron Microscope (FE-SEM; Zeiss Merlin, Germany) from membrane fragments after cryofracture with liquid nitrogen.

Electrochemical measurements. Carbon screen printed electrodes (SPE; Dropsens 110) integrating a carbon working-electrode (4mm of diameter), carbon counter-electrode and Ag pseudo-reference electrode were used throughout this work. Electrochemical measurements were performed using the µSTAT8000 multi-potentiostat (Dropsens, Spain) and Dropview 8400 software (Dropsens, Spain). Cyclic voltammetries were performed between -0.5 and 0.6 V using equimolar mixtures ferricyanide/ferrocyanide (1 mM) and a scan rate of 0.05 V·s^{-1}. Chronoamperometries were carried out at 0.4 V during 90 s. Under these experimental conditions, the measurements leveled out after 20 s and stable values were recorded at 40 s.

Ferricyanide respirometry. In ferricyanide respirometry a preincubation is carried out in which oxygen is substituted by ferricyanide as the final electron acceptor for bacterial respiratory metabolism (Morris et al. 2001, Catterall et al. 2010, Pujol-Vila et al. 2015, 2016; Vigués et al. 2018). Importantly, only living and metabolically active cells reduced ferricyanide to ferrocyanide and thus, the level of ferricyanide reduction can be used to determine the level of microbial activity. The assay was performed preincubating PES biosensors with 1mM ferricyanide solutions containing 0.2% glucose as the carbon source. Different preincubation times were tested (0, 30, 60 or 90 min) but 60 minutes was chosen to carry out most of the experiments. Ferrocyanide accumulation was determined as the stable current value recorded after 40 s of chronoamperometry at 0.4 V (vs Ag/Ag Cl). Biosensors containing dead bacteria (killed by addition of 4% glutaraldehyde) or live bacteria without carbon source were used as negative controls.

3.3. Results

3.3.1 Biocompatibility of the solvents used in the phase-separation process

To analyze the biocompatibility of several of the solvents available to dissolve PES we exposed *E. coli* cells to each of the solvents, as described in Methods, and checked their viability at different times. The results of the analysis are presented in Table 3.

Table 3. Study of the biocompatibility of organic solvents at different microorganism-solvent contact times (5 and 30 minutes, 24 hours).

	5 minutes		30 minutes		24 h	
	cfu·mL^{-1}	%viability	cfu·mL^{-1}	%viability	cfu·mL^{-1}	%viability
DMSO	9.2x10^{8}	74±4	2.0x10^{8}	18.1±1.2	<0.1	<8.3x10^{-9}
DMF	8.8x10^{8}	70±2	1.2x10^{8}	11±3	<0.1	<8.3x10^{-9}
Ethyl-lactate	8.0x10^{8}	64±3	3.9x10^{8}	36±4	<0.1	<8.3x10^{-9}
Methyl-lactate	9.5x10^{8}	76±2	3.4x10^{8}	32±3	<0.1	<8.3x10^{-9}
γ-butyrolactone	9.5x10^{8}	76±2	9.9x10^{7}	9±2	<0.1	<8.3x10^{-9}
control	1.2x10^{9}	100.0±0.8	1.1x10^{9}	91.6±0.9	1.0x10^{9}	89.1±1.1

Cell viability was affected by the solvents, but remained relatively high (64-76%) after short exposure times of 5 min. After 30 minutes of exposure, ethyl-lactate and methyl-lactate maintained a reasonable survival (36 and 32%), much higher than that obtained with DMSO (18%), DMF (11%) or γ-butyrolactone (9%). When the exposure time was extended to 24 h,

viability decreased below $8.3x10^{-9}$ % in all cases, a reduction of more than 10 orders of magnitude from the initial value, which suggested that all the organisms present in the sample were virtually death. From the tested solvents, DMSO was chosen because it allowed high survival at short times while providing the highest solubility of PES.

3.3.2 Characterization of PES-coated electrodes

Screen-printed carbon electrodes coated with porous PES membranes were obtained by non-solvent induced phase separation following the procedure described in the Methods section. The resulting membranes were analyzed by profilometry. The results, displayed in Figure 18a, show the existence of a circular structure with an elevated central area which tapers towards the exterior. Maximum thickness at the center was 300 μm. Membranes were cross-sectioned and visualized by SEM in order to analyze their inner structure (Figure 18b). The porous structure of the PES matrix changed with depth. Close to the surface (first 100μm), the membrane presented a homogeneous microporous structure. Beyond this superficial region, the bulk of the membrane presented much larger pores showing a sponge-like structure. This can be attributed to variations in the solvent exchange kinetics, much faster in the surface than inside the PES matrix (Guillen et al. 2011, Khorsand-Ghayeni et al. 2016).

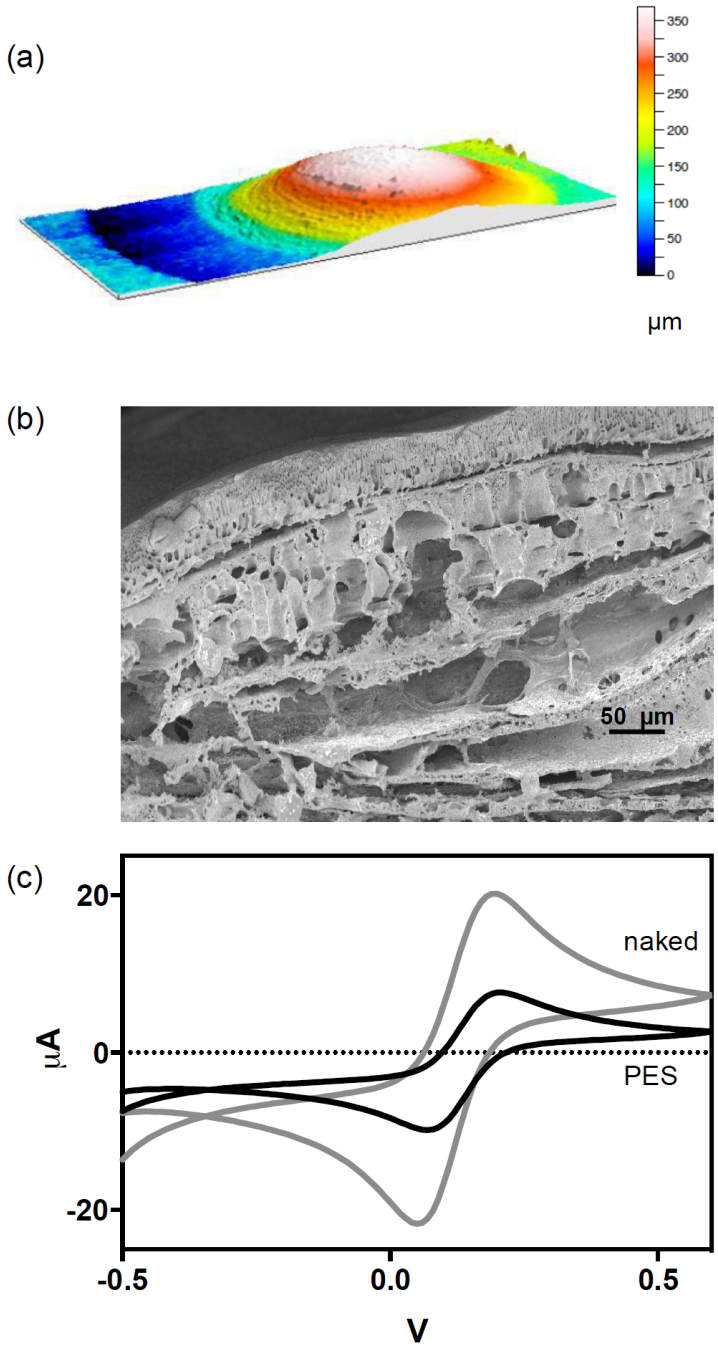

Figure 18. a) Optical profilometry results of the observations carried out on polyethersulfone-coated screen-printed electrode obtained. Maximum thickness at the center of the electrode is around 300 µm. **b)** Scanning electron microscopy cross-section of the PES membrane deposited on top of the electrode. **c)** Cyclic voltammograms of equimolar mixtures ferricyanide/ferrocyanide (1mM) with and without polyethersulfone membrane.

The electrochemical behavior of the PES-coated electrodes was studied by cyclic voltammetry. Electrodes were covered with 100 µL of an equimolar solution of ferricyanide and ferrocyanide (1 mM) and voltammetric measurements were performed after 5 min of incubation, to ensure

diffusional equilibrium. Recorded voltammograms are presented in Figure 18c. PES-coated electrodes showed a 60 % reduction of anodic and cathodic current magnitudes with respect to naked electrodes, as expected in membrane-modified electrodes due to layer isolation.

3.3.3 Biosensor fabrication and characterization

Bacterial entrapment in PES membranes was evaluated using DMSO as solvent and water suspensions of *E. coli* as non-solvent. The underlying mechanism consisted of the replacement of DMSO by water and the concomitant entrapment of cells inside the PES matrix (Figure 19).

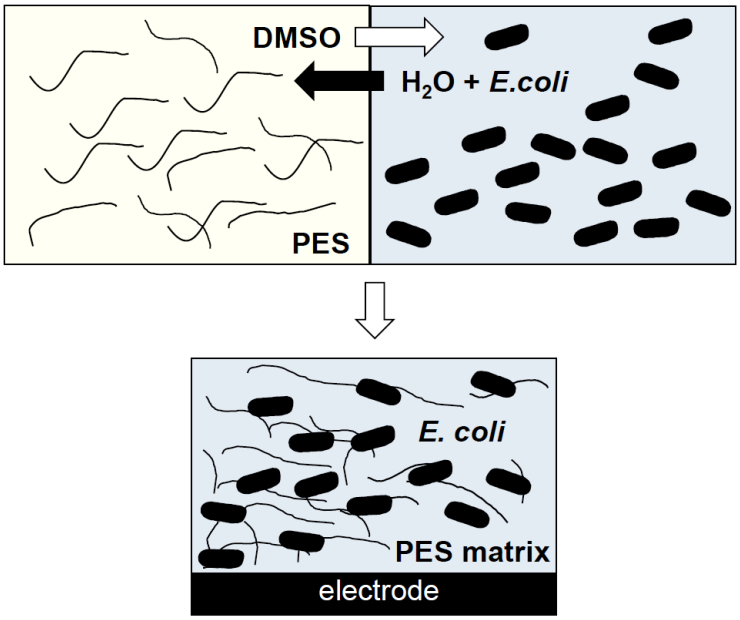

Figure 19. Entrapment of microbial cells on the biosensor surface by nonsolvent induced phase separation of a solution of polyethersulfone in DMSO. A water suspension of *E. coli* was used as a non solvent. During the non-solvent induced phase separation, the DMSO is substituted by the non-solvent, so that the membrane is formed with microorganisms trapped inside it.

PES membranes prepared by NIPS using suspensions of *E.coli* containing 5.6×10^9 cells·mL^{-1} (figure 20a) were compared to control samples obtained by the same protocol but using water without bacteria as non-solvent (figure 20b). In the first case, rod-shaped *E. coli* cells were clearly observed in the interstitial spaces between the membrane pores, confirming bacterial entrapment. No more relevant differences were observed when comparing both membranes.

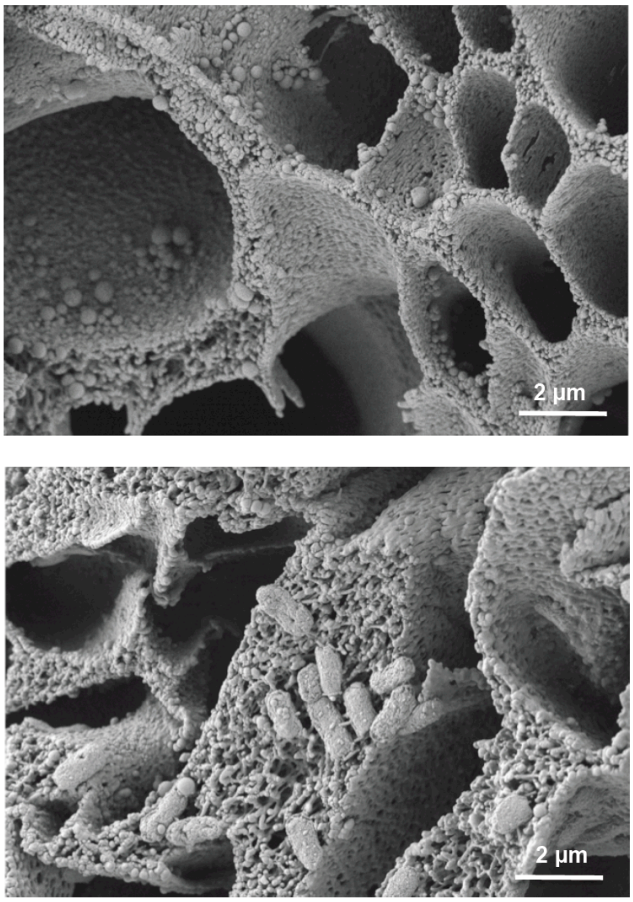

Figure 20. a) Cross-section image of a PES membrane obtained by Scanning Electron Microscopy. **b)** Image of a cross-sectioned PES membrane with microorganisms entrapped during phase separation.

Entrapment efficiency. Entrapment efficiency was determined by dissolving PES membranes in 100 µL of DMSO, staining with DAPI and counting bacterial cells by epifluorescence microscopy. An average of $2.3\pm0.1 \times 10^6$ cells were recovered from PES membranes. Taking into account that these membranes had a volume of 5 µL, this corresponded a concentration of 4.6×10^8 cells·mL^{-1} per membrane and thus, to an entrapment efficiency of 8.2%.

Biosensor response as a function of preincubation time and number of entrapped cells. To assess the effect of preincubation time on the response of the sensor, we performed ferricyanide-respirometry measurements of PES membranes containing 5.6×10^8 cells·mL^{-1} preincubated in the presence of 1 mM ferricyanide and 0.2 % glucose during 0, 30, 60 and 90 minutes. The results of the experiment are represented in Fig. 21a. As a rule, controls containing live cells without carbon source displayed detectable currents albeit very low. However, PES membranes containing live bacteria and incubated in the presence of glucose and ferricyanide displayed higher current values, which increased with the duration of the preincubation period up

to 60 minutes. Since extending preincubation from 60 to 90 minutes only provided a marginal increase in current, we decided to use 60 minutes preincubations in all future assays.

In order to assess the effect of the number of organisms immobilized on the response of the sensor, membranes were prepared containing different amounts of entrapped cells ($2.3x10^5$, $1.15x10^6$ and $2.3x10^6$ cells per membrane). The results, plotted in Fig. 21b, indicated the existence of a linear relationship between the number of organisms and the signal obtained. In this particular case, decreasing cell numbers below 10^5 cells per sensor provided currents in the nA range that fell below the detection limit of the equipment. Although increasing the number of cells per membrane may potentially increase the response of the sensor, this was not possible in practice since the microbial suspension used in the membrane preparation was already concentrated 100x from a fully grown culture (see Methods section 2.2).

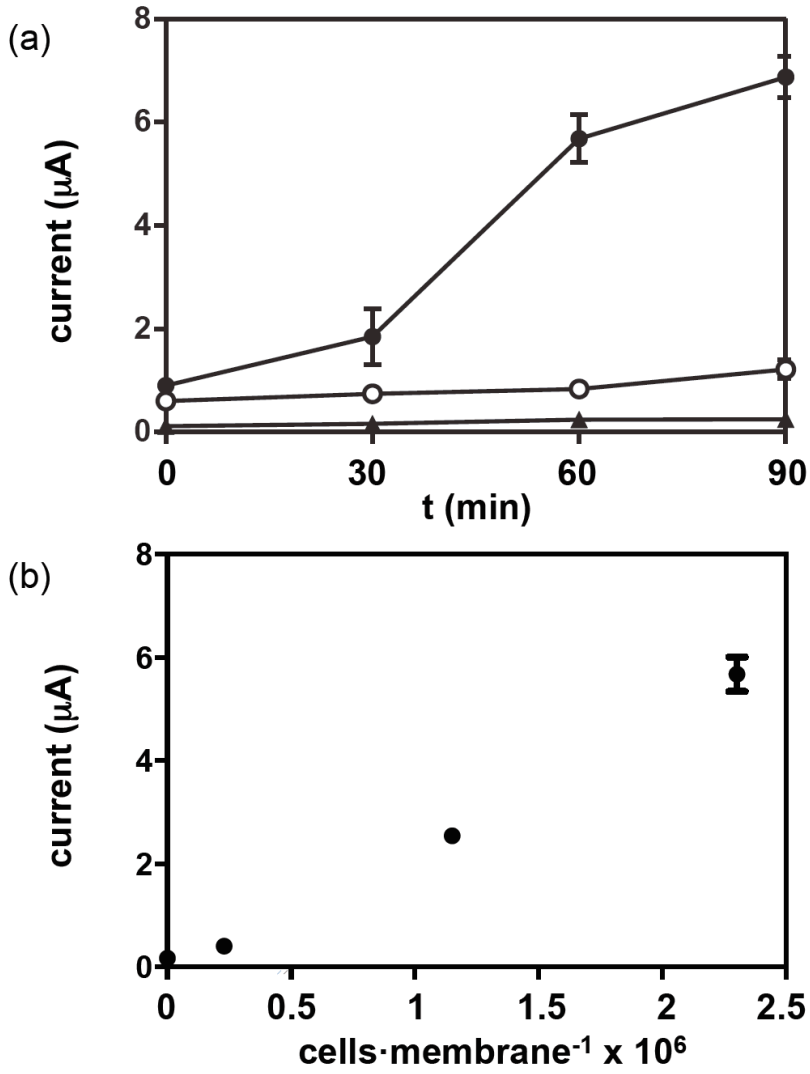

Figure 21. a) Stable chronoamperometry readings from *E. coli* containing PES membranes preincubated in the presence of Fe^{3+} for a period of 0, 30, 60 and 90 min. Metabolically active cells with glucose (●), without glucose (○) and glutaraldehyde-killed control (▲). **b)** Stable chronoamperometry readings from PES membranes containing different concentrations of *E. coli*, after a 60 min. preincubation in the presence of Fe^{3+} and glucose.

Stability of cell entrapment. In order to evaluate the stability of cell entrapment, *E.coli*-PES membranes were incubated in phosphate buffer at 37 ºC in a shaking incubator at 100 rpm. Under these conditions, in the absence of nutrients, cell growth was arrested, and we expected mechanical shaking to remove cells loosely attached to the membrane. The number of entrapped cells was again determined, after 2, 5 and 7 days, by dissolving the membrane in DMSO, and performing microscopy counts of DAPI stained samples. The results are plotted in Figure 22a. As shown, the total number of cells in the PES membrane remained almost constant along the

seven days of the experiment. This result demonstrates that cells were stably trapped within the membrane.

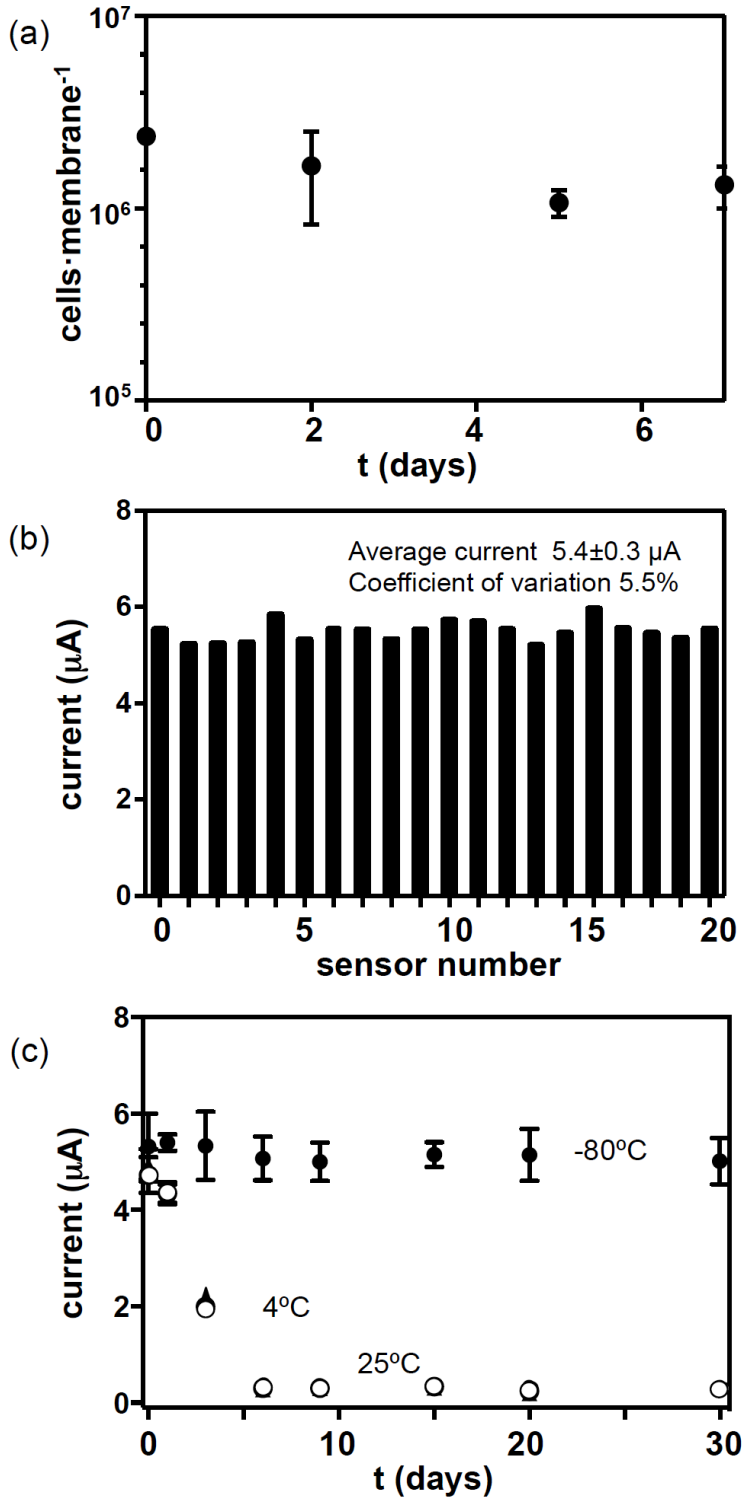

Figure 22. a) Variation of the number of organisms entrapped in PES membranes as a function of the time submerged in phosphate buffer with shaking. Numbers do not decrease significantly after one week indicating that entrapment is stable and cells are not easily removed from the PES matrix. **b)** Stable chronoamperometry readings from 20 different *E. coli* containing PES sensors after a 60 min. preincubation in the presence of Fe^{3+} and glucose. Repeatability is very

high with an average reading of 5.4 µA and a coefficient of variation of 5.4%. **c)** PES microbial biosensor stability determined by repeated measurements of the same sensors dried and stored at different temperatures (4ºC, 25ºC and -80ºC) over 30 days.

Sensor repeatability. Repeatability was determined by analyzing the metabolic response of 20 independent sensors by ferricyanide respirometry. The sensors were preincubated for 60 minutes in the presence of glucose and ferricyanide as described in Methods section. After that, the sensors were subjected to chronoamperometry and the stable current after 40 seconds was recorded. The whole procedure was repeated three times for each sensor (figure 22b). An average current of 5.4±0.3 µA was obtained, with a coefficient of variation of 5.5% well in the range of the values usually found in sensors based on biological processes.

Stability during storage. Finally, in order to establish the stability of the sensors when stored at different temperatures, a number of them were dried at 25 ºC for 1 hour and stored at 25, 4 and -80ºC for a period of 30 days. The sensors (4 at each temperature) were taken out of storage at different times, and their metabolic response was determined by ferricyanide respirometry. At the end of the measurements, the sensors were rinsed, dried for one hour at 25 ºC and stored again. As can be seen in figure 22c, when stored at -80ºC the biosensors preserved their metabolic response during the 4 weeks tested. Storage at 4 and at 25 ºC, however, was less successful. Although the sensors maintained most of their activity after three days storage at these temperatures, their activity decreased to less than 50% after 6 days and was virtually zero after 9 days of storage. According to these results, freezing the biosensor was the only option that guaranteed the stability and activity of the microbial biosensor during mid-term storage.

3.3.4 Toxicity assessment with PES biosensors

In order to assess the analytical performance of the PES-bacteria biosensors we applied them to the detection of toxicity in water samples using 3,5-dichlorophenol (DCP) as a model toxic agent. Screen printed electrodes containing live bacteria entrapped in PES were exposed for 30 min to different DCP solutions at concentrations ranging from 0.1 to 50 ppm. After that, the activity level of the sensors was monitored through ferricyanide respirometry.

Biosensor response was expressed in terms of inhibition percentage (I%) referred to sensors unexposed to the toxic agent. A concentration-response curve was obtained by plotting I% against DCP concentration, which is presented in Figure 23.

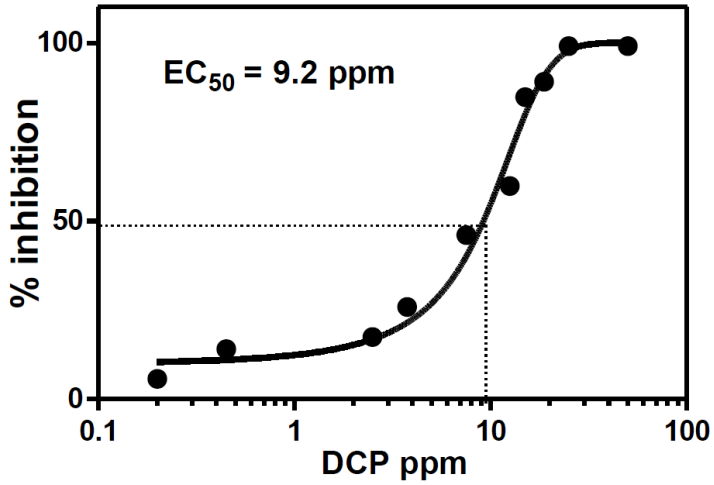

Figure 23. Application of the microbial-PES biosensor to toxicity determination using 3,5-DCP as a model compound. Percentage inhibition has been plotted as a function of 3,5-DCP concentration. The results indicate an EC_{50} of 9.2 ppm well in the range of previously reported values.

The biosensor response was concentration-dependent, from negligible inhibition at 0.2 ppm to 100% at 25 ppm of DCP. A half-maximal effective concentration (EC50) of 9.2 ppm was obtained, in agreement with the values reported for this toxic (ranging from 7 to 16.48 ppm) using other cell-based assays (Tizzard et al. 2004, Zhai et al. 2013, Qian et al. 2014, Gao et al. 2016, 2017). The results validate the suitability of non-solvent induced phase separation entrapment of bacteria in polyethersulfone matrices as a tool for the development of highly stable and reproducible microbial biosensors.

3.4. Discussion

In this work, we demonstrate how microorganisms can be successfully entrapped in polyethersulfone membranes produced by non-solvent induced phase separation, in order to obtain a microbial biosensor. We chose DMF as the reference standard solvent, together with four of the compounds proposed by Figoli et al. (2014) as non-toxic solvents for green NIPS membrane preparation (DMSO, γ-butyrolactone, ethyl-lactate and methyl-lactate). Despite being non-toxic, the biocompatibility of these solvents was in general low, killing virtually all the microorganisms present in the sample after a 24 h exposure. In general, non-toxic status is granted on the basis of toxicity or carcinogenicity tests carried out using low concentrations that mimic environmentally relevant values. In the case of microorganisms, however, resistance of microorganisms to full contact with organic solvents is related to the polarity of the solvent, represented as the logarithm of the partition coefficient of the solvent in a equimolar mixture of n-octanol and water (log P_{ow}) (Inoue et al. 1989). Log P_{ow} values in the range 1 to 4 are toxic to microorganisms at very low concentrations while solvents with log P_{ow} values lower than 1 display toxicity but only when their concentration is very high (Kusumawardhani et al. 2018). The solvents used in our work (DMSO, DMF, γ-butyrolactone, methyl-lactate and ethyl-lactate) have log P_{ow} values of -1.1, -0.85, 0.06, 0.165 and 0.15 (Sigma, Safety Data Sheets), all of them lower than 1. According to this and despite their reported biological safety, the use of these solvents undiluted, as occurs during NIPS, should have a negative effect on cell viability which was what we observed. Fortunately for our work, the lethality of the products decreased when reducing the duration of the exposure to 30 min and 5 min, indicating that we could use them with little loss of microbial viability, as far as contact was kept as short as possible.

The entrapment efficiency after phase separation (8.2%) was relatively low when compared to alginate-based methods, with entrapment efficiencies between 54 and 80% (Sandoval-Castilla et al. 2010, Pop et al. 2015), or to calcium-induced protein gels that provide entrapment efficiencies of 96% (Reid et al. 2005). Despite the lower entrapment efficiency, the use of a high concentration of cells in the non-solvent phase allowed an effective final concentration of 4.6×10^8 cells·mL^{-1} in the membrane, enough to produce a robust signal in the sensing process.

Regarding stability during storage, our results indicate a similar behavior when sensors were kept at 4 or at 25°C. In both cases, the response of the biosensors decreased steadily until becoming inoperative after 9 days. Storage stability data at 4°C reported by different authors using different immobilization matrices indicate much higher rates of survival. Thus, biosensors containing microorganisms entrapped in protein matrices crosslinked with glutaraldehyde were able to keep high activity levels (100%, 74% and 78%) after 20, 15 and 18 days of wet storage at 4°C (Choteau et al. 2005, Akylmaz et al. 2006, Sagiroglu et al. 2011). Similar data have been

reported for microorganisms adsorbed to glass fiber paper, collagen fibers or polyaniline (70%, 95% and 90% of activity retained after 120 days, 15 days and 7 days of dry storage at 4ºC) (Jha et al. 2009, Prathap et al. 2012, Emelyanova et al. 2017). During frozen storage, our sensors kept 100% of their initial activity during the first 30 days tested, despite repeated thaw/freeze cycles during intermediate measurements. The results suggested that the sensors could be produced and stored frozen until needed, providing at least 24 hours of stable operation after thawing.

3.5. Conclusions

Cell entrapment inside porous PES membranes by NIPS is here demonstrated and employed for the development of a PES-based microbial biosensor for general toxicity assessment. Screen printed electrodes have been coated with a 300 µm layer of polyethersulfone containing $2.3x10^6$ cells per electrode. Entrapment efficiency is high (8.2%) and both cell integrity and activity are maintained during the process. The procedure yields reproducible (CV 5.4%) and robust sensors that can be repeatedly used without loss of activity when subject to dry frozen storage without additional treatments.

Entrapped bacteria remain active and reduce ferricyanide to ferrocyanide in the presence of glucose, a process that can be detected electrochemically. When exposed to a toxic, microorganisms lose activity, ferrocyanide production stops and the electrochemical signal decreases. In order to evaluate their performance in a real application, the biosensors have been tested against DCP as a fast and simple method for the detection of toxicity. The biosensor provides a concentration-dependent response with an EC_{50} of 9.2 ppm for DCP, completely comparable to the values reported for the same compound using cell-based assays.

Successful immobilization of microorganisms in organic polymers susceptible of dry storage opens the door to the development of robust and durable microbial biosensors with potential use in industrial and environmental applications.

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CHAPTER 4
GENERAL DISCUSSION

4. GENERAL DISCUSSION

Development of microbial biosensors is subject to important technical limitations related mainly to: (i) poor reproducibility due to a highly variable immobilization protocols, (ii) low sensitivity by the difficulty of immobilizing high bacterial concentrations and (iii) short life-time due to poor survival during immobilization and storage. This thesis describes the development of two immobilization strategies that allow reproducible confinement of microorganisms at high densities and in a reproducible manner, while providing a physiological environment that allows adequate diffusion of nutrients, ensuring the functionality and viability of the trapped microorganisms.

In the first approach, (1) microbial cells are trapped in **graphite-doped alginate** while the polymeric matrix is electrodeposited on the surface of the electrode in very soft and biocompatible conditions (i.e. room temperature, aqueous medium, neutral pH, etc.). In the second approach, (2) microorganisms are entrapped in a **polyethersulfone** matrix through a phase inversion process where the material, initially dissolved in organic solvents, precipitates when in contact with bacterial suspension in aqueous medium. Both systems have been applied to surface modification (i.e. biofunctionalization) of electrochemical sensors to develop biosensors in which microorganisms act as the recognition element.

Alginate is a hydrogel that retains a large amount of water and maintains great flexibility while remaining well hydrated (Ahmed EM 2015). It presents good biocompatibility, low toxicity and easy gelation in very soft and physiological conditions (Lee and Mooney 2012), which makes this biomaterial one of the preferred matrices for immobilizing biological elements, e.g. microorganisms. The main problem with alginate is the difficulty to obtain consistent and reproducible membranes. In this thesis, we have developed an electrodeposition protocol for bacterial entrapment that allows reproducibility and an excellent control on the layer thickness and consequently, on bacterial content. The electrodeposition mechanism involves water splitting and the subsequent proton-mediated release of calcium from calcium carbonate particles, which induces the in situ formation of alginate hydrogels on the electrode surface through potential/current control. The thickness of the hydrogel depends on calcium release to the medium and therefore, on the electrodeposition time and on the applied potential. That is, longer electrodeposition times and applied potentials generate thicker alginate hydrogels capable to incorporate a larger number of bacteria, although the homogeneity of the hydrogel may be compromised due to bubbles formation. In the compromise conditions, 1.52×10^8 cfu·mL^{-1} of *E. coli* were trapped in a reproducible way, without loss of viability, 100% entrapment efficiency and very good repeatability. However, the structures dehydrate in a few hours and do not recover the original properties when rehydrated. This means that once formed, the bacteria-loaded matrix must be kept hydrated in order to preserve its properties.

Due to the non-conductive nature of such hydrogels, conducting particles were added to the precursor solution to produce conductive alginate hydrogels. From all the available carbon materials, graphene, graphene oxide, graphene nanosheets, carbon nanotubes (CNTs) and multiwalled nanotubes (MWCNTs) were discarded for presenting high bactericidal activity, mostly due to their nanometric size (Kang et al. 2008, Akhavan and Ghaderi 2010), or inhibit bacterial growth such in the case of graphene. In 2011, Liu et al. published an evaluation of antibacterial activity of different graphene-based materials and demonstrated that graphene is more bactericide than graphite. In our case, 20 μm graphite particles were selected for presenting low toxicity and not affecting *E.coli* viability.

The other matrix used in this work is polyethersulfone, a synthetic polymer used to make ultrafiltration membranes for water treatment (Zhu et al. 2015). This polymerized matrix is highly hydrophobic, which favours biofouling (Alenazi et al. 2017). This property has been widely exploited for microbial immobilization following protocols where these membranes are introduced in concentrated microorganisms solutions for hours (i.e. 16 hours in the work of Chung et al. 1998) to induce bacterial colonization of the membrane pores. Differing from previous publications, in this thesis the microorganisms have been entrapped inside the polyethersulfone while it was formed through a phase inversion process. Polyethersulfone, like all other asymmetric membranes, is formed by phase separation, concretely by a mechanism known as non-solvent induced phase separation (NIPS). In NIPS, the polymer initially dissolved in an organic solvent is extracted and precipitated by the use of a non-solvent, a liquid in which the polymer is insoluble (Tadros 2013). Any component present in the non-solvent solution during the formation of the membrane may be retained inside its porous matrix. Until now, only biorecognition elements such as enzymes and antibodies have been immobilized using this approach. This thesis demonstrates that, despite the toxic nature of most solvents, it is possible to immobilize microorganisms during the formation of the membrane with little loss of viability. The results indicate that microorganisms were retained within the membranes with an entrapment efficiency of 8.2%, achieving a concentration of $5.6x10^8$ cells·mL^{-1}. The stability of the trapped microorganisms and the repeatability of the measure were checked. It was found that the biosensors could be stored for 30 days at -80 Celsius degrees without losing functionality and maintaining good repeatability (coefficient of variation of 5.5%).

While both, alginate and polyethersulfone allow for reproducible immobilization of microorganisms at the electrode surface, the characteristics yielded by each polymer are remarkably different. Alginate is a highly biocompatible material that can be gelled in a controlled manner by means of electrodeposition providing highly active but short-lived membranes. On the other hand, immobilization of microorganisms in polyethersulfone by non-solvent induced phase separation requires toxic organic solvents and, as a result, involves lower bacterial survival.

However, it provides greater robustness and allows for long term storage without loss of viability or changes in the immobilization matrix.

To compare the efficiency of the immobilization protocols proposed in this thesis with those previously published is complicated due to the diversity and variability of the approaches. To help in this comparison, a number of publications addressing similar problems have been selected and summarized in Table 4, emphasizing the type of immobilization matrix, the entrapment efficiency, the concentration of trapped microorganisms, their survival during the immobilization process and their durability during storage.

Table 4. Types of immobilization matrices used in the immobilization of microorganisms emphasizing entrapment efficiency, survival during entrapment and long term storage.

Immobilization matrix	Entrapment efficiency (%)	Concentration of entrapment microorganisms	Microorganism survival during the entrapment process (%)	Long term storage	Reference
Al_2O_3 sol-gel solution	100%	25 mg of wet weight	---	30 days at room temperature	Chen et al. 2002
Alginate	---	15% w/v	100%	1-15 days at 4ºC wastewater	Kumlanghan et al. 2008
Alginate	76-91%	$1x10^8$ cfu·g^{-1}	---	---	Rahman et al. 2015
Alginate	60%	$1x10^{14}$ cfu·mL^{-1}	100%	---	Coelho-Rocha ND et al. 2018
Alginate	**100%**	**$1.52x10^8$ cfu·mL^{-1}**	**56%**	**few hours**	**Vigués et al. 2018**
Carbon paste	---	15 mg of dry weight	100%	21 days at 4ºC	Mulchandi et al. 2005
Cellulose acetate	100%	---	---	15 days at 4ºC	Yoshida et al. 2001
Chitosan and alginate	80%	10^7cells·g^{-1}	---	60 days at 4ºC	Chanratana et al. 2018
Gellan/xanthan	---	$6x10^6$ cells·mL^{-1}	7%	42 days	Landreau et al. 2016
Polyethersulfone (PES)	**8.2%**	**$5.6x10^8$ cells·mL^{-1}**	**---**	**30 days at -80ºC**	**Vigués et al. 2020**
Poly(glycerol) Silicate	---	1×10^6 to 1×10^7 cells·mL^{-1}	40%	1 day	Harper et al. 2011
Polyvinyl alcohol (PVA)/alginate	34%	----	---	---	Bae et al. 2014

Careful analysis of the data shown in Table 4 indicates that, as a rule, hydrogels (Al_2O_3, alginate, cellulose acetate or chitosan) present high entrapment efficiencies (60-100%) while, other matrices requiring more aggressive polymerization protocols, such as chemical, UV cross-linking or the use of organic solvents (PVA, PES), have low efficiencies in the range between 8-34%.

Several of the references included in Table 4 describing the use of aluminium oxide matrix, carbon paste, gellan/xanthan, poly(glycerol) silicate and PVA/alginate do not specify the efficiency of entrapment. Instead, these works provide information about the percentage of survival during immobilization. This percentage is between 7 and 34% in the last three matrices, whereas slightly higher in the first two. This is because both aluminium oxide and carbon paste polymerize spontaneously at room temperature while the others need chemical and/or physical reactions to produce and stabilize the polymeric matrix.

Another parameter of interest is survival rate during storage. When provided, storage data range from one to sixty days are reported, although most works describe the need to keep the matrix under refrigeration (e.g. fridge or freezer) in order to obtain a longer storage time.

Both immobilization protocols have been employed in the development of microbial biosensors for general toxicity assessment. In this type of sensor, the sensing mechanism is based on the metabolically mediated reduction of an external electron acceptor, i.e. ferricyanide, after incubation with toxic or non-toxic solutions (Catterall et al. 2010, Pujol-Vila et al. 2015, 2016, 2018). The evaluation of the presence of toxic substances in water is necessary to ensure human health and prevent their dispersion in the aquatic environment (Yang et al. 2018). In this sense, general toxicity assessment is of great importance since allows the evaluation of the action of the pollutants, even their synergistic effects, in a simple, portable and fast manner.

To validate the use of the two biosensors in toxicity testing we have chosen 3,5-dichlorophenol (DCP) as a model toxic agent. Chlorophenols are toxic molecules with important consequences to human health. Main problems are related to their absorption and accumulation in important organs, such as the liver or the kidneys, where they have been reported to be responsible of a number of cancer types (World Health Organization 2002). In the particular case of 3,5-dichlorophenol, long-term exposure in humans has been associated to the development of lymphomas as well as nasopharyngeal and lung cancer. Additionally, this compound has harmful effects on aquatic ecosystems. Probably for all these reasons, it has been used for a long time as a model in environmental toxicity studies.

In the last ten years, several works have been published using microbial biosensors for the detection of toxicity in water samples, which used DCP as a model toxic agent. In these works, different strategies have been used with the aim to immobilize microorganisms in matrices with a certain porosity to obtain microbial biosensors. The performance of the biosensors described

in these publications has been compared to the obtained in this thesis and summarized in Table 5.

Table 5. Values of EC_{50} ($mg \cdot L^{-1}$) of 3,5 DCP in different microbial biosensors toxicity assays.

Matrix	Microorganism	Transduction	EC_{50} ($mg \cdot L^{-1}$)	Storage	Reference
---	*E. coli*	optical	3.2		Zhai et al. 2013
PVA-alginate	*S. cerevisiae*	electrochemical	9.83		Qian et al. 2014
---	*P. aeruginosa*	optical and electrochemical	10-15		Yu et al. 2017
PVA	*E. coli*	electrochemical	9.62	10 days 4ºC	Liu et al. 2018
---	*S. oneidensis*	electrochemical	14.5		Yang et al. 2018
alginate	***E. coli***	**electrochemical**	**3.5**		**Vigués et al. 2018**
PES	***E. coli***	**electrochemical**	**9.2**	**30 days - 80ºC**	**Vigués et al. 2020**

All of these works have the same goal but differ in three aspects: the immobilized microorganism, the immobilization matrix and the transduction mechanism for biosensing. Although all contributions describe microbial biosensors, some of them do not use immobilization matrices to trap the microorganism on the sensor surface. All the works provide information about the limits of detection but very few provide data on the durability of the sensor neither during continued used nor during storage.

The most common bacterial model in the table is *Escherichia coli* (Zhai et al. 2013, Vigués et al. 2018 and 2020), but *Pseudomonas aeruginosa* (Yu et to the. 2017), *Shewanella oneidensis* (Yang et al. 2018) and *Saccharomyces cerevisiae* (Qian et al. 2014) have been also used.

According to the immobilization strategy, the use of PVA (Liu et al. 2018), PVA-alginate (Qian et al. 2014), and alginate (Vigués et al. 2018) stand out. Polyethersulfone (Vigués et al. 2020) is firstly presented in this thesis. All these matrices constitute good examples of synthetic polymers and hydrogels used as porous supports that allow cell entrapment and molecules diffusion. Three works that their authors mistakenly consider microbial biosensors do not immobilize microorganisms on the transducer but instead, use suspensions of microorganisms in liquid, therefore constituting a bioassay (Yu et al. 2017, Yang et al. 2018 and Zhai et al. 2013). We have included them on the table for the sake of discussing the EC_{50} values reported. Few works determine the efficiency of absorption or the percentage of living cells within the matrix, and very rarely address the stability of the matrix or the durability of the sensor during use and/or storage.

The third aspect in which these contributions differ is the mechanism of transduction: optical (Zhai et al. 2013), electrochemical (Yang et al. 2018, Qian et al. 2014, Liu et al 2018) or a combination of both (Yu et al. 2017). Some of the contributions agree on the use of redox mediators as metabolic indicators in the bioassay (Zhai et al. 2013, Liu et al. 2018, Vigués et al. 2018 and 2020).

The EC_{50} values from both optical and electrochemical transduction mechanisms range between 3.2 and 15 $mg \cdot mL^{-1}$. Specifically, optically-based sensors find an EC_{50} of 10$mg \cdot L^{-1}$ (Yu et al. using *Pseudomonas*) and 3.2 $mg \cdot L^{-1}$ (Zhai et al. 2013 with *E.coli*). Studies carried out using electrochemically-based sensors report EC_{50} values of 15$mg \cdot L^{-1}$ (Yu et al. 2017 with *Pseudomonas*), 14.5 $mg \cdot L^{-1}$ (Yang et al. 2018 with *Shewanella*), 9.83 $mg \cdot L^{-1}$ (Qian et al. 2014 using *Saccharomyces*). The remaining three electrochemical works immobilize *E.coli* obtaining EC_{50} of 9.62 $mg \cdot L^{-1}$ (Liu et al. 2018), 3.5 $mg \cdot L^{-1}$ (Vigués et al. 2018) and 9.2 $mg \cdot L^{-1}$ (Vigués et al. 2020). In general, most values are in the range of the values of EC_{50} for 3,5 DCP reported using standard methods such as Respiration Inhibition tests (4.5 to 10 $mg \cdot L^{-1}$) or Microtox (2 to 6.7 $mg \cdot L^{-1}$) (Wadhia and Thompson 2009).

In all these examples it is necessary that the microorganisms are alive so that, if the toxic is present the optical or electrochemical, signal decreases. In two of these works, i.e. Zhai et al. 2013 and Liu et al. 2018, electrochemical techniques and a redox mediator are used to detect the presence of toxic molecules in the water. Specifically, they used ferricyanide respiration inhibition, the same protocol used in the two papers presented in this thesis.

Zhai et al. developed a *E. coli* toxicity bioassay but microorganisms were not immobilized. In this case, they detected the metabolic reduction of ferricyanide to ferrocyanide and subsequent formation of Prussian Blue obtaining an EC_{50} for 3,5-DCP of 3.2 $mg \cdot L^{-1}$. In contrast, Liu C. et al. evaluate the influence of the polymeric matrix (agar, gelatin, an agar/gelatin mixture, chitosan, and polyvinyl alcohol (PVA)), with PVA providing better results. They obtained an EC_{50} of 9.62 $mg \cdot L^{-1}$ for 3,5-dichlorophenol. In this last work, the authors managed to store the sensor at 4ºC, maintaining acceptable stability even after 10 days of storage.

In this thesis, toxicity sensors were developed with *E. coli* entrapped in either graphite-doped alginate formed by electrodeposition or in polyethersulfone deposited through a phase inversion process. In both cases toxicity was detected through the inhibition of ferrocyanide microbial respiration. In the determination of 3,5-dichlorophenol, EC_{50} values obtained were 3.5 $mg \cdot L^{-1}$ in the graphite doped alginate sensor and 9.2 $mg \cdot L^{-1}$ in the polyethersulfone biosensor. Both values are in the range (3.2 - 15 $mg \cdot L^{-1}$) reported by different authors (Table 5). The two matrices used have different characteristics and therefore, the concentration of trapped microorganisms and their survival during storage are different. The alginate hydrogel efficiently entraps a large number

of microorganisms and can be generated reproducibly in situ. However, its durability is low due to the large amount of water retained in the structure and its sensitivity to drying. In contrast, the physicochemical characteristics of polyethersulfone allow the storage of biosensors for up to a month. In this case, however, the presence of toxic substances during membrane formation reduces the concentration of active microorganisms that can be trapped. Both processes provide an answer to the need for controlled immobilization of bacteria and pave the way to the development of microbial biosensors that match the requirements for reproducibility and durability currently required by the market.

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CHAPTER 5
CONCLUSIONS

5. CONCLUSIONS

1. Alginate microbial hydrogels can be deposited on the surface of polarized electrodes by applying a fixed potential of 0.5V in the presence of alginate and calcium carbonate. The thickness of the hydrogel layer can be reproducibly controlled by the duration of the potential applied in such a way that 60 seconds of polarization deposit a layer of 80 μm (CV = 0.3%). A high concentration of viable microorganisms ($2.8 \cdot 10^6$ cells$\cdot \mu L^{-1}$) is trapped with 100% efficiency and without loss of viability during the entrapment.

2. The response of the microbial alginate covered electrodes are improved by adding 4% w/v graphite particles (20μm) to the alginate matrix, making it conductive and increasing the electrochemically active area of the electrode. This conductive microbial hydrogel retains a high concentration of viable microorganisms trapped in a reproducible manner.

3. Alginate and alginate-conductive matrices with bacteria exposed to the air maintain hydration for no more than 90 minutes at room temperature. Hydrogel stability can be increased to a few hours during refrigerated storage in liquid. Freezing is not a viable storage alternative as the structure of the hydrogel is destroyed in the process.

4. Polyethersulfone has been used for the first time as a matrix to incorporate microorganisms during the formation of the membrane. This polymer precipitates through a process called NIPS (non-solvent induced phase separation). In this process, polyethersulfone dissolved in organic compounds comes into contact with water with a large amount of *E. coli* and a large part of microorganisms are trapped within the membrane without losing viability. Thus, high densities of *E.coli* can be reproducedly trapped with a trapping efficiency of 8.2%.

5. *E. coli*-containing PES membrane biosensors can be stored successfully for up to 1 month at -80ºC, in the absence of cryoprotectant and without loss of activity.

6. Alginate-graphite and polyethersulfone matrices have been used to entrap *E. coli* cells on the surface of screen-printed electrodes to develop a microbial biosensor for the detection of toxicity with a detection time of 90 minutes. Validation of these biosensors using 3,5 DCP provided values of EC_{50} (3.5 mg$\cdot L^{-1}$ using alginate-graphite and 9.2 mg$\cdot L^{-1}$ using polyethersulfone) comparable to the values obtained using standard methods such as Microtox or Respiration Inhibition tests.

ANNEXES

Published papers included in this thesis:

ANNEX 1. Electro-addressable conductive alginate hydrogel for bacterial trapping and general toxicity determination. 2018.

Vigués N, Pujol-Vila F, Marquez-Maqueda A, Muñoz-Berbel X, Mas J.

Published in Analytica Chimica Acta 7, 115-120.

ANNEX 2. Fast fabrication of reusable polyethersulfone microbial biosensors through biocompatible phase separation. 2020.

Vigués N, Pujol-Vila F, Macanás J, Muñoz M, Muñoz-Berbel X, Mas J.

Published in Talanta 206, 120192.